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## Removal of Heparan Sulfate from the Glomerular Basement Membrane Blocks Protein Passage

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### ABSTRACT

Heparan sulfate (HS) within the glomerular basement membrane (GBM) is thought to play a major role in the charge-selective properties of the glomerular capillary wall. Recent data, however, raise questions regarding the direct role of HS in glomerular filtration. For example, *in situ* studies suggest that HS may prevent plasma macromolecules from clogging the GBM, keeping it in an "open" state. We evaluated this potential role of HS *in vivo* by studying the passage of protein through the glomerular capillary wall in the presence and absence of HS. Intravenous administration of neuraminidase removed neuraminic acid—but not HS—from the GBM, and this led to albuminuria. Concomitant removal of HS with heparinase III, confirmed by ultrastructural imaging, prevented the development of albuminuria in response to neuraminidase treatment. Taken together, these results suggest that HS keeps the GBM in an open state, facilitating passage of proteins through the glomerular capillary wall.

*J Am Soc Nephrol* 18: 3119–3127, 2007. doi: 10.1681/ASN.2007020198

The glomerular capillary wall, which consists of fenestrated endothelial cells, the glomerular basement membrane (GBM), and podocytes with foot processes interconnected by slit diaphragms, forms the major filtration barrier of the glomerulus. It excludes plasma proteins with the size of albumin (69 kD, 3.6 nm) and larger from its filtrate.<sup>1,2</sup> Next to size, permeability of the glomerular capillary wall is controlled by charge interactions. Neutral and cationic molecules can more easily penetrate the GBM than anionic molecules, which encounter electrostatic repulsion by HS. In addition, the negative charges of the cell coat (glycocalyx) of the endothelial cells and podocytes may play a role in the charge-dependent glomerular filtration.<sup>3,4</sup> HS contains multiple carboxylic groups and negatively charged N-, 2-O-, 6-O-, and 3-O-sulfate groups.<sup>5,6</sup> Because the majority of HS resides in the GBM, it is assumed that GBM HS is responsible for the charge-selective properties of the glomerular capillary wall.<sup>3,7,8</sup> This is supported by a number of stud-

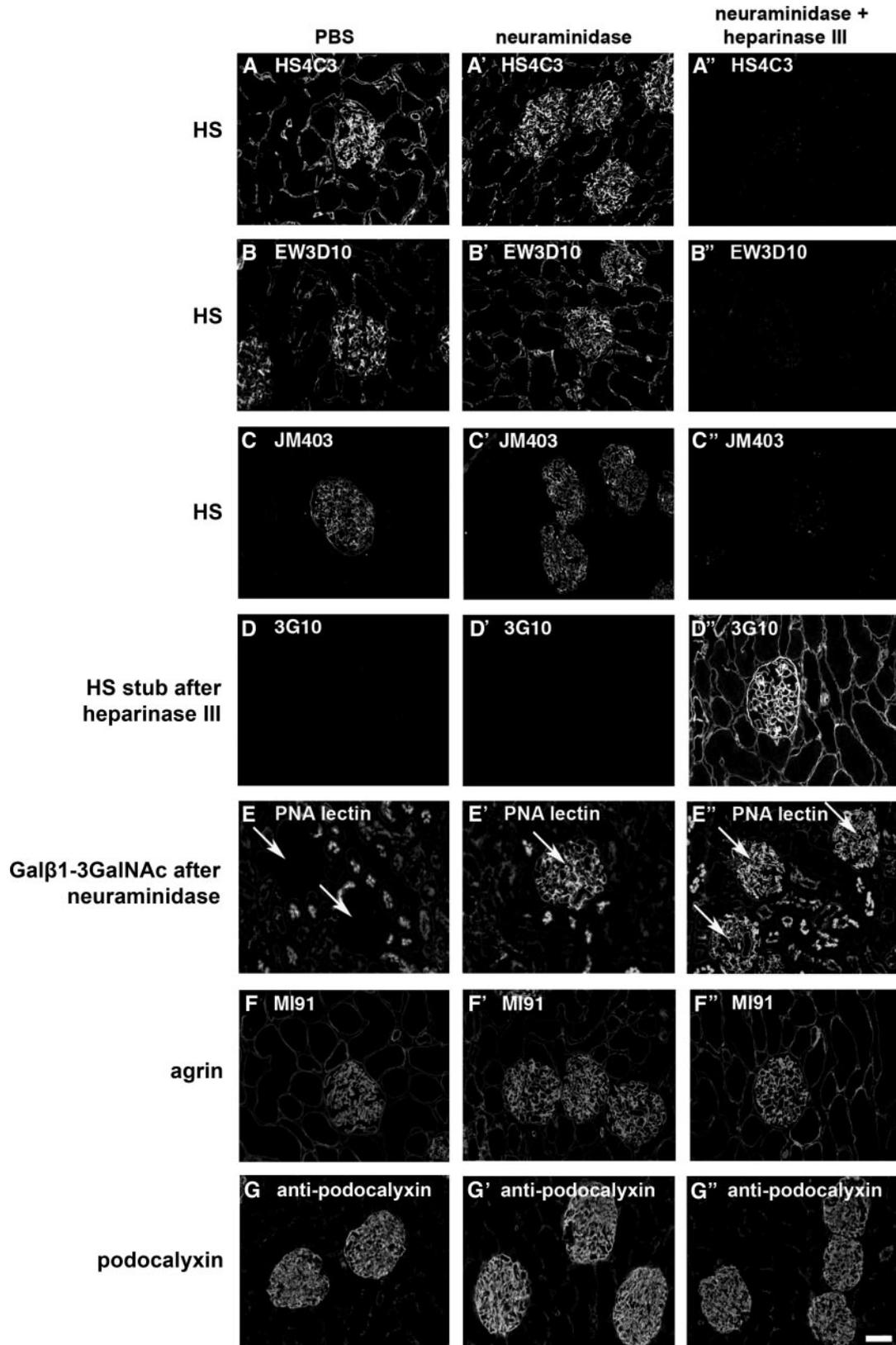
ies. First, a reduction in glomerular HS has been described in a number of human kidney diseases<sup>8–11</sup> and experimental animal models<sup>8,12–14</sup> characterized by proteinuria. Second, intravenous injection of rats with anti-HS antibody JM403 resulted in acute albuminuria.<sup>15</sup> Third, perfusion of isolated rat kidneys or glomeruli with the HS-degrading enzyme heparinase III resulted in penetration of ferritin and bovine serum albumin into the GBM and passage to the urinary space.<sup>16,17</sup> However, recent observations question the direct role of

Received February 14, 2007. Accepted July 9, 2007.

Published online ahead of print. Publication date available at [www.jasn.org](http://www.jasn.org).

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**Figure 1.** Evaluation of the efficacy of *in vivo* neuraminidase and heparinase III treatment on glomerular components. A through G: PBS; A' through G': neuraminidase; A'' through G'': neuraminidase + heparinase III. Staining for HS with anti-HS antibodies HS4C3 (A through A'), EW3D10 (B through B'') and JM403 (C through C'') was absent only after injection with neuraminidase + heparinase III (A'', B'', C''). Anti-HS stub antibody 3G10 (D through D''), reflecting heparinase III activity, only stained neuraminidase + heparinase

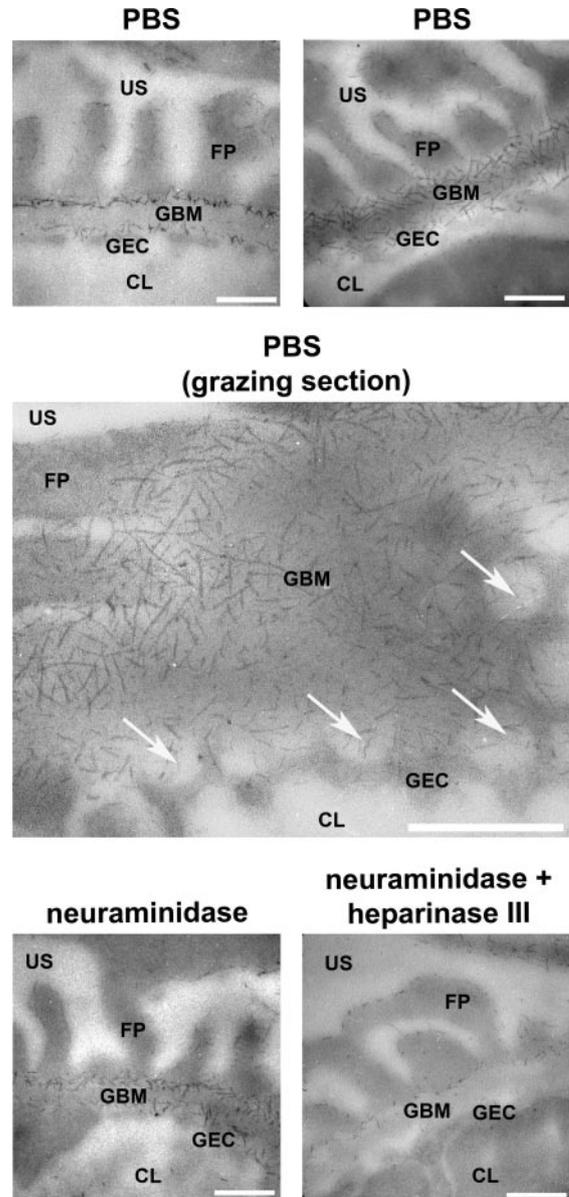
HS in glomerular filtration characteristics. *In vivo* removal of HS from the GBM by intravenous injection of rats with heparinase III did not result in acute proteinuria.<sup>18</sup> Podocyte-specific agrin (major heparan sulfate proteoglycan [HSPG] in the GBM<sup>19</sup>) knockout mice and mice lacking exon 3 of perlecan (HSPG expressed to a minor extent in the GBM<sup>8</sup>) do not develop proteinuria.<sup>20,21</sup> Podocyte-specific EXT1 (enzyme involved in HS polymerization<sup>5</sup>) knockout mice also showed no proteinuria before the age of 8 mo.<sup>22</sup> In addition, transgenic mice overexpressing the human endo- $\beta$ -D-glucuronidase heparanase showed only a two-fold increase in urinary protein.<sup>23,24</sup>

A study by Kanwar and Rosenzweig has indicated an alternative role for HS in the GBM. They found that neutralization of the negative charge of the GBM by *in situ* perfusion of rat kidneys with high molarity buffers resulted in accumulation of ferritin in the GBM.<sup>25</sup> It was suggested that the blockage of negative charges led to clogging of the GBM by circulating plasma macromolecules because a marked reduction in the permeability of insulin as well as inulin across the glomerular capillary wall was found. In this study we investigated this possible role of HS *in vivo* by evaluation of the effect of GBM HS on neuraminidase-induced proteinuria. It was found that acute removal of HS from the GBM stops proteinuria and that HS may thus have a role in facilitating protein transport over the GBM.

## RESULTS

### HS and Neuraminic Acid Are Removed from the Glomerulus by Heparinase III and Neuraminidase Treatment, Respectively

To test whether the injected heparinase III and neuraminidase enzymes had removed HS and neuraminic acid, respectively, presence of both components in the kidney was investigated with immunofluorescence staining. Renal HS staining with antibodies HS4C3 (Figure 1A) and EW3D10 (Figure 1B), which recognize sulfated HS epitopes, was abolished after injection of neuraminidase + heparinase III. Staining for the nonsulfated HS epitope defined by antibody JM403 (Figure 1C) also disappeared. No differences in staining for HS were found in the rats injected with neuraminidase alone. Staining for HS stubs, reflecting heparinase III activity, with antibody 3G10 (Figure 1D) was only visible after neuraminidase + heparinase III injection. Glomerular staining with *Peanut agglutinin* lectin (Figure 1E), reflecting neuraminidase activity, was intense in the rats



**Figure 2.** Electron microscopical detection of sulfated GAGs in the GBM of rats injected with PBS, neuraminidase, or neuraminidase + heparinase III, using cupromeronic blue in the critical electrolyte concentration mode. The grazing section demonstrates that GAGs in the GBM form a network. GAG staining in the GBM was greatly reduced after neuraminidase + heparinase III treatment, but not after treatment with neuraminidase alone. Bars = 250 nm. US, urinary space; FP, podocyte foot processes; GBM, glomerular basement membrane; GEC, glomerular endothelial cells; CL, capillary lumen. Arrows indicate fenestrae in the endothelium.

III-treated kidneys (D'). *Peanut agglutinin* lectin (E through E'), reflecting neuraminidase activity, showed a strong, linear staining along the glomerular capillary wall after neuraminidase and neuraminidase + heparinase III treatment (E', E'), whereas staining in the PBS-injected rats was negative (E) (arrows indicate glomeruli). Expression of the HSPG core protein agrin, visualized using antibody MI91 (F through F'), and the podocyte-associated, neuraminic acid-containing protein podocalyxin (G through G') was not affected by either neuraminidase or neuraminidase + heparinase III treatment (F', F', G', G'). Bar = 50  $\mu$ m; magnification is identical for each photograph.

treated with neuraminidase or neuraminidase + heparinase III, but negative in the PBS-injected rats. These results indicate that renal HS as well as neuraminic acid were removed after heparinase III and neuraminidase treatment, respectively. Expression of the core protein of agrin (Figure 1F), the major HSPG in the GBM, and of the protein part of podocalyxin (Figure 1G), a podocyte-associated neuraminic acid-containing protein, was not affected by enzyme treatments.

The effect of heparinase III was also studied at the electron-microscopic level using the cupromeronic blue staining procedure. This method visualizes sulfated glycosaminoglycans (GAGs) as electron-dense filaments caused by their collapse onto the core protein<sup>26</sup> (Figure 2).<sup>26</sup> There was a strong reduction of GAGs in the GBM after heparinase III + neuraminidase injection. A few short filaments were left, which may be caused by the presence of chondroitin sulfate, which is another class of sulfated GAGs present in small quantities in the GBM. HSPGs form a network in the GBM as deduced from grazing sections (Figure 2). The electron-dense filaments associated with the podocytic site of the GBM were larger and more intensely stained compared with the ones at the endothelial site. This indicates that the HSPGs from the podocytes and the endothelium are different.

Immediately after the first injection of neuraminidase + heparinase III, HS appeared in the urine (Figure 3A), indicating that heparinase III had cleaved HS. No HS was found at later time points (note that 8 h after the first injection a second injection was given), indicating that HS regeneration is not taking place within the time frame of the experiment (*i.e.*, 24 h). No HS was detected in the urine of the neuraminidase- and PBS-injected rats. Increased urinary levels of free neuraminic acid were detected in the rats injected with neuraminidase (data derived from Wijnhoven *et al.*<sup>18</sup>) and those with neuraminidase + heparinase III (Figure 3B). As can be seen, the increase in urinary excretion of neuraminic acid is retarded after combined injection with heparinase III. This suggests that penetration of neuraminidase into the glomerular capillary wall is retarded after cleavage of HS.

### Simultaneous Removal of HS and Neuraminic Acid Does Not Result in Proteinuria, Whereas Removal of Neuraminic Acid Alone Does

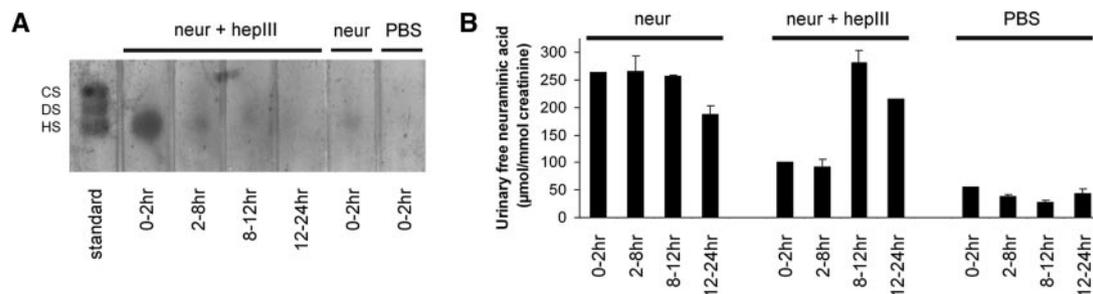
An increase in urinary albumin was detected in rats injected with neuraminidase alone as revealed by SDS-PAGE followed by Coomassie brilliant blue staining (Figure 4A). These results are in line with studies from Kanwar and Rosenzweig<sup>27</sup> and from Gelberg *et al.*<sup>28</sup> In contrast to neuraminidase-injected rats, the urinary protein profile of rats injected with neuraminidase + heparinase III was similar to that of the PBS-injected rats with only trace amounts of albumin. Albuminuria was further analyzed by ELISA (Figure 4B). Urinary albumin levels were greatly increased after injection of neuraminidase alone (*e.g.*,  $13.6 \pm 0.3$  versus  $1.4 \pm 1.1$  mg albumin/mg creatinine for neuraminidase- and PBS-injected rats at 12 to 24 h, respectively; (data derived from Wijnhoven *et al.*<sup>18</sup>). No increase in urinary albumin could be detected in the rats injected with neuraminidase + heparinase III (*e.g.*,  $0.9 \pm 0.3$  mg albumin/mg creatinine at 12 to 24 h).

Urinary volume was normal in the enzyme-treated rats (neuraminidase:  $8.2 \pm 1.1$  ml/24 h; neuraminidase + heparinase III:  $10.5 \pm 1.4$  ml/24 h; PBS:  $10.7 \pm 0.9$  ml/24 h). No major differences in creatinine clearance were detected (neuraminidase:  $2.8 \pm 0.3$  ml/min; neuraminidase + heparinase III:  $2.3 \pm 0.2$  ml/min; PBS:  $2.3 \pm 0.3$  ml/min).

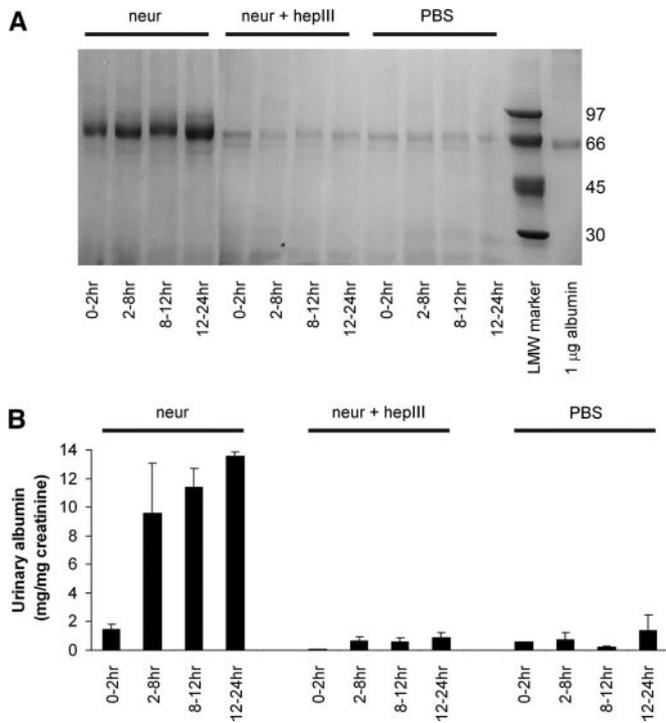
No alterations in renal morphology were observed for the enzyme-treated rats by light microscopy (Figure 5). No major abnormalities in the ultrastructure of the glomerular capillary wall were observed by electron microscopy either (data not shown). The three layers of the glomerular capillary wall (fenestrated endothelial cells, GBM, and podocyte foot processes) were clearly visible. There was no detectable foot process effacement. Presence of the GBM components type IV collagen and laminin, visualized by immunofluorescence staining, was not affected either (data not shown).

## DISCUSSION

The hypothesis that HS keeps the GBM in an open state, allowing proteins to pass through the barrier, was investigated *in*

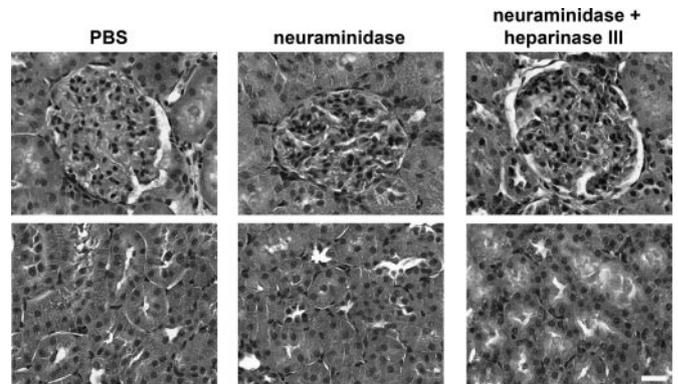


**Figure 3.** Urinary excretion of HS, demonstrated by agarose gel electrophoresis (A), and of neuraminic acid, demonstrated by an enzymatic method (B), in rats injected with neuraminidase, neuraminidase + heparinase III, or PBS. Values are given as mean  $\pm$  SEM ( $n = 2$  for neuraminidase and neuraminidase + heparinase III;  $n = 4$  for PBS). Immediately after injection of neuraminidase + heparinase III, HS was found in the urine. Increased levels of free neuraminic acid were detected in the urine of the rats treated with neuraminidase and neuraminidase + heparinase III. The standard contains 20 ng chondroitin sulfate (CS), 20 ng dermatan sulfate (DS), and 40 ng HS.



**Figure 4.** Urinary albumin, demonstrated by SDS-PAGE (A) and ELISA (B), in rats injected with neuraminidase, neuraminidase + heparinase III, or PBS. Values are given as mean  $\pm$  SEM. Note the increase in urinary albumin in the rats treated with neuraminidase, but not in the rats treated with neuraminidase + heparinase III. LMW, low molecular weight.

*in vivo* by intravenous injection of rats with neuraminidase in the presence and absence of HS. Glomerular HS and neuraminic acid were removed by heparinase III and neuraminidase, respectively, and both components could be detected in the urine. Rats injected with neuraminidase developed albuminuria, but only in the presence of HS. In its absence, no increase in urinary albumin was measured. From this we conclude that removal of HS from the GBM blocks passage of proteins through the GBM and that HS may thus have a role in facilitating protein passage. Because of its negative charge, HS attracts a large number of sodium ions, and as a consequence HSPGs attract large amounts of water, making the GBM a gel-like structure capable to withstand compressional forces.<sup>29</sup> Together with type IV collagen, which provides the structural backbone of the GBM,<sup>30</sup> HS therefore allows the GBM to withstand the dynamic glomerular blood flow pressure and to remain in an open state. This situation would be analogous to the role of GAGs in cartilage, where they form a hydrated gel trapped in a network of collagen fibrils providing pressure-absorbing properties. In addition, hydration of HS may allow molecules, which are also surrounded by a water coat, to slide along the sugar chain when passing from the capillary lumen to the urinary space. In such a way, the hydrated HS can be regarded as a kind of Teflon preventing plasma proteins from (nonspecific) interaction with other GBM molecules, such as

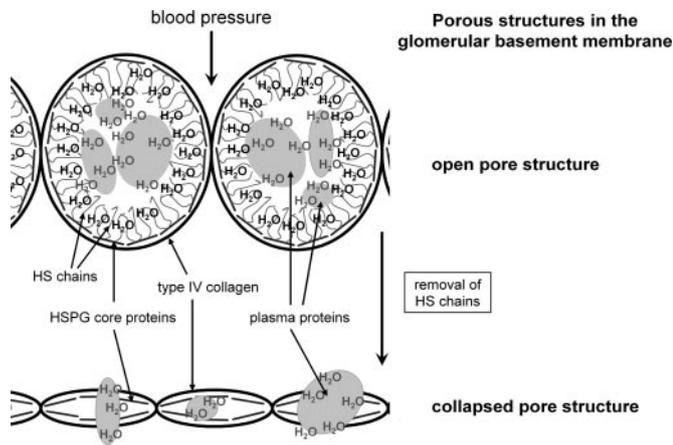


**Figure 5.** Delafield's hematoxylin and eosin staining of kidneys of rats injected with PBS, neuraminidase, or neuraminidase + heparinase III. Top: glomeruli; bottom: tubules. Renal morphology of the enzyme-injected rats was normal. Bar = 25  $\mu$ m; magnification is identical for each photograph.

type IV collagen and laminin. A constant flow of solutes through the GBM may thus be maintained with no plasma proteins being trapped in the GBM. Analogy of the GBM may be drawn with polysulfone membranes, which are used for dialysis therapy.<sup>31,32</sup> To prevent clogging by plasma proteins, the membranes contain sulfonates (equivalent to sulfate groups), which, like the sulfate groups in GBM HS, make the polymer polar, giving it a high affinity for water.<sup>33</sup> The idea to synthesize polysulfonated membranes was actually initiated by the notion that heparin (highly sulfated HS) acts as an antifouling agent and keeps the walls of blood vessels wet.<sup>34</sup>

Our data support the hypothesis of Kanwar and Rosenzweig that HS in the GBM serves as an anticlogging agent, facilitating protein permeability. Their hypothesis was based on *in situ* studies in which neutralization of the negative charge of the GBM was accomplished by perfusion of isolated rat kidneys with high-salt buffer, resulting in accumulation of ferritin in the GBM and in a marked reduction in the permeability of insulin as well as inulin across the glomerular capillary wall.<sup>25</sup> Their approach using high-salt conditions (up to 2.5 M NaCl), however, cannot be applied *in vivo*.

Taken together, we propose the following model (Figure 6): In the normal situation, type IV collagen forms a network, making the GBM a porous structure.<sup>30</sup> HSPGs are lining the type IV collagen scaffold and provide osmotic pressure<sup>29</sup> to keep the pores in an open state. In addition, they prevent passing plasma proteins from interacting with GBM components. When HS is removed, the GBM can not withstand the compressional forces caused by the blood flow, and the pore size will be reduced. This may lead to a reduced permeability of the GBM for larger molecules, which results in clogging and thereby compromises its ability to serve as an effective ultrafilter. Our experimental set-up does not allow a conclusion at which molecular size the permeability becomes compromised after acute HS removal. We can only conclude that the passage of water is not impaired because the urinary volume was not reduced after heparinase III injection. Also creatinine (113 Da)



**Figure 6.** Model illustrating the role of HS in the GBM in facilitating protein passage through the GBM. Top (open GBM): The GBM is composed of a basic network of type IV collagen to which negatively charged HSPGs are associated. Because of the abundance of negative charges, HS attracts large amounts of water, making the GBM a gel-like structure capable of offering counter pressure to the glomerular blood flow and keeping the GBM in an open state. In addition, the hydration of HS allows proteins to slide along the sugar chain from the capillary lumen to the urinary space. Bottom (collapsed GBM): After removal of GBM HS, the network structure collapses and proteins accumulate in the GBM (clogging). Proteins are no longer able to pass through the filtration barrier.

can still freely pass because creatinine clearance was not different after heparinase III injection.<sup>18</sup> Our data support the hypothesis of Kanwar and Rosenzweig<sup>25</sup> that HS in the GBM serves as an anticlogging agent for large proteins, facilitating their permeability, because neuraminidase-induced albuminuria was blocked after HS removal.

Loss of glomerular HS has been reported in a number of renal diseases with proteinuria, including diabetic nephropathy,<sup>9,11,35</sup> minimal change disease,<sup>10,11</sup> and membranous glomerulopathy.<sup>11</sup> How can this be explained? Although speculative, clogging of the GBM may ultimately lead to local disruption of the filtration barrier, resulting in leakage of plasma proteins into the urinary space and proteinuria. In this process, the proposed nondiscriminatory shunt pathway, which allows large proteins to pass the glomerular capillary wall and which is increased in patients with a nephrotic syndrome, may be involved.<sup>36–38</sup> It should be noted that proteinuria is not always associated with loss of GBM HS, as has been reported for IgA nephropathy<sup>11</sup> and congenital nephrotic syndrome of the Finnish type,<sup>39,40</sup> and which also indicates that GBM HS does not offer major resistance to proteins. This is in line with biophysical studies in which no differences in diffusion of albumin through matrices containing heparin, dextran sulfate, or uncharged dextran were found.<sup>41</sup> It should be noted, however, that prolonged absence of GBM HS may result in a number of effects including a reduction of HS-binding growth factors (fibroblast growth factor 2, vascular endothelial growth factor, transforming growth factor  $\beta$ , con-

nective tissue growth factor), structural GBM alterations, and disruption of cell-GBM interactions, leading to dedifferentiation/loss of podocytes and/or endothelial cells. In fact, a recent study of Chen *et al.*<sup>22</sup> points to renal pathology associated with prolonged absence of GBM HS. However, our experimental set-up, comprising an observation period of 24 h after enzyme injection, does not allow conclusions in this respect. Taken together, our results indicate that HS keeps the GBM in an open state, facilitating protein passage through the GBM.

## CONCISE METHODS

### Experimental Set-Up

Male Wistar rats (250 to 300 g) were housed in 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. Power analysis revealed that two rats per treatment group are sufficient to visualize a more than two-fold difference in urinary protein.

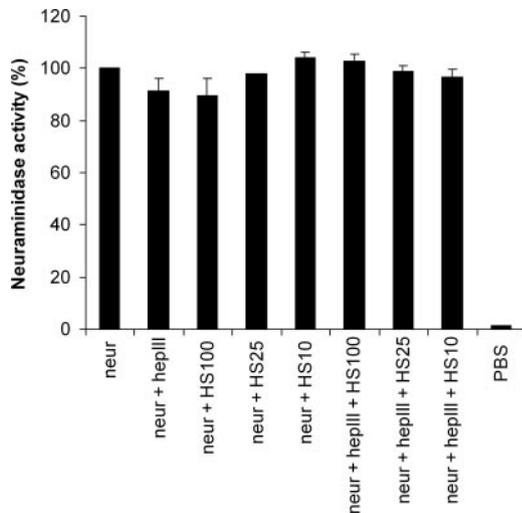
Rats were injected twice ( $t = 0$  h and  $t = 8$  h) *via* the tail vein with PBS (pH 7.3) containing 0.4 IU neuraminidase (E.C. 3.2.1.18; from *Vibrio cholerae*; 90 kD) (Calbiochem, La Jolla, CA) ( $n = 2$ ), PBS containing 0.4 IU neuraminidase and 0.3 IU heparinase III (E.C. 4.2.2.8; from *Flavobacterium heparinum*; 73 kD) (IBEX, Mt. Royal, Quebec, Canada) ( $n = 2$ ), or PBS alone ( $n = 4$ ). Rats were housed individually in metabolic cages to collect urine, and they were killed 24 h after the first injection. At the end of the experiment, blood was collected to obtain serum, and kidneys were removed and processed for general histology, immunofluorescence staining, and electron microscopy.

Using a protease fluorescence detection kit according to the manufacturer's protocol (Sigma, St. Louis, MO), it was checked whether the heparinase III and neuraminidase solutions contained protease activity. No protease activity was detected (data not shown).

To study if neuraminidase activity could be affected by heparinase III and/or HS, an *in vitro* assay was performed. An incubation mixture was applied containing 0.04 IU/ml neuraminidase, 0.15 mM 2'-(4-methylumbelliferyl)- $\alpha$ -D-acetylneuraminic acid (Sigma), and 0.167 M sodium acetate (pH 7.0). After incubation at 37°C for 2 h, the reaction was terminated by the addition of 0.8 M glycine buffer (pH 10.6) and 0.025% (vol/vol) Triton X-100. The amount of liberated 4-methylumbelliferone was measured spectrofluorometrically. The effect of heparinase III and/or HS on neuraminidase activity was investigated by adding 0.04 IU/ml heparinase III and/or 100, 25, or 10  $\mu$ g/ml HS to the incubation mixture. No inhibitory effect of heparinase III and/or HS on neuraminidase activity was detected (Figure 7). Neuraminidase activity was also studied *in vivo* (see below).

### Immunofluorescence Staining

To evaluate the effect of heparinase III, renal cryosections were incubated with antibodies as described previously.<sup>18</sup> The phage display-derived anti-HS antibodies HS4C3 and EW3D10 (produced in our laboratory<sup>42–44</sup>) were detected by incubation with



**Figure 7.** Evaluation of the effect of heparinase III and HS on neuraminidase activity *in vitro*. Activity of neuraminidase (0.04 IU/ml) in the absence of heparinase III and/or HS was set at 100%. Values are given as mean  $\pm$  SEM ( $n = 2$ ). No inhibition of neuraminidase activity was detected in the presence of heparinase III (0.04 IU/ml), HS (100, 25, and 10  $\mu$ g/ml), and heparinase III + HS. No activity was measured in the absence of neuraminidase (PBS control). Neur, neuraminidase; heplIII, heparinase III.-

mouse IgG anti-vesicular stomatitis virus (VSV) tag antibody P5D4 (1:10; Boehringer, Mannheim, Germany) and Alexa 488-conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR). Anti-HS antibody JM403<sup>45</sup> (1:400) was visualized using Alexa 488-conjugated goat anti-mouse IgM (1:200, Molecular Probes). Anti-HS stub antibody 3G10 (1:100; Seikagaku, Tokyo, Japan), which recognizes unsaturated uronates obtained after cleavage of HS,<sup>46</sup> was visualized using Alexa 488-conjugated goat anti-mouse IgG. Antibody MI91<sup>47</sup> (1:800), which stains the HSPG core protein agrin, was visualized using CY3-conjugated goat anti-hamster IgG (1:800; MP Biomedicals, Irvine, CA).

To evaluate the effect of neuraminidase, cryosections were incubated with *Peanut agglutinin* lectin (1:1000; Vector Laboratories, Burlingame, CA), which binds to an epitope that becomes available after removal of neuraminic acid (Gal $\beta$ 1-3GalNAc),<sup>48</sup> as described before.<sup>18</sup> Antibody ASD-86 (culture supernatant 1:20; provided by the Department of Pathology, Radboud University Medical Centre Nijmegen, Nijmegen, The Netherlands),<sup>18</sup> which stains the protein part of the podocyte-associated neuraminic acid-containing protein podocalyxin, was visualized using Alexa 488-conjugated goat anti-mouse IgG.

Expression of type IV collagen was studied using anti-type IV collagen antibody (1:250; Southern Biotech, Birmingham, AL), visualized using Alexa 488-conjugated donkey anti-goat IgG (1:200, Molecular Probes). Expression of laminin was studied using anti-laminin antibody (1:250; Sigma) and visualized using Alexa 488-conjugated goat anti-rabbit IgG (1:200, Molecular Probes). Staining of the sections was examined using a Leica CTR6000 microscope (Leica, Wetzlar, Germany).

## General Histology

Hematoxylin and eosin staining of paraffin-embedded kidney sections was performed as described previously.<sup>18</sup> Sections were examined using a Leica CTR6000 microscope.

## Electron Microscopy

To study the renal ultrastructure, kidney tissue was processed as described previously.<sup>18</sup> To visualize sulfated GAGs, kidney tissue was fixed in 25 mM sodium acetate buffer (pH 5.6) containing 2.5% (vol/vol) glutaraldehyde, 0.2 M MgCl<sub>2</sub>, and 0.2% (wt/vol) cupromeronic blue (Seikagaku, Tokyo, Japan), according to the critical electrolyte concentration method.<sup>18,26</sup> Ultrathin sections were prepared and studied using a Jeol TEM 1010 microscope (Jeol Ltd., Tokyo, Japan).

## Urine and Serum Analyses

Urinary creatinine, GAGs, neuraminic acid, and protein were determined in timed urine collections. Serum creatinine was determined from blood obtained at the time that the rats were killed.

### Creatinine

Urinary and serum creatinine were measured enzymatically as described previously,<sup>49</sup> using the Aeroset apparatus (Abbott, Hoofddorp, The Netherlands). All reagents were obtained from Roche Diagnostics (Almere, The Netherlands). Calibration was performed according to isotope dilution mass spectrometry in line with international agreements. Creatinine clearance (ml/min) was calculated as follows: [urinary creatinine ( $\mu$ mol/ml)  $\div$  serum creatinine ( $\mu$ mol/ml)]  $\times$  [urinary volume (ml)  $\div$  time (min)].

### Glycosaminoglycans

Urinary GAGs were determined by agarose gel electrophoresis. Staining was performed with combined azure A-silver treatment as described by van de Lest *et al.*<sup>50</sup>

### Neuraminic Acid

Urinary neuraminic acid was measured enzymatically using a colorimetric assay (Roche Diagnostics) according to the manufacturer's protocol.

### Proteins

Qualitative examination of urinary proteins of the injected rats was carried out by SDS-PAGE followed by Coomassie brilliant blue staining as described by Laemmli *et al.*<sup>51</sup> Urinary albumin was analyzed quantitatively using an ELISA kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol.

## ACKNOWLEDGMENTS

This study was supported by grants from The Netherlands Organization for Scientific Research (NWO) (program grant 902-27-292) and the Dutch Kidney Foundation (grant C05.2152). The authors thank Ron Wevers (Laboratory of Pediatrics and Neurology, Radboud Uni-

versity Nijmegen Medical Centre, Nijmegen, The Netherlands) for experimental advice, Ria Liebrand and Ine van den Berg (Laboratory of Pediatrics and Neurology, Radboud University Nijmegen Medical Centre) for practical assistance, and the Central Animal Facility of the Radboud University Nijmegen for technical support.

## DISCLOSURES

None.

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