Glomerular Endothelial Surface Layer Acts as a Barrier against Albumin Filtration

Martijn J.C. Dane,* Bernard M. van den Berg,* M. Cristina Avramut,* Frank G.A. Faas,† Johan van der Vlag,‡ Angelique L.W.M.M. Rops,§ Raimond B.G. Ravelli,∥ Bram J. Koster, † Anton Jan van Zonneveld,* Hans Vink,† and Ton J. Rabelink*

From the Einthoven Laboratory for Vascular Medicine,* Department of Nephrology, and the Department of Molecular Cell Biology, ‡ Leiden University Medical Center, Leiden; the Department of Physiology, § Maastricht University Medical Center, Maastricht; and the Department of Nephrology, § Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Accepted for publication January 15, 2013.

Address correspondence to Bernard M. van den Berg, Ph.D., Department of Nephrology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands, PO Box 9600, 2300 RC, Leiden, The Netherlands. E-mail: bmvandenberg@lumc.nl.

Glomerular endothelium is highly fenestrated, and its contribution to glomerular barrier function is the subject of debate. In recent years, a polysaccharide-rich endothelial surface layer (ESL) has been postulated to act as a filtration barrier for large molecules, such as albumin. To test this hypothesis, we disturbed the ESL in C57Bl/6 mice using long-term hyaluronidase infusion for 4 weeks and monitored albumin passage using immunolabeling and correlative light-electron microscopy that allows for complete and integral assessment of glomerular albumin passage. ESL ultrastructure was visualized by transmission electron microscopy using cupromeronic blue and by localization of ESL binding lectins using confocal microscopy. We demonstrate that glomerular fenestrae are filled with dense negatively charged polysaccharide structures that are largely removed in the presence of circulating hyaluronidase, leaving the polysaccharide surfaces of other glomerular cells intact. Both retention of cationic ferritin in the glomerular basement membrane and systemic blood pressure were unaltered. Enzyme treatment, however, induced albumin passage across the endothelium in 90% of glomeruli, whereas this could not be observed in controls. Yet, there was no net albuminuria due to binding and uptake of filtered albumin by the podocytes and parietal epithelium. ESL structure and function completely recovered within 4 weeks on cessation of hyaluronidase infusion. Thus, the polyanionic ESL component, hyaluronan, is a key component of the glomerular endothelial protein permeability barrier. (Am J Pathol 2013, 182: 1532 e1540; http://dx.doi.org/10.1016/j.ajpath.2013.01.049)
antithrombin-III, and bind extracellular superoxide dismutases, whereas after activation of the endothelial cell, they function as receptors for platelet and leukocyte adhesion.8–12

The pivotal role of the ESL is illustrated by the fact that, to our knowledge, no successful genetic mice models could be generated in which structural polysaccharide components of the ESL are lacking. These properties have put the ESL in the center of interest as a modulator of endothelial cell function.

The ESL has also been postulated to act as a barrier against albumin filtration. The combination of negatively charged heparan sulfates and the mesh-like structure of the less charged hyaluronan may theoretically prevent albumin filtration through the fenestrae.13 In fact, the Frank Starling filtration equilibrium in nonrenal microvasculature is generated by the inability of albumin to permeate the ESL and its resulting oncotic gradients over this layer.14 Moreover, acute infusion of high dosages of enzymes that degrade the ESL results in increased fractional albumin clearance, extrapolating this concept also to the kidney.15,16

In the current study, we explore the effects of long-term chronic disruption of the ESL on albumin filtration over the glomerular endothelium and the possible effects on glomerular morphological characteristics. To this end, we use a new electron microscopy technique that allows visualization of the ESL and albumin transport within the entire glomerular section at nanometer resolution.17 To study the functional role of the ESL with respect to prevention of albumin filtration, it was perturbed by long-term low-dose infusion of hyaluronidase, a hyaluronan degrading enzyme, which has been shown to increase the permeation of the ESL (eg, in the cremaster muscle).13

Materials and Methods

All materials are from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Mice and Experimental Groups

Experiments were performed using C57Bl/6 mice (B6, Charles River, France) at 14 weeks of age. Mice were randomly divided into two groups and implanted with an osmotic minipump (Alzet, Cupertino, CA) containing hyaluronidase (group 1, total n = 26) or inactivated hyaluronidase (group 2, total n = 22).

Anesthesia during surgical procedures was performed using an i.p. injection of 0.2 mL of a 1 mg/mL cocktail of midazolam (Roche Nederland BV, Woerden, The Netherlands), 50 µg/mL dexmedetomidine (Orion Corporation, Espoo, Finland), and 10 µg/mL fentanyl (Hameln Pharmaceuticals GmbH, Hameln, Germany) in H2O. After 4 weeks, six mice from each group were sacrificed, the pumps of six additional mice per group were removed, and these animals were allowed to recover for an additional 4 weeks. In a subset of control B6 mice (n = 2) and B6 mice with an osmotic minipump containing hyaluronidase for 4 weeks (n = 3), the glomerular ESL was stained using cupromeronic blue. In addition, 14-week-old B6 mice received an osmotic minipump containing various concentrations of hyaluronidase for 2 weeks to determine the optimal concentration (n = 15) or an osmotic minipump containing either hyaluronidase (n = 8) or inactivated hyaluronidase (n = 5) for 2 weeks for blood pressure measurements. All animals had free access to food and tap water.

The experimental protocol was approved by the local Animal Ethical Committee of Maastricht University (Maastricht, The Netherlands; hyaluronidase concentration determination and blood pressure measurements) and Leiden University (Leiden, The Netherlands; others). All animal work was performed in compliance with the Dutch government guidelines.

Hyaluronidase Solution

Bovine testicular hyaluronidase (in saline; 100 U/µL) was injected into an osmotic minipump, which releases its content at a rate of 0.25 µL/h (25 U/hour), into the right jugular vein for 4 weeks. The effect of protease contamination of the hyaluronidase solution is negligible after infusion into the circulation (data not shown). In a subset of animals, concentrations of 40 U/µL (10 U/hour), 100 U/µL (25 U/hour), and 400 U/µL (100 U/hour) in saline were used and released into the right jugular vein for 2 weeks. In parallel, heat-inactivated hyaluronidase (boiled for 30 minutes at 90°C, resuspended by vortex) was used as a control.18

Urine Collection and Analysis

Mice were kept in metabolic cages for 24 hours 2 days before pump implantation and 2 days before the end of the experiment (week 0 and week 4 or 8). Mice from the recovery groups were kept, in addition, in metabolic cages 2 days before pump removal (week 4) and 2 weeks before perfusion fixation (week 6). Urine was centrifuged and subsequently stored at −20°C. Rocket immunoelectrophoresis was used to quantify albumin levels in urine (protocol modified from Tran et al19). Urine creatinine levels were determined by a kinetic colorimetric assay using a commercially available kit (Creatinin Jaffé method; Roche Diagnostics, Mannheim, Germany) and a Cobas Integra 800 analyzer (Roche Diagnostics, Mannheim, Germany). Protein levels were determined by the TPU C3 protocol using a Cobas C analyzer (Roche Diagnostics).

Tissue Preparation

A subset of mice received 200 µL of ferritin (equine spleen, 25 mg/mL) via the tail vein, 15 minutes before the induction of anesthesia (previously described). In anesthetized mice, the abdominal aorta was exposed and cannulated distal to the renal arteries. The right renal artery was ligated at the renal stalk and immediately fixed by injecting 2% paraformaldehyde (PFA) in PBS before removal of the kidney. The kidney capsule of the unperfused excised right kidney
was removed, and the kidney was cut in half. One half was fixed overnight at 4°C in 2% PFA in PBS for detection of endogenous albumin and lectins, and the other half was placed in 1.5% glutaraldehyde (GA) and 1% PFA in PBS overnight at 4°C for detection of ferritin (described later). From six additional mice, three were treated with active and three with inactive hyaluronidase for 4 weeks. Both kidneys, unpurified, were isolated directly and snap frozen in liquid nitrogen for immunohistochemistry.

**Detection of the Endothelial Surface Layer**

For electron microscopic illustration of ESL in the glomerulus, the left kidney was perfused with 0.1% bovine serum albumin (BSA) and 5 U/mL heparin in HBSS at 2 mL/minute to remove blood, followed by 0.05% cupromeronic blue in sodium acetate buffer (25 mmol/L, pH 5.8) containing 1.5% GA, 1.0% PFA, and 25 mmol/L MgCl₂ for 5 minutes at 2 mL/minute. After excision of the left kidney, the capsule was removed and the kidney was sliced into small tissue samples (approximately 2 mm³) and incubated overnight at 4°C in the fixation solution containing cupromeronic blue, as previously described. These cupromeronic blue—stained samples were washed twice in 25 mmol/L sodium acetate buffer (pH 5.8) and incubated for 1 hour in 0.5% phosphotungstic acid in 50% ethanol. Samples were further dehydrated and embedded in epon. Transmission electron microscopy (TEM) data were collected on a Tecnai 12 (BioTWIN; FEI, Eindhoven, The Netherlands) from sections (100 nm thick), as previously described, using the same settings.

**Correlative Microscopy for Detection of Endogenous Albumin**

Kidney tissue was dissected into sections (100 μm thick) with a vibratome (Leica Microsystems BV, Rijswijk, The Netherlands). Parts of the slices are blocked with 10% normal goat serum and 0.3% Triton X-100 in PBS for 30 minutes on ice, followed by incubation at 4°C with horseradish peroxidase—conjugated goat anti-mouse albumin (diluted to 1:400; Bethyl Labs, Montgomery, TX) in 1% heat-inactivated normal goat serum in PBS. After washing, approximately 1 mL of 3,3'-diaminobenzidine reaction solution [15 mL of 3,3'-diaminobenzidine hydrochloride (DAB) solution + 10 to 15 μL H₂O₂, 30% (Dako, Glostrup, Denmark)] was added, incubated for 30 minutes at 4°C, washed again, and incubated for 1 hour with 1.5% GA and 1% PFA in cacodylate, rinsed in cacodylate, and post-fixed in 1% osmium tetroxide + 1.5% potassium ferrocyanide. Samples were dehydrated and processed further into epon. Sequential sections (100 nm thick) were mounted on a copper slot grid covered with Formvar support film and a 3-nm carbon coating for TEM, and on a water drop on a clean glass slide for reflection contrast microscopy (RCM). The sample was mounted with immersion oil (Immorsol 518F; Carl Zeiss, Göttingen, Germany) on an RCM-adapted microscope (reflection contrast device RV; Leica, Wetzlar, Germany). Images were recorded with a 100×, 1.25 numerical aperture objective. TEM sections were stained with an aqueous solution of lead citrate and uranyl acetate before visualization, and data were collected at an acceleration voltage of 120 kV, on a Tecnai 12 (BioTWIN) transmission electron microscope, equipped with an Eagle CCD camera (FEI Company, Eindhoven, The Netherlands).

**Ultrastructural Large-Scale Virtual Slides**

To place the presented high-power magnification images in their proper context, additional online ultrastructural large-scale virtual slides are provided (Cell Centered Database, http://ccdb.ucsd.edu/index.shtml, accession number P2133), as described previously by Faas et al. The virtual slides for the samples were recorded using automated data acquisition and stitching software with >25,740 magnification at the detector plane, corresponding to a 1.2-nm pixel size at the specimen level. This results in a slide with an overview of the glomerulus, in which one can zoom into high detail. By using this correlative microscopy approach, the DAB precipitate can be visualized with both RCM and TEM. Although RCM allows for the screening of many glomeruli from kidneys of different mice, TEM enables localization of the DAB precipitation at high resolution.

**Confocal Microscopy**

Remaining kidney tissue slices (see Correlative Microscopy for Detection of Endogenous Albumin) were washed twice with HBSS (Life Technologies Europe BV, Bleiswijk, The Netherlands) containing 0.5% BSA, 5 mmol/L HEPES, and 0.03 mmol/L EDTA (HBSS-BSA). Slices were stained overnight with 10 μg/mL of various fluorescently labeled lectins [ie, *Lycopersicon esculentum* (LEA) fluorescein isothiocyanate and *Bandeiraea simplicifolia* and *Triticum vulgare* (WGA) tetrarhodamine isothiocyanate] or 5 μg/mL monoclonal mouse anti-mouse CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in HBSS-BSA and, after a brief wash, with 10 μg/mL Alexa Fluor 488— or Alexa 568—conjugated goat anti-mouse IgG (Molecular Probes, Grand Island, NY). Slices in HBSS-BSA were fixed to the bottom of a Petri dish and were examined using a CLSM (710-NLO; Carl Zeiss, Göttingen, Germany) and a 40× objective lens (Plan Neo Fluar NA 1.3/oil differential interference contrast; Carl Zeiss). Confocal 12-bit gray-scale axial images (xy dimensions, 100 × 100 μm) of the glomerulus were recorded using ZEN-2009 Image software (Carl Zeiss). The images were analyzed with the public domain NIH ImageJ version 1.46 (http://rsb.info.nih.gov/ij). For each image, the amount and location of intensity profiles were quantified by calculating the distance from the peak of the CD31 signal to the half-width of the intraluminal or extraluminal lectin signal along a line of interest.

**Detection of Ferritin Particles**

Kidney samples fixed overnight at 4°C in 1.5% GA and 1% PFA in PBS were dissected into sections (100 μm thick)
with a vibratome. Slices were washed with PBS and 0.1 mol/L sodium cacodylate buffer, incubated for 1 hour with 1.5% GA and 1% PFA in sodium cacodylate buffer, rinsed in sodium cacodylate buffer, and post-fixed in 1% osmium tetroxide and 1.5% potassium ferricyanide. Samples were dehydrated and processed further into epon. TEM data were collected at an acceleration voltage of 120 kV on a Tecnai 12 (BioTWIN).

Immunohistochemistry

Frozen kidney sections (2 μm thick) were fixed in 2% PFA/0.3% Triton X-100 in PBS for 10 minutes, washed in PBS, and blocked for 30 minutes at room temperature in 2% fetal calf serum (Bodinco, Alkmaar, The Netherlands), 2% BSA, and 0.2% fish gelatin (Amersham Biosciences, Buckinghamshire, UK) in PBS. Sections were incubated for 45 minutes with 1 to 10 μg/mL primary antibodies against mouse synaptotodin (Progen Biotechnik GmbH, Heidelberg, Germany), nephrin (R&D Systems Europe Ltd, Abingdon, UK), or desmin (Tebu-Bio, Heerhugowaard, The Netherlands). Subsequently, sections were rinsed and incubated for 45 minutes with 5 μg/mL Alexa 488– or 594–conjugated secondary antibodies (Life Technologies Europe BV, Bleiswijk, The Netherlands). Finally, sections were rinsed with PBS, post-fixed with 1% PFA in PBS, and embedded in VectaShield mounting medium H-1000 (Brunschwig Chemie, Amsterdam, The Netherlands). The staining intensities of antibodies were scored in 50 glomeruli on a 0 to 10 scale (0, no staining; 1, 10% staining intensity of podocytes; 10, 100% staining intensity). Differences between groups were determined by U-test using GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA). P < 0.05 was considered statistically significant.

Systemic Tracer Dilution Determination

At 16 weeks of age, distribution volume determinations of labeled erythrocytes and 40-kDa high-molecular-weight dextrans were performed to assess systemic vascular red blood cell (V_{red}), plasma, ESL, and total vascular volume, as previously described.\textsuperscript{21} Short-term hyperglycemia increases ESL permeability and acutely decreases the linear density of capillaries with flowing RBCs.\textsuperscript{22} In brief, RBCs were labeled with carboxyfluorescein diacetate, succinimidyl ester (Life Technologies Europe BV) and mixed with a 10-mg/mL stock solution of 40-kDa Texas Red–labeled dextran. After i.v. injection of 0.1 mL of this tracer mix, blood samples were drawn at 2, 5, 10, 15, 20, and 30 minutes. Capillaries were centrifuged, hematocrit was determined, and plasma was stored at −20°C. Circulating labeled RBC fraction was determined using flow cytometry (FACSDiCalibur; Becton Dickinson, Franklin Lakes, NJ). Circulating vascular plasma was calculated using the following formula: [(1 − Ht) × V_{red}]/Ht.\textsuperscript{23,24} In each plasma sample, fluorescence was measured at 610 nm (40-kDa Texas Red-labeled dextran) using a spectrofluorophotometer (VICTOR; Perkin-Elmer, Groningen, The Netherlands). Concentration-time curves of D40-TR were fitted with a monoexponential function to determine initial tracer distribution volume.

Noninvasive Tail Blood Pressure Measurement

Blood pressure (BP) was measured in conscious animals using the tail-cuff method (CODA-2; Kent Scientific, Torrington, CT). Briefly, each session consisted of five acclimatization cycles, followed by 15 BP measurement cycles. On the data collection day, two sessions of 15 BP measurements were obtained; a set was accepted if the computer identified >50% successful readings. The average from one session was used for systolic BP, diastolic BP, mean BP, and heart rate in each individual mouse.

Statistical Analysis

Results are all presented as means ± SD, and morphometric data are given as means ± SEM. Differences between groups were determined by U-test using GraphPad Prism software version 5.0. P < 0.05 was considered statistically significant.

Results

Hyaluronidase Infusion Degrades the Glomerular Endothelial Surface Layer

Because complete removal of the ESL in vivo can result in massive thrombosis and instantaneous vessel occlusion, we aimed for a partial degradation to study the early effects of ESL loss on glomerular permeability properties. An osmotic minipump filled with bovine testicular hyaluronidase was implanted together with a catheter into the right jugular vein, to chronically degrade hyaluronan from the ESL within the murine blood circulation. Although a concentration of 100 U/hour resulted in massive vascular occlusions and poor prognosis within a week (data not shown), a concentration of 25 U/hour resulted in approximately a 50% reduction in systemic ESL volume after 2 weeks (Supplemental Figure S1A), as measured by the change in distribution volume of 40-kDa dextrans, which can freely access the ESL, minus the distribution volume of labeled erythrocytes, which cannot access the ESL. No behavioral changes, weight loss, or significant changes in BP or heart rate were observed in relation to infusion of either active or inactive hyaluronidase (Supplemental Figure S1B).

The changes in ESL were visualized at 4 weeks of long-term infusion of active hyaluronidase, with inactive hyaluronidase as a control. Renal sections were stained with lectins that bind to β-(1,4)-linked N-acetyl-glucosamine residues (LEA), α-D-galactosyl and N-acetyl-α-D-galactosamine residues (Bandicoria simplicifolia), or N-acetylneuraminic acids and N-acetyl-β-D-glucosamine (WGA) (Figure 1A). These lectins previously were shown to stain specific carbohydrate moieties in the ESL that correspond with intraluminal carbohydrates at
the ultrastructural level.\(^{25}\) To measure ESL thickness, the distance from the peak of the fluorescence signal of the endothelial cell membrane marker, CD31, to the half maximum intensity lectin signal was determined (Figure 1B). Compared with long-term infusion of inactivated hyaluronidase, both LEA (\(-0.14 \pm 0.06 \text{ versus } 0.73 \pm 0.14 \text{ µm}; P < 0.001\)) and BSI (\(0.34 \pm 0.11 \text{ versus } 0.90 \pm 0.08 \text{ µm}; P < 0.001\)) were significantly reduced after 4 weeks of long-term hyaluronidase infusion. After removal of the osmotic minipump and closure of the catheter, recovery of the glomerular ESL was monitored, and both lectins (LEA and BSI) returned to control levels (Figure 1C). In contrast, WGA predominantly bound to structures outside the endothelial perimeter when compared with CD31, which appeared podocyte specific (Figure 1, A–C). This extracapillary carbohydrate staining was unaffected by hyaluronidase infusion (\(0.66 \pm 0.13 \text{ versus } 0.40 \pm 0.31 \text{ µm}; P = 0.38\)) for 4 weeks and remained unchanged after 4 weeks of recovery (Figure 1C).

Ultrastructural Characterization of the Glomerular Endothelial Surface Layer

To identify changes in glomerular endothelial surface carbohydrate structures at the ultrastructure level, kidneys were perfused for a short period with the cationic dye, cupromeronic blue, and further processed for TEM. In this way, polysaccharides are fixed in a charge-dependent way. Throughout the glomerulus, cupromeronic blue staining was observed at various layers within the permeability barrier (Figure 2A). As may be appreciated from the high-resolution TEM images, compositionally different layers on the endothelial surface and the filled fenestrae could be observed (Figure 2, B and C). The

![Figure 1](https://example.com/fig1.jpg)  
**Figure 1**  Fluorescence-based lectin analysis in murine glomerulus. **A:** Cross-sectional average intensity projection of a murine glomerulus, stained with fluorescein-labeled lectins (LEA fluorescein isothiocyanate or WGA tetrarhodamine isothiocyanate) and merged projection. **B:** Example of fluorescence intensity plots explaining localization and quantification of lectin staining according to the endothelial cell position (marked with anti-CD31 antibody). Intraluminal localization is calculated by the distance of the CD31 peak (endothelial cell) to the half-width maximum intensity of the LEA peak (outer border ESL) (left and middle panels). WGA staining is present outside of the endothelial perimeter (right panel). **C:** Distribution of lectin intraluminal [LEA and Bandeiraea simplicifolia (BSI)] or extraluminal (WGA) surface coverage after 4 weeks of active (hyal) or inactive (ctrl) hyaluronidase treatment and after 4 weeks’ recovery on osmotic minipump removal. Values are means ± SEM (20 to 40 independent measurements per glomerulus of four kidneys). The difference in lectin coverage between the intraluminal and extraluminal compartment was assessed by nonparametric U-test. \(*P < 0.001\) versus control.
ESL staining showed dense plugs in the endothelial fenestrae and a dense layer covering the endothelial surface, which was coated by a more loosely attached layer (Figure 2, B and C). In the same capillary loop, the nonfenestrated endothelial part displayed a denser layer of stained material (Figure 2B), which resembled previous observations of ESL in other nonfenestrated vascular beds.26,27 The most striking difference between hyaluronidase-treated kidneys and their controls was the local absence of the dense plugs from the endothelial fenestrae (Figure 2C). Cupromeronic blue stained the remaining structures covering the fenestrated endothelial surface, whereas the densely stained structures covering the podocytes and their foot processes were not affected by long-term hyaluronidase infusion. Therefore, in the mild dosage used, hyaluronidase infusion appeared to have predominantly removed the electron-dense structures from the glomerular fenestrae.

Figure 2  Transmission electron microscopic images of a cupromeronic blue-stained glomerulus. A: Overview. B: Capillary section with staining on top of fenestrated (right panel) and nonfenestrated (left panel) endothelium. Electron microscopic detail of glomerular filtration barrier (insets). C: Detail of glomerular filtration barrier consisting of podocytic foot processes (FPs), GBM, endothelium (EC), and ESL covering the cell membrane and filling up the fenestrae (circled area, left panel). Long-term hyaluronidase treatment for 4 weeks removed ESL from within the fenestrae (circled area, right panel). Scale bars: 1 μm (A); 200 nm (B); 50 nm (insets, B); 100 nm (C).

Hyaluronidase Infusion Does Not Change Glomerular Basement Membrane Permeability

To exclude an effect of hyaluronidase on the glomerular basement membrane (GBM) barrier characteristics, retention of cationic ferritin by anionic (sulfated) binding sites in the GBM was measured. Total ferritin (2.3 ± 0.9 versus 2.9 ± 0.9 ferritin particles/10,000 nm² in the GBM) and subepithelial ferritin (4.5 ± 0.9 versus 5.3 ± 1.2 subepithelial ferritin particles/μm of GBM length) both remained unchanged, indicating that the hyaluronidase did not alter the composition and permeability characteristics of the GBM (Supplemental Figure S2).

Perturbation of the Glomerular Endothelial Surface Layer Results in Albumin Filtration

To determine the effect of the ESL perturbation by hyaluronidase on the filtration barrier for albumin, kidney slices were stained for endogenous albumin. The slices were stained with anti-albumin conjugated to horseradish peroxidase (HRP) and incubated with DAB to be able to visualize albumin with RCM and TEM simultaneously. Because the kidneys were fixed with paraformaldehyde alone, only bound albumin could be visualized. All unbound luminal proteins, including albumin, were washed away during staining and processing for TEM. Sections stained for bound endogenous albumin were first visualized using RCM (Supplemental Figure S3). Reflection of DAB was scanned, and glomeruli were counted positive when DAB reflection was visible at the glomerular filtration barrier. A striking 90% of the glomeruli were positive for albumin after hyaluronidase treatment (31 glomeruli per five kidneys), as observed by disperse reddish reflections outside the capillary loops (Supplemental Figure S3 and http://ccdb.ucsd.edu/index.shtm, accession numbers 9524 (control) and 9526 (hyaluronidase)). In contrast, no albumin staining was detected in the controls (22 glomeruli per three kidneys) and after the recovery period (15 glomeruli per three kidneys).

By using a newly developed technique to correlate the reflection found by RCM in high detail (at a 1.2 nm/pixel resolution), some matching glomeruli in consecutive virtual slides were imaged.17 The virtual slide allows digital analysis at nanometer resolution of a whole glomerular cross section in an unbiased approach (in this case, for DAB-stained albumin). The RCM images and the corresponding TEM virtual slides were correlated to determine the exact location of the bound endogenous albumin. In the hyaluronidase-treated group, albumin was mainly detected near the podocyte foot processes, especially in the subpodocytic spaces (Figure 3A and http://ccdb.ucsd.edu/index.shtm, accession number 9525). In contrast to the hyaluronidase-treated group, no albumin could be found past the endothelial layer and the glomerular basement membrane in control glomeruli (Figure 3A and http://ccdb.ucsd.edu/index.shtm, accession number 9523). Furthermore, after 4 weeks of recovery, the albumin that was found after the hyaluronidase treatment was no longer present (Figure 3A).
To be able to determine whether the perturbed filtration barrier shown with TEM also resulted in proteinuria and albuminuria, a 24-hour urine sample was collected. Samples were taken and measured before implanting the osmotic minipump (0 weeks), after 4 weeks of hyaluronidase treatment (4 weeks), and during the 4 weeks of recovery (6 and 8 weeks). Although albumin clearly was shown to be able to pass the filtration barrier in the TEM images after hyaluronidase, no differences could be found in albumin/creatinine (Figure 3B) or protein/creatinine (Figure 3C) levels. Because albumin also binds to podocytes and parietal epithelial cells in the hyaluronidase-treated group, this may suggest that uptake and degradation of albumin have occurred as well (Figure 4). To investigate whether albumin binding would also induce podocyte phenotype changes in response to injury, nephrin, synaptopodin, and desmin protein expression was studied (Supplemental Figure S4). Although there was a tendency toward a proliferating and migrating phenotype, such as can be observed during podocyte effacement (low nephrin, low synaptopodin, and high desmin) during hyaluronidase treatment, the model used was probably too mild to demonstrate this phenomenon clearly.

**Discussion**

Our data show that glomerular fenestrae are filled with dense negatively charged polysaccharide structures. On infusion of hyaluronidase, in a dosage that leaves the polysaccharide surfaces of other glomerular cells intact, but predominantly removes the polysaccharides in the endothelial fenestrae, albumin passage across the endothelium can be observed in almost all of the glomeruli. Such albumin passage was not observed for the control animals. Treatment with low-dose hyaluronidase is relatively selective in removing the ESL in the fenestrae, whereas the looser glomerular surface layer appears structurally unaltered. Rostgaard and Qvortrup have previously suggested the presence of sulfated carbohydrates in the glomerular fenestrae. Our current data indicate that the glomerular fenestrae are relatively enriched with easy, accessible hyaluronan. The fact that hyaluronan is less charged than the heparan sulfates in ESL may explain the ability to produce a dense mesh in the fenestrae.

Although hyaluronidase treatment theoretically could also affect the polysaccharide coating on other glomerular cells, the large-scale ultrastructural mapping, and the lectin staining, demonstrated no structural changes between the different treatment groups. Long-term removal of the ESL also did not affect the glomerular ultrastructure and basement membrane function, whereas the changes in ESL and albumin filtration were completely reversible on cessation of the hyaluronidase infusion. These observations lend further support to the notion that the glomerular ESL is dynamically regulated and that its changes reflect changes in endothelial function.
The trans-endothelial passage of albumin was not accompanied by albuminuria or proteinuria. This is in contrast to our earlier experiments in apolipoprotein E–deficient mice on a high-fat, high-cholesterol diet, which were infused with hyaluronidase. A possible explanation for this discrepancy is that the apolipoprotein E–deficient mice reflect a double hit, where loss of ESL was superimposed on pre-existing vascular and renal injury. This is supported by the fact that proteinuria in that model could only be provoked by co-administration of the high-fat, high-cholesterol diet. A recent study using multiphoton fluorescence imaging in spontaneous Munich-Wistar-Fromter rats also could relate widespread ESL loss to the occurrence of albuminuria. In the current study, we sought to determine the role of the ESL in physiological glomerular filtration and aimed at a selective reversible ESL disruption. At the tubular epithelium level, this bound albumin stick to these cells. However, both our cupromeronic blue and lectin analysis of the epithelial surface layer did not reveal any morphological changes after hyaluronidase treatment. At the tubular epithelium level, this bound albumin was not found. Tubular uptake can, however, not be excluded because, in contrast to the glomeruli, we could not survey the whole tubular compartment.

A limitation of our study was the fact that we did not selectively disrupt the glomerular ESL but affected the surface layer in other vascular endothelial beds. With respect to albumin filtration, the enzyme treatment did not result in changes in BP. At first sight, endothelial-specific inducible knockdown of the hyaluronidase-synthesizing enzyme perhaps might be a more specific approach to test the functional role of the glomerular ESL. However, our experimental work in progress so far would indicate that, in vivo, only minor modifications of the ESL are tolerated. Complete removal of ESL immediately results in severe thrombotic microangiopathy, as can also be observed with our experiments using high doses of hyaluronidase. In addition, enzymatic degradation of the podocytic surface layer has been shown to instantly result in podocyte detachment, together with massive amounts of ferritin in the urinary space. In this study, these morphological differences are not present yet. However, we do already see a perturbation of the glomerular filtration barrier. This suggests that the degradation of the endothelial surface layer presented in this study most likely reflects an early time point in the development of glomerular damage and albuminuria. In conclusion, the glomerular endothelial surface layer functions as a selective protein permeability barrier; a reduction of the key component, hyaluronan, within this layer facilitates albumin passage across the endothelial layer and the GBM toward the epithelial compartment.
Acknowledgments

We thank Daniel Potter and Hanneke Cobelens (Maastricht University Medical Center, Maastricht) and Frans Prins (Leiden University Medical Center, Leiden) for biotechnical support on the blood pressure measurements and all of the support for the use of the electron and reflection contrast microscopy, respectively.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.01.049.

References