Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier

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Jeansson, Marie, and Börje Haraldsson. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. Am J Physiol Renal Physiol 290: F111–F116, 2006. First published August 9, 2005; doi:10.1152/ajprenal.00173.2005.—In this study, we pursued the somewhat controversial issue whether the glycosaminoglycans (GAG) in the endothelial cell glycocalyx are important for glomerular size and charge selectivity. In isoflurane-anesthetized mice, Intralipid droplets were used as indirect markers of the glomerular endothelial cell-surface layer, i.e., the glycocalyx. The mice were given intravenous injections of GAG-degrading enzymes, which due to their high molecular weight remained and acted intra-vascularly. Flow-arrested kidneys were fixed and prepared for electron microscopy, and the distance between glomerular endothelial cells and the luminal Intralipid droplets was measured. The relative frequency of Intralipid droplets was calculated for each 50-nm increment zone up to 500 nm from the endothelial cell membrane surface as were the mean distances. Glomerular size and charge selectivity were estimated from the clearance data for neutral Ficolls (molecular radii of 12–72 Å), and albumin in isolated kidneys was perfused at 8°C. In enzyme-treated animals (hyaluronidase, heparinase, and chondroitinase; Intralipid droplets and the endothelium was decreased from 176 to 115–122 nm by enzyme treatment. These changes were accompanied by an increase in the fractional clearance for albumin. In conclusion, both morphological and functional measurements suggest the endothelial cell glycocalyx to be an important component of the glomerular barrier.

charge selectivity; hyaluronidase; heparinase; chondroitinase; Intralipid

The ability of the normal glomerular capillary wall to filter large volumes of plasma while retaining macromolecules such as albumin in the circulation is vital for renal function and a prerequisite for whole body homeostasis. Several studies, both in vivo and in vitro, have shown that the glomerular barrier restricts the passage of anionic macromolecules relative to uncharged molecules of similar size and configuration (11, 18, 23, 24, 27, 28, 33, 34). Other investigators argue that the barrier is uncharged (25). Anionic charges have been demonstrated, however, in all of the filtration barrier structures, i.e., the endothelial cell glycocalyx (1, 27), the glomerular basement membrane (GBM) (5, 20, 21, 27), and the podocyte glycocalyx (27). In earlier studies, where anionic probes were used, it was indicated that the GBM was the main glomerular barrier (27).

Functional studies on filtration across isolated GBM revealed, however, less charge selectivity than that proposed in vivo (3, 4, 8). This suggests that charge selectivity resides mainly at the endothelial or epithelial cells or that charged structures are lost during GBM isolation. We proposed a role for the endothelial cell glycocalyx in charge selectivity (2, 7, 14, 16, 18, 23, 34), an idea supported by others (1, 9). Briefly, endothelial cells are covered with a surface layer of membrane-associated proteoglycans, glycosaminoglycans (GAG), glycoproteins, glycolipids, and associated plasma proteins. This is known as the glycocalyx or the endothelial surface layer (26). The composition and the physical properties of the glycocalyx, including the effective thickness, seem to be influenced by the adsorption of plasma proteins (26). In other organs, the endothelial glycocalyx has a surprisingly large in vivo thickness as well as certain permselective properties as shown by Duling and co-workers (15, 35, 36) by combining intravital brightfield and fluorescence microscopy. These authors estimated the glycocalyx in cremaster muscle capillaries to be 400–500 nm thick by subtracting the width of the fluorescent tracer column from the anatomic diameter (36). Hence, the glycocalyx appears to be much thicker than estimated from electron microscopy, 50–100 nm (26), which likely underestimates the thickness due to the dehydration following tissue fixation. Problems in visualizing the glycocalyx are probably the reason why the glycocalyx has been overlooked when considering microvascular permeability and exchange. However, recent studies where vascular beds were perfused with a fluorocarbon-based oxygen-carrying fixative, followed by contrast enhancement preparation steps, showed a delicate ~60- to 300-nm-thick glycocalyx on the glomerular endothelium and its fenestrae (16, 31, 32).

Studies of the negatively charged components in the glomerular endothelial cell glycocalyx are incomplete, but there is indirect evidence for involvement of hyaluronic acid (1, 18), heparan sulfate (1), and sialoproteins (1). In addition, our group recently showed that human glomerular endothelial cells produce several different proteoglycans (2). Also, hyaluronic acid and/or chondroitin sulfate in the glomerular barrier are important for maintaining charge-selective properties (18). In this context, the glycocalyx comprises a stagnant endothelial cell-surface layer of loosely attached components.

This paper extends the findings of our recent paper that showed that GAGs are important for glomerular charge selectivity based on functional studies. In the present paper, the aim was to couple the functional alteration, i.e., decreased glomerular permselectivity, to a morphological change in the endo-

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theilial cell glycocalyx. This was done by measuring the effects of the high-molecular-weight enzymes heparinase, hyaluronidase, and chondroitinase on the endothelial cell glycocalyx. In addition, we provide new functional data on effects on the glomerular barrier by chondroitinase treatment.

MATERIALS AND METHODS

Experiments were performed in female C57BL/6 mice (B&K Universal Limited Grimston, Aldbrough, UK) on standard chow and with free access to water before the experiments. The experiments were approved by the Local Ethics Review Committee in Göteborg.

**Experimental setup.** Enzyme dissolved in saline or saline only (controls) was given as a bolus dose via the jugular vein and allowed to incubate in the animal according to Table 1. Digestion by all three enzymes, bovine testis hyaluronidase (Hya, H3506, Sigma, Stockholm, Sweden), *Flavobacterium heparinum* heparinase III (Hep, H8901, Sigma), and chondroitinase ABC from *Proteus vulgaris* (Chond, C2905, Sigma), was used in the morphological studies with Intralipid droplets. In addition, glomerular barrier function was studied in controls and chondroitinase-treated animals using the isolated, perfused kidney (cIPK) at 8°C. We previously published functional data from treatment with hyaluronidase and heparinase (18).

**General animal preparation.** Anesthesia was induced and maintained by inhalation of isoflurane (2-3% vol/vol, Isoflurane, Pharmacia & Upjohn, Stockholm, Sweden) mixed with air (~1 l/min) in an isoflurane vaporizer (Ohmeda Isotec 5, Simtec Engineering, Askim, Sweden). The body temperature of the mouse was kept at 37°C during the preparations by means of a thermostatically controlled heating pad.

**Intralipid experiments.** Intralipid (Pharmacia & Upjohn Sverige, Stockholm, Sweden) was prepared by discarding the top lipid layer of the solution after a night in a refrigerator. An enriched fraction of lipid droplets was obtained after centrifugation at 3,000 g for 10 min, and 100 μl were administered into the caval vein after the general animal preparation described above. After Intralipid was allowed to mix in the circulation for 10 min, the left renal artery and vein were clamped and the kidney was fixed by subcapsular injection of Karnovsky’s fixative (2.5% paraformaldehyde and 2% glutaraldehyde in 0.05 M Na-cacodylate buffer, pH 7.2). The kidneys were cut into 1-mm slices and placed in fixative before further processing. Tissue slices were postfixed in 1% OsO₄ and 1% K₄Fe(CN)₆ for 2 h at 4°C. After dehydration in ethanol, the specimens were embedded in epoxy resin (Agar 100) and heat-cured. Ultrathin tissue sections, 50–60 nm, were obtained with an ultramicrotome (Ultracut E, Reichert, Austria) fitted with a diamond knife. Sections were contrasted with lead citrate and uranyl acetate and examined in a Leo 912AB Omega electron microscope (Leo Electron Microscopy, Cambridge, UK).

**Intralipid morphometry.** Micrographs at a magnification of 8,000 were obtained from four to eight animals in each group giving a total of 624 glomerular capillaries for the measurements. For each micrograph, the distance between the Intralipid droplet and the endothelial cell membrane surface was measured in the zone 0–500 nm from the endothelial cell using EsiVision Pro (Soft Imagine System, Münster, Germany) computer software. In total, the distance for ~3,200 droplets was measured. Two different approaches were used to evaluate the morphological data. One was to calculate the relative frequency of droplets for each of a series of 50-nm increment zones. The other approach was to calculate the harmonic mean of the distances for each group to correct for possible differences in the section angle.

**Cooled isolated, perfused mouse kidneys.** After the general preparation described above, a cannula (PE-25) was put in the bladder for collection of urine. The aorta and caval vein were freed from surrounding tissue and clamped distal to the renal arteries. The aorta was cannulated in retrograde direction with a T-tube (PE-25), connected to a pressure transducer, a few millimeters distal to the clamp. The clamp was removed, allowing perfusion of the kidneys by means of a pulsatile pump (Ismatec, Zurich, Switzerland). The aorta was then ligated proximal to the renal arteries, and the caval vein was opened distal to the renal arteries for venous outflow. After a short period of equilibration, urine samples were collected and weighed. Perfusion pressure and urine weight changes were monitored by a computer using AcqKnowledge v 3.7.3 (Biopac Systems, Goleta, CA) computer software. Care was taken not to touch the kidneys and to provide adequate perfusion with either blood or perfusate during the preparation procedure. The temperature of the perfusate was kept at 8°C to inhibit tubular function as well as energy consumption and myogenic tone (10) without altering capillary permeability (24, 30).

**Perfusate.** Perfusate was prepared using a modified Tyrode solution with human serum albumin (HSA; 18 g/l; Immuno, Vienna, Austria), 51-Cr-EDTA, and FITC-labeled Ficoll in the size range 12–70 Å. The solution had the following composition: 113 mM NaCl, 4.3 mM KCl, 2.5 mM CaCl₂, 0.8 mM MgCl₂, 25.5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 5.6 mM glucose, 0.9 mM nitroprusside (Merck, Darmstadt, Germany), 10 mg/l furosemide, 200 mg/l FITC-labeled Ficoll (Bioflor, Uppsala, Sweden), and 0.16 MBq/l 51-Cr-EDTA (Amersham Pharmacia Biotech, Buckinghamshire, UK). All solutions were made with fresh distilled water (Millipore) with a resistivity of 18.2 MΩ cm. The perfusate (pH 7.4) was protected from light and gassed with 5% CO₂ in O₂.

**Albumin and 51-Cr-EDTA analysis.** Perfusate and urine samples from the cIPK were analyzed for 51-Cr-EDTA in a gamma-counter (Cobra, AutoGamma Counting systems, Packard Instrument, Meridan, CT), and albumin was analyzed using an immunoturbidimetric assay (Randox Laboratories, Antrim, UK). Glomerular filtration rate (GFR) was calculated from the urine over plasma concentration ratios (CU/CP) for 51-Cr-EDTA times urine flow (Qₜ). Fractional clearance of a solute is given by its clearance over GFR, where the solute renal clearance is calculated from the amount excreted in the urine over the perfusate concentration during a certain period of time.

**Analysis of Ficoll.** The fractional clearance for FITC-Ficoll of different radii was calculated by subjecting perfusate and urine samples to gel filtration (TSK-gel G4000PWXL, Tosoh Bioscience, Stuttgart, Germany) and fluorescence detection (Dionex fluorimeter RF-2000) for 51-Cr-EDTA times urine flow (Qₜ). Gel-membrane model. According to the gel-membrane model, the glomerular barrier is simplified to one charge-selective barrier (gel) and one size-selective barrier (membrane) in series. The gel is in contact with plasma and contains fixed negative charges, reducing the concentration of anionic solutes, such as albumin. The concentrations of a solute in the urine will depend on the effects of these two barriers as outlined below. Size-selective properties can be described by using

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dose, U/kg</th>
<th>Time, min</th>
<th>Molecular Weight, kDa</th>
<th>Type of Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>15</td>
<td>15</td>
<td>EM</td>
<td>cIPK</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>15,000</td>
<td>60</td>
<td>56</td>
<td>EM</td>
</tr>
<tr>
<td>Heparinase</td>
<td>82</td>
<td>15</td>
<td>71</td>
<td>EM</td>
</tr>
<tr>
<td>Chondroitinase</td>
<td>37–435</td>
<td>15</td>
<td>120</td>
<td>EM, cIPK</td>
</tr>
</tbody>
</table>

EM, electron microscopy; cIPK, cooled isolated, perfused kidneys.
Morphological experiments. The evaluation of micrographs revealed that digestion with either enzyme, i.e., hyaluronidase, heparinase, or chondroitinase, resulted in a significant increase ($P < 0.01$) in relative frequency of Intralipid droplets compared with controls, in the zone closest to the endothelial cells, 0–50 nm. Figure 1 shows the increase in relative frequency compared with controls for each 50-nm zone up to 500 nm. All relative frequencies of Intralipid droplets calculated can be compared with controls for each 50-nm zone up to 500 nm. All relative frequencies of Intralipid droplets calculated can be seen in Table 2. In addition, the harmonic mean of the distances between Intralipid droplets and the endothelium was calculated for each group (Fig. 2). The mean distance was found to be significantly decreased ($P < 0.01$, $P < 0.05$) from 176 (±10) nm in controls to 115 (±7), 115 (±8), and 122 (±10, −9) nm in hyaluronidase-, chondroitinase-, and heparinase-treated animals, respectively. Micrographs from the different groups are shown in Fig. 3. No ultrastructural changes were considered statistically significant.

**Table 2.** Relative frequency of Intralipid droplets

<table>
<thead>
<tr>
<th>Distance, nm</th>
<th>Control $(n = 8)$</th>
<th>Hya $(n = 4)$</th>
<th>Chond $(n = 6)$</th>
<th>Hep $(n = 5)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–50</td>
<td>0.07±0.02</td>
<td>0.18±0.02*</td>
<td>0.18±0.02*</td>
<td>0.16±0.01*</td>
</tr>
<tr>
<td>51–100</td>
<td>0.13±0.02</td>
<td>0.16±0.01</td>
<td>0.14±0.01</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>101–150</td>
<td>0.17±0.01</td>
<td>0.14±0.02</td>
<td>0.14±0.01</td>
<td>0.14±0.03</td>
</tr>
<tr>
<td>151–200</td>
<td>0.13±0.01</td>
<td>0.11±0.01</td>
<td>0.11±0.01</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>201–250</td>
<td>0.10±0.01</td>
<td>0.09±0.02</td>
<td>0.10±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>251–300</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>301–350</td>
<td>0.09±0.02</td>
<td>0.08±0.01</td>
<td>0.08±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>351–400</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.08±0.01</td>
<td>0.07±0.07</td>
</tr>
<tr>
<td>401–450</td>
<td>0.06±0.01</td>
<td>0.05±0.00</td>
<td>0.05±0.01</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>451–500</td>
<td>0.08±0.01</td>
<td>0.05±0.01</td>
<td>0.05±0.01</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>Intralipid droplets</td>
<td>904</td>
<td>530</td>
<td>901</td>
<td>947</td>
</tr>
<tr>
<td>Capillaries</td>
<td>210</td>
<td>91</td>
<td>152</td>
<td>171</td>
</tr>
</tbody>
</table>

Hya, hyaluronidase; Chond, chondroitinase; Hep, heparinase. *$P < 0.01$ compared with controls.
were found in other structures, i.e., endothelial cells, GBM, or podocytes.

Functional data of the glomerular barrier. Only two groups of animals were studied, namely, controls and chondroitinase-treated mice, since we reported functional data for the other enzymes in a recent paper (18). The GFR was similar in both groups, 0.072 (±0.017, −0.014) for controls and 0.045 (±0.005, −0.005) ml·min⁻¹·g wet wt⁻¹ for chondroitinase-treated animals. The fractional clearance for the negatively charged albumin, compared with controls, was found to be significantly (P < 0.001) increased after chondroitinase treatment (Fig. 3). There was no difference in fractional clearance for Ficoll 35.5 Å, the neutral counterpart of albumin. The fractional clearances for Ficolls 12–70 Å are shown in Fig. 4. To further describe the glomerular barrier, mathematical estimations were carried out using the data above and the fractional clearances for all Ficoll sizes (12–70 Å) in a gel-membrane model, including two-pore analysis and calculations of glomerular charge density (Fig. 5). The two-pore analysis showed a decrease in small-pore radius from 47.2 (±0.4) in controls to 45.0 (±0.6) after chondroitinase treatment (P < 0.01). The radius for the less frequent large pore was similar in all groups, 105.4 (±8.9, −8.2) in controls and 134.0 (±15.3, 13.8) after enzyme treatment. There was a significant reduction in charge density to 36.0 meq/l after chondroitinase treatment, compared with 55.1 meq/l in controls (P < 0.001). The large-pore fraction of the glomerular filtrate was similar in both

Fig. 3. Micrographs of glomerular capillaries with Intralipid droplets (*) in controls (A) and animals treated with hyaluronidase (B), heparinase (C), and chondroitinase (D).

Fig. 4. Means ± SE of the fractional clearance (θ) for albumin (open bars) and its neutral counterpart Ficoll 35.5 Å (filled bars) in controls and animals treated with Chond. Please note that they are plotted on different scales. *P < 0.001 compared with control.

Fig. 5. Means ± SE of the fractional clearances for Ficoll 12–70 Å in controls (■) and animals treated with chondroitinase (□).
DISCUSSION

In this study, we present the first evidence that morphological alterations in endothelial cell glyocalyx have functional consequences for glomerular permeability. Our main findings are as follows. First, digestion with either of the high-molecular-weight enzymes (hyaluronidase, heparinase, or chondroitinase) decreased the thickness of the glomerular endothelial cell glyocalyx, as estimated with electron microscopy and Intralipid droplets. Second, digestion of the endothelial cell glyocalyx with chondroitinase increased the fractional clearance for albumin. Third, chondroitinase treatment mainly affected charge selectivity as revealed by the gel-membrane model, as the fractional clearances for neutral Ficolls 12–70 Å were unchanged while albumin clearance was increased.

Digestion of the glyocalyx with either enzyme, i.e., hyaluronidase, heparinase, and chondroitinase, led to an increased relative frequency of Intralipid droplets 0–50 nm from the endothelial cell membrane as well as a decreased mean distance. In the present study, we were interested mainly in comparing controls with enzyme-treated animals rather than measuring the actual thickness of the glyocalyx. Hence, dehydration following tissue fixation is not a problem in this study. The kidneys were flow-arrested, which rules out a flow-dependent distribution of the Intralipid droplets. Also, Bowman’s capsule should protect glomerular capillaries from changed morphology due to an increased intrarenal pressure when subcapsular injections of fixative are used.

Functional aspects of glomerular filtration were studied in the cooled isolated, perfused kidney (cIPK) at 8°C. Cooling the kidneys during the perfusion inhibits tubular function (6, 10) and protease activity, giving a primary urine without tubular modifications. We previously discussed closely the advantages of the cIPK model compared with in vivo measurements of glomerular filtration (17, 18). In the present report, the functional parameters were studied after digestion with chondroitinase. Chondroitinase gave rise to an increased albumin clearance without any change in Ficoll 35.5 Å clearance, indicating reduced charge selectivity. This was further confirmed using the gel-membrane model where the estimated charge density of the glomerular barrier was decreased 35% after enzyme treatment. In a previous paper, hyaluronidase- and heparinase-treated animals exhibited a decreased charge selectivity as well (18). It is most unlikely that the enzymes used affect other parts of the barrier, as they are such large molecules (see Table 1).

Thus the enzyme concentration was highest intravascularly and the concentration gradient was expected to be 2–3 orders of magnitude across the glomerular barrier. Also, we found no ultrastructural changes in any of the groups of endothelial cells, GBM, or podocytes after enzyme treatment.

The endothelium has often been viewed as an insignificant part of the glomerular barrier, perhaps due to its fenestrations. However, we have in several papers (2, 7, 14, 16, 18, 23, 34) discussed the importance of the glomerular endothelial cell glyocalyx for charge selectivity. In fact, it is most likely that all structures in the barrier act in concert for the total selectivity of the glomerular barrier. Thus according to this “integrated view of the glomerular barrier,” proteinuria may result from an increased permeability in either layer (9, 14). For example, podocyte slit diaphragm proteins such as nephrin, podocin, and CD2-associated protein have been shown to be mutated in certain nephrotic syndromes. Hence, there is no doubt that the podocyte plays an important role in the maintenance of an intact glomerular barrier. It is, however, important not to overemphasize the role of the podocyte as there are other components of the glomerular barrier. Indeed, for most patients with nephrotic syndromes, the underlying causes and mechanisms still remain unknown, and the therapeutic arsenal is limited and unspecific.

Placing the charge barrier at the endothelial cell glyocalyx could explain the reversible alteration in charge selectivity when perfusate ionic strength is changed (7, 34). It has also been shown that glomerular permeability is affected by plasma composition (12, 19), supporting the idea of an intimate relationship between charge selectivity and plasma, probably exerted by the endothelial cell glyocalyx. Data in the literature regarding nephrotic syndromes in humans and the involvement of the endothelium and the endothelial glyocalyx are incomplete. However, proteinuria that accompanies preeclampsia most likely originates from the endothelium as the disorder...
seems to selectively affect the endothelium (22). Also, the endothelium has the potential of evoking a 10-fold increase in albumin clearance if we make the conservative assumption that it is as permeable as other capillaries (12).

In summary, we present for the first time both morphological and functional evidence that a decreased glomerular endothelial cell glycocalyx alters the charge-selective properties of the glomerular barrier. This suggests the endothelial cell glycocalyx to be an important component in the glomerular barrier and a possible target for therapy in certain nephrotic syndromes.

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