Glomerular Endothelial Glycocalyx Constitutes a Barrier to Protein Permeability

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ABSTRACT

Glycocalyx, composed of glycoproteins including proteoglycans, coats the luminal surface of the glomerular capillaries. Human heparanase degrades heparan sulphate glycosaminoglycans and is upregulated in proteinuric states. In this study, we analyze the structure of the human glomerular endothelial cell glycocalyx *in vitro* and examine its functional relevance, especially after treatment with human heparanase. Electron microscopy of conditionally immortalized glomerular endothelial cells revealed a 200-nm thick glycocalyx over the plasma membrane, which was also demonstrated by confocal microscopy. Neuraminidase treatment removed the majority of glycocalyx, reduced trans-endothelial electrical resistance by 59%, and increased albumin flux by 207%. Heparinase III and human heparanase specifically cleaved heparan sulphate: this caused no change in trans-endothelial electrical resistance, but increased the albumin passage across the monolayers by 40% and 39%, respectively. Therefore, we have characterized the glomerular endothelial cell glycocalyx and have shown that it contributes to the barrier to flux of albumin across the cell layer. These results suggest an important role for this glycocalyx in the restriction of glomerular protein passage *in vivo* and suggest ways in which human heparanase levels may be linked to proteinuria in clinical disease.

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The glomerular capillary wall, synonymous with the glomerular filtration barrier (GFB), is classically described as a three-layer structure. The two cellular components of this structure are podocytes with their foot processes and slit diaphragms and the fenestrated glomerular endothelial cells (GEnC). Between and also produced by the two cell types is the glomerular basement membrane (GBM). The permselective action of GFB is unique and has long intrigued scientists investigating its underlying basic mechanisms. In the past decade, interesting breakthroughs have been made in unraveling the biology of the podocyte slit diaphragm, leading to the belief that the podocyte is a major determinant of GFB permselectivity[1]; however, the contribution of the other cell involved in the GFB, the GEnC, has received less attention.[2]

Evidence of GEnC damage in conditions such as hemolytic uremic syndrome and preeclampsia, plus strong association of generalized endothelial dysfunction in diabetes and hypertension with the onset of microalbuminuria, suggest that GEnC play an important role in glomerular disease.[3,4] Further emphasizing the role of GEnC in glomerular phys-

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iology, we and others have shown that GEnC–podocyte communication *via* soluble mediators (including vascular endothelial growth factor [VEGF] and angiopoietin-1) is vital to maintaining the functioning of the GFB.[5–7]

The characteristic fenestrated phenotype of GEnC, along with increasing evidence of heterogeneity in endothelial behavior derived from different species and organs, indicates that it is vital to conduct these experiments in human GEnC.[8,9] Historically, GEnC have been difficult to study in culture because of their poor replicative potential leading to early senescence, in addition to nonavailability of representative GEnC lines. We have addressed this problem successfully by generating a conditionally immortalized (Ci) human GEnC line the behavior of which is comparable with primary culture GEnC, including the expression of fenestrations.[10]

The luminal aspect of all endothelia is covered by a meshlike hydrated structure known as glycocalyx, which comprises glycoproteins and, in particular, a special class of heavily glycosylated glycoproteins, proteoglycans (PG). Although glycocalyx is not visible on standard electron microscopy (EM), modification of the EM technique, for example by staining with cationic probes before fixation, overcomes this problem.[11,12] Reports of the thickness of the glycocalyx have varied between 50 and 300 nm, although these may be underestimations as a result of the dehydrating effect of EM fixation and processing. New innovative tools such as quantitative intravital microscopy have provided indirect evidence for a much thicker layer in the systemic microvasculature measuring up to 500 nm and known as endothelial surface layer.[13] In addition to the glycocalyx that is physically attached to the endothelial surface, endothelial surface layer includes a dynamic component made of adsorbed plasma proteins. In nonrenal microvascular beds, it is evident that thick glycocalyx has a significant impact on hemodynamics, oxygen transport, coagulation, inflammation,[14–18] and above all vascular permeability possessing both size and charge exclusion properties.[19]

With very few reports describing the relevance of glomerular endothelial glycocalyx in historical literature,[20] its role in glomerular microcirculation is yet to be elucidated. Until recently, the fenestrae of the GEnC, a requirement to support the high hydraulic conductivity across the GFB, were considered to be empty; however, scanning EM studies using sophisticated techniques to preserve glycocalyx structure have revealed that the GEnC glycocalyx measures up to 300 nm and covers both the fenestral and the interfenestral domains.[21] Such landmark studies confirm, first, that GEnC, like other microvascular endothelia, are covered with a thick coat of glycocalyx and, second, that the GEnC fenestrations are not empty but contain glycocalyx, which may contribute to the barrier to protein passage.

PG are an important constituent of the glycocalyx and have two components: A core protein and covalently bound glycosaminoglycan (GAG) side chains. Heparan sulfate (HS) PG, named after the attached HS GAG side chains, are the most abundant PG in the glycocalyx and are known to be produced by GEnC.[22] The contribution of HS GAG to the GFB has been studied in animal models, in which its enzymatic removal leads to increased permeability to macromolecules[23]; however, because of the presence of HS GAG in both the GBM and glycocalyx,[24] it is difficult to conclude from these *in vivo* studies whether the highly charge-selective restrictive properties of HS GAG are due to its presence in glycocalyx or GBM, most of which is produced by podocytes. Previous studies that have attributed this increase in macromolecular permeability to the loss of HS GAG from the GBM have not considered the possibility of a role for the GEnC glycocalyx.[25]

Human heparanase (HPSE-1) is an endo-β-d-glucuronidase that degrades HS GAG and is associated with extracellular matrix turnover and angiogenesis.[26,27] Overexpression of HPSE-1 in transgenic mice leads to early proteinuria and renal failure.[28] More recently, HPSE-1 expression was shown to be upregulated in a number of animal models of renal disease (passive Heymann nephritis,[29] puromycin aminonucleoside nephrosis,[30] and anti-GBM nephritis[31]) and in glomerular podocytes cultured in high glucose milieu.[32] In addition, increased HPSE-1 activity has been shown in urine from patients with early diabetes[33] (and microalbuminuria) and also in nondiabetic nephrotic syndrome.[34] This evidence suggests the involvement of HPSE-1 in diabetic and nondiabetic proteinuric renal disease.

We have now characterized the GEnC glycocalyx in detail and studied its contribution to the permeability properties of a GEnC monolayer using sophisticated *in vitro* culture systems. We have developed and tested the hypothesis that HPSE-1 can contribute to proteinuria through degradation of the GEnC glycocalyx.

RESULTS

Transmission EM

Transmission EM (TEM) demonstrated the presence of a 200-nm-thick layer coating the surface of the CiGEnC (Figure 1). This layer was enhanced by a cationic dye Alcian blue, hence suggestive of its anionic nature, and was taken to represent the CiGEnC glycocalyx.

Expression of Glycocalyx by Lectin Binding

Wheat germ agglutinin (WGA)-FITC binding demonstrated abundance of sugar residues on CiGEnC representing expression of heavily glycosylated glycoproteins present in glycocalyx compared with secondary only control (Figure 2, A and B). Further imaging of CiGEnC at higher magnification and using multicolor fluorescence labeling for actin and nucleus along with glycocalyx by WGA-FITC confirmed the cell surface distribution of glycocalyx expressed by these cells (Figure 2, C and D).

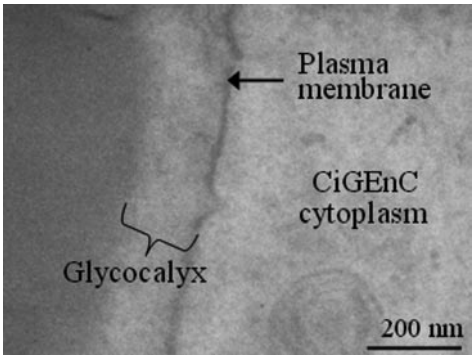

Figure 1. Transmission electron micrograph showing the presence of glycocalyx covering the CiGEnC measuring up to 200 nm. The glycocalyx is enhanced by Alcian blue staining before fixation.

Expression of PG Core Proteins

Western blotting for PG core proteins using lysates from CiGEnC demonstrated protein bands at molecular weights corresponding to syndecan-1, syndecan-4, glypican-1, versican, and perlecan. Comparisons between primary GEnC and nonproliferating CiGEnC (cultured at 37°C) showed similar levels of expression confirming the similarity of these cells to primary culture GEnC (Figure 3, lanes 1 and 3); however, proliferating CiGEnC (cultured at 33°C, Figure 3, lane 2) seemed to have reduced expression of glypican-1 and versican compared with other cells.

Structural Effects of Enzyme Treatments on GEnC Monolayers

Neuraminidase was the most potent enzyme at removing the sugar residues of glycocalyx demonstrated by marked reduction in WGA-FITC binding compared with untreated controls (Figure 4, A and B). Despite the effects on lectin binding, neuraminidase treatment had no effect on the morphology of CiGEnC monolayers as judged by phase contrast microscopy (Figure 4C). The loss of WGA-FITC binding was quantified by cell-based fluorescence (CBF) assay, showing a dosage-dependent effect of neuraminidase, independent of the control, actin (Figure 5). Expression (Figure 6) and distribution (Figure 6, A and C) of the intercellular adhesion molecule VE-cadherin was preserved after treatment of CiGEnC by neuraminidase (Figure 6, A and B).

CiGEnC also expressed HS GAG chains in abundance, exhibiting a mesh-like pattern on the cell surface compared with the control (Figure 7). Both Heparinase III and HPSE-1 treatments efficiently removed HS GAG from CiGEnC monolayers demonstrated by reduction in binding of anti-HS antibody (Figure 7) compared with control, further quantified using CBF assay (Figure 8). Again, removal of HS GAG by these enzymes did not seem to affect the cellular morphology or expression of VE-cadherin (Figure 7).

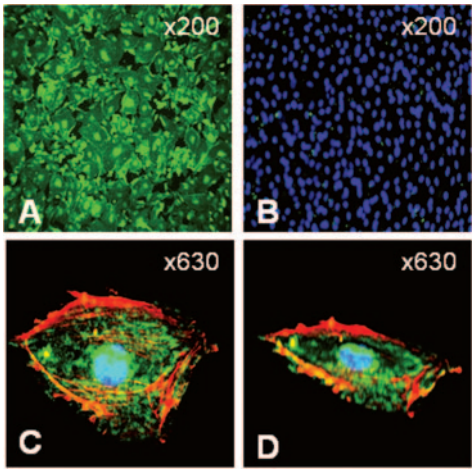

Figure 2. Fluorescence (A and B) and confocal laser scanning microscopy images (C and D) of CiGEnC. (A) Labeling of glycocalyx with WGA-FITC lectin (green). (B) Control (FITC alone) demonstrating nuclear staining with 4,6-diamidino-2-phenylindole (blue). (C and D) Images from three-dimensional reconstruction of *z* axis frames after multicolor labeling with WGA-FITC lectin (green), actin cytoskeleton (red), and nuclei (blue) demonstrating the cell surface distribution of glycocalyx.

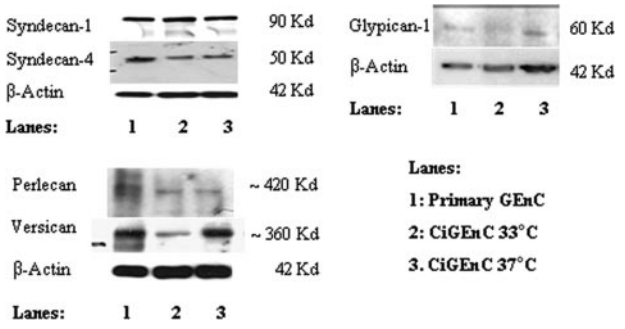

Figure 3. Western blotting analyses of protein extracted from primary culture GEnC (lane 1), proliferating CiGEnC at 33°C (lane 2), and nonproliferating CiGEnC after 7 d at 37°C (lane 3) demonstrating the expression of PG core proteins syndecan-1 and -4, glypican-1, perlecan, and versican. Images were derived from identical gels with each lane loaded with 20 μg of protein samples. Actin bands confirm the loading of comparable amounts of protein. Numbers indicate expected molecular weight of bands and correspond with molecular weights of marker proteins (data not shown).

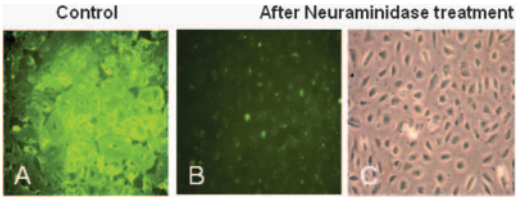

Figure 4. (A and B) Fluorescence microscopy demonstrating binding of WGA-FITC on CiGEnC monolayers. (A) Control. (B) After treatment with neuraminidase (1 U/ml for 60 min). (C) Phase contrast microscopy showing preserved CiGEnC monolayer after neuraminidase treatment. Magnification, ×200.

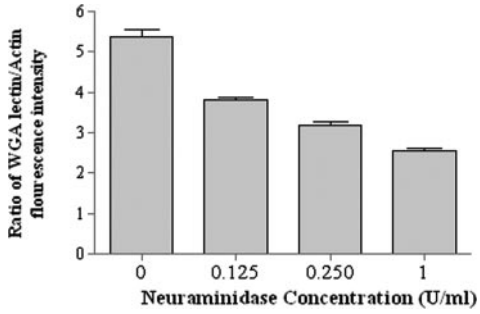

Figure 5. Chart showing effect of neuraminidase treatment on WGA-FITC binding to CiGEnC by a CBF assay. The *y* axis represents a ratio of fluorescence emission by WGA-FITC to control actin-FITC after treatment with various concentrations of neuraminidase shown at the *x* axis. Neuraminidase dosage-dependently reduces WGA-FITC binding. Bars show mean values ± SEM; $n = 15$; $P < 0.0001$ by ANOVA.

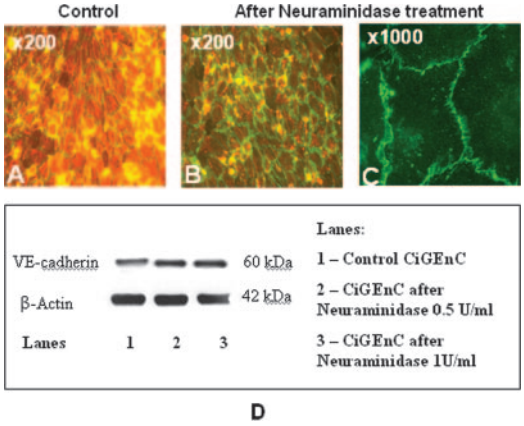

Figure 6. (A through C) Immunofluorescence microscopy showing co-labeling of CiGEnC monolayers with actin (red) and endothelial junctional protein VE-cadherin (green) in control monolayers (A) and after treatment with neuraminidase (60 min; B and C). (C) The junctional morphology assessed by expression and distribution of VE-cadherin is not affected by neuraminidase treatment. (D) Western blotting analyses of protein extracted from CiGEnC after treatment with neuraminidase. Control lysates were treated with vehicle alone. Each lane was loaded with 20 μg of the same protein samples. Actin bands confirm the loading of comparable amounts of protein. Numbers indicate expected molecular weight of bands and correspond with molecular weights of marker proteins (data not shown). Maintenance of VE-cadherin expression levels in neuraminidase-treated cells demonstrated by Western blotting compliments immunofluorescence findings. Magnifications: ×200 in A and B; ×1000 in C.

Effects of Glycocalyx Removal on CiGEnC Barrier Properties

Transendothelial electrical resistance (TEER) is inversely related to the fractional area of pathways across a cell monolayer open to water and small molecules. Neuraminidase caused a modest reduction in the mean TEER by 59% relative to the controls, equivalent to an increase in passage of water and small solutes through the monolayers (Figure 9A); however, analyzing the same pathways with TEER after

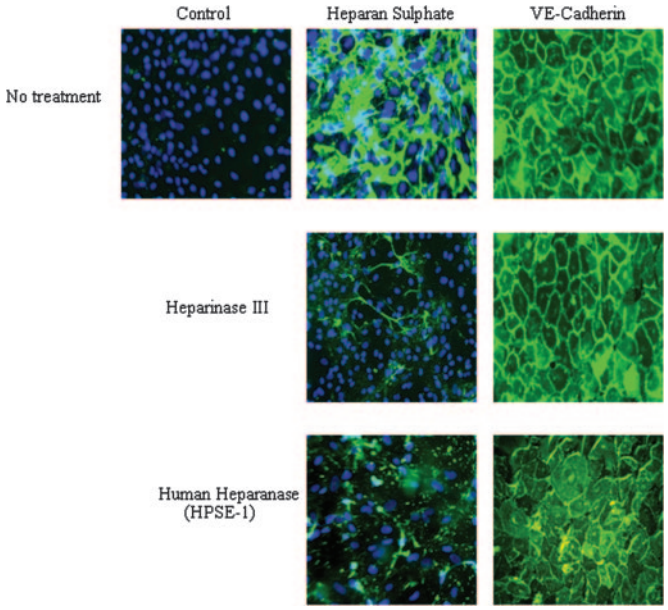

Figure 7. Immunofluorescence microscopy showing expression of extracellular HS GAG and VE-cadherin in control CiGEnC monolayers (top) and after treatment with heparinase III (middle) and HPSE-1 (bottom). These images demonstrate the removal of HS GAG with heparinase III and HPSE-1 while preserving the morphology of junctional protein VE-cadherin.

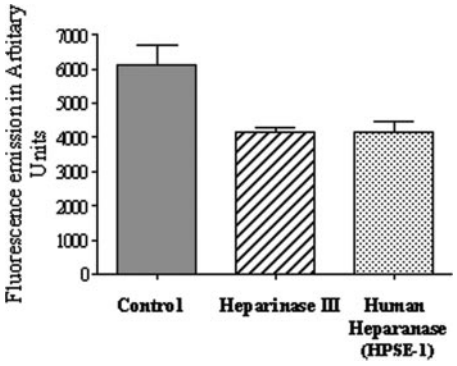

Figure 8. Effect of heparinase III and HPSE-1 treatment on the expression of HS GAG by CiGEnC using a CBF assay. HS GAG is proportional to the fluorescence emission. Compared with controls, heparinase III and HPSE-1 reduced HS GAG expression by 32 and 31%, respectively. Bars show mean values ± SEM; $n = 10$; $P < 0.0001$ by ANOVA.

treatment with enzyme heparinase III showed no significant change compared with vehicle only control (Figure 9B). In the same experiment cAMP medium (positive control) and thrombin (negative control) showed the expected increase and reduction in TEER. Similarly, HPSE-1 treatment did not induce any significant change in the TEER compared with controls (Figure 9C).

Neuraminidase treatment (at 1 U/ml) caused a 207% increase in the albumin flux across CiGEnC monolayers over a period of 3 h relative to untreated controls (Figure 10). Removal of HS GAG by heparinase III and HPSE-1 also

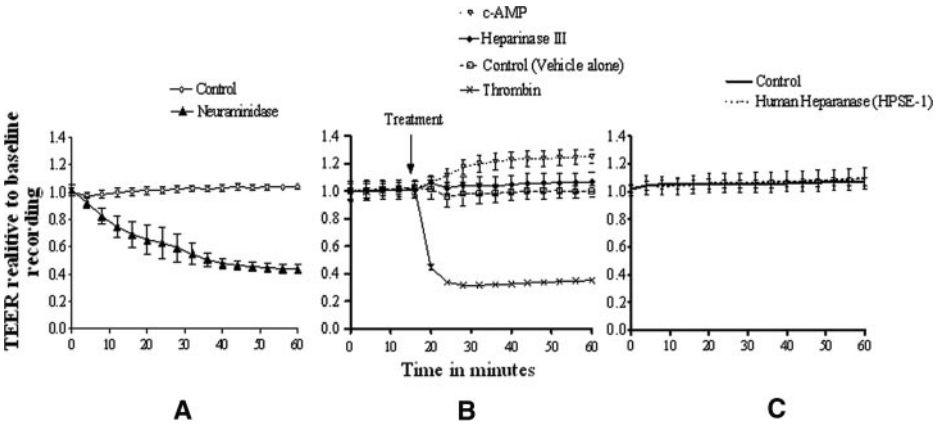

Figure 9. Effect of enzyme treatments on TEER of CiGEnC monolayers over time. (A) After neuraminidase (1 U/ml). Points represent means ± SEM; $n = 4$; $P < 0.005$ for the effect of treatment by repeated measures (rm) ANOVA. (B) After heparinase III (1 U/ml), vehicle only control, cAMP medium as a positive control, and thrombin (1 U/ml) as a negative control for the same period of time. Points represent means ± SEM; $n = 4$; $P < 0.005$ by rm ANOVA. *Post hoc* comparisons (Bonferroni) cAMP *versus* control and thrombin *versus* control both $P < 0.005$, heparinase III *versus* control and others NS. (C) After HPSE-1 (500 ng/ml). Points represent means ± SEM; $n = 4$; $P = 0.186$ for the effect of treatment by rm ANOVA. Neuraminidase reduced TEER by 59% relative to the control; however, heparinase III and HPSE-1 treatments showed no change compared with controls.

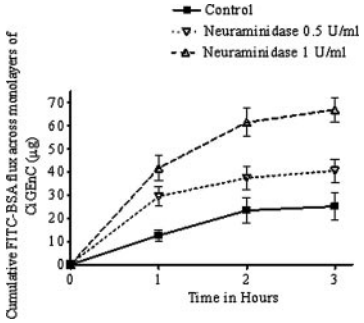

Figure 10. Effect of neuraminidase (0.5 and 1 U/ml) on the flux of albumin across CiGEnC monolayers over time. Each point represents mean ± SEM; $n = 10$; $P < 0.0005$ by rm ANOVA. *Post hoc* comparisons (Bonferroni): Neuraminidase 1.0 U/ml *versus* control $P < 0.05$ but others NS.

increased albumin flux 40 and 39%, respectively, over 3 h (Figure 11). The effects of neuraminidase and heparinase III on measures on GEnC barrier properties (TEER and FITC-BSA) were similar when performed in the presence of 100 ng/ml VEGF (data not shown).

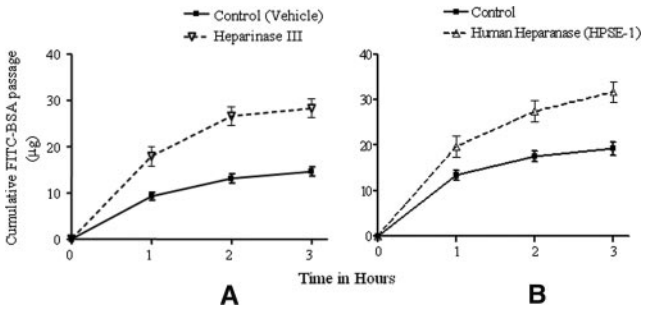

Figure 11. Effect of heparinase III (1 U/ml) and HPSE-1 (500 ng/ml) on albumin flux across CiGEnC monolayers over time in separate experiments. Each point represents mean ± SEM; $n = 12$; $P < 0.0005$ by rm ANOVA. *Post hoc* comparisons (Bonferroni): Heparinase III *versus* control and HPSE-1 *versus* control $P < 0.005$ for both experiments.

DISCUSSION

In this study, we provide evidence that structural alteration of human GEnC glycocalyx can have functional consequences for the passage of albumin. Furthermore and more interesting, specific degradation of HS GAG with recombinant HPSE-1 (known to be upregulated in some proteinuric disease states) also leads to an increased flux of albumin across the monolayer. In addition to being consistent with evidence from animal studies, this study presents a possible alternative mechanism of glomerular barrier dysfunction in renal disease.

The presence of glycocalyx on CiGEnC was confirmed by TEM and the lectin-binding experiments. Our findings of a glycocalyx thickness of 200 nm are consistent with estimates in published work on GEnC *in vivo*[35] and other endothelia *in vitro*.[13] The binding preferences of WGA lectin also indicate the predominance of sialic acid–rich glycoproteins and GAG chains in CiGEnC glycocalyx. Our Western blotting results confirm that nonproliferating CiGEnC express PG core proteins at levels comparable to primary culture GEnC and similar to the description in a recent publication.[23] In light of our results, it is plausible that the PG core protein perlecan present in the subendothelial (*lamina rara interna*) GBM[24] is produced by the GEnC, potentially also contributing to the anionic charge of GBM. This could also account for the loss of anionic charge (as a contributor to the onset of albuminuria) reported in conditions such as diabetes,[36] in which both generalized endothelial dysfunction and loss of perlecan in the GBM (perhaps also in glycocalyx) are early features.[36,37] Expression of HS GAG by CiGEnC suggests their potential of regulating interactions with the neighboring cells *via* the extracellular matrix. The method used to disrupt the glycocalyx using enzymes was based on two specific aims: First, in the case of neuraminidase, to remove the majority of the glycocalyx without disturbing the integrity of the cell monolayer, and, second, with heparinase III and recombinant HPSE-1, to degrade a specific constituent of glycocalyx (HS GAG, implicated in renal pathology).

The marked reduction of WGA binding after neuraminidase treatment suggests that sialic acid is a major constituent of

the sugar moieties present in CiGEnC glycocalyx. This was previously reported in GEnC and is also consistent with the reports from other microvascular beds.[13,38,39] Compared with neuraminidase, as expected, heparinase III treatment caused only a small reduction in WGA labeling; however, a specific antibody to HS GAG demonstrated a marked reduction in its expression, confirming the activity of the enzyme.

HPSE-1 has a different mechanism of action compared with heparinase III and cleaves HS GAG into larger fragments of 5 to 7 kD through a different cleavage site compared with the latter.[40] It is synthesized as a 65-kD latent enzyme that subsequently undergoes proteolytic cleavage, yielding a 50- and an 8-kD active heterodimer.[41] Lysosomes are the main storage site of active HPSE-1 because their acidic milieu (pH 5 to 6.5) is best suited to preserve its maximal activity. In addition to its intracellular housekeeping role in HS metabolism, active HPSE-1 is known to be transported by endosomes to the plasma membrane and extracellular space.[42] The recombinant HPSE-1 used in our study possessed maximal activity at pH 5 with a significant activity observed at pH 6.[43] The greater activity in an acidic milieu suggests that the majority of the HPSE-1 activity is intracellular, affecting the turnover of HS GAG, which could potentially lead to changes in both GEnC glycocalyx and GBM. This would also be consistent with the observations of high HPSE-1 activity coupled with loss of glomerular HS GAG in models of experimental diabetic nephropathy.[44]

TEER is used as an indicator of the pathways across a cell layer open to water and small molecules. The cAMP medium and thrombin provided good controls, demonstrating the ability of this system to detect changes in ion flux across CiGEnC monolayers. Our results show that removal of the majority of the glycocalyx by neuraminidase has a significant effect on both the passage of water and small solutes (as measured by TEER) and albumin flux; however, removal of HS GAG alone (using heparinase III or recombinant HPSE-1) has a significant effect on the albumin flux without affecting the pathways open to water and small solutes. We further tested the validity of these observations in the presence of exogenous VEGF, at a concentration and for a length of exposure shown previously to induce fenestrations.[10] The results were consistent with experiments performed in usual media, confirming the ability of the glycocalyx to contribute to protein restrictive properties of CiGEnC monolayers in a more fenestrated state as found *in vivo*.

The ability of HS GAG to restrict differentially the passage of albumin is probably due to its previously described charge-selective properties. This has been demonstrated *in vivo*, where removal of HS GAG has been shown to increase glomerular permeability to albumin.[23] Here glomerular permeability was estimated in cooled isolated perfused kidneys, a technique that inhibits tubular reabsorption and proteolytic degradation of albumin; however, in a recent study on intact rats,[45] removal of HS GAG by heparinase did not seem to cause proteinuria. This discrepancy could be a result of compensation of the increase in glomerular leakage by an increase in tubular reabsorption of protein (because this was not inhibited in this model). The evidence linking excess HPSE-1 to proteinuria is strongly suggestive of the contribution of HS GAG[27–30] in glomerular permselectivity; therefore, the study on intact rats[45] (which concluded that HS GAG in the GBM does not contribute to glomerular permselectivity) is suggestive of an important contribution from the GEnC glycocalyx.

Our results support the belief that enzyme treatments used in our experiments removed the surface glycocalyx rather than basement membrane components[23] because the basement membrane components were not directly exposed to enzymes. Furthermore, if the basement membrane components had been affected, then the integrity of the monolayers would have been compromised, yet, in our experiments, the monolayers seemed unchanged by phase contrast microscopy, retention of junctional morphology by VE-cadherin; therefore, the observed changes in macromolecular permeability are attributable to glycocalyx disruption rather than affects on basement membrane components

Our work confirms the presence of GEnC glycocalyx in culture, the structure of which is composed of sialic acid–rich glycoproteins and PG. The structure of this glycocalyx can be disrupted in culture by enzymes. Removal of HS GAG has a significant effect on albumin flux but not on passage of water and solutes. This finding is important for two reasons: First, it provides evidence to support the role of GEnC glycocalyx in selective permeability (restricting passage of macromolecules but allowing passage of water and small solutes); second, it suggests a mechanism by which HPSE-1 may increase permeability to macromolecules and hence contribute to the pathogenesis of proteinuria in clinical diseases. Blocking HPSE-1 in experimental disease may reduce proteinuria by preventing GEnC glycocalyx degradation instead of or as well as blocking effects on GBM. Blockade of HPSE-1 activity has therapeutic potential in a variety of glomerular diseases, particularly diabetic nephropathy, in which both proteinuria and upregulation of HPSE-1 have been described. Emerging data showing systemic loss of endothelial glycocalyx, along with increased microvascular permeability, in diabetes[46] both are consistent with the role of GEnC glycocalyx in glomerular permselectivity proposed here and suggest endothelial glycocalyx dysfunction as the missing common link between proteinuria and cardiovascular disease.

CONCISE METHODS

GEnC Culture

We used a normal human CiGEnC line as described in detail previously.[10] Briefly, primary culture GEnC were exposed to separate retroviral vectors transducing a temperature-sensitive mutant of SV40 large T antigen and the catalytic subunit of human telomerase. At the permissive temperature of 33°C, the tsSV40LT transgene is activated, causing cell proliferation (without telomere shortening), whereas at 37°C, the transgene is inactivated, rendering cells nonproliferative and quiescent.

CiGEnC were used for experiments after they were maintained at the nonpermissive temperature between 5 and 7 d. In some experi-

ments, primary culture GEnC (Applied Cell Biology Research Institute, Kirkland, WA) were used for comparison. All GEnC were cultured in endothelial growth medium 2 microvascular (EGM2-MV; Cambrex, Wokingham, UK) containing FCS (5%) and growth factors as supplied with the exception of VEGF, unless otherwise stated. Ci podocytes were cultured in RPMI 1640 as described previously.[47]

Phase Contrast Microscopy

Morphology of CiGEnC monolayers was examined by phase contrast microscopy after enzyme treatments and compared with monolayers exposed to vehicle only. All images were acquired using a digital camera (Nikon Coolpix E 4500; Nikon UK Ltd, Surrey, UK).

TEM

This technique has been described previously.[48] Briefly, monolayers of CiGEnC were fixed in freshly prepared solution of 2.5% glutaraldehyde and 0.5% Alcian blue in 0.1 M cacodylate buffer (pH 7.3). After 6 h, cells were washed three times in 0.1 M cacodylate buffer at 4°C and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer. Cells were washed again three times in cacodylate buffer and then distilled water before dehydration with ethanol. This was followed by embedding in Araldite resin. The cell layer was trimmed, and 0.5-μm survey sections were cut and stained with 1% toluidine blue in 1% aqueous borax for light microscopy. Cell-rich areas of the block were trimmed, and 50-nm-thick sections were cut and stained with 3% aqueous uranyl acetate and Reynolds lead citrate solution. Digital micrographs were taken on a Philips 100CS microscope.

Lectin Binding

Cells were grown to confluence on glass coverslips and fixed in 2% paraformaldehyde for 10 min and thereafter incubated with FITC-WGA (Sigma-Aldrich, St. Louis, MO) at 2 μl/ml for 30 min. WGA (from *Triticum vulgaris*) binds to sugar moieties (N-acetyl glucosamine and N-acetyl neuraminic acid) of glycoproteins present on the cell surface, the majority of which are likely to be PG constituents of glycocalyx. Nuclear staining was demonstrated using 4,6-diamidino-2-phenylindole. Coverslips were mounted in Vectashield aqueous mountant (Vector Laboratories, Peterborough, UK) and examined using a Leitz DMRB fluorescence microscope (Leica, Solms, Germany) and TCS-NT confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Immunofluorescence

After fixation as described previously, coverslips were either incubated directly in the blocking solution (5% FCS and 0.05% Tween20 in PBS) or permeabilized in 0.3% Triton X-100 before blocking. Cells were then incubated with antibodies to HS (HepSS-1; US Biologicals, Swampscott, MA), VE-cadherin (Santa Cruz Biochemicals, Santa Cruz, CA), or von Willebrand factor (vWF; DakoCytomation, Ely, Cams, UK) or with a Texas red–conjugated phalloidin to label actin (Molecular Probes, Eugene, OR). Primary antibody binding was detected using FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) except for HS (Alexa Flour 488 conjugated probe; Molecular Probes). Control cells were incubated with secondary antibodies only.

Generation of Recombinant Human Heparanase

The active 50-/8-kD heterodimer form of HPSE-1 protein was produced in an insect cell expression system as described previously.[43] Briefly, protein targeted for secretion was isolated by a one-step heparin-Sepharose chromatography procedure to give protein of >90% purity as judged by SDS-PAGE analysis. The recombinant enzyme behaved similarly to the native protein with respect to the size of HS fragments liberated on digestion, substrate cleavage specificity, and its preference for acidic pH.

Enzyme Treatments

Neuraminidase from *Clostridium perfringens* (EC 3.2.1.18; Sigma Aldrich) hydrolyzes the sialic acid residues of oligosaccharides and glycoproteins. Monolayers of CiGEnC were incubated in serum-free medium (SFM) with neuraminidase at concentrations from 0.125 to 1 U/ml for 60 min at 37°C. Controls were incubated in SFM alone under the same conditions.

Heparinase III from *Flavobacterium heparinum* (EC 4.2.2.8; Sigma Aldrich) cleaves HS GAG. Monolayers were incubated with concentrations ranging from 0.20 to 1 U/ml in SFM at 37°C for 4 h. Controls were incubated in SFM alone under the same conditions.

For some experiments, both control and test monolayers were cultured in 100 ng/ml recombinant human VEGF (VEGF-A_{165}; R&D Systems, Minneapolis, MN) for 24 h before enzyme treatments with neuraminidase and heparinase III. Using the previously mentioned protocol, we previously showed that VEGF increases the number of fenestrations expressed by CiGEnC.[10]

HPSE-1 cleaves HS GAG into larger fragments and was used at 200 to 1000 ng/ml in SFM pH 6.0 over 4 h. The control cells were incubated with SFM at pH 6. The optimal pH of incubation buffer (SFM) was determined by a set of preliminary experiments (data not shown), aiming to preserve HPSE-1 activity but with least effect on the integrity and permeability characteristics of CiGEnC monolayers. The results of these experiments confirmed higher activity of HPSE-1 at more acidic pH (pH 5 > 6 > 7).

Cell-Based Fluorescence Assay

A CBF assay, essentially as described previously,[6] was used to quantify changes in lectin binding and HS expression after treatments with neuraminidase (0.125 to 1 U/ml) or heparinase III (1.0 U/ml) and recombinant HPSE-1 (500 ng/ml).

Western Blotting

CiGEnC were lysed in Laemmli sample buffer, and protein concentrations were determined (bicinchoninic acid assay; Pierce Chemical Co., Rockford, IL). Lysates of primary GEnC were used as control. Samples were separated by SDS-PAGE and were blotted onto nitrocellulose or polyvinylidene fluoride (Immobilon-P; Millipore Corp., Billerica, MA) membranes. The membranes were blocked in 5% fat-free milk before incubation with antibodies to syndecan-1, syndecan-4, perlecan (all Zymed Laboratories, San Francisco, CA), glypican-1 (Santa Cruz), versican (R&D Systems, Morrisville, NC), and actin to confirm loading of comparable amounts of protein in each lane. After incubation with horseradish peroxidase–conjugated secondary antibodies, bands were detected by using Supersignal West

Femto maximum sensitivity chemiluminescence substrate (Pierce Chemical Co.) followed by imaging using a MultiDoc-It imaging System (UVP, Upland, CA).

Measurement of TEER

TEER was measured using an automated bioimpedance sensing system (ECIS; Applied Biophysics, Troy, NY). Compared with conventional tissue culture insert-based methods, this method has added advantages of sensitive, real-time TEER measurements in a controlled environment. The technical details of this method have been described previously.[49] CiGEnC were seeded at 100,000 cells/cm^2 in each well of the eight-well electrode slide (8W10E) supplied by the manufacturer. The base of an individual well has an array of 10 gold film electrodes that connect ECIS electronics to each of the eight wells. The resistance is reported in Ω, and the measurement from each well, at a given time point, is an average of the recordings from 10 electrodes.

The effects of a cAMP analogue and thrombin were examined as controls for the system. Increasing cAMP decreases permeability (hence, increases TEER) as opposed to thrombin, which increases permeability (reduction in TEER) of GEnC.[26] A cell membrane-permeable cAMP analogue, 8-(4-chlorophenylthio) pCPT-cAMP (Sigma), was used in combination with a cAMP-specific phosphodiesterase inhibitor, RO-20-1724 (CN Biosciences, Nottingham, UK) to elicit a maximal response. The medium in cell culture wells was replaced with SFM medium containing 20 μM RO-20-1724 and 300 μM pCPTcAMP (cAMP medium) or thrombin (1 U/ml) at the same time as enzyme treatments.

Culture of CiGEnC in Tissue-Culture Inserts

Polycarbonate supports (0.4 μm pore size, 0.5 cm^2 surface area) in tissue-culture inserts (1 cm diameter; Nalge Nunc Int., Rochester, NY) were seeded with CiGEnC at 100,000 cells/cm^2. Inserts were placed in 24-well plates, and media were changed three times a week.

Measurement of Transendothelial Protein Passage

Transendothelial permeability to macromolecules was assessed by measurement of passage of FITC-labeled BSA (Sigma) across the monolayer using tissue culture inserts as described previously.[6] Medium in both wells and inserts, containing CiGEnC monolayers, was replaced with SFM. After 1 h, the medium in the insert was replaced with 500 μl of SFM containing 0.5 mg/ml FITC-labeled BSA; that in the well was replaced with 500 μl of SFM containing 0.5 mg/ml unlabeled BSA. At 1, 2, and 3 h, 100-μl aliquots were removed and replaced with 100 μl of SFM containing unlabeled BSA (0.5 mg/ml). The fluorescence of the aliquots was measured as already described, and the amount of FITC-BSA passing through the monolayer was calculated by reference to a set of standard dilutions.

Statistical Analyses

GraphPad Prism-4 statistical software package (GraphPad Software, San Diego, CA) was used for all analyses, including SEM and ANOVA. $P < 0.05$ was taken to indicate statistical significance

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DISCLOSURES

None.

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