Basal lamina secreted by MDCK cells has size- and charge-selective properties

Nicholas Ferrell,1 Joseph Groszek,1 Lingyan Li,1 Ross Smith,1,2 Robert S. Butler,2 Christian A. Zorman,3 Shuvo Roy,4 and William H. Fissell1,5

Departments of 1Biomedical Engineering, 2Quantitative Health Sciences, 3Nephrology and Hypertension, Cleveland Clinic, Cleveland; 4Department of Electrical Engineering and Computer Science, Case Western Reserve University, Cleveland, Ohio; and 4Department of Biopharmaceutical Sciences, University of California, San Francisco, California

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Ferrell N, Groszek J, Li L, Smith R, Butler RS, Zorman CA, Roy S, Fissell WH. Basal lamina secreted by MDCK cells has size- and charge-selective properties. Am J Physiol Renal Physiol 300:F86–F90, 2011. First published October 27, 2010; doi:10.1152/ajprenal.00484.2010.—The role electrical charge plays in determining glomerular permeability to macromolecules remains unclear. If the glomerular basement membrane (GBM) has any significant role in permselectivity, physical principles would suggest a negatively charged GBM would reject similarly charged more than neutral species. However, recent in vivo studies with negative and neutral glomerular probes showed the opposite. Whether this observation is due to unique characteristics of the probes used or is a general physiological phenomenon remains to be seen. The goal of this study was to use the basement membrane deposited by Madin-Darby canine kidney epithelial cells as a simple model of a biologically derived, negatively charged filter to evaluate size- and charge-based sieving properties. Fluorescein isothiocyanate-labeled carboxymethylated Ficoll 400 (FITC-CM Ficoll 400) and amino-4-methyl-coumarin-labeled Ficoll 400 (AMC Ficoll 400) were used as negatively charged and neutral tracer molecules, respectively, during pressure-driven filtration. Streaming potential measurement indicated the presence of fixed, negative charge in the basal lamina. The sieving coefficient for neutral Ficoll 400 decreased by ~0.0013 for each 1-Å increment in solute radius, compared with a decrease of 0.0023 per Å for the anionic Ficoll 400. In this system, molecular electrostatic hindrance including a Donnan equilibrium between electrostatic barrier, including a Donnan equilibrium between...
It appears challenging to isolate GBM separately from other extracellular matrix components of the kidney. Extracellular matrix that has been solubilized from tumor matrix lacks collagen or contains collagen that is not crosslinked. However, polarized epithelial cells, such as Madin-Darby canine kidney (MDCK) cells, appear to deposit an organized basal lamina containing laminin, collagen, and heparan sulfate proteoglycans (9, 13, 16, 41–43). As such, cultured MDCK cells may offer an avenue to explore the role of basement membranes in size- and charge-dependent filtration. We now extended our prior models of filtration through extracellular matrix to include measurements of the effect of electrostatic charge on filtration.

It is also important to assess the electrical character of the membrane itself, as the presence of fixed anionic charges in the capillary wall is critical to models of glomerular permeselectivity that include an electrostatic barrier to albumin. Various efforts to define the distribution of charges have generally used cationic probes to stain and localize anionic structures in the capillary wall. The applicability of these histological data to transport physiology is not always clear or quantitative. Instead, we chose to measure a functional assessment of electrostatic contributions to transport, called the streaming potential. The flow of an electrolyte through a charged porous medium gives rise to an electrical potential gradient; similarly, application of a voltage across a charged porous structure bathed in electrolyte causes flow of fluid, called electroosmotic flow (31). Briefly, bulk electrical neutrality demands that immobile ions in a gel or filter must be balanced by counterions in solution. Thus, when there is fluid flow, there must be net flow of charge, as the counterions are free to move and the fixed charges are not. The magnitude of the voltage is limited by electrical conduction through the electrolyte, which dissipates the charge gradient. The measured streaming potential varies directly with charge density in the medium, and inversely with ion mobility in the electrolyte and inversely with pore size. Thus, streaming potential measurements provide insight into electrostatic contributions to hindered transport through a membrane. The potential measurement itself does not, however, describe size selectivity nor necessarily the magnitude of the electrostatic component to hindrance.

As an extracellular matrix model for the GBM incorporating crosslinked collagen, the basal lamina deposited by MDCK cells were prepared on porous substrates, decellularized, and mounted in ultrafiltration cells. Extracellular matrix charge was qualitatively estimated by measuring streaming potential through the decellularized basal laminae. Anionic and neutral Ficoll solutions were filtered through the extracellular matrix and sieving coefficients were measured.

**METHODS**

**Ficoll preparation and characterization.** Fluorescently labeled neutral and anionic polysaccharide probes were prepared as described (24). Generally, the carboxymethylation reaction of polysaccharides is done using strong NaOH and monochloroacetic acid (MCA) in aqueous medium at elevated temperature. Briefly, 1 g Ficoll 400 (Cat. no. 46326 and 46327, respectively; Fluka, St. Louis, MO) was mixed with 26.5 ml deionized water and stirred for 30 min at 40°C. A 10-M NaOH solution (20 ml) was slowly added. After that, MCA (15%, 53.5 ml) was added dropwise into the mixture. Thus, the reaction mixture was initially 1 M in chloroacetate and 2 M in NaOH. The mixture was stirred at 40°C for 3 h. Then, the mixture was neutralized with 5 M HCl and dialyzed against distilled water for 4 days. The solid carboxymethylated products were recovered by freeze-drying.

Labeling of Ficoll with FITC (fluorescein isothiocyanate) was performed after the method of Ohlson et al. (33). Ficoll was coupled with 7-amino-4-methylcoumarin (AMC) by reductive amination in the presence of sodium cyanoborohydride. Briefly, Ficoll was dissolved in a solution of AMC in DMSO/glacial acetic acid/sodium cyanoborohydride. The solution was then incubated at 80°C for 2 h. After conjugation with AMC, labeled Ficoll were precipitated in ethanol, centrifuged, and dissolved in deionized water. Labeled Ficoll was separated from free AMC with a PD-10 column. Labeled samples were analyzed by size-exclusion chromatography as previously described, except that the excitation and emission wavelengths of the fluorescence detector were set to the appropriate wavelengths for each fluorophore (19, 22).

**Basal lamina preparation and charge characterization.** MDCK epithelial cells were obtained from the American Type Culture Collection. Cells were grown in Ultra-MDMK medium (12–749Q; Lonza, Basel, Switzerland) with 10 ml/l antibiotic-antimycotic solution (15250; Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO2. Culture medium was replaced three times per week.

Ficolll was isolated from GBM using a similar method to that described previously (1, 42). MDCK cells were seeded on the rat tail collagen gels at 200,000 cells/insert. Cells were grown for 28–31 days with medium replacement three times weekly. MDCK cells were lysed with 10 mM Tris-HCl with 0.1% bovine serum albumin and 1 mM CaCl2 at pH 7.5. Cells were dissolved in 0.5% sodium deoxycholate (D6750; Sigma) in lysis buffer for 10 min at 37°C with fresh solution added after 5 min. Samples were washed with 0.5% IGEPAH (I3021; Sigma) in lysis buffer for 2 min. Samples were carefully rinsed with PBS three times.

**Filtration.** Filtration procedures for membranes and extracellular matrix have been described previously (18, 22). Briefly, after the extracellular matrix was decellularized, the Transwell membranes were cut from their supports and laid on a rigid 0.5-µm-pore size silicon frit. A second Transwell membrane was laid face-to-face on top of the first and the sandwich of extracellular matrix between two Transwell membranes was mounted in an ultrafiltration cell. A schematic of the sample configuration can be found in Fig. 1. A single
solution of 50 μg/ml each of FITC-labeled 400 (Ficoll) and AMC-labeled carboxymethyl Ficoll 400 (CM-Ficoll) in Dulbecco’s PBS (DPBS) with calcium chloride and magnesium chloride (D8662; Sigma) was introduced into the feed reservoir and continuously circulated through the feed side of the ultrafiltration cell by a peristaltic pump. The reservoirs and tubing were covered with aluminum foil to minimize photobleaching of the fluorophores. The feed side circuit was pressurized to two pounds per square inch (13.8 kPa, the same pressure at which the streaming potential was measured) with compressed air and ultrafiltrate samples were collected. The first sample collection was discarded as it may be diluted by the DPBS used to wet the membranes. Samples were collected in darkened microcentrifuge tubes and refrigerated. Sieving coefficients were calculated as the ratio of fluorescence in the ultrafiltrate divided by the fluorescence in the feed.

Statistics. Experiments were repeated in triplicate, except for chromatography of neutral Ficoll, which was performed in duplicate as results were identical to our previous extensive testing (20). Data were analyzed using linear mixed models incorporating molecular radius, charge, and radius-charge interaction. All analyses were run on SAS version 9.2 (SAS, Cary, NC).

RESULTS

Basement membrane characterization. MDCK cells grew to confluence rapidly and were maintained in cell culture for 28–31 days, and then removed as described. The underlying matrix was exceptionally smooth when observed by scanning electron microscopy compared with collagen gels (Fig. 2, A and B). Streaming potentials in basement membranes derived from MDCK cells using 10 mM KCl at 37°C average −0.05 ± 0.02 mV/kPa, consistent with the presence of fixed negative charges in the extracellular matrix. The results from the measurements taken with PBS at 37°C appear to demonstrate a slight negative charge as well with an average streaming potential measurement of −0.01 ± 0.01 mV/kPa (t-test, P < 0.02 vs. a slope of 0).

Permselectivity of MDCK-generated basement membranes. Basement membranes deposited by MDCK cells displayed size-dependent filtration of neutral Ficoll and charged Ficoll (Fig. 3). The rat tail collagen matrix also displayed faint trends towards size- and charge selectivity (Fig. 3). Linear mixed-model analysis of the sieving data indicated that charged and neutral populations were significantly different from each other (P < 0.0001) and there was a significant interaction between molecular radius and carboxymethylation (P < 0.0001).

DISCUSSION

It is generally accepted that each of the multiple layers of the glomerular capillary wall (GCW) contributes to the permeability barrier (26). The pathophysiology by which disease disrupts the barrier remains obscure, leaving mechanism-based treat-
ments for proteinuric renal disease out of reach. The reason why the permeability barrier remains persistently enigmatic is in part attributable to the fragility of the normal glomerulus. The endothelial glyocalyx has been thought a significant contributor to permselectivity in vivo but remains frustratingly evanescent in culture or biopsy samples (28, 35, 36). The basement membrane, long held to be a critical component of the permeability barrier, is much thinner than the wavelength of visible light, frustrating efforts at optical microscopy approaches to measuring spatial solute distribution. In addition, the ultrastructural appearance of the GBM seems sensitive to fixation, and the GBM cannot be isolated from biopsy samples independent of mesangial matrix (4, 5, 7, 11, 12, 14, 15, 30, 37, 38). Structural rearrangement of the glomerular podocyte almost invariably accompanies significant proteinuria, yet study of this intriguing cell has been limited as it does not appear to assume the same phenotype in vitro as it does in vivo, frustrating efforts to isolate the contribution of the slit diaphragm apparatus to the permeability barrier. In the face of these challenges, we sought to develop analogies to components of the capillary wall and explore the physical principles underlying transport within these structures (8, 10, 17–19, 21–23). The analogy may not be perfect, but the insights gained may assist in interpreting in vivo data.

To this end, we explored solute partitioning into Matrigel and bovine lens capsule basement membranes, and now we report filtration by a basal lamina produced by renal epithelial cells. The MDCK cell-generated basal lamina we employed has been described and used as a tool to investigate basement membrane assembly. In our hands, the basal lamina displayed charge selectivity, albeit weakly. This is consistent with the presence of fixed anionic charges within the basement membrane and tends to corroborate the physical intuition that most proteins, including constituents of basement membranes, are negatively charged at physiologic pH, and a gel of such proteins would be expected to reject anionic solutes based on charge as well as size. The size selectivity of this extracellular matrix model of the GBM is orders of magnitude less stringent than observed for the living kidney, or for isolated GBM as reported by Edwards et al. (14). This suggests that the transport properties of different capillary beds are sensitive to extracellular matrix composition.

The apparent range of pore sizes in the basal lamina tested in these experiments is probably an order of magnitude larger than those hypothesized to form the permeability barrier in the GCW. The polydisperse Ficoll 400 we used contains solutes that are hundreds of Ångstroms in radius, so we were able to carry out the experiment at this length scale. What does not scale, however, is the size of the electrical double layer. That we observed a charge effect at all seems remarkable given that the double layer might only be 3–5% of the size of the solute. This makes a strong case for more detailed modeling of charge effects in the complex medium of biologic gels. The measurements reported here suggest that the effect of electrostatics in glomerular permeability has the potential to be significant. This is consonant with a wealth of data in the literature stretching back several decades suggesting a role for charge, as well as size, in glomerular filtration.

It remains a challenge to integrate these in vitro data with animal data published on neutral and CM-Ficoll which appears to show enhanced transport of anionic Ficoll compared with neutral. Those observations were regarded as anomalous by the authors. We would speculate that having prepared and characterized our own anionic samples, there may have been a physicochemical difference between CM-Ficoll examined by others and the CM-Ficoll we examined.

The physiological relevance of these in vitro data to understanding of renal physiology and kidney disease is less tenuous than it might appear. Vigorous debate regarding structure-function relationships in the capillary wall persists and clouds development of mechanism-specific therapies for proteinuric renal disease. The publications by Guimaraes and Asgeirsson (2, 25) challenged conventional physical intuition about glomerular physiology, and in their aftermath, it has been debated whether the test solute they employed was in some way producing incongruous data or whether there was a new and unexplained phenomenon in the glomerular capillary that demanded explanation. Our choice to synthesize and characterize our solutes ourselves allowed us to confirm that the anionic solutes behave as expected in a highly structured silicon membrane (24), and now are retarded by a complex organized extracellular matrix secreted by epithelial cells. These data are consistent with the work of Deen and Ohlsen and others (11, 12, 32, 33) predicting that an anionic extracellular matrix will retard passage of anionic solutes compared with neutral ones. To our knowledge, we controlled other variables besides electrostatics (in particular, size and shape) that affect transport. Future work will lie in defining the charge densities in the extracellular matrix and the charge on the tracers themselves. These tools, in combination with molecular biologic approaches and novel imaging techniques, may allow investigators to probe dynamic interactions between layers of the filtration barrier in vivo.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
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