Commentary

Contribution of Proteoglycans Towards the Integrated Functions of Renal Glomerular Capillaries

A Historical Perspective

Yashpal S. Kanwar, Farhad R. Danesh, and
Sumant S. Chugh

*From the Departments of Pathology and Medicine,
Northwestern University, Chicago, Illinois*

Since the original colloidal iron tracer study by Farquhar and Palade almost 50 years ago,¹ the permeability characteristics of mammalian glomerular capillary wall have been the subject of extensive investigations.^{2,3} In the ensuing 2 to 3 decades, various studies were performed to delineate whether glomerular basement membrane or the slit diaphragm of the capillary wall is the primary filtration barrier.^{4,5} Amid this contentious issue, in the mid to late 1970s various clearance and additional tracer studies indicated that the glomerular capillary wall behaves as a size- as well as charge-selective barrier, and the latter biophysical properties were attributed to the anionic sites within the basement membrane that are enriched with sulfated proteoglycans (PGs).^{6–9} The article by Harvey et al¹⁰ in this issue of *The American Journal of Pathology* suggests that agrin, a basement sulfated proteoglycan, imparts an electronegative charge to the glomerular basement membrane (GBM), but it may not be responsible for its charge-selective permeability properties. In light of these findings, we deemed it necessary to revisit the tracer work performed during the past few decades and reconcile with recent observations made in various mutant mice keeping in perspective the complex ultrastructure and integrated functions of the glomerular capillary wall.¹¹

Structural Elements of the Glomerular Capillary Wall

The glomerular capillary wall, also described in the literature as the filtration unit of the glomerulus, fractionates the blood into an ultrafiltrate that is devoid of plasma proteins and cellular elements that are retained within the capillary lumina.¹¹ The ultrafiltration unit is a stratified

structure made up of GBM flanked on the inner and outer aspects by a highly attenuated fenestrated endothelium and interdigitating foot processes of the podocytes, respectively (Figure 1B). The endothelial fenestrae are quite large (~100 nm in diameter) and are devoid of diaphragm such that a bulk flow of plasma water solute can be channeled toward the GBM. On the other hand, the spaces between the interdigitating foot processes are spanned by a thin membrane, known as the slit diaphragm, that has a well-defined structure with 4×14 nm rectangular pores,¹² and it apparently also restricts the transcapillary passage of macromolecules. Conceivably, the GBM, an amorphous extracellular matrix scaffold of 300-nm width, serves as the major barrier for the passage of large macromolecules and is further stratified into a central dense layer known as lamina densa that is flanked on either side by relatively loose electron lucent layers termed as lamina rara interna and externa. The GBM is made up of high-molecular weight proteins, including type IV collagen, laminins, entactin/nidogen, and sulfated PGs.^{13–16} The latter include, agrin, perlecan, and collagen XVIII, and all these PGs are chimeric molecules with a wide variety of potential functional domains.^{14–16} In addition, glycosaminoglycans, made up of heparan (HS) and chondroitin sulfate chains, are attached to their respective core peptides of the PGs. These chains by virtue of having sulfate radicals endow the GBM with charge-selective properties besides size-selectivity as with laminin or type-IV collagen, concentrated in the lamina densa. On the other hand, the electronegative charge is mainly clustered in the lamina rarae, where its localization has been elucidated by various cationic probes, includ-

Supported by National Institutes of Health grants DK28492 and DK606035.

Accepted for publication April 11, 2007.

This commentary relates to Harvey et al, *Am J Pathol* 2007, 171:139–152, published in this issue.

Address reprint requests to Yashpal S. Kanwar, Northwestern University Medical School, Department of Pathology, Ward 6-076, 303 E. Chicago Ave., Chicago, IL 60611. E-mail: y-kanwar@northwestern.edu.

Figure 1. Electron micrograph of the ultrafiltration unit. This unit (**B**) is made up of GBM, which is stratified into lamina densa (LD) and lamina rara interna (LRI) and externa (LRE). The GBM is lined by the fenestrated (fn) endothelium (En) from inside and with foot processes (fp) of podocytes (Po) from outside. Space between the foot processes is occupied by slit diaphragm (Sd). Cap, capillary. **C:** The binding of cationic ferritin with the GBM anionic sites (arrowheads) enriched with heparan sulfate proteoglycans. US, urinary space. **A:** The colloidal iron-stained thick glycocalyx enriched with sialoglycoproteins (SGPs) covering the foot processes.

ing polyethylenimine, Alcian blue, ruthenium red, and cationic ferritin (Figure 1C).^{8,11} At present, it is unclear as to the amount of electrical charge contributed individually by each of these PGs, besides by other acidic proteins of the GBM.

The charge selectivity is also contributed by the sialic acid-rich glycocalyx coating the plasmalemma of the attenuated glomerular endothelium and podocyte foot processes (Figure 1A). Other plasmalemmal glycoproteins of the endothelium include podoendin, podocalyxin, and syndecans, the latter being cell surface-associated sulfated PGs, which may also contribute to the electrical charge of the capillary. The biochemical composition of the slit diaphragm, a prototype of shallow adherens junctions, is quite complex and has been extensively investigated in recent years. Its major components include nephrin, NEPH1, NEPH2, and podocin, in addition to junctional proteins like cadherins.¹⁷ The latter interact with actin cytoskeleton via linker proteins, such as ZO-1, CD2AP, CASK, and MAGI-2. Other plasmalemmal proteins that mediate interactions with the actin cytoskeleton include integrin $\alpha 6 \beta 1$ and α and β subunits of dystroglycan along with sarcospan and sarcoglycans. This entire

complex of plasmalemmal proteins interacts with E3 and E8 domains of laminin-1 and anchors the sole of the foot processes with the GBM and thus conceivably modulates the hydraulic flux across the filtration barrier.¹⁷ Interestingly, adhesion of foot processes is also mediated by sialic acid-rich glycocalyx because its enzymatic elimination leads to the detachment of the podocytes from the GBM, thereby resulting in the disruption of the hydraulic flux and consequential proteinuria.¹¹

Ultrastructural Basis of Filtration and Proteinuria

The seminal studies of the 1960s by Farquhar and Palade addressed this issue by using electron-dense tracer ferritin with biophysical properties similar to albumin.^{2,3} The ferritin introduced into the circulation was found restricted to the endothelial fenestrae and inner layers of the GBM.^{2,3} Similar localization was observed in *in situ* experiments where perfusate containing ferritin was directly introduced into the renal artery (Figure 2A), indicating the GBM as the primary filtration barrier. Subsequent work by

Figure 2. Electron micrographs of the capillary loops of kidneys perfused with native (pI ~4.9, **A** and **B**) and cationic (pI >7.5, **C** and **D**) ferritins. **B** and **D:** Kidneys treated with heparitinase and then perfused with ferritins. Increased permeation of native ferritin is seen in the GBM after heparitinase treatment (**B** versus **A**). The cationic ferritin binding is lost after the enzymatic treatment. Ferritin particles are seen forming a gradient within the GBM (**D**), and their distribution is similar to kidneys perfused with native ferritin (**B**). Cap, capillary; US, urinary space.

Karnovsky, Venkatachalam, and colleagues^{4,5} with peroxidatic tracers having varying isoelectric points and molecular weight revealed that some of them accumulated underneath the slit diaphragms following their introduction into the blood circulation. This meant that perhaps the slit diaphragm may be the ultimate filtration barrier. To resolve this issue, Caulfield and Farquhar used graded series of dextrans for further *in vivo* tracer studies in the early 1970s. The dextrans less than the molecular weight of albumin appeared in the urinary spaces, whereas the larger ones were retained within the capillary lumina. At no time point were the dextrans observed within the basement membrane; thus, the pendulum swung back to the notion that GBM is the primary filtration barrier. In support of this notion is also a recent elegant gene disruption study with laminin $\beta 2$ -deficient mice that develop proteinuria during the neonatal period associated with disorganization of the GBM components, especially the anionic sites.¹⁹

Because the dextrans are also well suited for *in vivo* physiological studies, Brenner and his colleagues⁷ measured renal clearances of dextrans of varying size and charge in the mid-1970s. The dextrans with similar molecular weight but with basic charge had higher clearance rate, establishing that the glomerular capillary behaves as the charge- as well as size-selective barrier. The charge selectivity was later found to be attributed to the GBM anionic sites enriched with heparan sulfate proteoglycans, because their *in situ* enzymatic removal led to an increased permeation of the native ferritin (pI ~4.9) within GBM (Figure 2B).⁹ Moreover, cationic ferritin (pI >7.5), which normally binds to the anionic sites in the lamina rarae (Figure 2C),^{9,11} formed a gradient within the basement membrane on the PGs' enzymatic degradation, with relatively high concentration of the tracer in inner layers of the basement membrane (Figure 2D), thus reinforcing the notion of the GBM being the primary filtration barrier with respect to its charge selectivity. Intriguingly enough, the cationic ferritin given intravenously was localized underneath the slit diaphragm within 1 to 2 hours (Figure 3A) and could be seen for up to 24 hours (Figure 3B). These findings meant that all of the strata of the capillary wall serve as charge- as well as size-selective barrier to varying degrees during the various stages of transglomerular passage of macromolecules, with the slit diaphragm acting as the final fence before the ultrafiltrate is formed in the urinary space. Conceivably, the disruption of the slit diaphragm would be expected to induce proteinuria, and that seems to be the case with nephrin-deficient mice and in patients with congenital nephrotic syndrome of Finnish type, in which mutations in the nephrin gene have been observed.²⁰ Such results can be reciprocated by the administration of nephrin monoclonal antibody to rats, which as a result develop a proteinuric state.²⁰ Thus, it seems that the biology of the slit diaphragm and/or of the podocytes are intricately linked to the pathogenesis of proteinuria as is further evidenced by the phenotypic changes seen in the Cd2ap-deficient mouse with widespread foot process effacement and albuminuria.¹⁹

Figure 3. Electron micrographs of glomerular capillary loops of a rat that received an intravenous injection of cationic ferritin (pI >7.5) after 2 (A) and 24 hours (B). Initially, the cationic ferritin is seen traversing the GBM and accumulating underneath the slit diaphragm (Sd). After 24 hours, the residual ferritin still can be seen underneath the slits. C: A kidney treated with neuraminidase followed by perfusion of native ferritin. A large number of ferritin particles are seen traversing the GBM and leaking into the urinary space in regions where foot processes (fp) have detached from the underlying GBM. Cap, capillary.

PGs and Glomerular Permeability

As indicated earlier, there are three major extracellular matrix-sulfated PGs, collagen XVIII, perlecan, and agrin, that have variable degree of expression within the GBM and mesangial matrix of the mammalian glomerulus.^{14–16} These PGs, besides being chimeric molecules, include both the HS and chondroitin sulfate glycosaminoglycan chains that impart variable degree of charge density to the GBM individually. The removal of PGs' chains with use of broad-spectrum glycosaminoglycan-degrading enzymes leads to an expected facilitated permeation of ferritin within the GBM (vide supra) and also increased fractional clearances (θ) of fluorescein isothiocyanate-Ficoll and albumin.²¹ Likewise, intravenous administration of a nonspecific monoclonal antibody to HS chains has been shown to induce selective proteinuria in rats.²² The fact that there is a generalized reduced expression of HS chains involving various pathogenetic mechanisms, eg, depolymerization and damage by reactive oxygen species, in a wide variety of renal glomerular diseases leads one to ponder which of the three PGs are lost in a given disease process and whether or not the disruption

of an individual PG leads to an altered permeability of the glomerular capillary wall.²³ These issues were addressed by recent gene disruption studies in mice. The collagen XVIII-deficient mice revealed expanded basement membrane matrices and elevation of serum creatinine, suggesting altered GBM composition conceivably due to the defective interactions with other glycoproteins.²⁴ Unfortunately, permeability studies related to the charge-selective properties were not performed. The knockin perlecan mice Hsph^{2Δ3/Δ3} lack HS chains but did not reveal any obvious ultrastructural abnormalities and had significantly increased urinary protein excretion following a protein overload.²⁵ The anionic sites were evaluated only in the lamina rara externa; thus, a detailed view of the renal phenotype in terms of charge characteristics of the glomerular capillary is lacking in this mouse model as well. Likewise, the current study by Harvey et al,¹⁰ although it may not be comprehensive, is noteworthy because it at least documents the absence of proteinuria in agrin-deficient mice, suggesting that the gene disruption of one class of sulfated PGs is not sufficient enough to alter the permeability of glomerular capillary wall. This may be because agrin is not highly glycosylated/sulfated to impart sufficient electronegativity, and moreover, the subendothelial anionic sites remained intact in neonatal agrin-deficient mice, suggesting a normal maturation of the GBM as far as the contribution of the endothelium is concerned. Another factor that should be borne in mind is that the disruption of one class of PGs may lead to minor increases in the fractional clearance of macromolecules that are insufficient to overcome tubular maximum. As a result, the proteins cannot be detected in the urine, which means the warranting of the essential tracer studies using electron microscopy as pioneered by Farquhar and Palade^{2,3} decades ago.

Concluding Remarks

In reconciliation with past tracer permeability experiments and current understanding of pathogenesis of proteinuria from knockout and knockin mice, it seems obligatory to conclude that the integrated functions of all strata of the glomerular capillary wall are essential to maintain its permeability characteristics. With the disruption of any component, either of slit diaphragm or GBM, one would anticipate a compromise in the barrier functions of the capillary wall. Moreover, a structural change in a given component at times induces conformational change in other components of the capillary wall. This is highlighted by the experiments in which reactive oxygen species-mediated disruption of the α -dystroglycan/matrix transmembrane complex led to a disordered organization of the fibrillar components of lamina rara externa as well as foot process effacement, which would conceivably result in detachment of the podocytes and ensuing proteinuria.²⁶ Such may be the case since neuraminidase-induced detachment *in situ* leads to increased permeability of ferritin (Figure 3B).²⁷ This notion is further supported by the fact that its intravenous administration results in an increased urinary albumin excretion.²⁸ The reactive oxy-

gen species that are generated in a wide variety of renal disease states do induce damage to the GBM, including the PGs, besides the structural perturbations in the slit diaphragm and podocytes.^{29,30} All in all, it means that all components of the capillary wall operate in synchrony to maintain normal homeostasis and integrated functions of the renal glomerulus.

References

1. Farquhar MG, Palade GE: Behavior of colloidal particles in the glomerulus. *Anat Rec* 1959, 133:378–379
2. Farquhar MG, Wissig SL, Palade GE: Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J Exp Med* 1961, 113:47–66
3. Farquhar MG, Palade GE: Glomerular permeability. II. Ferritin transfer across the normal glomerular capillary wall in nephritic rats. *J Exp Med* 1961, 114:699–716
4. Venkatachalam MA, Karnovsky MJ, Fahimi HD: Glomerular permeability: ultrastructural studies in experimental nephrosis using horseradish peroxidase as a tracer. *J Exp Med* 1969, 130:381–399
5. Venkatachalam MA, Cotran RS, Karnovsky MJ: An ultrastructural study of glomerular permeability in experimental nephrosis using catalase as a tracer protein. *J Exp Med* 1970, 132:1168–1180
6. Rennke HG, Cotran RS, Venkatachalam MA: Role of molecular charge in glomerular permeability. Tracer studies with cationized ferritins. *J Cell Biol* 1975, 67:638–646
7. Brenner BM, Hostetter TH, Humes HD: Molecular basis of proteinuria of glomerular origin. *N Engl J Med* 1978, 298:826–833
8. Kanwar YS, Farquhar MG: Presence of heparin sulfate in the glomerular basement membrane. *Proc Natl Acad Sci USA* 1979, 76:1303–1307
9. Kanwar YS, Linker A, Farquhar MG: Increased permeability of the glomerular basement membrane to ferritin after enzymatic removal of heparan sulfate. *J Cell Biol* 1980, 86:688–693
10. Harvey SJ, Jarad G, Cunningham J, Rops AL, van der Vlag J, Berden JH, Moeller MJ, Holzman LB, Burgess RW, Miner JH: Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. *Am J Pathol* 2007, 171:139–152
11. Kanwar YS, Venkatachalam MA: Ultrastructure of glomerulus and juxtaglomerular apparatus. *Handbook of Physiology*. Edited by EE Windhager. New York, Oxford University Press, 1992, pp 3–40
12. Rodewald R, Karnovsky MJ: Porous substructure of the glomerular slit diaphragm in rat and mouse. *J Cell Biol* 1974, 60:423–433
13. Miner JH: Renal basement membrane components. *Kidney Int* 1999, 56:2016–2024
14. Murdoch AD, Dodge GR, Cohen I, Tuan RS, Iozzo RV: Primary structure of the human heparin sulfate proteoglycan from basement membrane (HSPG2/Perlecan). *J Biol Chem* 1992, 267:8544–8557
15. Oh SP, Warman ML, Seldin MF, Cheng SD, Knoll JH, Timmons S, Olsen BR: Cloning of cDNA and genomic DNA encoding human type XVIII collagen and localization of the α 1(XVIII) collagen gene to mouse chromosome 10 and human chromosome 21. *Genomics* 1994, 19:494–499
16. Tsen G, Halfter W, Kroger S, Cole G: Agrin is a heparin sulfate proteoglycan. *J Biol Chem* 1995, 270:3392–3399
17. Pavenstädt H, Kriz W, Ketzler M: Cell biology of the glomerular podocyte. *Physiol Rev* 2003, 83:253–307
18. Cauffman JP, Farquhar MG: The permeability of glomerular capillaries to graded dextrans. Identification of the basement membrane as the primary filtration barrier. *J Cell Biol* 1974, 63:883–903
19. Jarad G, Cunningham J, Shaw AS, Miner JH: Proteinuria precedes podocyte abnormalities in *Lamb2*^{-/-} mice, implicating the glomerular basement membrane as an albumin barrier. *J Clin Invest* 2006, 116:2272–2279
20. Pätäri-Sampo A, Ihalmio P, Holthofer H: Molecular basis of glomerular filtration: nephrin and the emerging protein complex at the podocyte slit diaphragm. *Ann Med* 2006, 38:483–492
21. Jeansson M, Haraldsson B: Glomerular size and charge selectivity in

- mouse after exposure to glucosaminoglycan-degrading enzymes. *J Am Soc Nephrol* 2003, 14:1756–1765
22. van den Born J, van den Heuvel LP, Bakker MA, Veerkamp JH, Assmann KJ, Berden JH: A monoclonal antibody against GBM heparin sulfate induces an acute selective proteinuria in rats. *Kidney Int* 1992, 41:115–123
 23. Raats CJ, van den Born J, Berden JH: Glomerular heparin sulfate alterations: mechanisms and relevance for proteinuria. *Kidney Int* 2000, 57:385–400
 24. Utriainen A, Sormunen R, Kettunen M, Carvalhaes L, Sajanti E, Eklund L, Kauppinen R, Kitten G, Pihlajaniemi T: Structurally altered basement membranes and hydrocephalus in type XVIII collagen deficient mouse line. *Hum Mol Genet* 2004, 13:2089–2099
 25. Morita H, Yoshimura A, Inui K, Ideura T, Watanabe H, Wang L, Soininen R, Tryggvason K: Heparan sulfate of perlecan is involved in glomerular filtration. *J Am Soc Nephrol* 2005, 16:1703–1710
 26. Kojima K, Davidovitas A, Poczewski H, Langer B, Uchida S, Nagy-Bojarski K, Hovorka A, Sedivy R, Kerjaschki D: Podocyte flattening and disorder of glomerular basement membrane are associated with splitting of dystroglycan-matrix interaction. *J Am Soc Nephrol* 2004, 15:2079–2089
 27. Kanwar YS, Rosenzweig LJ: Altered glomerular permeability in aminonucleoside nephrosis as a result of focal detachment of the visceral epithelium. *Kidney Int* 1982, 21:565–574
 28. Wijnhoven TJ, Lensen JF, Wismans RG, Lamrani M, Monnens LA, Wevers RA, Rops AL, van der Vlag J, Berden JH, van den Heuvel LP, van Kuppevelt TH: In vivo degradation of heparin sulfate in glomerular basement membrane does not result in proteinuria. *J Am Soc Nephrol* 2007, 18:823–832
 29. Kashihara N, Watanabe Y, Makino H, Wallner EI, Kanwar YS: Selective decreased de novo synthesis of glomerular proteoglycans under the influence of reactive oxygen species. *Proc Natl Acad Sci USA* 1992, 89:6309–6313
 30. Greiber S, Muller B, Daemisch P, Panenstadt H: Reactive oxygen species alter gene expression in podocytes. *J Am Soc Nephrol* 2002, 13:86–95