Abnormal Glomerular Permeability Characteristics in Diabetic Nephropathy

Implications for the therapeutic use of low–molecular weight heparin

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The physicochemical characteristics of the glomerular capillary filtration membrane restrict the passage of macromolecules on the basis of molecular weight, charge, and shape. The proposed ionic charge permselectivity characteristics of the glomerular basement membrane (GBM) are determined by its chemical composition, primarily the highly sulfated glycosaminoglycan heparan. In diabetic nephropathy, the heparan sulfate content of the GBM is diminished. It has been proposed that decreased GBM heparan sulfate content causes decreased permselectivity to negatively charged macromolecules such as albumin, allowing this protein to leak into the urinary space. One possible explanation for decreased GBM heparan sulfate content in diabetic nephropathy is the observation that heparanase, an enzyme capable of degrading heparan sulfate, is upregulated in the glomerular epithelial cell (GEC) in response to increased glucose. Increased GEC heparanase activity has been demonstrated in glomeruli in diabetic kidneys, and increased urine heparanase has been observed in diabetic nephropathy. In vitro studies have shown that GEC heparanase activity depends on the glucose concentration of the culture medium. GEC heparanase activity can be inhibited by heparin compounds. Sulodexide, an orally active low–molecular weight heparin, has been shown to lower urine albumin excretion. The working hypothesis that has emerged is that sulodexide may be an in vivo heparanase inhibitor that reaches the glomerular capillary wall and prevents heparan sulfate degradation, thus allowing reconstruction of heparan sulfate content and restoration of GBM ionic permselectivity. Two clinical trials are currently being carried out to determine whether sulodexide is renoprotective in diabetic nephropathy.

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Alteration of the permeability characteristics of the glomerular capillary wall (manifest clinically as abnormal albuminuria) is an early expression of diabetic kidney disease. The normal selectivity permeability characteristics of the glomerular capillary wall allow a high permeability to water and small molecules. Despite the enormity of the amount of filtrate that is generated during the course of the day, the glomerular capillary wall efficiently and selectively prevents macromolecules from entering the urinary space. In normal humans, the 100 liters of filtrate that pass the filter each day require the retention of 4,000 g albumin. The sieving characteristics of the glomerular capillary wall have been studied extensively. A size-selective barrier exists that rejects the largest of proteins, such as albumin and immunoglobulins. In addition, a charge selective electrostatic barrier greatly restricts the passage of anionic molecules such as albumin (1–3). Experimental models using a variety of molecules of different radius and charge density have revealed that the glomerular capillary barrier is selective not only for size and charge of the molecule but also for shape (4). In the context of this sophisticated function of the glomerular capillary wall, one must emphasize that the remarkable ability of the structures that make up the filtration barrier carry out this function without becoming clogged or fouled. It is safe to say that no commercially available filtration membrane has these impressive functional characteristics. It is therefore not surprising to find that the known structure of the glomerular capillary wall is complex (Fig. 1) and getting even more complex with the passing months. The site of the actual barrier to the filtration of macromolecules has been debated. As noted in Fig. 1, the slit diaphragm that exists between the pedicels of the glomerular epithelial cells on the outer side of the glomerular basement membrane (GBM) has become increasingly well characterized (5). Disruption of this structure leads to the appearance of large amounts of protein in the urine. This was first shown by Tryggvason (6) and his colleagues who described nephrin, a critical component of the slit process structure, and showed that inherited absence of nephrin was associated with proteinuria in the Finnish form of congenital nephrosis (7).

CHARGE SELECTIVE BARRIER AND HEPARAN SULFATE

— Impedance of the glomerular capillary wall to macromolecular filtration probably occurs at sites in the glomerular capillary wall other than the glomerular filtration slits (8–11). Evidence supports the GBM serving an important function in this respect. An electrostatic impedance to the passage of negatively charged macromolecules appears to reside in the GBM and is attributed to the presence of glycosaminoglycans (9–15). The GBM has three layers that can be demonstrated ultrastructurally: the lamina rara interna, the lamina densa, and an outermost lamina rara externa. The negatively charged glycosaminoglycan content appears to be localized in the lamina rara interna and lamina rara externa. These anionic sites are composed of richly sulfated glycosaminoglycans, particularly hepa-

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Abbreviations: GBM, glomerular basement membrane.

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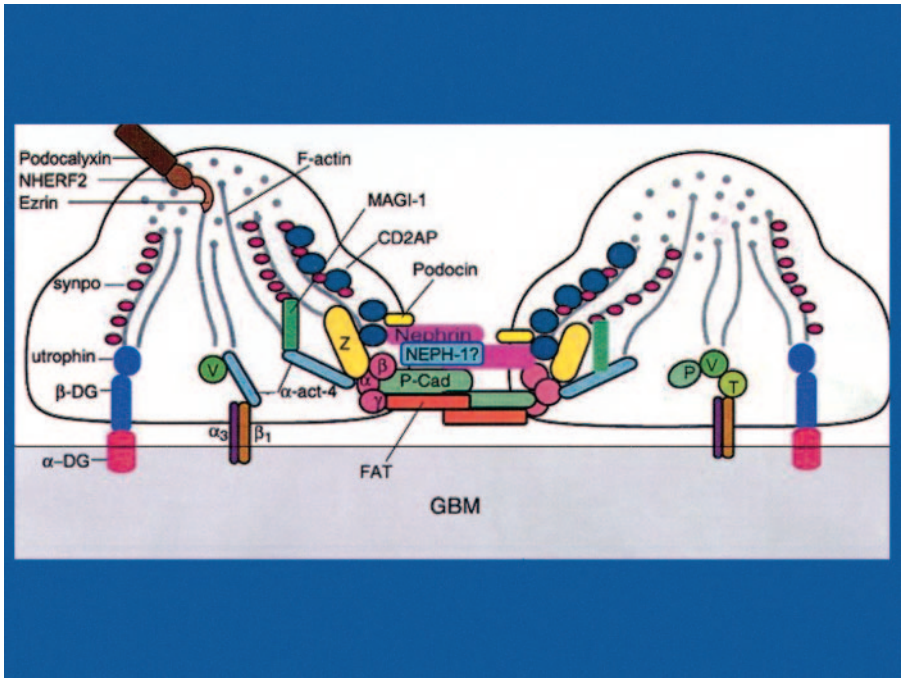

Figure 1— *A graphic reproduction of two adjacent glomerular epithelial cell foot processes with an interposed slit diaphragm. The molecular composition of the slit diaphragm interacts with the cytoskeleton of the epithelial cells. Among the known elements in this interaction are α-act-4 (α-actinin-4), α3 β1 (α3 β1 integrin), α-DG (α-dystroglycan), β-DG (β-dystroglycan), NHERF2 (sodium/hydrogen exchanger regulatory factor 2), P (Paxillin), P-cad (P-cadherin), synpo (synaptopodin), T (talin), V (Vinculin), CD2AP (CD2-associated protein), and GBM (glomerular basement membrane). Reproduced with permission from Mundel and Shankland (5).*

ran sulfate (16–18). Heparan is a glycosaminoglycan that consists of a regular repeating sequence of nonsulfated disaccharide units that are composed of D-glucosamine (or L-iduronic acid) and *N*-acetyl-D-glucosamine. The heparan sulfate disaccharide chain has up to 300 disaccharide units. This polysaccharide is highly sulfated and attached to a core protein. The most common basement membrane core protein associated is agrin, although perlecan and collagen XVIII have also been identified as associated core proteins. Agrin is a 212-kDa protein that has three heparan sulfate attachment sites. Heparan sulfate is not only present within the GBM but also can be found on the surface of endothelial and epithelial cells (19).

The neutralization of anionic sites within the glomerular capillary wall is associated with a loss of charge-dependent glomerular permselectivity. Hunsicker et al. (20) infused the polycation hexadimethrine into rats and showed an immediate loss of permselectivity with the appearance of albumin in the urine. With further infusion of this polycation, the permselectivity changes not only caused albuminuria but also the passage of larger molecules. These results revealed that the neutralization of the anionic structure of the GBM was not only responsible for charge selectivity characteristics, but also affected the size-selective pore-like behavior of the membrane. The infusion of specific enzymes, such as bacterial heparitinase, which is capable of selectively depleting the GBM of heparan-associated negative charges, was also associated with the appearance of abnormal amounts of albumin in the urine (12,21). Alteration of heparan sulfate quantitatively or functionally significantly alters glomerular permselectivity. The administration of a specific monoclonal antibody reactive with heparan sulfate causes albuminuria in rats (22). More recently, it has been shown that mice deficient in the basement membrane glycoprotein laminin developed albuminuria (23). The permselectivity defect in these laminin-deficient mice appeared to be due to disorganization in the distribution of anionic sites within the lamina rara interna and externa. These observations all served to emphasize the importance of intact glycosaminoglycan-dependent anionic sites for normal glomerular permselectivity.

GLOMERULAR HEPARAN SULFATE IN THE DIABETIC STATE

— The diabetic state is associated with a decrease in the glycosaminoglycan composition of the GBM (24–30). Decreased heparan sulfate content has been demonstrated biochemically and ultrastructurally in both human (23,26,27,29) and experimental (24,25,28) diabetes. In experimental diabetes, GBM heparan appears to be under-sulfated (31). These biochemical abnormalities correlate with functional studies that reveal both charge-selectivity and size-selectivity alterations in glomerular capillary wall permselectivity to be associated with albuminuria in the early diabetic state (32–35).

The cause of diminished GBM heparan sulfate content in diabetes remains a subject of substantial interest. Katz et al. (36) have proposed that heparan sulfate proteoglycan metabolism within the glomerulus may be altered by virtue of excessive activity of heparanase, which is an endo-β-D-glucuronidase that cleaves the heparan sulfate polysaccharide molecule. They reported increased heparanase activity in urine samples derived from patients with type 1 diabetic nephropathy. These investigators also observed that heparanase activity could be detected in lysates of cultures of glomerular epithelial and mesangial cells and implied that excessive urinary heparanase may have derived from these intrinsic glomerular cells, which could ultimately explain glomerular heparan sulfate deficiency in the diabetic state (36).

Xu et al. (37) focused attention on glucose-induced increased expression of the heparanase-1 gene in the glomerular epithelial cell. Incubation of cultured human glomerular epithelial cells in high-glucose medium was associated with increased heparanase-1 activity. A specific glucose receptor appears to activate the Ras/B Raf kinase system, which ultimately results in activation of the gene responsible for heparanase-1 (37–39) (Fig. 2). In accord with the proposal that the activity of this enzyme is enhanced in the diabetic state, heparanase-1 activity was readily demonstrated immunochemically in the glomerular epithelial cells in biopsy material taken from patients with diabetic nephropathy, but not in nondiabetic specimens (37–40) (Fig. 3). In addition, as noted by Katz et al. (36), abnormal amounts of heparanase

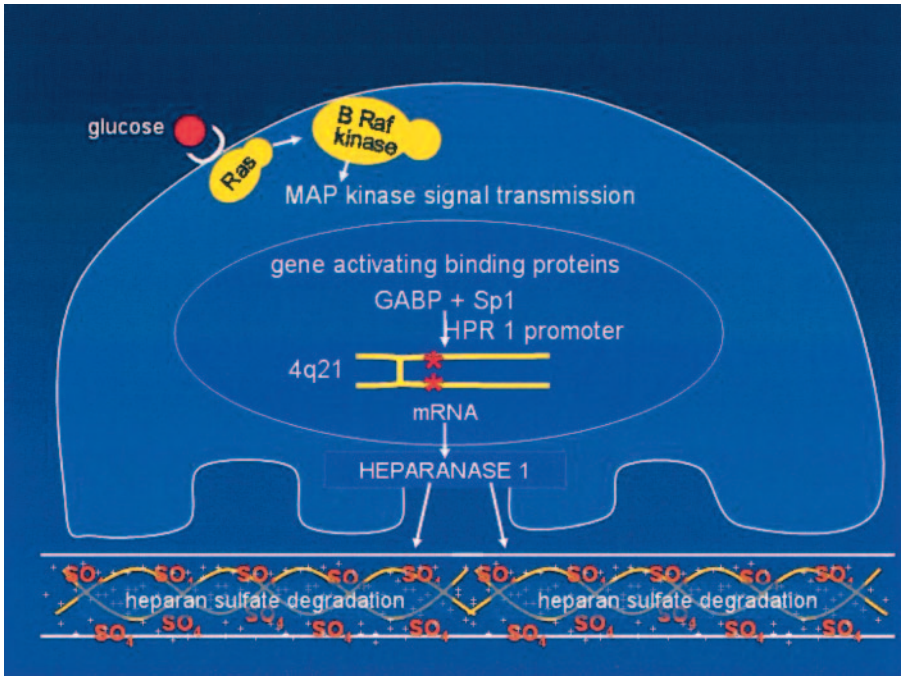

Figure 2—*Hypothetical pathogenesis of abnormal glomerular basement membrane permselectivity in diabetes: a graphic demonstration of the biochemical pathway linking high-glucose environment with the increased production of heparanase-1 by the glomerular epithelial cell. It is proposed that a specific cell-surface glucose receptor is present, which transmits a signal from Ras binding protein. The signal is then relayed to the BRaf kinase system, which activates the mitogen-activated protein (MAP) kinase signal transmission to the nucleus. This signal relay system results in activation of an intranuclear gene activating binding protein specific for the heparanase-1 promoter gene, which is present on the long limb of the fourth chromosome, with ultimate production of specific messenger RNA. It is proposed that glucose-induced increased heparanase-1 production by the glomerular epithelial cell results in degradation of glomerular basement membrane heparan sulfate. The + signs indicate the interaction of cations, particularly sodium, with the negative charges on the heparan sulfate molecule. The cationic "cloud" formed by these ions would be responsible for an osmotic force maintaining the gel structure of the glomerular basement membrane. The loss of heparan sulfate anionic charge decreases the cationic content of the glomerular basement membrane and may be responsible for alteration of gel structure and the loss of size selectivity as well as charge selectivity of this structure in this circumstance.*

were detected in the urine of patients with diabetic nephropathy (37).

In vitro cell culture studies have revealed that heparanase-1 gene expression is upregulated in cultured renal epithelial cells when they are placed in high-glucose media (37). To determine the effects of enhanced expression of heparanase-1 gene activity in a high-glucose environment, the cell surface heparan sulfate content of cultured glomerular epithelial cells was quantified in low- and high-glucose media (37). The results revealed that the cells cultured in high-glucose medium demonstrated a diminished cell surface heparan sulfate content and that this could be reversed by the addition of heparanase inhibitors including heparin and sulodexide (Fig. 4). Sulodexide is a soluble mixture of glycosaminoglycans derived from porcine viscera. The major components of sulodexide are low–molecular weight heparin sulfate (~80%) and dermatan sulfate (~20%).

When rat or human glomerular epithelial cells were grown on an artificial basement membrane that contained heparan sulfate proteoglycan (Matrigel), the permeability of the artificial membrane to albumin increased when the cells were incubated in high-glucose medium (37). This phenomenon was reversed by the addition to the medium of heparanase inhibitors including heparin or sulodexide.

These studies agree with recent reports of decreased heparan sulfate content in biopsy material from patients with type 2 diabetic nephropathy (40,41). An increase in glomerular heparanase activity can be demonstrated in experimental streptozotocin-induced diabetic nephropathy (41). This increased heparanase activity is associated with decreased glomerular heparan sulfate content and albuminuria (42,43).

Increased glomerular heparanase content has been described in several experimental nephropathies other than diabetes (44–47). The employment of an inhibitor of heparanase has been associated with decreased proteinuria in these experimental conditions (46,47).

HEPARIN AND HEPARINOIDS: THERAPEUTIC EFFECT

— Several investigators have administered heparin compounds, including oral sulodexide, and reported a decrease in the albumin excretion rate in patients with diabetic nephropathy (48–55). Gambaro et al. (56) carried out a study in 223 patients with type 1 or type 2 diabetes (the Diabetic Nephropathy and Albuminuria Study [DiNAS]) and either microalbuminuria or overt nephropathy. These investigators showed that treatment with 200 mg/day sulodexide for 4 months significantly lowered the albumin excretion rate in this patient population. They also reported that the decrease in albumin excretion was maintained for 2 months after the drug was stopped. The Collaborative Study Group carried out a pilot trial in 135 subjects with type 2 diabetes and a urine albumin excretion between 30 and 300 mg/day (57). The purpose was to confirm the findings of the DiNAS study and finalize a protocol for a large definitive trial. All patients in this pilot trial were treated with either an ACE inhibitor or an angiotensin receptor blocker in the highest dose approved by the Food and Drug Administration. In addition, careful attention was paid to blood pressure control. The employment of these therapeutic measures resulted in the patient population of this study having more advanced glomerular disease than previously reported for "microalbuminuria," since one would expect the urine albumin excretion rate to be significantly lowered by blood pressure control and renin-angiotensin system inhibitors before baseline measurements were taken. Patients received coded medication for 6 months with a follow-up of 2 months after cessation of treatment. The primary end point was the achievement of either normal albumin excretion or (at least) halving of the initial baseline albuminuria. In the preliminary analysis reported for this trial, patients receiving sulodexide achieved the primary end point over twice as often as those in the placebo group. Approximately 26% of

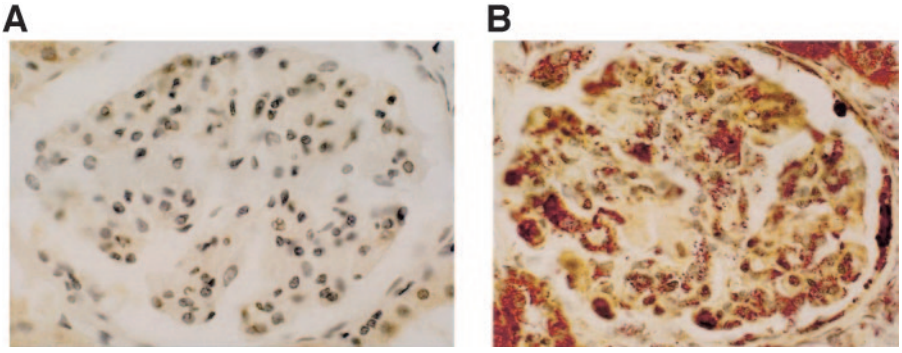

Figure 3—A *and* B: *Immunohistochemical staining of heparanase-1 expression in kidneys with or without diabetic nephropathy. In a study of autopsy or biopsy specimens from 50 diabetic patients (17 glomerulopathic patients and 12 normal kidney tissue specimens), heparanase-1 signals were intense in the visceral and parietal epithelial cells, glomeruli, and tubules of the cortex in kidneys with diabetic nephropathy* (B). *Heparanase-1 signals were absent or minimal in the glomeruli of biopsies from patients with a variety of glomerulopathies and autopsy specimens from normal kidneys (37).*

patients receiving sulodexide either achieved normoalbuminuria or halved the albumin excretion compared with 10.5% in the placebo group. The majority of patients achieving an end point maintained decreased albuminuria for 2 months after coded medication had been stopped.

It has been proposed that the results of the DiNAS study and the Collaborative Study Group pilot trial are in accordance with the observation of others that heparin therapy is capable of decreasing albuminuria in diabetic nephropathy. The demonstration that sulodexide appears to be orally active is of significant therapeutic consequence, and two large-scale clinical trials are currently being undertaken in populations of patients with type 2 diabetic nephropathy and "microalbuminuria" and in overt diabetic nephropathy.

CONCLUSION — The evidence that heparanase activity is increased in the glomerulus in diabetic nephropathy and GBM heparan sulfate decreased has led to the implication that the proposed mechanism for the action of sulodexide resides in its properties as a heparanase inhibitor. Certainly ample in vitro evidence supports this latter function of heparin and heparinoid compounds. Other potential mechanisms have also been considered. Heparin has several well-documented pharmacologic actions; among these is the ability to decrease transforming growth factor-β production. This effect has been documented in cultured mesangial cells, and the implication that this could explain efficacy in experimental diabetic nephropathy has been put forward (58–61). Heparanase inhibition and restoration of intrinsic capillary wall heparan sulfate content may well account for the observation of a period of delay before albumin excretion rate decreases after initiation of the drug and the continued decrease in albuminuria for months after the drug is stopped. If the larger definitive Collaborative Study Group trials support the preliminary observations that sulodexide is efficacious in reversing the abnormal glomerular permselectivity in the

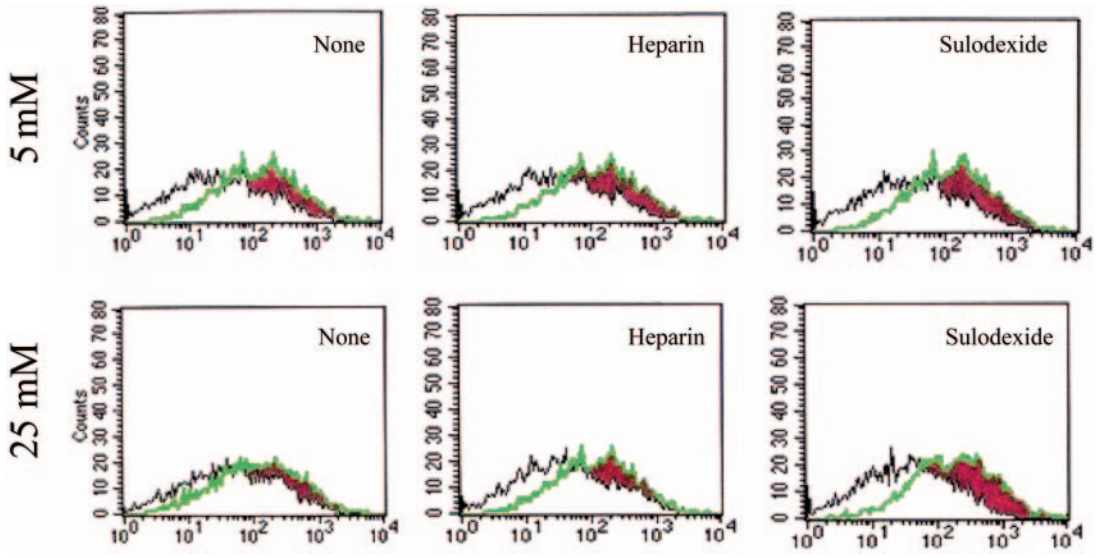

Figure 4— *Flow cytometric analysis of cell surface heparan sulfate expression of rat glomerular epithelial cells. Cell cultures were grown in complete medium containing 5 or 25 mmol/l glucose for 48 h in the presence of heparin or sulodexide or in their absence. Heparin and sulodexide concentrations were 50 μg/ml. Depicted are the comparisons between cell staining with control fluorescein-labeled mouse IgM (black curves) and staining with specific monoclonal antiserum to heparan sulfate (gray curves) (clone Hep SS; Seikagaku, Tokyo). The quantification of cell surface heparan sulfate by cell sorting was determined by the number of cells (horizontal axis) exhibiting a given intensity of fluorescein staining (vertical axis). The difference between the control and antibody-treated curves represents the quantification of the amount of heparan sulfate on the surface of the cells. When cells were incubated in high-glucose medium (25 mmol/l), substantially less surface heparan sulfate was measured (lower left) relative to cells grown in low-glucose medium (5 mmol/l)* (upper left, center, *and* right). *Heparin and sulodexide restored the cell surface heparan sulfate content* (lower middle *and* lower right). *These results imply increased heparanase-1 activity under conditions of high glucose, which is inhibited by heparin or sulodexide.*

diabetic state, we may well be observing the appearance of a new class of renoprotective agents—heparanase inhibitors.

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