Structure-Function Relationships Associated with Extracellular Matrix Alterations in Diabetic Glomerulopathy

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(J. Am. Soc. Nephrol. 1994; 5:1165–1172)

ABSTRACT
Proteinuria and progressive renal insufficiency are the primary manifestations of diabetic nephropathy. Accumulating evidence suggests that these clinical features can be linked, at least in part, to pathologic changes in the glomerular extracellular matrix. Most evidence suggests that glomerular basement membrane thickening and mesangial matrix expansion consist of at least three elements. These are (1) an accumulation of normal extracellular components; (2) an increase in the novel peptide chains of the normal components of Type IV collagen; and (3) an increase in matrix elements not normally expressed in the glomerulus. The pathogenetic features underlying these changes include increased synthesis and decreased degradation of matrix. Abnormal physicochemical interactions among these matrix elements likely contribute to alterations in three-dimensional structure, leading to proteinuria and loss of glomerular basement membrane filtering surface area. Many of these changes may be explained in whole or in part by direct or secondary effects of hyperglycemia, as well as by hemodynamic changes.

Key Words: Diabetic glomerulosclerosis, collagen, advanced glycosylation end products, extracellular matrix

Diabetic renal disease is characterized clinically by two major features—proteinuria and progressive renal insufficiency. A growing body of evidence suggests that each of these reflects, at least partly, pathologic changes in the glomerular extracellular matrix (ECM). This article will review what is currently known regarding the composition of the normal glomerular ECM, what biochemical changes occur in diabetes, proposed mechanisms responsible for these changes, and where possible, pathophysiologic correlations. In discerning the first of these clinical features, e.g., proteinuria, the focus of this review will be on the structure of the glomerular basement membrane (GBM) in diabetes. In examining the second, e.g., progressive renal insufficiency, the focus will be on the glomerular mesangium. Although tubulointerstitial ECM changes have been described both in experimental models (1,2) and in clinical diabetic nephropathy (3), this review will focus exclusively on the changes observed in the glomerulus.

BIOCHEMICAL COMPOSITION OF THE NORMAL GBM

Our understanding of the composition of the ECM continues to evolve. A number of detailed reviews on this subject have been published (4,5). The GBM and mesangial matrix are composed of a mixture of collagens, glycoproteins, proteoglycans, and glycosaminoglycans. The contribution of each varies somewhat in the two locations, making the GBM a composite entity similar, but not biochemically identical, to the mesangial matrix. Furthermore, the localization of particular ECM components in restricted areas of the GBM and mesangial matrix or regional differences in molecular isotypes in these areas further confer specialization of function at the microenvironmental level.

COLLAGEN

Type IV collagen provides the basic structural framework of the glomerular ECM (4,5). Composed of three peptide chains, each molecule has a long triple-helical component beginning at its amino terminus and a nonhelical globular domain at its carboxyl end (4). Both the amino and the carboxy terminals of the Type IV collagen molecule function to promote the cross-linking of collagen to form an ECM meshwork into which other ECM elements insert (4).

Classic Type IV collagen (composed of peptide chains COL4A1 and COL4A2), Types V and VI collagen, and fibronectin colocalize in a similar distribution in the glomerular subendothelial area and the mesangium (6,7). However, Type IV collagen traverses the entire width of the GBM (8–11). Recent data suggest that, unlike the collagen in the subendothelial region, Type IV collagen in the subepithelial region of the GBM is composed of novel peptide chains.
(COL4A3 and 4A4), potentially reflecting a difference in the cellular site of synthesis (7,12). COL4A3 and 4A4 are also present in the lamina densa. It has been proposed that the classic Type IV collagen of the GBM is synthesized by endothelial cells and the novel Type IV collagen is synthesized by visceral epithelial cells and that the classic and novel networks of Type IV collagen may be separate (7,8). This demonstrates the potential for local ECM glycoprotein isotypes to create unique microenvironments in the glomerulus. The precise distribution of the other Type IV collagen isotypes (e.g., COL4A5 and COL4A6) in the glomerulus remains to be defined.

**Fibronectin**

Fibronectin is a glycoprotein that is found in the plasma (13) as well as in the basement membrane and the mesangium of the glomerulus (14). Like Type IV collagen, it is capable of self-aggregation (15), although in the case of fibronectin, dimers are formed. It has specific recognition sites for the attachment of other ECM components, including collagen and glycosaminoglycans, and for the binding of serum proteins, such as fibrin (16). It is capable of binding cells via cell membrane integrins (17).

**Laminin**

The glycoprotein laminin is a third important component of the glomerular ECM. Laminin plays a major role in embryogenesis, cell growth, and differentiation (18), and has specific recognition sites for the binding of cells via integrin and nonintegrin receptors (19-21). The latter facilitates cell attachment and migration (21). Furthermore, laminin has additional binding sites for glycosaminoglycans (22) and attaches to collagen via a nidogen bridge (23). Laminin, like Type IV collagen and fibronectin, is capable of self-aggregation (24).

In the GBM, laminin is distributed predominantly in apposition to cells, reflecting its important cellular interactive functions. It is present in the subendothelial, subepithelial, and mesangial areas (6). Isotype distribution in the adult human glomerulus is not yet well established (25). Biochemists now hypothesize that, in the synthesis of basement membranes, laminin and Type IV collagen networks form independently and then intertwine (26).

The matrices of the glomerulus have a net negative charge. In the GBM, this is conferred predominantly by heparan sulfate proteoglycan (27), whereas in the mesangium, it largely reflects the presence of chondroitin sulfate proteoglycan. A large number of additional matrix elements have been found in basement membranes (28,29) and may also be present and functionally important in the glomerulus.

Basement membrane biochemists differ in opinion somewhat regarding the precise spatial orientation of the ECM components in the GBM. The prevailing view is that these ECM elements are arranged in a loose meshwork (4,24). Using these models, one may speculate that the type, amount, and charge of the ECM molecules that are present and the nature of their chemical binding define the physical properties of the GBM by modulating GBM pore size and charge. In so doing, to a large extent, the ECM of the GBM likely determines the size and the charge-selective properties of the GBM.

**Proteinuria: Structure—Function Relationships in the Diabetic GBM**

Proteinuria in diabetic nephropathy is a function of both structural and hemodynamic alterations. Thus, proteinuria is due to the increased permeability of the glomerular capillary wall coupled with intraglomerular hypertension. The latter may contribute in both the initiation and the exacerbation of the structural defect. Structurally, increased permeability may be due to capillary cellular dysfunction, basement membrane alterations, or both. Direct evidence for a cellular contribution to the increased glomerular capillary wall permeability in diabetic nephropathy is currently lacking. However, a high transcapillary albumin escape rate and impaired fibrinolytic capacity have been reported in diabetic patients with incipient and overt nephropathy, suggesting a diffuse defect of the vascular endothelium (30,31). Furthermore, although the role of capillary wall cells in maintaining glomerular size selectivity under physiologic conditions has been controversial, recent experimental work by Daniels et al. suggests a significant contribution of these cells in maintaining glomerular impermeability to large-molecular-weight substances (32). Finally, the occasional discordance between the amount of proteinuria observed in diabetic patients and the degree of GBM thickening in these individuals points to factors besides basement membrane structure as important determinants of proteinuria (33). Thus, indirect evidence suggests the possibility that a cellular contribution may be present in the increase of glomerular permeability in diabetic nephropathy.

Changes in the biochemical structure of the thickened GBM in diabetes have been more thoroughly investigated than the cellular alterations, and a relationship between proteinuria and the biochemical composition of the GBM has long been posited. In early and moderately advanced diabetic nephropathy, immunofluorescence microscopy showed that the thickened GBM is composed of an increase in Types IV and V collagen, laminin, and serum proteins (6). Less of the classic Type IV collagen peptide chains and more novel chains and plasma proteins appear to contribute to GBM thickening in severe diabetic nephropathy (6,34).

**The Contribution of Type IV Collagen to GBM Thickening**

Because of its importance in the overall structure of the GBM, it has been postulated for the last 30 yr that
GBM thickening in diabetes might be due to increased synthesis or decreased degradation of Type IV collagen. Evidence to support this hypothesis comes largely from either experimental animals or in vitro studies. These studies have often equated the products obtained from glomerular ECM extraction with GBM material and [3H]proline label incorporation with GBM synthesis. Because GBM and mesangial matrix extraction and synthesis cannot be differentiated with these kinds of experiments, some caution is appropriate in extrapolating these data to the GBM per se. However, glomeruli from diabetic rats incorporated more [14C]lysine into collagen than glomeruli from control rats (35,36). Increased amounts and/or activity of enzymes required for the posttranslational modification of Type IV collagen have been found in diabetic glomeruli (37). Increased glomerular [3H]proline incorporation has been noted in experimental diabetes in vivo, suggesting increased basement membrane synthesis (38,39). More hydroxyproline has been extracted from the GBM of diabetic patients than from controls (40). Finally, although not incontrovertibly reflective of basement membrane synthesis, serum and urinary peptide components of Type IV collagen are increased in diabetes in both experimental and clinical settings (39,41,42). However, evidence for increased synthesis in diabetes has not been universally found when sought (43,44), and relatively little corroborative evidence is available in human tissue.

Decreased degradation of Type IV collagen has also been suggested as a mechanism by which the GBM thickens in diabetes. In experimental models, a slow turnover rate of Type IV collagen has been described (45), as well as diminished activity and/or quantity of degradative enzymes (46–48).

**THE CONTRIBUTION OF NONENZYMATIC GLYCOXYLATION TO GBM THICKENING**

In recent years, a decrease in the turnover of ECM in diabetes has been attributed to the accumulation of proteins that have undergone nonenzymatic glycosylation (49,50). In this process, glucose initially binds reversibly to free amino groups on proteins forming Schiff bases, which arise within hours of the interaction of glucose with protein. Through a series of biochemical rearrangements, the glycosylated Schiff bases become more stable, forming Amadori products within days to weeks, which are partially irreversible. Further stabilization of the Amadori product over many weeks results in the chemically irreversible advanced glycosylation end product. It has been proposed that glycosylation of structural proteins in the ECM by this mechanism results in stable cross-linking. This is posited to inhibit the enzymatic degradation of ECM, thereby facilitating accumulation and GBM thickening.

Nonenzymatic glycosylation may contribute to GBM thickening by at least two other mechanisms besides its contribution to diminished degradation. First, nonenzymatic glycosylation of GBM proteins may alter the structure of the GBM by interfering with the function of specific recognition sites on ECM proteins that facilitate the interactions of ECM components. As an example, fibronectin binds less of the glycosaminoglycan heparin when it is glycosylated than when it is not glycosylated (51). In addition, laminin loses its classic cruciform shape when it is glycosylated, becoming a more dense, less elongated molecule, as seen by rotary shadowing electron microscopy (52). This glycosylated form of laminin advances more slowly on electrophoretic gels than does nonglycosylated laminin, demonstrating that the shape change confers an alteration in at least one physical property of the molecule (52). One may speculate that the shape changes induced by glycosylation might also conceal or diminish the availability of recognition sites that might be important in the three-dimensional assembly of the GBM. For instance, in laminin molecules, the sites for the binding of collagen through a nidogen bridge and for glycosaminoglycan binding are both present in relatively central positions that may be obscured by changes in shape (53,54). Finally, glycosylation may contribute to GBM thickening by facilitating the deposition of serum proteins at glycosylated sites on ECM proteins. In vivo, glycosylated collagen has been shown to bind more low-density lipoprotein than does nonglycosylated collagen (55). In vivo, the diabetic GBM is characterized by the "pseudolinear" deposition of albumin, immunoglobulin, and complement (56,57). It has been shown that this binding may be facilitated by nonenzymatic glycosylation (58). Currently, the physicochemical changes described have not been directly implicated as causative of GBM thickening. However, it is reasonable to posit that the thickened GBM in diabetes reflects numerous mechanisms. These likely include increased ECM synthesis, decreased ECM degradation, altered matrix interactions due to nonenzymatic glycosylation, altered matrix localization across the GBM, and nonspecific trapping of serum proteins.

**STRUCTURE-FUNCTION CORRELATIONS—SIZE-SELECTIVE PROTEINURIA**

Studies in patients with incipient or overt diabetic nephropathy demonstrate a correlation (albeit an imperfect one) between GBM thickening and proteinuria (59). Although there are patients with insulin-dependent diabetes mellitus who have little proteinuria in the face of thickened GBM, in general, there is a relationship between the magnitude of GBM thickening and the amount of urinary protein losses (59).

The qualitative nature of the proteinuria in diabetic patients was initially examined over a decade ago by studies in which the fractional clearance of injected neutrally charged dextrans of a wide range of molecular weights was measured. Compared with normal controls, patients with diabetic nephropathy exhibited a higher fractional clearance of larger molecular
weight neutral dextrans (e.g., ≈50 Å). By mathematical modeling, it was posited that the GBM of such individuals was characterized by uncharged "pores" of large size, which would permit the passage of these molecules (60). In recently published studies of the GBM in patients with diabetes and in controls, high-resolution scanning electron microscopy coupled with morphometry confirmed that the "pores" of the subendothelial and subepithelial GBM are indeed larger in diabetics than in controls (11). The mean pore size, however, was approximately 15 to 20 Å larger than those predicted by the clearance studies with neutral dextrans. There are at least two potential explanations for this size discrepancy. First, the pores accessible for morphometry by scanning electron microscopy were only the surface pores on the superficial aspects of the subendothelial and subepithelial GBM. It is possible that structurally deeper pores not accessible to scanning (and potentially of smaller diameter) may be present. Second, it is possible that consistent fixation artifact altered GBM pore size in the electron microscopy studies. Despite the lack of perfect agreement between physiologic and microscopic measurements of the pore size defect in diabetes, these morphologic studies nevertheless suggest a credible structural counterpart contributing to the functional size-selective defect previously noted in the diabetic GBM.

STRUCTURE-FUNCTION CORRELATIONS—CHARGE-SELECTIVE PROTEINURIA

More recently, a charge-selective defect has also been described in proteinuric diabetic patients. An increase in the fractional clearance of albumin compared with immunoglobulin G has been found in patients with overt diabetic nephropathy by one group of investigators (61) and in patients with incipient as well as overt diabetic nephropathy by another (62).

The charge-selective defect in diabetic nephropathy may also have a structural correlate in the biochemical composition of the GBM. The net negative charge of the glomerular capillary wall is conferred by the presence of anionic substances found predominantly on the cell membranes of glomerular endothelial and visceral epithelial cells, in regularly arranged sites along the subendothelial and subepithelial aspects of the GBM (26), and less abundantly, in the lamina densa. These are composed largely of heparan sulfate proteoglycan (HSPG) (27), molecules with a protein core and sulfated glycosaminoglycan side chains. In an experimental setting, the importance of HSPG in maintaining the charge-selective barrier function of the GBM has been demonstrated by comparing the penetration of injected anionic ferritin across the GBM before and after the enzymatic digestion of HSPG. The enzymatic removal of HSPG from the GBM facilitated the movement of anionic ferritin across the capillary wall (63).

It has been posited that the charge-selective defect in patients with diabetic nephropathy may be due to changes in GBM HSPG. However, it is still controversial whether consistent changes in HSPG ascribable to the diabetic milieu cause functional change. These controversies likely arise from the fact that information regarding HSPG in diabetic nephropathy has been acquired from animal models or human disease at various stages and also from cell culture studies. With these differing approaches, the results have not always been consistent. All of the following abnormalities in proteoglycan metabolism have been described in diabetes or in association with cellular exposure to high glucose concentrations: diminished HSPG synthesis or basement membrane HSPG content (10,64–69); abnormal distribution across the GBM (10); synthesis of subpopulations of HSPG with diminished sulfation (70,71); synthesis of HSPG with diminished GBM affinity (72,73) or with abnormal GBM-cellular interactions (74); and disturbed interactions with the GBM due to primary abnormalities in GBM ECM (72,73). Finally, diminished relative (rather than absolute) quantities of HSPG have been suggested to mediate functional change (75,76). In biopsy material from patients with diabetic nephropathy, anionic sites reflecting GBM HSPG content are diminished (77). However, the decrement is modest at most, appears late in the course of overt nephropathy, and does not occur in patients with microalbuminuria (77). The delay between a discernible absolute decrement in GBM HSPG and proteinuria suggests a lack of a cause and effect relationship, although the pathogenetic effect of early relative decrements of HSPG in thickened GBM cannot be excluded (76,77). Taken together, the data available support the hypothesis that the biochemical structure of the GBM profoundly affects the sieving properties of the glomerular capillary wall (Figure 1).
PROGRESSIVE RENAL INSUFFICIENCY: STRUCTURE-FUNCTION RELATIONSHIPS IN THE DIABETIC MESANGIUM

For most nephrologists, nodular glomerulosclerosis best typifies the histology of the advanced renal lesion of diabetes mellitus. However, this lesion is not common in diabetic patients, occurring in approximately 20% of patients with diabetic nephropathy whose kidneys are examined by biopsy or at autopsy, and in those, appearing in less than 25% of the glomeruli present (78). Thus, there is a poor correlation between the severity of clinical diabetic nephropathy and the presence of these lesions. The lesion most clearly associated with progressive loss of renal function is diffuse diabetic glomerulosclerosis, characterized predominantly by an expansion of mesangial matrix material (59,79). As the mesangium expands, its volume relative to the rest of the glomerulus increases, and it encroaches on the capillary lumen, diminishing the glomerular surface area available for ultrafiltration (59). In the nephropathy of insulin-dependent diabetes, the fractional volume of the mesangial matrix is negatively correlated with peripheral capillary surface area (59) and with creatinine clearance (59).

COMPOSITION OF THE EXPANDED MESANGIAL MATRIX AND MECHANISMS OF ACCRETION

The precise biochemical composition of the increased mesangial matrix material in diabetes is still being defined and remains controversial. Some investigators showed increases in mesangial Types IV, V, and VI collagen, laminin, and fibronectin in early and moderately advanced diabetic nephropathy, but decrements in most of these matrix elements with more advanced disease, and absence in diabetic nodules (6,80). However, others suggested that small increments in Types IV and V collagen, laminin, and fibronectin are present in advanced mesangial lesions and/or in nodules (79-82). Still others reported in human or experimental diabetes the presence of collagens not found in the mesangium under physiologic conditions, including Types I and III (83,84).

Studies of mesangial cells cultured in medium containing a high concentration of glucose suggest that increased synthesis and decreased degradation of matrix material may contribute to the mesangial expansion observed in diabetic nephropathy. Under these conditions, mesangial cells rapidly (in hours to days) increase levels of mRNA for Types I and IV collagen, laminin (85), and fibronectin (85,86) and synthesize more of the respective proteins (85,86) compared with culture conditions with low glucose levels. These data suggest that high concentrations of glucose relatively rapidly facilitate increased transcription and translation of ECM molecules. High-glucose culture conditions also appear to influence matrix accretion either by regulating the synthesis of enzymes that degrade matrix or by modulating the activity of those enzymes. Thus, mesangial cells grown in medium with a high concentration of glucose synthesized more tissue inhibitor of metalloproteinase mRNA (87). The increase in ECM mRNA levels seems to be glucose dependent and oncotic pressure independent; however, the effect on tissue inhibitor of metalloproteinase mRNA appears to be mediated by the oncotic forces exerted by glucose, because mannitol reproduced the glucose effect (87). In vivo, experimental diabetes in rats is also associated with decrements of mRNA levels for matrix metalloproteinases 1 and 3 (88).

The explanation for increased mesangial matrix material in diabetic nephropathy may be more complex, however, than the studies examining the acute effect of high glucose concentrations on mesangial cell ECM synthesis and degradation suggest. Studies in which mesangial cells were exposed to high concentrations of glucose for prolonged periods of time (weeks to months) failed to show the increased ECM mRNA and protein synthesis observed in the acute studies (89). Furthermore, when mesangial cells were incubated with bovine serum albumin that was experimentally modified by advanced glycosylation, there was only a transient increment in mRNA and protein synthesis for Type IV collagen (90). Increments of unknown duration in mRNA for laminin A, B1, and B2 were also measured (90).

Studies of experimental diabetes demonstrated increased mRNA levels for laminin B1 in whole kidney and glomeruli of rats (91,92), but not in the cortex of mice (93). Results for Type IV collagen mRNA are even more diverse, with studies of either whole kidney, cortex, or glomeruli being either unchanged or increased (91-93).

The heterogeneity of results in these in vitro and in vivo studies suggests that the increase in mesangial matrix in diabetic nephropathy may not be mechanistically monolithic and may vary over time. The relative balance between matrix synthesis and degradation in vivo is likely influenced by the composition of the constituent locally responsive cells, by the duration and the degree of hyperglycemia, by local elements influencing cytokine and growth factor secretion, and by insulin therapy and the effects of counterregulatory hormones.

Despite the current uncertainty regarding the precise biochemical composition of the expanded matrix in diabetic nephropathy and the mechanisms whereby this ECM accumulates, some experimental data support the hypothesis that mesangial matrix accretion in diabetes is potentially reversible. Although the advanced glycosylation end product is biochemically irreversible, the glycosylated proteins remain biodegradable, albeit more slowly than nonglycosylated proteins. Mononuclear cells bind glycosylated proteins via specific cell-membrane receptor sites and slowly internalize and degrade the ligand (94,95). Furthermore, whereas the fractional mesangial volume increases in rats rendered diabetic by streptozotocin, pancreatic islet transplantation returns the
fractional volume toward control values (96). Although the latter has not yet been demonstrated in humans, mean glomerular volume does seem to decrease slowly over years in patients undergoing pancreatic transplant compared with controls (97).

In summary, while our understanding of mechanisms continues to evolve, what emerges now are potential links between the functional changes we observe in our patients and the biochemical changes that appear to be responsible for them. Novel approaches to therapy are likely to arise as a result of the pursuit of these relationships.

ACKNOWLEDGMENT

Carlos Ayala provided excellent support in the preparation of the manuscript.

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