Differential Effects of Diabetes and Glomerulonephritis on Glomerular Basement Membrane Composition (43993)

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Abstract. **The hallmark of renal diseases involving the glomerulus is the presence of proteinuria. While the routes of pathogenesis of proteinuria have not been established, alterations in the barrier function of the glomerular basement membrane (GBM) have been implicated. We evaluated the effect of streptozotocin diabetes and passive Heymann nephritis (PHN) over time on the macromolecular composition of rat GBM to determine if changes in composition correlate with proteinuria. Six to twelve rats from each group (control, diabetic, and PHN) were sacrificed 1, 5, 28, 56, or 84 days after induction of disease. Identical amounts of GBM were subjected to a sequential extraction procedure, and type IV collagen, entactin, laminin, fibronectin, and anionic charge content were quantitated in the extracts. Type IV collagen and entactin content did not change with time or disease. Both laminin and fibronectin contents increased with time in GBM in all groups, but this increase was significantly greater in diabetic GBM. A significant decrease in anionic charge content of GBM coincided with the onset of albuminuria at Day 28 in diabetes, but no change was seen in PHN. In diabetic rats, the increase in laminin content over control preceded the onset of albuminuria, while the increase in fibronectin was not apparent until after albuminuria was present. In PHN, no differences in type IV collagen, entactin, laminin, fibronectin, or anionic charge content of GBM were found compared with control, even though profound albuminuria was evident from Day 5 through 84. Thus, while alterations in laminin and fibronectin content may contribute to the loss of glomerular permselectivity in streptozotocin diabetes, such changes apparently are not involved in PHN.**

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The glomerular filtration barrier is a complex structure composed of endothelial cells, basement membrane, and epithelial cells; it allows free movement of water and small solutes, but restricts the passage of plasma proteins. Proteinuria is the clinical consequence of a defect in the restrictive permeability of the glomerulus. The relative contribution of the cellular and noncellular components of the glomerular barrier to this selective permeability has been an area of some controversy (1). The endothelial cell layer is highly fenestrated and probably does not exhibit significant resistance to passage of plasma proteins. Epithelial cells are proposed to contribute to the glomerular barrier selectivity at the level of the filtration slit and slit diaphragm. The noncellular extracellular matrix, which constitutes the GBM and mesangial matrix, serves as the filtration barrier between the lumen of the glomerular capillaries and the Bowman's space within the renal glomerulus. The biochemical composition of GBM and the characterization of its components has been studied extensively in the last 15 years (reviewed in Refs. 2–5).

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Diabetic nephropathy is characterized by microalbuminuria that progresses over time to overt proteinuria. Glomerular basement membrane (GBM) thickening has been reported to occur early in the course of diabetic nephropathy, and mesangial matrix expansion and sclerotic obsolescent glomeruli, which are observed in more severe disease, have been correlated with proteinuria (6–8). Proteinuria is closely correlated to the degree of glomerular sclerosis and is used as an indirect marker of renal injury (9–11).

Passive Heymann nephritis (PHN) is a rat model of membranous glomerulonephritis characterized by nephrotic range proteinuria. In contrast to diabetic nephropathy, the thickening of the GBM in PHN is not associated with increased expression of mRNA for the ECM proteins, laminin, fibronectin, or type IV collagen (12, 13).

The association between glomerular sclerosis and proteinuria suggests that altered ECM composition may contribute to the loss of permselectivity (7, 9, 14). Alternatively, the glomerular events that ultimately result in proteinuria and loss of GFR may also initiate sclerosis as an independent but concurrent event. It is important to note that most morphometric studies have been performed after severe sclerosis and proteinuria are both present and do not address the question of whether early changes in glomerular ECM composition accompany the expression of proteinuria.

The present study was designed to compare the relationship of alterations in GBM composition to glomerular barrier dysfunction in these two different models of proteinuric renal disease.

Materials and Methods

Experimental Animals. One hundred sixty-two age-matched male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN), were divided randomly into three groups. Diabetes was induced in the first group by a single intravenous injection of streptozotocin (60 mg/kg body wt in sodium citrate buffer, pH 4). Passive Heymann nephritis was induced in the second group by a single intraperitoneal injection of sheep anti-Fx1A antibody (4 ml/kg body wt). The third group of 54 rats served as controls, receiving a single intraperitoneal injection of saline (4 ml/kg body wt). For each of the three groups, rats were sacrificed at the following times post-injection: 1 day (n = 12); 5 days (n = 6); 28 days (n = 12); 56 days (n = 10–12); and 84 days (n = 9–12). The following measurements were made prior to death: body weight, serum glucose using a glucose analyzer 2 (Beckman, Palo Alto, CA), and 24-hr urinary albumin excretion rate using an inhibitory ELISA. Serum glucose levels were measured weekly in the diabetic rats. To maintain serum glucose levels between 500 and 600 mg/dl, all diabetic rats for the 28-, 56-, and 84-day time points received a daily low-dose subcutaneous injection of 0.1 unit Lente insulin (Eli Lilly and Co., Indianapolis, IN). All animals were housed in the same room, received the same standard chow and had free access to water. Rats were anesthetized with sodium pentobarbital prior to removal of kidneys and then sacrificed by cervical dislocation.

Isolation and Extraction of Glomerular Basement Membrane. All isolation and extraction procedures were performed at 0–4°C in the presence of the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml; aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Kidneys were excised, decapsulated, and the cortex cut away; four to six kidneys were pooled to yield two samples of cortex for each time point and treatment group. Glomeruli were separated from cortices using a standard sieving procedure (15). Briefly, tissue was minced, forced through a #170 stainless steel sieve (pore size: 90 μm), while being rinsed frequently with ice-cold phosphate-buffered saline (PBS), and finally sievings were poured onto sequential sieves of 180-, 150-, 75-, and 63-μm pore sizes with the glomeruli collecting on the 75- and 63-μm sieves. When viewed, the glomerular fraction contained a mixture of capsulated and decapsulated glomeruli with less than 5% of particles being small tubular fragments.

Glomerular basement membrane was isolated as previously described (16). GBM is being used to refer to glomerular matrix, which should be mostly basement membrane, but which may contain some mesangial matrix. Briefly, glomeruli were resuspended in PBS containing 1% sodium deoxycholate at a volume of 1 ml/50 mg tissue wet wt. Samples were sonicated at 20 W for 1 min/3 ml volume using a Cole-Parmer model 4710 ultrasonic homogenizer with a 2-mm probe. The sonications were performed stepwise for 1 min followed by 1 min of cooling, with the sample tube always on ice. After sonication the samples were centrifuged at 48,000g for 30 min; the sonication procedure was carried out two more times, and the resulting basement membrane pellets were transferred to 1.5-ml centrifuge tubes, washed three times with water, and repelleted each time at 16,000g. The final GBM pellets were lyophilized and weighed.

Glomerular basement membrane was subjected to sequential extractions as previously described (16). Briefly, lyophilized GBM (2 mg) was suspended in 1 ml of 5 M guanidine hydrochloride, sonicated for 1 min at 20 W, and extraction continued for 3 hr at room temperature on an end-over-end rocker. Samples were centrifuged at 16,000g for 20 min, and the supernatants decanted. The pellets were resuspended in 0.5 ml of 5 M guanidine with 0.02 M dithiothreitol (guanidine/DTT), and kept at room temperature for 3 hr on an

end-over-end rocker, and centrifuged. Finally, the pellets were resuspended in 0.5 ml of 2% SDS, 0.02 *M* DTT, and incubated for 1 hr at 100°C. After cooling, the samples were centrifuged at 16,000*g* for 20 min, and the SDS/DTT extract was decanted leaving small, insoluble pellets.

Analysis of Extracts. Total protein content of the three extracts was measured using a micro-bicinchoninic acid assay (BCA; Pierce, Rockford, IL) with bovine serum albumin as the standard. Aliquots of the guanidine and guanidine/DTT extracts were dialyzed for 72 hr against 3 *M* urea to remove guanidine and DTT, which was shown to interfere with the micro BCA assay. Aliquots of the SDS/DTT extracts were dialyzed for 72 hr against PBS at room temperature to remove the DTT and most of the SDS.

SDS polyacrylamide gel electrophoresis was performed using vertical slab gels (17). Samples were electrophoresed on 6% gels with an overlying 4% stacking gel. Proteins were visualized by reducing silver or transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) and immunoblot analysis was performed (18).

Type IV collagen, laminin, fibronectin, and entactin were quantitated using inhibitory ELISA (19). Briefly, 96-well microtiter plates (plate #3590; Costar Corp., Cambridge, MA) were coated with antigen. In other plates, specific antibody was incubated overnight with known quantities of the antigen or dilutions of unknown samples. After washing the coated plates, aliquots of incubated antibody were added to the coated plates and incubated. The wells were rinsed and then anti-IgG conjugated to horseradish peroxidase was added. Bound antibody was detected colorimetrically using 2-2 azino-di-(3 ethyl benzothiazolin). Amounts of type IV collagen, laminin, fibronectin and entactin were calculated from standard curves using AssayZap 2.0 (BIOSOFT, Ferguson, MO). The anti-type IV collagen, anti-laminin, anti-fibronectin, and anti-entactin antibodies were shown not to be cross-reactive in the inhibitory ELISA (Fig. 1).

Anionic charge content was measured using an alcian blue dot-blot assay and scanning densitometry (20). Known amounts of heparan sulfate (Sigma Chemical Co., St. Louis, MO) and samples from the 5 *M* guanidine extracts were reacted with Immobilon-N membrane (pore size: 0.45 μm; Millipore Corp., Bedford, MA) in a dot-blot apparatus for 15 min. Following a brief rinse with PBS under vacuum, the membrane was removed from the apparatus and allowed to soak for 30 min in PBS containing 0.5% Tween 20, pH 7.4. The membrane was then stained for 20 min with the following: 0.2% alcian blue 8GX, 0.05 *M* Na acetate, 0.05 *M* $MgCl_2$, and 0.05 *M* NaCl, pH 5.7. After drying, the membrane was digitized using a flatbed scanner (HSD Scan-X, HSD) and a Macintosh IIfx computer. Densitometric analysis was performed using the Image 1.41 program (National Institutes of Health, Bethesda, MD).

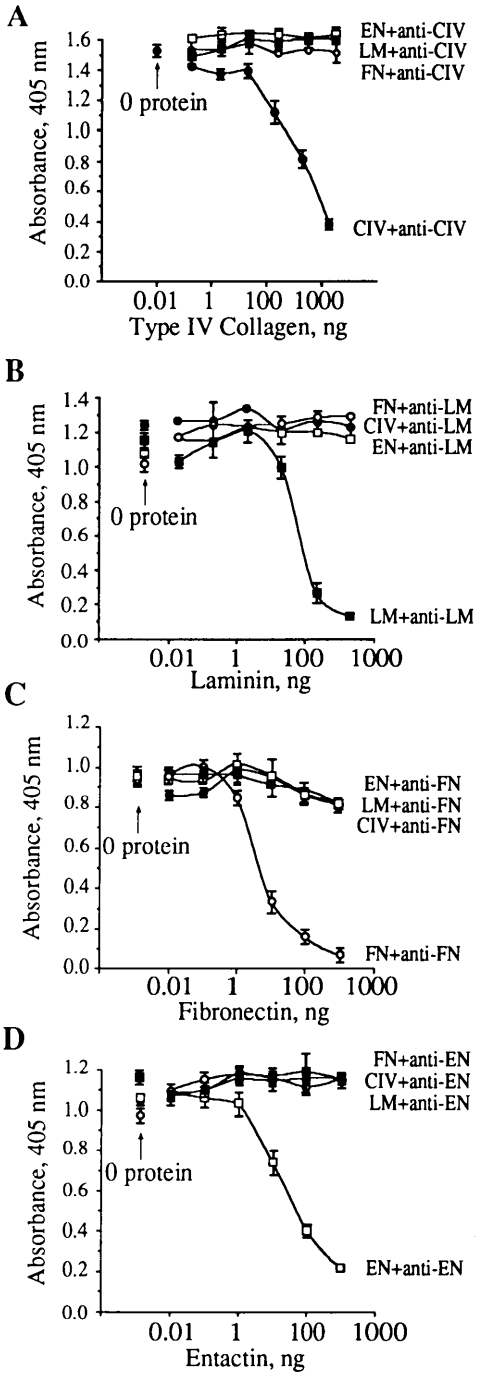

Figure 1. Typical inhibitory ELISAs and demonstration of lack of cross-reactivity of antibodies to type IV collagen (CIV), laminin (LM), fibronectin (FN), and entactin (EN) on plates coated with type IV collagen (A), laminin (B), fibronectin (C), or entactin (D).

Source of Antigens and Antibodies. Rat serum albumin (used for albumin ELISA) was from Sigma. Polyclonal antibodies to rat serum albumin were generated in rabbits using rat serum albumin. Polyclonal antibodies to mouse type IV collagen were the generous gift of Dr. Kevin McCarthy, University of Ala-

bama at Birmingham. Polyclonal antibodies to mouse laminin were the generous gift of Dr. Dale R. Abrahamson, University of Alabama at Birmingham. Rat fibronectin and anti-human fibronectin were from Telios Pharmaceuticals (San Diego, CA). Mouse entactin and anti-mouse entactin were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-rabbit IgG was from Pierce.

Statistical Analysis. Comparisons among the different groups at each time point and over the course of the study were made by analysis of variance or by the Tukey-Kramer HSD test, as implemented in JMP (SAS Institute Inc., Cary, NC) and significance was assigned at the $P < 0.05$ level. All data are expressed as mean ± SEM.

Results

Animal Models. Mean values from rats 1, 5, 28, 56, and 84 days after injection for body weight, serum glucose, and kidney weight to body weight ratios are summarized in Table I. At the time of injection, the body weights of the control, diabetic, and PHN rats were not different (217 ± 1 g). Weight gain in the diabetic and PHN rats was significantly less than that of control rats from Day 28–84; however, the reduced growth rate was more apparent in the diabetic rats. Twenty-four hours after injection of streptozotocin in the diabetic rats, serum glucose levels were elevated compared with control and PHN rats. Glucose levels stabilized between 500 and 600 mg/dl with the low-dose insulin therapy throughout the study. Histological evaluation (data not shown) of diseased kidneys showed morphology that was consistent with these models of disease progressing as expected. Consistent with observed renal hypertrophy, kidney weights were greater in both the diabetic and PHN versus control rats from Day 28 through 84.

Development of Albuminuria. Urinary albumin excretion rate increased with time in both control and diabetic rats (Fig. 2); however, the increase was 2-fold greater in diabetic rats beginning on Day 28 (control: 4.7 ± 0.4 mg/day; diabetic: 8.0 ± 2.2 mg/day; $n = 12$, $P < 0.05$). In PHN albuminuria was evident at Day 5 achieving a value 500-fold greater than in control or diabetic rats. Urinary albumin excretion rate increased further between Day 5 and 28 when it reached a plateau in the range of 500–600 mg/day.

Analysis of Extracts. The percentage of total GBM protein extracted at each step from control, diabetic, and PHN rats is shown in Figure 3. In comparing the total protein extracted from the dry GBM, apparently only 70% ± 3% of the GBM was solubilized ($n = 30$ groups; protein measurements were corrected for poor GBM reactivity with the BCA assay, which was found to be 0.66 of the reactivity of albumin, comparing well to similar data for the Lowry method [21]). Some of the missing protein may have been lost during dialysis to remove the extraction agents, but at least a small amount of the GBM remained as a visible, insoluble pellet at the completion of the three extraction procedures. This result is consistent with the known presence of nonreducible cross-links in the GBM (22).

Electrophoresis of GBM extracts showed complex electrophoretic protein profiles among the guanidine, guanidine/DTT, and SDS/DTT extracts. Silver staining of the guanidine extracts revealed numerous bands ranging from 220 kDa and smaller (Fig. 4). Among these bands were those that migrated alongside laminin B chains, fibronectin, and entactin. The guanidine/DTT extracts revealed numerous bands including

Table I. Physiological Parameters in Control, Diabetic, and Passive Heymann Nephritis (PHN) Rats at Different Numbers of Days after Injection of Either Streptozotocin (Diabetes) or Anti-Fx1A (PHN)

Day	Injection	Body weight (kg)	Serum glucose (mg/dl)	Kidney weight (g/kg body wt)
1	Control	224.4 ± 3.2	102.3 ± 6.9	6.18 ± 1.09
	Diabetic	233.5 ± 5.6	255.4 ± 16.9[a]	5.31 ± 0.77
	PHN	208.0 ± 3.3[a]	79.3 ± 4.1	6.79 ± 0.13
5	Control	249.7 ± 4.1	68.7 ± 2.0	4.94 ± 0.45
	Diabetic	225.0 ± 5.8	175.0 ± 25.6[a]	5.67 ± 0.53[a]
	PHN	234.8 ± 4.0	73.5 ± 10.4	5.86 ± 0.50[a]
28	Control	366.8 ± 4.5	152.4 ± 4.1	4.58 ± 0.26
	Diabetic	232.4 ± 12.9[a]	536.9 ± 18.6[a]	6.86 ± 0.84[a]
	PHN	319.8 ± 11.8[a]	149.2 ± 5.2	6.11 ± 0.66[a]
56	Control	411.2 ± 5.5	148.2 ± 3.2	3.99 ± 0.10
	Diabetic	303.3 ± 10.4[a]	589.4 ± 25.4[a]	5.46 ± 0.01[a]
	PHN	387.6 ± 9.9	184.2 ± 10.6	4.47 ± 0.27[a]
84	Control	431.8 ± 7.0	167.8 ± 5.9	4.70 ± 0.11
	Diabetic	332.2 ± 7.1[a]	604.1 ± 16.7[a]	7.96 ± 0.38[a]
	PHN	399.8 ± 4.1[a]	191.3 ± 11.6	5.00 ± 0.34[a]

[a] $P < 0.05$ versus control group.

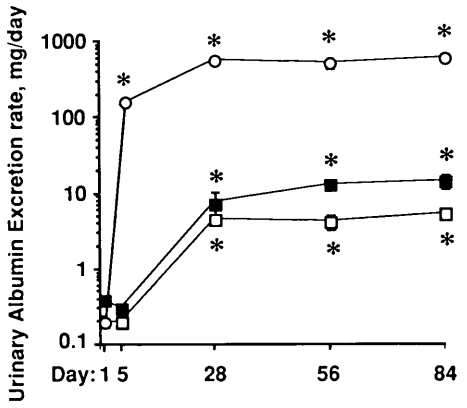

Figure 2. Urinary albumin excretion rate in control (open squares), diabetic (solid squares), and passive Heymann nephritis (PHN) (open circles) rats from 1 to 84 days postinjection. Note log scale. Values are means ± SEM. *Significantly different from control and Day 1.

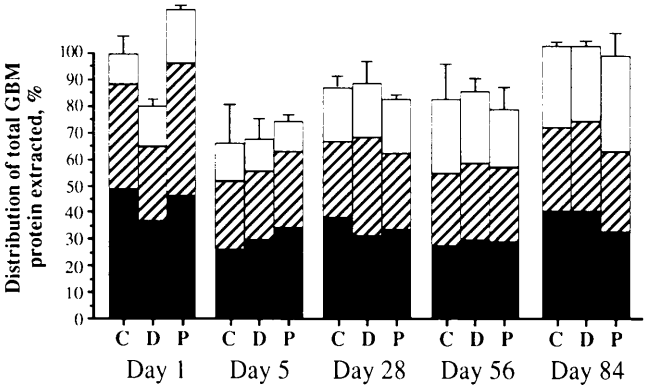

Figure 3. Distribution of protein relative to control values on Day 1 for sequential extracts of GBM. Note that total protein extracted (shown by total height of bars) is not affected by time or by treatment (C, control; D, diabetes; P, passive Heymann nephritis). Shading within the bars shows relative amounts of protein extracted in the three sequential steps: GBM was first treated with 5 *M* guanidine (solid areas), followed by 5 *M* guanidine/20 m*M* DTT (hatched areas), and then by 2% SDS/DTT (open areas), as described in Materials and Methods. $n = 6$ for each.

larger proteins up to 400 kDa. Two intensely stained bands, one at 190 and the other at 210 kDa were observed in all three groups. The 210-kDa band migrated alongside the EHS laminin B chains and the 190-kDa band migrated alongside a large fragment of fibronectin. A doublet at 400–440 kDa was observed in all three groups; these two bands corresponded to the migration of EHS laminin A chain (400 kDa) and dimeric fibronectin (440 kDa): The SDS/DTT extracts showed bands that ranged from 350 down to 120 kDa. Among these were two bands that migrated alongside the α-chains of type IV collagen. No differences in staining of these protein profiles were detected with time or among control, diabetic, and PHN rats.

Immunoblot analysis (not shown) revealed that among the complex mixture of proteins, type IV collagen was solubilized exclusively in the guanidine/DTT and SDS/DTT extracts while laminin, fibronectin, and entactin were solubilized completely in the guanidine and DTT extracts, as previously shown (16). Based upon these immunoblots, ELISAs were performed on the extracts to obtain quantitative results.

The amount of type IV collagen was measured against mouse protein standards in both the guanidine/DTT and SDS/DTT extracts as shown in Figure 5A. The total amount of type IV collagen did not change as a function of time or disease; the apparent type IV collagen content for control, diabetic, and PHN at all five time points taken together averaged 29.1 ± 1.2 μg/mg dry GBM. Even though no significant differences were apparent for type IV collagen, there was some variability in the measured amounts between control, diabetic, and PHN GBM.

Figure 5B shows that the laminin content increased with time in all groups: control, 4.6 ± 0.5 to 8.2 ± 0.4 (Days 1 to 84); diabetic, 3.5 ± 0.7 to 12.0 ± 0.7; PHN, 3.6 ± 0.3 to 10.5 ± 0.8 μg/mg GBM, using mouse laminin as standard. The increase in laminin was greater in magnitude for the diabetic GBM compared with control and PHN starting on Day 5. Although the first measurable increase in laminin in diabetic GBM occurred prior to the onset of proteinuria (Day 28, Fig. 2), the greatest increase occurred between Day 28 and 56, just after proteinuria was observed. By Day 84, the increased laminin content appeared to have peaked, paralleling the plateau observed for proteinuria. The laminin content in PHN was not significantly different than that in control GBM at any time point.

The fibronectin content of the diabetic GBM increased significantly at Day 56, coinciding with the dramatic increase in laminin but occurring after the establishment of proteinuria (Fig. 5C). The fibronectin content continued to increase and exceeded the amount of type IV collagen (by 20%) by Day 84 (fibronectin content, day 1 vs 84: 4.3% ± 0.4% to 35.0% ± 5.7%, using mouse fibronectin as standard). The fibronectin content of PHN GBM revealed no significant differences compared with control GBM at any time point (Day 1 to 84: control, 7.2% ± 2.3% to 9.0% ± 3.3%; PHN, 7.2% ± 1.8% to 8.9% ± 3.4%).

The apparent entactin content of GBM did not change as a function of time or disease (Fig. 5D). The entactin content for control, diabetic, and PHN averaged 1.9 ± 0.06 μg/mg dry GBM, using mouse entactin as standard.

Anionic charge content of the GBM was estimated by densitometric analysis of dot blots stained with alcian blue as illustrated by a typical blot in Figure 6. Alcian blue staining was significantly decreased in diabetic GBM compared with control and PHN GBM.

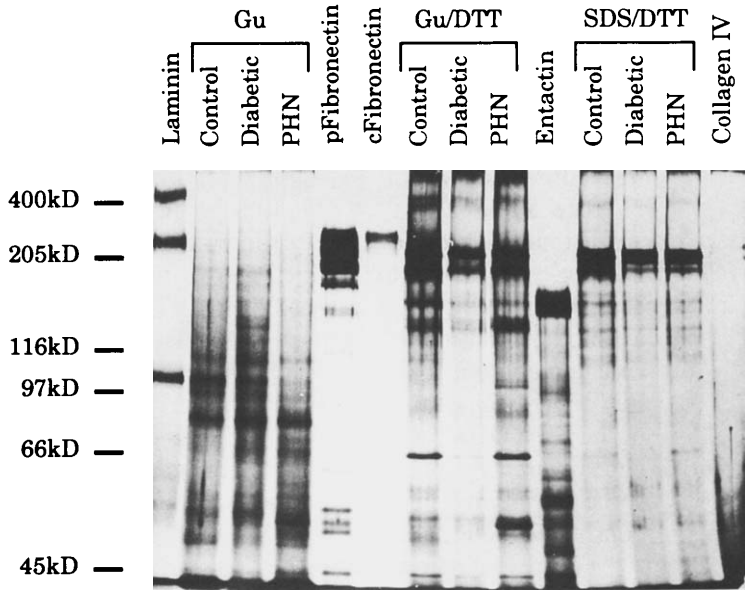

Figure 4. Representative gel showing solubilized components of GBM from control, diabetic, and PHN rats (at Day 56 after injection). Normalized volumes (about 2 μg/lane) of guanidine (Gu), guanidine/DTT (Gu/DTT), and SDS/DTT sequential extracts of GBM were electrophoresed on 6% vertical polyacrylamide slab gels in the presence of a reducing agent.

This decrease was evident by Day 28 and correlated with the onset of proteinuria in diabetic rats. Alcian blue staining remained significantly diminished at subsequent time points in the diabetic GBM. The densitometric analysis showed that the anionic charge content from the PHN or control GBM did not change with time (Fig. 6). Selective degradation of heparan sulfate by nitrous acid treatment did not greatly decrease the alcian blue staining in diabetic GBM samples; however, nitrous acid pretreatment revealed markedly decreased staining for control and PHN GBM samples (data not shown).

Discussion

Diabetes. A characteristic feature of insulin-dependent diabetes mellitus is thickened GBM. It has been suggested that increased synthesis and accumulation of type IV collagen, laminin, and fibronectin are responsible for the thickening and contribute to the progressive renal dysfunction characteristic of this disease (9, 23). However, the relationship between the development of glomerular barrier dysfunction that is manifested in albuminuria and the accumulation of these extracellular matrix proteins has not been examined previously.

In the present study, diabetes for up to 3 months had no effect on type IV collagen content of GBM. Similarly, other studies that examined early stages of diabetes did not find evidence of induction of type IV collagen synthesis. Ledbetter *et al.* (24) measured the mRNA levels of the classical chains of type IV collagen (α1[IV], α2[IV]) in the cortices of spontaneously diabetic KKAy mice; mRNA levels were unchanged after 1 and 2 months with a 2-fold increase detectable only after 4 months. Ihm *et al.* (25), using isolated glomeruli from streptozotocin diabetic rats, detected no changes in glomerular α1(IV) mRNA levels at 1 week or 1 month. In contrast, results reported by other investigators who examined human and experimental diabetic glomeruli measured increased amounts of type IV collagen content (23, 26–28). However, results of changes in type IV collagen reflect long-term effects of diabetes on glomeruli from both human and experimental rats, as opposed to the more acute effects examined in the present study.

It has been shown that diabetic GBM becomes less soluble with long-term progression of disease. Specifically, type IV collagen has been shown to be more resistant to proteolysis and extraction techniques (28, 29). In contrast, we found no differences in the amounts of total protein extracted from control, diabetic and PHN GBM in early stages of disease (Fig. 3). Although SDS-PAGE revealed complex protein profiles among the guanidine, guanidine/DTT, and SDS extracts, there were no differences in these profiles among control, diabetic and PHN extracted GBM. Furthermore, there were no differences among these electrophoretic protein profiles with time. Collectively, these results suggest that the absence of differences in type IV collagen contents with the acute stage of disease were not due to proteolysis or decreased solubility of GBM or collagen.

In contrast to type IV collagen, laminin content was found to be increased 35% in GBM of diabetic rats after three months of experimental diabetes. Laminin content has also been shown to be altered in human diabetic GBM. Immunostaining for laminin is increased in early and intermediate stages of diabetes, with diminution in this staining during the late severe stages of diabetic nephropathy (23, 30, 31). In cultured

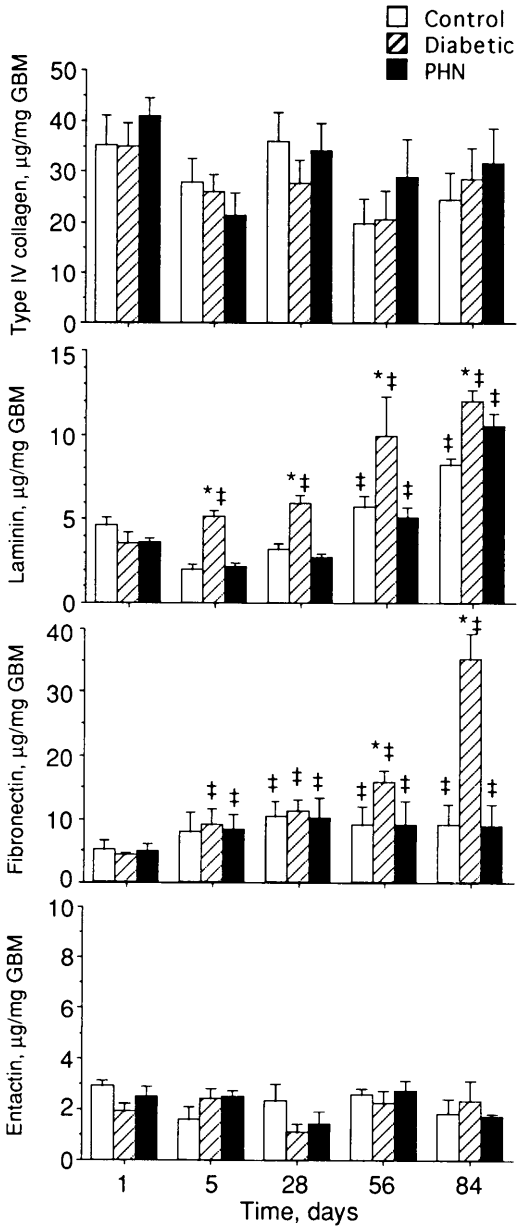

Figure 5. Type IV collagen, laminin, fibronectin, and entactin content in GBM from control, diabetic, and PHN rats relative to mouse protein standards. (A) Type IV collagen measured in both the guanidine/DTT and SDS/DTT extracts. (B) Laminin in the combined guanidine and guanidine/DTT extracts. (C) Fibronectin in the combined guanidine and guanidine/DTT extracts. (D) Entactin in the combined guanidine and guanidine/DTT extracts. n = 8 for each bar. *‡Significantly different from control and Day 1, respectively.

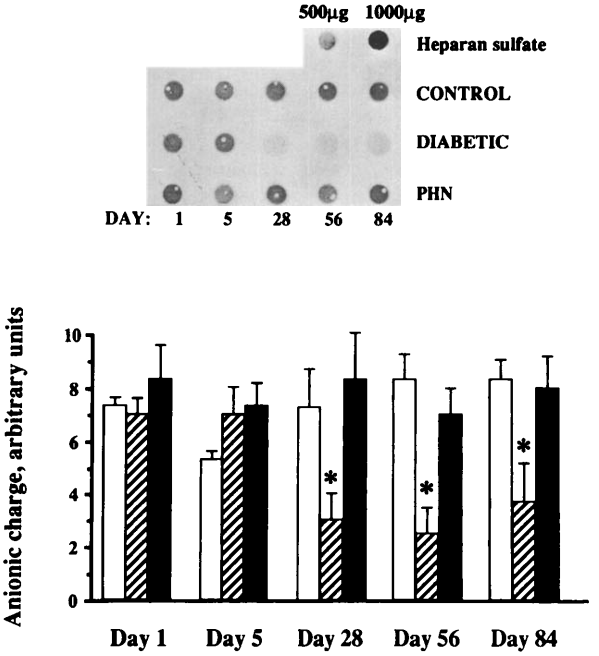

Figure 6. Anionic charge content of the GBM as estimated by densitometric analysis of dot blots stained with alcian blue. Results from samples of the 5 *M* guanidine extracts from control (open bars), diabetic (hatched bars), and PHN (black bars) are shown. The inset is a typical blot revealing the marked decrease in staining for the diabetic samples. Also shown are amounts of heparan sulfate glycosaminoglycan samples serving as controls. n = 3 for each bar. *Significantly different from control.

rat mesangial cells grown in high glucose, laminin B1 chain mRNA after one week was increased by 40–50% (32). Thus, the results described herein suggest that, unlike type IV collagen, synthesis of laminin is stimulated early in the course of diabetes. Although the increased laminin content was initially detectable at day 5 in the present study, a more pronounced increase occurred during the interval immediately following the development of albuminuria (Table II). Interestingly, the laminin content of the control GBM was also increased on Day 56 and 84. Age-related albuminuria was observed in the control rats, and, like in the diabetic rats, was evident prior to the increase in laminin content.

Similar to laminin, the fibronectin content was found to be increased in GBM of diabetic rats. We found a nearly 2-fold increase in fibronectin content, which occurred after the onset of albuminuria (Table II). While some workers have reported significant increases after 6 weeks of diabetes (26), others reported no changes in the amount of fibronectin in late, severe stages (31). Furthermore, mesangial cells grown in

Table II. Alterations in Laminin and Fibronectin Content and Anionic Charge of Diabetic GBM with Respect to Appearance of Albuminuria

	Day 1	Day 5	Day 28	Day 56	Day 84
Laminin	—	↑	↑	↑	↑
Fibrnectin	—	—	↑	↑	↑ ↑
Anionic charge	—	—	↓	↓	↓
Albuminuria	—	—	↑	↑	↑

Note. —, not different from control or Day 1; ↑, significantly greater than control and Day 1; ↓, significantly less than control and Day 1.

high glucose concentration revealed increased fibronectin secretion and mRNA levels after 1 week, suggesting hyperglycemia has a direct effect on fibronectin production (32). Collectively, these findings suggest that altered fibronectin production increases after the onset of proteinuria with subsequent decreases in the late severe stages of the disease. The concomitant increases in both laminin and fibronectin after the onset of albuminuria may be the result of some compensatory mechanism to regain normal glomerular permselectivity or may be induced by the presence of proteinuria.

In the present study, diabetes had no effect on entactin/nidogen content of GBM. In the only previous work on entactin/nidogen in diabetic GBM, immunohistochemical studies showed prominent entactin/nidogen staining only in the mesangial matrix where mesangial expansion was present (33). This mesangial staining disappeared with progression of the disease; however, thickened GBM showed an increased entactin/nidogen staining. Thus, in conjunction with these immunohistochemical studies, entactin/nidogen content of GBM does not appear to be altered during early stages, but may be affected during later progression of diabetes.

The physiological relevance of type IV collagen, laminin, fibronectin, and entactin in the glomerular barrier is unknown. However, the functional significance of the anionic charge barrier with the normal filtration properties of the GBM has been demonstrated (34). Resistance to filtration of negatively charged proteins such as albumin has been attributed in part to the anionic charge density of the GBM, based on the observations that administration of polycations which bind to anionic sites on the GBM or antibodies directed to polyanionic heparan sulfate proteoglycan in GBM induce acute and reversible albuminuria (35, 36). It is commonly accepted that the content of heparan sulfate glycosaminoglycan chains and heparan sulfate proteoglycans are diminished in the early stages of diabetes (37–41). Thus, our finding of markedly decreased anionic charge content is consistent with findings in human and experimental diabetes. Furthermore, the association of decreased GBM anionic charge content with concomitant albuminuria (Table II) supports the hypothesis that loss of anionic charge of GBM contributes to impaired glomerular barrier function.

PHN. In contrast to diabetes, passive Heymann nephritis resulted in rapid and severe albuminuria 5 days after injection of anti-Fx1A antibody. Albuminuria peaked at 1 month and persisted throughout the study. Despite profound albuminuria at Day 5, no measurable changes were detected in type IV collagen, laminin, fibronectin, entactin, or anionic charge content in the GBM of PHN rats. In two other separate studies, no changes were found in the mRNA levels of type IV collagen, laminin B2 chain, S-laminin, and fibronectin after 15 days (13) or 3 months (12) of PHN even though rats displayed marked proteinuria by Day 5, as in the present study. Recently, Minto *et al.* (42) demonstrated the synthesis and appearance of type I collagen, a collagen not normally found in the glomerulus, in the glomeruli of rats with PHN. These investigators suggested that the accumulation of glomerular extracellular matrix in PHN is perhaps a result of the induction and incorporation of type I collagen. The thickened GBM in PHN might be due to other extracellular matrix components not yet assessed, or to abnormal induction of isoforms of extracellular matrix macromolecules.

Summary. The present work shows that the effects of streptozotocin diabetes and PHN on GBM composition are quite different. Diabetes affects the GBM macromolecular composition. A significant reduction in anionic charge content correlated with significant albuminuria, and increases in both laminin and fibronectin occurred in the period immediately following the onset of albuminuria. Therefore, it is possible that the decreased anionic charge content resulted in albuminuria, while the increase in laminin and fibronectin were a compensatory response to repair the damaged glomerular barrier. Although overt albuminuria was evident after 5 days with passive Heymann nephritis, this disease had no effect on the GBM macromolecular composition. No changes in type IV collagen, laminin, fibronectin, entactin, or anionic charge content were detected. Thus, the stimuli that lead to changes in GBM composition in diabetes are likely related to those which result in albuminuria. However, there appears to be no relationship between the changes in the major GBM constituents and overt albuminuria in PHN.

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