

Studies on Macromolecular Components of Human Glomerular Basement Membrane and Alterations in Diabetes

Decreased Levels of Heparan Sulfate Proteoglycan and Laminin

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SUMMARY

Treatment of human glomerular basement membrane (GBM) with 4 M guanidine HCl resulted in a preferential extraction of noncollagenous components including laminin, fibronectin, entactin, and heparan sulfate proteoglycan, whereas effective solubilization of type IV collagen required exposure to denaturing solvents in the presence of reducing agents. The guanidine HCl-solubilized constituents were identified by immunochromatography, polyacrylamide gel electrophoresis, CL-6B filtration, and DEAE-cellulose chromatography. Two immunologically related heparan sulfate proteoglycans (M_r ~350,000 and 210,000) were observed by electrophoresis, with the higher-molecular-weight form being predominant. An examination of the two proteoglycans after heparitinase digestion or chemical deglycosylation indicated that heparan sulfate chains and other carbohydrate units are attached to core proteins with M_r ~140,000 and 110,000, respectively.

Radioimmunoassays indicated that human diabetic GBM contained significantly lower ($P < .005$) amounts of heparan sulfate proteoglycan and laminin with average values that were 30 and 60%, respectively, of nondiabetic controls; the fibronectin content of the diabetic GBM, however, was not significantly different from the normal. These findings, together with previous studies showing increases in GBM collagen, indicate that an alteration in the macromolecular architecture of this basement membrane occurs in diabetes that may be responsible for the filtration defect and the ultimate glomerular occlusion. *Diabetes* 36:374-81, 1987

Recognition that the glomerular basement membrane (GBM) undergoes alterations in diabetes that impair the functioning of the renal filtration barrier has stimulated interest in a search for the biochemical changes that are responsible for this pathology (1-3). By compositional (4-6) and metabolic (7) studies in

humans and experimental animals, respectively, such investigations have provided evidence that a chemical rearrangement involving collagen and noncollagenous constituents is indeed characteristic of the diabetic GBM.

Recent advances in the understanding of basement membranes as highly organized multicomponent structures consisting of collagen, proteoglycans, and glycoproteins (8-10) and the attendant development of procedures for extracting and quantitating these molecules have provided the possibility of expanding the examination of diabetic glomerulopathy by probing the macromolecular organization of the GBM in this disorder. We undertook such an exploration of the human GBM with emphasis on the proteoglycan and some of the major noncollagenous glycoprotein components that could be extracted away from most of the type IV collagen with guanidine HCl. Laminin, entactin, fibronectin, and heparan sulfate proteoglycan were identified among the solubilized constituents; the proteoglycan was found in two immunologically related forms (M_r ~350,000 and 210,000), which was in contrast to the situation in bovine GBM, where only the smaller-molecular-weight species was observed (10). Quantitation by immunochemical procedures indicated the GBM from human diabetic subjects contained significantly decreased amounts of heparan sulfate proteoglycan and laminin compared with that from nondiabetic individuals. Together with previous studies (4-6) indicating that the diabetic GBM is enriched in collagen, these findings suggest that a molecular imbalance in basement membrane constituents is a consequence of this disease state.

MATERIALS AND METHODS

Preparation of GBM. Human kidneys from diabetic and nondiabetic subjects were obtained at autopsy from the New

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England Deaconess Hospital and stored at -20°C until the GBM was prepared by the procedures previously described (4,6). GBM from individual kidneys was studied from six nondiabetic and eight diabetic subjects whose ages ranged from 50 to 72 yr (mean \pm SE 65 ± 4 yr) and from 44 to 76 yr (mean 56 ± 4 yr), respectively, and who were equally divided between men and women. All of the diabetic individuals had been on insulin therapy, and the duration of their disease ranged from 15 to 40 yr (mean 28 ± 3 yr). Histological evidence of varying degrees of glomerulopathy was evident in the diabetic kidneys, whereas the glomeruli from the nondiabetic subjects, all of whom had died from causes unrelated to renal disease, were within normal limits by microscopic examination. Phosphorus analyses indicated that cellular material had been effectively removed from the nondiabetic and diabetic preparations (1.5 ± 0.3 and 1.7 ± 0.4 $\mu\text{g}/\text{mg}$ dry membrane, respectively.)

For large-scale preparations of normal GBM (40–80 mg), the glomeruli from several nondiabetic subjects (mean age 66 yr) without renal pathology were pooled, and these basement membranes were used for characterization studies.

Solubilization of GBM components. Basement membranes (3 mg/ml) were suspended in 4 M guanidine HCl, 0.05 M sodium phosphate, pH 7.0, and stirred with a small magnet for 3 h at room temperature. The residue obtained after centrifugation ($1000 \times g$ for 20 min at 4°C) was reextracted with the same volume of guanidine HCl reagent for 2 h at room temperature, and after centrifugation the two supernatants were combined as extract 1. Further treatment of the insoluble basement membrane material was carried with an equal volume of the guanidine HCl reagent containing 20 mM dithiothreitol (DTT) for 3 h at room temperature to yield a supernatant after centrifugation (extract 2). After a wash with 0.05 M sodium phosphate, pH 7.0, the GBM residue was heated at 100°C for 60 min in the phosphate buffer containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 5% (vol/vol) 2-mercaptoethanol (ME) to bring most of the remaining protein into solution (extract 3).

All extractions of GBM and subsequent fractionations were conducted in the presence of a mixture of protease inhibitors consisting of 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 0.4 mM *N*-ethylmaleimide, 0.01 mM *p*-chloromercuriphenyl sulfonic acid, and 10 mM EDTA.

Gel filtration and DEAE-cellulose chromatography of GBM components. The guanidine HCl-solubilized material (extract 1) from nondiabetic human GBM was fractionated at 4°C on a Sepharose CL-6B column (1.5×80 cm) equilibrated and eluted with 4 M guanidine HCl in 0.05 M sodium phosphate, pH 7.0. The guanidine HCl extract, after extensive dialysis at 4°C against 8 M urea in 0.02 M sodium phosphate, pH 7.0, in the presence of protease inhibitors, was also fractionated on a DEAE-cellulose column (DE-52 microgranular) in a manner similar to that previously described (10), except that the linear gradient ranged from 0.05 M to 1.0 M NaCl. Aliquots of the column fractions were analyzed for various components by solid-phase radioimmunoassay. The tubes from the DEAE-cellulose column that contained the proteoglycan were pooled for further study and brought to a small volume in 0.02 M sodium phosphate, pH 7.0, by repeated centrifugation in a Centricon-10 microconcentrator (Amicon). Bovine GBM heparan sulfate proteoglycan

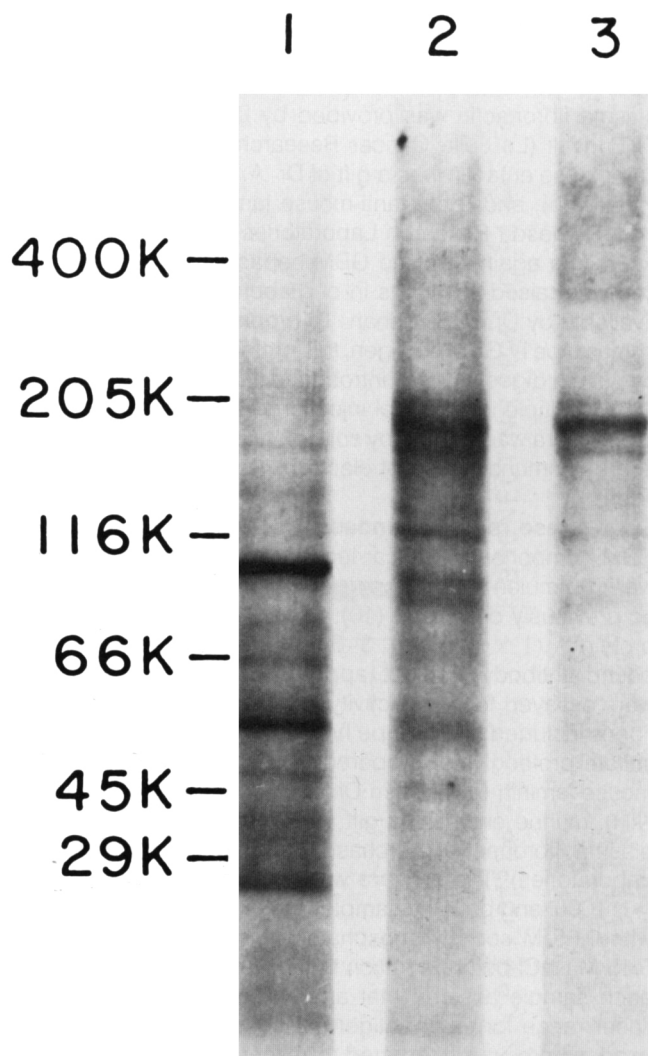


FIG. 1. SDS-PAGE of guanidine HCl (lane 1), guanidine HCl-dithiothreitol (DTT) (lane 2), and SDS-2-mercaptoethanol (ME) (lane 3) extracts of normal human glomerular basement membrane (GBM). Each lane was loaded with 30 μg of protein, and electrophoresis was conducted as described in text; components were visualized with Coomassie Blue. Designated molecular-weight markers were mouse laminin heavy chain (400,000), rabbit muscle myosin (205,000), *E. coli* β -galactosidase (116,000), bovine serum albumin (66,000), hen ovalbumin (45,000), and bovine erythrocyte carbonic anhydrase (29,000).

prepared in a similar manner was made available by Dr. A. S. B. Edge of this laboratory.

Radiolabeling of proteoglycan. Reductive alkylation (11) with [^{14}C]formaldehyde (53 mCi/mmol; New England Nuclear, Boston, MA) and sodium cyanoborohydride was carried out in the manner previously described (12); the reagents were removed by ultrafiltration (Centricon-10) to yield a product with a specific activity of 7500 dpm/ μg peptide.

Radioiodination was achieved with the lactoperoxidase-glucose oxidase system employing carrier-free ^{125}I -Na (Amersham, Arlington Heights, IL) and Enzymobeads (Bio-Rad, Richmond, CA) according to the manufacturer's direction. The reaction was stopped by centrifugation of the beads and filtration of the supernatant on a Sephadex G-25 column (0.7×20 cm) equilibrated with 0.15 M sodium chloride/0.05

M sodium phosphate, pH 7.0; the specific activity of the iodinated proteoglycan was 3.3×10^7 cpm/ μ g peptide.

Source of antisera. Rabbit antiserum against human plasma fibronectin was provided by Drs. E. Ruoslahti and E. Engvall (La Jolla Cancer Research Foundation); rabbit anti-mouse entactin was a gift of Dr. A. Chung (University of Pittsburgh), and rabbit anti-mouse laminin was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Antiserum against bovine GBM heparan sulfate proteoglycan was raised in rabbits in our laboratory (10) and made available by Dr. P. S. Mohan. To prepare antiserum against human type IV GBM collagen, this protein (100 μ g), prepared by pepsin digestion, was introduced into rabbits by a schedule of multiple intradermal injections (13). The specificity of the antisera was verified by solid-phase radioimmunoassays and by immunoblotting of electrophoretically separated antigens.

Solid-phase radioimmunoassays. Quantitation of various GBM components was carried out on flat-bottom microtiter wells (Immulon 2, Removawells, Dynatech, Alexandria, VA) as previously described (10); in this procedure 125 I-labeled protein A (1×10^5 cpm, 3–5 ng) was used to detect the bound antibody, and the appropriately washed wells (10) were assayed for radioactivity in a γ -counter. Standard antigens included human type IV collagen and bovine heparan sulfate proteoglycan prepared in our laboratory as well as mouse laminin (a gift from Drs. H. Kleinman and G. Martin, NIH), murine entactin (a gift of Dr. A. Chung), and human plasma fibronectin (purchased from Bethesda Research Laboratories). The antisera were employed at a dilution of $>1:1000$, and both the samples and standards were diluted with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl before addition to the wells. Multiple assays of each sample assured that analysis was performed in the linear range for each antigen (up to 80 ng); at the dilution used, as previously reported (10), there is no interference in this assay by the guanidine HCl, guanidine HCl–DTT, urea, or SDS–ME reagents.

Immunoprecipitation of proteoglycan. Radiolabeled heparan sulfate proteoglycan was immunoprecipitated by a procedure with antiserum and protein A–Sepharose (14). After heating the proteoglycan in a solution of 4% SDS (~25 μ l) at 100°C for 4 min, 4 vol of 0.05 M Tris–HCl buffer, pH 7.4, containing 5% Triton X-100, 0.19 M sodium chloride, 6 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 U/ml Trasylol were added, followed by 5 μ l of antiserum. After an overnight incubation at 4°C, the mixture was cleared by centrifugation ($1000 \times g$, 5 min) and then shaken at room temperature for 2 h with 30 μ l of 50% suspension of protein A–Sepharose CL-4B (Sigma, St. Louis, MO). After washing by centrifugation, the beads were eluted with 3% SDS–5% ME in 60 mM Tris–HCl buffer, pH 6.8, for electrophoretic examination.

Enzymatic and chemical removal of carbohydrate from proteoglycan. Immunoprecipitated radiolabeled proteoglycan bound to protein A–Sepharose beads was incubated with 5 mlU heparitinase (EC 4.2.2.8) (Miles, Elkhart, IN) in 100 μ l 0.05 M Tris–HCl buffer, pH 7.2, containing 0.02 M calcium acetate, 1 mM phenylmethylsulfonyl fluoride, and a mixture of other protease inhibitors (15) at 37°C for 16 h. After heating at 100°C for 4 min in the SDS–ME–containing

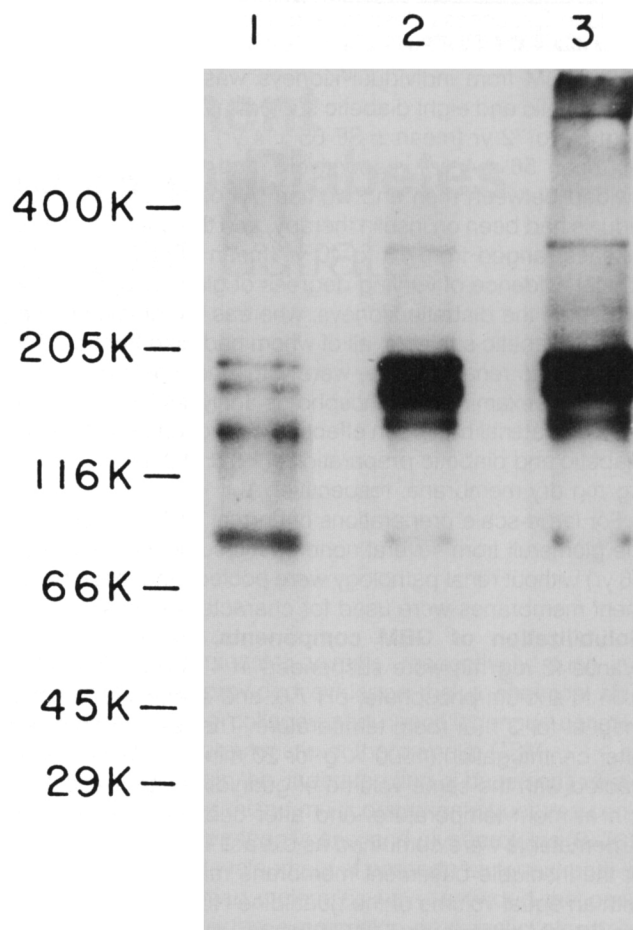


FIG. 2. Immunological identification of type IV collagen in electrophoretically resolved guanidine HCl (lane 1), guanidine HCl–DTT (lane 2), and SDS–ME (lane 3) extracts of normal human GBM. Conditions of electrophoresis and molecular-weight markers were same as in Fig. 1. Resolved proteins were immunoblotted with anti-human type IV collagen serum as described in text. Bound antibody was detected with 125 I-labeled protein A and visualized by autoradiography.

buffer, the eluant from the beads was submitted to polyacrylamide gel electrophoresis (PAGE).

Deglycosylation of radiolabeled proteoglycan, after removal of salt by ultrafiltration and lyophilization, was carried out by the trifluoromethanesulfonic acid (TFMS) procedure (16). After treatment the sample was dialyzed against 0.02 M ammonium bicarbonate, pH 8.0, at 4°C and lyophilized before examination by electrophoresis.

Polyacrylamide gel electrophoresis. Electrophoresis was performed in SDS on vertical polyacrylamide slab gels (3 mm thick) according to the procedure of Laemmli (17) with a linear 4–15% acrylamide gradient separating gel overlaid by a 3.5% stacking gel. Before application the samples were heated for 4 min at 100°C in 0.06 M Tris–HCl buffer, pH 6.8, containing 3% SDS and 5% ME. The proteins were visualized by staining the gels with Coomassie Blue; 125 I-labeled components were detected by autoradiography, whereas 14 C-labeled samples were submitted to fluorography after treatment with EN³HANCE (New England Nuclear) using, in each case, X-Omat AR film (Eastman Kodak, Rochester, NY).

Electrophoretic transfer of proteins to nitrocellulose sheets was achieved by the methods of Towbin et al. (18), and

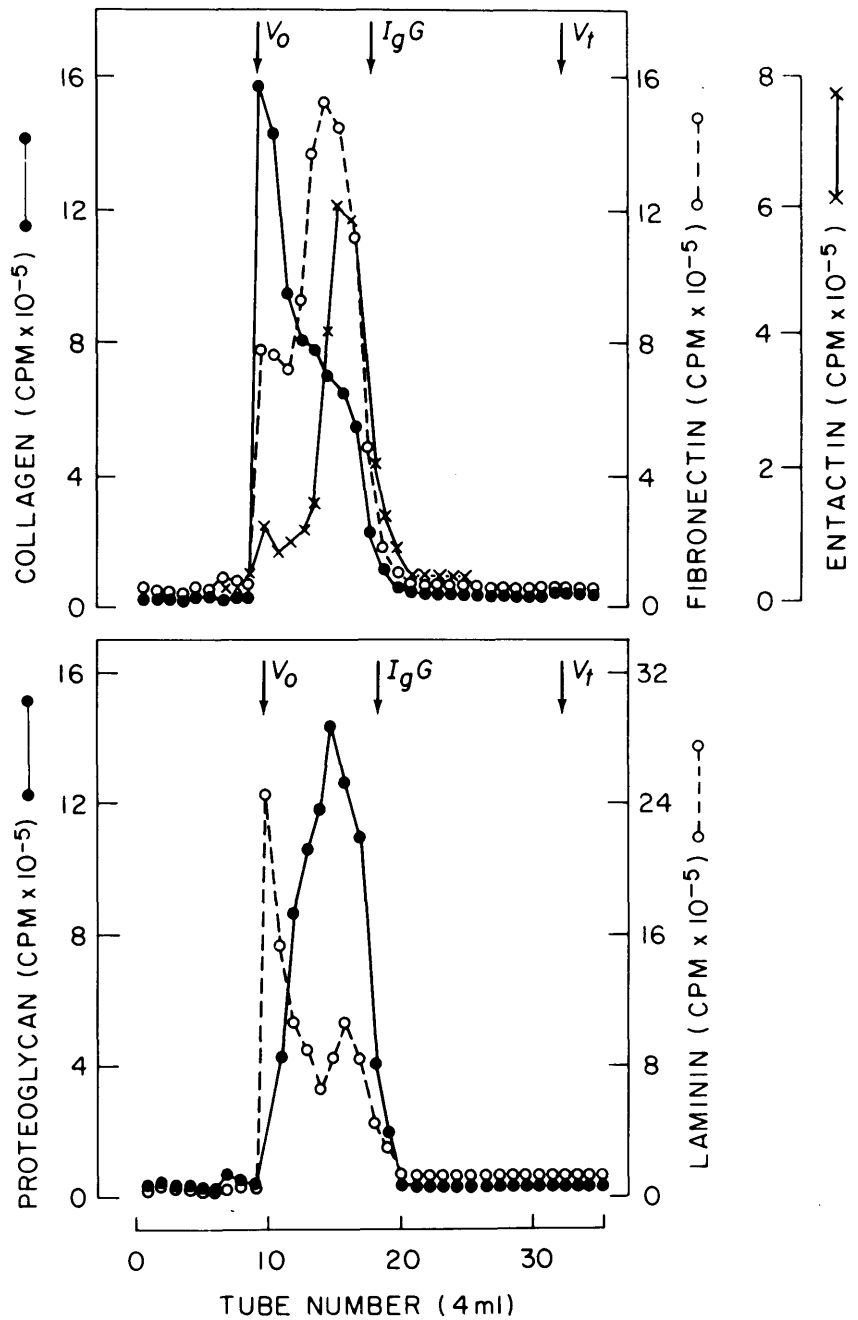


FIG. 3. Filtration on Sepharose CL-6B of guanidine HCl extract of normal human GBM. Sample (1.8 mg protein) originating from 15 mg GBM was loaded on 1.5×80 -cm column equilibrated and eluted with 4 M guanidine HCl in 0.05 M sodium phosphate, pH 7.0, at 8 ml/h. Emergence of indicated proteins was followed by solid-phase radioimmunoassay in which ^{125}I -labeled protein A was used to quantitate bound antibody as described in text; values are expressed per ml of each tube. Void volume (V_0) and total volume (V_t) as well as elution position of standard IgG ($M_r = 150,000$) are indicated; calf thyroglobulin ($M_r = 670,000$) was excluded from column.

immunological identification was then carried out with anti-serum followed by ^{125}I -labeled protein A and autoradiography as previously described (10). The location of standard proteins was determined by staining a second nitrocellulose sheet from the transblotted gels with India ink (19).

Chemical analysis. Protein was estimated by amino acid analyses (12) or by a modification of the Lowry procedure (20).

RESULTS

Electrophoretic examination of guanidine HCl-solubilized GBM proteins. Guanidine HCl extraction (extract 1) of pools of normal human GBM led to the solubilization of 10–13 mg protein/100 mg GBM dry wt, and subsequent treatment of the residue with guanidine HCl in the presence of

DTT (extract 2) brought additional protein (12–14 mg/100 mg GBM) into solution. On SDS-PAGE, staining with Coomassie Blue revealed many components in the guanidine HCl extract, whereas a simpler pattern in which three polypeptides (M_r 350,000; 190,000; and 170,000) predominated was seen in the guanidine HCl–DTT and SDS-ME (extract 3) solubilized fractions (Fig. 1). Immunoblotting with anti-type IV collagen serum indicated that these three bands were the major collagenous components presumably representing the α_1 - and α_2 -subunits as well as a higher-molecular-weight aggregate; faster moving constituents that reacted with the anticollagen serum were also evident particularly in the guanidine HCl extract (extract 1). A comparison of the Coomassie Blue-stained (Fig. 1) and immunoreactive (Fig. 2) bands indicated that, whereas the components solubilized

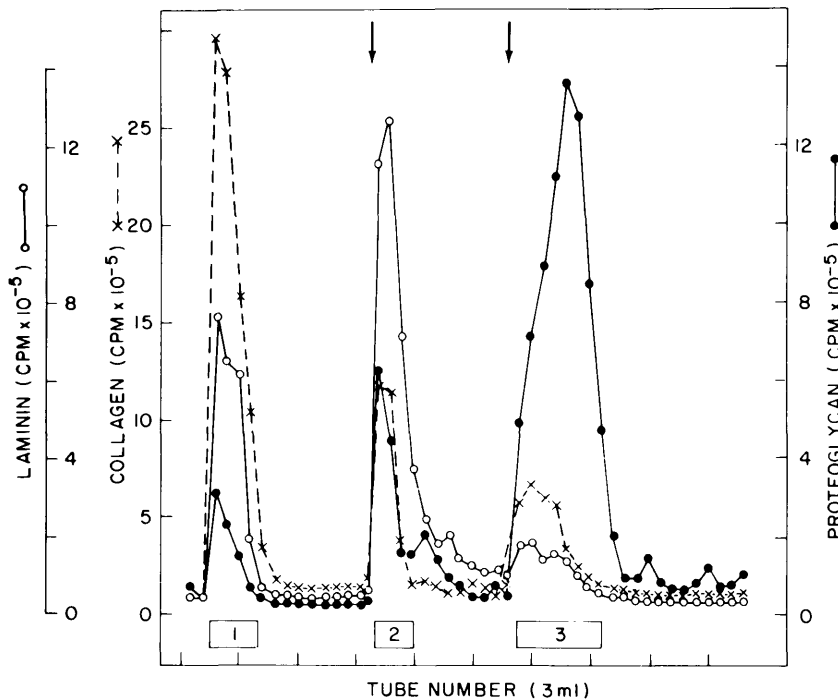


FIG. 4. Chromatography on DEAE-cellulose of guanidine extract of normal human GBM. Sample (2.5 mg protein), after dialysis against 8 M urea, 0.02 M sodium phosphate, pH 7.0, was placed on 1 x 6-cm column equilibrated with same buffer. At tube 16, 0.05 M NaCl was added to this buffer, and at tube 27, a linear NaCl concentration gradient was started as described in text. Elution of indicated components was followed by solid-phase radioimmunoassays; values are expressed per ml of each tube. Fibronectin and entactin were also analyzed (elution not shown). Numbered areas designated tubes that were combined; analyses of these pools are shown in Table 1.

by guanidine HCl-DTT and SDS-ME (extracts 2 and 3) were primarily collagenous, the guanidine HCl-solubilized material (extract 1) consisted largely of noncollagenous polypeptides as has previously been observed in bovine GBM (10). Indeed the hydroxylysine contents of the proteins in extracts 1, 2, and 3 that were found to be 8.6, 22.3, and 33.1 residues/1000 total amino acid residues, compared with 26.5/1000 residues in the intact GBM, were consistent with this uneven distribution of collagen among the GBM extracts. Digestion with bacterial collagenase of the protein remaining insoluble after guanidine HCl-DTT extraction under the conditions previously defined (21) resulted in the disappearance of all of the major bands observed on electrophoresis of the undigested SDS-ME extract (data not shown).

Fractionation of guanidine HCl-solubilized proteins by gel filtration and DEAE-cellulose chromatography. Filtration of the guanidine HCl extract of the human GBM on Sepharose CL-6B under nonreducing conditions revealed the presence of fibronectin, entactin, laminin, and heparan sulfate proteoglycan in addition to the type IV collagen as determined by radioimmunoassay (Fig. 3). Most of the collagen and laminin appeared in the void volume, whereas the other components primarily entered the gel. A second laminin peak coincided with the entactin ($K_{av} = 0.28$) and is believed to represent the lack of specificity already noted

for the antilaminin serum (10). The proteoglycan eluted ($K_{av} = 0.22$) at an earlier position than that previously observed (10) on a similar column for the bovine GBM proteoglycan ($K_{av} = 0.30$).

TABLE 1
Distribution of guanidine HCl-solubilized components of human glomerular basement membrane in DEAE-cellulose peaks

| Peak* | Proteoglycan | Fibronectin | Laminin | Entactin | Collagen |
|-------|--------------|-------------|---------|----------|----------|
| 1 | 4 | 56 | 30 | 73 | 60 |
| 2 | 11 | 37 | 64 | 18 | 20 |
| 3 | 85 | 7 | 6 | 9 | 20 |

Values indicate percent of component present in peaks from DEAE-cellulose column as measured by solid-phase radioimmunoassays. *Peaks are designated in Fig. 4.

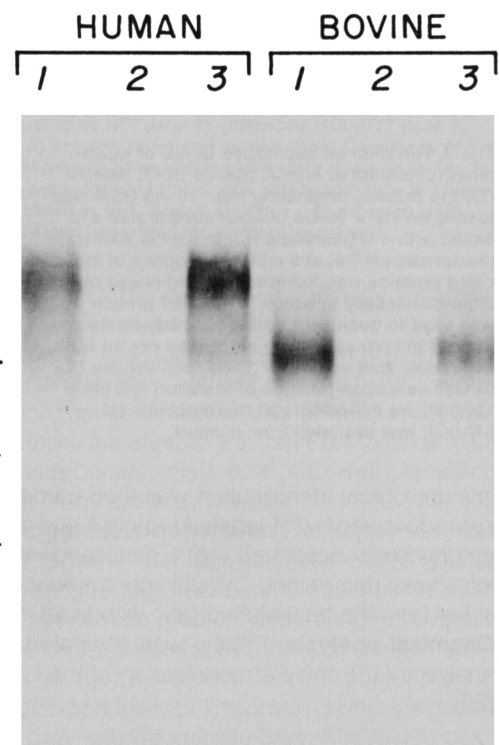


FIG. 5. Comparison of electrophoretic migration of immunoprecipitated normal human and bovine GBM heparan sulfate proteoglycans. SDS-PAGE was carried out on ¹⁴C-labeled (2000 dpm) human and bovine DEAE-cellulose-purified proteoglycan before (lanes 1) and after (lanes 2 and 3) immunoprecipitation with anti-bovine GBM heparan sulfate proteoglycan serum as described in text. Immunoprecipitates from nonimmune serum were loaded on lanes 2. Components were visualized by fluorography; molecular-weight markers are described in Fig. 1.

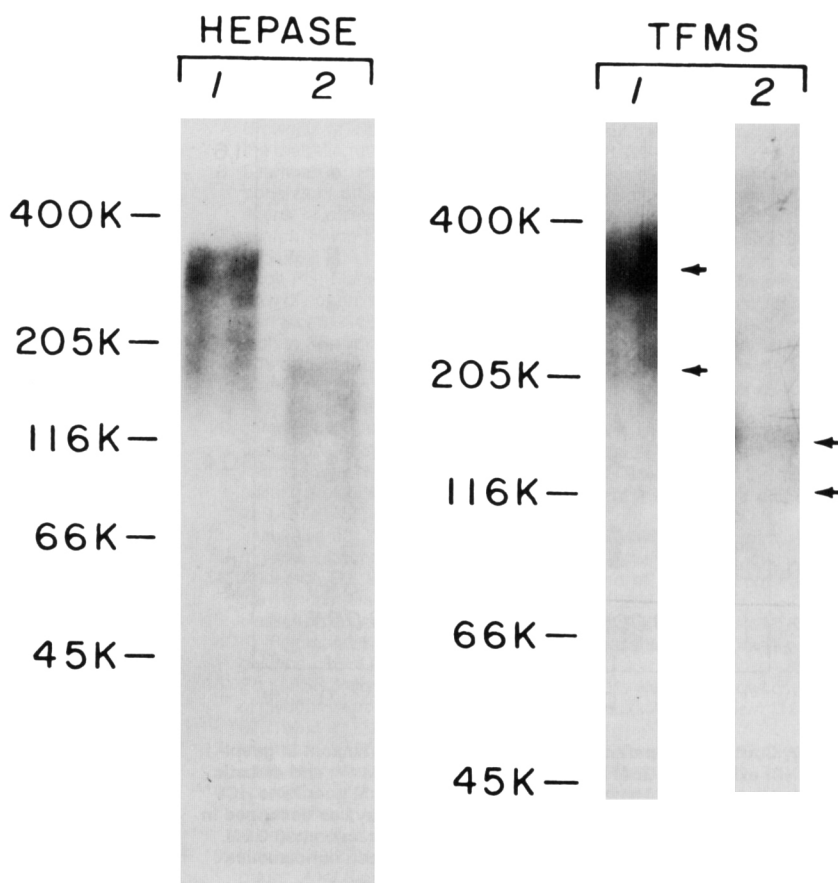


FIG. 6. Effect of heparitinase digestion (left) and TFMS treatment (right) on electrophoretic migration of heparan sulfate proteoglycan from normal GBM. SDS-PAGE was carried out on ^{125}I -labeled human proteoglycan after digestion without (lane 1) and with (lane 2) heparitinase (HEPASE) as well as before (lane 1) and after (lane 2) TFMS treatment. Visualization was achieved by autoradiography; arrows indicate major components evident before and after TFMS deglycosylation. Molecular-weight markers are described in Fig. 1.

Chromatography on DEAE-cellulose provided a procedure for resolving the guanidine HCl-solubilized proteins into three major peaks that represented components not retained (peak 1), eluted with 0.05 M NaCl (peak 2), and emerging with the NaCl gradient (peak 3) (Fig. 4). Whereas the proteoglycan was eluted primarily in peak 3, the fibronectin, laminin, and entactin were only weakly adsorbed to the column and emerged (>90%) in peaks 1 and 2 (Table 1). Most of the guanidine HCl-solubilized type IV collagen was recovered in peaks 1 and 2, but a substantial portion emerged with the salt gradient in a manner similar to that previously observed in bovine GBM (10).

Characterization of proteoglycan component. Polyacrylamide gel electrophoresis of the DEAE-cellulose purified human proteoglycan, after ^{14}C -labeling by reductive alkylation, indicated the presence of two components consisting of a major (M_r , ~350,000) and minor (M_r , ~210,000) band (Fig. 5, lane 1). The electrophoretic pattern of the human proteoglycan immunoprecipitated with anti-bovine GBM proteoglycan serum (Fig. 5, lane 3) was identical to that of the untreated preparation, whereas no components were evident when preimmune serum was substituted for the specific antiserum (Fig. 5, lane 2). In contrast, the bovine proteoglycan showed only a single band on electrophoresis, both before and after immunoprecipitation (Fig. 5, lanes 1 and 3), which had a similar migration to that of the lower-molecular-weight component of the human GBM. Electrophoresis without ME reduction did not change the two-banded pattern of the human proteoglycan preparation (data not shown).

Digestion with heparitinase reduced the molecular weight

of the two components substantially (M_r , ~165,000 and 125,000), consistent with the presence of heparan sulfate chains in these glycoconjugates (Fig. 6, left). To achieve complete deglycosylation, treatment with the TFMS reagent was undertaken, which resulted in the appearance of bands with M_r , ~140,000 and 110,000 (Fig. 6, right).

Comparison of diabetic and nondiabetic GBM components. The yield of GBM isolated from diabetic kidneys (mean \pm SE 65 ± 18 mg/100 g kidney cortex) was substantially greater than that from nondiabetic cases (26 ± 4 mg/100 g cortex).

Similar amounts of protein were solubilized from diabetic and nondiabetic GBM by guanidine HCl treatment (extract 1) with means \pm SE of 9.1 ± 0.8 and 10.5 ± 0.8 mg/100 mg GBM, respectively. Radioimmunoassays, however, indicated that there was a highly significant decrease in the amount of heparan sulfate proteoglycan in the extract of the diabetic GBM, with the level of this component being reduced to 30% of normal (Table 2; Fig. 7). The content of laminin was also found to be significantly less in the diabetic samples (60% of normal), whereas the amount of fibronectin was statistically indistinguishable from the controls (Table 2; Fig. 7).

Immunoanalysis of the guanidine HCl-DTT-solubilized proteins (extract 2) indicated the occurrence of only small amounts of proteoglycan and laminin that represented < 6% of that present in the guanidine HCl extract; furthermore, the level of these two components in the SDS-ME extract (extract 3) was below the reliable range of detection. Fibronectin occurred in the more substantial amounts in extract 2 (dia-

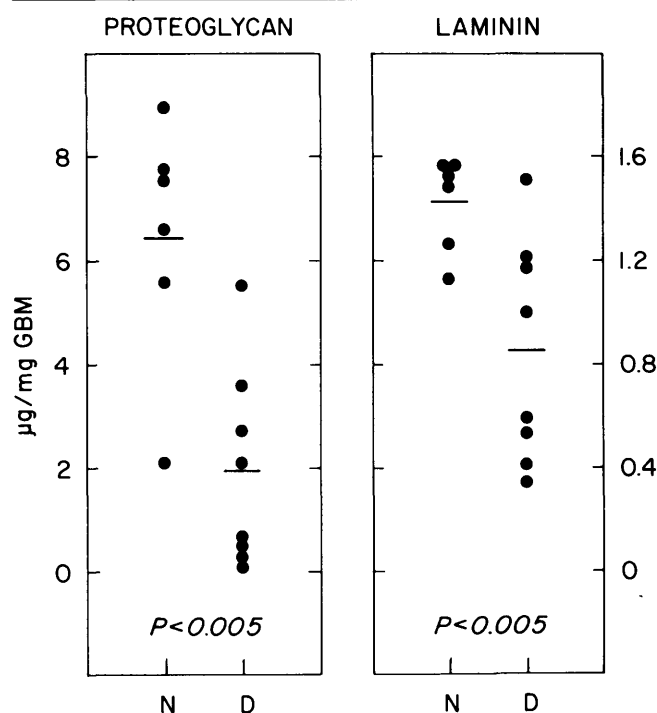


FIG. 7. Content of heparan sulfate proteoglycan and laminin in guanine HCl extracts of GBM from individual nondiabetic (N) and diabetic (D) human subjects. Analyses were carried out on 4 M guanidine HCl-solubilized fraction by solid-phase radioimmunoassays as described in text; values are expressed per mg dry weight of unfractionated GBM. Mean is indicated for each group, and P value between nondiabetics and diabetics is shown.

betic, 0.7 ± 0.2 µg/mg GBM; nondiabetic, 0.6 ± 0.1 µg/mg GBM), but the differences between the diabetic and nondiabetic samples were not statistically significant.

DISCUSSION

This investigation indicates that human GBM, like the basement membranes of bovine glomeruli (10), although consisting predominantly of type IV collagen, contains several of other components including heparan sulfate proteoglycan, laminin, fibronectin, and entactin. These can to a large extent be solubilized by guanidine HCl under nonreducing conditions while leaving most of the collagen in the residue. Guanidine HCl in the presence of DTT was somewhat more effective in bringing type IV collagen into solution, but it required SDS-ME to achieve a substantial extraction of this protein as previously observed in studies on bovine GBM (10,22). Indeed, examination of the electrophoretic pattern by immunoblotting indicated that the predominant bands observed by Coomassie Blue staining in the guanidine HCl-DTT and SDS-ME extracts were α₁- and α₂-subunits of type IV collagen as well as a slower moving, apparently cross-linked aggregate of these chains; in contrast the material solubilized by guanidine HCl alone contained many non-collagenous components.

Because of the postulated role of heparan sulfate proteoglycan as a major constituent of the glomerular anionic filtration barrier (23), we undertook a characterization of this glycoconjugate from human GBM. Our studies indicated the

occurrence of two heparan sulfate proteoglycans with a high-molecular-weight form (M_r ~350,000) predominating over a smaller species (M_r ~210,000). This contrasts to the situation in bovine GBM in which we observed only a lower-molecular-weight component (M_r ~200,000) as previously reported from this laboratory (10,12). Because antiserum raised against the bovine GBM proteoglycan was found to react with both human glycoconjugates, it appears that common antigenic determinants most likely occur in the core proteins of these molecules to which the antiserum is primarily directed (A. S. B. Edge and R. G. S., unpublished observations). The two human proteoglycans are apparently not associated by disulfide bonds, because they could be seen as distinct entities on SDS-PAGE in the absence of reducing agent. Because two components, each of decreased molecular weight, were still evident after heparitinase digestion (M_r ~165,00 and 125,000) and after complete deglycosylation by TFMS treatment (M_r ~140,000 and 110,000), their difference cannot be attributed solely to variation in the number or size of carbohydrate units. Indeed, the smaller proteoglycan may arise from a proteolytic processing of the core protein of the larger component as has been suggested to explain the interrelationship between two forms of proteoglycan in the Engelbreth-Holm-Swarm (EHS) tumor (15). The substantial reduction in molecular weight by TFMS treatment of both proteoglycan species beyond that achieved by heparitinase digestion indicates that the human GBM glycoconjugates, like those from the bovine basement membranes (12,24), contain N-linked and/or small O-linked carbohydrate units in addition to the glycosaminoglycan chains.

The immunochemical analyses reported here, together with previous studies (4-6), indicate that the human GBM undergoes profound alterations in its macromolecular composition in the diabetic state. The marked reduction in the proteoglycan content of the diabetic GBM that we observed is consistent with previous chemical measurements of the heparan sulfate chains associated with this glycoconjugate (6). Indeed, a decreased incorporation of [³⁵S]sulfate into GBM glycosaminoglycan has been observed in experimental diabetes (25,26), and a reduced level and synthesis of extracellular matrix heparan sulfate proteoglycan from EHS tumors implanted in diabetic mice has been reported (27,28). Although the laminin content of the human diabetic GBM was not decreased to the same extent as that of the

TABLE 2
Content of several noncollagenous components in guanidine HCl extract from nondiabetic and diabetic human glomerular basement membrane (GBM)

| Subject | N | Proteoglycan (µg/mg GBM) | Laminin (µg/mg GBM) | Fibronectin (µg/mg GBM) |
|-------------|---|--------------------------|---------------------|-------------------------|
| Nondiabetic | 6 | 6.4 ± 1.0 | 1.4 ± 0.1 | 2.9 ± 0.2 |
| Diabetic | 8 | 1.9 ± 0.7 | 0.85 ± 0.15 | 2.2 ± 0.7 |
| P value | | <.005 | <.005 | <.2 |

Analyses were performed on 4 M guanidine HCl-solubilized fraction (extract 1) by solid-phase radioimmunoassays employing antisera and standard antigens described in text. Each component is reported as mean ± SE/mg dry GBM; protein content of guanidine HCl extracts for nondiabetic and diabetic subjects was 10.5 ± 0.8 and 9.1 ± 0.8 mg/100 mg GBM.

heparan sulfate proteoglycan, the diabetic values differed from normal in a highly significant manner. Such an effect of the diabetic state has not been noted in the murine EHS tumor, which was found to have either normal [streptozocin-treated mice (28)] or elevated [*db/db* mice (27)] levels of this glycoprotein. These disparate observations may well be a function of the biological difference between the mature basement membrane of the glomerulus and the extracellular matrix of the implanted tumor, which under usual conditions contains as much as 20% of its protein in the form of laminin.

The fibronectin level of the diabetic GBM was not significantly altered from normal, which would suggest that the substantial reduction in proteoglycan and laminin are indeed of a specific nature.

Whereas it has not been feasible to obtain reliable immunochemical measurements of the collagen content of basement membranes because of the difficulty of quantitatively solubilizing this protein, it has become evident from metabolic studies (7,29) as well as from amino acid and sugar analyses (4–6) that the diabetic GBM has an increased amount of this protein.

It is tempting to attribute the increased protein permeability of the diabetic glomerulus to the decreased heparan sulfate proteoglycan content of the GBM, because this glycoconjugate has been implicated as a major component of the anionic filtration barrier (23,30); however, the overproduction of basement membrane collagen may be an equally deleterious phenomenon, ultimately leading to the occlusion of the renal glomerulus by basement membrane material.

In evaluating the origin of the molecular imbalance we observed in the human diabetic GBM, it must be appreciated, that contrary to most basement membranes, three distinct cell types are believed to contribute to its biogenesis and degradation (23). The macromolecular architecture of this basement membrane could therefore be altered by a differential response of these cells to the conditions imposed by the diabetic milieu.

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REFERENCES

- Brown DM, Andres GA, Hostetter TH, Mauer SM, Venkatachalam MA: Kidney complications. *Diabetes* 31 (Suppl. 1):71–81, 1982
- Brownlee M: Microvascular disease and related abnormalities: their relation to control of diabetes. In *Joslin's Diabetes Mellitus*. 12th ed. Marble A, Krall LP, Bradley RF, Christlieb AR, Soeldner JS, Eds. Philadelphia, PA, Lea & Febiger, 1985, p. 185–216
- Sternberg M, Cohen-Forster L, Peyroux J.: Connective tissue in diabetes mellitus: biochemical alterations of the intercellular matrix with special reference to proteoglycans, collagens and basement membranes. *Diabetes Metab* 11:27–50, 1985
- Beisswenger PJ, Spiro RG: Studies on the human glomerular basement membrane: composition, nature of the carbohydrate units, and chemical changes in diabetes mellitus. *Diabetes* 22:180–93, 1973
- Spiro RG: Search for a biochemical basis of diabetic microangiopathy. *Diabetologia* 12:1–14, 1976
- Parthasarathy N, Spiro RG: Effect of diabetes on the glycosaminoglycan component of the human glomerular basement membrane. *Diabetes* 31:738–41, 1982
- Brownlee M, Spiro RG: Glomerular basement membrane metabolism in the diabetic rat: in vivo studies. *Diabetes* 28:121–25, 1979
- Szarfman A, Hassell DR, Rohrbach DH, Stanley JR, Martin GR: Components of basement membranes: their properties, functions and alterations in disease states. In *New Trends in Basement Membrane Research*. Kuhn K, Schöne H, Timpl R, Eds. New York, Raven, 1982, p. 265–75
- Scott PG: Macromolecular constituents of basement membranes: a review of current knowledge on their structure and function. *Can J Biochem Cell Biol* 61:942–48, 1983
- Mohan PS, Spiro RG: Macromolecular organization of basement membranes. Characterization and comparison of glomerular basement membrane and lens capsule components by immunochemical and lectin affinity procedures. *J Biol Chem* 261:4328–36, 1986
- Jentoft N, Dearborn DG: Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J Biol Chem* 254:4359–65, 1979
- Parthasarathy N, Spiro RG: Isolation and characterization of the heparan sulfate proteoglycan of the bovine basement membrane. *J Biol Chem* 259:12749–55, 1984
- Vaitukaitis JL: Production of antisera with small doses of immunogen: multiple intradermal injections. *Methods Enzymol* 73:46–52, 1981
- Anderson DJ, Blobel G: Immunoprecipitated proteins from cell-free translations. *Methods Enzymol* 96:111–20, 1983
- Hassell JR, Leyshon WC, Ledbetter SR, Tyree B, Suzuki S, Kato M, Kimata K, Kleinman HK: Isolation of two forms of basement membrane proteoglycans. *J Biol Chem* 260:8098–105, 1985
- Edge ASB, Faltynek CR, Hof L, Reichert LE, Jr., Weber P: Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal Biochem* 118:131–37, 1981
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* 227:680–85, 1970
- Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–54, 1979
- Hancock K, Tsang VCW: India ink staining of proteins on nitrocellulose paper. *Anal Biochem* 133:157–62, 1983
- Peterson GL: A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 83:346–56, 1977
- Cammarata PR, Spiro RG: Identification of noncollagenous components of calf lens capsule: evaluation of their adhesion-promoting activity. *J Cell Physiol* 125:393–402, 1985
- Hudson BG, Spiro RG: Studies on the native and reduced alkylated renal glomerular basement membrane. Solubility, subunit size, and reaction with cyanogen bromide. *J Biol Chem* 247:4229–38, 1972
- Farquhar MG: The glomerular basement membrane. A selective macromolecular filter. In *Cell Biology of the Extracellular Matrix*. Hay ED, Ed. New York, Plenum, 1981, p. 335–78
- Edge ASB, Spiro RG: Distribution of sulfate groups and disaccharide sequences in heparan sulfate chains of glomerular basement membrane proteoglycan (Abstract). *Fed Proc* 44:1789, 1985
- Cohen MP, Surma ML: [³⁵S]sulfate incorporation into glomerular basement membrane glycosaminoglycans is decreased in experimental diabetes. *J Lab Clin Med* 98:715–22, 1981
- Brown DM, Klein DJ, Michael AF, Oegema TR: ³⁵S-glycosaminoglycan and ³⁵S-glycopeptide metabolism by diabetic glomeruli and aorta. *Diabetes* 31:418–25, 1982
- Rohrbach DH, Hassell JR, Kleinman HK, Martin GR: Alterations in the basement membrane (heparan sulfate) proteoglycan in diabetic mice. *Diabetes* 31:185–88, 1982
- Rohrbach DH, Wagner CW, Star VL, Martin GR, Brown KS, Yoon JW: Reduced synthesis of basement membrane heparan sulfate proteoglycan in streptozotocin-induced diabetic mice. *J Biol Chem* 258:11672–77, 1983
- Hasslacher C, Reichenbacher R, Gechter F, Timpl R: Glomerular basement membrane synthesis and serum concentration of type IV collagen in streptozotocin-diabetic rats. *Diabetologia* 26:150–54, 1984
- Spiro RG, Parthasarathy N: Studies on the proteoglycan of basement membranes. In *New Trends in Basement Membrane Research*. Kuhn K, Schöne H, Timpl R, Eds. New York, Raven, 1982, p. 87–98