

GLOMERULOPATHY IN RATS WITH STREPTOZOTOCIN DIABETES

Accumulation of Glomerular Basement Membrane Analogous to Human Diabetic Nephropathy*

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Although the accumulation of glomerular basement membrane (GBM)¹ and basement membrane-like material in human diabetes is well documented (1-4), evidence supporting the occurrence of similar changes in animal models with experimental diabetes is not uniformly accepted. Several investigators have observed increased glomerular basement membrane thickness in monkeys (5) and rats (6, 7) with chronic alloxan diabetes and in rats with long-term streptozotocin diabetes (8), but other workers have been unable to find pathologic alterations in glomeruli from experimentally diabetic rats (9, 10). Similarly, changes in parameters of basement membrane synthesis in glomeruli from diabetic animals have been found in some, but not all, studies. For example, Wahl et al. (11) could not demonstrate increased incorporation of labeled glucose into GBM when glomeruli from alloxan diabetic animals were compared with those from nondiabetic animals. Beisswenger (9) reported that the incorporation of ¹⁴C-lysine and appearance of hydroxy-¹⁴C-lysine in GBM of incubated glomeruli from streptozotocin-diabetic rats was not significantly different from that of nondiabetic glomeruli. Other investigators, on the other hand, have found increased ¹⁴C-hydroxyproline (12) and ¹⁴C-hydroxylysine (13) synthesis in isolated glomeruli from diabetic rats, as well as an increase in the activities of the glomerular lysyl hydroxylase (14, 15) and renal glucosyltransferase (16) enzyme systems, both of which are intimately involved in GBM biosynthesis.

As part of a series of experiments examining basement membrane synthesis in glomeruli isolated from normal and streptozotocin-diabetic rats, we have been able to accurately assess glomerular size and basement membrane content in glomeruli obtained from >200 such animals. The findings presented below demonstrate that glomeruli from streptozotocin-diabetic rats are larger and contain more basement membrane than do non-diabetic glomeruli.

Materials and Methods

Experimental Animals and Tissue Preparation. Kidneys were obtained from male white rats immediately after sacrifice by anesthetic overdose or by placement in a carbon dioxide chamber, and the renal cortex was separated by gross dissection. Diabetic animals were sacrificed 35-45

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¹ Abbreviations used in this paper: GBM, glomerular basement membrane; SDS, sodium dodecyl sulfate.

d after tail vein injection of streptozotocin, 85 mg/kg of body weight. Streptozotocin was administered when the rats weighed between 80 and 120 g, and noninjected litter mates served as controls. The renal cortical tissue from normal and diabetic animals was pooled for glomerular isolation; cortices from 9 to 34 animals per group were employed for each experiment.

Glomeruli were isolated essentially as described by Spiro (17), sequentially using stainless-steel meshes of the following pore sizes: 149, 250, and finally, 63 μm . A wash solution of cold buffered saline (0.85% NaCl) was employed. With this process, connective tissue and tubular fragments are retained by the first two sieves, whereas glomeruli are retained by the final sieve. The preparation is routinely almost completely devoid of tubular elements and consists of glomeruli $>63 \mu\text{m}$ but $<149 \mu\text{m}$ in diameter, which has been repeatedly confirmed by direct microscopic measurement. Using a Pasteur pipette and small amounts of buffered saline, glomeruli are collected from the final sieve by repeated rinsing and aspiration, followed by centrifugation at 1,000 rpm for 10 min, and suspension of the glomerular pellet in cold buffered saline. Glomerular yield was determined by counting the number of glomeruli in three separate 0.002-ml samples of each preparation under the light microscope at $\times 100$ power. Glomerular preparations were well agitated immediately before removal of each aliquot to assure uniform suspension, and the total number of glomeruli in the preparation was calculated from an average of the three samples counted. Material retained by the first two sieves (149 and 250 μm), as well as a sedimented concentrate of the wash through the final sieve were routinely examined after suspension in buffered saline. The 250- μm retentate contains only larger tissue fragments, whereas the 149- μm retentate is comprised of tubular elements with occasional embedded glomeruli. Glomeruli isolated by this procedure were intact and discrete, with sharp borders and microscopically visible refractile cellular elements. These preparations actively incorporate label into protein and collagen when incubated in vitro (18).

Sample Analysis. Glomeruli were suspended in 10–50 ml (depending on total number of glomeruli in the preparation) of cold buffered saline containing proteolytic inhibitors (19) and disrupted in 10–20-ml aliquots with a sonicator providing about 300 W of energy (Biosonik IV, Braun Co., San Mateo, Calif.) by 1-min bursts for a total of 10 min. The preparations were monitored light microscopically for completeness of disruption, which was typically $>90\%$ in normal samples. When disruption was judged incomplete, ultrasound treatment was repeated for 5 min with 1-min bursts, and the preparation re-examined. Glomeruli from diabetic animals sometime required at least one such additional 5-min period of sonic disruption. Paired experiments with normal and diabetic glomeruli showed that extension of the sonication time, as reported by Spiro (20), did not lead to any change in the composition of the basement membranes, but did result in more complete collection of GBM in diabetic samples. Adequate disruption was important to ensure quantitative collection of basement membranes because the membranes of undisrupted diabetic glomeruli could not be recovered for subsequent analysis. The basement membranes were then rapidly collected by low-speed centrifugation (1,200 g); centrifugation time was kept short (1–2 min) to avoid contamination with intracellular elements and the supernate was removed with great care. In earlier experiments (Table I, normal animal groups 1–3 and diabetic animal groups 8–9), the membranes were washed several times with 0.1 M acetic acid at 4°C, whereas in the remainder of the experiments membranes were washed three times with a solution of 1 M NaCl, 0.05 M Tris containing protease inhibitors (19). Use of the latter solution was initiated to minimize endogenous proteolytic activity which could artifactually alter the relative solubility of the membrane proteins. Washing with one of these solutions is essential to remove contaminating intracellular proteins and adherent interstitial collagen but the chemical identity of normal and diabetic glomerular basement membrane prepared after treatment with either of these solutions is the same. Because glomerular basement membrane is insoluble in dilute acetic acid or salt, this process allows for its isolation as a gelatinous residue which is relatively pure when subjected to amino acid analysis (13). A differential effect of either washing procedure on normal and diabetic basement membrane was not observed. The supernate from either of these washes is devoid of 3-hydroxyproline and hydroxylysine and contains none of the characteristic absorption peaks of rat GBM when analyzed by gel chromatography after reduction and denaturation.²

² Some peptide-bound hydroxy-¹⁴C-lysine is found in the acetic acid extract of basement membrane prepared from isolated glomeruli which have been incubated for 2 h with ¹⁴C-lysine (18). This material,

Aliquots of the glomerular basement membrane preparations were assayed for protein by an adaptation of the method of Lowry in which the protein was solubilized with 1 N NaOH for 30 min at room temperature. Relative color yields with the Lowry technique of normal and diabetic glomerular basement membrane, determined by absorption curves on identical amounts of lyophilized material, were the same. The remainder of the samples was reduced and denatured by heating at 100°C for 2 min in 2 ml of 1% (wt/vol) sodium dodecyl sulfate (SDS) and 5% (vol/vol) 2-mercaptoethanol in 0.1 M sodium phosphate buffer (pH 7.4). Samples were then incubated for 2 h at 37°C and dialyzed at room temperature against two changes of 500 ml of 0.1% SDS in 0.1 M sodium phosphate buffer (pH 7.4). Unsolubilized material was removed by centrifugation at 3,000 *g* for 10 min. Comparable amounts (by protein determination) of normal and diabetic preparations were chromatographed at room temperature on a 1.5 × 95-cm column of 6% agarose (Biogel, Bio-Rad Laboratories, Richmond, Calif. A-5m; 200–400 mesh) which was equilibrated and eluted with 0.1 M sodium phosphate buffer containing 0.1% SDS. Void volume was determined with blue dextran, and the column was calibrated with the following protein standards: ³H-procollagen prepared from chick embryo calvaria (21), collagen α-chains prepared from lathyritic rat sternal cartilage, bovine serum albumin, and trypsin. Effluent fractions were monitored spectrophotometrically for UV absorption at 230 nm.

Results

Pertinent data concerning the experimental animal groups are presented in Table I. Streptozotocin-diabetic rats were markedly hyperglycemic and exhibited stunted growth at the time of sacrifice ≈6 wk after injection. Individual values for the amount of renal cortex and basement membrane protein, and for the number of glomeruli obtained in each animal group, are also given in Table I.

The number of glomeruli obtained with the differential sieving technique employed directly reflects that proportion of all glomeruli with a diameter between the 63- and 149-μm limits of the sieving system. Experiments in our own and other (22) laboratories have established that glomerular size is related to body weight, with the mean glomerular diameter (determined by micrometry) proportional to body weight in animals 75–300 g. It is therefore necessary to consider the weight of the animals when comparing glomerular or basement membrane yields between nondiabetic and diabetic rats. As seen in Fig. 1 A, glomerular yields (number of glomeruli per gram of renal cortex per gram of body weight) were significantly greater in diabetic preparations. Although this finding could in part represent a decrease in the nonglomerular mass of diabetic rats, it is also consistent with an increased mean glomerular diameter in diabetic kidneys. This interpretation was confirmed by optical estimates in which the first 20 glomeruli in each sample were measured with a micrometer ($N = 118 \pm 6\mu$; $Db = 132 \pm 8\mu$).

The yield of basement membrane, expressed either as micrograms of basement membrane protein per gram of renal cortex ($N = 27.83 \pm 5.76$; $Db = 55.62 \pm 9.92$; $P < 0.05$) or as 10^{-8} g of basement membrane protein per gram of renal cortex per gram of body weight was also significantly greater in diabetic preparations (Fig. 1 B). Calculation of the amount of basement membrane protein per glomerulus (Fig. 1 C) demonstrated that this finding actually represented glomerular basement membrane accumulation rather than the (unlikely) possibility that diabetic samples have more glomeruli. The amount of basement membrane was greatest in animal groups with

which represents newly synthesized basement membrane collagen-like protein produced *in vitro*, appears to have different solubility characteristics from mature basement membrane which, as demonstrated by several investigators, is insoluble in acetic acid (23).

TABLE I
Experimental Animal Data

Animal group	Number animals	Body wt	Blood glucose	Cortex	No. glomeruli	Basement membrane (protein)
		<i>g</i>	<i>mM/liter</i>	<i>g</i>	$\times 10^5$	μg
1 Normal	10	383	<8.0	19.2	3.060	450
2 Normal	10	380	<8.0	18.4	2.816	627
3 Normal	18	300	<8.0	30.5	4.500	650
4 Normal	27	407	<8.0	78.5	14.920	728
5 Normal	24	366	<8.0	52.3	37.600	2,204
6 Normal	30	230	<8.0	57.0	10.800	770
7 Normal	34	370	<8.0	52.9	16.800	2,700
Mean \pm SEM		348 \pm 23	<8.0	44.1 \pm 8.4	12.928 \pm 4.636	1,161 \pm 340
8 Diabetic	10	211	21.89	22.6	4.258	880
9 Diabetic	9	203	22.00	22.8	4.648	1,040
10 Diabetic	17	273	23.44	52.3	25.100	1,872
11 Diabetic	18	249	22.28	32.9	26.400	2,355
12 Diabetic	26	243	25.39	32.1	12.700	2,770
Mean \pm SEM		236 \pm 13*	23.0 \pm .66*	32.5 \pm 5.4	14.621 \pm 4.791	1,783 \pm 365

Body weights and blood glucose values recorded are the averages for each group. * $P < 0.01$ compared to nondiabetic values.

the longest duration of diabetes (Table I, groups 10 and 11), but statistical correlation could not be made with the available data.

About 65% of both normal and diabetic basement membrane protein was solubilized by reduction and denaturation in the presence of SDS. This finding is in agreement with other studies on the solubility of nondiabetic glomerular basement membrane (23). Hydroxylysine (24) and hydroxyproline (25) assay of the solubilized material was performed to determine if this procedure resulted in the solubilization of similar polypeptide components in normal and diabetic samples. Molecular sieve chromatography of the reduced and denatured basement membrane revealed several major absorbance peaks, including one at the void volume. Elution profiles of normal and diabetic samples were similar, with neither the presence nor absence of any specific peak noted in the diabetic preparations (Fig. 2). Reference to a calibration curve indicated that these components had apparent molecular weights ranging from $\approx 30,000$ to $>300,000$ daltons.

Discussion

The role of genetic versus environmental factors in the pathogenesis of diabetic nephropathy and in relation to capillary basement membrane thickening remains controversial. Although many authorities believe that the presence of chronic hyperglycemia critically contributes to the putative processes which lead to microangiopathic lesions (20, 26), it is difficult to obtain conclusive evidence in support of this relationship in human populations. These problems could more easily be approached in animal models with experimental diabetes where investigation of glomerular

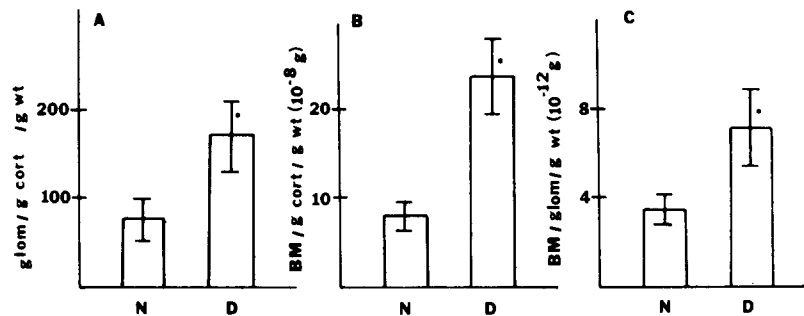


FIG. 1. Glomerular and basement membrane yields in normal and diabetic rats. 1 A, The number of glomeruli recovered per gram of renal cortex relative to the body weight in grams. 1 B, The amount of basement membrane protein obtained per gram of renal cortex relative to the body weight in grams. Values reported as 10^{-8} g of protein. 1 C, The amount of basement membrane protein calculated per glomerulus relative to body weight. Values reported as 10^{-12} g of protein. Values represent mean \pm SEM of the results obtained with pooled renal cortical tissue in each animal group as listed in Table I. * $P < 0.05$ compared to nondiabetic values. N, nondiabetic; D, diabetic.

pathology and biochemistry under controlled conditions could define the effects of nutritional, hormonal, and other factors on glomerular metabolism and basement membrane deposition. There is considerable reservation, however, regarding the occurrence of nephropathic changes in experimental diabetes that correspond to those found in human diabetes, and these reservations continue to cloud the significance and acceptance of chemical and biosynthetic alterations which have been found in glomeruli from such animals.

The elegant studies of Osterby and her colleagues have established that human diabetic nephropathy begins after the onset of juvenile diabetes, with measurable thickening of the peripheral capillary basement membrane occurring by 2 yr after the manifestation of clinical disease (4, 27, 28). The gradual progression of this process leads to the accumulation of large amounts of basement membrane in the glomerular tuft with ultimate occlusion of the capillary network. Glomerular enlargement is an associated change, occurring in part as a result of the excess deposition of solid material and, in part, due to a compensatory hypertrophy of functioning open glomeruli (29–32). Certainly the present study, which documents increased glomerular size and increased basement membrane in rats with streptozotocin diabetes, now establishes that the glomerular expansion and accumulation of basement membrane which are characteristic of human diabetic nephropathy occur in this experimental animal model. Furthermore, these changes occur despite weight loss and attendant loss of protein and fat in other tissues. Several studies have shown that renal protein synthesis paradoxically increases in diabetes, in contrast to the effect of insulin deficiency in other tissues (13, 33, 34). It is suggested that this increase contributes to the accumulation of glomerular basement membrane in diabetes. Although we cannot completely explain the difference between our results and those of Beisswenger (9), who reported that the yield of glomerular basement membrane from streptozotocin diabetic rats was not different from that of normal, it is noted that the basement membranes analyzed in that portion of the study contained $<2\%$ hydroxylysine, suggesting considerable contamination.

Chemical analyses of glomerular basement membrane have not provided satisfac-

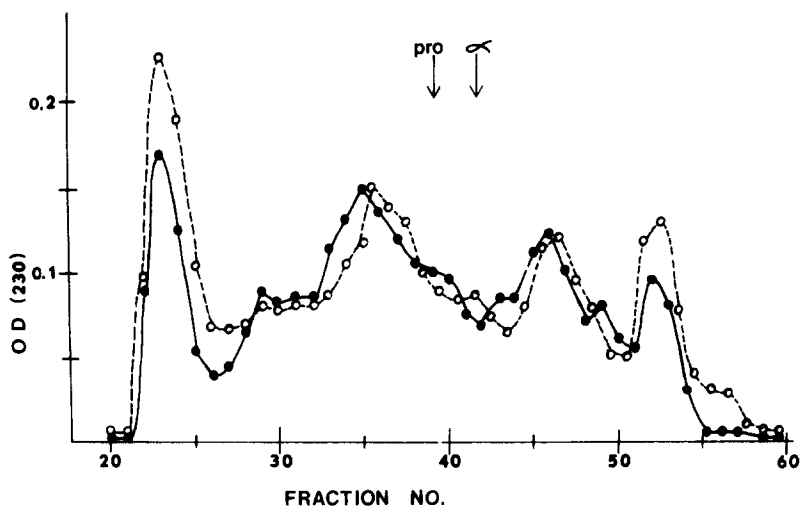


FIG. 2. Gel filtration on SDS-agarose of normal and diabetic rat glomerular basement membrane. The basement membranes were purified by treatment with cold 1 M NaCl, 0.05 M Tris-HCl (pH 7.5) containing protease inhibitors as described in the text, and solubilized by reduction and denaturation in the presence of SDS. Approximately 500 μ g (protein) of this material was chromatographed on a 1.5 \times 95-cm calibrated column of agarose A-5m. The void volume was at fraction 22 and the total volume at fraction 70. Arrows indicate the elution positions of chick embryo calvaria procollagen prepared according to the method of Monson et al. (21) and of α -chains prepared from lathyritic rat sternal cartilage. The elution profiles depicted above represent the average absorption of four separate runs performed in each of the experimental groups (normal animal groups 4-7; diabetic animal groups 10-12). ●, nondiabetic; ○, diabetic.

tory insight into the problem of diabetic nephropathy. Glomerular basement membrane from diabetic patients has been reported to contain more hydroxylysine residues and hydroxylysine-linked disaccharide units by some (35, 36) but not all (37, 38) investigators. There is now considerable evidence supporting the heterogeneous nature of basement membranes, which includes the presence of multiple collagen-like and non-collagen components (39-43). Increased or decreased proportions of one of these components would not necessarily alter the amino acid pattern of unfractionated glomerular basement membrane unless the composition of these components were strikingly different from the rest of the basement membrane. This problem cannot be resolved until the absolute amounts of all the subunits in normal and diabetic glomerular basement membrane are determined. Each of the peaks identified on agarose gel analysis of normal and diabetic basement membrane in the present study probably contains many components, and it is not surprising that the chromatographic patterns were similar. Even if abnormal components were present, dilution of such by the still present normal basement membrane with slow turnover might have obscured minor differences (20, 44). This analysis, however, at least represents a step toward resolution of the controversy surrounding basement membrane chemistry in diabetes, and meaningful data should evolve from further delineation of the normal and diabetic basement membrane components. Results from our most recent experiments support the concept that diabetic glomerular basement membrane, although quantitatively increased, is qualitatively similar to that of normal.

Evidence is accumulating that biosynthetic abnormalities related to glomerular basement membrane production can be demonstrated in experimental diabetes

(12–16). That these changes have pathologic significance is confirmed in the present report. Although one must always exercise caution in directly applying data from experimental models to human disease, it is concluded that use of the streptozotocin-diabetic rat is suitable for the study of mechanisms and factors contributory to the development and progression of diabetic nephropathy.

Summary

Glomeruli from streptozotocin-diabetic and age-matched nondiabetic rats were quantitatively isolated by a differential sieving technique. The insoluble glomerular basement membranes were purified following sonic disruption in the presence of proteolytic inhibitors. The yield of glomeruli and of glomerular basement membrane relative to the amount of renal cortex and the body weight of the animals, as well as the calculated amount of basement membrane per glomerulus, were all significantly greater in diabetic rats when compared to non-diabetic controls. Glomerular basement membranes from normal and diabetic rats were solubilized by reduction and denaturation in the presence of SDS and subjected to agarose gel analysis. About 65% of both normal and diabetic basement membrane was solubilized by this procedure, and the elution profiles of non-diabetic and diabetic preparations were similar. These results suggest that rat renal basement membrane is qualitatively similar but quantitatively increased in streptozotocin-diabetes. Since glomerular enlargement and accumulation of basement membrane are characteristic of human diabetic nephropathy, the findings also suggest that the streptozotocin-diabetic rat is an appropriate animal model for studies relating to the pathogenesis of this complication of diabetes.

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