Glomerular Selective Permeability to Macromolecular Neutral Dextrans in Experimental Diabetes

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Summary. Rats with streptozotocin-induced chronic diabetes mellitus develop a glomerulopathy functionally manifested by proteinuria. The ability of the glomerular capillary wall to retard filtration of macromolecules was examined in 5 chronically diabetic Munich-Wistar rats exhibiting excessive proteinuria (39 ± 7 mg/24 h, mean $\pm$ SEM) and 5 age-matched normal Munich-Wistar rats without increased proteinuria (4.7 ± 0.2 mg/24 h). Urinary albumin excretion was not increased in the diabetic rats (2.0 ± 0.6 mg/24 h vs 1.6 ± 0.3 mg/24 h) suggesting that the normal net electronegative charge of the glomerular capillary wall was not altered. Fractional clearances of macromolecular neutral dextrans were similar in diabetic and normal rats throughout a wide range of molecular size (18–42 Å). Glomerular filtration rate was the same in the two groups of rats (2.77 ± 0.16 ml/min in diabetics and 2.72 ± 0.11 ml/min in normals) suggesting that renal haemodynamic factors did not influence fractional clearances of neutral dextrans in diabetic rats. We conclude that the proteinuria exhibited by these chronically diabetic rats is not attributable to alterations of size-selective properties of the glomerular capillary wall, such as increases in the size or the number of pores.

Key words: Streptozotocin diabetes, proteinuria, glomerular permeability, glomerular filtration rate, fractional clearances of neutral dextrans, glomerular pores

Proteinuria is the predominant abnormality of renal function in rats with experimental diabetes mellitus [1–4]. The pathogenesis of proteinuria in this experimental model of diabetic nephropathy has not been elucidated. The glomerulus normally functions as an

effective barrier to filtration of serum proteins based on their size, net electrical charge [5], and steric configuration [6]. Structural alterations of the glomerular capillary in diabetes have been postulated to enhance porosity of the glomerulus, thereby diminishing its effectiveness as a selective filter [7, 8]. In this study, we have examined the fractional clearances of a polydisperse population of neutral dextrans for the purpose of determining whether alterations in the size-selective properties of the glomerular capillary wall contribute to the proteinuria of chronic experimental diabetes.

Materials and Methods

Two groups of female Munich-Wistar rats were studied [1] 5 rats with streptozotocin-induced (50 mg/kg) diabetes mellitus (DM) of 7–10 months duration, and [2] 5 age-matched normal control (NC) rats. Free access was provided to standard laboratory chow and tap water. Prior to study, all rats were placed in individual metabolic cages for monitoring of 24-hour excretion rates of urinary volume, glucose, and protein.

Rats were surgically prepared for clearance studies under light ether anaesthesia. Cannulae were placed in a jugular vein for infusions and in a femoral artery for sampling of blood and measurement of blood pressure. A soft silastic catheter was passed transurethraally into the bladder, and a snugly tied suture was placed circumferentially around the external urethra in order to prevent leakage of urine around the catheter. Rats were then placed in a plastic restraining cage and permitted to awaken and stabilise for 2–3 h. During that time, a priming dose (8 μ Ci) of 14 C-inulin (New England Nuclear, Boston, Mass.) was administered and followed by an infusion at 0.02 ml/min of 0.154 mol/l saline containing 4 μ Ci/ml 14 C-inulin. Additional volumes of 0.154 mol/l saline were infused as needed to maintain the rate of fluid delivery equal to the rate of urine flow.

After urine flow had stabilised, two consecutive 15 minute clearance studies were performed, with blood being sampled at the midpoint of each. During a third 15 minute period, fractional clearances of neutral dextrans were determined in the following manner. Tritiated dextrans of a wide molecular size distribution, prepared according to the method of Chang et al. [9], were

Table 1. Comparison of metabolic and renal function data in normal and chronically diabetic rats

	Diabetic (n = 5)	Control (n = 5)	p ^a
Body weight (g)	174±10	214±2	<0.005
Plasma glucose (mg/dl)	440±81	132±10	<0.001
Urine volume (ml/24 h)	103±11	20±1	<0.001
Urine glucose (g/24 h)	9.2±1.4	0	<0.001
GFR (ml/min)	2.77±0.16	2.72±0.11	NS
GFR/100 g body weight (ml/min)	1.59±0.02	1.27±0.04	<0.001
Urine protein (mg/24 h)	39±7	4.7±0.2	<0.005
Urine albumin (mg/24 h)	2.0±0.6	1.6±0.3	NS
Urine albumin/protein (%)	5.5±1.0	33.3±5.0	<0.001
Urine albumin (mg/24 h/100 g body weight)	1.24±0.31	0.74±0.11	NS

Values are mean±SEM

NS, not statistically significant ($p>0.05$); n = number of rats

^a significance of the difference between normal and diabetic rats

administered IV as a bolus injection (5 μ Ci in 0.5 ml) followed immediately by infusion of the same solution at 0.045 ml/min. About 90 s after completing injection of the priming dose, continuous collection of blood from the femoral artery was begun by means of a withdrawal pump (Model 944, Harvard Apparatus Co., Willis, Mass.) at a constant rate equal to the infusion rate of ³H-dextran. Collection of urine was begun about 90 s after beginning the continuous withdrawal of femoral arterial blood and finished at the same time interval after stopping the 15-minute collection of blood. The time intervals between administration of the priming dose and initiation of blood collection and between collections of blood and urine were identical in individual rats and were equal to the "transit time" for an IV bolus (0.05 ml) of 3% Lissamine Green to appear in the urine. This clearance method is recommended by Chang et al. [9] for the purpose of enhancing accuracy of dextran clearance measurements. The percent deviation from the mean for the 3 GFR determinations in individual rats was 6.9±0.5% (SEM), and the mean coefficient of variation for the 10 rats was 9.4%.

Analytical Methods

The 24 h urine samples and plasma samples collected prior to the clearance studies were analysed for glucose using a Beckman Glucose Analyzer (Beckman Instruments, Inc., Fullerton, Ca.). Urinary total protein was measured by the Coomassie blue protein-dye binding assay of Bradford (10) (Bio-Rad Laboratories, Richmond, California). The urinary excretion of albumin per 24 h was assayed by electrophoresis on a 10% polyacrylamide slab gels by the method of Kahn and Rubin [11]. After staining the gel with Coomassie brilliant blue, each specific albumin band was scanned (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), and the area under each uniform peak was measured with an electronic graphics calculator (Numonics Corp., Lansdale, Pennsylvania). The quantity of urinary albumin was derived from a standard curve of peak areas for five different quantities (from 50–1000 ng) of bovine serum albumin electrophoresed concurrently with the urine samples. Analysis of 17 consecutive protein solutions detected 103.9±2.2% (SEM) of the albumin present.

Samples of urine and plasma from the three clearance periods were analysed for ¹⁴C-inulin by liquid scintillation (Packard Tri-

Carb Spectrophotometer Model 3390, Packard Instruments, Inc., Downers Grove, Illinois).

The chromatographic methods of Chang et al. [9] were employed for separating dextrans of varying molecular size. The G-100 Sephadex columns were calibrated using dextran T₁₀, T₂₀, T₄₀, T₈₀ and as many as seven different biological proteins with molecular weights ranging from 12,500 to 150,000. The fractional volume available to test dextrans and test proteins (k_{av}) was plotted against the log of the respective Stokes-Einstein radii yielding a linear relationship identical to that reported by others [9]. Samples of 100 μ l of plasma and urine obtained during the third clearance period were mixed with 200 μ l of 0.01 mol/l ammonium acetate buffer (pH 7.0) and 1.0 mg of blue dextran and chromatographed on a calibrated 95 × 1.5 cm column of Sephadex G-100. Each 2 ml fraction collected in a position corresponding to the elution of molecules with a size from 18 to 42 angstroms (Å) were individually added to 7 ml Aquasol (New England Nuclear, Boston, Mass.) in a counting vial and thoroughly mixed so that a gel formed. The ³H-dextran and ¹⁴C-inulin in each fraction were counted, making appropriate corrections for quenching and cross-over.

Calculations

GFR was calculated as the urine-to plasma (U/P) concentration ratio of inulin times the urinary flow rate. The fractional clearances of dextrans were calculated as the U/P ratio for each molecular size dextran divided by the U/P ratio of inulin. Statistical analyses were performed using the Student t test [12]. Data are expressed as the mean±SEM.

Results

Metabolic and renal function data for diabetic and age-matched control rats are shown in Table 1. The diabetic rats weighed less than the normal rats (174 vs 214 g, $p<0.005$). Intermittent assays of nonfasting blood glucose indicated that comparable levels of hyperglycaemia persisted throughout the course of diabetes in each rat (460±79 at one month of diabetes and 440±81 at 7–10 months). At the time of the studies, the rats also were found to be intensely glycosuric (9.2±1.4 g/24 h) and polyuric (103±11 ml/24 h).

Proteinuria in diabetic rats was greater than in normal rats (39 vs 5 mg/24 h, $p<0.005$). Urinary excretion of albumin was similar in the two groups (2.0±0.6 mg/24 h in diabetics and 1.6±0.3 mg/24 h in normals). If corrected for differences in body weight, diabetic rats exhibited slightly higher albumin excretion (1.24±0.31 versus 0.74±0.11 mg/24 h/100 g body weight), but the difference between the groups failed to attain statistical significance. The contribution of albumin to the total urinary protein was much less in the diabetic group (5.5% versus 33.3%, $p<0.001$).

The mean values for absolute GFR were virtually identical for each group of rats (2.77±0.16 ml/min in diabetics vs 2.72±0.11 ml/min in normals); however,

Table 2. Fractional clearances^a of neutral dextrans of varying effective molecular radii in normal and chronically diabetic rats

	18Å	20Å	22Å	24Å	26Å	28Å	30Å	32Å	34Å	36Å	38Å	40Å	42Å
Normal (n = 5)	1.01 ±0.03	0.99 ±0.03	0.96 ±0.03	0.91 ±0.02	0.83 ±0.02	0.71 ±0.03	0.59 ±0.02	0.46 ±0.02	0.34 ±0.02	0.22 ±0.01	0.14 ±0.01	0.07 ±0.01	0.03 ±0.01
Diabetic (n = 5)	0.94 ±0.08	0.93 ±0.08	0.90 ±0.08	0.82 ±0.06	0.75 ±0.07	0.64 ±0.06	0.52 ±0.04	0.40 ±0.03	0.29 ±0.03	0.20 ±0.02	0.12 ±0.01	0.07 ±0.01	0.04 ±0.01
p	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data are mean ± SEM

NS, not significant ($p > 0.05$); n = number of rats^a Fractional clearance refers to the ratio of the renal clearance of neutral dextran to the simultaneously determined clearance of inulin **Fig. 1.** Fractional clearances of neutral dextrans in normal and chronically diabetic rats. See Methods for information regarding determination of effective molecular radii of neutral dextrans and the measurement and calculation of fractional clearances

if corrected for body weight, the mean value for GFR was slightly but significantly greater in diabetic rats (Table 1).

Data for the fractional clearances of neutral dextrans in both normal and diabetic rats are illustrated in Figure 1 and Table 2. In normal hydropenic rats, restriction of dextran filtration was not measurable until the effective molecular radius exceeded 22 Å. The fractional clearances of neutral dextrans progressively decreased as molecular size increased. The fractional clearances of macromolecular neutral dextrans were not different in normal hydropenic rats and chronically diabetic rats with proteinuria.

Discussion

Rats given streptozotocin develop the metabolic aberrations characteristic of human diabetes mellitus and a glomerulopathy histologically manifested by increased mesangium [1, 2, 4, 13, 14] and thickening of the glomerular basement membrane [14], and functionally manifested by proteinuria [1, 2, 4, 16]. We recently reported that rats with severe, long-term experimental diabetes have normal glomerular filtra-

tion rates and renal plasma flow [4]. In the present study, the mean value of GFR in rats chronically diabetic for 7–10 months again was found to be the same as in age-matched normal rats, although slightly higher if factored by body weight. However, correcting renal clearance data for body weight in diabetic rats is of dubious validity since GFR and body weight are likely to be independent variables separately affected by metabolic aberrations of the diabetic process. Further obscuring any possible relationship between body weight and GFR is the observation that diabetic rats may have larger kidneys and larger glomeruli than normal rats [17]. Nevertheless, this experimental model of diabetic nephropathy is characterised functionally by proteinuria unaccompanied by renal insufficiency.

Although proteinuria has emerged as the primary functional abnormality of the kidneys in diabetic rats [1–4], the responsible pathogenic mechanisms have not been elucidated. The present study was undertaken to evaluate whether the proteinuria observed in chronically diabetic rats resulted from alteration in the size-selective properties of the glomerular capillary wall. Macromolecules of the size of inulin (~14Å), or smaller, readily traverse the

glomerulus so that they appear in the fluid within Bowman's space in the same concentration as in plasma water [9]. Dextran, like inulin, is filtered and neither reabsorbed nor secreted by the tubules so that the urinary excretion of macromolecular dextrans of varying molecular size is a function of filtration by the glomerulus [9]. As the effective molecular radius of the dextrans increases, passage through the glomerulus becomes progressively restricted [9]. Accordingly, the ratio of the clearance of neutral dextran of a given molecular size to the clearance of inulin provides an index of the size selective properties of the glomerular capillary wall. In normal animals, the ratio is 1.0 for neutral dextrans of low molecular size (≤ 20 Å) and it progressively falls towards zero for larger dextran molecules (≤ 42 Å) (Fig. 1 and Table 2).

In experimental diabetic glomerulopathy, an increase in either the number or size of glomerular pores could result in an impaired ability to restrict filtration of serum proteins. Finding an increase in the fractional clearances of macromolecular neutral dextrans would provide support for this hypothetical mechanism of proteinuria in diabetes. However, in this study the fractional clearances of neutral dextrans were similar in diabetic and age-matched normal rats. Thus, proteinuria in our chronically diabetic rats cannot be attributed to an increase in number or size of glomerular pores.

Mogensen found normal fractional clearances of neutral dextrans in human subjects with early diabetes, although they were not clinically proteinuric [18]. On the other hand, Carrie and Myers recently reported that diabetic patients with heavy proteinuria (>2 g/d) and albuminuria exhibited depressed fractional clearances of neutral dextrans with effective molecular radii of 24–36 Å while fractional clearances of smaller and larger molecules were normal [19]. These results are compatible with a reduction in size or number of glomerular pores in patients with diabetic nephropathy and heavy proteinuria.

Since the fractional clearances of macromolecules may be influenced not only by the selective permeability properties of the glomerular capillary wall per se but also by haemodynamic factors that determine glomerular filtration, an alternative interpretation of our results is possible. In confirmation of their theoretical analysis [20], Chang and colleagues have shown that the fractional clearances of intermediate-sized (25–35 Å) neutral dextrans are diminished when glomerular plasma flow, and filtration rate, are increased [9]. Hence, it is conceivable that an upward displacement of the fractional clearances of neutral dextrans owing to increased number and/or size of glomerular pores could have been obscured by off-

setting influences of hyperfiltration in the diabetic group. This possibility seems unlikely as GFR was the same in both groups of rats.

The glomerular capillary wall normally possesses a net negative electrical charge owing to the presence on the epithelial surface of glycoproteins containing sialic acid [21, 22]. These glomerular polyanions appear to retard filtration of negatively charged macromolecules (such as albumin) to a greater extent than uncharged macromolecules of equivalent size [23]. In both clinical and experimental renal diseases manifested by heavy proteinuria, fractional clearances of uncharged macromolecules have been decreased while excretion of negatively charged macromolecules were increased, suggesting diminished charge-selectivity of the glomerular barrier, possibly resulting from reduced glomerular polyanion [24–27]. If the loss of negative glomerular charges that has been found in people with advanced diabetic nephropathy [28, 29] were also to occur in diabetic rats, then one might expect them to demonstrate a substantial increase in the excretion of negatively-charged proteins, such as albumin. Increases in albumin excretion of small magnitude have been observed previously in chronically diabetic rats [16, 30]. Albumin, however, has not been shown to be the dominant urinary protein in chronically diabetic rats [3]. We found no difference in absolute rates of albumin excretion in diabetic and normal rats. Factoring by body weight suggests greater albuminuria in diabetic than normal rats, but the difference fails to attain statistical significance and is of doubtful biological significance, particularly as albumin comprised only about 6% of the total protein in diabetic urine. Hence, to the extent that albuminuria per se may serve as an index of enhanced filtration of negatively charged macromolecules, it seems unlikely that a substantial impairment of charge-selectivity of the glomerulus underlies the proteinuria observed in our diabetic rats.

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