Original Article

Podocyte Detachment and Reduced Glomerular Capillary Endothelial Fenestration in Human Type 1 Diabetic Nephropathy

Masao Toyoda,[1] Behzad Najafian,[2,3] Youngki Kim,[2] M. Luiza Caramori,[4] and Michael Mauer[2,4]

The aim of this study was to investigate the structural characteristics of podocytes and endothelial cells in diabetic nephropathy. We studied 18 patients with type 1 diabetes (seven normoalbuminuric, six microalbuminuric, and five proteinuric), and six normal control subjects. Groups were not different for age. Type 1 diabetic groups were not different for diabetes duration or age at diabetes onset. Podocyte foot process width (FPW), fraction of glomerular basement membrane (GBM) surface with intact nondetached foot processes (IFP), fraction of glomerular capillary luminal surface covered by fenestrated endothelium [S_S(Fenestrated/cap)] and classic diabetic glomerulopathy lesions were morphometrically measured. Albumin excretion (AER) and glomerular filtration (GFR) rates were also measured. GFR correlated inversely and AER directly with GBM and mesangial measurements in diabetic patients. FPW correlated inversely with GFR ($r = -0.71$, $P = 0.001$) and directly with AER ($r = 0.66$, $P = 0.003$), GBM, and mesangial parameters. The GBM fraction covered by IFP was decreased in proteinuric versus control subjects ($P = 0.001$), normoalbuminuric patients ($P = 0.0002$) and microalbuminuric patients ($P = 0.04$) and correlated with renal structural and functional parameters, including AER ($r = -0.52$, $P = 0.03$). Only 78% of GBM was covered by IFP in proteinuric patients. S_S(Fenestrated/cap) was reduced in normoalbuminuric ($P = 0.03$), microalbuminuric ($P = 0.03$), and proteinuric ($P = 0.002$) patients versus control subjects. S_S(Fenestrated/cap) correlated with mesangial fractional volume per glomerulus ($r = -0.57$, $P = 0.01$), IFP ($r = 0.61$, $P = 0.007$), and FPW ($r = -0.58$, $P = 0.01$). These novel studies document that podocyte detachment and diminished endothelial cell fenestration are related to classical diabetic nephropathy lesions and renal function in type 1 diabetic patients and support a need for further studies of podocyte/GBM adherence and podocyte/endothelial cell functional interactions in diabetic nephropathy. *Diabetes* 56:2155–2160, 2007

Strong correlations between morphometric measures of mesangial expansion and glomerular basement membrane (GBM) width and functional parameters in diabetic nephropathy have long been known (1–2). More recently, however, interest in the possible role of podocytes in the development and/or progression of diabetic nephropathy has grown (3). Podocytes are important in maintaining glomerular permselectivity, and the development of proteinuria is associated with morphological changes in these cells, including foot process effacement (4). Podocyte detachment and GBM denudation have been documented in diverse renal conditions associated with severe proteinuria, such as idiopathic focal and segmental glomerulosclerosis (FSGS), puromycin nephrosis, amyloidosis, and reflux nephropathy (5–8). Foot process effacement and decreased podocyte number and/or density per glomerulus have been reported in patients with type 1 and type 2 diabetes (9–14). There is, however, no previously reported direct visualization and quantitation of podocyte detachment and GBM denudation in human diabetic nephropathy. On the other hand, we have recently shown that tuft to Bowman's capsule adhesions (TBCA), known to be associated with GBM denudation of podocytes (15), is common in proteinuric patients with type 1 diabetes (16).

Diabetic nephropathy has also been associated with biomarker changes consistent with generalized endothelial dysfunction (17). With the emergence of studies implicating podocytes and vascular endothelial growth factor in the pathogenesis of diabetic nephropathy (18), endothelial cells and their possible cross-talk with podocytes (19) deserve more attention. However, knowledge of endothelial structural changes in diabetic nephropathy is extremely limited (20,21).

This study, for the first time, demonstrates and quantitates direct histological evidence of podocyte detachment and GBM denudation in patients with type 1 diabetes with various albumin excretion rates (AERs) and describes the relationships to other glomerular structural and renal func-

From the [1]Division of Nephrology and Metabolism, Department of Internal Medicine; Tokai University School of Medicine, Kanagawa, Japan; the [2]Division of Pediatric Nephrology, Department of Pediatrics; University of Minnesota, Minneapolis, Minnesota; the [3]Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota; and the [4]Department of Medicine, University of Minnesota, Minneapolis, Minnesota.

Address correspondence and reprint requests to Michael Mauer, MD, Professor of Pediatrics and Medicine, University of Minnesota, 420 Delaware St. SE, MMC 491, Minneapolis, MN 55455. E-mail: mauer002@umn.edu.

Received for publication 8 January 2007 and accepted in revised form 12 May 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 29 May 2007. DOI: 10.2337/db07-0019.

This manuscript and its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

AER, albumin excretion rate; FPW, foot process width; FSGS, focal and segmental glomerulosclerosis; GBM, glomerular basement membrane; GFR, glomerular filtration rate; L_S(Slit/PGBM), slit diaphragm length density per PGBM; L_S(Slit/MGBM), slit diaphragm length density per MGBM; MGBM mesangial GBM; PGBM, peripheral GBM; S_S(IFP/PGBM), fraction of PGBM covered by intact foot processes; S_S(IFP/MGBM), fraction of MGBM covered by intact foot processes; S_S(Fenestrated/cap), surface density of fenestrated endothelium per glomerular capillary lumen; S_V(PGBM/glom), surface density of PGBM per glomerulus; TBCA, tuft to Bowman's capsule adhesions; V_V(MC/glom), mesangial cell fractional volume per glomerulus; V_V(Mes/glom), mesangial fractional volume per glomerulus; V_V(MM/glom), mesangial matrix fractional volume per glomerulus.

TABLE 1
Clinical and renal functional data of normal control subjects and normoalbuminuric, microalbuminuric, and proteinuric type 1 diabetic groups

	C	NA	MA	P	*P* value
n	6	7	6	5	
Sex (male/female)	5/1	3/4	2/4	2/3	NS
Age (years)	39 ± 11	29 ± 5	35 ± 13	38 ± 10	NS
Age at type 1 diabetes onset (years)	—	12 ± 6	11 ± 6	15 ± 5	NS
Type 1 diabetes duration (years)	—	16 ± 6	24 ± 12	24 ± 6	NS
A1C (%)	‡	7.6 ± 4.2	8.6 ± 0.9	9.0 ± 0.7	NS
Hypertension	0	2	3	5	0.002,* 0.03†
SBP (mmHg)	‡	124 ± 15	130 ± 14	136 ± 8	NS
DBP (mmHg)	‡	69 ± 12	73 ± 11	77 ± 4	NS
GFR (ml/min per 1.73 m^2)	‡	112 ± 7	89 ± 37	68 ± 22	0.008†
AER (μg/min)	‡	7.6 (1.8–10.2)	47.9 (20.5–117.5)	387.2 (268.0–869.1)	DD

Data are means ± SD or median (minimum–maximum). Only *P* values <0.05 are shown. *P vs. C; †P vs. NA. ‡Not available. C, control subjects; DBP, diastolic blood pressure; DD, different by definition; MA, microalbuminuric; NA, normoalbuminuric; P, proteinuric; SBP, systolic blood pressure.

tional parameters. Also measured was the extent of glomerular capillary endothelial fenestration as related to glomerular function and to diabetic glomerulopathy lesions, including podocyte abnormalities.

RESEARCH DESIGN AND METHODS

Eighteen research subjects (11 women) with type 1 diabetes, including seven normoalbuminuric, six microalbuminuric, and five proteinuric patients and six control normal living kidney donors were studied. Patients with type 1 diabetes who volunteered for research percutaneous renal biopsies and renal function studies were admitted to the General Clinical Research Center (GCRC) at the University of Minnesota for these studies. Normal living kidney donor research biopsies were obtained at transplant surgery while the kidney was still in situ in the donor. Type 1 diabetic groups, matched as closely as possible for age, duration of diabetes, and age at onset of diabetes, did not differ statistically for these parameters (Table 1). Subjects with type 1 diabetes in this study were also compared for classic diabetic glomerulopathy lesions to 137 research renal biopsy subjects with similar type 1 diabetes duration and AER to test whether the subjects reported here were representative. All studies were performed with permission of the Committee on the Use of Human Subjects in Research at the University of Minnesota and after informed consents were obtained.

Clinical studies. AER was measured in timed urine collections as described previously (22). Patients were classified into three groups based on AER values in at least two of three consecutive urine samples (normoalbuminuric: AER <20 μg/min; microalbuminuric: AER 20–200 μg/min; and proteinuric: AER >200 μg/min). Glomerular filtration rate (GFR) was estimated from the clearance of iothalamate or iohexol, both shown to be interchangeable with the clearance of inulin (23). A1C was measured by high-performance liquid chromatography. Blood pressure was the mean of multiple measurements by General Clinical Research Center nurses over ≥2 days using automated calibrated equipment. Hypertension was defined as blood pressure >130/80 mmHg or the use of antihypertensive drugs. All hypertensive patients were receiving ACE inhibitors.

Renal tissue processing. Renal biopsies were processed for electron microscopic stereological studies as detailed elsewhere (24). In brief, tissues were fixed in 2.5% glutaraldehyde in Millonig buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in PolyBed 812 (PolySciences, Warrington, PA). Semithin (1-μm-thick) sections were prepared to identify randomly acquired profiles of glomeruli. The center-most nonsclerosed glomerular profiles were selected. Intact glomeruli in each of three electron microscopic blocks were photographed using a JEOL 100 CX electron microscope (JEOL, Peabody, MA). No biopsy was excluded from this study because less than three appropriate glomeruli were available.

Stereological studies. Overlapping digital electron microscopic images at ×3900 magnification were used to construct a montage of an entire profile of each glomerulus studied. These montages were evaluated by two masked observers, and glomeruli with fixation or mechanical artifacts, such as glomerular tuft compression, extensive extrusion of proximal tubular cell material into glomerular urinary or capillary luminal spaces, or rupture of GBM or Bowman's capsule, were excluded. Also excluded (albeit rarely encountered) were glomerular cross-sections containing FSGS lesions. All structural measurements were obtained by a single masked observer.

Mesangial fractional volume per glomerulus [V_V(Mes/glom)] was estimated by superimposing a point-counting grid consisting of coarse and fine points over each montage. Course points were 19.2 μm apart. There were 4 fine points for each course point (24): 205 ± 51 course points and 178 ± 105 fine points were counted per biopsy. Surface density of peripheral GBM (PGBM) per glomerulus [S_V(PGBM/glom)] was estimated by superimposing a point and line grid over each montage (24). Points were 19.2 μm apart, and each point represented 9.6 μm of line length: 205 ± 51 course points and 193 ± 58 intercepts between the grid line and PGBM were counted.

Additional electron microscopic images were obtained at ×11,700 and ×34,600 magnification using a systematic uniform random sampling protocol. Mesangial matrix fractional volume per glomerulus [V_V(MM/glom)] and mesangial cell fractional volume per glomerulus [V_V(MC/glom)] were measured by superimposing the same point-counting grid over the ×11,700 images: 187 ± 110 and 125 ± 49 fine points per biopsy fell on mesangial matrix and mesangial cells, respectively. GBM width was estimated using the orthogonal intercept method on these same images (24): 120 ± 25 measurements were made per biopsy.

Images at ×34,600 were used to classify and quantitate podocyte-GBM interfaces and capillary lumen coverage. A line drawn where the GBM and capillary lumina lost their parallelism was used to define PGBM versus mesangial GBM (MGBM) (24). Podocyte-GBM interfaces were classified on the basis of the presence and arrangement of foot processes on GBM as follows: *1*) areas with intact foot processes, where foot processes interconnected with slit diaphragms were continuously covering GBM; *2*) areas with no foot process coverage (detached); and *3*) areas where a mixture of intact and detached foot processes produced an intermediate appearance (Fig. 1). Coverage of capillary lumina was classified into areas where endothelial cytoplasm was fenestrated or nonfenestrated. Where fenestrated, the endothelial cell cytoplasm was uniformly thin and regularly perforated by open spaces. In nonfenestrated areas, the endothelial cell cytoplasm was at least twice the thickness of the surrounding cytoplasm and free of perforations (Fig. 2). Tangential cuts of endothelial cells containing clear circular spaces were classified as fenestrated.

An unbiased 6.7 × 5.8 μm counting frame with inclusion and exclusion sides was superimposed on each image, leaving a 2-μm surrounding guard zone to allow for correct identification of structures approaching the periphery of images (Fig. 3). Each counting frame contained 20 vertical lines (288 nm apart) for estimating surface densities (Fig. 3). The number of intercepts between the vertical lines and areas with intact, detached, or mixed intact and detached foot processes were counted on PGBM and MGBM separately. The fractional surface of each specific area was estimated as

$$S_S(X/Z) = \frac{\sum I_X}{\sum I_Z} \quad (1)$$

where *X* represents areas with intact, detached, or mixed intact and detached foot processes, *Z* is the reference surface (PGBM or MGBM), and *I* is the number of intercepts. Similarly, the fraction of these surfaces affected by artifacts, defined by the rupture of cell membranes and release of cell organelles into Bowman's space, was measured. The average number of intercepts between the vertical lines and the PGBM per biopsy was 592 ± 235 and between the vertical lines and the MGBM per biopsy was 273 ± 111. Slit diaphragm length density per PGBM [L_S(Slit/PGBM)] and MGBM [L_S(Slit/MGBM)] were measured only in intact foot process areas. These parameters were estimated as

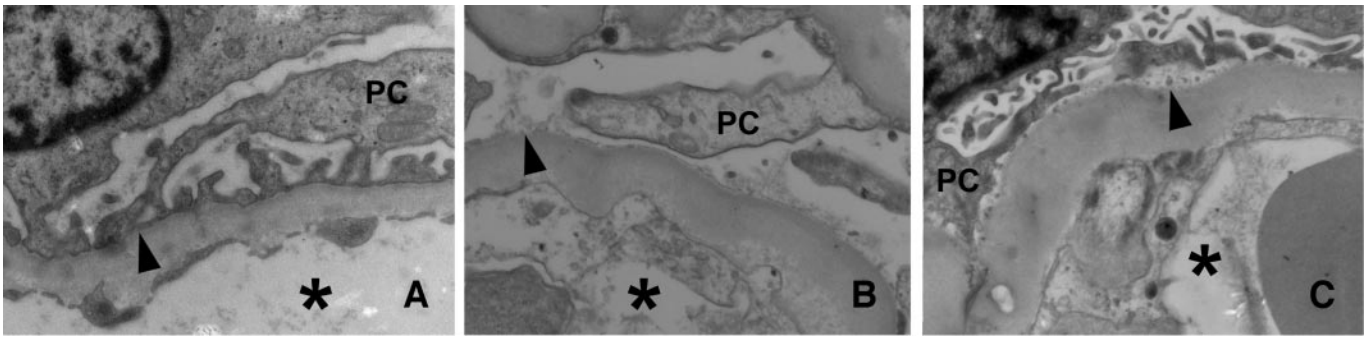

FIG. 1. Podocyte (PC)-GBM interfaces (arrowheads) are classified into areas with intact foot processes (*A*), areas with no foot process coverage (*B*), and areas with a mixture of intact and detached foot processes (*C*). *Capillary lumen.

$$L_S(Slit/Z) = \frac{\sum Q_{Slit}}{\sum I_{\text{intact FP}} \cdot \text{d}} \quad (2)$$

(25) where Z is the reference surface (PGBM or MGBM), Q_{Slit} is the number of filtration slit profiles in the counting frame, and $I_{\text{intact FP}}$ is the number of line intercepts with areas with intact foot processes, and d is the distance between the grid lines. The reciprocal of this parameter is the average foot process width (FPW). The average number of intercepts between the vertical lines and PGBM with intact foot processes per biopsy was 539 ± 235 and between the vertical lines and MGBM with intact foot processes was 248 ± 113.

The fractional surfaces of fenestrated and nonfenestrated capillary luminal coverage were estimated using this same grid and eq. 1, where X represents fenestrated or nonfenestrated areas and Z (reference surface) is the capillary luminal surface. An average of 520 intercepts per biopsy were counted over the capillary/PGBM interface and 303 per biopsy over the capillary/MGBM interface.

Statistics. Data are expressed as means ± SD, unless otherwise specified. AER values were logarithmically transformed before analysis. Group differences were evaluated by ANOVA and least significant difference post hoc test when indicated. Frequency differences were compared with the χ^2 test, and Fisher's exact test was performed if indicated. Relationships between the variables were evaluated using simple correlation. P values < 0.05 were considered statistically significant.

RESULTS

The groups were not statistically significantly different for age, and the groups with type 1 diabetes did not differ significantly for age at diabetes onset, diabetes duration, systolic or diastolic blood pressure, or A1C (Table 1). Hypertension was more frequent in proteinuric than normoalbuminuric ($P = 0.03$) patients or control subjects ($P = 0.002$), and GFR was lower in proteinuric than in normoalbuminuric patients ($P = 0.008$) (Table 1).

Electron microscopic measurements revealed classic changes of diabetic glomerulopathy in patients with type 1 diabetes, including an 88% increase ($P = 0.004$) in GBM width, a 100% increase ($P = 0.01$) in V_V(Mes/glom), and a 165% increase ($P = 0.008$) in V_V(MM/glom) compared with control subjects. GBM width was greater in proteinuric patients versus control subjects ($P = 0.00003$) or normoalbuminuric patients ($P = 0.005$) and in microalbuminuric ($P = 0.002$) and normoalbuminuric ($P = 0.03$) patients versus control subjects. V_V(Mes/glom) was greater in proteinuric patients versus control subjects ($P < 0.00001$), normoalbuminuric patients ($P = 0.00001$), or microalbuminuric patients ($P = 0.001$) (Table 2) and in microalbuminuric patients versus control subjects ($P = 0.001$) or normoalbuminuric patients ($P = 0.04$). Group values and statistical differences for V_V(MM/glom) and V_V(MC/glom)

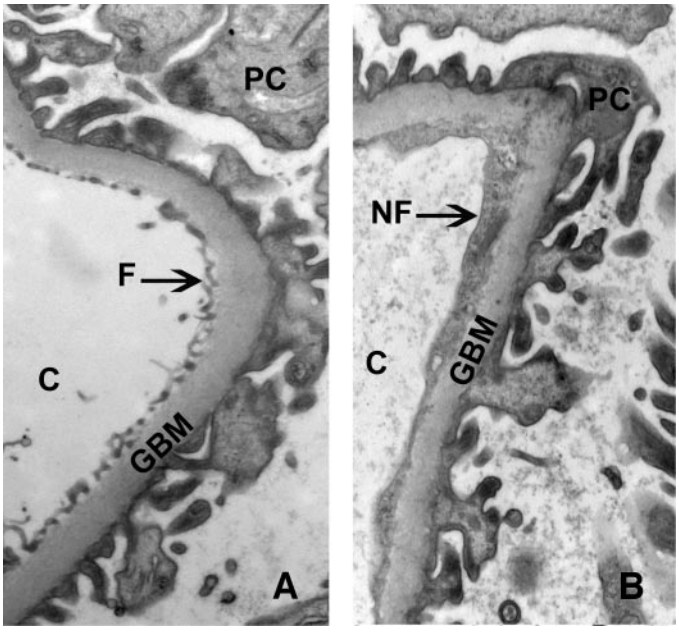

FIG. 2. Capillary endothelial coverage is classified into fenestrated (F) (*A*) and nonfenestrated (NF) (*B*) areas. C, capillary lumen; PC, podocyte.

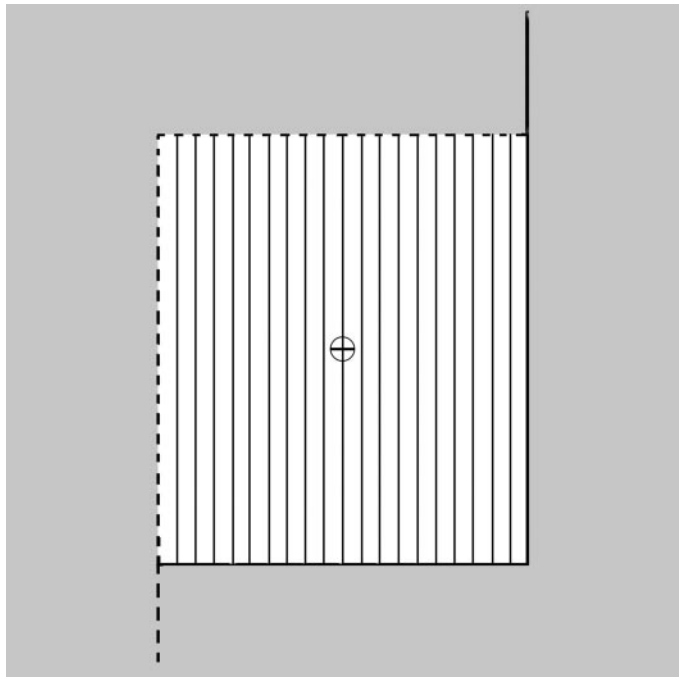

FIG. 3. The unbiased counting frame used for estimating length and surface densities. The gray area represents the guard zone (see text). Dashed lines represent inclusion sides; bold lines represent exclusion sides. Actual size: 6.7 × 5.8 μm. Number of vertical lines per frame = 20.

TABLE 2
Classic glomerular structural parameters in control subjects and normoalbuminuric, microalbuminuric, and proteinuric type 1 diabetic groups

	C	NA	MA	P	*P* value
n	6	7	6	5	
GBM width (nm)	361 ± 62	548 ± 116	639 ± 217	807 ± 111	0.03,* 0.002,† 0.00003,‡ 0.005§
V_V(Mes/glom)	0.16 ± 0.03	0.22 ± 0.04	0.30 ± 0.06	0.50 ± 0.11	0.001,† <0.00001,‡ 0.00001,§ 0.001,‖ 0.04¶
V_V(MM/glom)	0.07 ± 0.02	0.12 ± 0.02	0.16 ± 0.03	0.30 ± 0.17	0.04,* 0.0004,† <0.00001,‡ <0.0001,§ <0.0001,‖ 0.03¶
V_V(MC/glom)	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.11 ± 0.04	0.007,‡ 0.03§

Data are means ± SD. Only *P* values <0.05 are shown. *NA vs. C; †MA vs. C; ‡P vs. C; §P vs. NA; ‖P vs. MA; ¶MA vs. NA. C, control subjects; MA, microalbuminuric; NA, normoalbuminuric; P, proteinuric.

are also shown in Table 2. There were no statistically significant differences for any of these structural parameters between the diabetic study subjects and the normoalbuminuric ($n = 84$), microalbuminuric ($n = 32$), or proteinuric ($n = 21$) comparison groups with similar type 1 diabetes duration and AER (data not shown).

GFR in patients with type 1 diabetes, as expected, correlated inversely with V_V(Mes/glom) ($r = -0.67$, $P = 0.002$), V_V(MM/glom) ($r = -0.72$, $P = 0.001$), and GBM width ($r = -0.56$, $P = 0.02$). AER_{log} correlated directly with V_V(Mes/glom) ($r = 0.65$, $P = 0.003$), V_V(MM/glom) ($r = 0.73$, $P = 0.001$), and GBM width ($r = 0.55$, $P = 0.02$) and inversely with S_V(PGBM/glom) ($r = -0.49$, $P = 0.04$).

FPW in proteinuric patients was increased by 62%, compared with that in control subjects ($P = 0.002$), and by 53% ($P = 0.006$), compared with that in normoalbuminuric patients (Table 3). FPW was not statistically significantly different in control subjects versus normoalbuminuric or microalbuminuric patients or in proteinuric versus microalbuminuric patients. FPW correlated directly with AER_{log} ($r = 0.66$, $P = 0.003$) and inversely with GFR ($r = -0.71$, $P = 0.001$) in patients with type 1 diabetes. FPW also correlated directly with V_V(Mes/glom) ($r = 0.50$, $P = 0.03$), V_V(MM/glom) ($r = 0.61$, $P = 0.008$), and GBM width ($r = 0.68$, $P = 0.002$) in these patients.

The fraction of PGBM covered by intact foot processes [S_S(IFP/PGBM)] was reduced in proteinuric patients compared with that in control subjects ($P = 0.001$), normoalbuminuric patients ($P = 0.002$), and microalbuminuric patients ($P = 0.04$) (Table 3) with, on average, >20% of PGBM surface associated with abnormalities in podocyte attachment in the proteinuric patients. S_S(IFP/PGBM) correlated inversely with AER_{log} ($r = -0.52$, $P = 0.03$) and directly with GFR ($r = 0.66$, $P = 0.003$) in patients with type 1 diabetes. S_S(IFP/PGBM) also correlated inversely with V_V(Mes/glom) ($r = -0.62$, $P = 0.006$) and directly with S_V(PGBM/glom) ($r = 0.60$, $P = 0.008$) in patients with type 1 diabetes. S_S(IFP/MGBM) intergroup differences (Table 3) and correlations (data not shown) were very similar to those for S_S(IFP/PGBM). Surface density of GBM affected by definitive artifact was small, was not different among groups, and was not correlated with S_S(IFP/PGBM) (data not shown).

Of the glomerular capillary endothelial surface, 41% was fenestrated [S_S(Fenestrated/cap)] in control subjects. S_S(Fenestrated/cap) was reduced in normoalbuminuric ($P = 0.03$), microalbuminuric ($P = 0.03$), and proteinuric ($P = 0.002$) patients versus control subjects (Table 4). S_S(Fenestrated/cap) correlated inversely with V_V(Mes/glom) ($r = -0.57$, $P = 0.01$), V_V(MM/glom) ($r = -0.60$, $P = 0.009$), V_V(MC/glom) ($r = 0.47$, $P = 0.05$), and FPW ($r = -0.58$, $P = 0.01$) and directly with S_S(IFP/PGBM) ($r = 0.61$, $P = 0.007$) and S_V(PGBM/glom) ($r = 0.67$, $P = 0.002$) in patients with type 1 diabetes.

DISCUSSION

Foot process effacement, a regular concomitant of proteinuria, was recognized in the earliest human glomerular electron microscopic studies (26). However, the complex nature of foot process effacement (27) and a direct and consistent cause and effect relationship between this lesion and proteinuria (28) has been difficult to unravel. FPW highly correlated with AER and paralleled diabetic glomerular lesions in this and in previous studies in patients with type 1 diabetes (10,25,29). However, more recent studies have identified increased FPW in normoalbuminuric adolescent patients with type 1 diabetes compared with living kidney donors (11), which increased in 5-year follow-up biopsies (30). Although a trend toward increased FPW from control subjects to normoalbuminuric, microalbuminuric, and proteinuric patients was also observed in the present study, group differences were statistically significant only between proteinuric patients and control subjects or normoalbuminuric patients.

The initial aim of this study was to investigate podocyte morphological alterations, including FPW, in patients with type 1 diabetes with a wide range of AERs. The reciprocal of the L_S(Slit/GBM) (25) can be taken as mean FPW, if GBM coverage by foot processes is not interrupted. Examination of systematic random high magnification images of glomeruli revealed areas of GBM with detached foot processes, rendering this method of FPW estimation

TABLE 3
Podocyte structural parameters in control subjects and normoalbuminuric, microalbuminuric, and proteinuric type 1 diabetic groups

	C	NA	MA	P	*P* value
n	6	7	6	5	
FPW (nm)	390 ± 76	412 ± 56	510 ± 154	630 ± 154	0.002,* 0.006†
S_S(IFP/PGBM) (%)	98 ± 3	96 ± 2	89 ± 8	78 ± 18	0.001,* 0.002,† 0.04‡
S_S(IFP/MGBM) (%)	97 ± 4	95 ± 4	90 ± 11	75 ± 18	0.003,* 0.003,† 0.02‡

Data are means ± SD. Only *P* values <0.05 are shown.*P vs. C; †P vs. NA; ‡P vs. MA. C, control subjects; MA, microalbuminuric; NA, normoalbuminuric; P, proteinuric.

TABLE 4
Glomerular capillary endothelial fenestration in control subjects and normoalbuminuric, microalbuminuric, and proteinuric type 1 diabetic groups

	C	NA	MA	P	*P* value
n	6	7	6	5	
S_S(Fenestrated/cap) (%)	41 ± 11	32 ± 5	32 ± 3	25 ± 8	0.03,* 0.03,† 0.002‡

Data are means ± SD. *NA vs. C; †MA vs. C; ‡P vs. C. Only *P* values <0.05 are shown. C, control subjects; MA, microalbuminuric; NA, normoalbuminuric; P, proteinuric.

inapplicable in those areas. This inapplicability led to the development of the definitions of intact and detached podocyte areas used here.

Podocyte detachment, unlike foot process effacement, is not a necessary concomitant of proteinuria. In some animal models, proteinuria onset coincides with podocyte detachment and urinary podocyte loss was a better marker of glomerular damage than proteinuria in rodent models of glomerular injury (31,32). Areas of foot process detachment have also been observed in glomeruli from nephrotic patients with idiopathic FSGS, but predominantly in the segmental sclerosis lesions (5); such segmental sclerosis lesions were not studied here. However, FSGS lesions may have been present at other levels and would be anticipated in >50% of glomeruli, located at or close to the glomerular tubular junction in the proteinuric subjects (16). Podocyte detachment has also been reported in other pathologic conditions associated with proteinuria, including amyloidosis (7) and reflux nephropathy (8), whereas it was not observed in a careful study of congenital nephrotic syndrome or minimal change disease (33). Podocyte detachment has been observed in streptozocin-induced diabetes in rats (34), but to our knowledge, the present study is the first report of this phenomenon in human diabetes. In vitro experiments have shown decreased expression of the $\alpha_3\beta_1$ integrin, the principal adhesion complex that attaches the podocyte to the GBM, in rat and human podocytes cultured in high glucose media (35,36). These observations were confirmed by in vivo studies showing that the $\alpha_3\beta_1$ integrin was decreased in the podocytes of diabetic humans and rats (37,38). Moreover, increased numbers of podocytes in the urine of microalbuminuric and proteinuric patients with type 1 diabetes (39) is also consistent with in vivo separation of podocytes from the GBM in patients developing diabetic nephropathy. However, separation of podocytes from GBM does not necessarily imply GBM denudation, as the denuded areas could be covered by other podocytes. In fact, a reduction in podocyte number has been reported in normoalbuminuric patients with type 1 diabetes, i.e., before podocyte detachment is morphologically detectable (9). Surprisingly, ~22% of GBM in proteinuric patients was not covered by intact foot processes. Trends toward this finding were also identifiable in normoalbuminuric (~4%) and microalbuminuric (~11%) patients compared with control subjects (~2%) and the fraction of GBM with intact foot process coverage correlated inversely with AER_{log} and directly with GFR.

Podocyte detachment has been linked to TBCA, FSGS, and nephron destruction (15). TBCA, common in proteinuric patients with type 1 diabetes (16), is largely restricted to the glomerular-tubular junction area (15). Although a linkage between podocyte detachment and TBCA in proteinuric patients can be posited, the distribution of podocyte detachment in glomeruli was not determined in the current studies.

The number and/or density of podocytes per glomerulus are reportedly reduced in patients with type 1 and type 2 diabetes (9–14). White et al. (10) found a negative correlation between podocyte number and AER in normotensive proteinuric type 1 diabetic patients. The decreased number of podocytes per glomerulus in Pima Indians with type 2 diabetes was considered the strongest structural predictor of progression in albuminuria followed closely by mesangial fractional volume (40). A recent report of reduced numerical density of podocytes per glomerulus in microalbuminuric and proteinuric Northern Italian patients with type 2 diabetes argued that decreased podocyte number density but not decreased podocyte number per glomerulus was highly correlated with albuminuria (14). White et al. (13) also reported a significant inverse correlation between proteinuria and both podocyte number and density per glomerulus in mostly hypertensive microalbuminuric and proteinuric research subjects with type 2 diabetes (13). Concordant with these studies and alluded to above, Nakamura et al. (39) showed that podocytes were excreted in the urine of 53% of microalbuminuric and 80% of proteinuric patients with type 2 diabetes, whereas normoalbuminuric patients and healthy control subjects had no detectable urinary podocytes (39).

Endothelial dysfunction, as assessed indirectly through measures such as von Willebrand factor, factor VII activity, plasminogen activator inhibitor-1, vascular adhesion molecules, and nitric oxide–related vascular reactivity, has been hypothesized to contribute to the risk for diabetic nephropathy and to the concordance of this risk and the risk of macrovascular disease in both type 1 and type 2 diabetes (17). The morphology of glomerular capillary endothelial cells in diabetes, however, has not been extensively studied. Evan and Luft (20) first reported reduced size and number of glomerular endothelial fenestrae in alloxan-induced diabetic rats studied by scanning electron microscopy, and these findings have more recently been confirmed in STZ-induced diabetic rats (21). The present study, in which unbiased stereological measurements were used, is the first to report decreased endothelial fenestration in diabetic humans. Because diabetic rats of a given strain all tend to develop glomerular lesions at similar rates, the findings in these animals cannot be definitively attributed to diabetic nephropathy and may simply reflect the diabetic state. Moreover, reduced endothelial fenestration has also been reported in experimental cyclosporin A nephrotoxicity (41), serum sickness nephritis (42), and the remnant kidney model (43) and is thus not specific to diabetes. Nonetheless, our findings that reduction in glomerular capillary endothelial cell fenestration correlates with severity of diabetic glomerulopathy and podocyte lesions link this new finding to diabetic nephropathy risk and support a need for additional studies to investigate the importance of this phenomenon in the development and progression of diabetic nephropathy.

In summary, this study for the first time documents progressive foot process detachment and reduced endo-

thelial fenestration in human patients with type 1 diabetes. Both findings are associated with other important glomerular structural changes of diabetic nephropathy. These observations suggest that all cellular components of glomeruli are affected in diabetic nephropathy. A more detailed picture of the orchestrated glomerular cell structural and functional changes is required to understand the development of these interrelated lesions.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) Grants P01-DK-13083 and R01-DK-51975 and by grants from the Juvenile Diabetes Research Foundation (JDRF) International. This publication was also made possible by support from Grant M01 RR00400 from the National Center for Research Resources (NCRR), a component of the NIH. M.T. was supported by a training grant from his institution, and B.N. was supported by a JDRF fellowship grant. M.L.C. is currently supported by a JDRF Career Development Award.

We thank Patricia L. Erickson and Sandra Cragg for assistance with manuscript preparation and Thomas Groppoli and John Basgen for help with the renal structural studies.

REFERENCES

1. Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–1155, 1984
2. Caramori ML, Kim Y, Huang C, Fish AJ, Rich SS, Miller ME, Russell G, Mauer M: Cellular basis of diabetic nephropathy: 1. Study design and renal structural-functional relationships in patients with long-standing diabetes. *Diabetes* 51:506–513, 2002
3. Wolf G, Chen S, Ziyadeh FN: Capillary wall toward the center of disease. *Diabetes* 54:1626–1634, 2005
4. Pavenstadt H, Kriz W, Kretzler M: Cell biology of the glomerular podocyte. *Physiol Rev* 83:253–307, 2003
5. Grishman E, Churg J: Focal glomerular sclerosis in nephrotic patients: an electron microscopic study of glomerular podocytes. *Kidney Int* 7:111–122, 1975
6. Whiteside CI, Cameron R, Munk S, Levy J: Podocytic cytoskeletal disaggregation and basement-membrane detachment in puromycin aminonucleoside nephrosis. *Am J Pathol* 142:1641–1653, 1993
7. Katafuchi R, Taguchi T, Takebayashi S, Harada T: Proteinuria in amyloidosis correlates with epithelial detachment and distortion of amyloid fibrils. *Clin Nephrol* 22:1–8, 1984
8. Tada M, Jimi S, Hisano S, Sasatomi Y, Oshima K, Matsuoka H, Takebayashi S: Histopathological evidence of poor prognosis in patients with vesicoureteral reflux. *Pediatr Nephrol* 16:482–487, 2001
9. Steffes MW, Schmidt D, McCrery R, Basgen JM: Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int* 59:2104–2113, 2001
10. White KE, Bilous RW, Marshall SM, El Nahas M, Remuzzi G, Piras G, De Cosmo S, Viberti G: Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes* 51:3083–3089, 2002
11. Torbjornsdotter TB, Perrin NE, Jaremko GA, Berg UB: Widening of foot processes in normoalbuminuric adolescents with type 1 diabetes. *Pediatr Nephrol* 20:750–758, 2005
12. Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, Coplon NS, Sun L, Meyer TW: Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 99:342–348, 1997
13. White KE, Bilous RW, Diabetes Study Group: Structural alterations to the podocyte are related to proteinuria in type 2 diabetic patients. *Nephrol Dial Transplant* 19:1437–1440, 2004
14. Dalla Vestra M, Masiero A, Roiter AM, Saller A, Crepaldi G, Fioretto P: Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes* 52:1031–1035, 2003
15. Kriz W, Gretz N, Lemley KV: Progression of glomerular diseases: is the podocyte the culprit? *Kidney Int* 54:687–697, 1998
16. Najafian B, Crosson JT, Kim Y, Mauer M: Glomerulotubular junction abnormalities are associated with proteinuria in type 1 diabetes. *J Am Soc Nephrol* 17:S53–S60, 2006
17. Stehouwer CDA: Endothelial dysfunction in diabetic nephropathy: State of the art and potential significance for non-diabetic renal disease. *Nephrol Dial Transplant* 19:778–781, 2004
18. Khamaisi M, Schrijvers BF, De Vriese AS, Raz I, Flyvbjerg A: The emerging role of VEGF in diabetic kidney disease. *Nephrol Dial Transplant* 18:1427–1430, 2003
19. Ballermann BJ: Glomerular endothelial cell differentiation. *Kidney Int* 67:1668–1671, 2005.
20. Evan AP, Luft FC: Effect of alloxan-induced diabetes on the glomerular filtration barrier of the rat. *Ren Physiol* 3:257–264, 1980
21. Davila-Esqueda ME, Vertiz-Hernandez AA, Martinez-Morales F: Comparative analysis of the renoprotective effects of pentoxifylline and vitamin E on streptozotocin-induced diabetes mellitus. *Ren Fail* 27:115–122, 2005
22. Chavers BM, Simonson J, Michael AF: A solid phase fluorescent immunoassay for the measurement of human urinary albumin. *Kidney Int* 25:576–578, 1984
23. Gaspari F, Perico N, Remuzzi G: Measurement of glomerular filtration rate. *Kidney Int* 63:S151–S154, 1997
24. Fioretto P, Steffes MW, Mauer SM: Glomerular structure in non-proteinuric insulin-dependent diabetic patients with various levels of albuminuria. *Diabetes* 43:1358–1364, 1994
25. Bjorn SF, Bangstad HJ, Hanssen KF, Nyberg G, Walker JD, Viberti GC, Østerby R: Glomerular epithelial foot processes and filtration slits in IDDM patients. *Diabetologia* 38:1197–1204, 1995
26. Farquhar MG, Vernier RL, Good RA: An electron microscopic study of the glomerulus in nephrosis, glomerulonephritis and lupus erythematosus. *J Exp Med* 106:649–660, 1957
27. Asanuma K, Kim K, Oh J, Giardino L, Chabanis S, Faul C, Reiser J, Mundel P: Synaptopodin regulates the actin-bundling activity of α-actinin in an isoform-specific manner. *J Clin Invest* 115:1188–1189, 2005
28. Kalluri R: Proteinuria with and without renal glomerular podocyte effacement. *J Am Soc Nephrol* 17:2383–2389, 2006
29. Ellis EN, Steffes MW, Chavers B, Mauer SM: Observations of glomerular epithelial cell structure in patients with type I diabetes mellitus. *Kidney Int* 32:736–741, 1987
30. Perrin NE, Torbjornsdotter TB, Jaremko GA, Berg UB: Follow-up of kidney biopsies in normoalbuminuric patients with type 1 diabetes. *Pediatr Nephrol* 19:1004–1013, 2004
31. Whiteside C, Prutis K, Cameron R, Thompson J: Glomerular epithelial detachment, not reduced charge density, correlates with proteinuria in Adriamycin and puromycin nephrosis. *Lab Invest* 61:650–660, 1989
32. Yu D, Petermann A, Kunter U, Rong S, Shankland SJ, Floege: Urinary podocyte loss is a more specific marker of ongoing glomerular damage than proteinuria. *J Am Soc Nephrol* 16:1733–1741, 2005
33. Lahdenkari AT, Lounatmaa K, Patrakka J, Holmberg C, Wartiovaara J, Kestila M, Koskimies O, Jalanko H: Podocytes are firmly attached to glomerular basement membrane in kidneys with heavy proteinuria. *J Am Soc Nephrol* 15:2611–2618, 2004
34. Boyd RB, Thompson VW, Atkin J: Alterations in glomerular permeability in streptozotocin-induced diabetic rats. *J Am Podiatr Med Assoc* 86:57–62, 1996
35. Kretzler M: Regulation of adhesive interaction between podocytes and glomerular basement membrane. *Microsc Res Tech* 57:247–253, 2002
36. Kitsiou PV, Tzinia AK, Stetler-Stevenson WG, Michael AF, Fan WW, Zhou B, Tsilibary EC: Glucose-induced changes in integrins and matrix-related functions in cultured human glomerular epithelial cells. *Am J Physiol* F671–F679, 2003
37. Regoli M, Bendayan M: Alterations in the expression of the $\alpha_3\beta_1$ integrin in certain membrane domains of the glomerular epithelial cells (podocytes) in diabetes melliitus. *Diabetologia* 40:15–22, 1997
38. Chen HC, Chen CA, Guh JY, Chang JM, Shin SJ, Lai YH: Altering expression of $\alpha_3\beta_1$ integrin on podocytes of human and rats with diabetes. *Life Sci* 67:2345–2353, 2000
39. Nakamura T, Ushiyama C, Suzuki S, Hara M, Shimada N, Ebihara I, Koide H: Urinary excretion of podocytes in patients with diabetic nephropathy. *Nephrol Dial Transplant* 15:1379–1383, 2000
40. Meyer TW, Bennett PH, Nelson RG: Podocyte number predicts long-term urinary albumin excretion in Pima Indians with type II diabetes and microalbuminuria. *Diabetologia* 42:1341–1344, 1999
41. Kobayashi M, Takaya S, Koie H, Nagai K Glomerular endothelial changes in cyclosporine A-treated rats: Scanning and transmission electron microscopic studies. *Jpn J Surg* 21:210–215, 1991
42. Chowdhury AK: Participation of endothelial cells in the development of glomerulosclerosis: a study on murine serum sickness nephritis with mitomycin C. *Pathol Int* 46:173–182, 1996
43. Faraj AH, Morley AR: Remnant kidney pathology after five-sixth nephrectomy in rat. II. Electron microscopy study. *APMIS* 101:83–90, 1993