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

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Short Running Title:  Podocyte foot process detachment in diabetic nephropathy
Abstract

Objective: To study the structural characteristics of podocytes and endothelial cells (EC) in diabetic nephropathy (DN).

Research Design and Methods: We studied 18 patients with type 1 diabetes (T1DM) [7 normoalbuminuric (NA), 6 microalbuminuric (MA), 5 proteinuric (P)] and 6 normal controls (C). Groups were not different for age. T1DM 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 non-detached foot processes (IFP), fraction of glomerular capillary lumenal surface covered by fenestrated endothelium [$S_{(Fenestrated/cap)}$] and classic diabetic glomerulopathy lesions were morphometrically measured. Albumin excretion (AER) and glomerular filtration (GFR) rates were also measured.

Results: 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 P vs. C ($p=0.001$), NA ($p=0.0002$) and MA ($p=0.04$) patients 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 P patients. $S_{(Fenestrated/cap)}$ was reduced in NA ($P=0.03$), MA ($p=0.03$) and P ($p=0.002$) vs. C. $S_{(Fenestrated/cap)}$ correlated with $V_v(Mes/glom)$ ($r=-0.57$, $p=0.01$), IFP ($r=0.61$, $p=0.007$) and FPW ($r=-0.58$, $p=0.01$).

Conclusions: These novel studies document that podocyte detachment and diminished EC fenestration are related to classical DN lesions and renal function in T1DM and support a need for further studies of podocyte/GBM adherence and podocyte/EC functional interactions in DN.

Key Words: diabetes, diabetic nephropathy, podocyte, glomerular endothelial cell, albuminuria, human
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<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
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<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
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<td>FSGS</td>
<td>Focal segmental glomerulosclerosis</td>
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<td>AER</td>
<td>Albumin excretion rate</td>
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<tr>
<td>NA</td>
<td>Normoalbuminuric</td>
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<td>Microalbuminuric</td>
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<td>C</td>
<td>Control</td>
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<td>HbA₁c</td>
<td>Hemoglobin A₁c</td>
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<td>Electron microscopy</td>
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<td>Fine points</td>
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<td>FPW</td>
<td>Foot process width</td>
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<td>Vv(Mes/glm)</td>
<td>Volume fraction of mesangium per glomerulus</td>
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<tr>
<td>Sv(PGBM/glm)</td>
<td>Surface density of peripheral GBM per glomerulus</td>
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<tr>
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<tr>
<td>Vv(MC/glm)</td>
<td>Volume fraction of mesangial cells per glomerulus</td>
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<td>Peripheral GBM</td>
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<tr>
<td>MGBM</td>
<td>Mesangial GBM</td>
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<td>Length density of slit diaphragm per PGBM surface</td>
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<td>Lₛ(Slit/MGBM)</td>
<td>Length density of slit diaphragm per MGBM surface</td>
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<tr>
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<td>Surface density of areas with intact foot-processes per PGBM</td>
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<td>Surface density of areas with intact foot-processes per MGBM</td>
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<tr>
<td>Ss(Fenestrated/cap)</td>
<td>Surface density of fenestrated endothelium per glomerular capillary lumen</td>
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Introduction
Strong correlations between morphometric measures of mesangial expansion and glomerular basement membrane (GBM) width and functional parameters in diabetic nephropathy (DN) have long been known (1-2). More recently, however, interest has grown in the possible role of podocytes in the development and/or progression of DN (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 (T1DM) and type 2 (T2DM) diabetes (9-14). There is, however, no previously reported direct visualization and quantitation of podocyte detachment and GBM denudation in human DN. On the other hand, we have recently shown that tuft to Bowman’s capsule adhesions, known to be associated with GBM denudation of podocytes (15), are common in proteinuric patients with T1DM (16).

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

This study, for the first time, demonstrates and quantitates direct histologic evidence of podocyte detachment and GBM denudation in patients with T1DM with various albumin excretion rates (AER), and describes the relationships to other glomerular structural and renal functional parameters. Also measured was the extent of glomerular capillary endothelial fenestration as related to glomerular function and to diabetic glomerulopathy lesions, including podocyte abnormalities.

Materials and Methods
Subjects
Eighteen (11 females) research subjects with T1DM, including 7 normoalbuminuric (NA), 6 microalbuminuric (MA) and 5 proteinuric (P) patients, and 6 control (C) normal living kidney donors were studied. Patients with T1DM 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 (U of MN) for these studies. Normal living kidney donor research biopsies were obtained at transplant surgery while the kidney was still in-situ in the donor. T1DM groups, matched as closely as possible for age, duration and age at onset of diabetes were not statistically different for these parameters (Table 1). Subjects with T1DM in this study were also compared for classical diabetic glomerulopathy lesions to 137 research renal biopsy subjects with similar T1DM 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 U of MN and after informed consents were obtained.

Clinical Studies
AER was measured in timed urine collections as previously described (22). Patients were classified into three groups based on AER values in at least 2 of 3 consecutive urine samples: NA: AER <20µg/min; MA: AER 20-200µg/min and P: AER >200µg/min. GFR was estimated from the clearance of iothalamate or iohexol, both shown to be interchangeable with the clearance of inulin (23). Hemoglobin A₁c (HbA₁c) was measured by high performance liquid chromatography. Blood pressure (BP) was the mean of multiple measurements by GCRC nurses over two or more days using automated calibrated equipment. Hypertension was defined as BP >130/80mmHg or the use of anti-hypertensive drugs. All hypertensive patients were receiving ACE inhibitors.
Renal Tissue Processing
Renal biopsies were processed for electron microscopic (EM) 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, Inc., Warrington, PA). Semi-thin (1µm thick) sections were prepared to identify randomly acquired profiles of glomeruli. The center-most non-sclerosed glomerular profiles were selected. Intact glomeruli in each of 3 EM blocks were photographed using a JEOL 100 CX electron microscope (JEOL, USA, Peabody, MA). No biopsy was excluded from this study because less than three appropriate glomeruli were available.

Stereological Studies
Overlapping digital EM images at 3,900 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 and mechanical artifacts, such as glomerular tuft compression, extensive extrusion of proximal tubular cell material into glomerular urinary or capillary lumenal spaces, or rupture of GBM or Bowman’s capsule were excluded. Also excluded (albeit rarely encountered) were glomerular cross-sections containing large FSGS lesions. All structural measurements were obtained by a single masked observer.

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

Additional EM images were obtained at 11,700 and 34,600 magnification using a systematic uniform random sampling protocol. Mesangial matrix \([Vv(MM/glm)]\) and mesangial cell \([Vv(MC/glm)]\) fractional volumes per glomerulus were measured by superimposing the same point counting grid over the 11,700X images: 187±110 and 125±49 FP per biopsy fell on MM and MC, respectively. GBM width was estimated using the orthogonal intercept method on these same images (24): 120±25 measurements were made per biopsy.

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

An unbiased 6.7 x 5.8 µm counting frame with inclusion and exclusion sides was superimposed on each image, leaving a 2µm surrounding guard zone to allow for correct identification of structures approaching the periphery of images (Figure 3). Each counting frame contained 20 vertical lines (288nm apart) for estimating surface densities (Figure 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

\[ S_s(X/Z) = \sum \frac{I_x}{I_Z} \]

(equation 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 the Bowman’s space were 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 \( L_s(\text{Slit/PGBM}) \) and MGBM \( L_s(\text{Slit/MGBM}) \) were measured only in intact foot process areas. These parameters were estimated as

\[ L_s(\text{SD}/Z) = \sum \frac{Q_{\text{Slit} \times \text{mag}}}{I_{\text{intact FP}} \times FP \times d} \]

(25), where Z is the reference surface (PGBM or MGBM), \( Q_{\text{Slit}} \) is the number of filtration slit profiles in the counting frame, mag is the magnification, \( I_{\text{intact FP}} \) 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 intact FP over PGBM per biopsy was 539±235, and between the vertical lines and intact FP over MGBM was 248±113.

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

Statistics
Data are expressed as mean±SD, unless otherwise specified. AER values were logarithmically transformed prior to analysis. Group differences were evaluated by ANOVA and LSD post-hoc test when indicated. Frequency differences were compared with the Chi-square test and Fisher 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 T1DM did not differ significantly for age at diabetes onset, diabetes duration, systolic or diastolic blood pressure or HbA1c (Table 1). Hypertension was more frequent in P than NA (p=0.03) patients or C (p=0.002) and GFR was lower in P than NA patients (p=0.008) (Table 1).

Electron microscopic measurements revealed classical changes of diabetic glomerulopathy in patients with T1DM, including an 88% increase (p=0.004) in GBM width, a 100% increase (p=0.01) in \( V_\text{v(Mes/glom)} \) and a 165% increase (p=0.008) in \( V_\text{v(MM/glom)} \) compared to C. GBM width was greater in P vs. C (p=0.00003) or NA (p=0.005), and in MA (p=0.002) and NA (p=0.03) vs. C. \( V_\text{v(Mes/glom)} \) was greater in P vs. C (p=0.00001), NA (p=0.00001) or MA (p=0.001) patients (Table 2) and in MA patients vs. C (p=0.001) or NA patients (p=0.04). Group values and statistical differences for \( V_\text{v(MM/glom)} \) and \( V_\text{v(MC/glom)} \) 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 NA (n=84), MA (n=32), or P (n=21) comparison groups with similar T1DM duration and AER (data not shown).

GFR in patients with T1DM, as expected, correlated inversely with \( V_\text{v(Mes/glom)} \) (r= -0.67, p=0.002), \( V_\text{v(MM/glom)} \) (r= -0.72, p=0.001) and GBM width (r= -0.56, p=0.02). \( \text{AER}_{\log} \) correlated directly with \( V_\text{v(Mes/glom)} \) (r=0.65, p=0.003), \( V_\text{v(MM/glom)} \) (r=0.73, p=0.001) and GBM.
width \( (r=0.55, p=0.02) \) and inversely with \( \text{Sv(PGBM/glom)} (r=-0.49, p=0.04) \).

FPW in P patients was increased by 62%, compared to C \( (p=0.002) \) and by 53% \( (p=0.006) \), compared to NA patients (Table 3). FPW was not statistically significantly different in C vs. NA or MA patients, or in P vs. MA patients. FPW correlated directly with \( \text{AER}\log (r=0.66, p=0.003) \) and inversely with \( \text{GFR} (r=-0.71, p=0.001) \) in patients with T1DM. FPW also correlated directly with \( \text{Vv(Mes/glom)} (r=0.50, p=0.03) \), \( \text{Vv(MM/glom)} (r=0.61, p=0.008) \) and GBM width \( (r=0.68, p=0.002) \) in these patients.

The fraction of PGBM covered by intact foot processes \( \text{S} \) \( \text{s} \) (IFP/PGBM) was reduced in P patients compared to C \( (p=0.001) \), NA \( (p=0.002) \) and MA \( (p=0.04) \) patients (Table 3) with, on average, more than 20% of PGBM surface associated with abnormalities in podocyte attachment in the P patients. \( \text{S} \) \( \text{s} \) (IFP/PGBM) correlated inversely with \( \text{AER}\log (r=-0.52, p=0.03) \) and directly with \( \text{GFR} (r=0.66, p=0.003) \) in patients with T1DM. \( \text{S} \) \( \text{s} \) (IFP/PGBM) also correlated inversely with \( \text{Vv(Mes/glom)} (r=-0.62, p=0.006) \) and directly with \( \text{Sv(PGBM/glom)} (r=0.60, p=0.008) \) in patients with T1DM. \( \text{S} \) \( \text{s} \) (IFP/MGBM) inter-group differences (Table 3) and correlations (data not shown) were very similar to those of \( \text{S} \) \( \text{s} \) (IFP/PGBM). Surface density of GBM affected by definitive artifact was small, not different among groups, and was not correlated with \( \text{S} \) \( \text{s} \) (IFP/PGBM) (data not shown).

The initial aim of this study was to investigate podocyte morphological alterations, including FPW, in patients with T1DM with a wide range of AER. The reciprocal of the length density of slit diaphragm per GBM surface \( \text{Ls(Slit/GBM)} \) (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 inapplicable in those areas. This 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 (32, 33). Areas of foot process detachment have also been observed in glomeruli from nephrotic patients with idiopathic FSGS, but predominantly in the segmental sclerosis lesions (34), but such segmental sclerosis lesions were not studied here. However, FSGS lesions may have

Discussion

Foot process effacement, a regular concomitant of proteinuria, was recognized in the earliest human glomerular EM 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 T1DM (10, 25, 29). However, more recent studies have identified increased FPW in NA adolescent patients with T1DM compared to living kidney donors (30) which increased in 5-year follow-up biopsies (31). Although a trend toward increased FPW from controls to NA, MA, and P patients was also observed in the present study, group differences were statistically significant only between P patients and C or NA patients.
been present at other levels, and would be anticipated in more than 50% of glomeruli, located at or close to the glomerular tubular junction, in the P subjects (16). Podocyte detachment has also been reported in other pathologic conditions associated with proteinuria, including amyloidosis (7) and reflux nephropathy (8), while it was not observed in a careful study of congenital nephrotic syndrome or minimal change disease (35). Podocyte detachment has been observed in STZ-induced diabetes in rats (36), 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 α3β1 integrin, the principal adhesion complex that attaches the podocyte to the GBM, in rat and human podocytes cultured in high glucose media (37, 38). These observations were confirmed by in vivo studies showing that the α3β1 integrin was decreased in the podocytes of diabetic humans and rats (39, 40). Moreover, increased numbers of podocytes in the urine of MA and P patients with T1DM (41) is also consistent with in vivo separation of podocytes from the GBM in patients developing DN. However, separation of podocytes from GBM does not necessarily imply GBM denudation, as the denuded areas could be covered by other podocytes. In fact, reduction in podocyte number has been reported in NA patients with T1DM, i.e., before podocyte detachment is morphologically detectable (9). Surprisingly, approximately 22% of GBM in P patients was not covered by intact foot processes. Trends towards this finding were also identifiable in NA (~6%) and MA (~12%) patients compared to controls (~2%) and the fraction of GBM with intact foot process coverage correlated inversely with AERlog and directly with GFR.

Podocyte detachment has been linked to tuft to Bowman’s capsule adhesions (TBCA), FSGS and nephron destruction (15). TBCA, common in P patients with T1DM (16), are largely restricted to the glomerular-tubular junction area (15). While a linkage between podocyte detachment and TBCA in P patients can be posited, the current studies did not determine the distribution of podocyte detachment in glomeruli.

The number and/or density of podocytes per glomerulus are reportedly reduced in patients with T1DM and T2DM (9-14). White et al found a negative correlation between podocyte number and AER in normotensive proteinuric T1DM patients (10). The decreased number of podocytes per glomerulus in Pima Indians with T2DM was considered the strongest structural predictor of progression in albuminuria followed closely by mesangial fractional volume (42). A recent report of reduced numerical density of podocytes per glomerulus in MA and P Northern Italian patients with T2DM argued that decreased podocyte number density, but not decreased podocyte number per glomerulus, was highly correlated with albuminuria (14). White et al also reported a significant inverse correlation between proteinuria and both podocyte number and density per glomerulus in mostly hypertensive MA and P research subjects with T2DM (13). Concordant with these studies and alluded to above, Nakamura et al showed that podocytes were excreted in the urine of 53% of MA and 80% of P patients with T2DM, while NA patients and healthy C subjects had no detectable urinary podocytes (41).

Endothelial dysfunction, as assessed indirectly through measures such as von Willebrand factor, factor VII activity, PAI-1, vascular adhesion molecules and nitric oxide related vascular reactivity, has been hypothesized to contribute to DN risk and to the concordance of this risk and the risk of macrovascular disease in both T1DM and T2DM (17). The morphology of glomerular capillary endothelial cells in diabetes, however, has not been extensively studied. Evan et al first reported reduced size and number of glomerular endothelial fenestrae of alloxan-induced diabetic rats studied by scanning EM (20) and this has more recently been confirmed in STZ-induced diabetic rats (21). The present study, which employed unbiased stereological measurements, is the first to report decreased endothelial fenestration in diabetic humans. Since 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 DN and may simply reflect the diabetic state. Moreover, reduced
endothelial fenestration has also been reported in experimental cyclosporine A nephrotoxicity (43), serum sickness nephritis (44) and the remnant kidney model (45) 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 DN risk and support a need for additional studies to investigate the importance of this phenomenon in the development and progression of DN.

In summary, this study for the first time documents progressive foot process detachment and reduced endothelial fenestration in human patients with T1DM. Both findings are associated with other important glomerular structural changes of DN. These observations suggest that all cellular components of glomeruli are affected in DN. A more detailed picture of the orchestrated glomerular cell structural and functional changes is required to understand the development of these interrelated lesions.

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References


Table 1. Clinical and renal functional data of normal controls (C) and normoalbuminuric (NA), microalbuminuric (MA), and proteinuric (P) type 1 diabetic (T1DM) groups.

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<th></th>
<th>C (n=6)</th>
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<th>MA (n=6)</th>
<th>P (n=5)</th>
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<td>Male/Female</td>
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<td>Age (yrs)</td>
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<td>24±12</td>
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<td>GFR (ml/min/1.73m²)</td>
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<td>AER [median (min-max); µg/min]</td>
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<td>47.9</td>
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Abbreviations: HbA₁c=hemoglobin A₁c, SBP=systolic blood pressure, DBP=diastolic blood pressure, GFR=glomerular filtration rate, AER=albumin excretion rate, #=not available, * P vs. C, † P vs. NA, NS=not significant, DD=different by definition. Only p-values <0.05 are shown.
Table 2. Classical glomerular structural parameters in controls (C) and normoalbuminuric (NA), microalbuminuric (MA) and proteinuric (P) type 1 diabetic (T1DM) groups.

<table>
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<th>NA (n=7)</th>
<th>MA (n=6)</th>
<th>P (n=5)</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM width (nm)</td>
<td>361±62</td>
<td>548±116</td>
<td>639±217</td>
<td>807±111</td>
<td>0.03*, 0.002†, 0.00003‡,</td>
<td>0.005§</td>
<td></td>
</tr>
<tr>
<td>V_Mes/glom</td>
<td>0.16±0.03</td>
<td>0.22±0.04</td>
<td>0.30±0.06</td>
<td>0.50±0.11</td>
<td>0.001†, &lt;0.00001‡,</td>
<td>0.00001§, 0.001‖, 0.04‖</td>
<td></td>
</tr>
<tr>
<td>V_MM/glom</td>
<td>0.07±0.02</td>
<td>0.12±0.02</td>
<td>0.16±0.03</td>
<td>0.30±0.17</td>
<td>0.04*,0.0004†,</td>
<td>&lt;0.00001‖, &lt;0.0001§,</td>
<td>&lt;0.0001‖, 0.03‖</td>
</tr>
<tr>
<td>V_MC/glom</td>
<td>0.07±0.02</td>
<td>0.08±0.02</td>
<td>0.08±0.02</td>
<td>0.11±0.04</td>
<td>0.007‖, 0.03§</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: GBM=glomerular basement membrane; V_Mes/glom, V_MM/glom and V_MC/glom=fractional volumes of mesangium, mesangial matrix and mesangial cells per glomerulus, respectively, * NA vs. C, † MA vs. C, ‡ P vs. C, § P vs. NA, ‖ P vs. MA, ‡‡ MA vs. NA. Only p-values <0.05 are shown.
Table 3. Podocyte structural parameters in controls (C) and normoalbuminuric (NA), microalbuminuric (MA) and proteinuric (P) type 1 diabetic (T1DM) groups.

<table>
<thead>
<tr>
<th></th>
<th>C (n=6)</th>
<th>NA (n=7)</th>
<th>MA (n=6)</th>
<th>P (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPW (nm)</td>
<td>390±76</td>
<td>412±56</td>
<td>510±154</td>
<td>630±154</td>
<td>0.002*, 0.006†, 0.04‡</td>
</tr>
<tr>
<td>$S_{S}(IFP/PGBM)$ (%)</td>
<td>98±3</td>
<td>96±2</td>
<td>89±8</td>
<td>77±18</td>
<td>0.001*, 0.002†, 0.04‡</td>
</tr>
<tr>
<td>$S_{S}(IFP/MGBM)$ (%)</td>
<td>97±4</td>
<td>95±4</td>
<td>90±11</td>
<td>75±18</td>
<td>0.003*, 0.003†, 0.02‡</td>
</tr>
</tbody>
</table>

Abbreviations: PGBM=peripheral glomerular basement membrane (GBM); MGBM=mesangial GBM; FPW=foot process width on PGBM; IFP=intact foot process; $S_{S}(IFP/PGBM)$ and $S_{S}(IFP/MGBM)$=fractional surfaces of IFP per PGBM and MGBM, respectively, *P vs. C, †P vs. NA, ‡P vs. MA. Only p-values <0.05 are shown.
Table 4. Glomerular capillary endothelial fenestration in controls (C) and normoalbuminuric (NA), microalbuminuric (MA) and proteinuric (P) type 1 diabetic (T1DM) groups.

<table>
<thead>
<tr>
<th></th>
<th>C (n=6)</th>
<th>NA (n=7)</th>
<th>MA (n=6)</th>
<th>P (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sₖ(Fenestrated/cap) (%)</td>
<td>41±11</td>
<td>32±5</td>
<td>32±3</td>
<td>25±8</td>
<td>0.03*, 0.03†, 0.002‡</td>
</tr>
</tbody>
</table>

Abbreviations:  
Sₖ(Fenestrated/cap)= surface density of fenestrated endothelium per capillary lumen,  *NA vs. C, †MA vs. C, ‡P vs. C.  Only p-values <0.05 are shown.
Figure Legends

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

Figure 2. Capillary endothelial coverage is classified into (left) fenestrated (F) and (right) non-fenestrated (NF) areas. (C) Capillary lumen; (PC) Podocyte; (GBM) Glomerular basement membrane.

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

Appendix Figure 4

Classical glomerular structural parameters of diabetic nephropathy. Values for glomerular basement membrane (GBM) width and mesangial $[V_{\text{V}}(\text{Mes/glm})]$; mesangial matrix $[V_{\text{V}}(\text{MM/glm})]$ and mesangial cell $[V_{\text{V}}(\text{MC/glm})]$ fractional volumes in controls (C), normoalbuminuric (NA), microalbuminuric (MA) and proteinuric (P) type 1 diabetic patients. □=mean; SE=standard error; SD=standard deviation; ○=statistical outlier. Only p-values <0.05 are shown.

Appendix Figure 5

Structural parameters of podocytes and endothelial fenestration. Values for foot process width; surface density of intact podocyte foot processes on peripheral glomerular basement membrane $[S_{\text{s}}(\text{IFP/PGBM})]$; surface density of intact podocyte foot processes on mesangial glomerular basement membrane $[S_{\text{s}}(\text{IFP/MGBM})]$ and surface density of endothelial fenestration per glomerular capillary lumen $[S_{\text{s}}(\text{Fenestrated/cap})]$ in controls (C), normoalbuminuric (NA), microalbuminuric (MA) and proteinuric (P) type 1 diabetic patients. □=mean; SE=standard error; SD=standard deviation; ○=statistical outlier. Only p-values <0.05 are shown.
Figure 3