Is Podocyte Injury Relevant in Diabetic Nephropathy?

Studies in Patients With Type 2 Diabetes

Michele Dalla Vestra, Alessandra Masiero, Anna Maria Roiter, Alois Saller, Gaetano Crepaldi, and Paola Fioretto

Podocyte structural changes have been suggested to be involved in the pathogenesis of albuminuria in diabetes. We evaluated podocytes density, number, and structure in 67 white patients with type 2 diabetes: 21 normoalbuminuric (NA), 23 microalbuminuric (MA), and 23 proteinuric (P). Kidney function and biopsy studies were performed; 20 kidney donors served as control subjects. Electron microscopic morphometric analysis was used to estimate numerical density of podocytes per glomerulus [Nv(epi/glom)], filtration slit length density per glomerulus (FSLv/glom), and foot process width (FPW). The number of podocytes per glomerulus (Epi N/glom) was obtained by multiplying Nv(epi/glom) by mean glomerular volume. Nv(epi/glom) was significantly decreased in all type 2 diabetic groups compared with control subjects and was lower in MA and P than in NA (P < 0.0001, ANOVA). Epi N/glom was lower in MA and P patients compared with control subjects (P < 0.002, ANOVA); however, there were no significant differences among the type 2 diabetic groups. In addition, MA and P had decreased FSLv/glom and increased FPW compared with NA (P < 0.005 for both, ANOVA). The albumin excretion rate was inversely related to Nv(epi/glom) and FSLv/glom and directly to FPW (P < 0.0005 for all), whereas there was no correlation with Epi N/glom. In conclusion, changes in podocyte structure and density occur since the early stages of diabetic nephropathy and might contribute to increasing albuminuria in type 2 diabetic patients. These findings also suggest that in white type 2 diabetic patients, the density of podocytes may be functionally more relevant than the absolute number. Diabetes 52:1031–1035, 2003

The pathogenesis of microalbuminuria and overt proteinuria in type 2 diabetes is still unclear. Mesangial expansion and glomerular basement membrane (GBM) thickening, two classic parameters of diabetic glomerulopathy, are related to renal dysfunction in type 2 diabetes (1–5); however, the magnitude of these lesions does not completely explain the development of abnormal albuminuria in these patients (3–5). For example, despite persistent microalbuminuria or proteinuria, some patients with type 2 diabetes do not have diabetic glomerulopathy (5–7). Podocytes contribute to glomerular permeability, and the development of proteinuria is associated with marked morphological changes in these cells. In fact, the relationships between the changes in podocyte foot process structure and proteinuria, first documented in nil lesion nephrotic syndrome (8), have also been described in diabetes. Thus, changes in epithelial cell structure have been observed in patients with type 1 diabetes both with microalbuminuria and overt proteinuria (9,10). A decrease in the number of podocytes per glomerulus was found in Pima Indians with type 2 diabetes and clinical nephropathy (11), and the authors suggested that this phenomenon was causally related to the progression of diabetic nephropathy (11,12). The present study evaluated podocytes’ structural parameters in relation to albumin excretion rate (AER) and glomerular filtration rate and to other glomerular structural parameters in white patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Patients. Inclusion criteria were type 2 diabetes, diagnosed according to World Health Organization criteria (1985), for at least 2 years; age 70 years or less; serum creatinine <180 μmol/l; and adequate renal biopsy tissue for electron microscopic morphometric analyses. Patients were excluded when they had renal biopsy contraindication or known nondiabetic renal disease. All biopsies were performed on the basis of a research protocol, not for clinically indicated diagnostic purposes. These studies were approved by the Ethical Committee of the University of Padova, and each patient gave written informed consent before each study. Patients were admitted to the Division of Internal Medicine at the University of Padova Hospital, where percutaneous renal biopsy and renal functional studies were performed. Overall, three patients were not receiving antihypertensive treatment; among the remaining, all but seven patients were receiving ACE inhibitors or angiotensin receptor blockers alone or associated with dihydropyridinic calcium antagonists and/or diuretics and/or α- and β-blockers.

Antihypertensive treatment was withdrawn for 3–5 days before and during admission and then resumed. Urinary AER was determined on at least three in-hospital sterile 24-h urine collections. Glomerular filtration rate (GFR) was determined by the plasma clearance of 51Cr-EDTA from 12 blood samples over 300 min. AER <20 μg/min was defined as normoalbuminuria, 20–200 was defined as microalbuminuria, and >200 was defined as proteinuria, when present in at least two of three urine collections.

Normal control subjects. Twenty normal subjects served as control subjects for the morphometric studies. They were living related kidney donors at the University of Minnesota.

Renal function studies. AER was measured by an immunoturbidimetric method. GFR was measured by modeling analysis of plasma decay of 51Cr-EDTA (13). Blood pressure was measured at least 10 times with the patients in the supine position, and the values provided are the mean of these repeated measurements. HbA1c was measured by high-performance liquid chromatography (DIAMAT Analyzer, BIO-RAD, CA).

Renal structure studies. Kidney biopsies were performed under ultrasound guidance by an experienced investigator (P.F.). Tissue was immediately
PODOCYTE STRUCTURE IN TYPE 2 DIABETES

TABLE 1
Clinical features of the type 2 diabetic patients

<table>
<thead>
<tr>
<th></th>
<th>NA</th>
<th>MA</th>
<th>P</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>12 M/9 F</td>
<td>19 M/4 F</td>
<td>19 M/4 F</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61 ± 5</td>
<td>55 ± 8*</td>
<td>59 ± 7</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28 ± 4</td>
<td>27 ± 3</td>
<td>29 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>D2 duration</td>
<td>11 ± 8</td>
<td>12 ± 5</td>
<td>16 ± 6†</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.5 ± 1.4</td>
<td>8.8 ± 1.5†</td>
<td>9.0 ± 1.5†</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>GFR (ml·min⁻¹·1.73 m⁻²)</td>
<td>98 ± 21</td>
<td>106 ± 36</td>
<td>86 ± 30</td>
<td>NS</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>81 ± 2</td>
<td>108 ± 8</td>
<td>108 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>AER (µg/min)</td>
<td>9 (3–138)</td>
<td>48 (20–138)</td>
<td>722 (237–394)</td>
<td>—</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± 1 SD, except for AER presented as median (range). MBP, mean blood pressure; *P < 0.05 vs. NA and P; †P < 0.01 vs. NA.

processed for light, electron, and immunofluorescence microscopy. Electron microscopic examination was conducted on tissue fixed in 2.5% glutaraldehyde and embedded in Polybed (14). Tissue for light microscopy was embedded in paraffin, sectioned at 2–3 µm, and stained with periodic acid-Schiff.

Electron microscopy. Morphometric analysis was performed on three open glomeruli per biopsy. Photographs obtained at ×3,000 were assembled into photomontages of the entire glomerular profile (14).

Podocytes were defined as residing within the glomerular tuft but outside the GBM. Only visceral epithelial cells (podocytes) were considered, whereas parietal epithelial cells were not.

Podocyte density per glomerulus [Nv(epi/glom)] was calculated using the Weibel and Gomez method (15). Area density of podocyte nuclei per glomerulus [Na(epi-nuclei/glom)] was determined by point counting: Na(epi-nuclei/glom) = N × (avgp × (d/mag)²), where N is the number of nuclei, avgp is the number of points hitting the glomerular profile, d is the distance between coarse grid points, and mag is the magnification. The volume density of podocyte nuclei per glomerulus [Vv(epei-nuclei/glom)] was then calculated: Vv(epei-nuclei/glom) = Σp × (avgp × 25), where Σp is the number of fine grid points over nuclei and 25 is the number of fine points corresponding to each coarse point. Finally, the numerical density of podocytes [Nv(epi/glom)] was calculated: Nv(epi/glom) = (1/1.55) × [Na(epei-nuclei/glom)/Vv(nuclei/glom)]⁻², where 1.55 is the shape factor for the nuclei (16). The same montages were used to estimate mesangial fractional volume [Vv(mes/glom)] by point counting (14).

A set of micrographs at 12,000× was used to estimate GBM width by the orthogonal intercept method (17). Another set of micrographs, obtained at 20,000× by systematically sampling, was used to estimate filtration slit length density per glomerulus (FSLv/glom), surface density of the peripheral GBM [Sv(PGBM)], and foot process width (FPW) over the peripheral GBM (9,10).

Unbiased counting frame, to avoid the edge effects, was superimposed on each micrograph.

FSLv/glom = 2 × Q/K² × P, where Q is the number of filtration slits, K is the distance between points divided for the magnification, and P is the number of points hitting the glomerulus.

Sv(PGBM) = 2 × K(d/mag) × P, where K is the number of intersections between the surface trace and the test lines and d is the test line length per point.

FPW = Sv(PGBM)/FSLv/glom

Light microscopy. Periodic acid-Schiff-stained paraffin-embedded light microscopy slides were used to estimate mean glomerular volume (MGV) by the method of Weibel-Gomez (15), only when at least 10 glomerular profiles were available. The number of glomeruli was adequate in 45 patients (15 normoalbuminuric [NA], 16 microalbuminuric [MA], and 14 proteinuric [P]).

PODOCYTE STRUCTURE IN TYPE 2 DIABETES

TABLE 2
Gluomeral morphometric parameters in the type 2 diabetic patients and control subjects

<table>
<thead>
<tr>
<th>Control subjects</th>
<th>NA</th>
<th>MA</th>
<th>P</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nv(epi/glom) (n/× 10⁶ µm⁻³)</td>
<td>263 ± 110†</td>
<td>175 ± 72*</td>
<td>115 ± 45</td>
<td>98 ± 46</td>
</tr>
<tr>
<td>Epi N/glom</td>
<td>833 ± 184‡</td>
<td>654 ± 265</td>
<td>592 ± 283</td>
<td>554 ± 311</td>
</tr>
<tr>
<td>FSLv/glom (µm/µm³)</td>
<td>NA</td>
<td>0.27 ± 0.08*</td>
<td>0.21 ± 0.07</td>
<td>0.17 ± 0.07</td>
</tr>
<tr>
<td>FPW (nm)</td>
<td>NA</td>
<td>560 ± 88§</td>
<td>663 ± 110</td>
<td>724 ± 210</td>
</tr>
<tr>
<td>MGV (× 10⁶ µm³)</td>
<td>3.5 ± 1.0‡</td>
<td>4.1 ± 1.5</td>
<td>5.2 ± 2.6</td>
<td>5.4 ± 1.8</td>
</tr>
<tr>
<td>Vv(mes/glom)</td>
<td>0.19 ± 0.03†</td>
<td>0.23 ± 0.04§</td>
<td>0.25 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>GBM width (nm)</td>
<td>310 ± 38‡</td>
<td>405 ± 49§</td>
<td>477 ± 93§</td>
<td>584 ± 139</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± 1 SD. NA, not available; *P < 0.01, NA vs. MA and P; †P < 0.0001, control vs. all D2; ‡P < 0.01, control vs. MA and P; §P < 0.05, NA vs. MA and P < 0.005, NA vs. P; ‖P < 0.005, MA vs. P.
patients (Table 2 and Fig. 1). The number of podocytes per glomerulus (Epi N/glom) was lower in MA and P patients compared with control subjects but was not significantly different among the type 2 diabetic groups (Table 2). MGV was greater in MA and P patients compared with control subjects, with no significant differences among the type 2 diabetic groups (Table 2).

FSLv/glom was lower in MA and P compared with NA patients with a trend toward lower values in P compared with MA patients (P = 0.059) (Table 2 and Fig. 1). Similarly, FPW was higher in MA and P compared with NA patients with no differences between the MA and the P groups (Table 2 and Fig. 1).

**Podocyte structure, diabetic glomerulopathy, and renal function.** Nv(epi/glom) and Epi N/glom correlated with GBM width (r = −0.40, P < 0.01 and r = −0.45, P < 0.002, respectively) and Vv(mes/glom) (r = −0.34, P < 0.01 and r = −0.47, P < 0.003, respectively).

FPW correlated directly with GBM width (r = 0.50, P < 0.0005) and Vv(mes/glom) (r = 0.55, P < 0.0005). FSLv/glom was inversely related to GBM width (r = −0.55, P < 0.0005) and Vv(mes/glom) (r = −0.61, P < 0.0005).

AER was inversely related to Nv(epi/glom) (r = −0.52, P < 0.0005) (Fig. 2) but not to Epi N/glom. AER was inversely correlated with FSLv/glom (r = −0.50, P < 0.0005) (Fig. 2) and directly with FPW (r = 0.44, P < 0.0005) (Fig. 2). AER also correlated with Vv(mes/glom) (r = 0.55, P < 0.0005) and GBM width (r = 0.59, P < 0.0005).

In contrast GFR, inversely related to Vv(mes/glom) (r = −0.52, P < 0.0005), was only weakly related to FSLv/glom (r = 0.29, P < 0.05) and was not correlated with Nv(epi/glom), Epi N/glom, and FPW. Blood pressure was not correlated with any structural parameter.

**Podocyte structure in patients with normal Vv(mes/glom).** We compared podocyte structural parameters in 16 patients with abnormal AER (12 MA and 4 P) and normal Vv(mes/glom) (≤0.25, mean ± 2 SD in normals) with that of 16 NA patients with normal Vv(mes/glom). Patients with increased AER had lower Nv(epi/glom) (119 ± 50 n/μm² × 10⁶) and FSLv/glom (0.20 ± 0.05 μm/μm³) than NA patients (168 ± 74, P < 0.05 and 0.29 ± 0.07, P < 0.02, respectively). There was no significant difference in Epi N/glom between the two groups (654 ± 265 and 791 ± 330). FPW was greater in patients with increased AER than in NA patients (654 ± 95 vs. 549 ± 87 nm; P < 0.01). GBM width was similar in the two groups (422 ± 74 vs. 305 ± 50 nm; NS).

**DISCUSSION**

This study demonstrates that changes in podocyte structure occur at the early stages of diabetic nephropathy in patients with type 2 diabetes and are related to AER. Although other glomerular lesions, especially mesangial expansion and GBM thickening, have received extensive attention and have demonstrated strong association with renal dysfunction in diabetes (1–5,21), these structural parameters can account for only 30–50% of the variability.
in AER and GFR, leaving much of these functional variables unexplained. In recent years, podocyte alterations have been considered as potential contributors to the pathogenesis of diabetic nephropathy (9–12,16,22,23). Podocytes are an integral part of the filtration barrier, and changes in their structure have been observed in a broad range of proteinuric glomerular diseases, including diabetes (9–12,20). At variance with other glomerular cells, podocytes are considered to have limited capacity to replicate, at least postnatally (16,22,24–26). Thus, the loss of podocytes would necessarily require the residual cells to cover a larger area of GBM. This could cause foot process widening and reduce the ability of the podocytes to remain attached to GBM, with consequent areas of bare GBM, which are potential starting points for glomerulosclerosis.

Studies in rats with reduced nephron number and consequent glomerular hypertrophy have demonstrated that podocytes suffer progressive injury when forced to cover a larger surface area. Initially, these rats develop proteinuria and foot process widening and subsequently glomerular sclerosis (24,25). Very few studies have explored podocyte number in humans; low podocyte number has recently been observed in IgA nephropathy (27). Steffes et al. (16) reported decreased podocyte numbers in patients with type 1 diabetes and normal AER, even in those with short diabetes duration. White et al. (20) in contrast observed similar numbers of podocytes in normal individuals and in normotensive patients with type 1 diabetes and abnormal AER, although there was a trend toward fewer podocytes in the diabetic cases. Moreover, this study found a correlation between podocyte number and AER only in the P patients, whereas there was no correlation when the MA and P patients were pooled together (20). Low podocyte number has been described in Pima Indians with type 2 diabetes and P (11). Pagaltunan et al. (11) described broadening of foot processes associated with a reduction in podocyte number and density per glomerulus in P patients, suggesting that podocytopenia and podocyte damage may contribute to the progression of diabetic nephropathy. More recently, longitudinal studies of the MA Pima Indians, including sequential renal biopsies, strengthened the concept that loss and lesions of podocytes are involved in the progression of diabetic nephropathy (12). However, the pathogenetic mechanisms underlying diabetic renal disease in the ethnically homogeneous Pima Indians may not be extrapolated to white patients with type 2 diabetes.

Indeed, we have described structural heterogeneity in white type 2 diabetic patients, with only 30–50% of patients presenting mesangial expansion and GBM thickening, the remaining having predominantly tubulointerstitial and/or vascular changes, or essentially normal renal structure (5,6). Therefore, in type 2 diabetes, the “classical” lesions of diabetic glomerulopathy alone often do not explain the presence of abnormal AER and podocytes could have an important role in causing altered glomerular permeability.

Thus, we studied podocyte structure, density, and number in a large number of white patients with type 2 diabetes. We found that changes in podocyte structure and density are detectable in the early stages of diabetic nephropathy and that these lesions become more severe with increasing AER. The numerical density of podocytes was significantly decreased in all type 2 diabetic groups, more markedly in MA and P compared with NA. Also, the number of podocytes per glomerulus was reduced in MA and P compared with control subjects without significant differences among the type 2 diabetic groups. AER was related to podocyte numerical density but not to number per glomerulus. Thus, particularly at these early stages of the disease, the numerical density of podocytes is related to the magnitude of the permselectivity defect, whereas the absolute number is not. These findings may seem somehow different from previous reports in diabetes, where the interest was on podocyte number rather than on density; however Steffes et al. (16) reported only on podocyte number and not density. White et al. (20) described a reduction in podocyte density but not in number in P patients with type 1 diabetes. Podocyte density was more markedly reduced than number in Pima Indians, but the authors mainly emphasized the reduction in number (11). There was no information on the relationships between AER and structural parameters in this article (11).

In a subsequent longitudinal study, the numbers of podocytes were reported to be related to the progression to proteinuria in the MA group, but information on density was not reported (12). Thus, on the basis of the published data, it is not known whether podocyte density and number provide similar information and are equally relevant; indeed, podocytes only recently have been investigated in diabetes, and, certainly, a clearer answer will be forthcoming as future investigations are completed.

A decrease in numerical density can be consequent to 1) enlargement in glomerular volume, 2) decrease in the absolute number/glomerulus, or 3) a combination of both factors. Our results are in keeping with the third possibility and demonstrate that a decrease in the absolute number/glomerulus is not necessary for a decrease in density to occur.

At the present time, on the basis of the findings of our study, we propose that podocyte numerical density is more functionally relevant than the podocyte number per glomerulus. Indeed, the number of podocytes per glomerulus may not provide important information on glomerular architecture. Thus, the same number of podocytes in a patient with large glomeruli would be forced to cover a larger area of peripheral GBM than in a patient with smaller glomeruli and be subjected to increased mechanical stress and injury. In addition to the finding of lower podocyte numerical density in patients with increased AER than in those with NA, we observed a significant inverse correlation between podocyte numerical density and AER, suggesting a link between podocyte density and glomerular permeability to albumin. Similarly, FPW was increased and filtration slit length density was decreased in MA and P compared with NA, and both were significantly related to AER. In contrast, GFR was marginally related only to filtration slit length density and was not correlated with FPW, podocyte number, or density.

Compared with the findings in Pima Indians (11), we found differences in all podocyte parameters between NA and MA patients; this is likely to be due to the larger number of patients in our study. In contrast, we did not find the significant differences between MA and P patients observed in the Pima Indians (11), probably because our P
patients had lower AER levels. In keeping with our observations, Bjorn et al. (10) described an increase of FPW over the peripheral GBM in patients with type 1 diabetes and abnormal AER compared with NA patients, without significant differences between MA and P patients.

It has been suggested that podocyte injury in diabetes may be related to mesangial expansion, which can cause closure of capillary loops and obliteration of podocytes (11). Indeed, in the present cohort of patients, there were strong associations between the degree of diabetic glomerulopathy (mesangial volume fraction and GBM width) and podocyte density and number per glomerulus, FPW, and filtration slit length density. It is not possible to dissect from this cross-sectional study whether these glomerular lesions all develop in parallel or one antedates the other. Nevertheless, to understand the structural basis for albuminuria in patients without diabetic glomerulopathy, we compared podocyte density and structure in two groups of patients with normal Vv(mesglomer): the first with NA, the second with increased AER. The numerical density but not the number of podocytes and filtration slit length density were decreased and FPW was increased in patients with abnormal AER compared with NA. These findings may help in understanding the structural basis for increased AER in these patients without diabetic glomerulopathy and suggest that podocyte injury, as a primary event, or altered glomerular capillary permeability with podocyte change, as a secondary event, may develop independently from mesangial expansion. Whether the podocyte changes described here contribute to the increased permeability of the filtration barrier to proteins or is the increased permeability to protein that causes podocyte damage is unknown.

In conclusion, this study demonstrates that changes in podocyte structure and numerical density are present at the early stages of diabetic nephropathy and, along with mesangial expansion and GBM thickening, might contribute to renal functional abnormalities in white patients with type 2 diabetes. Moreover, podocyte structural changes could explain abnormal albuminuria in patients without diabetic glomerulopathy. Whether podocyte injury is consequence or cause of abnormal AER will be addressed only by longitudinal studies with repeated measures of renal structure and function.

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REFERENCES


