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Glomerular distribution of type IV collagen in diabetes by high resolution quantitative immunochemistry

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Glomerular distribution of type IV collagen in diabetes by high resolution quantitative immunochemistry. We examined type IV collagen distribution and density in human diabetic kidneys by quantitative immunogold electron microscopy. We studied normal kidney transplant donors and "slow-track" and "fast-track" insulin dependent diabetic (IDDM) patients. The "slow-track" patients had IDDM for ≥ 20 years and mesangial volume fraction (VvMes/glom) of ≤ 0.32. The "fast-track" patients had IDDM for ≤ 20 years and VvMes/glom ≥ 0.37. Renal biopsies were embedded in Lowicryl, reacted with polyclonal anti-type IV collagen (in the distribution of the classical α1(IV) and α2(IV) collagen chains) and monoclonal anti-α4(IV) collagen chain antibody followed by gold conjugated secondary antibody. We found, by morphometric techniques, a decrease in the immunogold densities of anti-type IV collagen in the subendothelial zone of the GBM in the "fast-track" IDDM patients. There was a trend towards a decrease in mesangial matrix (MM) particle density in the "fast-track" ($P = 0.07$) but not in the "slow-track" patients. However, because of the marked increase in MM in the "fast-track" patients, the per glomerulus estimated quantity of these antigens in MM was increased. In contrast, the density of α4(IV) collagen chain was increased in the epithelial zone of the GBM in the "fast-track" IDDM patients. It is not known whether these changes in glomerular type IV collagen represent markers of advanced diabetic lesions or whether these changes might be detected earlier in diabetic patients destined for the later development of serious lesions.

The hallmark of the renal structural changes of diabetic nephropathy is extracellular matrix (ECM) accumulation in glomerular, tubular and interstitial sites. The main glomerular ECM component is type IV collagen although type V and VI collagens are also present. Type IV collagen consists of at least five distinct chains: "classical" [α1(IV), α2(IV)] and more recently described "novel" [α3(IV), α4(IV) and α5(IV)] chains. Additional ECM components include the laminins, fibronectin, heparan sulphate proteoglycans, and other minor glycoproteins [1–6]. Biochemical analyses of human glomerular basement membrane (GBM) preparations have suggested increased amounts of type IV collagen and decreased amounts of laminin in diabetes [7, 8]. However, since preparations of GBM, by current procedures, contain both GBM and mesangial matrix (MM) [9], biochemical determinations could reflect the changes which occur in either or both of these structurally and immunohistochemically distinct regions of the ECM [1–4, 9–14].

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Recent studies of diabetic patients indicate that alterations in ECM components are not equally distributed throughout the glomerulus and that alterations in MM may occur independently of those in GBM [7, 10].

Antibodies to α1(IV) and α2(IV) collagen chains of type IV collagen localize along the subendothelial aspect of the GBM and throughout the MM, whereas antibodies to α3(IV) and α4(IV) collagen chains localize throughout the entire thickness of the GBM but not in MM [(3, 10–12, 15–18]. The localization of these ECM components in normal human kidney does not appear to be the same as that proposed by animal studies [19–23].

It is well known that not all diabetic patients progress to overt renal disease. This suggests that it may be important to differentiate those renal structural changes which occur in all diabetic patients, independent of nephropathy risk, from the structural changes occurring only in patients highly susceptible to the development of serious lesions. In the current study, we have performed quantitative immunogold electron microscopy to estimate the density of type IV collagen chain distributions in peripheral GBM and MM in insulin-dependent diabetes mellitus (IDDM) patients and normals. We asked whether there are site specific alterations in type IV collagen chain distribution patterns or density of labeling which distinguish IDDM patients who are developing lesions rapidly from patients developing lesions slowly, if at all. We found decreased density of localization of antibodies to type IV collagen in the distribution of α1(IV) and α2(IV) chains (subendothelial zone of GBM and MM) in the "fast-track" IDDM patients. In contrast, there was increased gold particle density for α4(IV) collagen chains in the epithelial zone of the peripheral GBM in the "fast-track" IDDM patients.

Methods

Patients

Kidney biopsy tissues were chosen from those available from a large group of IDDM patients, studied as part of a protocol for evaluating patients being considered for pancreas transplantation. The IDDM patients in the current study were selected from a subset of patients that had tissue appropriately fixed at the time of biopsy for immunogold electron microscopy. All patients with such tissues meeting the inclusion criteria were studied. The criteria were based on severity of glomerular

Table 1. Clinical characteristics of the study groups

	Sex F/M	Age (years)	Duration (years)	C_{Cr} ml/min/1.73/m²	UAE mg/24 hr Median (Range)
Control ($N = 6$)	2/4	38 ± 13	—	—	—
"Slow-track" IDDM ($N = 6$)	4/2	32 ± 14	26 ± 7	123 ± 32	8 (1.5, 1059)
"Fast-track" IDDM ($N = 6$)	4/2	34 ± 5	14 ± 5	72 ± 27	544 (9, 1226)
P				< 0.01	< 0.001

Abbreviations are: C_{Cr}, creatinine clearance; UAE, urinary albumin excretion; IDDM, insulin-dependent diabetes mellitus; F/M, female/male.

lesions determined by morphometric analysis and on duration of diabetes in order to create two nonoverlapping groups as follows: (1) "slow-track" patients ($N = 6$): duration of IDDM ≥ 20 years, volume fraction of mesangium (VvMes/glom) ≤ 0.32; (2) "fast track" patients: duration of IDDM ≤ 20 years, VvMes/Glom ≥ 0.37. Six biopsy tissues were obtained from age-matched kidney donors at the time of transplantation surgery and were used to quantify the normal patterns. Characteristics of the normal and the two patient groups are presented in Tables 1 and 2.

Measurement of glomerular volume

The point counting method of Weibel and Gomez [24] was used to measure glomerular volume (Vglom) on light microscopy tissues which were fixed in Zenkers solution, embedded in paraffin, and sectioned and stained with periodic acid-Schiff. Six to 82 (26 ± 21; $\bar{X}$ ± SD) glomerular profiles per patient were measured.

Preparation and measurement of tissue for electron microscopy

Routine stereologic techniques, previously described in detail [6, 25–27] were used to measure mesangial fractional volume (VvMes/glom), Vv mesangial matrix (MM)/glom), surface density of the peripheral GBM (SvPGBM), and GBM width. Peripheral GBM surface per glomerulus (S/G) is the product of SvPGBM and Vglom.

Antibodies

The antibodies employed in this study are listed in Table 3.

Preparation of tissue for immunogold electron microcopy

Renal tissue obtained by percutaneous needle biopsy in the IDDM patients or by needle biopsy at transplant surgery in the controls was immediately examined using a dissecting microscope to ensure that glomeruli were in the tissue samples. Small cubes (1 mm^3) of cortical kidney tissue were immediately fixed in 4% periodate-lysine paraformaldehyde (PLP) solution for two hours at 4°C and then washed with 0.025 M phosphate buffer containing 6% sucrose [14]. The samples were dehydrated in a graded ethanol series at progressively lower temperatures, down to −35°C, and infiltrated with Lowicryl K4M (Chemiche Werke Lowi; Waldkraiburg, Germany). The resin was polymerized at −35°C with long wave ultraviolet radiation. Ultrathin silver-to-gold interference contrast sections were cut and mounted on Formvar coated, 100-mesh nickel grids. The grids were then incubated overnight at 4°C with primary antibody. Excess unbound primary antibody was then washed off the grids using PBS containing 1% BSA and 0.05% Tween-20. Afterwards, they were exposed to 10 nm gold-conjugated secondary antibodies for two hours at room temperature and washed, first with PBS, and then water. The sections were then stained for 10 minutes with uranyl acetate and five minutes with lead citrate.

Observation was done with a JEOL 100CX electron microscope. Twenty to 30 photomicrographs were randomly taken for each glomerulus and enlarged to a final magnification of about 42,000×. Limitations in the amount of tissue or antibody available for these studies resulted in not all IDDM patients or controls being studied with all of the immunohistochemical probes.

Measurement of gold particle densities

The density of labeling was expressed as the number of gold particles per unit area (particles/μm^2). Peripheral GBM was defined, as previously described, as that part of the GBM covered on one side by epithelial cells and on the other side by endothelial cells with no interposition of mesangial cells or matrix [27]. Segments of peripheral GBM were randomly chosen and arbitrarily divided into three zones of equal width (endothelial, middle and epithelial zones; Fig. 1A). This was done because the limits of the lamina rara interna, lamina densa and lamina rara externa could not be precisely determined. Although the inner and outer thirds of the GBM contain the lamina rarae interna and externa, respectively, some of the lamina densa was also regularly included in both of these zones. On the other hand, the middle zone appeared to include only the lamina densa. A grid consisting of 0.8 cm squares was placed over each micrograph. The area of each zone of peripheral GBM sampled was estimated by adding the number of squares in which the lower left hand corner intersected a given GBM zone. The number of gold particles labeling each zone of the GBM was then counted. Mesangial GBM was defined as that part of the GBM in which one side was covered with epithelial cells and other side interfaced with mesangial matrix and cells. The boundary between the mesangial GBM and the mesangium was roughly drawn to extend from the endothelial edge of the peripheral GBM, approximating the width of the peripheral GBM, and paralleling the epithelial edge of the mesangial GBM. Matrix material internal to these boundaries and to endothelial cells was considered to be MM. Non-matrix material was considered mesangial cells (MC). A grid of 1 cm squares was used to measure MM and MC. The area sampled of each component was determined by counting the number of times the lower left hand corner of a square fell on a given component. The number of gold particles labeling a given component within the squares was counted. The same grid was used to calculate the density of background staining. The densities of gold particles within the glomerular capillary lumenal and

Table 2. Morphometric analysis of the study groups

	GBM width *nm*	VvMes/glom	VvMM/glom	MM/Mes	SvPGBM $\mu m^2/\mu m^3$	Vglom $\times 10^6 \mu m^3$	S/G $\times 10^6 \mu m^2$
Control ($N = 6$)	327 ± 39	0.19 ± 0.05	0.08 ± 0.02	0.51 ± 0.09	0.133 ± 0.01	1.375 ± 0.46	0.185 ± 0.066
"Slow-track" IDDM ($N = 6$)	660 ± 253	0.27 ± 0.06	0.16 ± 0.05	0.68 ± 0.09	0.104 ± 0.02	1.87 ± 1.06	0.177 ± 0.062
"Fast-track" IDDM ($N = 6$)	706 ± 164	0.45 ± 0.08	0.30 ± 0.04	0.72 ± 0.06	0.079 ± 0.02	2.35 ± 0.67	0.182 ± 0.068
ANOVA	0.005	0.0001	0.0001	0.005	0.0008	NS	NS
C vs. S	0.05	NS	0.05	0.05	0.048	—	—
C vs. F	0.05	0.0001	0.0001	0.005	0.008	—	—
S vs. F	NS	—	0.0001	NS	NS (0.1)	—	—

Abbreviations are: IDDM, insulin-dependent diabetes mellitus; C, controls; S, "slow-track" patients; F, "fast-track" patients; NS, not statistically significant; GBM, glomerular basement membrane; VvMes/glom, mesangial volume fraction per glomerulus; VvMM/glom, mesangial matrix volume fraction per glomerulus; MM/Mes, the ratio of mesangial matrix volume to total mesangial (matrix + cell) volume; SvPGBM, surface density of peripheral GBM; Vglom, glomerular volume; S/G, peripheral GBM surface per glomerulus.

Table 3. Characteristics of antibodies used in this study

Antibodies	Specificity	References/source
Monoclonal antibodies		
Mab 85	Human α4(IV) collagen chain	[10, 13]
Polyclonal antibodies		
Goat anti-type IV[a]	Pepsin digested human Placental basement membrane	Southern Biotechnology

Abbreviations are: Mab, monoclonal antibody; NC, noncollagenous domain.

[a] Reactivity of this antibody in human kidneys is identical to those of antibodies against α1(IV) and α2(IV) collagen chains [3, 12]

urinary spaces were calculated and expressed as background density and subtracted from the densities in the other locations.

Immunogold density values derived here are not presumed to represent an exact concentration of an antigen in a tissue since: there is not a 1:1 ratio of gold particles to IgG molecules; there are differences in antibody affinities and/or titers; and there may be a partial denaturation of antigenic sites by fixation [28–30]. However, our methods should allow the comparison of relative site specific densities among the various groups if the conditions are standardized [31, 32]. To minimize potential problems caused by day to day variations in fixation schedules, embedding media and incubation protocols, one normal biopsy was randomly selected to be used as a control to be run each time that a set of tissues was studied for a given antigen. It was necessary to overlap these controls and then switch from the first to the second as we ran out of tissue on the first. Gold particle densities were corrected for the original control value by multiplying the densities counted for a set of tissues studied at a given time by the ratio of the control value first obtained and the control value obtained with that set of tissues. For the polyclonal type IV collagen antibody we used the particle density of mesangial matrix to obtain this ratio. We used mesangial GBM particle density to standardize for anti-α4(IV) collagen chain antibody. Results expressed as particle density have been standardized as described here. The number of particles per glomerulus were estimated for peripheral GBM zones as:

$$\text{SvPGBM} \times 1/3 \text{ GBM width} \times \text{V glom} \times \text{standardized particle density}$$

where SvPGBM = surface density of the peripheral GBM and Vglom = glomerular volume.

The number of particles per glomerulus were estimated for mesangial matrix as:

$$\text{VvMM/glom} \times \text{Vglom} \times \text{standardized particle density}$$

where VvMM/glom = volume fraction of mesangial matrix per glomerulus.

Immunofluorescent microscopy studies have suggested that antigens for some antibodies, such as anti-α4(IV) collagen chain, may be unmasked by pretreatment of frozen tissue sections with 6 M urea, resulting in increased staining intensity [10]. For this reason we treated Lowicryl sections from two normal individuals for 20 minutes, 1, 2, 4 and 24 hours with 6 M urea before incubating them with primary antibody to α4(IV) collagen chain. We found no significant effect of the 6 M urea treatment on gold particle densities for α4(IV) collagen chain.

Statistics

Comparisons of the labeling densities and density percentiles for a given antigen and for a given zone of the GBM mesangial matrix utilized analysis of variance (Super ANOVA, Abacus Concepts, Berkeley, California, USA) and differences between groups evaluated by the Scheffes' test with probabilities for multiple comparisons. $P < 0.05$ was considered significant. Values of $P > 0.05 < 0.1$ were considered indicative of a trend. Urinary albumin excretion rates, because these data are not normally distributed, were compared using a nonparametric (Mann-Whitney U test) test.

Results

Clinical characteristics

Four of six IDDM patients in both the "slow-" and "fast-track" group were female while two of six controls were female (Table 1). The ages were similar in all three groups. Duration of IDDM was, by design, greater in the "slow-track" patients and

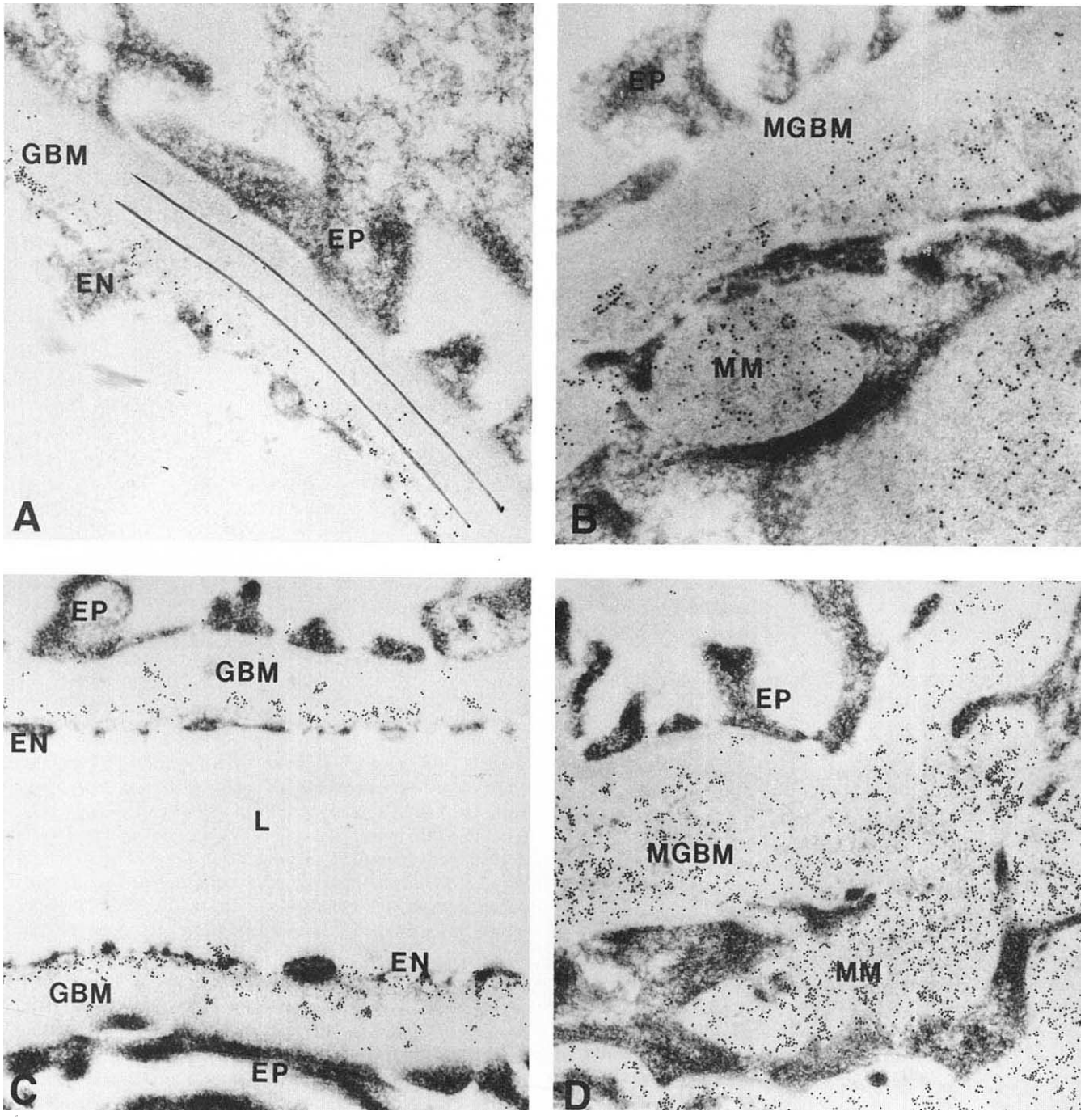

Fig. 1. A. *EM photomicrograph of gold labeled antibody to polyclonal anti-type IV collagen antibody in a normal human kidney embedded in Lowicryl demonstrating the pattern of distribution of the classical [α1(IV), α2(IV)] chains of type IV collagen.* Note concentration of the gold particles in the endothelial (EN) zone compared to the middle and epithelial (EP) zone of the glomerular basement membrane (GBM). **B.** EM photomicrograph of gold labeled antibody to polyclonal anti-type IV collagen in the mesangial region of a normal glomerulus with distribution of gold particles on the inner aspect of the mesangial GBM (MGBM) and in mesangial matrix (MM). **C.** Electron photomicrograph of distribution of polyclonal type IV collagen antibody in the peripheral capillary wall in a "fast-track" IDDM patient showing persistence of the EN concentration of the gold particles. L = lumen. **D.** Electron photomicrograph of polyclonal type IV collagen antibody in the mesangial region of a "fast-track" IDDM patient showing persistence of the normal pattern of concentration of gold particle distribution on the inner aspect of the MGBM and in MM. A ×35,400; B ×49,700; C, D ×29,700.

thus not analyzed statistically. Creatinine clearances were lower ($P < 0.01$) and urinary albumin excretion rates were higher ($P < 0.001$) in the "fast-track" compared to the "slow-track" diabetic patients. Five in the "slow-track" group had normal values for UAE (22 mg/24 hr or less), and one had overt proteinuria (UAE > 200 mg/24 hr). In contrast, only one in the

Table 4. Peripheral glomerular basement membrane polyclonal anti-type IV collagen particle density

	Endothelial zone	Middle zone	Epithelial zone	Total peripheral GBM particles/glomerulus
	particles/μm^2			
Control (N = 6)[a]	141 ± 39	57 ± 21	30 ± 4	4539 ± 1811
"Slow-track" IDDM (N = 5)	86 ± 34	35 ± 19	26 ± 15	6906 ± 3975
"Fast-track" IDDM (N = 5)	55 ± 30	27 ± 17	28 ± 17	4293 ± 1453
ANOVA	0.02	NS (0.07)	NS	NS
C vs. S	NS	NS	—	—
C vs. F	0.03	NS (0.09)	—	—
S vs. F	NS	NS	—	—

Abbreviations are: GBM, glomerular basement membrane; IDDM, insulin-dependent diabetes mellitus; C, controls; S, "slow-track" patients; F, "fast-track" patients; NS, not statistically significant; N, number of subjects studied.

"fast-track" group had normal UAE, one had microalbuminuria (UAE 45 to 200 mg/24 hr), and four overt proteinuria.

Morphometric analysis

GBM width was increased in both diabetic groups but was similar between the "slow-" and "fast-track" IDDM patients (Table 3) VvMes/glom was significantly increased in the "fast-track" IDDM patients compared to controls, but this was not true of the "slow-track" IDDM patients. The two diabetic groups differed in VvMes/glom by design. VvMM/glom was increased in both diabetic groups compared to controls and was greater in the "fast-" compared to the "slow-track" IDDM patients (Table 2) The ratio of mesangial matrix to total mesangial volume (matrix + cell) was increased in both diabetic groups compared to controls but was similar in the two diabetic groups (Table 2). SvPGBM was decreased in both IDDM groups compared to normals, more so in the "fast-track" patients (Table 2). There were no group differences in Vglom or S/G.

Immunogold studies

Polyclonal type IV collagen. Labeling of polyclonal antibody agains classical type IV collagen was concentrated in the endothelial zone of the peripheral GBM and decreased towards the epithelial zone in all three groups (Table 4, Fig. 1 A and C). The labeling appeared uniform throughout the mesangial matrix and to be more intense than in the GBM (Fig. 1 B and D). The density of gold particles for polyclonal anti-type IV collagen was decreased in the endothelial zone of GBM in the "fast-track" IDDM patients compared to controls (Table 4). Although a similar direction of change was seen in the "slow-track" patients, this was not statistically significant. The number of gold particles per glomerulus in each of the three zones was not different between the groups (data not shown). Also, there was a trend towards a decrease in density for polyclonal anti-type IV collagen in the middle zone of the GBM in the "fast-track" patients versus controls (Table 4). There was no difference between the groups in the total number of particles per glomerulus, including all three zones of the peripheral GBM (Table 4). There was a trend towards a decrease in MM particle density in the "fast-track" IDDM patients compared to controls, but this was not quite significant (P = 0.07; Table 5). Despite this, because of the increased volume of MM per glomerulus, the total number of particles per glomerulus labeling for polyclonal anti-type IV collagen was increased in the "fast-track" IDDM patients (Table 5).

Table 5. Mesangial matrix polyclonal anti-type IV collagen particle density

	Matrix density particles/μm^2	Mesangial matrix particles/glomerulus
Control (N = 6)	176 ± 26	4710 ± 2008
"Slow-track" IDDM (N = 5)	154 ± 43	14928 ± 6773
"Fast-track" IDDM (N = 5)	100 ± 53	21675 ± 14915
ANOVA	NS (0.07)	0.03
C vs. S	NS	NS
C vs. F	NS (0.07)	0.03
S vs. F	NS	NS

Abbreviations are: IDDM, insulin-dependent diabetes mellitus; C, controls; S, "slow-track" patients; F, "fast-track" patients; NS, not statistically significant, N, number of subjects studied.

Table 6. Peripheral glomerular basement membrane anti-α4(IV) NC domain particle density

	Endothelial zone	Middle zone	Epithelial zone
	particles/μm^2		
Control (N = 5)	13.8 ± 4.8	58.6 ± 10.1	41.6 ± 12.5
"Slow-track" IDDM (N = 6)	15.3 ± 8.1	54.1 ± 19.2	42.3 ± 19.8
"Fast-track" IDDM (N = 6)	21.0 ± 10.0	54.8 ± 23.8	68.8 ± 14.2
ANOVA	NS	NS	0.02
C vs. S	—	—	NS
C vs. F	—	—	0.05
S vs. F	—	—	0.04

Abbreviations are: IDDM, insulin-dependent diabetes mellitus; C, controls; S, "slow-track" patients; F, "fast-track" patients; NS, not statistically significant; N, number of subjects studied.

α4(IV) collagen chain. Particle density labeling for anti-α4 (IV) collagen chain was concentrated in the middle and epithelial zones of the peripheral GBM in all three groups (Table 6; Fig. 2 A and C). However, it was increased on the epithelial side of the GBM in the "fast-track" IDDM patients compared with the "slow-track" IDDM patients and the controls (Table 6). Peripheral GBM particles per glomerulus were increased in the "fast-track" IDDM patients compared to controls in all three zones and in the total peripheral GBM, which included all three zones (Table 7). There was a trend towards an increase in the number of particles per glomerulus in the epithelial zone in "fast-track" versus "slow-track" IDDM patients, but this did not reach significance ($P < 0.09$, Table 7). There was no labeling for anti-α4(IV) collagen chain in the MM in the normals or in the two diabetic groups (Fig. 2 B and D).

Discussion

We applied quantitative immunohistochemistry using unbiased sampling methods based upon stereologic principles to examine the site specific distribution and to quantify the relative density of various type IV collagen chains in human biopsy materials from normal and from IDDM patients with slow and

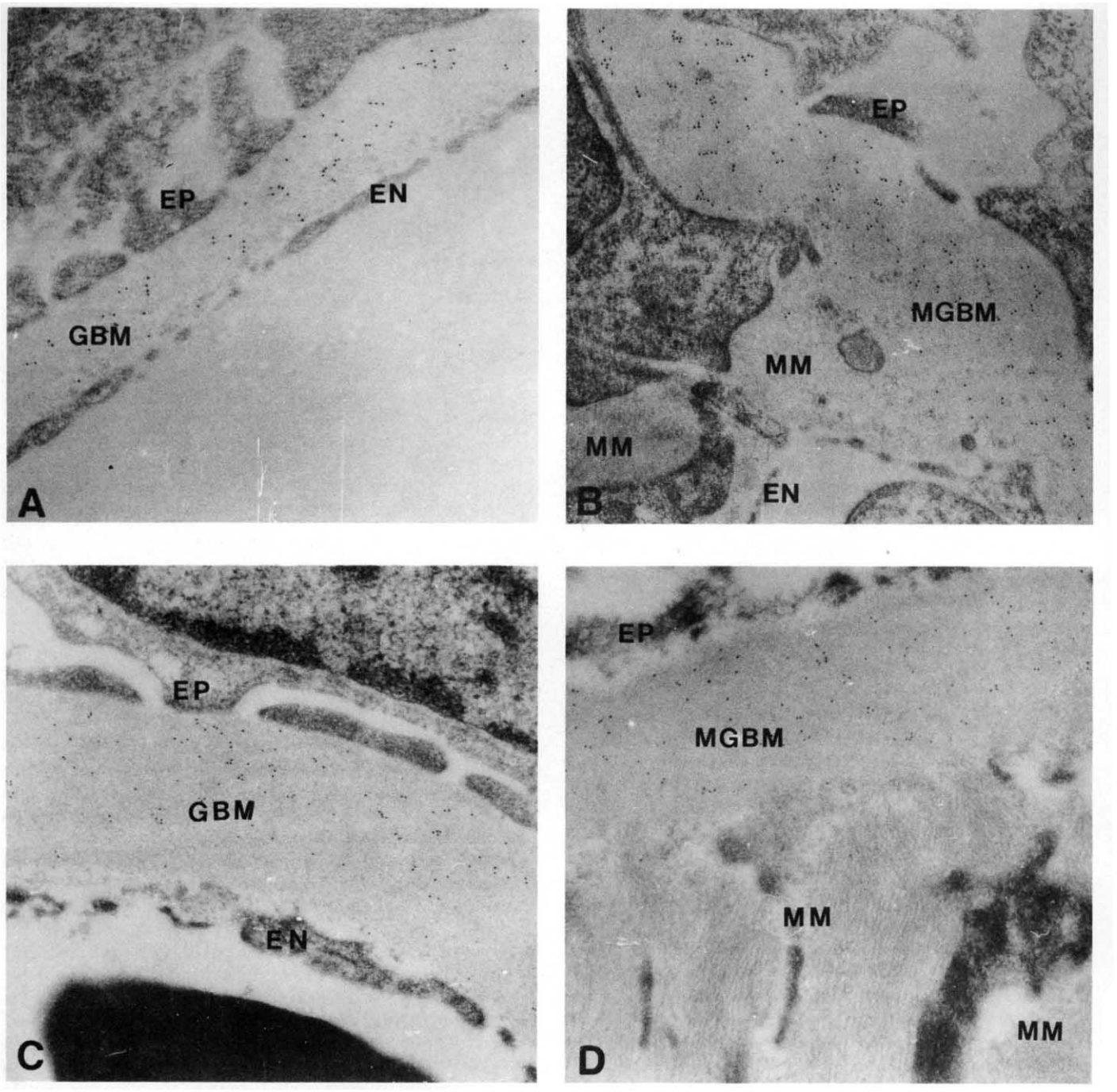

Fig. 2. A. *Electron microscopic (EM) photomicrograph of an immunogold marker of the distribution of anti-α4(IV) collagen in the peripheral capillary wall of a normal human kidney embedded in Lowicryl.* Gold particles are distributed throughout the glomerular basement membrane (GBM) but concentrated in the middle zone and are relatively sparse in the endothelial (EN) and epithelial (EP) zones of the GBM. **B.** EM photomicrograph of an immunogold marker of the distribution of anti-α4(IV) collagen in mesangial region of a normal human glomerulus. Note that gold particles are restricted to the mesangial GBM (MGBM) and completely absent from mesangial matrix (MM). **C.** EM photomicrograph of an immunogold marker of the distribution of anti-α4(IV) collagen in the thickened GBM of a "fast-track" IDDM patient. Note distribution of gold particles mainly in the EP and middle zones of the GBM with less particles in the EN zone of the GBM. **D.** EM photomicrograph of an immunogold marker of the distribution of anti-α4(IV) collagen in a "fast-track" diabetic patient with restriction of the gold particles to the MGBM and, as in the normal, absence of gold particles from MM. A ×28,100; B ×29,700; C, D ×32,400.

with rapid development of a crucial lesion of diabetic nephropathy [6].

A polyclonal antibody to type IV collagen, which localized exclusively in the pattern of α1(IV) and α2(IV) collagen chains [9, 11, 12] was found to be concentrated in normals in the endothelial third of the GBM and to decrease in density towards

Table 7. Anti-α4(IV) NC domain peripheral glomerular basement membrane particles per glomerulus

	Endothelial zone	Middle zone	Epithelial zone	Total peripheral GBM
	particles/glomerulus			
Control ($N = 5$)	157 ± 66	1178 ± 567	735 ± 439	2141 ± 935
"Slow-track" IDDM ($N = 6$)	621 ± 332	1603 ± 761	1654 ± 840	3878 ± 1890
"Fast-track" IDDM ($N = 6$)	828 ± 483	2724 ± 1142	2948 ± 1106	6540 ± 2680
ANOVA	0.03	0.04	0.005	0.02
C vs. S	NS	NS	NS	NS
C vs. F	0.03	0.05	0.005	0.02
S vs. F	NS	NS	NS (0.09)	NS

Abbreviations are: GBM, glomerular basement membrane; IDDM, insulin-dependent diabetes mellitus; C, controls; S, "slow-track" patients; F, "fast-track" patients; NS, not statistically significant; *N*, number of subjects studied.

the epithelial third of the GBM. Also, this antibody localized in normals uniformly throughout the MM. These findings confirmed previous reports from animal [31] and human [11, 33] studies.

These distribution patterns were maintained in IDDM patients. However, the density of gold particles for classical type IV collagen was significantly decreased in the endothelial zone of the peripheral GBM, and tended to be decreased in the middle zones of the GBM and in MM of the "fast-track" IDDM patients. The particles per glomerulus in the MM increased in the "fast-track" IDDM patients while particles per glomerulus in each of the three GBM zones remained unchanged. These data are consistent with the idea that classical type IV collagen makes up a smaller proportion of the mass of the MM and the subendothelial zone of the peripheral GBM in IDDM patients with the rapid development of marked mesangial expansion. These data also suggest that other ECM molecules are accumulating in the expanding MM and GBM of IDDM patients. Candidate molecules for MM include fibronectin, laminin, type VI collagen, and late in the course of the disease types I and III collagen. Despite the decrease in particle density, the total number of gold particles per glomerulus signaling the localization of polyclonal anti-type IV collagen antibody was increased in MM in the "fast-track" IDDM patients, reflecting the marked MM expansion in these patients and indicating an accumulation of these molecules at this site in patients with severe lesions.

Makino et al performed quantitative immunogold electron microscopy with anti-bovine type IV collagen antibody in five non-insulin dependent diabetic patients whose disease categorizations were said to range from mild to severe [34]. They found decreased particle density in the thickened GBM and particle density which was unchanged from normal in the expanded mesangium of these patients. However, these studies are difficult to compare with ours because the site specificity in normals of the anti-type IV collagen antibody of Makino et al showed much greater particle density in the middle zone of the GBM than in the region of the lamina rara interna. This differs markedly from our findings and from the known distribution of classical type IV collagen chains in normals using immunofluorescence microscopy [3, 10] or immunogold electron microscopy with highly specific monoclonal reagents (Zhu D et al, unpublished data).

Previous immunofluorescence [4, 10] and immunohistochemical [35] studies have suggested increased amounts of type IV collagen in the mesangium in diabetic patients, which diminish only as the lesions, especially of the nodular type, became far advanced. Here we will try to reconcile these observation with those of the current study where the density of MM gold particles indicating the presence of "classical" type IV collagen chains is not increased in the "slow-track" IDDM patients. We have previously shown that the fraction of glomerular mesangium which is occupied by MM as opposed to mesangial cells is increased in longstanding IDDM patients whether or not VvMes/glom is increased [36]. Thus, even though the VvMes/glom was not significantly increased in the "slow-track" patients in the present study, MM volume fraction and the ratio of MM volume to total mesangial volume (matrix + cells) was increased. At the resolution of the light microscope we hypothesize that this structural change in the "slow-track" patients provides for the subjective perception of greater intensity of staining, while in fact, antigen density in mesangial matrix, as revealed by quantitative immunogold EM, is not changed. If correct, this hypothesis suggests that great caution is required in the interpretation of subjective semiquantitative light microscopic antibody labeling studies of extracellular matrix material in diabetes and in other diseases.

It is not likely that the changes in antigen density found in the present studies are due to modifications of the type IV collagen molecules, for example, by glycosylation. Firstly, there are no studies suggesting that glycation of ECM molecules interferes with their ability to interact with antibodies. Secondly, it would be difficult for the hypothesis that ECM glycation causes interference with ECM-antibody interactions to explain why there was decreased density of polyclonal anti-type IV collagen antibody localization in the endothelial zone and unchanged density in the epithelial zone of the GBM. Glucose is so small a molecule as to be expected to be unhindered across the entire glomerular capillary wall. Similarly, this hypothesis would not explain why density of α4(IV) collagen chain is increased in the GBM of the "fast-track" IDDM patients.

Anti-α4(IV) collagen chain antibody localized predominantly in the middle zone, and less in the epithelial and endothelial zones of peripheral GBM in both normal and "slow-track" IDDM patients. This is true despite the marked thickening of the GBM in the "slow-track" IDDM patients. In contrast α4(IV) collagen chain was present predominantly on the epithelial zone of the thickened GBM and gradually decreased towards the endothelial zone of the GBM in the "fast-track" IDDM patients. Since GBM width was increased similarly in the "slow-track" and "fast-track" IDDM patients, it is unlikely that the increased α4(IV) collagen chain density in the epithelial zone or particle counts per glomerulus in the peripheral GBM of the "fast-track" patients could alone explain GBM thickening. The fact that the "slow-track" patients with even longer duration of IDDM than the "fast-track" patients had similar densities of α4(IV) collagen chains in the three GBM zones as the normal group suggests that the alterations seen in the "fast-track" IDDM patients were not duration dependent.

Either the increased epithelial zone α4(IV) collagen chain density in the "fast-track" patients is somehow consequent to the more severe mesangial expansion in this group, or this abnormality may be an indicator or premonitor of glomerular biochemical alterations which result in the development of marked MM expansion. Only longitudinal studies involving biopsies spaced five or more years apart can answer this question. Also these cross sectional studies cannot answer whether these alterations are due to increased production, decreased degradation or both of α4(IV) collagen chain in the epithelial zone.

We have previously shown that α3(IV) and α4(IV) collagen chains have identical glomerular distributions in normal human kidney tissue [3, 12]. Desjardins et al found polyclonal anti-α3(IV) localized in both the GBM and the MM in normal and diabetic rats [31]. These results suggest that there are important differences in the site specific distribution of ECM components in rats compared to humans and illustrate the serious limitations of generalizations from animal to human studies. It is thus likely that the altered dynamics of ECM production and/or breakdown which lead to diabetic nephropathy in humans will need to be explored in humans for a full understanding of the mechanisms involved.

In summary, there is a decrease in the immunohistochemical electron microscopic estimates of the density of classical type IV collagen in MM and in the subendothelial zone of the GBM in IDDM patients rapidly developing serious lesions of diabetes. In contrast, the density of α4(IV) collagen chain was increased in the epithelial zone of the GBM in these "fast-track" IDDM patients. It is not known whether these alterations are markers of advanced diabetic lesions or whether earlier detection of these abnormalities in diabetic patients could predict the later development of serious lesions. These studies suggest limitations of conventional immunohistochemical methods of studying ECM alterations in disease, illustrate important differences between animals and humans, and emphasize the importance of studies in humans.

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