Diabetologia (1997) 40: 15–22

Diabetologia

Alterations in the expression of the $\alpha_3\beta_1$ integrin in certain membrane domains of the glomerular epithelial cells (podocytes) in diabetes mellitus

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Summary In view of the major alterations which take place at the level of the extracellular matrix of the glomerular wall in diabetes mellitus and the key roles played by β_1 integrins in cell-to-matrix interactions, it is imperative to understand the role played by integrins in the development of diabetic glomerulosclerosis. In the present study, we revealed by immunocytochemistry the ultrastructural distribution of the $\alpha_3\beta_1$ at the level of the plasma membrane of the different renal glomerular cells from short- and long-term diabetic rats. For the endothelial cells, the labelling present on both the luminal and abluminal plasma membranes was low. For the podocyte epithelial cells, the labelling was present on both the luminal and basal plasma membranes, the former being concentrated at points of contact between podocyte foot processes. The labelling on the basal plasma membrane was more significant and similar in domains facing either the glomerular basement membrane or the mesangial matrix. The plasma membrane of mesangial cells also exhibited $\alpha_3\beta_1$. The labelling was recorded under diabetic conditions, at the same sites, with similar intensities, alongside that of the basal plasma membrane of podocytes facing the glomerular basement membrane, the density of which decreased significantly. This decrease in labelling was similar in renal tissues from short- and long-term diabetic animals. These results demonstrate that $\alpha_3\beta_1$ present at the podocyte basal plasma membrane facing the glomerular basement membrane, which undergoes important alterations in diabetes, could be involved in the major dysfunctions of the glomerular wall characteristic of diabetic glomerulosclerosis. Since the changes in integrin were found to occur as early as after 1 month of hyperglycaemia, when morphological alterations of the glomerular basement membrane are not yet established, we propose that they constitute an early event which precedes the onset of diabetic nephropathy. [Diabetologia (1997) 40: 15–22]

Keywords Integrins, diabetes mellitus, glomerular wall, immunocytochemistry.

Integrins form a large family of protein receptors involved in cell-to-cell and cell-to-matrix interactions. They are transmembrane heterodimeric glycoproteins composed of α and β subunits; 8 different β and 16 α subunits have been described [1–3] and have been divided into subfamilies according to the β subunits.

The β_1 subfamily includes specific receptors for laminin, such as $\alpha_6\beta_1$ and $\alpha_7\beta_1$ and for fibronectin, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ [1]. Some β_1 integrins, such as the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are able to bind either collagens or laminin, while others, such as the $\alpha_v\beta_1$ can bind either fibronectin or vitronectin [1]. At any rate, the receptor showing the greatest versatility is the $\alpha_3\beta_1$ which binds collagen molecules [4, 5], laminin [6], fibronectin [4], entactin [7] and epiligrin [8]. In addition $\alpha_3\beta_1$

Received: 20 June 1996 and in revised form: 24 September 1996

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Abbreviations: ECM, Extracellular matrix; GBM, glomerular basement membrane; PBS, phosphate buffered saline; TGF-β1, transforming growth factor-β1.

has been involved in cell-cell adhesion via homophilic and heterophilic interactions [9–12], although such function is being challenged [13].

The β_1 integrins show a ubiquitous distribution throughout tissues and are expressed in cells arising from ecto-, endo- and mesodermal origins [14]. In kidney, various β_1 integrins have been found and their distribution as well as modifications during nephrogenesis [15, 16] and nephropathies [17, 18] have been reported. Studies on β_1 distribution in kidney have mainly been carried out by light microscopy [15–22]; a few have been performed by transmission electron microscopy [18, 19, 22] demonstrating the presence of $\alpha_3\beta_1$ on podocytes, mesangial and endothelial cells of human glomeruli [15, 17–22]. In addition, α_1, α_2, α_5 are expressed by mesangial and endothelial cells as well [15, 17, 20–22]. Regarding the expression of the β_1 integrins in rat kidney, studies in situ were restricted to light microscopy [23] most of the available information arising from cell culture systems [24–26]. Rat podocytes and glomerular endothelial cells in culture seem to display the same β_1 pattern of distribution as those in situ [24, 26]. Rat podocytes in culture have been found to present only $\alpha_3\beta_1$ [24], although, according to Cybulsky et al. [25] they would also express the α_2 subunit. Thus, the $\alpha_3\beta_1$ appears to be very important since it is probably the only β_1 integrin expressed by podocytes.

In long-term diabetes a progressive alteration of the glomerular extracellular matrix (ECM) compartments leads to an expansion of the glomerular basement membrane (GBM) [27, 28]. During diabetes the GBM not only thickens but the spatial distribution of some of its components (i. e. type IV collagen) is markedly altered [29–31]. Since long-term diabetes is associated with significant alterations of the ECM and since integrins appear to be the key proteins mediating cell-ECM relationships, it is important to understand the role of integrins in the onset of diabetic nephropathy.

In the present study we investigated the cellular distribution of the $\alpha_3\beta_1$ integrin in the glomerulus of normal as well as short- and long-term diabetic rats. This was carried out by quantitative immunocytochemistry, demonstrating that the pattern of distribution of this integrin on podocytes is markedly altered in diabetes with a significant decrease in its expression at the level of the foot processes facing the GBM.

Materials and methods

Animals. An experimental chronic hyperglycaemic state was induced in male Sprague-Dawley rats (approximately 100 g body weight) by an intraperitoneal injection of streptozotocin (70 mg/kg body weight) dissolved in 100 mmol/l citrate buffer, pH 4.5. Control animals received the citrate buffer alone. The hyperglycaemic state developed within the first 48 h after the injection and continued throughout the entire experiment; this was demonstrated by regular measurements of glycosuria and glycaemia carried out using Multistix and Dextrostix reagent strips (Miles, Ames, Ontario, Canada). The animals were not insulin treated. Control animals remained normoglycaemic. Four groups, each comprising three animals, were created. Group 1: short-term diabetic rats (1 month); group 2: long-term diabetic rats (12–13 months); group 3 and 4 age-matched controls. Blood glucose levels averaged 20.0 ± 1.7 mmol/l for the long-term hyperglycaemic animals vs 7.1 ± 0.5 mmol/l for the age-matched controls; 25.5 ± 1.1 mmol/l for the short-term hyperglycaemic animals vs 6.5 ± 0.2 mmol/l for their age-matched controls.

Tissue processing. Animals were anaesthetised and the kidneys were immediately fixed in situ with 1% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4). Upon excision, the tissues were further fixed by immersion in the same fixative for an additional 2 h at 4°C. After rinsing in the same buffer, the tissue fragments were dehydrated in methanol and embedded in Lowicryl K4M at –20°C [32]. Ultrathin sections were cut, mounted on Parlodion-carbon coated nickel grids and processed for immunocytochemistry.

Immunocytochemistry. Tissue sections were incubated on a drop of 0.15 mol/l glycine in 0.01 mol/l phosphate buffered saline (PBS) for 20 min, and transferred on a drop of PBS containing 1% ovalbumin for an additional 20 min. Subsequently, the grids were incubated on a drop of a rabbit anti-human integrin α_3 polyclonal antiserum (Chemicon Int., Temecula, CA., USA) at room temperature for 2 h. After rinsing in PBS, the sections were incubated with 1% ovalbumin in PBS for 20 min, and transferred onto a drop of 10 nm gold particle conjugate goat anti-rabbit IgGs (British BioCell Int. Cardiff UK.) for 30 min at room temperature. The grids were then washed with PBS, rinsed in distilled water and allowed to dry. Before examination with a Philips 410 electron microscope (St.-Laurent, Quebec, Canada), the grids were stained with uranyl acetate. The anti-α_3 antibody was used at a 1:200 dilution and the second antibody (goat anti-rabbit IgG) was diluted at 1:10. The specificity of the labelling was tested by replacing the rabbit anti-α_3 antibody by a normal rabbit serum and by performing the labelling protocol omitting the primary specific antibody. On the other hand, the specificity of the antibody and its reactivity towards the glomerular proteins were tested by immunoblotting. Homogenates of rat glomeruli purified by a sieving method [33], were run on 7.5% SDS-PAGE. After transfer onto nitrocellulose and incubation with the antibody, only one band in the range of approximately 135 kDa was detected (results not shown).

Morphometric analysis. The distribution of the labelling present on the different plasma membrane domains of the glomerular cells was analysed. The labelling on the endothelial cell plasmalemmal membrane was evaluated on the luminal and abluminal domains. The labelling on the podocyte plasma membrane was also evaluated on the luminal and basal sides. On the latter, two subdomains were selected; the plasma membrane of the foot processes facing the GBM and that facing the mesangial matrix. For the quantitative evaluation 50 to 100 micrographs (magnification × 31 000) of each different glomerular component were recorded for each animal. The quantitation was carried out by measuring the length of each plasma membrane domain and counting the number of gold particles located directly on this same membrane. The labelling density was reported in terms of number of gold particles/μm ± SD.

Statistical analysis. An image processing system (Videoplan 2, Carl Zeiss Inc., Toronto, Canada) was used for this evaluation

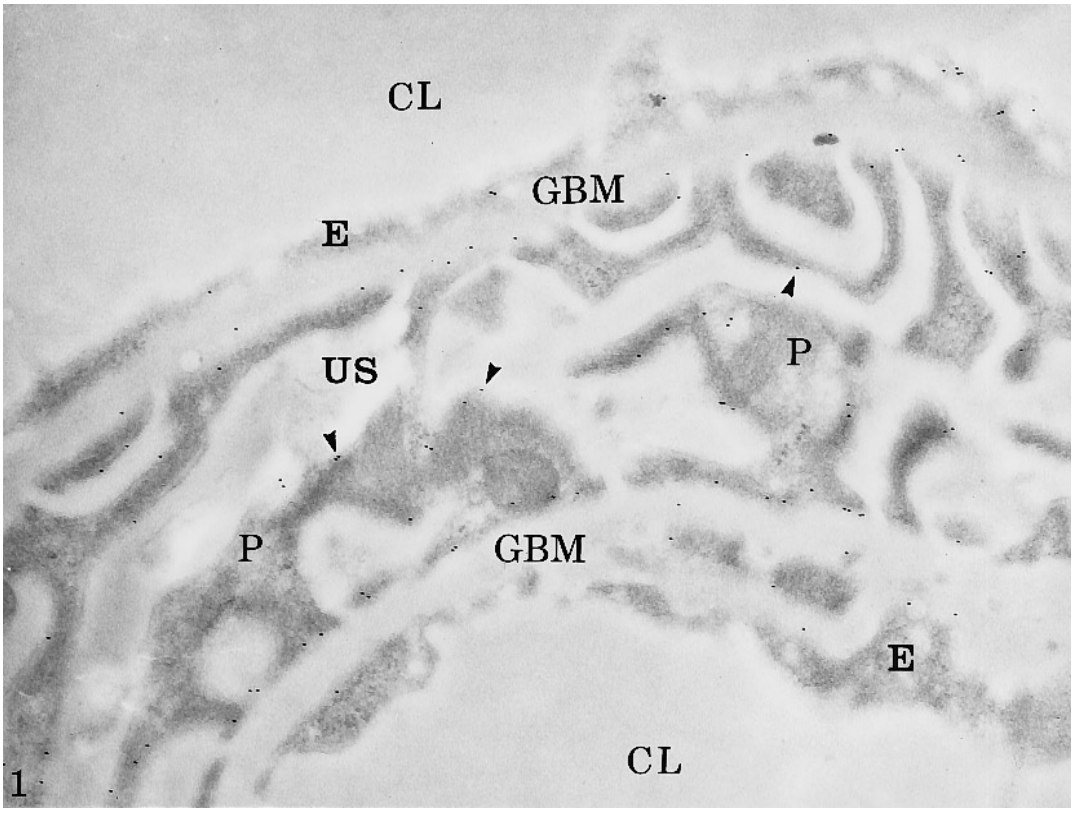

Fig. 1. Normoglycaemic rat. The α_3 subunit, as revealed by gold particles, is mainly expressed on the podocytes (P). A strong reaction is localised on the basal plasma membrane domain of the foot processes along the glomerular basement membrane (GBM). Few gold particles are also present on the luminal membrane domain of podocytes (▶). The endothelial cells (E) show a faint reaction with scattered gold particles along the GBM. US, Urinary space; CL, capillary lumen. × 31 000

and its basic statistical analysis. First, all the data collected from different glomerular membrane compartments were tested using one-way analysis of variance. When significance was attained ($p < 0.01$), between-group differences were established using Bonferroni's *t*-test.

Results

Upon application of the anti-α_3 antibody on rat tissue, a labelling by gold particles was obtained in all the cells composing the glomerulus. In each case, the labelling was restricted to the cell plasma membrane with no intracellular signal (Figs. 1–5). However, the labelling varied according to the cell type examined.

The podocytes of the normoglycaemic animals showed an intense and continuous labelling for the α_3 on the basal plasma membrane facing either the GBM (Figs.1 and 2) or the mesangial matrix (Fig. 3). However, the labelling on the foot processes was not restricted to the basal plasma membrane domain, but was also present, although moderate, on the luminal plasma membrane facing the urinary space (Figs. 1 and 2). When favourable angles of sectioning were obtained, the areas of contacts between neighbouring podocyte foot processes appeared to be preferentially labelled (Fig. 2). The glomerular endothelial cells showed a weak reaction compared to the podocytes (Fig. 1). Scattered gold particles were observed on the abluminal side of the endothelium and, as previously reported in human glomerulus [22], a very faint reaction was also detected on the luminal side. The α_3 subunit was also observed on the plasma membrane of the mesangial cells (Fig. 3). As is well-known, the complex shaped mesangial cells are provided with long cytoplasmic processes spreading from the cell body into the mesangial matrix. The α_3 was found along the plasma membrane of the cell body as well as that of the processes with a similar pattern of distribution.

The labelling obtained on tissues from normoglycaemic and on long-term hyperglycaemic animals was similar (Figs. 4 and 5); only the distribution of the gold particles on the basal plasma membrane of the podocytes was markedly altered in diabetic rats. The continuous labelling observed in tissues of normoglycaemic animals was replaced by a rather weak and patchy reaction, the gold particles being scattered along the basal plasma membrane (Figs. 4 and 5). Moreover, the reaction detected along the basal plasma membrane of podocytes was not uniform, since areas facing the mesangial matrix displayed higher intensities (Fig. 5). The labelling on the basal plasma membrane of the podocytes from the diabetic

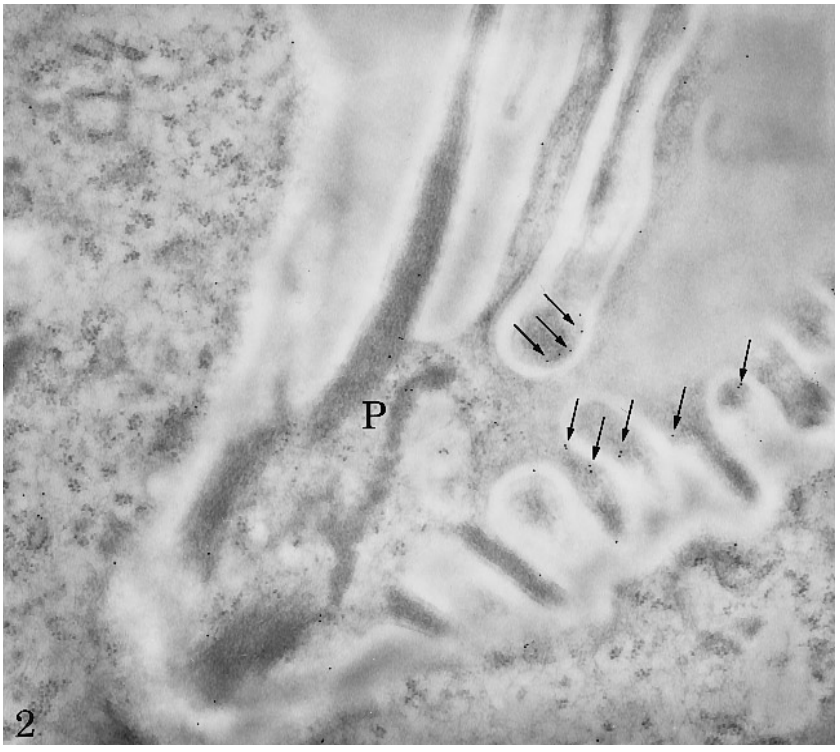

Fig. 2. Normoglycaemic rat. High magnification demonstrating a labelling particularly at sites of contact between podocyte (P) foot processes (→). × 23 000

rats appeared thus to decrease only in those sites where the foot processes faced the GBM.

Quantitative evaluation of the density of labelling confirmed these observations and demonstrated that in tissues of normoglycaemic animals the basal plasma membrane of the podocytes was the most intensely labelled (Table 1). The labelling on mesangial and endothelial cells was lower than that of the podocytes. Regarding the tissues from long-term diabetic animals, the most striking result was the significant reduction in labelling registered at the level of the basal plasma membrane of the podocytes, particularly in the domain facing the GBM. The labellings on the other domains and in the plasma membrane of the other cell types remained similar to those of the normoglycaemic animals (Table 1).

The distribution of α_3 was also analysed on the basal membrane domain of the podocytes in short-term (1 month) diabetic rats and in their age-matched controls. The results obtained and the pattern of the labelling were comparable to those found in the long-term diabetic animals. Similar reduction of the labelling was registered at the level of the podocyte basal plasma membrane facing the GBM (0.448 ± 0.16 particles/μm vs 0.706 ± 0.20 particles/μm for the age-matched controls).

The specificity of the immunolabelling was assessed by two different control experiments and in both cases, the labelling by the α_3 antibody was almost abolished, demonstrating the significance of our results.

Discussion

In view of the alterations occurring at the level of the ECM in glomeruli of diabetic animals and of the key role played by β_1 integrins in the mediation between the ECM and the glomerular cells, the presence of the ECM receptors on podocytes and endothelial cells at the GBM interface must be considered extremely important in the overall renal filtration function and alterations in diabetes. In the present study, we investigated the detailed distribution of the $\alpha_3\beta_1$ integrin in glomeruli from normal, short- and long-term diabetic rats using the immunocytochemical approach at the electron microscope level.

The α_3 subunit was present in all the glomerular cell types in both normal and diabetic rats along the cellular plasma membranes facing the GBM and the mesangial matrix. It was found in moderate levels in luminal plasma membranes of podocytes and endothelial cells. The mesangial cells also showed a moderate reaction and the morphometrical evaluation showed no significant changes between normo- and hyperglycaemic animals. Similar results were obtained on endothelial cells with no major changes between normo- and hyperglycaemic animals.

However, the labelling on the podocyte plasma membranes was more significant. Our data revealed the presence of α_3 not only at the basal plasma membrane of the foot processes along the GBM, but also along the luminal plasma membrane domain facing the urinary space. This finding contradicts the hypothesis of the sole expression of the $\alpha_3\beta_1$ at the

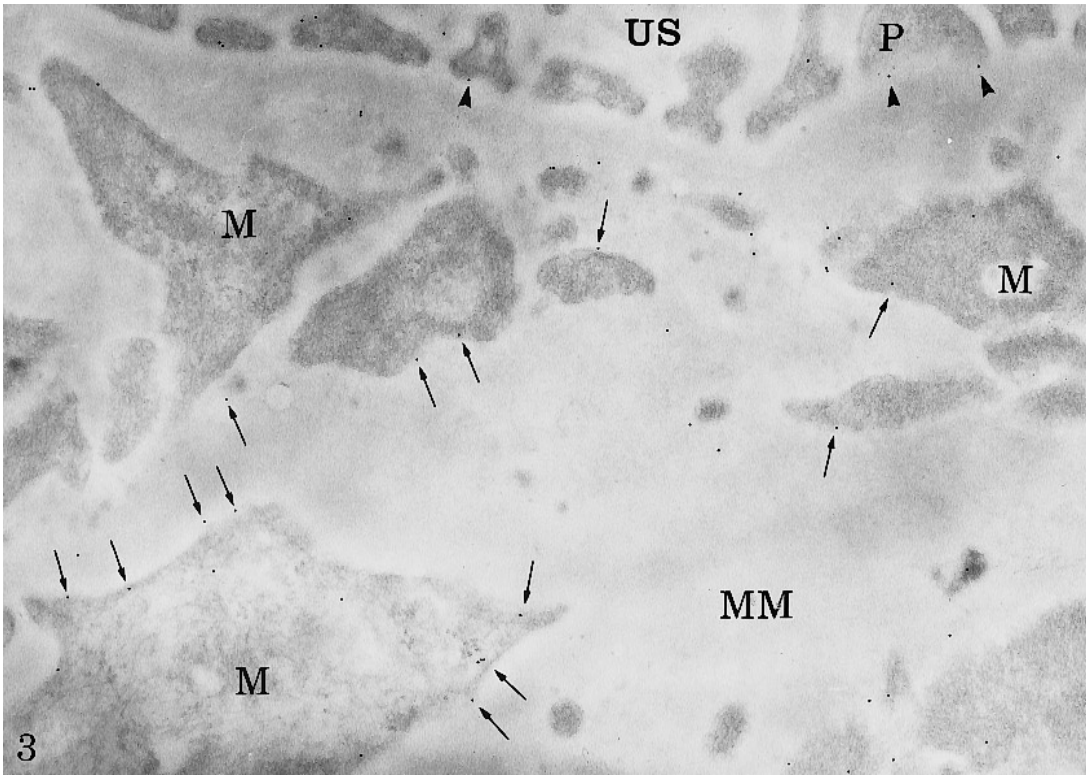

Fig. 3. Normoglycaemic rat. Mesangial area. The gold particles revealing α_3 are located on the mesangial cell bodies (→) and on their cytoplasmic processes. Fewer particles are seen at the level of the podocyte (▶). MM, Mesangial matrix; M, mesangial cells; US, urinary space; P, podocytes. × 34 000

base of the foot processes along the GBM [34] but should be expected from the well-known role of the β_1 integrins in cell-to-cell interactions [9, 10, 12]. Our results are supported by the $\alpha_3\beta_1$ immunolocalization at the sites of cell-cell contacts, either in situ or in vitro [9, 10] and by the induction of cell aggregation with the use of anti-α_3 antibodies [11, 35, 36]. The structure of the podocytes in the context of the glomerular loops is complex; the foot processes arising from one cell are tightly overlapped with those deriving from neighbouring cells. Little is known about podocyte biology and even less about interactions among foot processes [37]. However, the complex structure of podocytes must be maintained, and depends on specific interactions between adjacent foot processes which could involve membrane receptors such as integrins.

The most intense labelling for α_3 was recorded on podocyte basal plasma membrane. Tissues from normoglycaemic rats demonstrated strong and continuous labelling at this site and the intensities of labelling were similar in domains facing either the GBM or the mesangial matrix (Table1). In contrast, the pattern of labelling distribution and intensity for α_3 along the GBM decreased significantly in diabetes, displaying a discontinuous pattern. Indeed, in

Table 1. Intensity of labelling for α_3 subunit on different glomerular cell types in long-term diabetic rats and their age-matched controls

	GEC/GBM n = 100/animal	GEC/MM n = 50/animal	GEC/US n = 50/animal	Endo. luminal n = 50/animal	Endo. ablum. n = 50/animal	Mesangium n = 50/animal
Control 1	0.706 ± 0.24[b]	0.659 ± 0.25	0.485 ± 0.11	0.286 ± 0.10	0.356 ± 0.16	0.594 ± 0.20
Control 2	0.708 ± 0.30[c]	0.688 ± 0.26	0.470 ± 0.09	0.292 ± 0.09	0.354 ± 0.13	0.556 ± 0.17
Control 3	0.724 ± 0.24[d]	0.790 ± 0.25	0.460 ± 0.09	0.280 ± 0.09	0.400 ± 0.13	0.583 ± 0.16
Diabetic 1	0.406 ± 0.19[a]	0.775 ± 0.25	0.490 ± 0.10	0.266 ± 0.09	0.364 ± 0.10	0.611 ± 0.16
Diabetic 2	0.378 ± 0.18[a]	0.745 ± 0.23	0.468 ± 0.09	0.271 ± 0.09	0.371 ± 0.10	0.595 ± 0.17
Diabetic 3	0.451 ± 0.26[a]	0.741 ± 0.21	0.469 ± 0.10	0.265 ± 0.06	0.358 ± 0.11	0.619 ± 0.14

GEC/GBM, Podocyte basal side facing the glomerular basement membrane; GEC/MM, podocytes basal side facing the mesangial matrix; GEC/US, luminal side of podocytes; Endo. luminal, glomerular endothelial cells luminal side; Endo. ablum, glomerular endothelial cells abluminal side.

The intensity of labelling is expressed as gold particles/ μm ± SD

Control 1, 2, 3; Age-matched control rats; Diabetic 1, 2, 3; long-term diabetic rats.

[a] $p < 0.01$ vs b, c, d

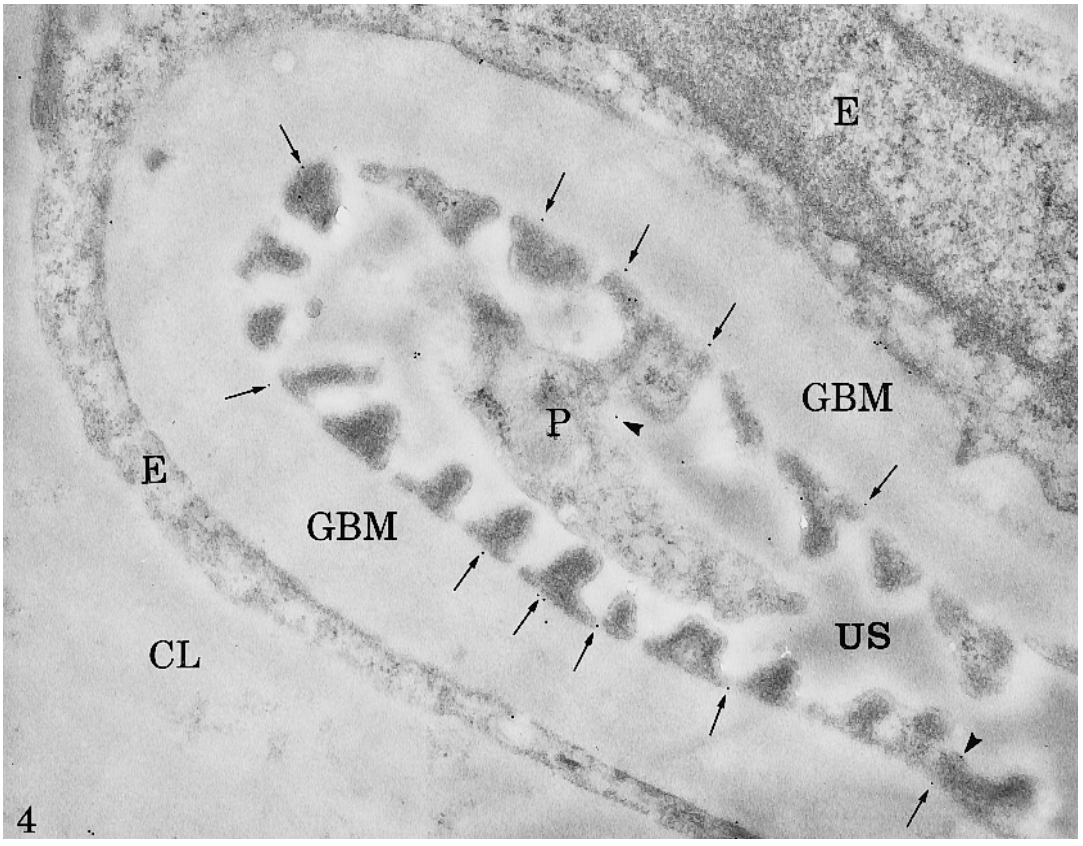

Fig. 4. Hyperglycaemic rat. Labelling for the α_3 on the foot processes facing the glomerular basement membrane (GBM) is weak (→). Very few gold particles (▶) are located on the luminal membrane domain of the podocytes (P). The endothelium (E) shows a low reaction with scattered gold particles along the GBM. US, Urinary space; CL, capillary lumen. × 29 000

regions facing the GBM, the labelling exhibited by tissues of diabetic animals was significantly reduced while that of the region facing the mesangial matrix remained unchanged (Table 1). It is well established that the $\alpha_3\beta_1$ integrin, probably the most abundant if not the only β_1 integrin expressed by podocytes [38], has a large spectrum of ligands among the ECM components [4–8]. In vitro studies have shown that $\alpha_3\beta_1$ is able to mediate adhesion of cultured podocytes to collagen molecules, laminin and fibronectin [24], and, with the exception of type I collagen, rat GBM does contain all these ECM components [39, 40]. Thus, the reduced expression of α_3 on podocyte plasma membrane facing the GBM could be related to the asymmetrical distribution of type IV collagen on the subendothelial side of the glomerular wall established during diabetic glomerulosclerosis [29–31]. To test such hypothetical correlation, we examined the expression of α_3 in tissues of 1-month hyperglycaemic rats which do not yet show any morphological modification of their GBM. A reduction in α_3 labelling, similar to that found in tissues of long-term hyperglycaemic animals, was recorded at the base of the foot processes facing the GBM, suggesting that the altered expression of the α_3 integrin could be directly due to the diabetic state rather than to the modifications of the GBM. Two main factors have been shown to alter the glomerular integrity: high blood glucose concentration and transforming growth factor-β1 (TGF-β1). High glucose levels and TGF–β1 seem to act on glomerular cells by increasing production of ECM components [41–43] and by reducing the expression and activity of metalloproteinases [44] and cathepsins [44, 45]. An overexpression of the TGF–β1 has been reported to occur in diabetic rat kidneys as early as 2–3 days after the induction of hyperglycaemia [46]. Although there is a large body of evidence correlating hyperglycaemia and TGF-β1 with the ECM accumulation in diabetic glomerulopathy [41–43], little is known about the action of these factors on β_1 integrin expression in renal cells [47, 23]. The specific action exerted by TGF-β1 on β_1 integrin expression has not been explored in detail, and seems to vary according to the cell types under study [48]. However, a down-regulation of the α_3 by TGF-β1 has been reported in glomeruli from nephrotic rats [23], and rat alveolar epithelial cells cultured in the presence of this factor [49]. Our finding of a decreased expression of the α_3 in podocytes of hyperglycaemic animals, might thus be considered as an early effect of the high glucose levels and/or overexpression of TGF-β1 [46].

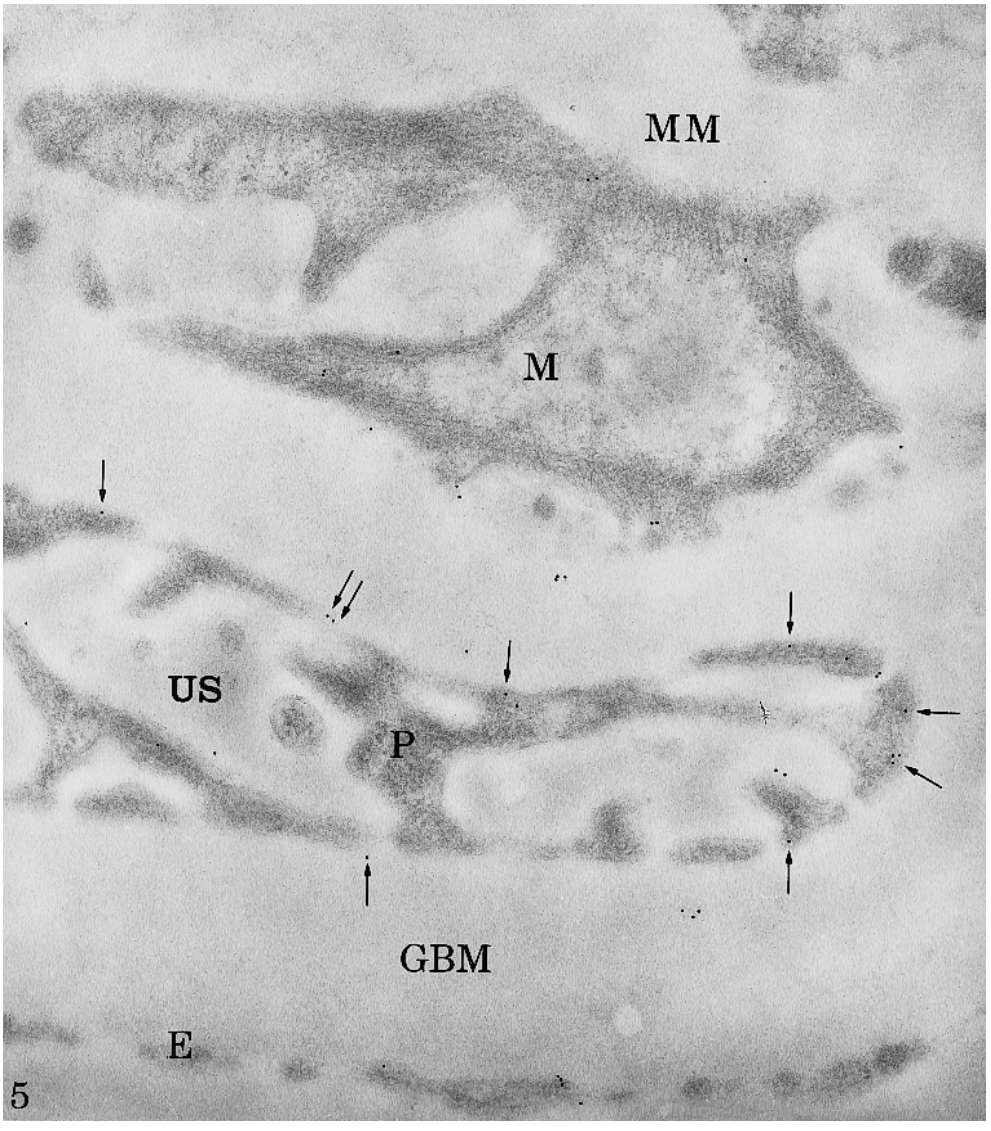

Fig. 5. Hyperglycaemic rat. In the mesangial area labelling for α_3 is preferentially located on the podocytes (P). The reaction is more intense (→) on the side of the mesangial matrix (MM) as compared to the side of the glomerular basement membrane (GBM). The mesangial cell (M) and the glomerular endothelium (E) show scattered gold particles. US, Urinary space. × 39 000

Early alteration of podocyte integrins in diabetes and particularly in the domain facing the GBM could influence the alterations occurring in the glomerular wall and in the progress of the diabetic nephropathy. Since the podocytes are the main producers of GBM components in adult glomeruli, it is possible that a significant alteration of their most important integrin may interfere with the normal ECM deposition. Moreover, α_3 has been reported to exert an active role on ECM assembly [50]. Thus, the early modification of this receptor on podocytes in diabetes may create problems in the organisation of the different extracellular components leading to significant alterations in GBM structure and function.

Acknowledgements. This work was supported by grant MT-9702 from the Medical Research Council of Canada. The technical assistance of Mr. G. Mayer and Ms. D. Gingras is highly appreciated.

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