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Alterations in the distribution of plasma fibronectin and the ultrastructure of podocytes in the peripheral glomerular loops in nephrotic rats

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Abstract Glomerular distribution of rat plasma fibronectin was examined during the course of puromycin (PAN)- and daunomycin (DM)-induced nephrosis. In control animals, fibronectin was detected in the mesangial matrix and along the glomerular basement membrane (GBM), closely associated with the plasma membrane of glomerular cells. In peripheral loops, immunoprecipitates were preferentially distributed in the laminae rarae externa and interna. Fibronectin was densely precipitated in a glomerulosclerotic lesion induced by DM at 8 weeks after the injection. In peripheral loops, loss and reconstruction of epithelial foot processes occurred in PAN nephrosis but the change was accompanied by negligible perturbation of fibronectin distribution in the lamina rara externa. In contrast, a remarkable decrease of fibronectin was observed in DM nephrosis, unrelated to the presence or absence of foot processes. The decrease in immunoreactivity for fibronectin in the lamina rara externa seemed to have no association with podocyte attachment to or detachment from the GBM. Plasma fibronectin distributed in the lamina rara externa is not directly involved in the modification of podocyte configuration or podocyte attachment, although its spatial distribution may have some functional significance for preserving the ultrastructure of the GBM.

Key words Fibronectin · Glomerular basement membrane · Proteinuria · Podocyte

Introduction

The glomerular visceral epithelial cell, the podocyte, resting on the glomerular basement membrane (GBM) is a highly specialized cell having unique morphological features. Its cell body extends primary and secondary cytoplasmic processes, which branch into terminal foot processes, and the terminal processes arising from two adjacent podocytes display a regular alternate arrangement. In proteinuria this fern-leaf pattern of the podocyte is usually retracted into broad and flattened cytoplasmic processes. In the advanced nephrotic stage, exfoliation of podocytes from the GBM is often observed, eventually resulting in segmental glomerular sclerosis and/or hyalinosis [18, 23]. Thus, an intimate interrelationship exists between podoycte morphology and glomerular functions. Although the factors participating in maintenance of the characteristic podocyte architecture are unknown, they are thought to be related to intracytoplasmic cytoskeletal elements and/or certain specific properties of the cell membrane.

Recent in vitro studies have indicated that extracellular matrix macromolecules have a striking effect on a variety of cell behaviours [25]. In particular, fibronectin fibrils are thought to be connected with the intracytoplasmic cytoskeleton via transmembrane linkers, or integrins, and this system may control cell shape, stabilize cell attachments to the substrate, and regulate cell locomotion, polarity, metabolism, and differentiation [19, 20, 33]. However, the relevance of these findings has not been widely tested in the setting of glomerular disease.

We investigated the interrelationship between alterations in the distribution of fibronectin and foot process abnormalities during the course of experimental nephropathies induced in rats by puromycin aminonucleoside (PAN) and daunomycin (DM).

Materials and methods

Male Sprague-Dawley rats weighing 200–220 g were kept in metabolic cages with free access to standard chow and water. After 1 week of acclimatization, 14 animals received a single tail-vein injection of 15 mg PAN (Sigma Chemical Co., St Louis, Mo.) as 1.5% saline solution. Eight rats were killed 10 days after the injection and the others 25 days after the injection. Five animals received a single intravenous injection of 7.5 mg/kg of daunomycin (Daunorubicin, Farmitalia, Italy) as 0.2% saline solution and were sacrificed 8 weeks after the injection. Three normal rats were used as controls (no injection). All experiments were carried out with

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due consideration for the "Principles of Laboratory Animal Care" (NIH Publication no. 85-23, revised 1985).

Urine samples were collected and analysed for protein contents by the quantitative sulfosalicylic acid method.

Under ether anaesthesia, the left kidney was cannulated and perfused in situ, first with 0.05 mol/l phosphate-buffered saline pH 7.2 (PBS) to remove the circulating blood, and subsequently with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.4, containing 8% sucrose. The perfusion pressure and flow rate were kept constant in all experiments: the perfusion pressure was 120 mmHg, and the flow rate was 3 ml/min. Small blocks of the cortex were immersed in the same fixative for 30 min to 2 h.

For immunohistochemistry, the fixed samples were embedded in paraffin. Tissue sections were incubated overnight with the polyclonal antibody against rat plasma fibronectin (Chemicon, Temecula, Calif.) at 4°C. Bound antibody was detected by the streptavidin–biotin immunoperoxidase method.

Small blocks of the renal cortex for transmission electron microscopy were postfixed in 1% osmium tetroxide for 90 min. They were dehydrated with ascending concentrations of ethanol and embedded in Epon 812.

The specimens fixed for immunoelectron microscopy were rinsed in 0.1 mol/l phosphate buffer, dehydrated through graded ethanol and embedded in LR white (London Resin Co., Woking, UK). Ultrathin sections mounted on nickel grids were first incubated overnight with anti-fibronectin antibody at 4°C. Sections treated with the primary antibody were followed by the secondary antibody coupled to 5- or 10-nm colloidal gold particles (Biocell Research Ltd., Cardiff, UK) for 1 h at room temperature. Before each incubation, sections were incubated for 15 min in 1.0% bovine serum albumin in 0.01 mol/l PBS, and they were washed between incubations with PBS.

Ultrathin sections were contrasted with uranyl acetate and lead citrate prior to the examination with a JEM 1200-EX electron microscope at 80 kV. At least 5 glomeruli from each animal were examined, and immuno-gold particles per 1000 nm length of the GBM in a precise cross section were enumerated in at least 10 areas of each glomerulus.

Results

In control rats, the total urinary protein excretion did not exceed 6 mg/day. Immunohistochemically, glomerular mesangial areas were labelled weakly with the antibody against plasma fibronectin and a weak linear distribution pattern was observed along the peripheral glomerular capillary walls (Fig. 1A). Immunoelectron microscopic examination revealed that fibronectin, visualized as gold particles, is precipitated in the GBM and mesangial matrix, closely associated with the basal surface of podocyte foot processes and with mesangial cells (Figs. 2, 3). In the peripheral loops, immunoprecipitates mostly appeared to be distributed in the laminae rarae externa and interna, leaving only a few granules in the lamina densa. The labelling density was heterogeneous (Fig. 2A, B) and this pattern was more pronounced in grazing sections (Fig. 2C). The numbers of 10-nm gold particles per 1000-nm length of the GBM in the laminae rarae externa and interna varied from 5 to 18 (10.3±5.0 : mean±SD) and from 1 to 7 (2.3±1.1), respectively. There was no evidence to indicate that the particles were distributed in a certain regular pattern throughout the GBM. Thus, the limited number and the heterogeneous distribution of immunogold particles prevent a precise analysis in the lamina rara interna.

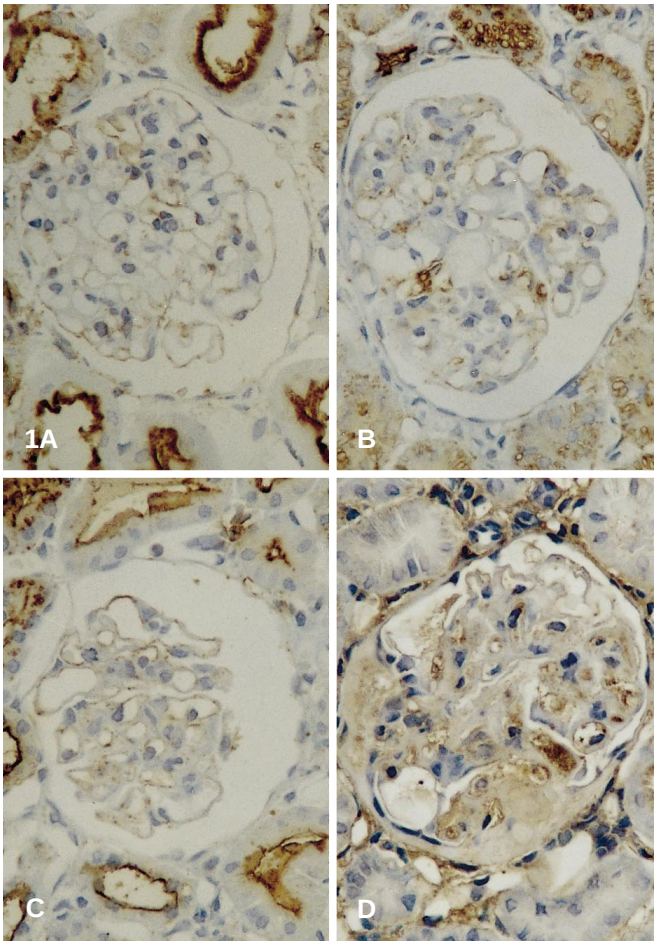


Fig. 1A–D Intraglomerular localization of rat plasma fibronectin detected immunohistochemically. **A** Fibronectin is localized along the capillary loops and in the mesangial areas of normal rats. **B**, **C** No significant differences are noted between the **A** normal and **B, C** PAN-treated rats (10 days and 25 days after the injectin of PAN, respectively). **D** In DM treated rat fibronectin is labelled more intensely in the mesangium and sclerotic lesion than in normal controls. ×370

The experimental animals that had received an injection of PAN had a mean urinary protein excretion of 248 mg/day at 10 days. The striking ultrastructural finding at this stage was loss of the normal arrangement of interdigitating podocyte foot processes so that the GBM was covered by flattened podocyte cytoplasm. In addition, the podocytes had an increased number of cytoplasmic vacuoles. The regular fenestrated pattern of the endothelium tended to become obscure. These morphological alterations were observed in all rats killed at this stage. In 6 animals killed at 25 days, urinary protein values had declined to below 20 mg/day. The glomerular ultrastructure returned to an almost normal appearance: notably, the branching pattern of podocyte cytoplasmic processes had been restored. Immunohistochemical and immunoelectron microscopic examination revealed no significant change with regard to the distribution of rat plasma fibronectin (Figs. 1B, C, 4). This observation is further supported by a semi-quantitative assessment of

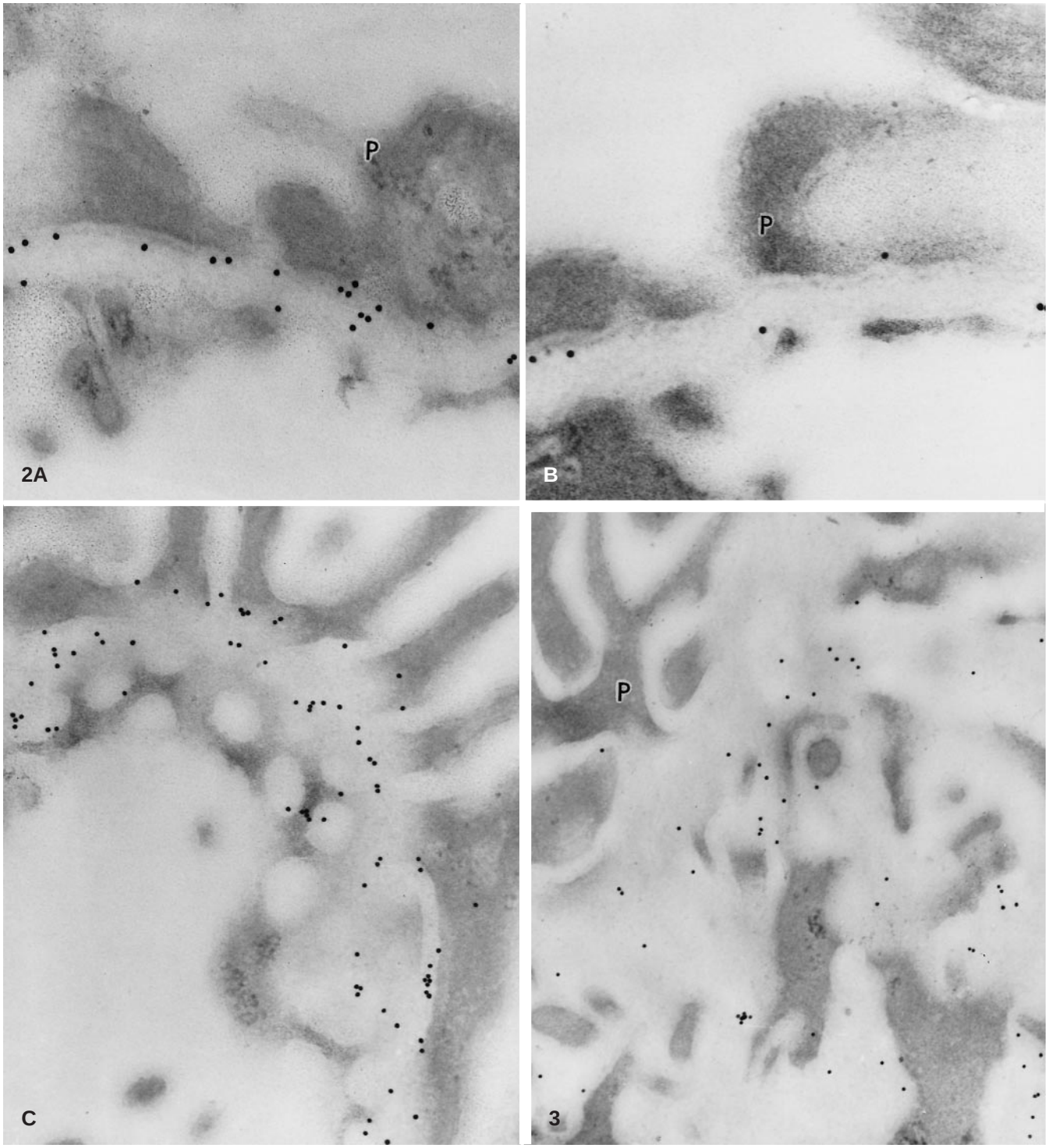


Fig. 2A–C Ultrathin sections of the normal glomerular capillary wall labelled with an antibody against plasma fibronectin. The gold particles are preferentially located over the laminae rarae externa and interna of the GBM, with a few over the lamina densa. **A**, **B** The labelling density is heterogeneous, and **C** this is more pronounced in grazing sections of the GBM. (*P* podocytes). **A** ×100,000, **B** ×113,000, **C** ×75,000

Fig. 3 In control rats the labelling in the mesangium is closely associated with the plasma membrane of mesangial cells. ×60,000

immunogold particles in the lamina rara externa per unit length of the GBM, the values being 9.4±4.4 (2–19) at 10 days and 10.4±4.6 (6–17) at 25 days.

The rats receiving injections of DM exhibited mean proteinuria of 270.9 mg/day at 8 weeks. Histologically, focal and segmental glomerular tuft distortion developed in association with progressive mesangial thickening owing to an increase in the matrix, which was sometimes accompanied by capsular adhesions. Swelling of podocytes was observed consistently, and they often contained

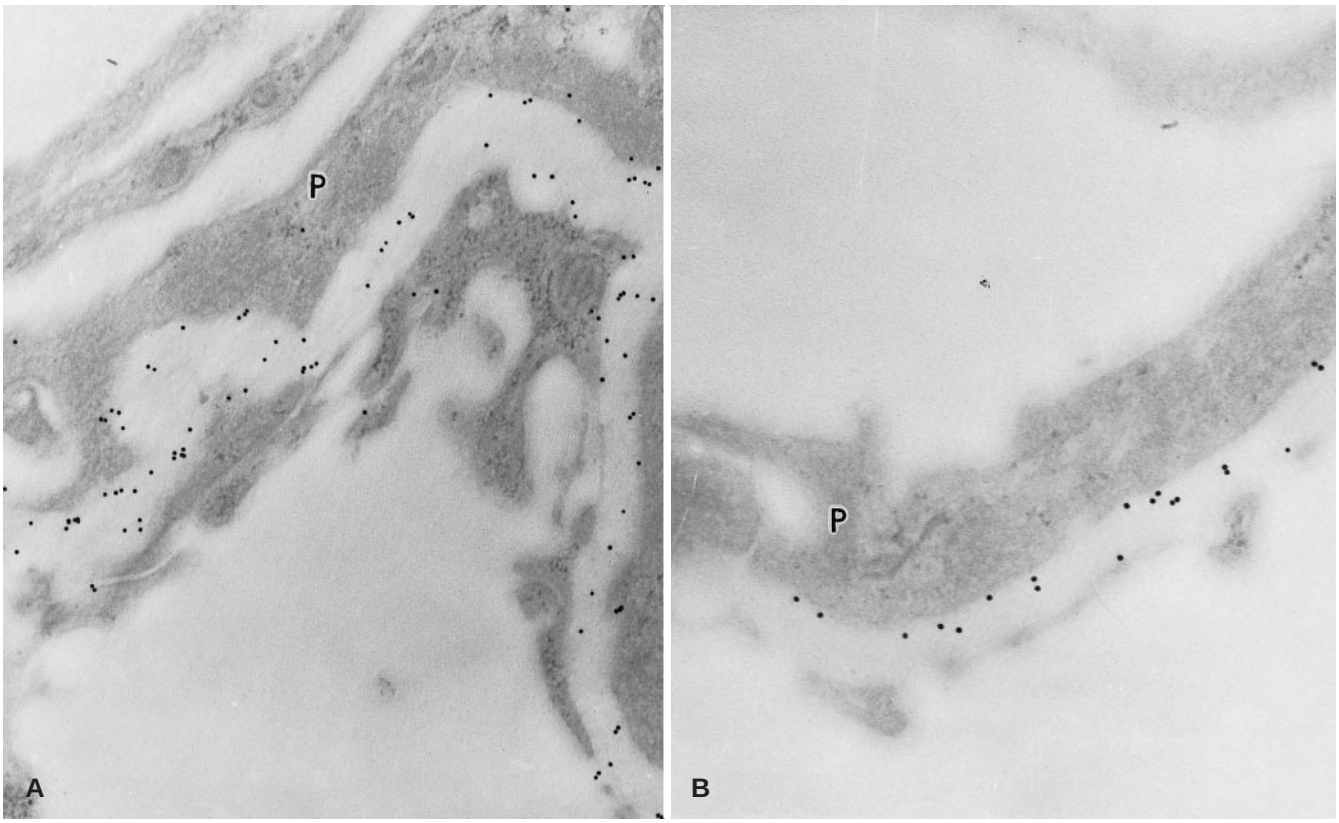
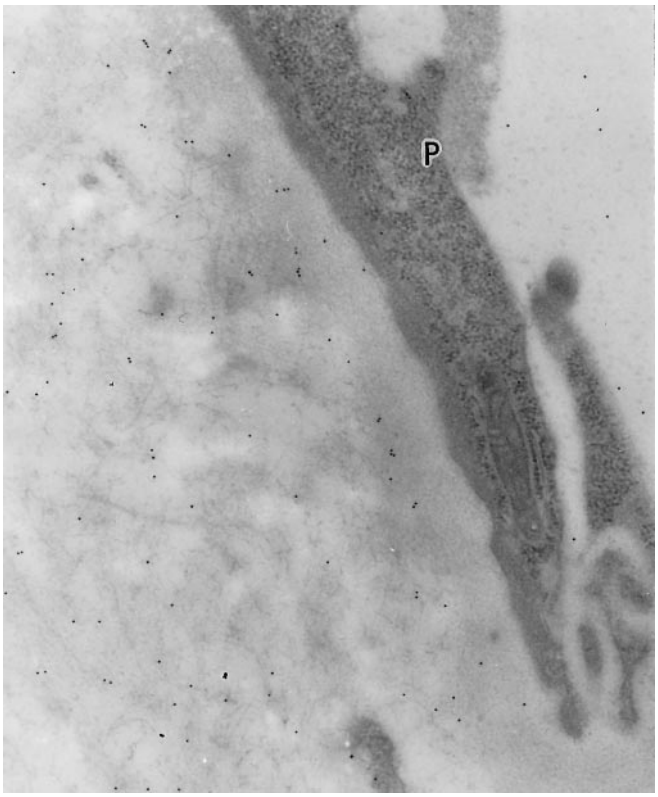


Fig. 4 Localization of the antibody against plasma fibronectin in the glomerular capillary wall 10 days after the PAN injection. Irrespective of the structural alterations of podocytes, no significant changes in the distribution of fibronectin are noted. **A** ×51,000, **B** ×71,000



variable numbers of hyaline droplets and large cystic vacuoles. Ultrastructural examination revealed various degrees of foot process loss. Endothelial cells were somewhat swollen, resulting in localized loss of fenestrations. Moreover, segmental detachment of endothelial cells and of podocyte cytoplasm from the GBM were observed. Some glomeruli persisted with minimal alterations in which normal arrangements of foot processes were largely preserved. However, in the severely damaged glomeruli, segmental or lobular tuft obliteration developed with deposition of dense amorphous material and the appearance of foam cells. The GBM showed frequent wrinkling and thickening, with a fibrillar appearance at higher magnification in some parts.

Immunohistochemically, rat plasma fibronectin was condensed in the mesangium, particularly in the sclerotic lesions, while it was faintly distributed in the peripheral loops (Fig. 1D). Immunoelectron microscopic examination revealed that immunogold precipitates corresponding to plasma fibronectin increased in number and were irregularly distributed in the sclerotic lesions, except for the subepithelial areas (Fig. 5). However, no significant immunogold precipitates occurred in the peripheral loops. In fact, they had dramatically reduced in number throughout the GBM, even in those loops in which the

Fig. 5 Anti-rat plasma fibronectin antibody coupled to 5 nm gold particles is densely and irregularly deposited in glomerulosclerotic and obsolescent lesions. However, gold particles are largely reduced in the subepithelial areas. ×30,000

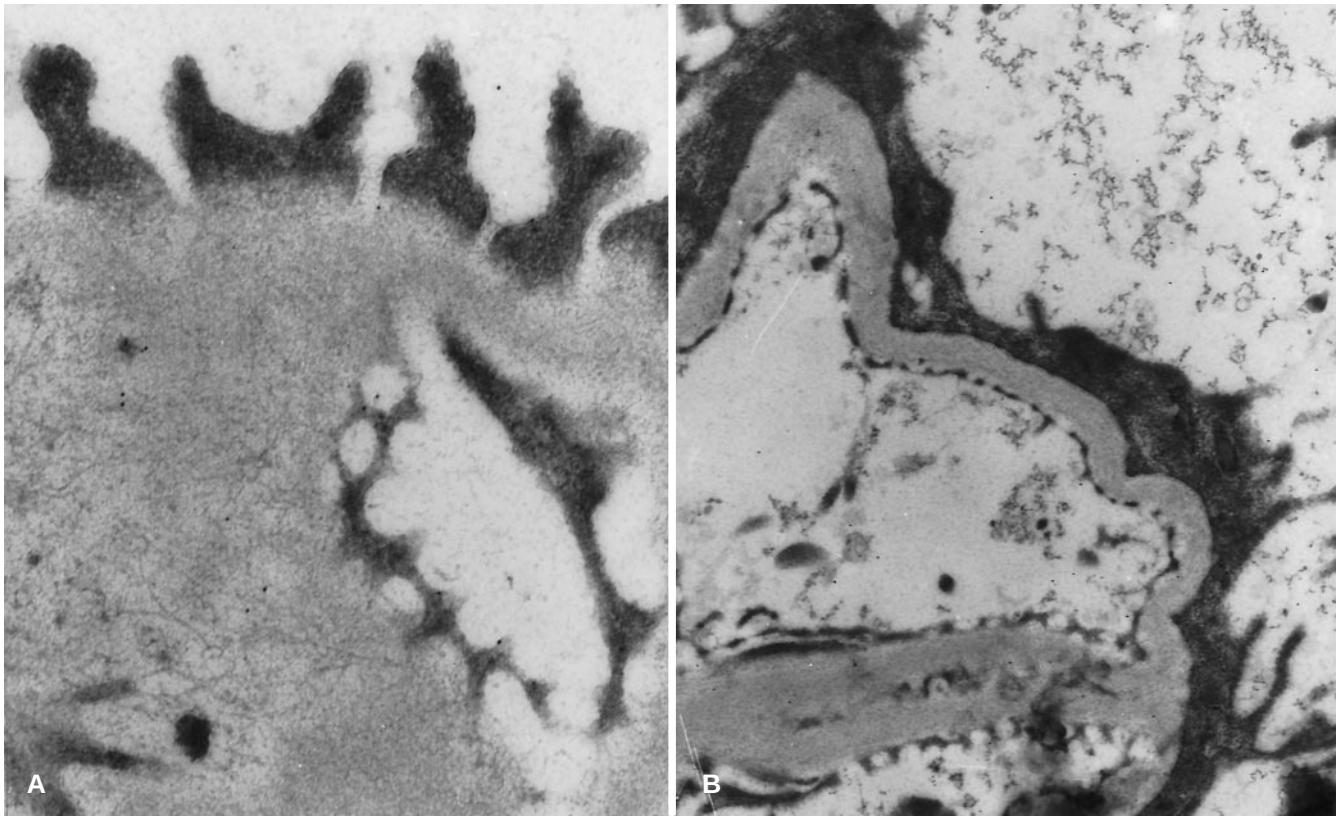


Fig. 6A, B Distribution of the antibody against plasma fibronectin in the peripheral glomerular loops 8 weeks after the injection of DM: 5-nm gold particles are heavily reduced in number throughout the GBM, both in the loops where the foot processes remain discrete and where the foot processes are blunted. **A** ×46,000, **B** ×21.000

foot processes remained discrete (Fig. 6). The number of particles in the lamina rara externa per unit length of the GBM was estimated as 0.5±0.9.

Discussion

Fibronectin is one of the major components of the glomerular extracellular matrix. However, the immunohistochemical localization of this glycoprotein in glomerular capillary walls is still a matter of controversy: some workers have demonstrated fibronectin in the lamina densa of the GBM [24, 36] while others have reported its presence in both the subendothelial and the subepithelial areas of the GBM [14]. Moreover, early investigators found no labelling at all in glomerular capillary walls [31, 35]. In the present study, the antibody against plasma fibronectin was precipitated in both the GBM and the mesangial matrix of control rats, basically along the plasma membranes of glomerular cells. This discrepancy could be attributable to differences in the methods and/or antibodies used.

The distribution of plasma fibronectin along the cell membranes in the normal glomerulus suggests that it acts as an adhesion molecule, stabilizing attachments of glomerular cells to the extracellular matrix and maintaining the cell shape.

We found that loss and reconstruction of podocyte foot processes occurred without any definite alterations in the distribution of fibronectin in PAN nephrosis, but that fibronectin had decreased remarkably in DM nephrosis, a change bearing no relation to foot process changes. This indicates that fibronectin distributed in the lamina rara externa does not participate directly in the maintenance of foot process morphology. It is well known that heparan sulfate proteoglycan in the GBM [5] and sialic acid on the podocyte cell surface [26] have a crucial role in preserving both the functional and the morphologic integrity of the glomerulus. Orci et al. [29] also emphasized that specific plasma membrane domains, which were detected only at the basal part of foot processes by *Helix pomatia* lectin, were related to the maintenance of the complex foot process architecture, but that such a function would be the result of specific interactions of the molecules with the exrtracellular matrix represented by the GBM.

Detachment of podocytes from the GBM is frequently encountered in various glomerular diseases and results in glomerular sclerois and/or hyalinosis. Fibronectin has been considered to be a component involved in the fixation of podocytes and endothelial cells onto the GBM, because enzymatic digestion of glomerular sialic acid induces broad detachments of these cells from the GBM [21]. We saw a significant decrease in immunoreactivity for fibronectin in the lamina rara externa in DM nephro-

sis, but attachment of podocytes to the GBM was still largely preserved. Our results thus suggest that plasma fibronectin is not decisively involved in podocyte attachment to the GBM, although it still seems possible that fibronectin could act as an adhesion molecule even after the loss of its antigenicity.

Our result is a significant negative, because the main integrin in the glomerulus is $\alpha3\beta1$, which has fibronectin as a major ligand. However, $\alpha3\beta1$ is not the only glomerular integrin [2] and fibronectin is not its only ligand [15]. In fact, co-distribution of $\alpha3\beta1$ integrin and laminin in a linear pattern along the GBM has been demonstrated [3], and exposure of podocytes to PAN and Adriamycin (identical to DM) in vitro resulted in a loss of the $\beta1$ integrin focal adhesions, concomitant with an altered distribution and decreased expression of laminin and heparan sulfate [12]. Kemeny et al. [22] also suggested that alterations of the glomerular distribution and expression of $\alpha5$ integrin and $\alpha3\beta3$ integrin (vitronectin receptor) contribute to the detachment of podocytes from the GBM. On the other hand, podocyte adhesion mediated by $\alpha3\beta1$ integrin to types I and IV collagen was shown to be more effective than adhesion to laminin and fibronectin in vitro [1]. Whatever the events involved further investigations seem to be necessary to clarify the nature and location of constituent(s) responsible for the firm attachment of podocytes to the GBM.

Our morphological observations were confirmed by a semi-quantitative method, although statistical analysis could not be carried out because of the limited number of experimental animals. For exact analysis serial sections would be advisable, to allow evaluation of the three-dimensional distribution of immunoprecipitates in a glomerular loop.

It has been demonstrated by a sequential extraction procedure that the increase in fibronectin content in the GBM leads to the loss of glomerular permselectivity in streptozotocin diabetes [8]. This process has not been demonstrated in other experimental nephritides, including nephrotoxic serum nephritis [36] and passive Heymann nephritis [8]. Our results clearly indicate that the distribution of fibronectin in the GBM is unrelated to the development of proteinuria, as far as PAN nephrosis is concerned.

Recent studies have revealed increased accumulation of fibronectin in glomerulosclerotic lesions [6, 9, 28]. Various growth factors and/or cytokines have been claimed to be capable of stimulating glomerular cells to produce extracellular matrix, for example fibronectin production by mesangial cells [17, 27]. Bergijk et al. [7] reported that the abundance of fibronectin was not reflected in the mRNA levels, and antibodies against the cellular form of fibronectin failed to stain glomerulosclerotic lesions, suggesting specific accumulation rather than de novo synthesis of fibronectin. In DM nephrosis, we observed a large amount of plasma fibronectin in the sclerotic lesions in contrast to its decrease at the peripheral loops. We speculate that, in DM nephrosis, plasma fibronectin is excreted into the urine from the peripheral loops under the conditions of increased transglomerular permeability due to disturbances of the size-selective barrier [34]. The enhanced binding capacity of fibronectin in the sclerotic lesions may be explained by an increase in collagen and/or denaturation of collagen [13, 16]. The possibility of fibronectin migration from the GBM to the sclerotic lesions can also be considered. Such mechanisms may not be operative in PAN nephrosis, in which modifications of the charge-selective barrier are thought to be responsible for a dramatic increase in the glomerular permeability [11].

The spatial distribution of plasma fibronectin in the GBM demonstrated may have a functional significance and its perturbation may cause alterations to the molecular structure of the GBM. As fibronectin can be alternatively spliced at three regions, which potentially give rise to functionally distinct variants [4, 10, 30, 32], the target of the antibody will have to be more accurately defined.

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