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Reduced content and abnormal distribution of anionic sites (acid proteoglycans) in the diabetic glomerular basement membrane

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Summary. Sulfated proteoglycans (fixed anionic sites) on the glomerular basement membrane (GBM) of kidneys from diabetic and nondiabetic patients have been demonstrated by electron microscopy using polycationic dyes (ruthenium red, polyethyleneimine). These substances were used for immersion fixation of renal biopsy specimens.

The thickened GBM of diabetics revealed a reduced proteoglycan content within both the narrowed laminae rarae, where normally particles were seen at 60 nm intervals. Proteinuria was observed in all such cases, but no immunopathological alterations of the basement membranes were seen. With both tracer substances anionic sites were also demonstrated in different segments of the thickened lamina densa in diabetics. In polyethyleneimine-treated biopsies some segments of the membrane showed increased anionic moieties at the junction of the basement membrane and the epithelial and endothelial cell membranes. These are probably acid glycoproteins linked to the cell membrane and the synthesis of these basement membrane components may represent a compensatory mechanism seeking to restore normal permeability.

Key words: Diabetes – Glomerular basement membrane – Proteoglycans

Introduction

The glomerular basement membranes (GBM) represents a thin extracellular matrix which separates epithelial and endothelial cells and is rich in fixed anionic charges. The functional role of these anionic sites was established mainly by studies showing a reduced glomerular clearance of negatively charged dextrans in comparison to neutral dextrans of the same size (Chang et al. 1975). Other workers have demonstrated an enhanced penetration

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Dedicated to Prof. Dr. W. Gerok, on occasion of his 60th birthday

of cationized ferritin molecules across the glomerular capillary wall compared with anionic or neutral ferritin molecules of the same size (Rennke et al. 1975).

Glomerular permeability to anionic serum proteins is markedly influenced by fixed negative charges, mainly represented by proteoglycans rich in sulfate groups (Kanwar and Farquhar 1979). In diabetics, besides a marked generalized thickening of the GBM, there is an increased porosity to macromolecules (Steffes and Mauer 1984). In the work reported here the content and distribution of sulfated proteoglycans (anionic sites) in the GBM of long-term diabetics has been investigated by electron microscopy.

Materials and methods

Renal biopsy specimens from five patients suffering from insulin-dependent diabetes for more than 10 years were investigated. All the patients had proteinuria of more than 3.5 g/day and no immune deposits were observed in the glomerular basement membrane by immunofluorescence and conventional electron microscopy. Normal kidney tissue from nephrectomy specimens were used as controls.

Acid proteoglycans were stained with the cationic substances ruthenium red (RR) and polyethyleneimine (PEI) using the immersion methods developed by Luft (1971) and Schurer et al. (1977, 1978) as follows. Immediately following needle biopsy blocks of renal tissue smaller than 0.2 mm in diameter were cut and immersed in the fixative. For the ruthenium red (RR) method the blocks were fixed for 1 h in cold glutaraldehyde (1.2%) in cacodylate buffer (0.07 M, pH 7.3, 450 mOsm) containing 0.05% RR. Blocks were rinsed in three changes of 0.15 M buffer over a 10 min period and immersed for 3 h at room temperature in OsO_3 1.7% cacodylate buffer 0.07 M and 0.05% RR as described by Luft (1971) but modified in that a reduced RR concentration was used.

For the polyethyleneimine (PEI) method small tissue blocks were prefixed in cold glutaraldehyde (1.0%) in cacodylate buffer (0.2 M, pH 7.4, 400 mOsm) for 1 h and then immersed for 30 min in a 0.5% PEI solution (MW 1800; pH 7.4, 400 mOsm), washed with the same cacodylate buffer and reimmersed in 2% phosphotungstic acid (PTA) and 0.1% GA mixture (pH 7.4) to obtain insoluble precipitates of PEI bound to the glomerular proteoglycans. Postfixation was performed with 1% osmic acid at room temperature for 2 h.

The blocks were embedded in Epon and unstained sections were studied with the electron microscope. To allow free access of cationic dye to the glomerular tuft only glomeruli with an open Bowman's space were selected for electronmicroscopic evaluation.

Results

1. Distribution of ruthenium red (RR) and polyethyleneimine (PEI) in the normal GBM

Sections through the GBM stained with RR contained rows of small RR-positive granules (~20 nm in diameter) arranged at intervals of approximately 60 nm (Fig. 1a). Sometimes these granules seemed to be staggered, indicating deposition in different planes. On average there appeared to be more granular anionic sites in the lamina rara externa than in the lamina rara interna (LRI). The distribution was not related to the endothelial fenestrations or to the epithelial slit pores. The distribution of the granules in the LRI and LRE appeared to be mostly regular in the different regions

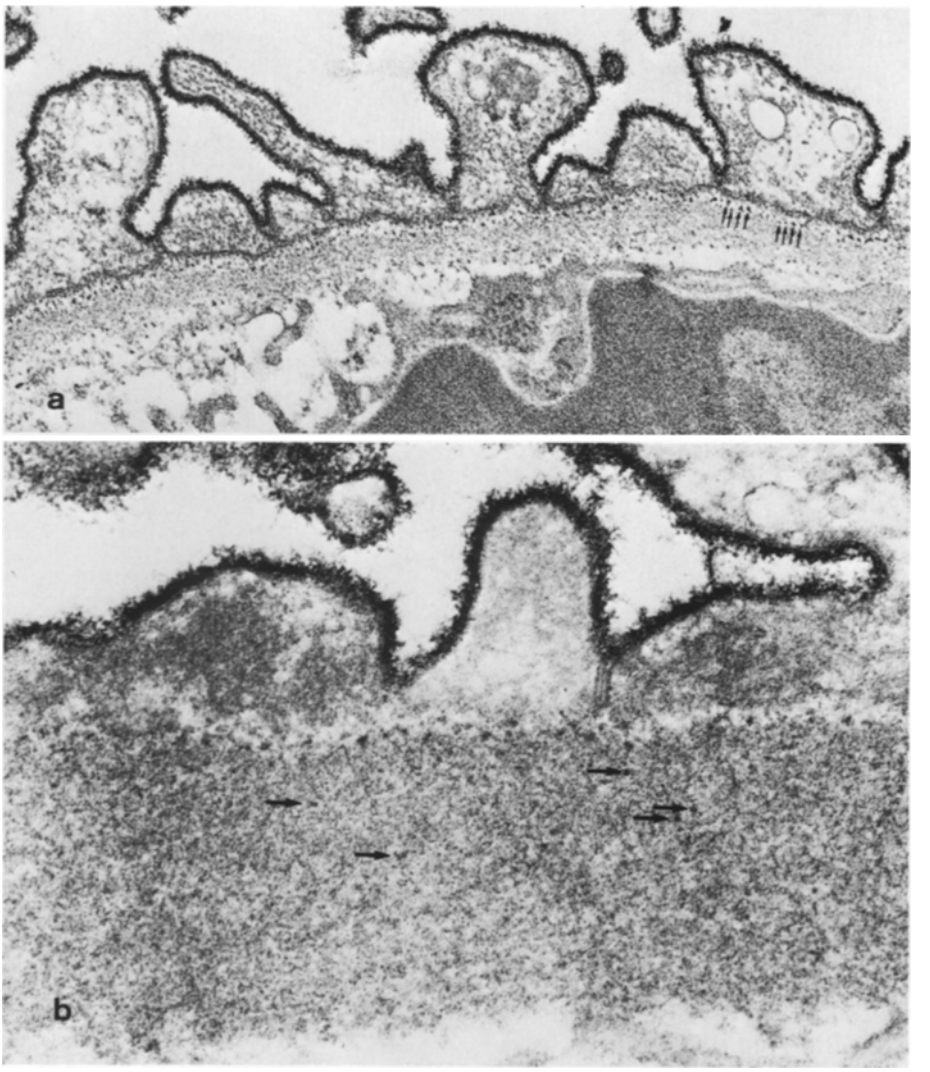

Fig. 1a, b. Electron micrographs of RR-stained GBM of normal **a** and diabetic **b** kidneys. **a** RR-stained anionic sites are distributed at more or less regular intervals (*arrows*) along the lamina rara interna and externa (×60,000). **b** RR granules in the diabetic kidney are diffusely reduced and sometimes appear within the lamina densa (×73,000)

of the capillary loop i.e. in peripheral and central (mesangial) parts. In PEI immersion studies PEI granules (Fig. 3a), indicating the presence of anionic sites, were seen in the LRE, less often in the LRI and only in traces in the lamina densa. These granules seemed to be connected by fine filaments from one site to the other or extended either to the epithelial or endothelial cells or into the lamina densa. The distribution of the granules was not related to adjoining endothelial or epithelial cells.

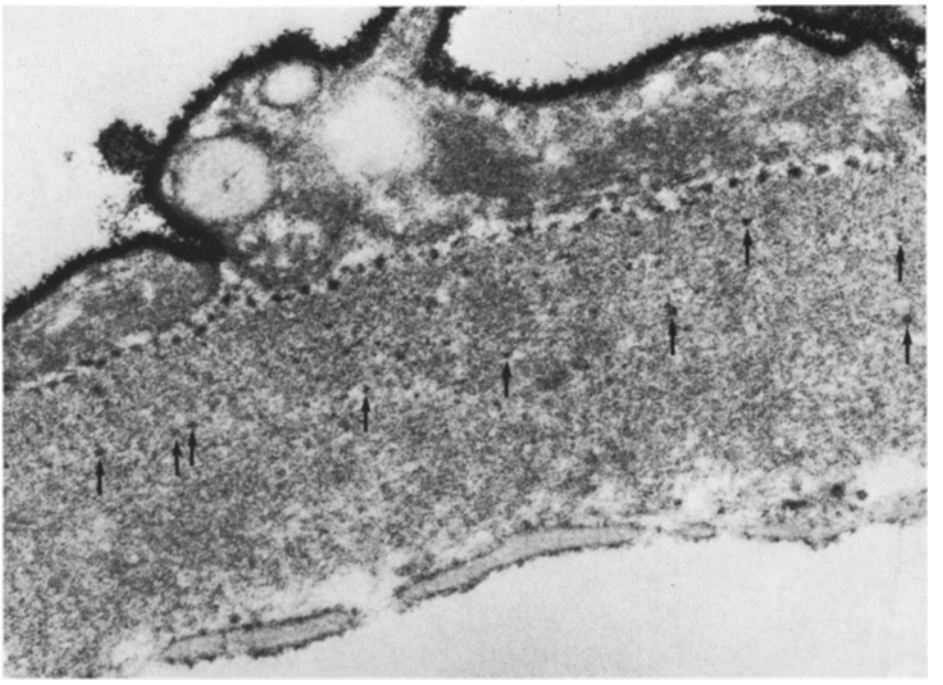

Fig. 2. Electron micrograph of a diabetic kidney with reduced RR granules along the lamina rara externa and interna, and irregularly distributed anionic sites within the lamina densa (× 70,000)

2. Distribution of ruthenium red (RR) and polyethyleneimine (PEI) in the diabetic GBM

The GBM of patients with long-standing insulin-dependent diabetes revealed a moderate or frequently pronounced thickening compared with normal GBM (Fig. 1b).

The number of RR-stained anionic sites was significantly reduced in both laminae rarae (LRI and LRE). The reduction of visible granules appeared to be diffuse in all regions of the GBM, but some segments of the GBM revealed only a slightly reduced number of anionic sites (Fig. 1b). Surprisingly, anionic sites were seen in various central parts (lamina densa) of the thickened basement membrane in an irregular distribution (Fig. 2).

PEI immersion-staining revealed a similar reduction of anionic sites in the diabetic GBM (Fig. 3b). Anionic sites were generally reduced in all membrane layers but in certain sections a laminar increase of PEI was observed in subepithelial as well as in central parts of the basement membrane. Adjacent endothelial and epithelial cell processes appeared to be flattened or fused.

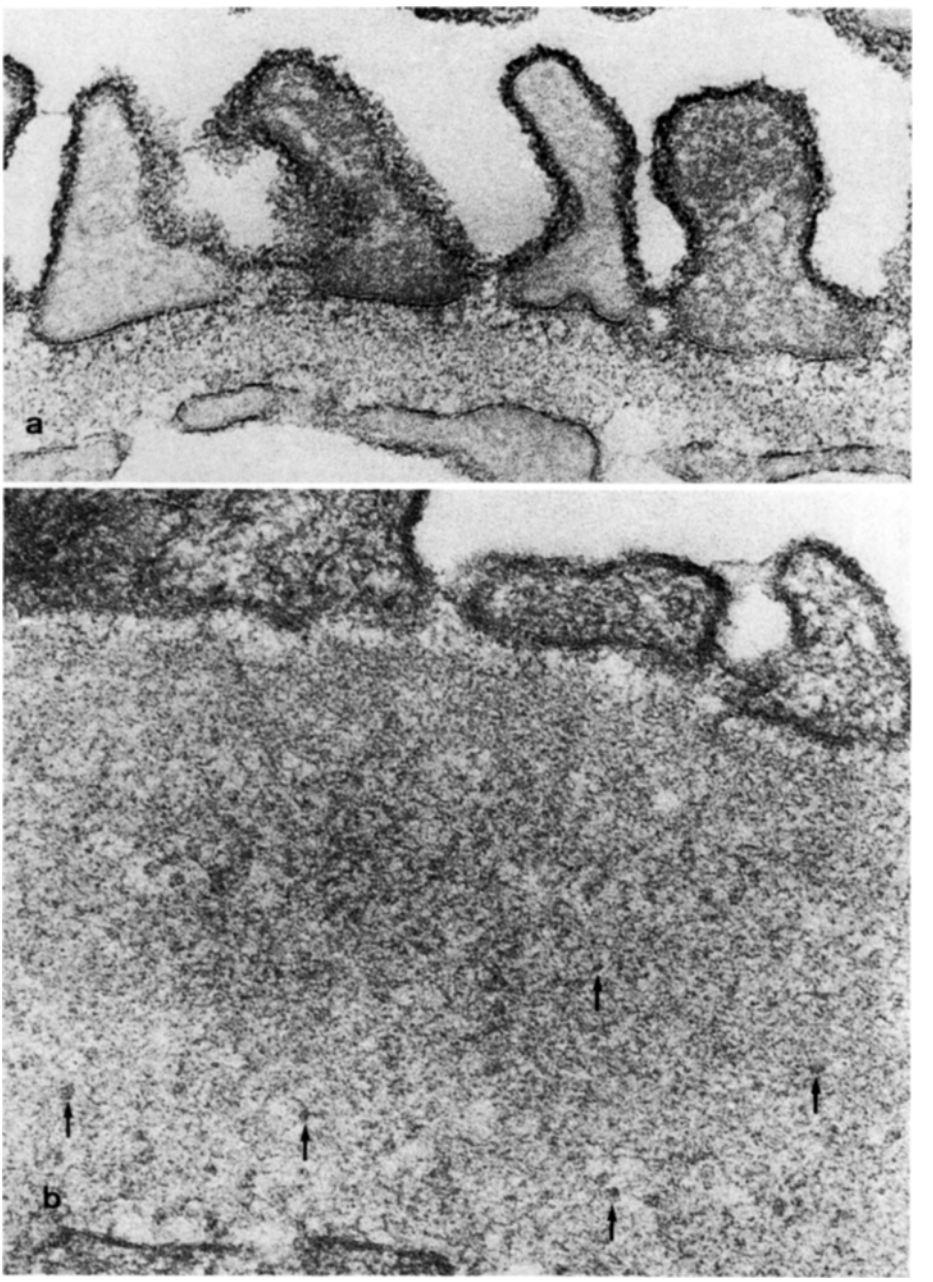

Fig. 3a, b. Electron micrographs of PEI-stained GBM from normal **a** and diabetic **b** kidneys. **a** PEI-stained anionic sites are distributed along the lamina rara externa and interna (× 90,000). **b** PEI granules in the diabetic kidney are diffusely reduced within the lamina rara externa and interna; some are seen within the lamina densa (*arrows*; × 90,000)

Discussion

Structure and proteoglycans of the normal GBM

The GBM represents a size- and charge-selective barrier for macromolecular filtration. This sieving property of the glomerular capillary wall results in an ultrafiltrate that is nearly free of plasma proteins, including anionic albumin. The GBM is a part of the glomerular extracellular matrix and consists mainly of type IV collagen as a structural protein to which negatively charged components are attached. These include laminin, entactin (a recently observed sulfated glycoprotein) and other sulfated proteoglycans. Since glycoproteins like laminin and entactin are attached to the cell membrane of epithelial and endothelial cells, sulfated glycosaminoglycans linked to a core protein make up the predominant part of the sulfated polyanionic macromolecules (mainly heparan sulfate proteoglycan) (Kanwar et al. 1981; Lemkin and Faquhar 1981). They represent the anionic sites in the GBM. Other investigations with different cationic dyes (ruthenium red, Alcian blue) or cationic molecules (lsyzoyme, cationised ferritin) have revealed that these sites are distributed in a lattice-like arrangement in the lamina rara interna (LRI) and the lamina rara externa (LRE) of the GBM (Caulfield and Farquhar 1976; Farquhar 1980). In addition, Laurie et al. (1984) have shown by different immunostaining methods after releasing hidden antigenicity, that five different substances including heparan sulfate proteoglycans are also uniformly localized throughout the lamina densa.

The results presented in this paper confirmed a more or less regular distribution of anionic sites localized at intervals of 60 nm on both sides of the lamina densa in the normal human GBM. This distribution pattern was observed with polycationic dyes (ruthenium red, polyethyleneimine) as well as by others using monocationic dyes (Alcian blue $+ MgCl_2$, Safranin O) (Reale et al. 1983).

Proteoglycans in the diabetic GBM

In insulin-dependent diabetes (IDD) a marked thickening of the GBM is observed coinciding gradually with an impairment of its function as a selective filtration barrier (Brown et al. 1982). From the onset of diabetes a slightly increased urinary albumin excretion (Østerby-Hansen 1965) may be observed for some years during which the initially normal GBM gradually increases in thickness. This basement membrane thickening is also age-related in humans and animals and appears in spontaneously diabetic rats at the age of 12 weeks (Yagihashi et al. 1978). In a six-month experiment with "well-controlled" diabetic rats, neither an increased basement membrane width nor an increase in mesangial area was found (Rasch 1980). An increase in the GBM width as a result of massive type IV collagen accumulation was found in diabetic rodents (Rasch 1979; Steffes et al. 1979), diabetic dogs (Steffes et al. 1982) and in diabetic humans with intact or transplanted kidneys (Østerby 1972; Mauer et al. 1983). Among the factors

which may influence the increase in basement membrane width, the blood insulin level is more important than the blood glucose level (Federlin and Bretzel 1981) though others believe that there is a close relationship between blood glucose level and glomerular basement membrane thickness (Fox et al. 1973).

Different investigations in human diabetics, in experimentally induced diabetic rats and in diabetic mice with transplanted basement membrane-producing tumours have revealed important changes in the protein composition and synthesis of the GBM. These mostly biochemical studies demonstrate an increased amount and synthesis of type IV collagen which explains the thickening of basement membranes. Enzyme activites (prolylhydrolase, glucosyl-galactosyltransferase), inducing modifications in collagen, were found to be elevated in diabetic mice (Rohrbach and Martin 1982). Others have confirmed a reduced amount and synthesis of basement membrane (heparan sulfate) proteglycan (Spiro and Parthasaraty 1982; Cohen and Surma 1981; Rohrbach et al. 1982), and the acid glycoprotein laminin, a cell attachment factor, was found to be elevated in diabetic mice. This reduction in sulfated proteoglycans in the diabetic GBM seems to be responsible for the increased permeability to albumin.

The present investigation was undertaken to determine if and how the content and distribution of anionic sites (sulfated proteoglycans) are changed in the GBM. It was prompted by the discovery that a widespread reduction in the anionic sites in both laminae rarae was present in moderately or severly thickened GBM in five patients suffering from IDD for more than 10 years. From the results of the biochemical investigations cited such changes would be expected. The reduction occurs mainly in a diffuse manner but there was a surprising additional irregular accumulation of anionic sites in the middle (lamina densa) of the GBM which does not appear to have been reported previously in the normal or diseased GBM. This accumulation can probably be explained as a localized attempt to compensate for the functionally defective GBM the porosity of which is increased. An increased binding of PEI in the subepithelial and sometimes in the subendothelial regions of some moderately thickened segments of the GBM cannot be explained. Since these PEI aggregations are intimately joined to cell membranes it is possible that where the relationship of cells to the GBM is disturbed there is an excessive synthesis of acid glycoproteins (Carlin et al. 1981) which have an extraordinary affinity for PEI.

Most reports dealing with the ultrastructural distribution and architecture of proteoglycans in the GBM have used kidney perfusion with a fixative solution containing cationic dyes (Reale et al. 1983; Suzuki et al. 1984). This procedure results in a good staining of all parts fo the GBM. The immersion technique used here has the disadvantage that it avoids a possible limited diffusion of cationic molecules by using very small pieces (less than 0.2 mm in diameter) of kidney tissue. In order to exclude or minimize the possible influence of inadequate diffusion, only those glomeruli with open Bowman's spaces were evaluated. That the tracer substances also reach the inner parts of the basement membranes under the conditions used is

demonstrated by the good impregnation of anionic moieties representing glycoproteins along the endothelial cell membranes.

These studies extend our knowledge of alterations in the GBM of diabetic patients with proteinuria.

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