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Ultrastructural changes of the glomerular basement membrane in diabetic nephropathy revealed by newly devised tissue negative staining method.

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Abstract

In order to clarify the mechanism of proteinuria in diabetic nephropathy, ultrastructural changes of the glomerular basement membrane (GBM) in patients with diabetic nephropathy were examined by electron microscopy using our newly devised "tissue negative staining method". The normal human GBM showed a fine meshwork structure consisting of fibrils forming the small pores. The diameter of these pores was slightly smaller than that of human albumin molecules. The GBM in patients with diabetic nephropathy showed irregular thickening. At higher magnification, hitherto unknown cavities and tunnel structures, which were not seen in normal controls, were observed in the thickened GBM. In some portions, these cavities presented a honeycomb-like appearance. The diameters of the cavities and tunnels were far larger than the dimensions of albumin molecules. These enlarged structures are believed to allow serum protein molecules to pass through the GBM from the capillary lumen to the urinary space. These results suggest that the cause of massive proteinuria in diabetic nephropathy is the disruption of the size barrier of the GBM.

KEYWORDS: glomerular basement membrane, diabetic nephropathy, tissue negative staining, nephrotic syndrome, ultrastructure

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Ultrastructural Changes of the Glomerular Basement Membrane in Diabetic Nephropathy Revealed by Newly Devised Tissue Negative Staining Method

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In order to clarify the mechanism of proteinuria in diabetic nephropathy, ultrastructural changes of the glomerular basement membrane (GBM) in patients with diabetic nephropathy were examined by electron microscopy using our newly devised "tissue negative staining method". The normal human GBM showed a fine meshwork structure consisting of fibrils forming the small pores. The diameter of these pores was slightly smaller than that of human albumin molecules. The GBM in patients with diabetic nephropathy showed irregular thickening. At higher magnification, hitherto unknown cavities and tunnel structures, which were not seen in normal controls, were observed in the thickened GBM. In some portions, these cavities presented a honeycomb-like appearance. The diameters of the cavities and tunnels were far larger than the dimensions of albumin molecules. These enlarged structures are believed to allow serum protein molecules to pass through the GBM from the capillary lumen to the urinary space. These results suggest that the cause of massive proteinuria in diabetic nephropathy is the disruption of the size barrier of the GBM.

Key words : **glomerular basement membrane, diabetic nephropathy, tissue negative staining, nephrotic syndrome, ultrastructure**

In diabetes mellitus, nephropathy is one of the major complications which determines the prognosis. Clinically, the patients with diabetes mellitus often manifest nephrotic syndrome and renal failure. Histologically, characteristic changes of diabetic nephropathy are thickening of the glomerular basement membrane (GBM) and increase of mesangial matrix. However, the mechanism of proteinuria in diabetic nephropathy is unclear. The ultrastructural changes in molecular level of the GBM also have not been clarified.

Glomerular ultrafiltration is considered to occur in the GBM (1-3). In 1977, we observed purified unfixed bovine GBM by electron microscopy after routine negative staining and demonstrated a three-dimensional meshwork structure of the GBM consisting of fibrils that formed pores with dimensions slightly less than those of albumin molecules (4-6). This fine meshwork structure was believed to play a critical role as a size barrier against serum protein molecules in ultrafiltration in the glomerulus. More recently, we developed a "tissue negative staining method" that permitted observation of the molecular-level ultrastructures *in situ* of ultrathin sections that had been routinely prepared for electron microscopy (7, 8). In this study, we demonstrated the existence of tunnel structures and cavities in the GBM of patients with membranous nephropathy and lupus nephritis. These structures may bear a causal relationship to free passage of plasma protein through the GBM.

In this study, in order to clarify the mechanism of proteinuria in diabetic nephropathy, the ultrastructure of the GBM in patients with diabetic nephropathy manifesting nephrotic syndrome was examined by means of our tissue negative staining method.

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Subjecs and Methods

Subjects. The specimens were obtained by renal biopsies of three patients (cases 1-3) with non-insulin dependent diabetes mellitus manifesting nephrotic syndrome who excreted more than 3.5 g protein into their urine per day. All of these specimens were examined by the routine methods including immunofluorescence, light and electron microscopic studies, and showed uniform thickening of the GBM (Fig. 1), nodular lesions, and exudative or hyalinosis lesions. As normal controls, specimens from normal portions of the kidneys of three patients operated on due to renal carcinoma, and a specimen by renal biopsy of a patient with urological bleeding were used. We confirmed that these normal controls have no pathological change by the same routine methods. Thickness of the normal GBM and diabetic GBM were about 300 nm and 741.5 ± 152.4 nm, respectively.

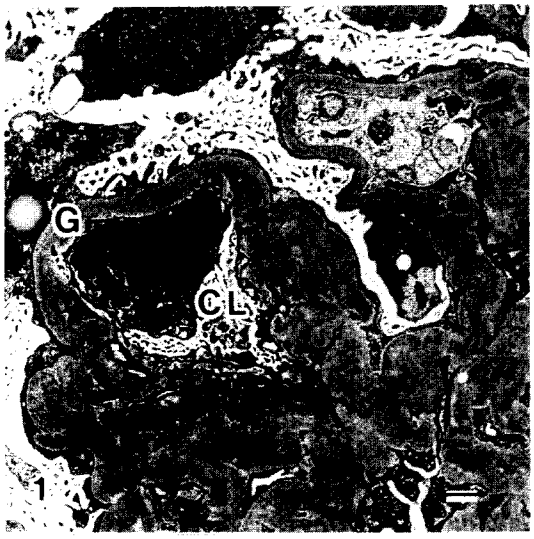

Fig. 1 Ultrathin section of glomerulus in the patient with diabetic nephropathy (case 1). Thickened glomerular basement membrane (G) and collapsed capillary lumen (CL) are observed. Bar = 1 μm. × 4,000.

Tissue negative staining. The tissue negative staining was performed as previously described by us (7, 8). Briefly, the specimens were fixed in 2.5 % glutaraldehyde, post-fixed in 1 % OsO_4 in 0.1 M cacodylate buffer (pH 7.4), and embedded in Epon 812. After embedding, removal of Epon 812 was performed by a modification of Lane's method (9). After hydrophilization, a drop of 1 % phosphotungstic acid (pH 7.3) was placed on the section, and excessive solution was blotted with a blotting paper. These sections were observed and photographed using transmission electron microscope (Hitachi H-700) at an acceleration voltage of 75 KV at magnifications of 2,000 to 30,000.

Morphometric study. Measurement of the diameters of small pores and fibrils was performed at 50 sites on electron micrographs magnified 300,000 times. The short diameters and long dimensions of pores were measured from one point of the internal surface of the fibril to the opposite point using a loupe with a scale. The short diameter of the pores was measured first and the long dimension was measured perpendicular to the short diameter.

To estimate the degree of the structural changes, the 5 perpendicular lines across the GBM in 5 glomeruli were drawn at random on electron micrographs of the capillary (× 60,000), and the sum of total diameters of all pores and cavities on those lines was measured.

Results

Normal human GBM. The normal human GBM showed a definite three-dimensional meshwork structure resembling a crystal lattice (Fig. 2). This meshwork consisted of fine fibrils forming numerous nearly uniform-sized round or oval pores. This structure was observed all over the GBM, and seemed to be uniform almost everywhere in the GBM. Subepithelial space and subendothelial spaces were clearly observed. At higher magnification, many small pores were demonstrated more clearly (Fig. 3). The short diameter of the pores was 2.5 ± 0.4 nm (mean ± SD) and long dimension was 2.8 ±

Fig. 2 Tissue negative staining of the capillary wall in normal human kidney. Epithelial cell (EP), the GBM (G), endothelial cell (ED), subepithelial space (long arrow), subendothelial space (short arrow) are clearly seen. Bar = 100 nm. × 40,000.

Fig. 3 Tissue negative staining of the capillary wall in normal human GBM at higher magnification. Many small pores (arrowheads) are demonstrated clearly. Bar = 10 nm. × 300,000.

Fig. 4 Tissue negative staining of the GBM in diabetic nephropathy (case 3). Numerous large round or irregularly shaped defects are observed. These cavities (arrowheads) are approximately 10-50 nm in diameter. Bar = 100 nm. × 20,000.

Fig. 5 Tissue negative staining of the GBM in diabetic nephropathy (case 3) at higher magnification. Many cavities are aggregated and show a honeycomb-like appearance (arrowheads). Bar = 100 nm. × 60,000.

Fig. 6 Tissue negative staining of the GBM in diabetic nephropathy (case 1) at higher magnification. Numerous cavities (arrowheads) are also observed. Bar = 100 nm. × 60,000.

Fig. 7 Tissue negative staining of the GBM in diabetic nephropathy (case 1) at higher magnification. Many large cavities (arrowheads) are clearly seen in the thickened GBM. Bar = 100 nm. × 60,000.

0.5 nm. The diameter of the fibrils was 1.9 ± 0.4 nm.

GBM in patients with diabetic nephropathy. The GBM in patients with diabetic nephropathy showed irregular thickening (Fig. 4). Numerous large round or irregularly shaped defects that were able to be described as cavities and tunnels were observed in thickened GBM.

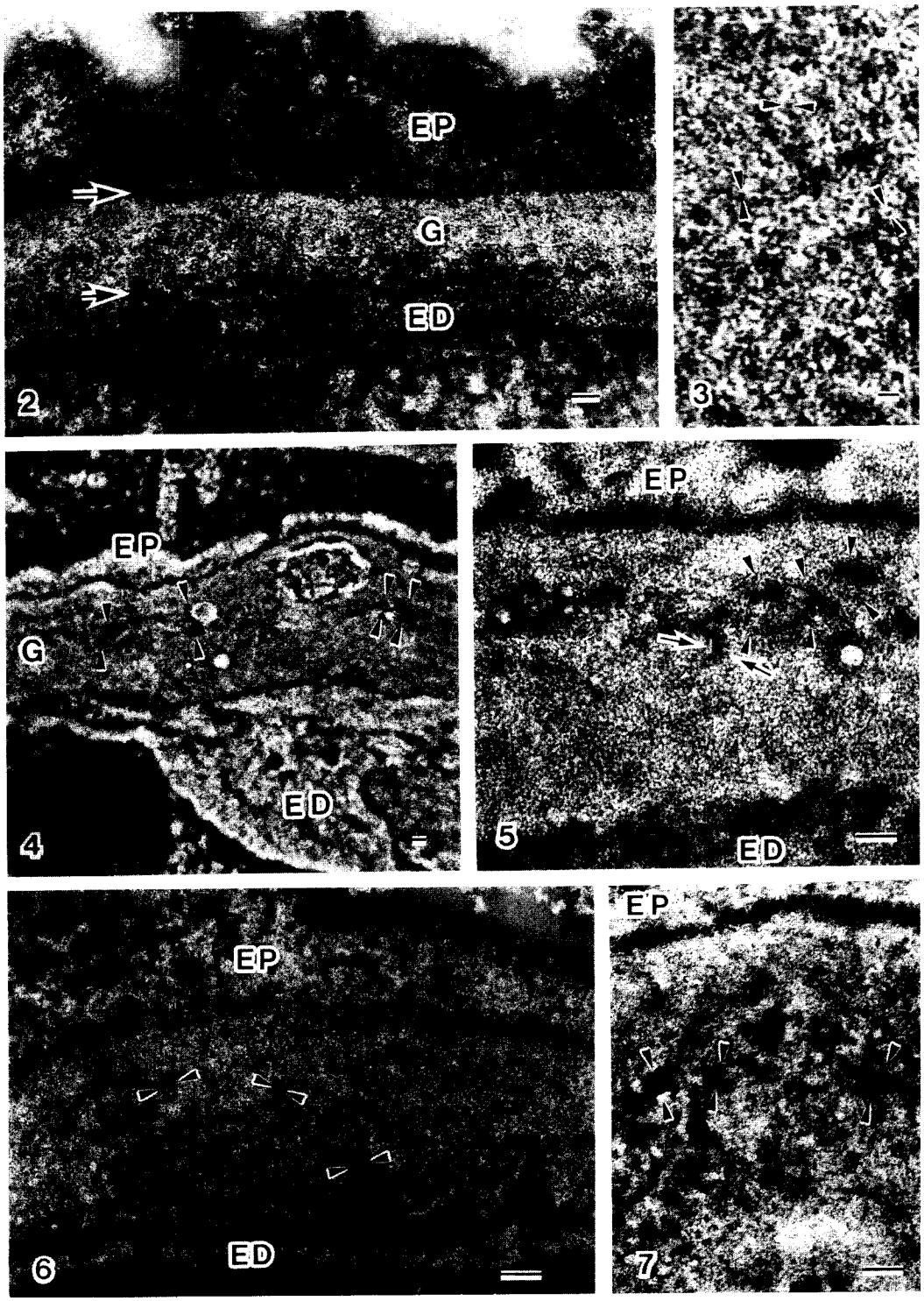

Table 1 The sum of total diameters of the pores, cavities and tunnels, and urine protein volume per day of the patients and control

	Case 1	Case 2	Case 3	Normal control[a]
Total diameter of the pores, cavities and tunnels[b] (nm/100 nm)	66.3 ± 1.6*	64.0 ± 3.5*	70.6 ± 3.7*	56.6 ± 0.4
Urine protein per day[c] (g/day)	4.3 ± 0.8	3.6 ± 1.2	9.3 ± 2.2	< 0.1

All results are expressed as the mean ± SD. *; Significantly different from control ($p < 0.01$).

a: Specimens from normal portions of the kidneys of the three patients operated on due to renal carcinoma, and a specimen by renal biopsy of a patient with urological bleeding. *b:* The sum of total diameters of all pores, cavities and tunnels on the five perpendicular lines across the GBM on electron micrographs (× 60,000) were measured. *c:* Average urine protein volumes per day during two weeks before renal biopsy.

Some of these cavities seemed to be arranged in layers which were located almost parallel to the edge of the GBM. At higher magnification (Figs. 5–7), in some portions of thickened GBM, many cavities had aggregated and showed honeycomb-like appearance. The diameters of the cavities and tunnels were approximately 10–50 nm. In the morphometric study, the sum of total diameters of pores, cavities and tunnels on the perpendicular lines across the GBM were 56.6 ± 0.4 nm (mean ± SD) per 100 nm GBM in normal human specimens, 66.3 ± 1.8 nm in case 1, 64.0 ± 3.5 nm in case 2, 70.6 ± 3.7 nm in case 3 per 100 nm GBM in the specimens from diabetic nephropathy patients (Table 1). This value of diabetic nephropathy was significantly larger than that of normal controls ($p < 0.01$, Wilcoxon test).

Discussion

Patients with diabetic nephropathy often manifest nephrotic syndrome. Morphologically, the characteristic changes of the glomerulus in patients with diabetic nephropathy are thickening of the GBM and mesangial matrix expansion.

The GBM is believed to be the main filtration barrier against serum protein molecules in the glomerulus (1, 2). Concerning the ultrastructure of the GBM, we observed purified GBM by routine negative staining method and demonstrated the fine meshwork consisted of fibrils in the GBM as a schematic representation in Fig. 8 (4–6). Numerous small pores about 3 nm in diameter were present in the meshwork structure. Some of the pores had aggregated to form larger dark spots. These spots resembled irregular bunches of grapes and were less well-defined than the cavities and tunnels. We proposed that this structure functions as the size barrier in ultrafiltration in the glomerulus.

The major components of the GBM are type IV collagen, laminin and heparan sulfate proteoglycan. Timpl *et al.* (10) and Yurchenco *et al.* (11) reported that type IV collagen forms a meshwork of irregular polygons. We also demonstrated that this meshwork structure of the GBM was composed of type IV collagen by immunoelectron microscopy after chemical treatment (12).

Recently, we established the tissue negative staining method which enables the observation of structures at the molecular level *in situ* (7, 8). We observed the morphological changes of the GBM in patients with nephrotic syndrome by this method and demonstrated the presence of the cavities and the tunnel structures in the GBM in patients with membranous nephropathy and lupus nephritis (8). The diameters of these cavities and tunnels were far larger than the diameter of human albumin molecules (Fig. 9) (8). Thus, we proposed that these pathological changes of the GBM caused proteinuria (7, 8).

In diabetic nephropathy, it is reported that an increased production of glomerular extracellular matrix components and a decreased turnover of extracellular matrix presumably leads to a thickening of the GBM and expansion of the mesangial matrix (13). It is also reported that non-enzymatic glycation interferes with the interaction of matrix components (14). Biochemical study indicated that heparan sulfate proteoglycan (HS-PG) decreased and type IV collagen increased in the GBM in diabetic nephropathy. Beisswenger and Spiro (15) found increased levels of hydroxyproline and hydroxylysine contents in diabetic GBM and Karttunen *et al.* found increased levels of extractable type IV collagen (16). Rohrbach *et al.* reported that the HS-PG was reduced in EHS (Engelbreth-Holm, Swarm) tumors implanted in diabetic mice (17). They inferred that the loss of proteoglycans might lead to an increase in the porosity of the

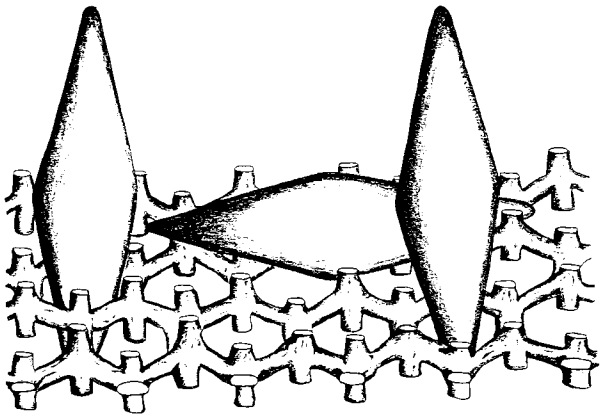

Fig. 8 The schema of albumin molecules and molecular sieve of normal GBM.

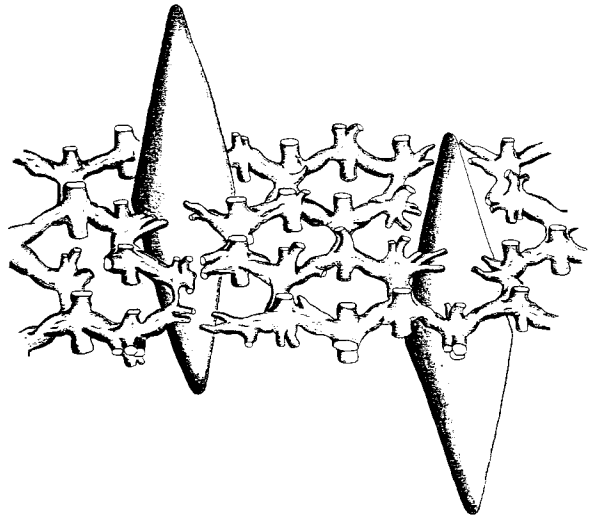

Fig. 9 The schema of the mechanism of proteinuria. Albumin molecules and the enlargement of molecular sieve.

structure of the GBM with compensatory increase in type IV collagen and laminin. Immunohistochemical study suggested that type IV collagen was increased in the glomerulus of the patients with diabetic nephropathy (18). Our recent immunoelectron microscopic study of the diabetic GBM demonstrated the decreased density of type IV collagen in spite of an increase in the absolute number of gold particles reacting with type IV collagen due to the thickening of the GBM (19).

In our present study, we demonstrated the hitherto unknown morphological changes of the GBM in patients with diabetic nephropathy by our tissue negative staining method. Numerous large round or irregular defects formed the cavities and tunnel structure. The diameter of these structures are 10–50 nm. The human albumin molecule has a molecular weight of 66,248 and a prolate ellipsoid with a short axis of 3.8 nm and long axis of 15.0 nm (20). The dimensions of albumin molecule are much smaller than those of the cavities and tunnels in the GBM of diabetic patients. This discrepancy in size allows the serum albumin molecule and other larger serum protein molecules to pass across the GBM in diabetic nephropathy. These changes were not observed in normal GBM. In addition, in patients with diabetic nephropathy, the ratio of total diameters of small pores, cavities and tunnels to the thickness of the GBM was significantly larger than that of normal GBM. These results indicate that the disruption of size barrier of the GBM leads to proteinuria in patients with diabetic nephropathy. As the ratio increases, the total amount of protein in proteinuria appears to increase. Although the cause of these structural changes is unclear, non-enzymatic glycation may interfere the formation of normal meshwork structure of type IV collagen molecules. In order to produce proteinuria by this mechanism it is necessary that the cavities and tunnels continue throughout the entire GBM from the inner surface to the outer surface. In membranous nephropathy, the nephrotic tunnels obviously penetrated the entire GBM (8). In diabetic GBM, these findings have not yet been demonstrated. In the future, serial sections should be used in addition to the tissue negative staining method to clarify these changes.

We conclude that the disruption of size barrier of the GBM permits massive proteinuria in patients with diabetic nephropathy.

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