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## Abstract

We developed a "tissue negative staining method" to observe the molecular-level ultrastructure in situ in any portion of the ultrathin sections routinely prepared for electron microscopy. This method was used in electron microscopy of the glomerular basement membranes (GBM). The GBM in patients with nephrotic syndrome was discovered to possess a tunnel structure, designated as "nephrotic tunnel", with lumen large enough to allow free passage of protein molecules. This tunnel seemed to be involved in the etiology of nephrotic syndrome. This new method appears to be applicable to a variety of purposes in biological studies.

**KEYWORDS:** mechanism, proteinuria, nephrotic syndrome, electron microscopy, negative staining

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— Brief Note —

# Mechanism of Proteinuria in Nephrotic Syndrome

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**We developed a "tissue negative staining methed" to observe the molecular-level ultrastructure *in situ* in any portion of the ultrathin sections routinely prepared for electron microscopy. This method was used in electron microscopy of the glomerular basement membranes (GBM). The GBM in patients with nephrotic syndrome was discovered to possess a tunnel structure, designated as "nephrotic tunnel", with lumen large enough to allow free passage of protein molecules. This tunnel seemed to be involved in the etiology of nephrotic syndrome. This new method appears to be applicable to a variety of purposes in biological studies.**

*Key words :* **mechanism, proteinuria, nephrotic syndrome, electron microscopy, negative staining**

Glomerular ultrafiltration is believed to occur in the glomerular basement membrane (GBM). However, the existence of morphological filtration pores postulated by Pappenheimer (1) was unclear. In 1977, we observed purified unfixed bovine GBM by electron microscopy after negative staining and demonstrated a three-dimensional meshwork structure of the GBM consisting of the fibrils that formed pores with dimensions slightly smaller than an albumin molecule (2-5). We also observed similar pores in routine ultrathin sections that had received chemical treatment (6). In the present study, we describe the tissue negative staining method we developed to observe the ultrastructure of the GBM *in situ*, and clearly visualized the molecular-level ultrastructure of the GBM. Observation of GBM from patients with nephrotic syndrome showed irregular tunnels penetrating the GBM and honeycomb structures. These pathologic changes may bear a causal relationship to proteinuria in nephrotic syndrome.

*Bovine descemet's membrane.* Fresh bovine Descemet's membrane was routinely fixed, and embedded, and cut into ultrathin sections. Some sections were prepared for electron microscopy by the routine method, and the others by our newly devised tissue negative staining method (Fig. 1), which was performed as follows.

The sections were, for removal of Epon 812 by a modification of Lane's method (7), placed in a 400-mesh nickel grid covered with a carbon film, immersed for 3 h in a 1/10 dilution of 100 ml of ethanol containing 4 g of NaOH, immediately prepared before use, and washed in distilled water. The grid was then floated in distilled water in a beaker with the ultrathin sections immersed for more than 15 min for hydrophilization. A

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drop of 1% phosphotungstic acid (pH7.3) was placed on the section, and excessive solution was blotted with a blotting paper. These sections were observed and photographed using a Hitachi H-700 transmission electron microscope at acceleration voltage of 75KV at magnifications of 2,000, 5,000, 10,000 and 30,000.

Descemet's membrane prepared by each method showed a three-dimensional meshwork structure consisting of units of equilateral triangles formed by nodes connected by fibrils. When 50 sites were measured using a loupe with a scale on micrographs (× 50,000), the distance between the nodes was 128 ± 8.6nm in sections prepared by the routine method and 127.0 ± 5.4 nm in sections prepared by our tissue negative staining method. These findings are consistent with the results in our previous study using negative staining of unfixed Descemet's membrane (8).

*Purified bovine GBM.* Observation of bovine GBM purified by Spiro's method (9) after our tissue negative staining showed a three-dimensional meshwork structure consisting of fibrils forming numerous round or oval pores nearly uniform in size (Fig. 2). Fifty sites were measured using a loupe with a scale on micrographs (× 300,000). The diameter of the fibrils was 1.9 ± 0.3nm. The short diameter of the pores was 3.0 ± 0.5nm, and the long dimension was 3.5 ± 0.6nm. These findings are consistent with the results of our previous studies using negative staining of unfixed bovine GBM purified by the same method (2-5). Slight differences in values may be due to the differences in the methods.

Thus, our new tissue negative staining method allows observation of the molecular-level ultrastructure.

*Normal bovine and human GBMs in situ.* To evaluate the usefulness of our new method for observing the ultrastructure of the GBM *in situ*, normal bovine glomeruli and normal human glomeruli in biopsy specimens from 3 patients with urological bleeding were observed after our tissue negative staining. As shown in Fig. 3, the GBM, epithelial cells, and endothelial cells were clearly differentiated. Ultrastructurally, bovine GBM and human GBM, as shown in Fig. 4, showed a clear three-dimensional meshwork structure consisting of fibrils forming nearly uniform pores. Fifty sites were measured on micrographs (× 300,000). The diameter of the bovine fibrils was 1.9 ± 0.5nm. The short diameter of the bovine pores was 3.3 ± 0.3nm, and the long dimension was 3.5 ± 0.4nm. In humans, the fibril diameter was 1.9 ± 0.4nm, and the short diameter of the pores was 2.5 ± 0.4nm, and the long dimension, 2.8 ± 0.5nm. In cattle, these findings were consistent with those in the purified GBM.

Thus, our tissue negative staining allows ultrastructural observation of tissue *in situ*.

*Human GBM in situ in nephrotic syndrome.* Renal biopsy specimens were obtained from 3 previously untreated patients with membranous

**Fig. 1** Tissue negative staining of bovine Descemet's membrane. Micrograph shows a meshwork structure consisting of equilateral triangles composed of fibrils connecting nodes. × 50,000.

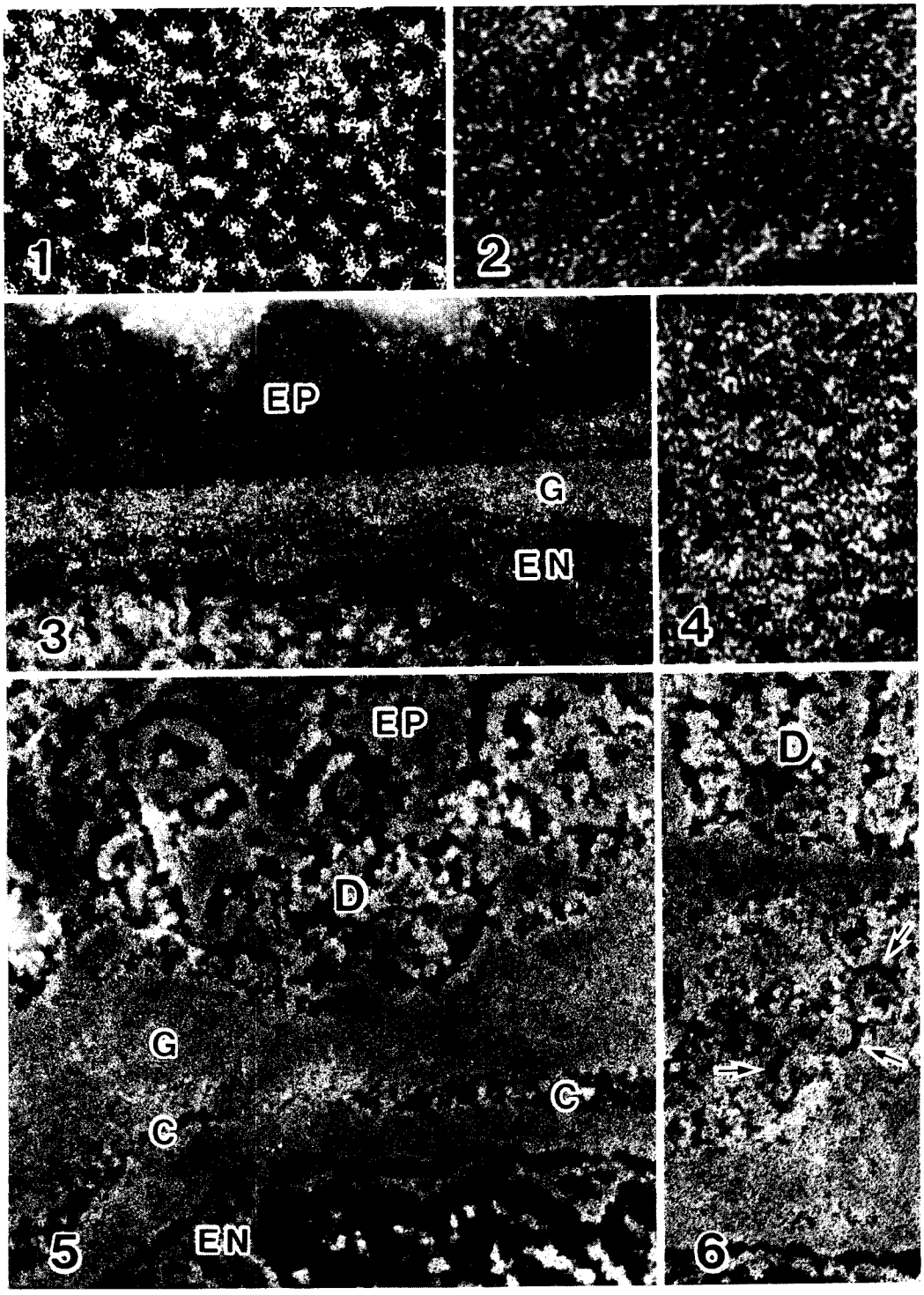
**Fig. 2** Tissue negative staining of purified bovine GBM, showing meshwork structure consisting of fibrils forming numerous pores. × 240,000.

**Fig. 3** Tissue negative staining of the capillary wall in a normal human glomerulus. The GBM (G), epithelial cells (EP), and endothelial cells (EN) are clearly differentiated. × 40,000.

**Fig. 4** Tissue negative staining of normal human GBM. Numerous nearly uniform-sized round or oval pores formed by fibrils are observed. × 300,000.

**Fig. 5** Tissue negative staining of the glomerular capillary wall in membranous nephropathy. GBM (G), epithelial cells (EP), endothelial cells (EN), deposits (D), and numerous cavities (C) can be differentiated. × 40,000.

**Fig. 6** Tissue negative staining of the GBM in membranous nephropathy. Irregular tunnels (arrows) with a width of 20-50nm penetrating the GBM are observed. A part of the tunnels forms cavities. D: deposit. × 80,000.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nephropathy, and 2 with lupus nephritis showing nephrotic syndrome. These specimens were observed after the routine ultrathin sectioning and our tissue negative staining. The GBM was irregularly thickened, and deposits seen in the sections prepared by the routine method were observed as sparse conglomerates of molecules in sections prepared by our tissue negative staining method (Fig. 5). The GBM in nephrotic syndrome specimens showed round or irregular-shaped defects. At a higher magnification, parts of the defects were observed as irregular tunnel-shaped defects penetrating the GBM from the endothelium to the epithelium (Fig. 6). These tunnels were very irregular and branched, forming scattered large round or irregular-shaped cavities. The diameter of the tunnel was about 20–50 nm, and that of the largest cavity was more than 100 nm. At sites in the GBM showing more marked changes, numerous cavities (20–200 nm in diameter) were aggregated, presenting a honeycomb appearance. Such pathologic changes were observed at nearly every site of the GBM in the nephrotic syndrome specimens. The degree of these changes seemed to be related to the degree of proteinuria. Similar changes were observed in lupus nephritis accompanied by nephrotic syndrome. In the GBM seen in nephrotic syndrome specimens, the most basic change visible was tunnel formation. Thus, we termed the structure "nephrotic tunnel".

Our investigations are apparently the only published reports of studies to evaluate the basement membrane by negative staining or tissue negative staining. The negative staining method has been generally accepted to be a gentle and excellent method to reveal fine structures of purified viruses and macromolecules. Our new tissue negative staining method is appropriate for observing tissue at the molecular level and may allow observation *in situ* in nearly every tissue. This method provides much higher resolution than the deep-etch replica method and other methods, and can be widely applied.

The GBM observed *in situ* by tissue negative staining had a three-dimentional meshwork structure consisting of fibrils forming numerous pores similar to the structure in the purified GBM previously observed by routine negative staining (2–5). Since we reported the meshwork structure of the GBM and other basement membranes in 1977 (2), some studies have been made using the deep-etch replica method (10, 11) and the conventional ultrathin sectioning method (12). However, the pores of the fibril meshwork in these studies were far larger than in ours, and had irregular shapes and varied in size. This may have been because the resolution provided by their methods was inadequate to observe the ultrastructure of several nanometers. The primary component of the GBM is type IV collagen. Timpl *et al.* (13) and Yurchenco *et al.* (14) reported that type IV collagen formed a meshwork of irregular polygons. It is probable that the regular meshwork in the GBM observed in this study consists of type IV collagen.

The bovine albumin molecule has a molecular weight of 66,210 and is a prolate ellipsoid with a short axis of 4 nm and a long axis of 14 nm (15). The human albumin molecule has a molecular weight of 66,248 (15) and a prolate ellipsoid with a short axis of 3.8 nm and a long axis of 15.0 nm (16). In the meshwork structure of the normal bovine GBM, the short diameter of the pore was $3.3 \pm 0.3$ nm, and the long dimension was $3.5 \pm 0.4$ nm. In normal human GBM, the short diameter of the pore was $2.5 \pm 0.4$ nm, and the long dimension was $2.8 \pm 0.5$ nm. Therefore, albumin molecules can not pass through the pore. Thus, the three-dimensional meshwork structure of the GBM acts as a size barrier, a molecular sieve. In the specimens from patients with membranous nephropathy and lupus nephritis showing nephrotic syndrome, the GBM appeared abnormal at most sites. Nephrotic tunnels penetrating the GBM and honeycomb structure were observed. These pathologic changes, never seen in normal control specimens, were large enough to allow passage of albumin molecules, hereby fostering the condition of proteinuria. We previously proposed a hypothesis of molecular sieve disrup-

tion (5), which we believe is confirmed in this study.

One cause of these ultrastructural pathologic changes is destruction of the GBM by immunological mechanisms. Another cause may be the formation of incomplete meshwork in the GBM due to a failure in the assembly of the incomplete fibril molecules that are produced intracellularly or extracellularly.

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## References

1. Pappenheimer JR: Passage of molecules through capillary walls. Physiol Rev (1953) **33**, 387-423.
2. Ota Z, Makino H, Miyoshi A, Hiramatsu M, Takahashi K and Ofuji T: Electron microscopic demonstration of meshwork structure in human and bovine glomerular basement membranes. Acta Med Okayama (1977) **31**, 339-342.
3. Ota Z, Makino H, Miyoshi A, Hiramatsu M, Takahashi K and Ofuji T: Molecular sieve in glomerular basement membrane as revealed by electron microscopy. J Electron Microsc (Tokyo) (1979) **28**, 20-28.
4. Ota Z, Makino H, Takaya Y and Ofuji T: Molecular sieve in renal glomerular and tubular basement membranes as revealed by electron microscopy. Renal Physiol (Basel) (1980) **3**, 317-323.
5. Ota Z, Makino H, Takaoka M, Soda K and Suzuki S: Mechanism of proteinuria in rats with chronic serum sickness; in Glomerular Dysfunction and Biopathology of Vascullar Wall, Seno, Copley, Venkatachalam, Hamashima and Tsujii eds, Academic Press, Tokyo (1985) pp 165-174.
6. Shikata K, Makino H, Ichiyasu A and Ota Z: Three-dimensional meshwork structure of glomerular basement membrane revealed by chemical treatment. J Electron Microsc (Tokyo) (1990) **39**, 182-185.
7. Lane BP and Europa DL: Differential staining of ultrathin sections of Epon-embedded tissue for light microscopy. J Histochem Cytochem (1965) **13**, 579-582.
8. Makino H, Toyofuku H, Mino Y, Takaoka M and Ota Z: Molecular sieve in bovine Descemet's membrane as revealed by negative staining. Acta Med Okayama (1983) **37**, 155-157.
9. Spiro RG: Studies on the glomerular basement membrane. Preparation and chemical composition. J Biol Chem (1967) **242**, 1915-1922.
10. Kubosawa H and Kondo Y: Ultrastructural organization of the glomerular basement membrane as revealed by a deep-etch replica method. Cell Tissue Res (1985) **242**, 33-39.
11. Takami H, Naramoto A, Shigematsu H and Ohno S: Ultrastructure of glomerular basement membrane by quick-freeze and deep-etch methods. Kidney Int (1991) **39**, 659-664.
12. Inoue S and Leblond CP: Three-dimensional network of cords: The main component of basement membranes. Am J Anat (1988) **181**, 341-358.
13. Timpl R, Wiedermann H, Van Delden V, Furthmayr H and Kuhn K: A network model for the organization of type IV collagen molecules in basement membrane. Eur J Biochem (1981) **12**, 203-211.
14. Yurchenco PD, Tsilibary EC, Charonis AS and Furthmayr H: Models for the self-assembly of basement membrane. J Histochem Cytochem (1986) **34**, 93-102.
15. Peter T Jr: Serum albumin; in The Plasma Protein, Vol 1, Putnum ed., Academic Press, New York (1975) pp 133-181.
16. Oncley JL, Scatchard G and Brown A: Physical-chemical characteristics of certain of the proteins of normal human plasma. J Phys Colloid Chem (1947) **51**, 184-198.

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