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Ultrastructure of glomerular basement membrane by quick-freeze and deep-etch methods

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Ultrastructure of glomerular basement membrane by quick-freeze and deep-etch methods. The glomerular basement membrane of rat kidneys were three-dimensionally observed by quick-freeze and deep-etch replica methods at high resolution. The middle layer (lamina densa) was composed of 6 to 10 nm fibrils which formed a meshwork structure. The space between the fibrils had polygonal shape. The average long dimension of the space between fibrils was 17 nm and the short one was 13 nm. At the outer layer (lamina rara externa), fibrils connected podocytes perpendicularly with the meshwork of the middle layer. At the inner layer (lamina rara interna), similar perpendicular fibrils also connected endothelial cells with the meshwork of the middle layer. This is the first report to visualize the three-dimensional meshwork structure of the middle layer (the lamina densa) in situ. The function of anchoring podocytes to the lamina densa was suggested in the perpendicularly arranged fibrils of the outer layer. The quick-freeze and deep-etch method is useful in analyzing filamentous ultrastructure in glomeruli, and will be applied to clarifying pathological ultrastructure in kidney diseases.

Basement membranes are thin extracellular layers which underlie epithelia and surround muscle and fat cells as well as nervous system [1–3]. The scaffolding of basement membranes is formed by a self-assembly of type IV collagen, heparan sulfate proteoglycan and laminin [4–7]. Basement membranes act as providing physical support to structures and cell attachment. Besides, glomerular capillary wall restricts the transmural passage of large plasma proteins while offering little resistance to the filtration of water and small solutes, by a charge as well as a size barrier [8–11]. The conventional ultrathin section in the field of electron microscopy shows that the glomerular basement membrane (GBM) is composed of three layers: an electron-dense lamina densa with less dense zones on either side, the lamina rara externa and the lamina rara interna. Fine fibrils of variable widths have been described in all layers of the GBM [12, 13]. However, three-dimensional connection of these fibrils in the GBM was not well defined by thin sections. It is known that deep-etching method is high versatile of visualizing cells [14–17] and extracellular matrices [18]. Previously, the ultrastructural difference between GBM and mesangial matrix has been studied by the quick-freeze and deep-etch method [19]. The purpose of this study is to clarify the three-dimensional ultrastructure of the GBM in situ by the same methods at high resolution.

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Methods

Male Fischer rats weighing between 200 and 250 g. (Charles River Co., Wilmington, Massachusetts, USA) were perfused with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for five minutes through the aorta [19]. The renal cortices were cut into small pieces (2 × 2 × 4 mm) with razor blades and washed in the phosphate buffer for 20 to 30 minutes in order to remove soluble proteins in the glomeruli. These tissues were postfixed with 0.25% glutaraldehyde in the phosphate buffer for 30 minutes. Then they were immersed in 10% methanol and quickly frozen in an isopentane-propane mixture (about −190°C) cooled in liquid nitrogen [15, 20].

The frozen tissue surfaces were carefully fractured in liquid nitrogen by a scalpel and transferred into an Eiko FD-3S freeze-fracture machine [14, 19]. They were deeply etched at the temperature of −95°C under the vacuum condition of 2 to 6 × 10^{-7} Torr for 15 to 20 minutes, and rotary shadowed with platinum to a total thickness of 2 nm at an angle of 35°. They were additionally coated with carbon at an angle of 90°. After being taken from the machine, the replica specimens were coated with 2% collodion in amylacetate and the kidney tissues were dissolved in household bleach (sodium hypochlorite). The replica membranes were put on grids, immersed in amylacetate solution to dissolve the collodion films and observed in a Hitachi H-700 transmission electron microscope.

All figures were printed from inverted negative films so that platinum deposits appear white. The long and short dimensions of pores and the diameters of fibrils were measured on electron micrographs. A long diameter was measured first, followed by measuring a short dimension perpendicularly to it. The thickness of the platinum deposits was included in the calculations of fibril diameters. These data were described as mean ± standard deviation in the results. Some stereo-pairs were taken at tilting angles of ± 5°.

Results

The replica electron micrograph three-dimensionally showed a three layered structure of the glomerular capillary wall with the width of 250 to 280 nm (Figs. 1, 2). However, it is not clear at present whether these layers are completely identical with those observed in conventional, epoxy-resin embedded, ultrathin sections, that is, lamina rarae externa and interna, and

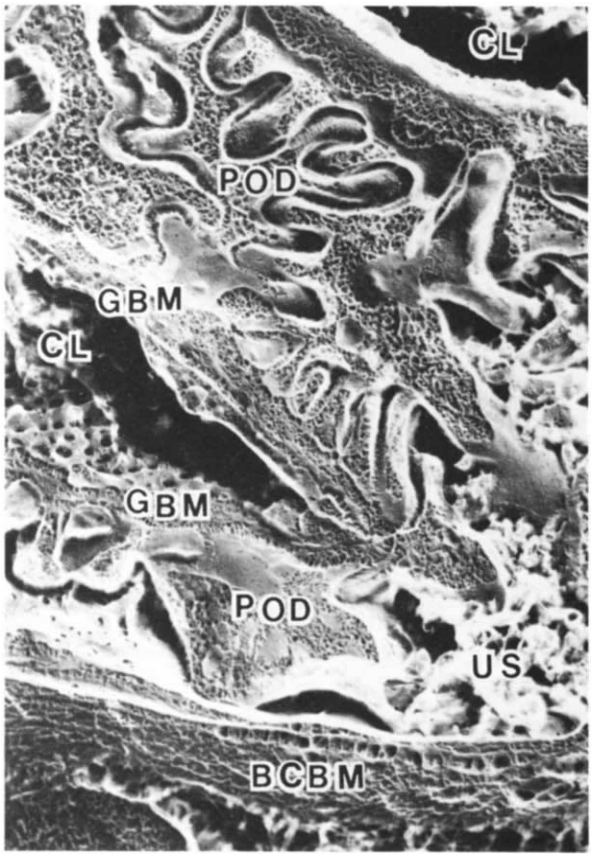


Fig. 1. *Replica electron micrograph of the glomerulus.* Cytoskeleton of podocytes (POD) and the glomerular basement membrane (GBM) are demonstrated. BCBM; basement membrane of Bowman's capsule, CL; capillary lumen, US; urinary space. ×19,200

lamina densa. To avoid the confusion with the terminology used with ultrathin sections, we describe the layers observed with the deep-etching replica method as outer, middle and inner layers.

The outer layer was 30 to 40 nm wide and consisted of fibrils, which connected podocyte plasma membranes perpendicularly with the meshwork of the middle layer (Fig. 2). But these perpendicular fibrils were not uniformly distributed by stereoscopic observation (Fig. 4). Podocyte plasma membranes were sometimes fractured in the middle of their structures, enabling the observation of E-faces (Fig. 3) or P-faces (Fig. 4). On the outer true surfaces, abundant granules of glycocalyces were seen, in contrast to rather smooth P-faces and E-faces. In the podocytes, cytoskeletal meshwork interconnected organelles with inner true surfaces.

The middle layer was 160 to 200 nm wide and had a polygonal meshwork structure which was composed of fibrils, 6 to 10 nm wide (Fig. 2). On the capillary loop, the middle layer was connected with the outer and inner layers by perpendicularly arranged fibrils. The spaces between fibrils of the meshwork had the long diameter of 16.8 ± 8.7 nm and the short one of 12.0 ± 6.2 nm in 70 counting points (Fig. 3). Stereo-pairs demonstrated that the meshwork had a three-dimensional organization (Fig. 4).

The inner layer was 30 to 40 nm wide and perpendicular fibrils (9.0 ± 2.2 nm in diameter) connected true outer surfaces of endothelial cells with the middle layer in a way similar to those of the outer layer. When the capillary wall was fractured parallel to the plasma membranes of endothelial cells, their fenestrae were three-dimensionally observed at the P-faces (Fig. 5). The mean size of these fenestrae was 55.9 ± 14.5 nm (N = 20).

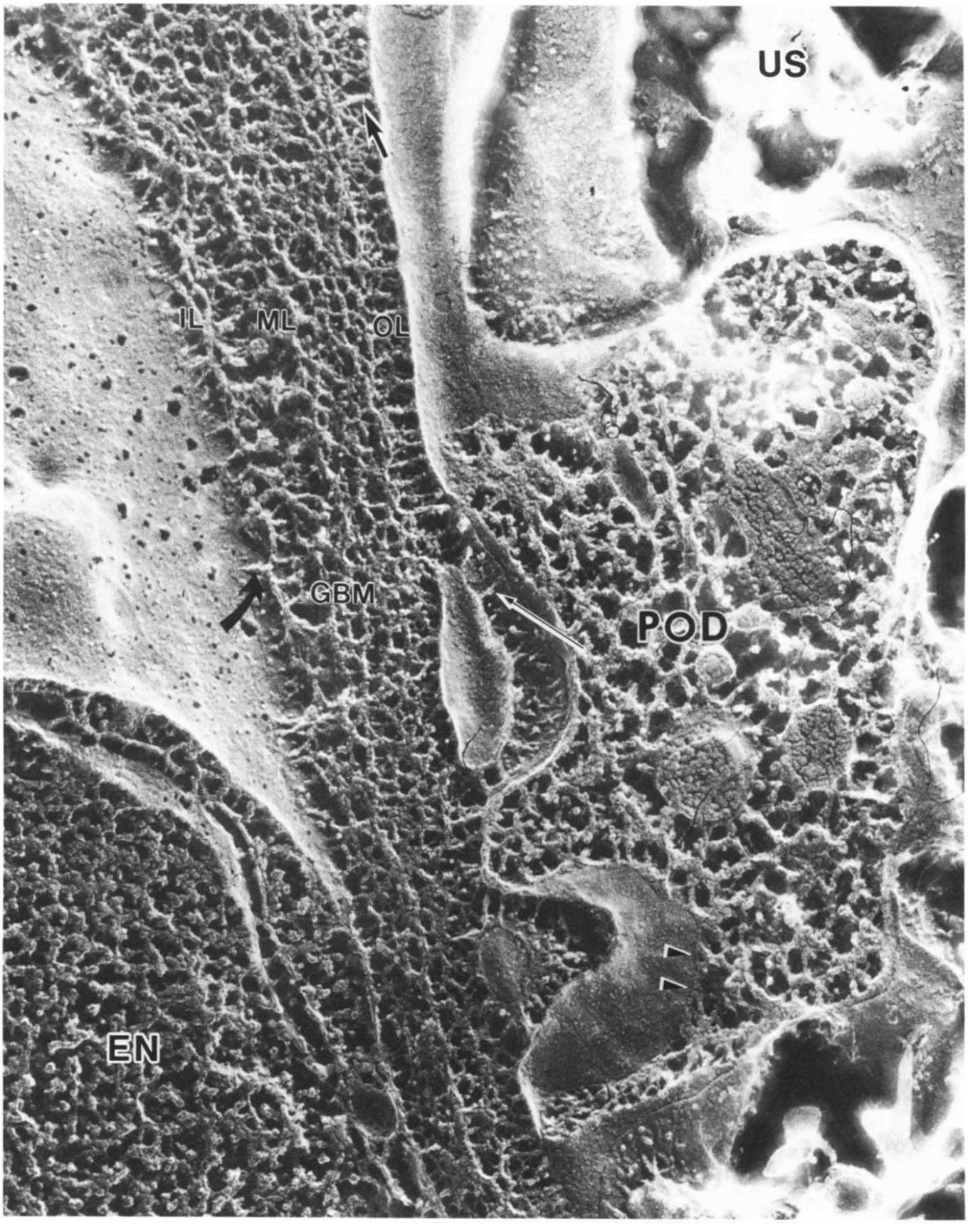
Discussion

Quick-freeze, deep-etch and rotary-shadow electron microscopy has proven valuable in providing high resolution views of cells [14–17] and extracellular matrices [18–20]. Using this method, we have shown the three-dimensional fibrillar and meshwork structure of the glomerular basement membrane in situ and found that it is composed of three layers. They seem to fit with the ultrastructure that is observed in the conventionally sectioned glomerular basement membrane, that is, laminae rarae externa and interna, and lamina densa, from the distribution pattern and the proportion of these three layers.

Immunohistochemically, the GBM has been assumed to contain type IV collagen, laminin, entactin, heparan sulfate proteoglycan and fibronectin [21]. These components were demonstrated to undergo spontaneous aggregation to form orderly networks [4–7], which may provide the framework of the middle layer. Leblond and Inoue showed the type IV collagen network of Reichert's membrane by plasmin digestion [3]. The average length of the segments was 12.1 nm. This length is close to the meshwork size of the GBM, 16.8 × 12.0 nm.

The GBM has been clearly identified as a size restrictive structure, and structural determinants of permeability for macromolecules have been analyzed in some detail [22, 23]. Initially, it was assumed that the GBM acted as a molecular sieve, filtering molecules on the basis of their size and shape. Pappenheimer first applied hydrodynamic models of solute transport through GBM [24]. This model envisions transport of solutes as taking place through large numbers of identical cylindrical pores. Macromolecular solutes are postulated to behave as solid spheres moving in a fluid continuum. Using this approximation, the normal glomerular capillary wall acts as if it had a membrane with uniform cylindrical pores with radii of approximately 5 nm [25]. More recent data [4, 7] and our present datum do not support the cylindrical pore model and instead indicate that the BM has a polygonal mesh-like structure. The

Fig. 2. *Replica electron micrograph of crossly fractured glomerular capillary wall.* The middle layer (ML) of the GBM is composed of fibrils, which form a polygonal meshwork structure. At the outer layer (OL), fibrils (6 to 8 nm in diameter) connect the meshwork structure of the middle layer with the true surface of podocytes perpendicularly (large arrow). At the inner layer (IL), fibrils also connect the middle layer with the true surface of the endothelial cells (curved arrows). The arrowheads indicate a fractured line between inner true surfaces and E-faces. Fibrils interconnect the adjoining foot processes (long arrow). In the podocytes intermediate filaments and microfilaments form cytoskeletal network. EN; endothelial cell, POD; podocyte, US; urinary space. ×87,500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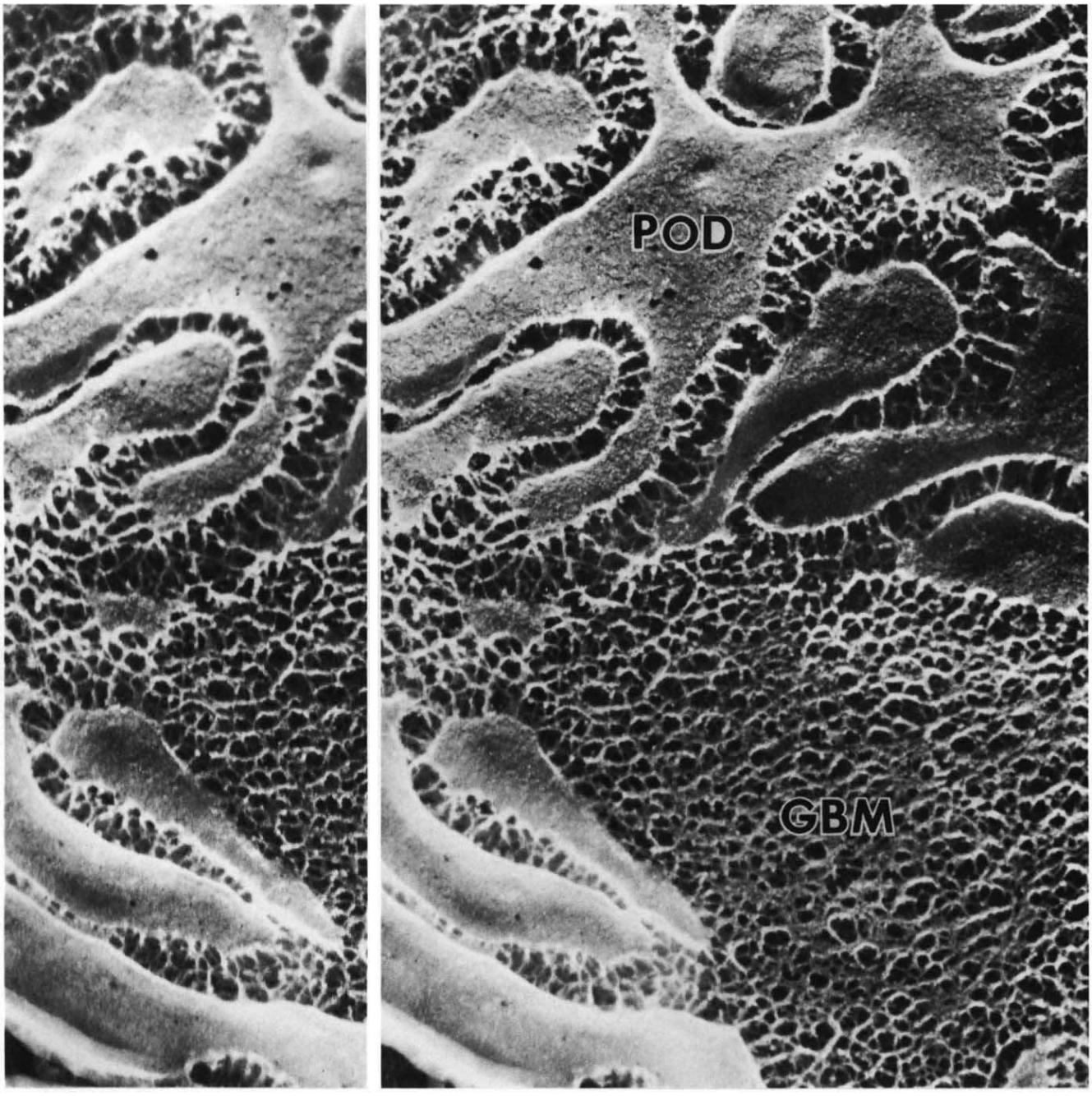

Fig. 3. *Replica electron micrograph of the glomerulus obliquely fractured to the basement membrane.* The middle layer of the GBM has meshwork structure. POD; podocyte. ×77,100 No overlapping views are included in this stereo-pair in order to enlarge the field of view.

space between mesh-like fibrils (16.8 × 12.0 nm) does not appear to produce a sufficiently tight sieve to exclude albumin. We don't have enough data to explain how the mesh-like structure of the GBM acts as molecular sieve. Charge-sieving provided by heparan sulfate acts as another molecular barrier [26]. Perfusion pressure and hemorheology [27] play some roles in the filtration of the GBM. However, it is not known if the meshwork structure of the GBM alters by high perfusion pressure or increased blood viscosity. Anyway the meshwork structure demonstrated in this study is not seemingly a rigid framework, but rather a flexible structure imparted by superhelix formation of type IV collagen [7].

The laminae rarae externa and interna are seen as electron lucent layers by conventional ultrathin sections. It has been known that anionic sites, composed of heparan sulfate proteoglycan, were distributed throughout the laminae rarae externa and interna, by using cationized ferritins, ruthenium red [26] and the high iron diamine method [28]. Caulfield showed that 9

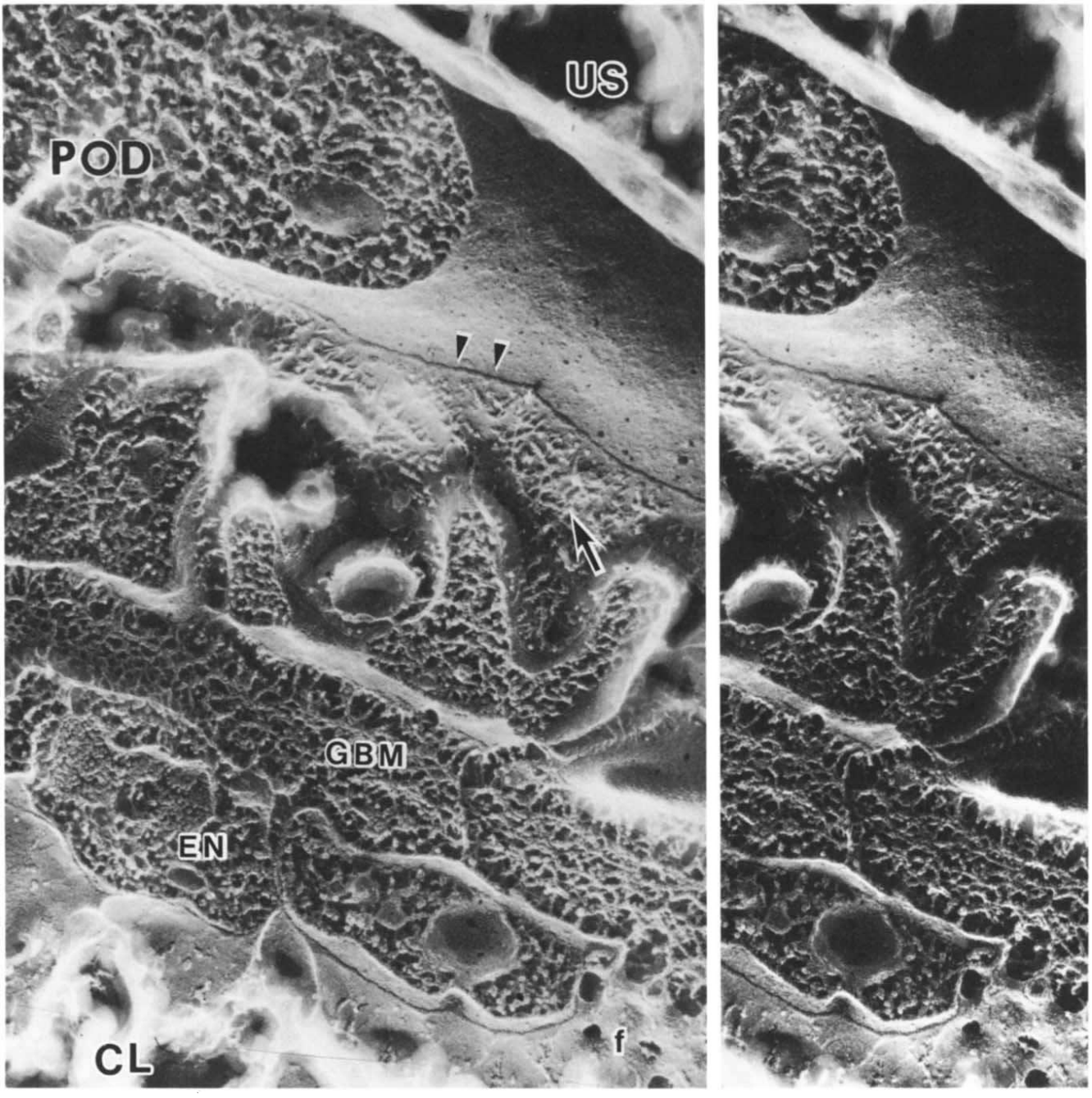

Fig. 4. *Stereo-pictures of a crossly fractured peripheral region of a glomerular capillary wall.* The arrowheads show a fracture line between the outer true surface and the P-face of a unit membrane. Abundant glycocalyces of the podocytes (POD) are seen (arrow). US; urinary space, EN; endothelial cell, f; endothelial fenestra, CL; capillary lumen. ×69,000

nm fibrils ran perpendicularly to the plasma membrane of podocytes by Alcian Blue staining. These perpendicular fibrils were also found at the lamina rara interna [29]. In the present study, the fibrils with mean diameters of 8.1 nm connected the outer true surface of the podocytes with the meshwork of the lamina densa. These 'perpendicular fibrils' could be made of protein backbones of glycosaminoglycan [30]. Functionally, these fibrils seem to anchor podocytes, because the podocytes have been known to receive the glomerular transcapillary hydraulic pressure difference, approximately 35 mm Hg [31]. Pathologically, we have reported partial interruption of 'perpendicular fibrils' using serum sickness nephritis [32].

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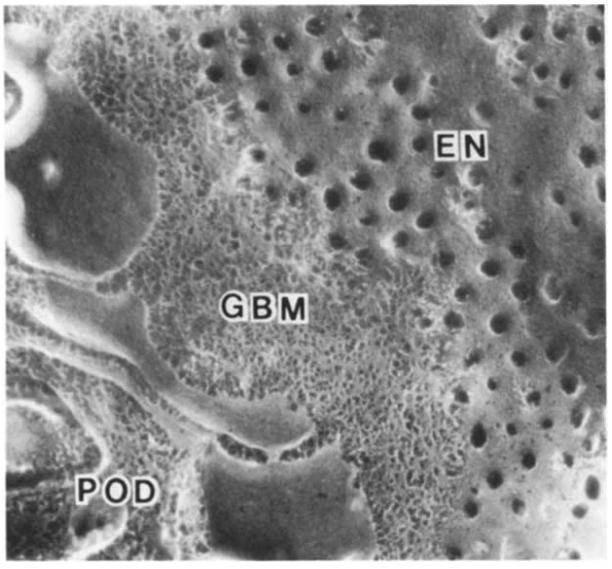

Fig. 5. *Replica electron micrograph of obliquely fractured GBM.* Fenestrae of the endothelial cells are clearly seen. POD; podocyte, EN; endothelial cell. ×25,800

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