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Abstract

Isolated rat glomerular basement membrane was treated with elastase and observed by transmission electron microscopy. The treatment with elastase revealed the fundamental structure of the glomerular basement membrane quite clearly, and enabled the observation of a sieve structure within the glomerular basement membrane. This sieve structure may play a major role in the filtration of blood as well as in the production of urine. Treatment with antibody showed that the sieve was mainly constituted of type IV collagen.

KEYWORDS: glomerular basement membrane, enzymatic digestion, meshwork structure, type IV collagen, elastase

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Molecular Sieve of the Rat Glomerular Basement Membrane: A Transmission Electron Microscopic Study of Enzyme-Treated Specimens

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Isolated rat glomerular basement membrane was treated with elastase and observed by transmission electron microscopy. The treatment with elastase revealed the fundamental structure of the glomerular basement membrane quite clearly, and enabled the observation of a sieve structure within the glomerular basement membrane. This sieve structure may play a major role in the filtration of blood as well as in the production of urine. Treatment with antibody showed that the sieve was mainly constituted of type IV collagen.

Key words : **glomerular basement membrane, enzymatic digestion, meshwork structure, type IV collagen, elastase**

The detailed structure of the glomerular basement membrane (GBM) was unclear until several years ago. In 1951, Pappenheimer presumed the existence of small pores of 7.0-8.2 nm in diameter within the GBM from his physiological data (1, 2). Other researchers have also speculated the existence of small pores of 6-10 nm in diameter in the GBM using tracers such as ferritin and dextran (3, 4). However, the pores in the GBM and their fine structure had not been observed directly by electron microscopy until recently (5, 6). In 1977, Ota *et al.* observed human, bovine, and rat GBM by transmission electron microscopy (TEM), using negative staining, and demonstrated for the first time that the GBM was composed of a porous meshwork made of fibrils (7-9). Negative staining is advantageous in obtaining a higher resolving power and a clearer image at a high magnification, and it enables one to observe unfixed specimens, thus avoiding changes to the tissues during fixation and embedding (10). On the other hand, negative staining requires the preparation of a purified specimen. Alternative techniques to reveal the meshwork structure of the GBM are required. Ordinary transmission electron microscopy of ultrathin sections of fixed specimens from untreated GBM have demonstrated three layers (lamina rara externa, lamina densa and lamina rara interna). Each layer appears amorphous or granular, and not fibrillar. Some investigators have previously attempted to observe the GBM after chemical treatments, including enzymatic digestion, but they could not get any conclusion on the reality of the molecular sieve because their treatment might have been insufficient (12).

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In this experiment, we observed isolated elastase-digested and negatively stained rat GBM by conventional TEM. Elastase causes hydrolysis of peptide, amide and ester bonds of neutral and non-aromatic amino acids (13), and removes some substances other than collagen. Thus, the elastase digestion revealed a skeletal structure of collagen, which was suited to our purpose. The present study based on this enzymatic digestion method extended our previous works (14-16) and showed clearly that a meshwork made of fibrils is the fundamental structure of the GBM. Immunohistochemical methods further showed that these fibrils were mainly composed of type IV collagen.

Materials and Methods

Isolation and treatment of GBM. GBM was isolated from male Wistar rat kidneys by Sipro's method (17). Cortices of kidneys were cut into small pieces with scissors. They were carefully pressed and mashed with the bottom of a beaker on a No. 80 metal mesh (Ikemoto Co., Ltd., Tokyo, Japan) and washed through with an ice-cold 0.05 M phosphate buffer solution (PBS). They were further filtrated through No.100, 120, 150 metal meshes, and finally collected on a No.170 mesh. By observation using a phase contrast microscope, the collected samples were confirmed to be pure glomeruli without contamination by cell debris, broken tubules or Bowmann's capsules. The glomeruli thus isolated were suspended in a solution of 1 M NaCl, and sonicated (Heat Systems—Ultrasonics, Inc., Farmingdale, NY, USA) intermittently for 5 min at 0℃. The sediment was resuspended in 1 M NaCl solution and centrifuged at 3000 rpm for 30 min to wash out cell debris. This procedure was repeated 3 times. In addition, the sediment was washed with distilled water three times in the same manner. Specimens obtained in this way were observed under a phase contrast microscope, and they were confirmed to be almost pure GBM. A part of the specimen was used for the control study, and the rest was processed for enzymatic treatment. Five ml of 0.05 M PBS containing 0.01% elastase (Sigma chemical Co., St. Louis, MO, USA; extracted from porcine pancreas) were added to 0.1 ml of GBM and stirred for 5 h at 37℃. After repeating the washing and centrifuging cycle 3 times, the digested GBM was suspended in cold 0.05 M PBS and submitted to electron microscopy immediately.

Observation by ultrathin section method. Non-treated GBM, as a control, and digested GBM were fixed with 2% glutaraldehyde at 5℃ for 1 h, postfixed with 1% osmium tetroxide for another hour, dehydrated in a graded ethanol series, and embedded in Epon 812. Ultrathin sections were stained with 2% uranyl citrate and 2% lead citrate and were observed by TEM (Hitachi H-700) at 100 kV.

Observation by negative staining. No.400 meshes were covered with a collodion membrane and thinly coated with carbon. Drops of the suspensions of the control GBM and the digested GBM were applied to these meshes for 2 min and were blotted with filter paper. Another drop of 2% phosphotungstic acid (PTA), pH 7.2, was applied for 3 min as a staining solution. PTA was blotted with filter paper, and the specimens were immediately observed by TEM.

Measurement of the diameter of pores and the width of fibrils. Five electron micrographs obtained from five different specimens, which were taken at the original magnification of 1×10^5, were ultimately enlarged 2×10^5. Long and short dimensions, and the width of 20 pores per electron micrograph, 100 pores in all, were measured with a scaled loupe.

Identification of the fibrils by enzyme-labeled antibody method (18). Firstly, 50 μl of 1% rabbit anti-type IV collagen antibody was added to 0.1 ml of digested GBM suspended in PBS, and incubated at 25℃ for 60 min. The suspension was washed 3 times with 0.05 M PBS at 5℃ and was incubated with the second antibody, 5 μl of 1% peroxidase conjugated sheep anti-rabbit IgG antibody (Zymed Laboratories, Inc., South San Francisco, CA, USA), at room temperature for 120 min. The suspension was washed with PBS three times, and reacted with 10ml of 0.03% 3, 3'-diaminobenzidine (DAB)-H_2O_2 solution (0.1 ml 5% H_2O_2+100 ml Tris buffer containing 0.03% DAB, pH 7.6) for 5 min at 20℃. After washing with Tris buffer, the suspension was fixed with 2% glutar-

aldehyde, postfixed with 1% osmium tetroxide and then processed for regular ultrathin sectioning as described above. As a control the digested GBM was reacted with normal rabbit serum instead of anti-type IV collagen rabbit antibody. Neither of these specimens was subjected to the electron staining.

Results

Observation of sectioned samples. Ribbon-like GBM was seen in the control specimen (Fig. 1). The GBM appeared as a somewhat granular and amorphous single layer. No fibrils were seen on the surface of the GBM, though it was rough to some extent. After digestion with elastase numerous fibrils were observed inside and outside the GBM (Fig. 2). Fibrils inside the GBM were equal in width and formed a uniform meshwork which had a spongy appearance (Fig. 3). Fibrils had the same density whether in the center or periphery of the GBM. This observation indicated that the GBM is composed of a single layer. Fibrils outside of the GBM (arrowheads in Fig. 2) were crisp, string-shaped, about 200-300 nm in length and 3 nm in width, and linked to one another, and were not observed in undigested specimens. Some nodule-like structures 10 nm in diameter were observed on the fibrils (arrows). Most of the fibrils outside were linked with those on the surface of the GBM, which seemed to be drifting away from the surface of the GBM, with the lattice of the meshwork being disentangled (Fig. 3). Fibrils just leaving the GBM were folded many times finely, keeping the morphological characteristics inside of the GBM.

Observation of negatively-stained samples. Under TEM using negative staining, control undigested GBM looked like small sheets of folded paper with linear contours and angular ends as was reported by Ota *et al.*(8). At low magnification, the surface of the GBM was felt-like or spongy in appearance. At higher magnification, it was composed of a meshwork structure made up of fibrils (Fig. 4). Digested GBM appeared almost the same as control GBM. Thick fragments of GBM looked like pieces of paper and had small change on the surface. However, when thin fragments of GBM were observed, the fibrils forming the meshwork structure appeared more clearly than those of control GBM (Fig. 5). This fundamental structure appeared throughout all parts of the GBM. Fibrils were folded many times and formed small polygonal pores (arrows). Around the periphery of the GBM, the lattice of the meshwork was loose, and fibrils with nodules were observed. The fibrils were almost equal in width and the pores formed by the fibrils were almost the same in size.

Measurements of fibrils and pores. The size of fibrils, and the long and short dimensions of pores are shown in histograms (Fig. 6). The width of fibrils determined by the ultrathin section method was 2.9 ± 0.5 nm; the long dimension of pores was 3.5 ± 0.8 nm, and the short dimension of pores was 3.1 ± 0.9 nm. Those determined by the negative staining method were 3.2 ± 0.5, 3.4 ± 0.5 nm, and 2.3 ± 0.7 nm, respectively.

Identification of fibrils by reaction with the enzyme-labeled antibody. Though the control specimen was so faint that it was hard to identify the GBM (Fig. 7), the experimental specimen appeared electron dense with fine granules both inside and outside the GBM at a low magnification (Fig. 8). As electron staining was not performed, the fine structure of the GBM was not observed. However, at a higher magnification, fine fibrils appeared faintly.

Discussion

Whether or not the ultrastructure of the GBM is related to the cause of protein-

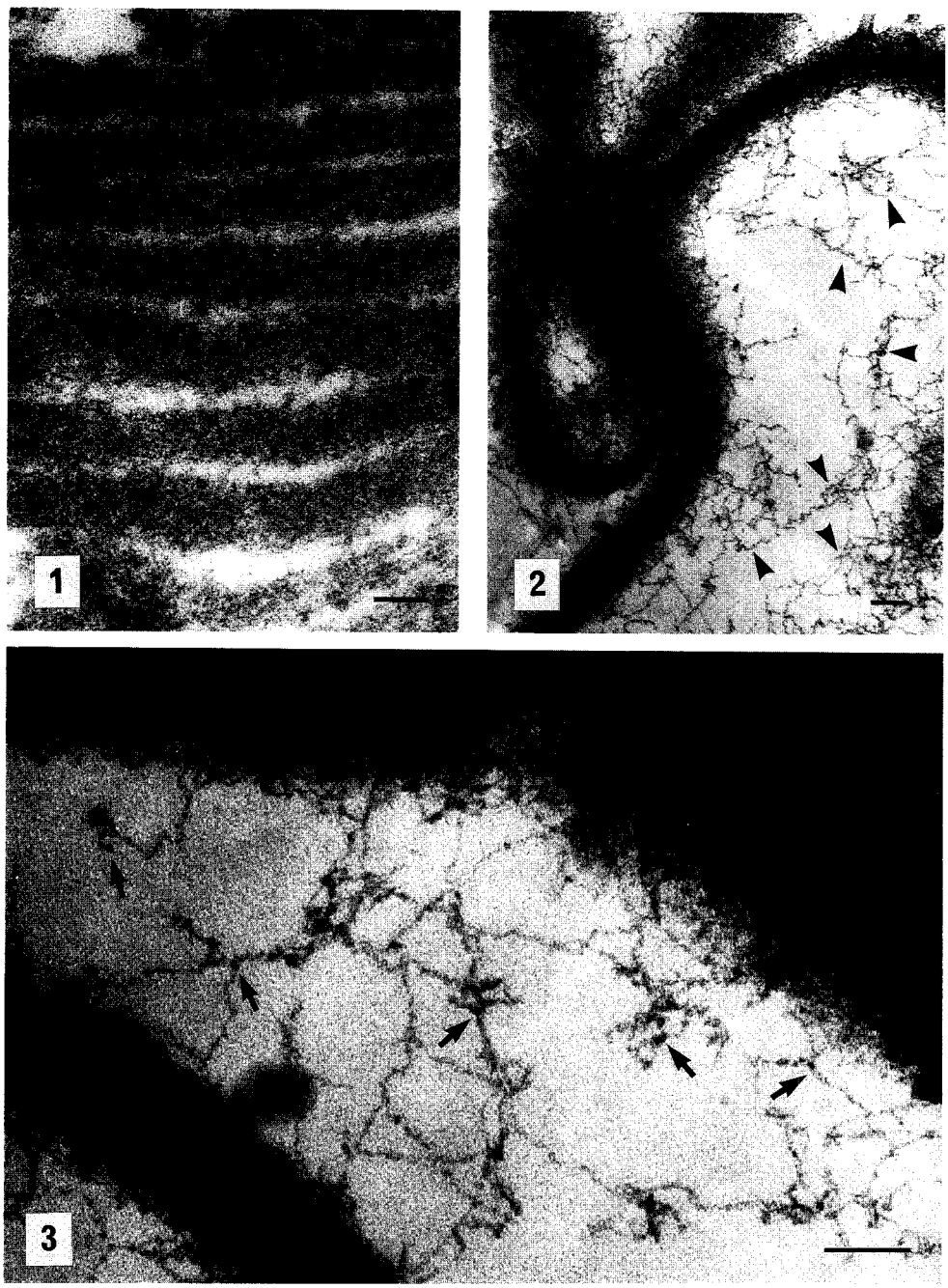

Fig. 1 An ultrathin section of untreated isolated glomerular basement membrane (GBM) composed of a granular and amorphous single layer. No fibrils are seen either inside or outside of the GBM. Bar=100 nm, ×80,000.

Fig. 2 An ultrathin section of glomerular basement membrane (GBM) after elastase treatment. Many fibrils are seen both inside and outside of the GBM. Fibrils derived from the GBM are shown by arrowheads. Bar=100 nm, × 50,000.

Fig. 3 An ultrathin section of glomerular basement membrane (GBM) after elastase treatment. Fibrils drifting away from the surface of the GBM and node-like structures are seen on the fibrils (arrows). Bar=100 nm, ×130,000.

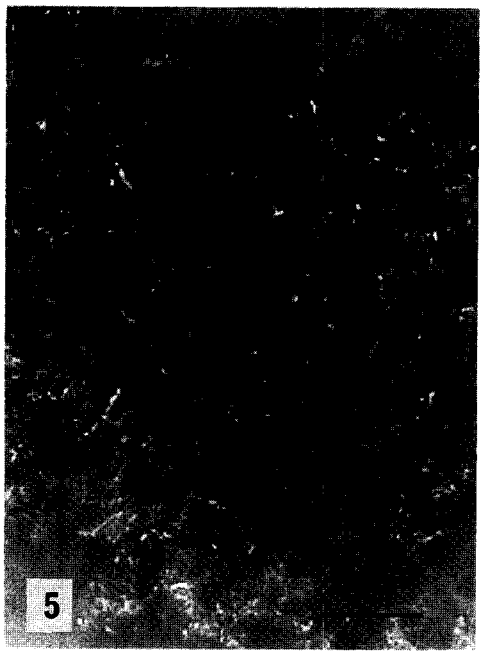

Fig. 4 Negative staining of untreated glomerular basement membrane using 1% phosphotungstic acid. Note the polygonal pores. Bar=100 nm, ×100,000.

Fig. 5 Negative staining of digested glomerular basement membrane using 1% phosphotungstic acid. Fibrils form a meshwork structure. Polygonal pores are observed (arrows). Bar=100 nm, ×150,000.

uria has been a matter of controversy for a long time (19). The kidneys filtrate blood and yield urine, and the general hypothesis has been that filtration is done mainly at the GBM according to molecular size (5). That is, molecules larger than albumin can not pass the GBM, though smaller ones can. Therefore, the GBM theoretically should have pores slightly smaller than albumin molecules. However, the pores had not been electron micrographically demonstrated until several years ago, when we observed purified GBM by TEM after negative staining, and found that the GBM is composed of a three-dimensional meshwork composed of fibrils which form numerous small pores 3-4 nm in diameter (7). Based upon these findings, we proposed a molecular sieve theory (8). The values obtained in the present study are consistent with our previous one. It is well known that the albumin molecule is a prolate ellipsoid with axes of approximately 15.0 by 3.8 nm in man, 12.9 by 3.9 nm in the cow, and probably within the same range in the rat (20). Therefore, this meshwork can be thought to play the role of a molecular sieve. Furthermore, using the same method, we reported that the proteinuria which accompanies experimental glomerulonephritis or diabetic nephritis might be caused by the enlargement of these pores (21-23).

It was difficult to demonstrate the fibrillar structure and small pores of the GBM by traditional TEM of sectioned samples. By the deep-etch method, Kubosawa *et al.* demonstrated fibrils interconnected to form a three-dimensional network (11). To the best of our knowledge, we are the first to demonstrate the GBM to be composed of a

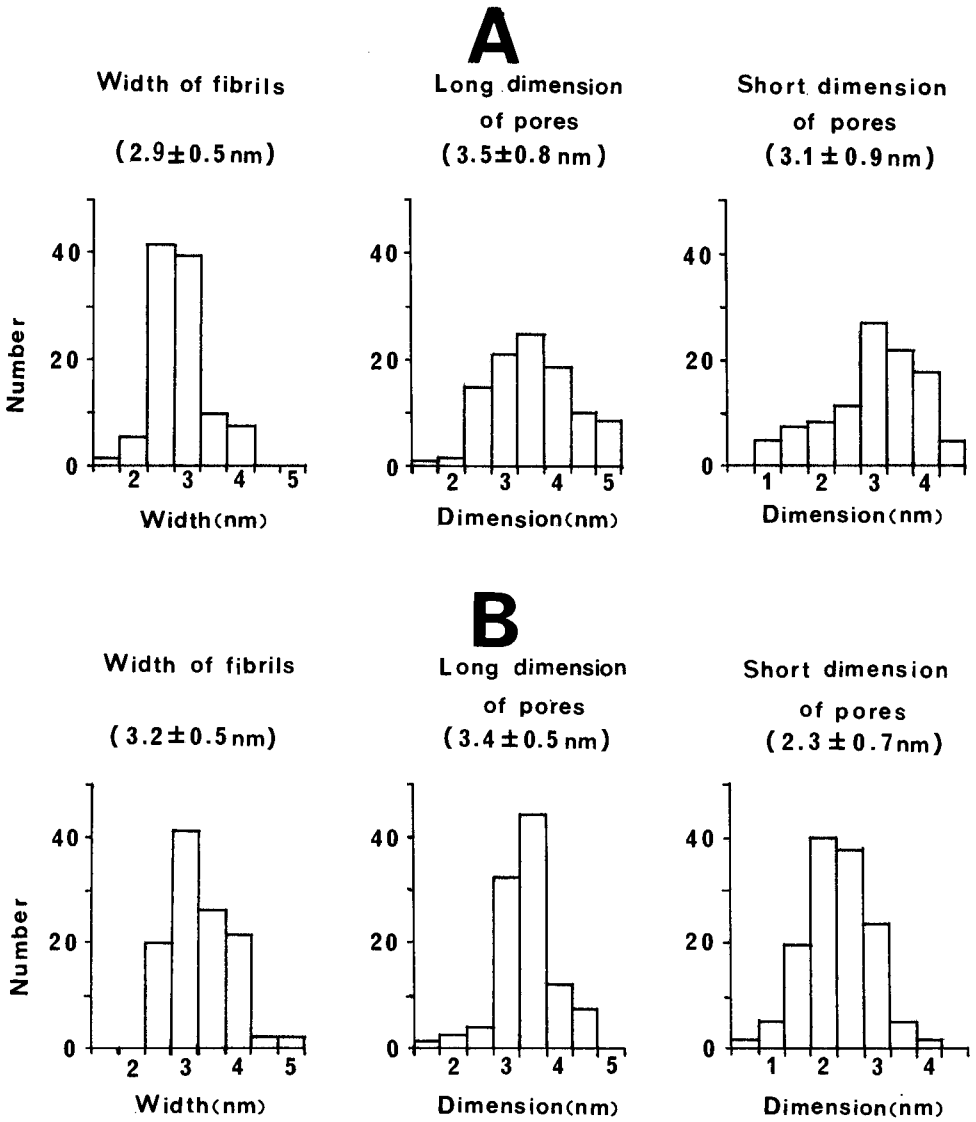

Fig. 6 Width of fibrils, and long and short dimensions of pores as measured by the ultrathin section method (A), and negative staining (B). Each mean ± SD is indicated in the parentheses.

meshwork of fibrils by an ultrathin sectioning method (15, 16). In contrast to the theory that the sieving of macromolecules is done by the pores of the GBM, the existence of an electric charge barrier of the glomerular capillary wall has attracted attention (24). Serum protein is negatively charged, and the surface of the glomerular epithelium and endothelium, and most of the GBM are negatively charged, because they contain sialic protein which is an electrically negative substance. Also, both the laminae rara externa and interna of the GBM contain heparan sulfate proteoglycan which is negatively charged (25, 26). However, it might be difficult to explain the mechanism of fil-

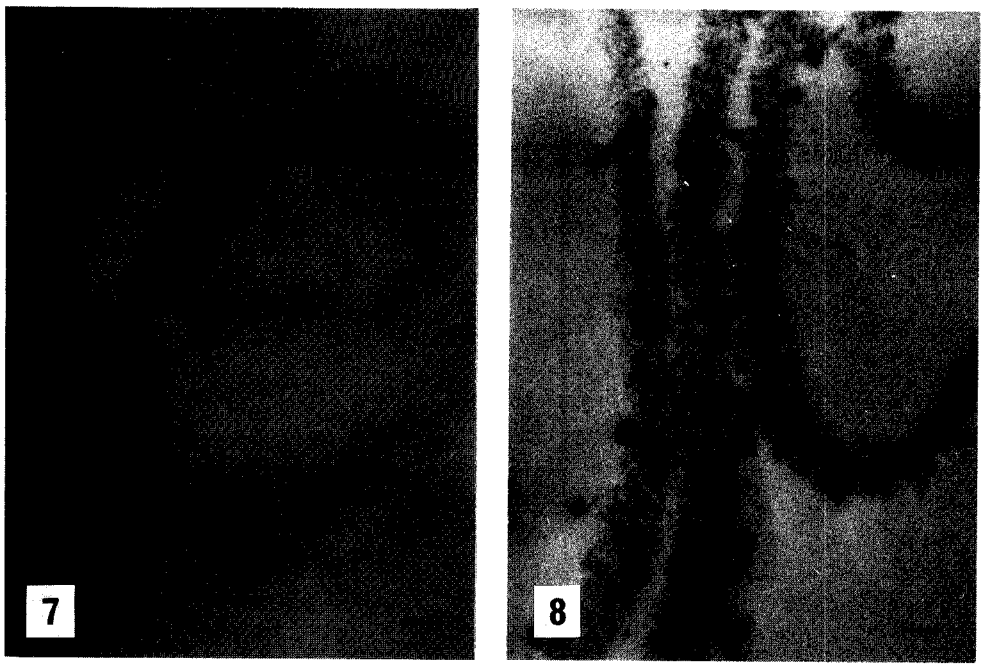

Fig. 7 Immunoelectron micrograph of digested glomerular basement membrane (GBM) incubated with normal rabbit serum and peroxidase conjugated anti-rabbit IgG antibody. GBM was not stained. Bar=100 nm, ×60,000.
Fig. 8 Immunoelectron micrograph of digested glomerular basement membrane (GBM) incubated with rabbit anti-type IV collagen antibody and peroxidase conjugated anti-rabbit IgG antibody. GBM was strongly stained. Bar=100 nm, × 60,000.

tration by the glomerulus only by the effect of a charge barrier. The electrical repellency may be effective only in the restricted space which the size barrier determines.

By reacting the first and second antibody with digested GBM in the test tube, and further reacting with 3,3′-diaminobenzidine, we avoided denaturation and loss of immunogenicity and volume, as GBM was not affected by special fixation, freezing or drying. As the GBM itself is only 200 nm thick, and it became thinner after digestive treatment, the permeability of antibody and 3,3′-diaminobenzidine seemed to be excellent. It is said that type IV collagen of the GBM is distributed equally in the lamina densa (27), and this concept is consistent with our electron micrographs obtained by the enzyme-labeled antibody method.

As to why the fibrillar structure can not be found in undigested GBM but can be found in digested GBM by TEM, it was thought that some substance, which was stained by electron staining, surrounded fibrils of type IV collagen. The substance might make the meshwork structure of the fibrils invisible by ultrathin sectioning, although fluid or small substances can pass through the space among the substance. This is the reason why the staining solution could penetrate the GBM easily by negative staining, and reveal the meshwork successfully. This substance might have been removed by the effect of elastase in our experiment.

Type IV collagen, which is one of the fundamental components of the GBM, appears to have a triple helical structure 2-3 nm in width and 400 nm in length. It looks like a curly string under the electron microscope. It bears a globular NC_1 domain

11.2-12.5 nm in diameter at one end, and a rod-like 7 s domain 30 nm in length at the other end. Many collagen molecules form a large network by linking one another in a characteristic manner, as recently reported by Timpl *et al.* (28, 29). Furthermore, when isolated pure type IV collagen molecules are incubated, they combine with one another, and self-aggregation occurs. Through the observation of self-aggregation, the structure of type IV collagen molecules in the GBM was recently proposed to be a three dimensional meshwork by some researchers (30, 31). They only speculated the fibrillar structure of type IV collagen in GBM from their data *in vitro,* and the conclusion was not obtained yet. Their models of GBM supported our molecular sieve theory (7, 8) of the GBM. In this experiment, the meshwork structure of fibrils was observed by TEM observation of regular ultrathin sections, and the mechanism of the molecular sieving was further demonstrated.

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