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Abstract

In order to explore the pathogenic mechanism of proteinuria in glomerulonephritis, ultrastructural changes of the glomerular basement membrane were investigated in rats with chronic serum sickness induced by repeated intravenous injections of bovine serum albumin (experimental rats). Rats injected with saline served as controls. The animals were sacrificed and examined 13 weeks after treatment, when the mean urinary protein of experimental animals reached 206 mg/24h/100g body weight. Enhanced transcapillary passage of anionic ferritin was observed in experimental rats. Purified glomerular basement membranes of control and experimental rats were examined by electron microscopy after negative staining. The glomerular basement membrane of experimental rats had enlarged pores. The results suggest that an increase in the radius of glomerular pores may be responsible for proteinuria in glomerulonephritis.

KEYWORDS: glomerular basement membrane, proteinuria, glomerular permeability, ultrastructure, glomerulonephritis

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Changes in the Molecular Sieve of the Glomerular Basement Membrane of Rats with Chronic Serum Sickness

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In order to explore the pathogenic mechanism of proteinuria in glomerulonephritis, ultrastructural changes of the glomerular basement membrane were investigated in rats with chronic serum sickness induced by repeated intravenous injections of bovine serum albumin (experimental rats). Rats injected with saline served as controls. The animals were sacrificed and examined 13 weeks after treatment, when the mean urinary protein of experimental animals reached 206 mg/24h/100g body weight. Enhanced transcapillary passage of anionic ferritin was observed in experimental rats. Purified glomerular basement membranes of control and experimental rats were examined by electron microscopy after negative staining. The glomerular basement membrane of experimental rats had enlarged pores. The results suggest that an increase in the radius of glomerular pores may be responsible for proteinuria in glomerulonephritis.

Key words : **glomerular basement membrane, proteinuria, glomerular permeability, ultrastructure, glomerulonephritis**

The glomerular basement membrane (GBM) normally functions as both a size and charge barrier in the filtration of macromolecules (1-4). Fractional clearance studies of experimental nephritis have suggested the impairment of size-selective properties of the filtration unit in association with charge defects (5). The structural equivalence of charge changes has been demonstrated by the loss of anionic sites with the use of various cationic probes (1, 5-8). In contrast to the significance of electric charge, ultrastructural changes in the GBM relating to its function as a size barrier have not been elucidated in detail. Recently we demonstrated that the GBM of normal rats (9) consisted of a molecular sieve with uniform pores, and that the GBM of rats with Masugi nephritis (10) had enlarged pores. We presumed that an increase in the radius of glomerular pores may be responsible for proteinuria. The present study deals with ultrastructural changes of the GBM in an immune complex type of nephritis in rats.

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Materials and Methods

Induction of chronic serum sickness. Chronic serum sickness (CSS) was induced according to the method of Arisz *et al.* (11) in 25 female Fischer rats weighing approximately 100 g. Brief-

ly, each animal was preimmunized subcutaneously with 3 mg of bovine serum albumin (Cohn Fraction V, Sigma Chemical Company, St. Louis, U.S.A.) in complete Freund's adjuvant. Each animal received daily intravenous injections of 2 mg of bovine serum albumin 2 weeks after the third subcutaneous immunization. Similarly, 25 control rats were injected with saline instead of bovine serum albumin. Rats were kept in individual cages with free access to standard chow and water. The protein content of the urine was measured by the Tonein TP method (Otsuka Chemical Co., Tokushima, Japan). Morphological studies were performed on the 13th week after the initial immunization.

Histological procedures. Renal cortex for light microscopic study was fixed in Bouin's fluid and embedded in paraffin. Sections 4-μm thick were stained with periodic acid-Schiff and periodic acid silver methenamine. For immunofluorescence study, renal tissue was snap frozen in n-hexane cooled to −70°C in a dry ice acetone bath. Frozen sections cut at 4-μm were processed for direct immunofluorescence using fluorescein isothiocyanate conjugated (FITC) rabbit antiserum to BSA and FITC-goat antiserum to rat IgG and C3 (Cappel Laboratories, Malvern, U.S.A.).

For electron microscopy, renal cortex was fixed in 2.5% glutaraldehyde in cacodylate buffer and post fixed in osmium tetroxide and processed as described before (12).

Tracer studies. Five control and five experimental animals were used for *in vivo* tracer studies utilizing native anionic ferritin (twice crystallized horse spleen ferritin, Sigma Chemical Co., pI 4.3-4.4). To reduce its cadmium content, ferritin was dialyzed against ethylenediamine tetraacetic acid solution followed by phosphate buffer (13). Immediately before injection, ferritin was passed through a 0.45 μm Millipore filter. Rats were anesthetized with an intraperitoneal injection of sodium pentbarbital (Nembutol, Abbott Laboratories, Chicago, U.S.A.), and the ferritin solution (100 mg/ml) was administered via the tail vein over a 3-5 min period at a dose of 50 mg/100g body weight. Renal cortical biopsies were performed at 5 min, 15 min, 30 min, 1 h, 2 h, and 3 h after the delivery of the tracer. The renal cortex was fixed in 2.5% glutaraldehyde in cacodylate buffer and processed as described above. The sections were stained with alkaline bismuth (14).

Preparation and observation of rat GBM. Rat GBM was isolated essentially by the method of Spiro (15) as described before (9, 10). After each step of isolation of GBM, the material was examined under a phase-contrast microscope. The purity of a part of the final pellet of GBM was confirmed by ultrathin sectioning. The rest of the purified GBM was studied by electron microscopy (Hitachi H700), using negative staining with 1% phosphotungstic acid, pH 7.3, as previously described (9, 10).

Measurement of pores and strands was also done as described before (9, 10). Briefly, long and short (measured perpendicular to the long dimension) dimensions of the pores and width of the strands were measured on micrographs with a magnification of 300,000-1,000,000 which were enlarged 10 times from the negatives.

Results

Urinary protein. By the 9 th week, an increase in urinary protein excretion above normal levels was observed in experimental rats. By the 13 th week, all experimental rats excreted more than 80 mg/24h/100g body weight of protein, and the average reached 206 mg/24h/100g. In contrast all control rats excreted less than 5 mg/24h/100g of protein.

Histological findings. At the 13th week, diffuse proliferation of endocapillary cells, accumulation of leukocytes, thickening of glomerular capillary walls, and segmental fibrinoid necrosis were seen by light microscopy. Fine to coarse granular deposits of rat IgG, C3 and BSA were mostly localized along the glomerular capillary walls, and few deposits were seen in the mesangium by immunofluorescence. Electron-dense deposits of varying size were localized mainly in the subepithelium (Fig. 1), but intramembranous, subendothelial and mesangial deposits were also observed by electron microscopy. Effacement of epithelial cell foot processes,

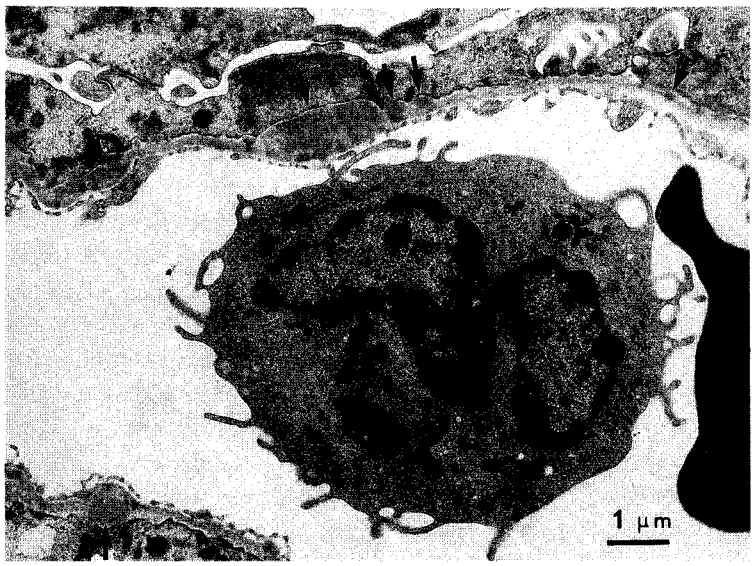

Fig. 1 Ultrathin section of a glomerulus of an experimental rat 13 weeks after the initial immunization. Variously sized electron dense deposits (arrows) are mainly localized subepithelially. A monocyte is attached to an endothelial cell. ×8,300

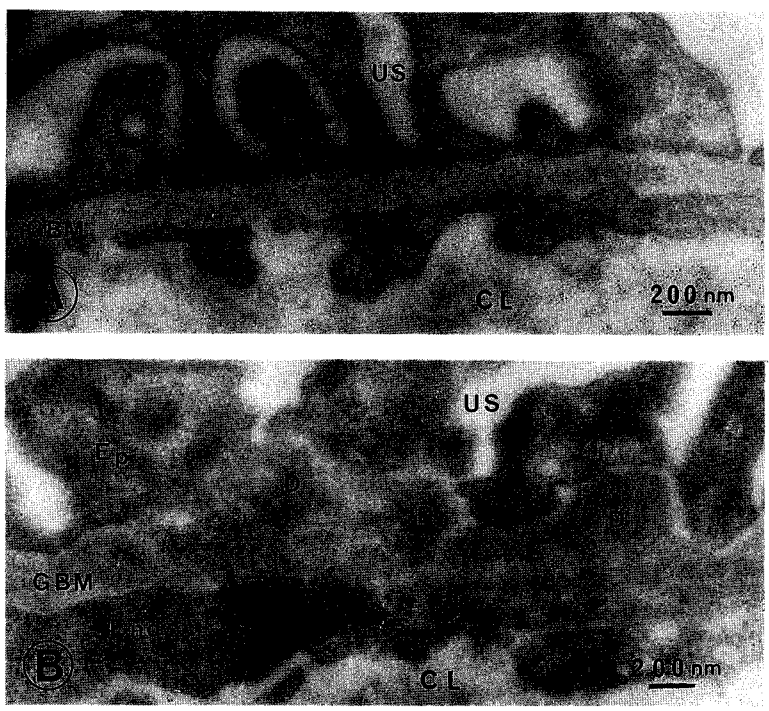

Fig. 2 Ferritin distribution in a segment of a glomerular capillary loop. A: Control rat 30 min after the tracer injection. Ferritin molecules are seen predominantly along the inner aspect of the glomerular basement membrane (GBM). ×41,000. B: Experimental rat 15 minutes after the injection of ferritin. Note the marked thickening of the GBM as a result of deposits (D). ×29,000 End, endothelial cell; Ep, epithelial cell; US, urinary space; CL, capillary lumen.

protein reabsorption droplets and vacuoles in epithelial cells were observed (Fig. 1). Infiltration of monocytes was seen in many of the capillaries (Fig. 1), and a few polymorphonuclear leukocytes were also seen. No significant pathologic change was seen in control rats.

Tracer studies. In control rats, ferritin distribution was investigated at two stages. From 5 to 30 min after the injection of anionic ferritin, most of the capillary luminal space and endothelial fenestrae were filled with ferritin. Ferritin particles mostly accumulated in the lamina rara interna and inner aspect of the lamina densa of the GBM with few particles found beyond this layer (Fig. 2A). An hour after the ferritin injection, the tracer concentration was decreased in the lumen, slightly increased in the lamina rara interna and inner aspect of lamina densa, and unchanged beyond these layers, as compared to the earlier stage. In rats with CSS, ferritin molecules were seen throughout the width of the GBM at all time points studied. Ferritin particles were usually partially or entirely excluded from electron dense deposits (Fig. 2B). Ferritin particles did not accumulate in epithelial slits or below slit diaphragms.

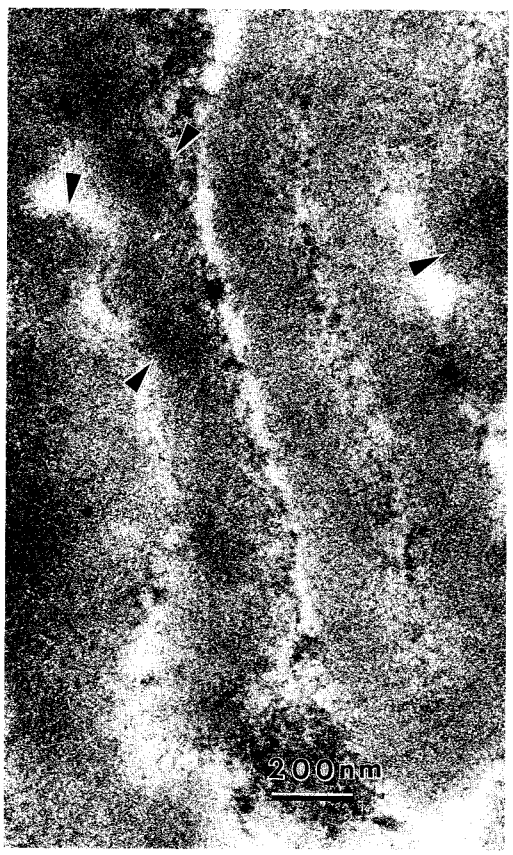

Fig. 3 Ultrathin section of isolated GBM from rats with chronic serum sickness. Part of the basement membrane appears electron dense (arrowheads). ×55,000.

Ultrastructure of GBM. Observation of ultrathin sections of GBM pellets confirmed the presence of basement membranes with little contamination (Fig. 3). A part of the periphery of the basement membrane had a serrated and dense appearance.

With negative staining, the surface of the isolated GBM appeared felt-like or spongy under low magnification both in experimental and control rats. Higher magnification revealed a molecular sieve composed of pores and strands in both groups. In the GBM of control rats, the strands were almost equal in width measuring 3 ± 1 nm. Pores were round or oval, measuring 4 ± 1 nm across the long diameter and 3 ± 1 nm across the short diameter (average of 100 pores) (Fig. 4A). In experimental rats, most of the fragments of GBM showed destruction and enlargement of the molecular sieve (Fig. 4B). Although some of the pores remained similar in size to the control, many of them were enlarged or elongated. The long diameter

Table 1 Pores and strand sizes of glomerular basement membrane of rats with chronic serum sickness (CSS)[a]

	Control	CSS
Long dimension, nm	4 ± 1	9 ± 5*
Short dimension, nm	3 ± 1	6 ± 3*
Width of strand, nm	3 ± 1	4 ± 1

a: Values are the means ±SD of 100 pores or strands. *Statistically significant difference from controls ($p < 0.001$).

was 9 ± 5 nm, the short diameter was 6 ± 3 nm, and the width of the strand was 4 ± 1 nm (average of 100 pores). Long and the short diameters of pores from rats with CSS nephritis differed significantly from those of control rats (Table 1).

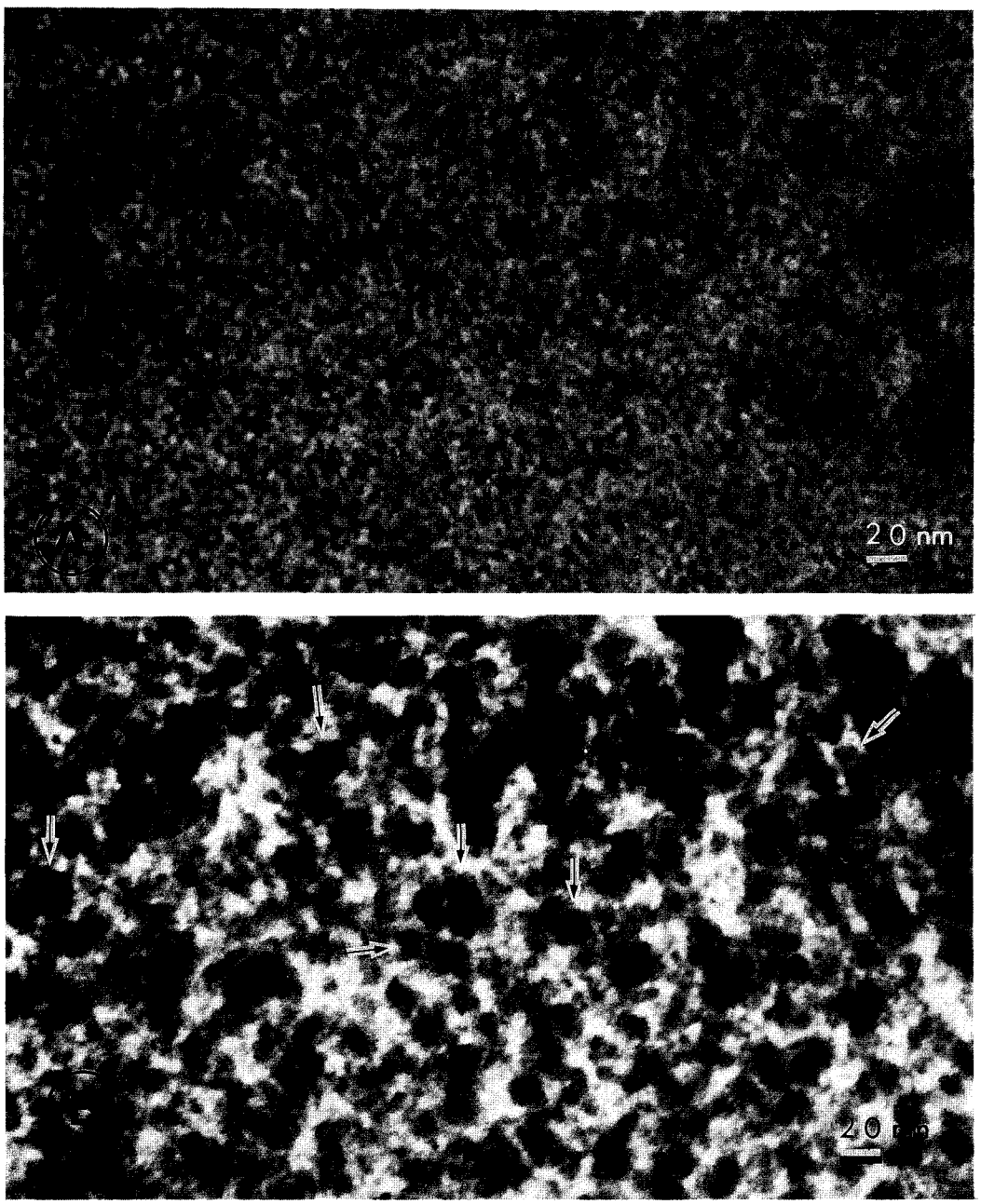

Fig. 4 Negative staining of rat glomerular basement membrane with 1% PTA. A: control rat GBM greatly magnified. ×300,000. B: Experimental rat GBM. Note enlarged pores (arrows). ×300,000.

Discussion

In this investigation, increased glomerular permeability was observed in rats with CSS by tracer studies, and enlargement of the radius of pores of GBM was revealed by negative staining of isolated GBM. These results indicate that the increase of the glomerular porosity is responsible for proteinuria in rats with CSS.

Glomerular permeability to macromolecules is influenced by the molecule-filter interaction with respect to charge, size, shape and hemodynamic factors (2, 4). So far no single definitive defect that can account for all states of proteinuria has been discovered. We have concentrated our efforts on the ultrastructural changes of the GBM which could affect the function of the GBM as a size barrier. Negative staining has an advantage over conventional thin sectioning with respect to resolving power (9, 16), and with this method, we could reveal changes in the molecular sieve of the GBM in rats with CSS.

CSS, which is one of the most common experimental models of the immune complex type of glomerulonephritis, was induced in rats with great uniformity. All rats became nephrotic 4 weeks after the intravenous injection of BSA. They showed similar morphological changes: mesangial increase, epithelial cell injury with massive obliteration of foot processes, microvilli and bleb formation, electron dense deposits mainly in the subepithelium, and monocyte infiltration. All of these alterations were compatible with the findings of previous investigators (11, 17, 18).

Our tracer findings in control and experimental rats were similar to those reported previously (7, 8, 13), and clearly demonstrate increased permeability in CSS. Schneeberger *et al.* (8) found a significant number of anionic ferritin particles beyond the lamina densa in rats with autologous immune complex nephritis which excreted more than 10 mg/24h urinary protein. Kelly and Cavallo (7) demonstrated large amounts of ferritin particles among immune complex deposits in the basement membrane of proteinuric NZB mice. In our experimental rats, which excreted massive amounts of protein, the distribution of ferritin particles was very similar to that described by Kelly and Cavallo. These findings suggest a generalized increase in glomerular permeability rather than a focal and nonuniform defect (19), and are consistent with the ultrastructural findings obtained by negative staining.

The pathogenesis of the increased glomerular permeability in CSS is thought to be due to the action of inflammatory mediators from monocytes (17, 18). Monocytes contribute not only to the elimination of immune complexes by endocytosis, but also to the increase in vascular permeability via the release of lysosomal enzymes and platelet-activating factor (18). Monocytes have also been shown to have a role in acute serum sickness (20). Reduction in the number of monocytes by means of total body irradiation, or antimacrophage serum, was reported to result in the inhibition of glomerular infiltration by monocytes and pronounced reduction of proteinuria. As described above, monocytes were observed in substantial number in the glomerular capillaries, often in direct contact with endothelial cells or the GBM.

Ferritin is a relatively large molecule with an effective molecular radius of 6.1 nm. The albumin molecule is stated to be ellipsoid with axes of approximately 14 by 4 nm (21) and an effective molecular radius of 3.6 nm. From our experiments, the average size of pores in the GBM of control and experimental rats was 4 by 3 nm and 9 by 4 nm, respectively. So while neither albumin nor ferritin molecules could pass through the

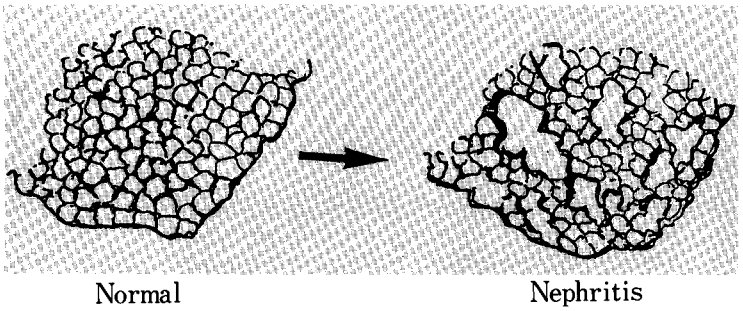

Fig. 5 Schematic representation of ultrastractural changes of the glomerular basement membrane in glomerulonephritis. In normal control rats the glomerular basement membrane has a meshwork structure with uniform pores, whereas it has enlarged pores in glomerulonephritis.

molecular sieve of the GBM of control rats, both molecules could pass easily through the enlarged molecular sieve of the GBM of rats with CSS nephritis. Hence, the enlargement of the pores in the GBM is likely to be responsible for proteinuria in rats with CSS. Combining the results of the present experiment and the previous ones concerning Masugi nephritis (10), aminonucleoside nephrosis (22) and diabetic nephropathy (23), the increase in the radius of glomerular pores appears to be responsible for proteinuria in various types of glomerulonephritis (Fig. 5).

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