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Cellular Processes of Glomerular Adhesion in Aged Rats

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Summery. **The process of glomerular adhesion to Bowman's capsule was morphologically investigated in aging rats. The results showed that segmentally hyperplastic parietal epithelial cells of the Bowman's capsule extended their cytoplasms to the segments of the glomerular capillary and covered the areas of the denuded part of the glomerular basement membrane from which the glomerular epithelial cells had been detached. The separation of the glomerular epithelial cells from the underlying glomerular basement membrane allowed local passage of macromolecular substances across the capillary wall. In conclusion, datachment and eventual cell death of the glomerular epithelial cells, together with various factors in exuded macromolecular substances relate directly to the subsequent proliferation of parietal epithelial cells. Thus, glomerular adhesion should be considerd as a multistep process.**

INTRODUCTION

Adhesion or synechia of the glomerular capillary to Bowman's capsule is one of cardinal lesions of progressive glomerular diseases, not only in the case of primary focal segmental glomerulosclerosis,[1] but also in other human chronic glomerunephritides.[1,2] Adhesion is frequently seen in experimental nephritis and nephrosis,[3] including aged rats.[4,5] According to the definition, adhesion may be fibrinous or fibrous, it may occur with or without proliferation of epithelial cells, and may be associated with pseudotubules.[6] The definition omits the type and origin of epithelial cells with which to reach adhesion. Fibrous adhesions are easily detectable in preparations of periodic acid-Schiff (PAS) and periodic acid methenamine silver (PAM) stains as a connective tissue bridging the glomerular capillary and Bowman's capsule. They must be the final form of cellular adhesion processes. Processes leading to the ultimate fibrous adhesion bring up several as yet unanswered questions as to which type of epithelial cell participates in the process of adhesion and how they produce ultimately fibrous connective tissue in such a limited place as Bowman's space. As the first step toward solving these questions, a sequence of cellular events of glomerular adhesions occurring in aged rats was studied by light microscopic, immunofluorescent and immunoelectron microscopic methods. The results showed that hyperplastic parietal epithelial cells (PECs) first reached their cytoplasms across Bowman's space for segments of the glomerular basement membrane (GBM), of which the glomerular epithelial cells (GECs) had been denuded by undetermined mechanisms. Various amounts of extracellular matrix (ECM) were present in spaces between the PECs. Subsequently the ECM bridged the capillary tuft and Bowman's capsule. Thus, glomerular adhesion should be considered a multistep process.

MATERIALS AND METHODS

Spontaneously hypertensive rats (SHR) of both sexes were used. They were kept on regular rat chow with free access to water. From the 3rd month to the end of the experiment, each rat was housed in a metabolic cage and 24 hr-urine samples were collected once every month and the amounts of protein excretion in the urine determined by turbidity with trichloroacetic acid using a nephelometer. At 5, 13, 15,17 and 19 months of age, the rats were anesthetized with ether, the abdominal aorta was cannulated below the renal arteries, and the kidneys were perfused with 0.5% of polyethyleneimine-1800 (PEI) in 0.1%

cacodylate buffer at pH 7.4 for 5 min according to the method by Schurer.[7] Excised kidneys were processed for three morphological studies, light and electron microscope, and immunofluorescence. Coronal sections of kidneys were fixed in buffered formalin and processed for light microscopic study. PAS and PAM were stained on 2 μ sections and adhesion rates were obtained. From representative kidneys of rats at 15, 17 and 19 months, series of more than 100 sections were uninterruptedly cut, orderly numbered and stained with PAS. Profiles of glomerular adhesions were searched both upward and downward of a given section. For ordinary electron microscopy, the tissue was cut into 1 mm cubes fixed in 2% glutaraldehyde/0.1 M phosphate buffer, pH 7.4 at 4°C for 12 hr. It was postfixed in OsO4 in the same buffer for 1 hr and embedded in Epon 812 by standard techniques. For PEI demonstration, 1 mm cubes of renal cortex were placed in 2% phosphotungustic acid/0.1% glutaraldehyde for 1 hr and refixed in 2.5% glutaraldehyde for 2 hr. After osmification for 2hr, they were embedded in Epon 812. The number of anionic sites per 1,000 nm in length and the thickness of the GBM were measured in male and female rats in their 5th and 19th months of age. On electron micrographs which were enlarged at the final magnification of 40,000, 50 sites were selected from each rat. The measurement of the thickness was aided by a vernier caliper. Another part of the kidneys was frozen in N-hexan precooled at -70°C and cryosectioned at 4 μ. Cryosections were reacted first with anti-rat Ig G, Ig M, C3, fibrinogen antibodies, anti-human type I and IV collagen (the kind gift of Dr. Ohshima, Medical College of Wakayama), anti-laminin, and fibronectin (purchased from Advance Biofacture, U.S.A.), heparan sulfate proteoglycan (a gift of Drs. Ohkura and Mastuo, Nagoya University School of Medicine) and monoclonal anti-gp 330 (a gift of Dr. Ronco, Paris, France). Other antibodies used in this experiment were prepared in this laboratory. The sections were then stained with fluorescine labeled anti-rabbit Ig G or anti-mouse Ig G. They were examined under an Olympus Vanox-T.

For immunoelectron microscopy another group of rats consisting of three each at 13, 15 and 17 months were perfused via the abdominal aorta with saline first and then paraformaldehyde-lysine-periodate (PLP). One mm pieces cut from the cortex were further fixed in PLP at 4°C for 4 hr, dehydrated with -20°C cooled dimethylformamide and embedded in hydrophilic methacrylate resin (GMA) at -20°C under ultraviolet light.[8] Glomeruli with cellular adhesions were selected from semithin sections stained with toluidine blue and ultrathin sections were prepared. For immunohistochemical electron microscopy, ultrathin sections mounted on nickel grids were incubated in the appropriately diluted ($\times$ 200-$\times$4,000) antibodies as described above at 4°C overnight and then reacted with protein A-colloidal gold at room temperature for 1 hr.[9,10] All grids were stained with uranium acetate and lead citrate. They were carbon coated and examined under Hitachi HS-9 and H-600 electron microscopes.

RESULTS

1. Urinary protein excretion

The amounts of urinary protein excretion are shown in Fig. 1. From the experimental 13th month, rats began to produce larger amounts of protein in the urine, being over 100 mg/day on the average during the 18th month. Female rats remained in the range below 15 mg/day throughout the course of the experiment, an amount which was less than that of the males at the start of the experiment.

2. Light microscopic findings

Renal histology of aged SHR, especially males, fell into the category of focal segmental glomerular sclerosis (FSGS). In the advanced state of FSGS, adhesion could not be separated from glomerular sclerosis. Kidneys of aged rats showed a mixture of early and advanced stages of glomerular sclerosis. When a lesion of either collapsed capillaries, hyalinosis or mesangial sclerosis was seen in a given section without any evidence of adhesion, an upward or downward search of sequential sections usually demonstrated the presence of adhesions. The morphology of the adhesions was of full variety in regard to their locations in Bowman's space and the proportion of cellular to fibrous components. A great many of them were fibrous, indicating that they were of old adhesions. Trapped glomerular capillaries in the fibrous lesion were frequently collapsed, containing hyalinosis and vacuolated cells in collapsed lumina (Fig. 2). Less fibrous adhesion were of a mixture of undetermined cells and fibrinous components. Epithelial cells around fibro-cellular adhesions were hyperplastic, often vacuolar and lined in a row on a connective tissue mass. The percentage of fibrous and fibrocellular adhesions of each rat was obtained by observing all glomeruli in a given section. Adhesion rates were from 7.9% to 12.5% in males in their 19th month, and 0 to 1.2% in female rats of the corre-

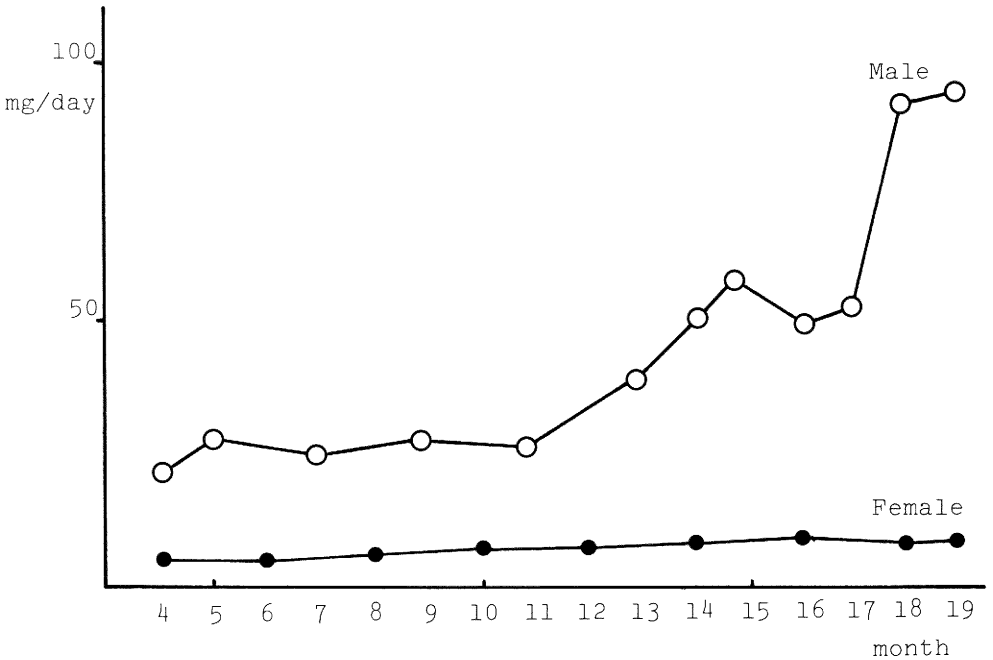

Fig. 1. Urinary excretion of protein of spontaneously hypertensive rats, both male and female during the course of the experiment. Male rats began to produce large amounts of proteinuria from the 13th month. Female rats did not excrete significant protein in the urine.

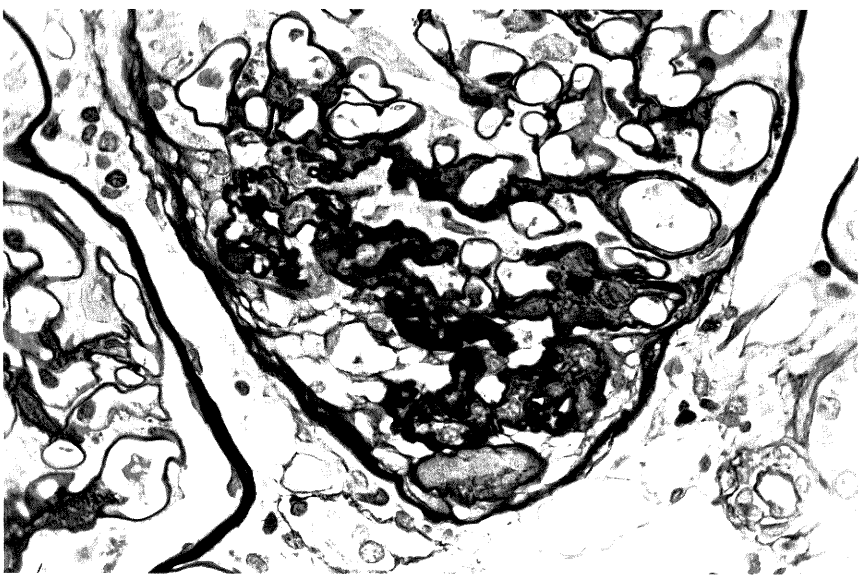

Fig. 2. The glomerular capillaries which were trapped in the fibrous adhesion showed collapsed segments of the capillary wall, hyalinosis and increased mesangial matrix. (PAS, ×400)

sponding age.

By observing serial sections of rats from the 15th, 17th and 19th month groups, we came across sets of early cellular process of glomerular adhesion which were definitely separable from mesangial sclerosis and presented several possibilities to account for their occurrence. The GEC changes in aged male SHR varied with the age; they were virtually normal in appearance until the 13th month, showed a few epithelial changes with PAS positive droplets in some

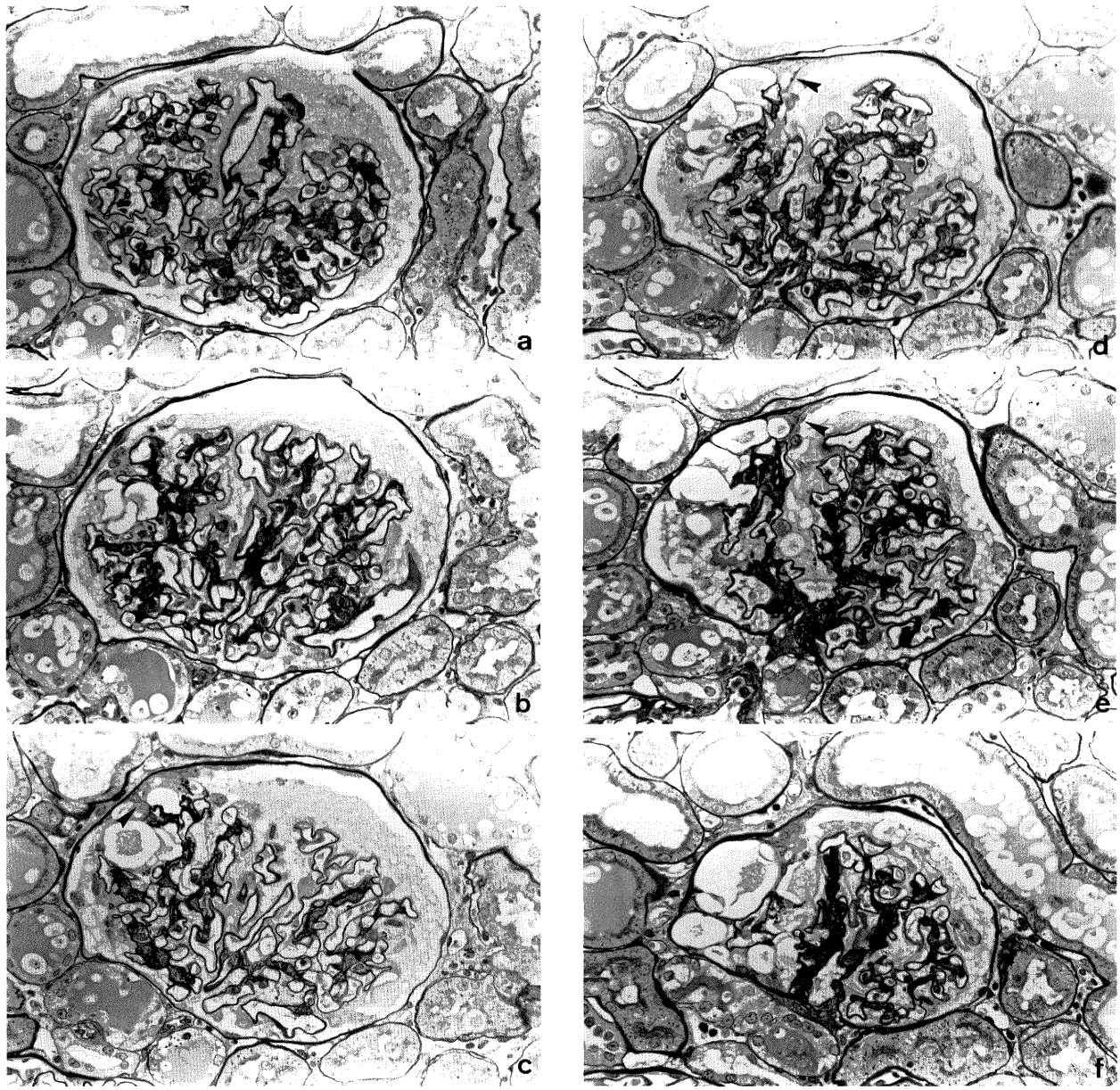

Fig. 3. A set of cellular adhesion was shown from a to f. Parietal cells which segmentally proliferated in Boman's space (b) produced a thin thread of connective tissue across Bowman's capsule to the capillary wall (c, arrowhead). Another thread is seen in Fig. 3d and 3e (arrowhead). Some epithelial cells were highly vacuolated. (PAS, ×200)

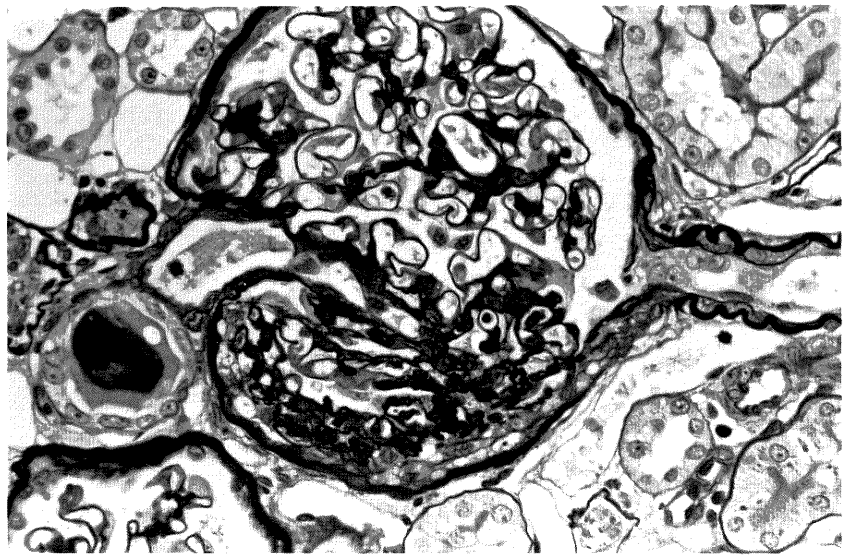

Fig. 4. A partially sclerotic glomerulus which was sectioned through its vascular and urinary poles, showing atrophic proximal tubule and the presence of cast in the distal tubule. (PAS, ×200)

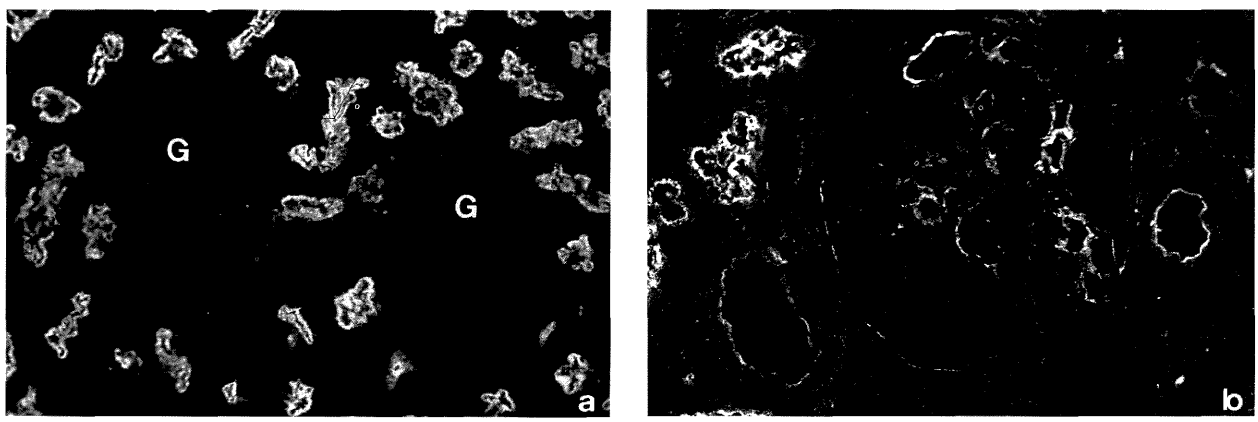

Fig. 5. Indirect immunofluorescent microscopy of normal (a) and experimental rat (b) kidneys with anti-gp 330 antibody. Tubular brush border was positive but those cells in the glomerulus (G) were negative in the normal rat (a) The same antibody reacted with some of the parietal cells of Bowman's capsule and highly vacuolated cells in the cellular adhesion of experimental rat (b). A few cells covering the glomeruar capillary are also positive. ×200

glomeruli in the 15 and 17 month-olds, and had segmental collections of highly vacuolated (bleb-like) epithelial cells in the 19 months-olds. In Figure 3a-f, an example of cellular events leading to ultimate adhesion is shown. Epithelial cells which segmentally proliferated in Bowman's space as in Fig. 3a were turned into cells of large vacuolated cells of bleb-like appearance in Fig. 3b. A thread of PAS-positive tissue ran between either proliferated or highly vacuolated cells from Bowman's capsule to one of the glomerular capillaries, as in Fig. 3c. At least three threads were seen in this paticular lesion (Fig. 3d-f). Most of the capillary loops adjacent to group of bleb-like cells were widely patent, and neither a segment of hyalinosis nor collapsed capillaries were found. Glomeruli without adhesions had hypertrophic epithelial cells which frequently contained many reabsorbed granules in their cytoplasms. Glomeruli with smaller adhesions were not usually accompanied by tubular atrophy, but a glomerulus with large adhesions was often seen with atrophic proximal tubules and the presence of cast in the lumen of its distal tubule when the vascular pole was sectioned (Fig. 4).

3. Immunofluorescent findings

Sites of adhesion were reactive with anti-type IV collagen, laminin, fibronectin and fibrinogen antibodies on immunofluorescent microscopy. The reaction of anti-type I collagen antibody was not seen in the glomeruli. The immunofluorescent patterns of each antiserum on glomeruli without adhesions were essentially the same as in those of 5-month-old rats, with the exceptions of reactions to anti-IgM and C3 in sclerotic mesangium and anti-fibrinogen in adhesions in aged rats. Anti-gp 330 antibody reacted with the brush border of the proximal convoluted tubules and PECs of Bowman's capsule, but GECs were negative (Fig. 5a). Cellular portions of glomerular adhesion consisted of those cells reactive to anti-gp 330 in a pattern of pseudotubular structure. It was noteworthy that some of pseudotubules in a separate position were also positive (Fig. 5b).

4. Electron microscopic and immunohistochemical findings

The electron microscopic examination of the glomeruli of rats at the 5th and 13th months revealed that GEC were normal in appearance. The GECs under-

Table 1. The thickness of the GBM and the number of anionic sites per unit length

	5 m male	5 m female	19 m male	19 m female
thickness of GBM in nm (n=50)	177.5±22	170±25	465.5±77.5	344±78.6
anionic sites per 1,000 nm of the GBM (n=50)	16.4±2.2	16.1±2.1	15.6±2.6	15.0±2.2

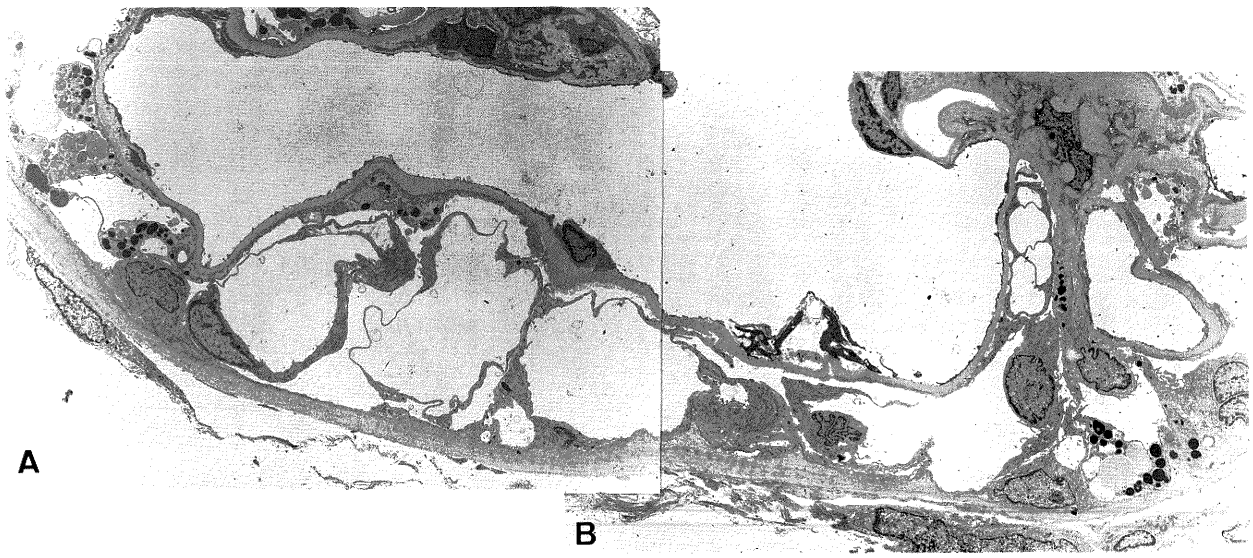

Fig. 6. Highly vacuolated epithelial cells were seen in Bowman's space. Cells which had a broad base on Bowmn's capsule represent parietal cells in origin. (×1,000)

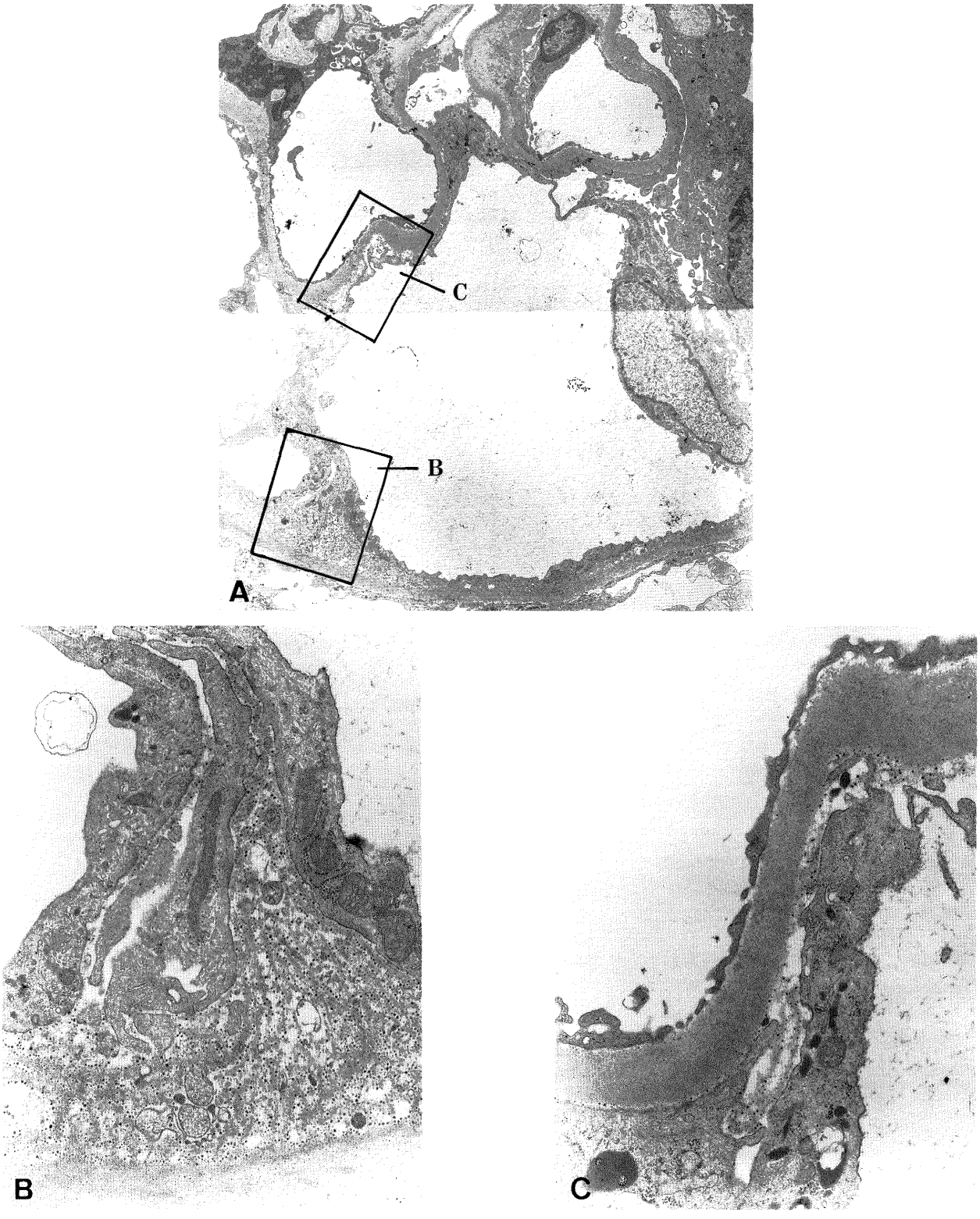

Fig. 7. Vacuolated epithelial cells which based on the capsule of Bowman extended their cytoplasms to the glomerular basement membrane (A). In narrow space between cells various amounts of connective tissue with PEI particles were present (B and C). (A, ×2,000, B and C, ×10,000)

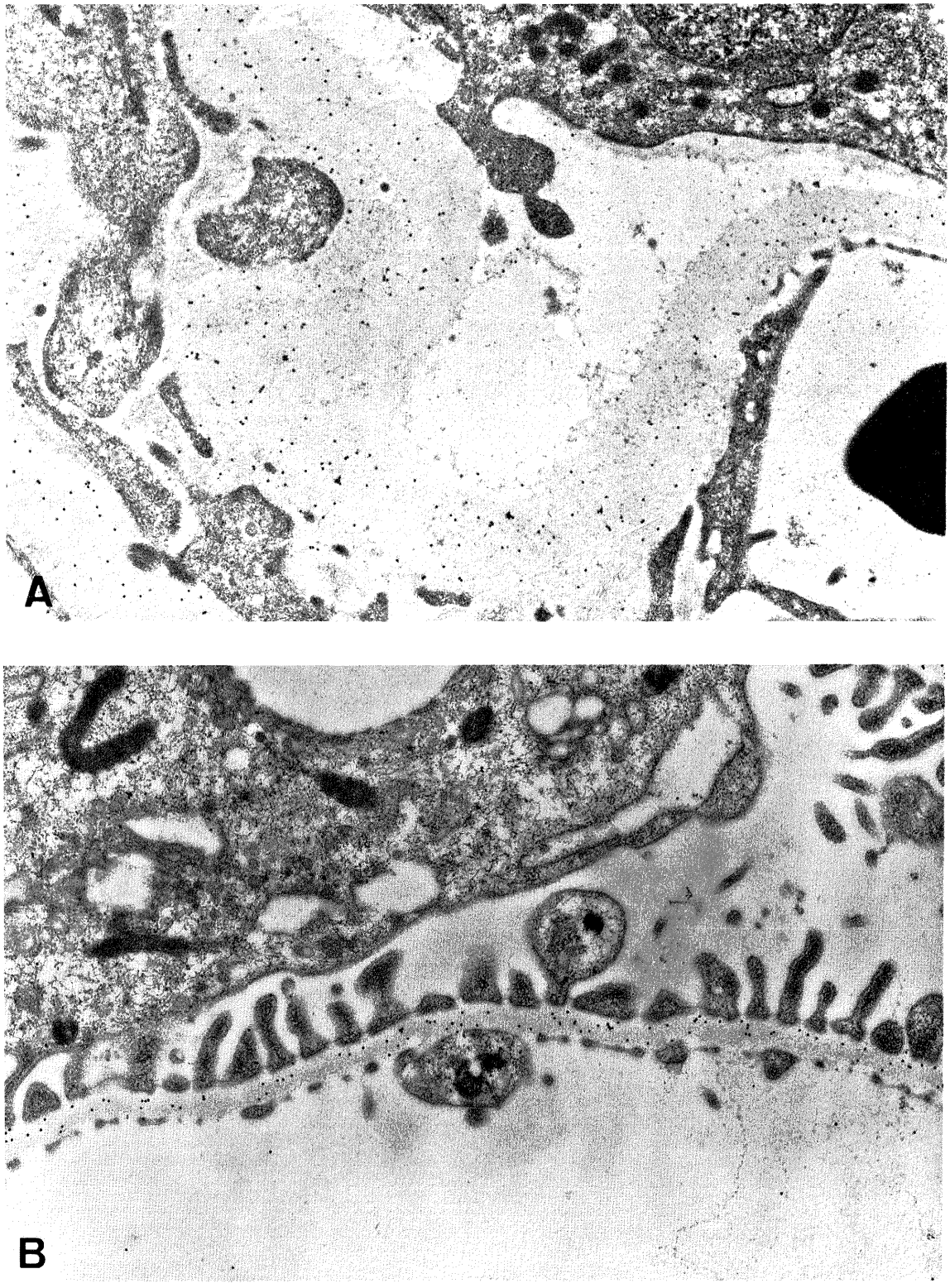

Fig. 8. In the segment of the glomerular basement membrane from which the visceral epithelial cells separated, the reaction against anti-laminin antibody was preserved (A). B was the normal rat. Immunogold electron microscopy against anti-laminin antibody. (×10,000)

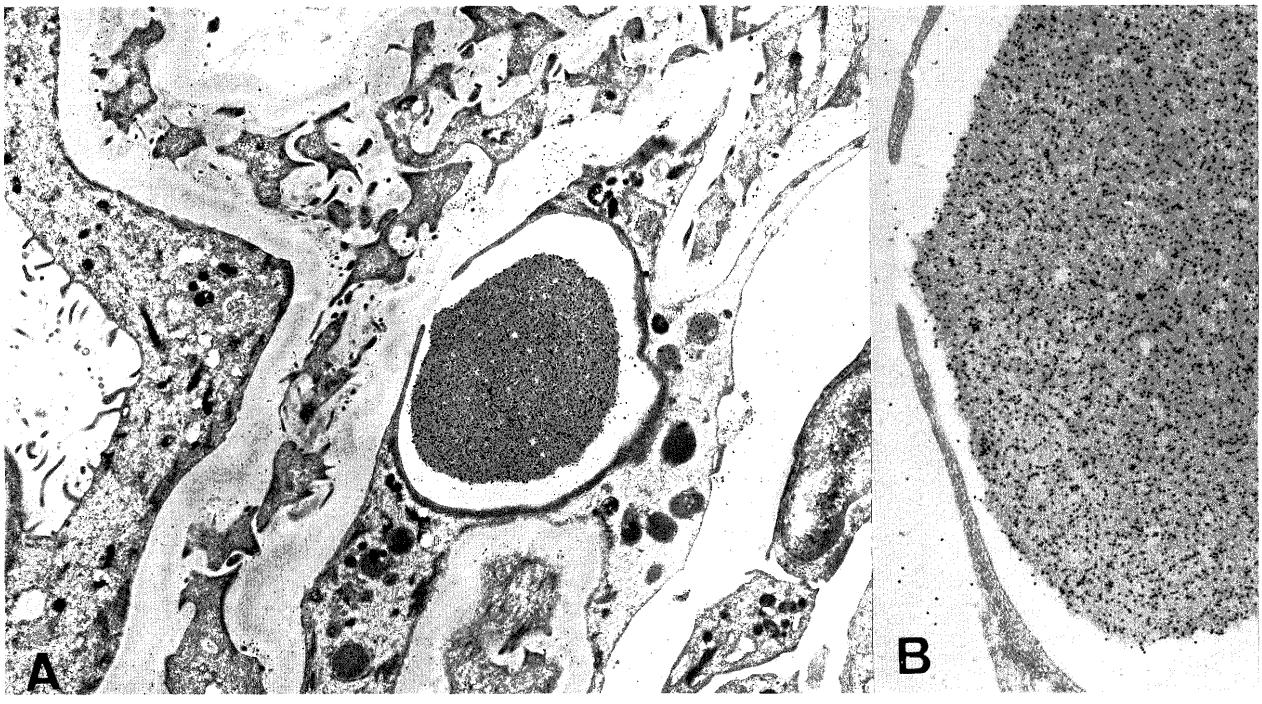

Fig. 9. In the pocket made by visceral epithelial cell and the glomerular basement membrane contained the material which was strongly reactive with anti-fibrinogen antibody. Immunogold method (A, ×3,000, B, 10,000)

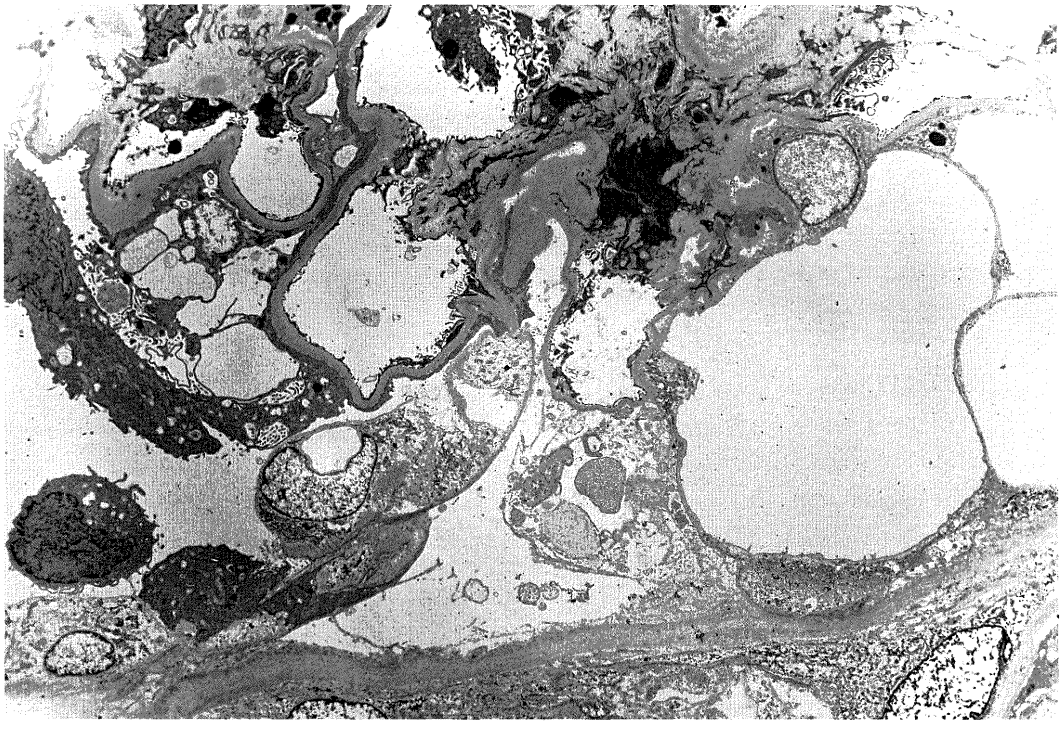

Fig. 10. A row of parietal epithelial cells with small amounts of connective tissue united the segment of the glomerular basement membrane from which visceral epithelial cells detached to Bowman's capsule. ×3.000

went some nonspecific structural alterations as the experimental month continued. These changes were focal obliteration, a simplification of foot processes, the distortion of the slit diaphragm, an increase in the number of lysosomal granules, vesicular or bleb-like enlargement and focal detachment of the GECs from underlying GBM. With the start of overt proteinuria, some GECs had multiple osmiophilic lysosomes of various sizes, shapes and electron densities. The vesicles either contained a granular proteinacious material or appeared as clear space. The amounts of cytoplasmic elements of GECs varied; some cells had a slender cytoplasm with sparse cytoplasmic organellae with indented nuclei, while in others the organellae were abundant. In the 19th month the number of the GESs with numerous granules was small and GECs of a vacuolated appearance increased.

One of the sites selected as a possible early cellular adhesion from multiple semi-thin sections is shown in Fig. 6a and b. Epithelial cells were highly ballooned. One cell contained both lysosomes and blebs but many of them were less in organellae and highly ballooned up. Several cells were separated from the underlying GBM. The cells which were based broadly on Bowman's capsule were also vacuolar but hyperplastic and suggested PECs in origin. Although there was a small amount of ECM as shown by PEI labeling in the narrow spaces between the PECs and GECs (Fig. 7a-c), bridging between the capillary loops and Bowman's capsule was not completed. The thickness of the GBM and the number of anionic sites per unit length in the 5-month-old rats were the same for males and females. Of rats in the 19th month, male rats showed a considerable increase in the thickness of the GBM. The number of anionic sites did not differ between male and female rats in the 19 month (Table 1), a fact which was also confirmed by immunoelectron microscopy for anti-heparan sulfate proteoglycan antibody. At the portions of the GBM from where the GEC detached, the number of anionic sites varied. A fair number of anionic sites remained where GECs separated focally from the GBM. Widely denuded segments of the GBM lost their anionic sites. Reaction to anti-laminin, and fibronectin in the segments of denuded GBM was not different from those with normal glomeruli as shown in Fig. 8a and 8b. Staining with anti-fibrinogen demonstrated: 1) an increase of reaction product in the closed pockets made by the GBM and a partially detached epithelium; and 2) focal and segmental reaction with anti-fibrionogen in a sclerotic mesangial matrix and overlying GBM (Fig. 9a and 9b). PECs varied in shape; most of them were atrophic but often hypertrophic, and contained membrane-bound granules of a considerable density. In the periphery of such lesions there were lines of PECs in a row which attached to a part of the denuded GBM (Fig. 10) in the appearance of a cellular bridge between the denuded GBM and Bowman's capsule. There were various amounts of cell debris in the space made by these bridges.

DISCUSSION

It has been recognized that aging male rats are prone to the development of proteinuria, biochemical abnormalities characteristic of the nephrotic syndrome and FSGS.[10-12] Since human renal diseases, including FSGS, occur without any loss of renal mass, it is reasonably important to examine the natural course of rat glomerular sclerosis, particularly epithelial changes associated with aging. The renal epithelial lesions in aged male SHR varied with the age; a normal appearance persisted until the 13th month of the experiment, a few epithelial changes appeared with PAS positive droplets in some glomeruli at the 15 and 17 months, and either segmental bleb-like epithelial cells or adhesion with tubulointerstitial changes were noted in the 19 month. These findings are compatible with previous observations on GECs of various rat strains.[10,12-16] Using a reconstructive approach, we have been able to show earlier cellular processes of adhesion without obvious glomerular sclerosis in light and electron microscopy.

The three main changes are seen in the sites of cellular adhesion: 1) a sharp reduction in the number of fully developed reabsorption droplets from GECs which took on a bleb-like appearance; 2) the detachment of GECs from the GBM and epithelial cell death; and 3) the production of various amounts of extracellular matrix. The first to be considered is epithelial cell changes. Progressive epithelial changes progressing further beyond a generalized foot process loss of GECs in such a disease as FSGS have been less thoroughly explored, but the suggestions from rats with nephrosis are instructive.[17] A quantitative and qualitative assessment of glomerular epithelial alterations in rats with aminonucleoside nephrosis demonstrated an early increase in the number of lysosomes and phagosomes followed by a reduction on their numbers at a later stage. A corresponding increase in the number of phagosomes was seen with no concentration of their contents, namely a vacuolar or bleb-like appearance. These late epithelial changes were considered to be manifestations of epithelial cell injury; a defect in endocytosis, inavailability or

delivery of lysosomal enzymes ("exhaustion" of energy supply) were not likely to be reversible.[17] Morphological sequences in our observations similar to those in rats with aminonucleoside nephrosis suggest a decrease or loss of the incorporation and disposal of protein uptake by GECs has occurred focally and segmentally in aged rats. We did not encounter any evidence in this study suggesting the presence of cell proliferation among adjoining GECs around the denuded GBM. The second change is the detachment of bleb-like cells in a group from the GBM. This implies a defect in the synthesis of either GBM or epithelial surface components by which epithelial cells and GBM normally adhere to one another. Although a generalized loss of colloidal iron staining to the epithelial cell membrane was shown in nephrotic rats with aminonucleoside,[18] the mechanism by which GEC detachment occurs segmentally is unknown. The overall GBM width of male rats was thicker than that of female rats at 19 months of age, but immunological reactions to anti-type IV collagen, laminin, fibronectin and heparan sulfate proteoglycan antibodies were the same in both males and females. At the sites of epithelial detachment from the GBM, anionic sites decreased or virtually disappeared; however, the same reactions with antibodies for type IV, laminin and fibronectin preserved. The heparan sulfate proteoglycans have been known to play a role in charge selectivity of the GBM,[19-20] their role is apparently minor as far as the detachment of the GEC is concerned. The GBM, though thick, may not be the cause for GEC exfoliation. GECs with normal foot processes and slit diaphragms present a structural basis for a restriction to the hydraulic flow across the glomerular capillary wall.[21,22] Structural and functional derangement of epithelial cells and the resultant detachment of cells from GBM are likely to invite a local increase of convectional flow across the GBM. The accumulation of fibrinogen in pockets made by detached epithelial cells and a denuded segment of the GBM indicates that increased passage of macromolecular substances across the GBM has occurred. A combined mixture of cell debris, fibrinogen and loose connective tissue elements are signs of the eventual cell death of detached GEC and the process of organization.

The third is the production of connective tissue and pseudotubular structures. In light microscopy, a row of PECs was frequently observed at the sites of adhesion. These cells often form a pseudotubuolar structure. Immunofluorescent findings with the use of anti-gp 330 strongly indicates they are PECs in origin, because none of the GECs are positive to this antibody. It is interesting that many PECs proliferate along the GBM which might have been collapsed or sclerotic. Such proliferating PECs become frequently vacuolar in and around the adhesion. Presumably the vacuolation of PECs derives from mechanisms similar to alterations of GECs because the finding of hypertrophic PECs with a full development of membrane-bound dense granules is not rare. A few vacuolated PECs may also go into ultimate cell death, which is considered to stimulate another cell renewal system of PECs. It is necessary to stress that the glomerular adhesion is the first and preceding lesion seen in aged male rats. The close correlation of hyalinosis, collapsed capillaries and the increase of a mesangial matrix and deposition of macromolecular substances in the mesangium indicates they are the lesions of subsequent occurence. Thus, adhesion is an indication of complexed cellular events initiated by glomerular epithelial alterations. The defective endocytosis and resultant formation of multiple vacuoles and the final appearance of bleb-like cells of GECs are not merely the result of persistent proteinuria, but appear to be specific functional damages to GECs associated with the development of the glomerular lesion, namely adhesion to Bowman's capsule. The detachment and eventual cell death of GECs relate directly to the subsequent proliferation of PECs and final production of an extracellular matrix which binds both glomerular capillary loops and Bowman's capsule. It is not inappropriate to consider that the segmental trapping of glomerular capillaries in adhesion disturbes intraglomerular microcirculation. The turbulent flow of the circulation and increased luminar shear stress allow further alterations locally in intrinsic mesangial properties such as reactions to macromolecular substances, platelet adherence to damaged glomerular endothelial cells, and coagulation mechanisms. Each of these final morphological expressions is observed in glomeruli with focal segmental sclerosis.

Finally, massive renal ablation models may be an appropriate manipulation for the progression of renal disease that follows a significant loss of nephron mass[23-28] but their relevance to the progression of glomerular diseases of insidious onset and slow progression is less clear. A careful interpretation and extrapolation to human diseases should be necessary with the consideration of species differences that exist between rats and dogs,[29,30] and humans, too.[31]

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