Abnormality of glomerular basement membrane in aging brachymorphic mice.

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Abstract
Brachymorphic mice have a chondrodystrophia induced by a point mutation in bifunctional sulfurylase kinase polypeptide functioning as ATP-sulfurylase and adenosine-phosphosulfate kinase. This disorder in the mice arises from the undersulfation of glycoconjugates in a numerous kind of organs and tissues.
Unexpectedly, the aged female mouse (114 weeks old) with hematuria was found in brachymorphic mice. After the ultrastructural examination, pathological findings were identified in renal glomerular basement membrane. Consequently, attempts have been made to search the aging changes of ultrastructure in the renal glomerulus in aged normal and brachymorphic mice. In both aged normal and brachymorphic mice, humped intramembranous dense transformation and thickened glomerular basement membrane with multilayered matrix in lamina densa and narrowed lamina rara were shown in varying degrees. These pathological findings of glomerular basement membranes are markedly in brachymorphic mice than in normal mice. The disorders of renal glomerular basement membrane are thought to result from functional changing of aged glomerular basement membrane organizing cells (podocyte and endothelial cells) and undersulfated heparansulfate.

Key word : Brachymorphic mouse, undersulfation, glomerular basement membrane, anionic site, electron microscopy

introduction

Brachymorphic mice have a chondrodystrophia induced by a recessive gene on the autosomal chromosome that generates a point mutation in bifunctional sulfurylase kinase polypeptide functioning as ATP-sulfurylase and adenosine-phosphosulfate kinase. This disorder in the mice arises from the undersulfation of cartilage tissues that induced by the decreasing synthesis of sulfate donor, generated by the abnormal sulfurylase kinase. As a result, brachymorphic mice are characterized by inheritable dwarfism with disproportionally shortened limbs, tail and trunk[1-13]. Moreover, undersulfation of sulfated glycoconjugates are found widely in numerous kind of organs and tissues in brachymorphic mice[13].

Unexpectedly, the aged female (114 weeks old) with hematuria was found in brachymorphic mice. After the examination by means of transmission electron microscopy, irregularly thickened basement membrane with dense deposits and proliferative mesangial matrix were observed in renal glomerulus in this mouse. Moreover, its structural findings resemble to that of membranous glomerulonephritis. Consequently, attempts have been made to search the aging changes of ultrastructure in the renal glomerulus in aged normal and brachymorphic mice.

Materials and Methods

Experimental animals:

The mice were housed in a temperature-, humidity- and light-controlled room (almost 25℃ , 50% humidity, and 12 hours of light per day), were permitted cage activities, and were given standard amounts of pelleted food and clean water. These procedures were reviewed and approved by the animal care and use committees of Nagoya Bunri University.

For the experiments, normal and brachymorphic female mice were sacrificed at ages of 8, 15, 30, 50 and 80 weeks after the birth.

Tissue preparation:

After undergoing the sodium pentobarbital anesthesia, the mice were perfused via the left cardiac ventricle with physiological saline solution followed by perfusion fixation of 0.05M phosphate buffered 2.5% glutaraldehyde-2.0% paraformaldehyde solution (pH7.4) with or without

0.05% ruthenium red[14,15]. After the perfusion fixation, kidney was dissected out and cut to the tiny pieces. Tiny pieces of tissue blocks were immersed at 4℃ for 60 min in 0.05M phosphate buffered 2.5% glutaraldehyde-2.0% paraformaldehyde solution (pH7.4) with or without 0.05% ruthenium red and then, rinsed in the same phosphate buffer (pH7.4) without fixatives. After rinsing with phosphate buffer, tissue blocks were incubated in 1.0% osmium tetraoxide in 0.05M phosphate buffer (pH7.4), rinsed with same buffer without osmium tetraoxide, dehydrated in an ethanol series of ascending concentrations and embedded in Quetol 651[16,17]. Ultrathin sections were cut at a thickness of approximately 100nm on an ultramicrotome (LKB 4802A), mounted on cupper grids and subjected to the following procedures.

Staining procedure:

Ultrathin sections were counterstained with 2.0% uranyl acetate solution and lead salts mixture solution at room temperature for 2-5 min each[18,19]. After the staining, ultrathin sections were rinsed sufficiently with distilled water and coated with carbon using vacuum evaporator.

Observation:

All ultrathin sections were examined in a transmission electron microscope (JEM 200CX, JEOL), and photographs of glomerulus were captured. For the research of the thickness of glomerular basement membrane, approximately 10 or more photographs of renal glomerulus (magnification of 10,000) were taken randomly, and measured by using the Motic IMAGES 2000 (Micro-Optic Industrial Group Co. LTD.).

Results

When renal glomeruli of the aged female brachymorphic mouse (114 weeks after birth) were observed with light and electron microscope, irregularly thickened glomerular basement membrane with intramembranous

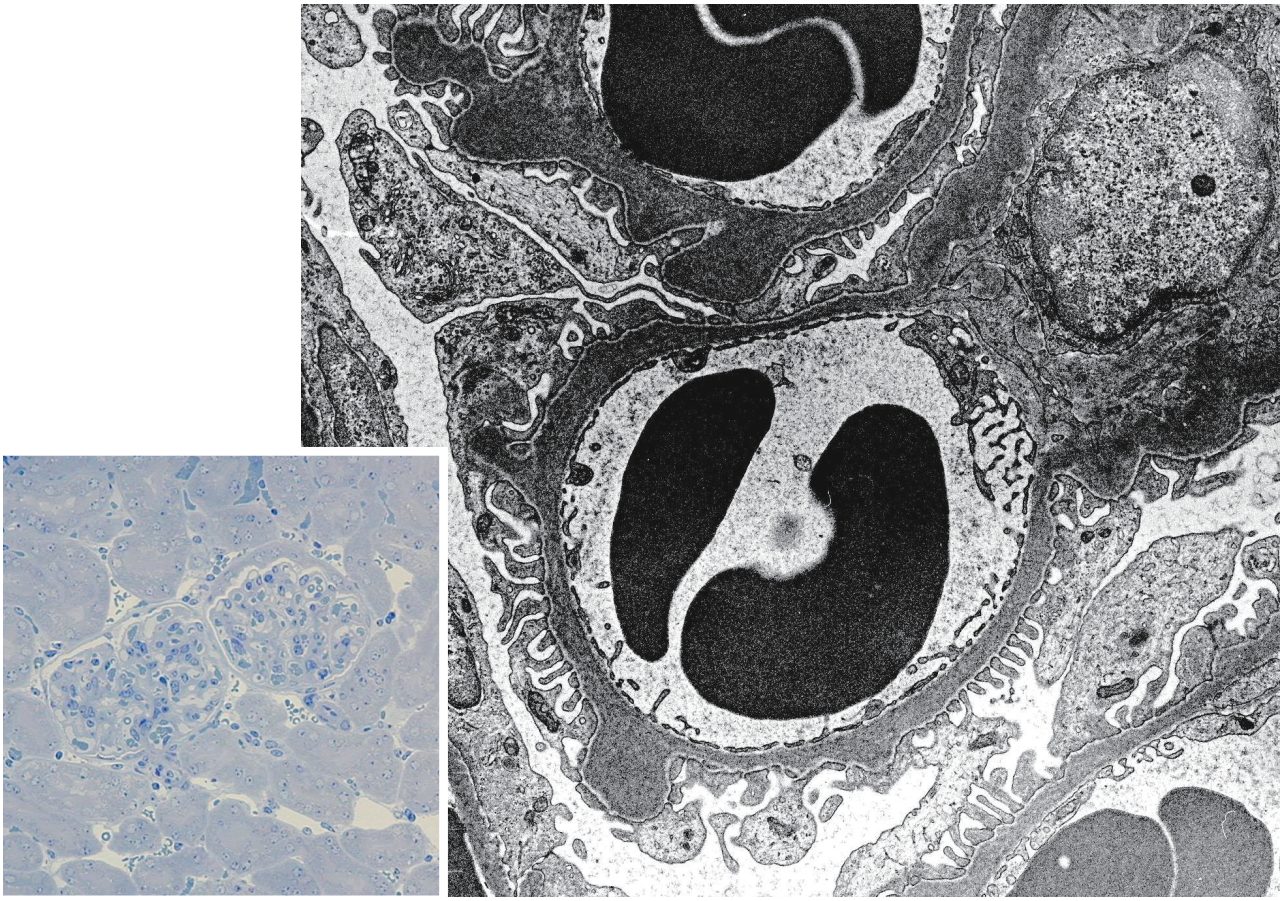

Figure 1: Light and electron microscopic images of renal glomerulus in female brachymorphic mouse with albuminuria at age of 114 weeks. Magnification of 200 (light micrograph) and 3,000 (electron micrograph) folds.

dense transformation, proliferative mesangial matrix and narrowed cavity of capillary vessels were shown (Fig. 1).

In normal renal glomerulus in 8 weeks after birth (Fig. 2, left side), proper glomerular basement membrane which are clearly separated with 3 layer involving lamina densa (LD), lamina rara externa (LRE) and interna (LRI), regularly lined fenestra of podocyte (PC, FP), compactly packaged mesangial cells (MC) and its matrix and wide cavity of glomerular capillary vessel (C) were observed. Correspondingly, in renal glomerulus in 8 weeks after birth of brachymorphic mice (Fig. 2, right side), ultrastructures of glomerulus such as glomerular basement membrane, fenestrated podocyte, mesangial area and cavity of capillary vessel were almost similar to that of normal mice without any pathological changes. However, the thickness of glomerular basement membrane in brachymorphic mice was feebly narrower than that of normal mice, especially in lamina densa.

In normal mice in 15 weeks after birth, ultrastructure of renal glomerulus was as same as the structure of normal mice in 8 weeks after birth (Figs. 2 and 3, left sides). However, in brachymorphic mice of 15 weeks after birth, glomerular basement membrane became irregularly thickened(Fig. 3, right side, asterisk). In other words, a few number of small humpy intramembranous dens transformation, diffused and multilayered lamina densa and markedly narrowed lamina rara in glomerular basement membrane were shown. Moreover, pathological ultrastructural changings of renal glomerulus such as a few number of fused foot process of podocyte (arrow head) and slightly proliferated mesangial matrix were observed (Fig. 3, right side).

In normal mice in 30 weeks after birth, ultrastructural components of renal glomerulus described above were almost same to those in 15 weeks after birth (Fig. 4, left sides). In brachymorphic mice in 30 weeks after birth,

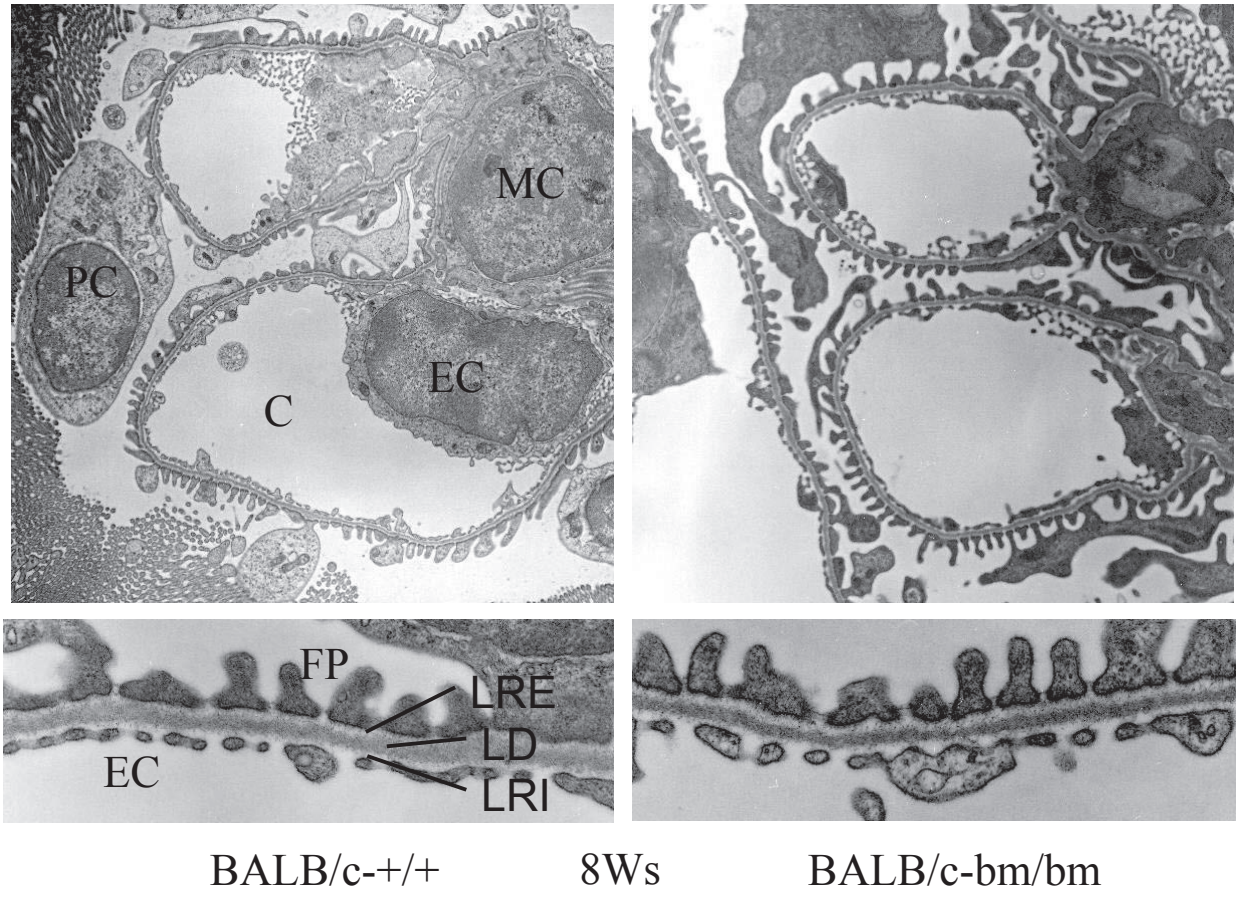

Figure 2: Electron microscopic images of renal glomerulus in normal (left side) and brachymorphic (right side) mice. C: capillary vessel, PC: podocyte, EC: endothelial cell , MC: mesangial cell, FP: foot process of podocyte, LRE: lamina rara externa, LD: lamina densa, LRI: lamina rara interna. Magnification of ,3000 (upper micrographs) and 10,000 (lower micrographs) folds.

similarly, the changed ultrastructural components of renal glomerulus resemble to the those of brachymorphic mice in 15weeks after birth (Fig. 4, right sides).

When the ultrastructural microphotographs of renal glomerulus in normal mice of 50 weeks after birth were searched (Fig. 5, left side), humped intramembranous dense transformation (asterisk) and thickened glomerular basement membrane with multilayered matrix in lamina densa and markedly narrowed lamina rara in glomerular basement membrane were shown in patches. Additionally, moderately proliferated mesangial matrices were widely observed even in normal mice. In brachymorphic mice in same age (Fig. 5, right side), markedly thickened glomerular basement membranes with humped intramembranous dense formation (asterisks) were numerously observed. Additionally, fused foot process of podocyte (arrow head) and enhanced proliferative mesangial matrix were commonly shown in renal glomeruli of aged brachymorphic mice.

In 80 weeks after birth, glomerular basement membranes were markedly thickened with irregularly widened and multilayered lamina densa and narrow lamina rara in both normal and brachymorphic mice (Fig. 6). Then, numerous humped intramembranous dese transformation in glomerular basement membrane and fused foot process of podocyte were observed in both mice, popularly. Additionally, in both mice, endothelial cells had many cell process (square) and fused fenestrate in this age (Fig. 6).

Figures 7 and 8 indicate the graphs of the thickness of glomerular basement membrane in normal and brachymorphic mice with aging. Thickness of glomerular basement membrane was gradually increased with aging. Although, the degree of thickness was larger in brachymorphic mice than normal mice (Fig. 7). Furthermore, when the thickness of sublayers in glomerular basement membrane with aging was searched,

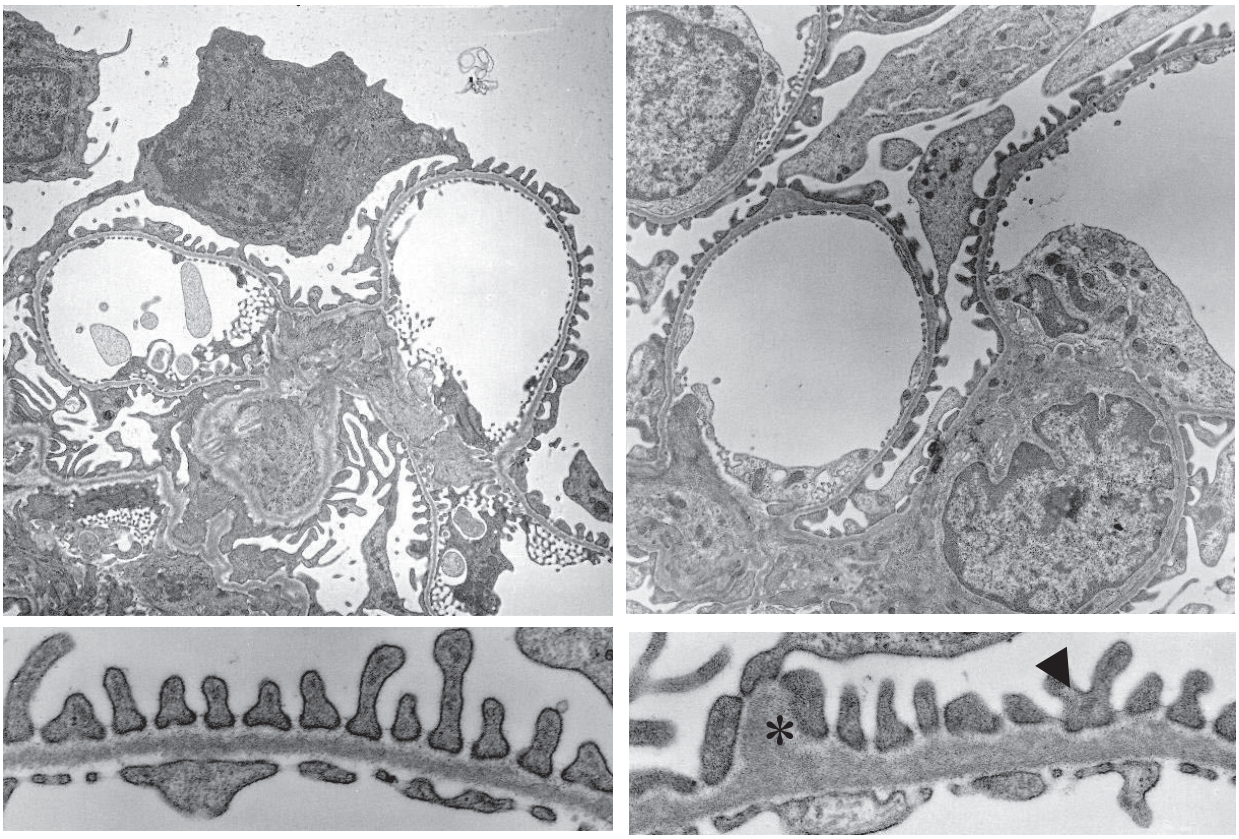

Fig. 3 : BALB/c-+/+ 15Ws BALB/c-bm/bm

Figures3-6: Electron microscopic images of renal glomerulus in normal (left side) and brachymorphic (right side) mice. Asterisks: humped intramembranous dense formation in glomerular basement membrane. Arrow head: fused foot process of podocyte. Square: cell process in endothelial cell. Magnification of ,3000 (upper micrographs) and 10,000 (lower micrographs) folds.

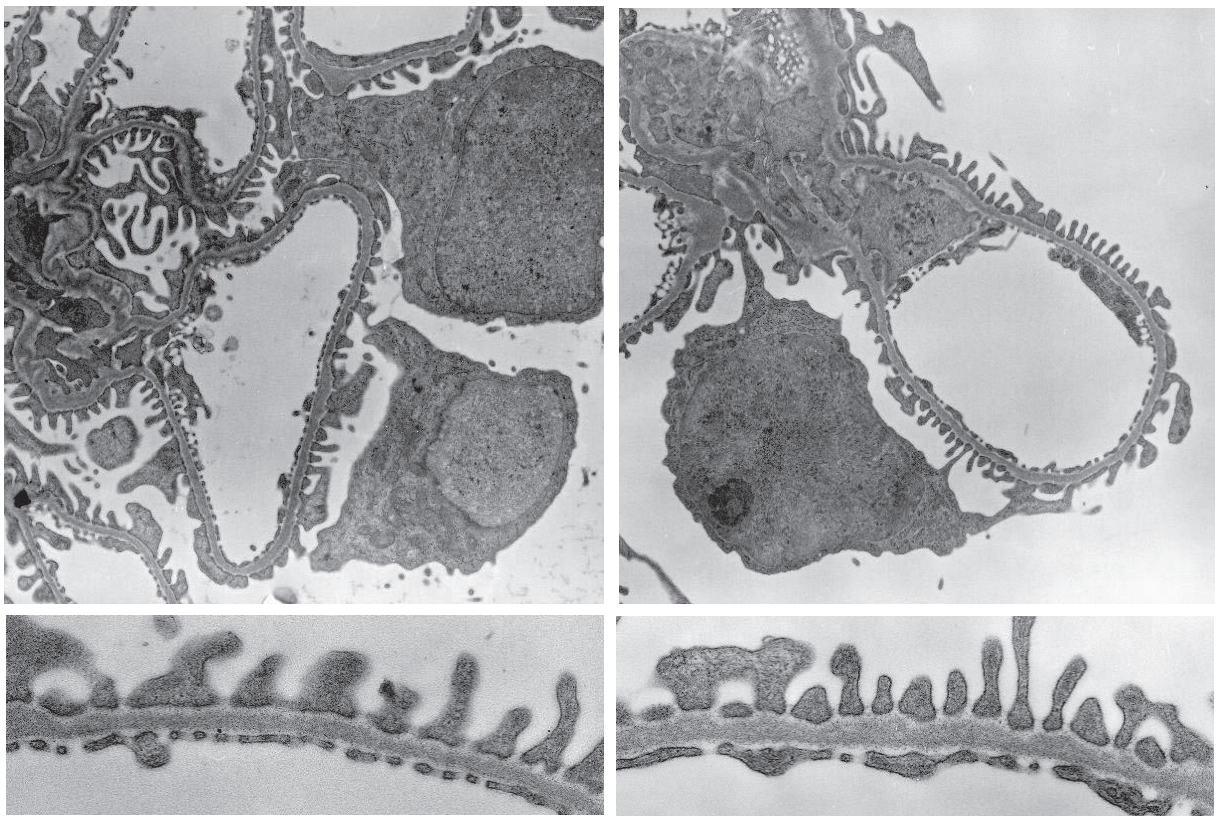

Fig. 4: BALB/c-+/+ 30Ws BALB/c-bm/bm

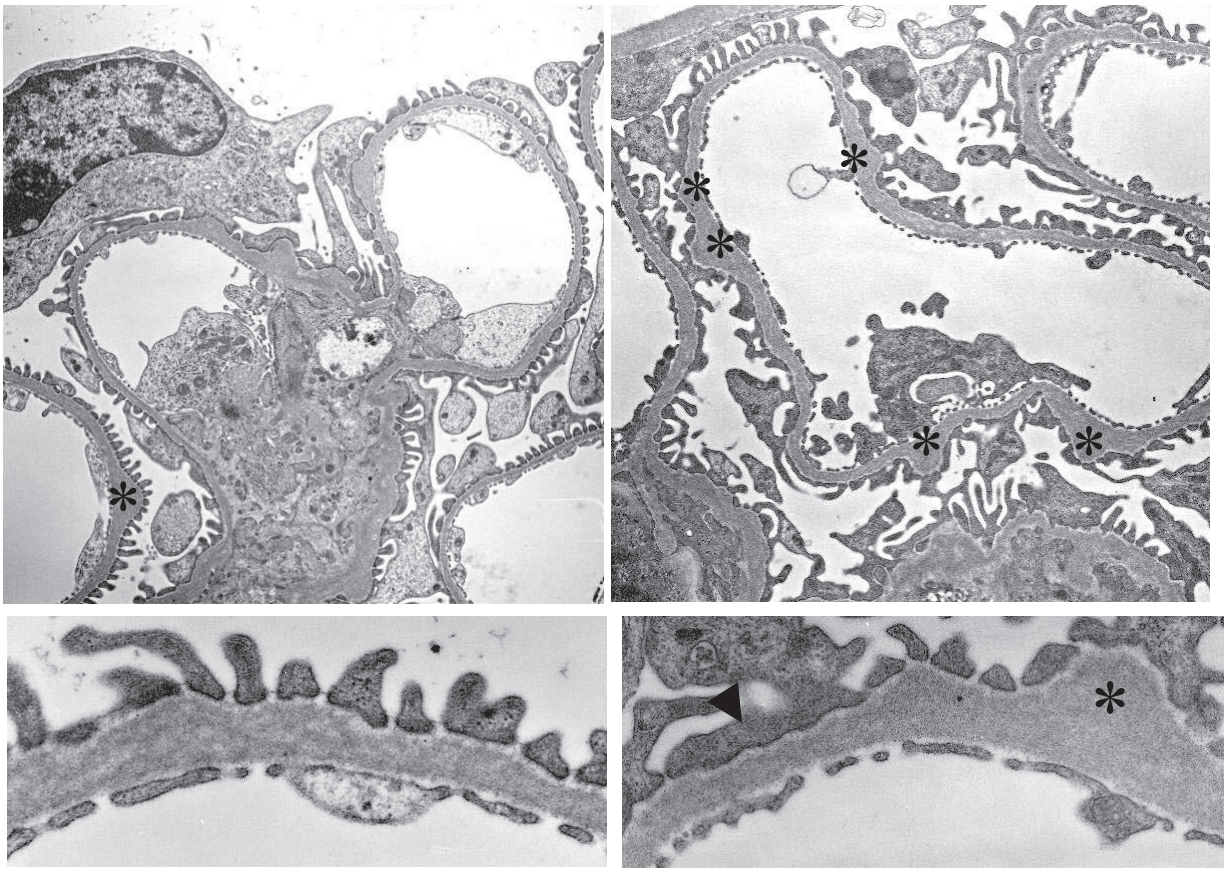

Fig. 5: BALB/c-+/+ 50Ws BALB/c-bm/bm

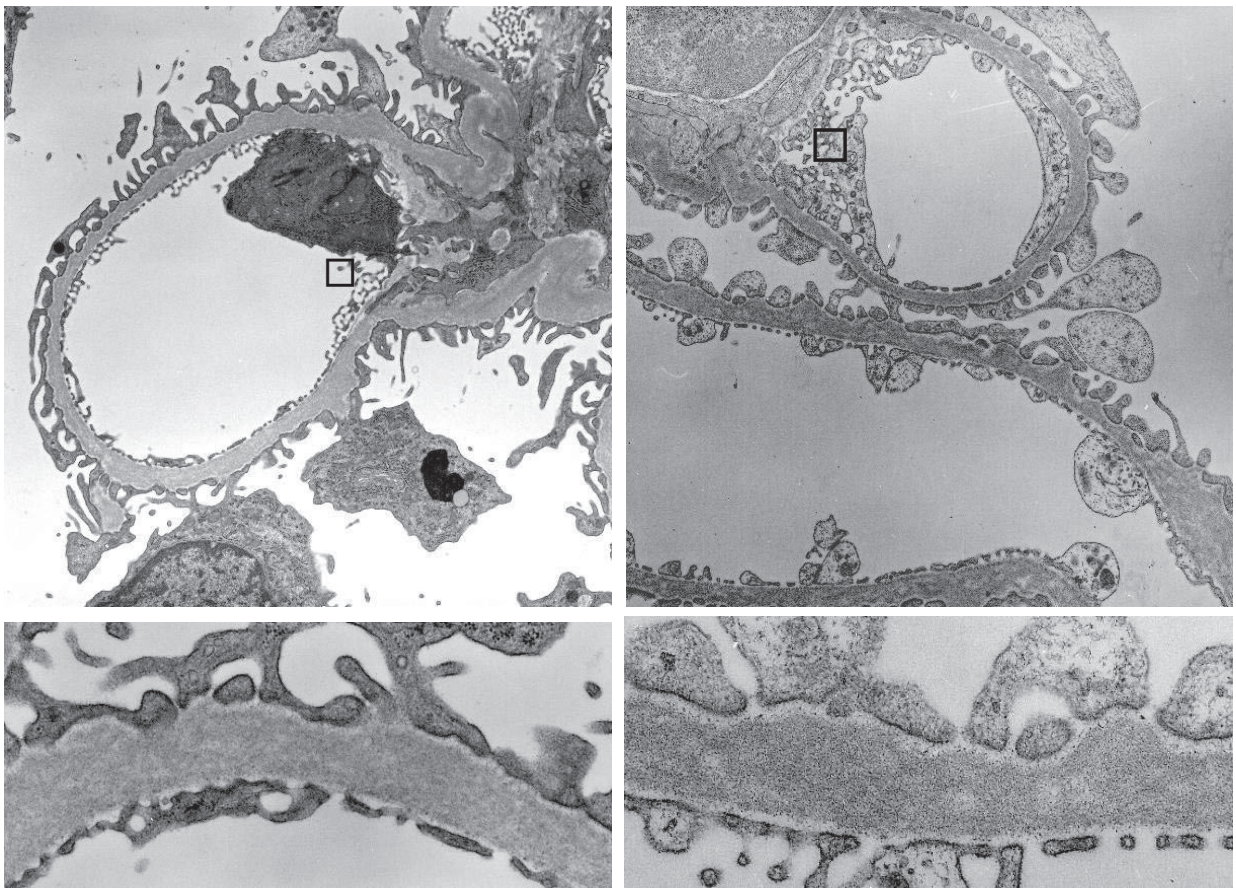

Fig. 6: BALB/c-+/+ 80Ws BALB/c-bm/bm

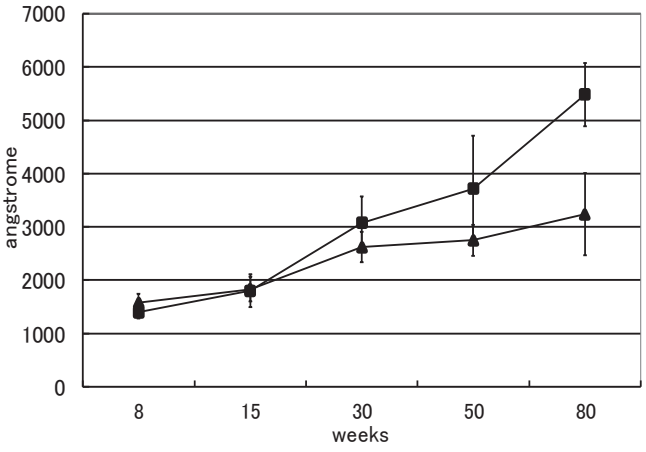

Figure 7: A graph of thickness of glomerular basement membrane (BM) in normal (▲) and brachymorphic (■) mice with ageing.

lamina densa was particularly thickened than lamina rara externa and interna (Fig. 8).

When stained with ruthenium red, ruthenium red-reacted strong positive black particles were regularly distributed in lamina rara interna and externa of renal glomerular basement membrane in normal mice in 15 weeks after birth (Fig. 9, upper left side, arrow heads). In brachymorphic mice in same ages, ruthenium red-reacted weak black particles were irregularly scattered in lamina rara externa and interna of renal glomerular basement membrane (Figs.9, upper right side, arrow heads). In normal mice in 50 weeks after birth, ruthenium red-reacted black particles were existed in lamina rara externa and interna of glomerular basement membrane and a few particles were scattered in to lamina densa (Fig. 9, lower left side, arrow heads). On the other hand, in brachymorphic mice, ruthenium red-reacted black particle indicated exceedingly feeble reaction in broad basement membrane irregularly (Fig. 9, lower right side, arrow heads).

Discussion

Kidney is bean-shaped organ, located in retroperitoneum. In the kidney, there is almost two million of structural and functional unit, called nephron, that consist of renal corpus and renal tubules. In the nephron, plasma in capillary

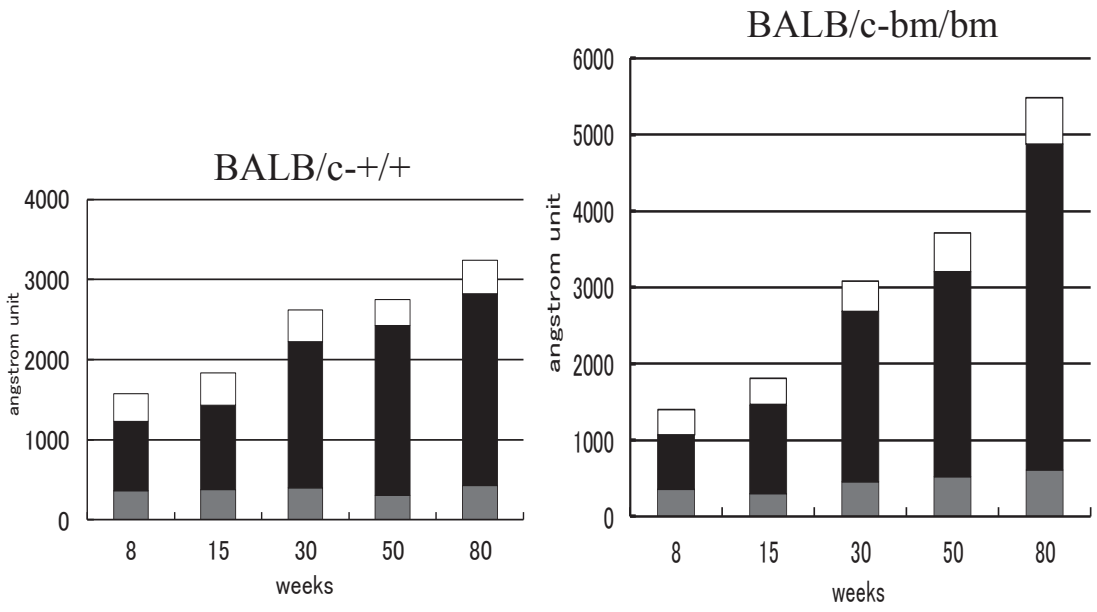

Figure 8: A graph of thickness of sublayers in glomerular BM in normal (left side) and brachymorphic (right side) mice. White: lamina rara externa. Black: lamina densa. Grey: lamina rara interna

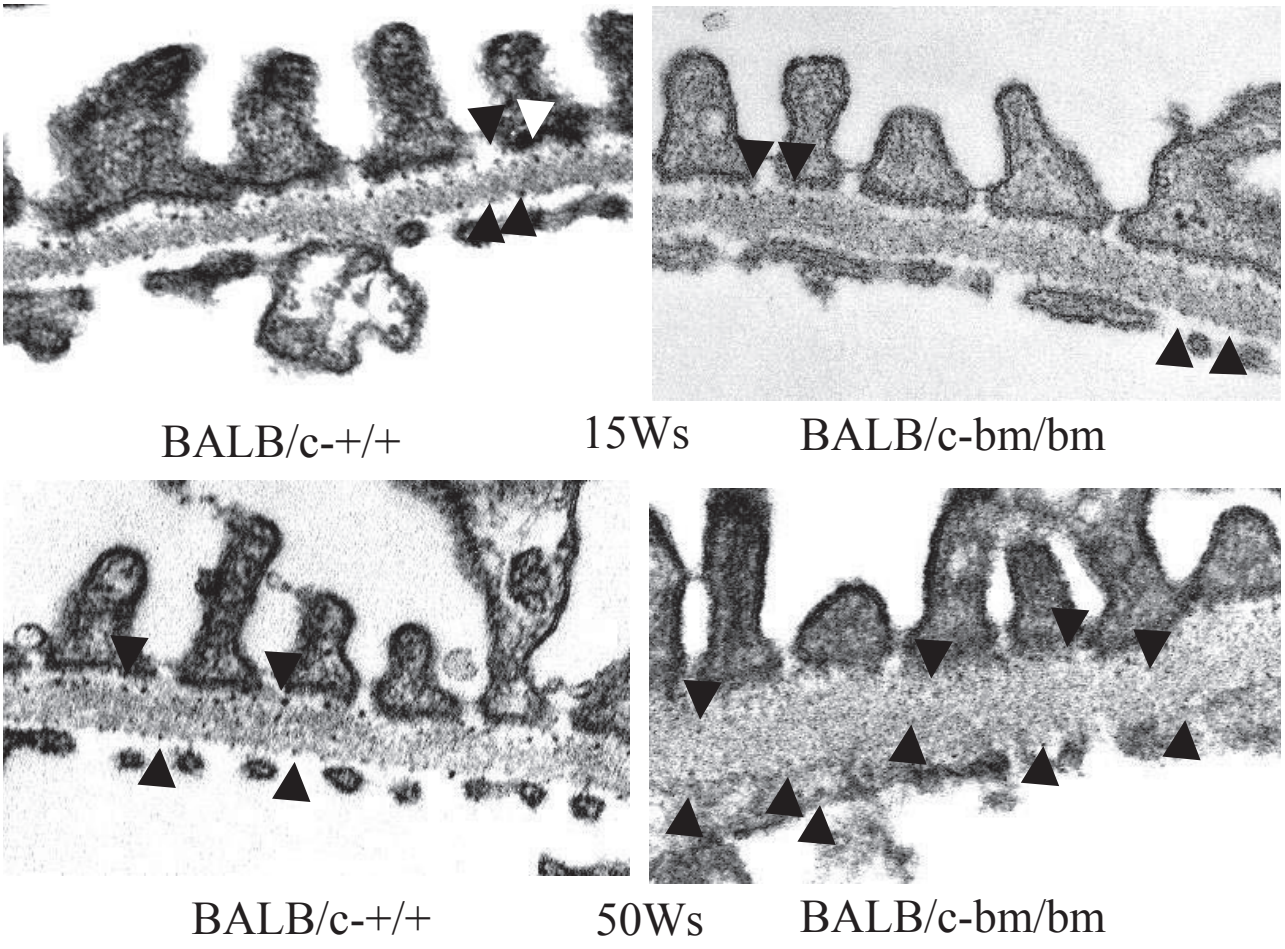

Figure 9: Electron microscopic images of renal glomerular basement membranes at ages of 15 weeks (upper micrographs) and 50 weeks (lower micrographs), stained with ruthenium red. Arrowheads: ruthenium red-positive anionic sites. Magnification of 10,000 folds.

vessels is filtrated to be primitive urine (glomerular filtrate) in renal corpus and then many kinds of materials are resorbed from or secreted to primitive urine in renal tubules, and, finally, urine is produced. Renal corpus is containing renal glomerulus and surrounding Bowman's capsule, lined by capsular epithelium (parietal wall). Moreover, renal glomerulus is composed with vascular endothelial cell, podocyte, mesangial cell and its matrix, and glomerular basement membrane[20]. The glomerulus is the filtration apparatus in the kidney. In the structure of the glomerulus, the vascular side is lined by fenestrated endothelial cells. Then, glomerular capillary vessels are separated from the urinary space by the glomerular basement membrane. Urinary space side of the glomerular basement membrane is lined by epithelial cells, known as podocytes. The whole structure of glomerulus is supported by the mesangial cells and their mesangial matrix[21].

According to McCarthy et al.[22], glomerular basement membrane and its associated cells are critical elements in the renal ultrafiltration process. The anionic charge associated with several carbohydrate moieties in the glomerular basement membrane are thought to form a charge selective barrier that restrict the transmembrane flux of anionic proteins across the glomerular basement membrane into the urinary space. The charge selective function, along with the size selective component of the basement membrane, serves to limit the efflux of plasma proteins from the capillary lumen. Heparan sulfate glycosaminoglycans are known to be anionically charged carbohydrate structures attached to proteoglycan core proteins and have a role in establishing the charge selective function of the glomerular basement membranes.

Aging is associated with structural and functional changes in the kidney. Structural changes include glomerulosclerosis, thickening of the basement membrane, increase in mesangial matrix, tubulointerstitial fibrosis and arteriosclerosis[23]. Other functional changes in aging include an increase in glomerular basement permeability and decreased ability to dilute or concentrate urea. Moreover, a loss of glomerular basement membrane heparan sulfate chains is associated with proteinuria in several glomerular diseases and may contribute to the underlying pathology[24].

Ruthenium red is known to the one of cationic dye and used for research to visualize the anionic site such as heparan sulfate binding site in tissues[14, 15, 25]. In the glomerular basement membrane, ruthenium red positive anionic sites are located in lamina rara with a reticular pattern.

In this study, glomerular basement membrane became gradually thickening with aging. Especially, in brachymorphic mice, thickening in glomerular basement membrane is markedly. Additionally, staining pattern of anionic sites in glomerular basement membrane with ruthenium red was more irregular in brachymorphic mice than in normal mice. The reason of this finding is speculated that anionic barrier is defective and the transmembrane flux of anionic proteins across the glomerular basement membrane into the urinary space is increase, because anionic site of glomerular basement membranes is weaker in brachymorphic mice than in normal mice.

In otherwise, it is thought that glomerular basement membrane was made by endothelial cell and podocyte. According to resent articles, in early glomerular development, both endothelial cell and podocyte are organizing each sides of glomerular basement membrane. However, in adult, podocyte maintains glomerular basement membrane mainly[23, 26]. Resultingly, it is presumed that the pathological findings of glomerular basement membrane in this paper are induced by functional changes of aged glomerular basement membrane organized cells, especially podocyte, in both normal and brachymorphic mice and undersulfated heparansulfate in brachymorphic mice. Additionally, progressive disorder of glomerular basement membrane discussed in this paper thought to induce collapse of renal glomerulus, resulting albuminuria and hematuria.

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