Morphological diagnosis of Alport syndrome and thin basement membrane nephropathy by low vacuum scanning electron microscopy

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ABSTRACT

Alport syndrome (AS) and thin basement membrane nephropathy (TBMN) are genetic disorders caused by mutations of the type IV collagen genes COL4A3, COL4A4, and/or COL4A5. We here aimed to investigate the three-dimensional ultrastructure of the glomerular basement membrane (GBM) in order to introduce a novel method of diagnosing AS and TBMN. The subjects were 4 patients with AS and 6 patients with TBMN. Conventional renal biopsy paraffin sections from AS and TBMN patients were stained with periodic acid methenamine silver (PAM) and observed directly under low vacuum scanning electron microscopy (LVSEM). The PAM-positive GBMs were clearly visible under LVSEM through the overlying cellular components. The GBMs showed characteristic coarse meshwork appearances in AS, and thin and sheet-like appearances in TBMN. At the cut side view of the capillary wall, the GBMs in AS appeared as fibrous inclusions between a podocyte and an endothelial cell, while the GBMs in TBMN showed thin linear appearances. These different findings of GBMs between AS and TBMN were easily observed under LVSEM. Thus, we conclude that three-dimensional morphological evaluation by LVSEM using conventional renal biopsy paraffin sections will likely be useful for the diagnosis of AS and TBMN, including for retrospective investigations.

Alport syndrome (AS) is a genetic disorder caused by mutations of the type IV collagen genes COL4A3, COL4A4, and COL4A5. It is characterized by renal, cochlear, and ocular involvement, and AS patients are moreover predisposed to developing end-stage renal disease (1, 6, 12). On the other hand, thin basement membrane nephropathy (TBMN) results from mutations of the COL4A3 and COL4A4 genes, and is associated with few extra-renal abnormalities and a good renal prognosis. One of the characteristic features of AS and TBMN is morphological changes of the glomerular basement membranes (GBMs). A combination of type IV collagen genes, which produce different constitutions of the alpha chain of collagen type IV, is very important in the construction of normal GBMs. Renal findings under transmission electron microscopy (TEM) of AS include partially thickening, splitting, fragmenting, and basket-weave appearances of the GBMs; whereas TBMN is characterized by a reduction of the thickness of the GBMs. Although the diagnosis of AS or TBMN is generally established by detailed family history, urinalysis, biopsy of the kidney or skin, and molecular genetic analysis, the differential diagnosis is often difficult in both these diseases, owing largely to insufficiency of the biopsy samples and the complexity of molecular genetic analysis. Furthermore, if there are no glomeruli in the biopsy samples for immunofluorescence and TEM analyses,
a diagnosis of AS or TBMN can often not be made. Moreover, while AS is generally accompanied by atypical findings of the type IV collagen alpha chains α5 (IV) and α2 (IV) upon immunofluorescence analysis of renal or skin biopsy samples, a mosaic or normal pattern does not always indicate AS or normal findings, respectively. Furthermore, molecular genetic analyses of the type IV collagen genes COL4A3, COL4A4, and COL4A5 do not always detect mutations in AS patients (13). Therefore, another efficient and helpful method for diagnosing AS and TBMN is urgently needed.

Adding to the TEM observation of GBM structure, conventional scanning electron microscopy (SEM) of acellular glomeruli in human glomerulonephritis, combined with cell extraction techniques, was developed to visualize the surface structure of the GBM that is present between podocytes and endothelial cells (2, 4). Using this technique, some researchers have investigated GBM alterations in a three-dimensional manner (3, 5, 15, 17); however, to our knowledge, the three-dimensional findings of GBMs in AS or TBMN have not yet been reported.

We have previously demonstrated the usefulness of low vacuum scanning electron microscopy (LVSEM) to evaluate the histological findings of glomeruli by using conventional renal biopsy paraffin sections for the rapid three-dimensional analysis of glomerular structure (10, 14, 16). Especially, the GBMs could be visualized through the overlying cellular components without removal, by staining with periodic acid methenamine silver (PAM) and by using the backscattered electron (BSE) mode of LVSEM, owing to the fact that the PAM stain contains silver, a heavy metal, which enhances the BSE signals. In addition, LVSEM enabled us to investigate intact GBMs without the need for cell lysis, and allows detailed and efficient three-dimensional GBM observation of renal biopsy paraffin specimens more easily than conventional SEM.

In the present study, we investigated the three-dimensional morphological findings of GBMs in AS or TBMN using renal biopsy samples and demonstrate the potential use of LVSEM in the diagnoses of both these diseases.

MATERIALS AND METHODS
A total of 4 (3 males and 1 female) and 6 (1 male and 5 females) patients with AS and TBMN, respectively, were identified. The ages of the patients with AS and TBMN ranged from 3 to 16 years (median age: 12.0 years) and 8 to 19 years (median age: 10.0 years), respectively. All patients received renal biopsies at the Tottori University Hospital under the approval of the Ethics Committee of Tottori University (Permission No. 2107), and were diagnosed by pediatric nephrologists based on the histological results. Paraffin sections of 5 μm in thickness were created from all renal biopsy samples according to the standard procedure, and stained with hematoxylin and eosin (H & E), periodic acid-Schiff, and PAM for light microscopy. The diagnosis of AS or TBMN was confirmed by renal immunofluorescence staining of collagen type IV α5 (IV) and α2 (IV) chains. For immunofluorescence staining, the fresh renal tissue samples were placed into optimal cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and cut into 4-μm-thick sections from the OCT compound blocks. Direct immunofluorescence staining (11) was performed at the Shigei Medical Research Institute (Okayama, Japan).

The specimens for the LVSEM observation of the GBM structure were prepared as described previously (9, 10). Briefly, renal biopsy paraffin sections of 5 μm in thickness (same as those prepared for light microscopy) were used. The sections on the slides were deparaffinized with xylene and subsequently transferred to distilled water through an alcohol descending series. Next, the sections were stained with PAM but without following H & E staining. After washing with distilled water for 1–2 min, the sections on the slides were deparaflnized with xylene and subsequently transferred to distilled water through an alcohol descending series. Next, the sections were stained with PAM but without following H & E staining. After washing with distilled water for 1–2 min, the sections on the slides were directly observed by LVSEM without cover slips. Observations were made with LVSEMs (Hitachi TM-1000 or TM3030; Hitachi Co., Ltd., Tokyo) at an acceleration voltage of 15 kV with 30 Pa. For TEM observation of GBM in some biopsy samples, ultrathin epoxy resin sections were created, stained with uranyl acetate and Pb, and observed using a TEM (Hitachi H-7100; Hitachi Co., Ltd., Tokyo) at an acceleration voltage of 70 kV.

RESULTS
Under LVSEM, the three-dimensional ultrastructure of the GBMs of AS (Fig. 1) and TBMN (Fig. 2) were examined in detail. At the lower magnifications (less than ×1,000), it was difficult to distinguish the differences of the GBMs in the paraffin sections of AS and TBMN (Fig. 1a, Fig. 2a), whereas at higher magnifications, differences in the structure and brightness of the GBMs between AS and TBMN were easily detectable. The GBMs showed characteristic coarse meshwork appearances in AS and thin and sheet-like appearances in TBMN.

Figure 1 shows representative LVSEM and TEM
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fibrous inclusions between a podocyte and an endothelial cell at the cut side view of the capillary walls (Fig. 1b, c). The surface appearances of the GBMs observed from the endothelial side showed distinct coarse meshwork structures. Figure 1d shows a TEM image obtained from another AS biopsy sample. In this case, the GBM displayed a basket-weave appearance with partial thickening, splitting, or fragmenting of the lamina densa. The fibrous inclusions or coarse meshwork structures observed under LVSEM were thought to correspond to the lamina densa of the GBMs observed under TEM. Furthermore, focal effacements of foot processes of podocytes were also noted.

Figure 2 shows LVSEM and TEM images of glomeruli observed in AS specimens. Positively PAM-stained GBMs, Bowman’s basement membrane, and the mesangial matrix of the glomeruli were clearly observed in bright appearances when contrasted to PAM-negative cellular components such as podocytes, endothelial cells, and mesangial cells, which were rarely observed under LVSEM (Fig. 1a–c). At higher magnifications (more than ×2,000), LVSEM allowed detailed investigation of the intact subsurface aspects of the capillary walls, which indicated intact GBMs, through the thin parts of the overlying endothelial cells or podocyte foot processes by detecting strong BSE signals from the PAM-positive GBMs (Fig. 1b). The bright GBMs showed irregularities in thickness, and appeared as fibrous inclusions between a podocyte and an endothelial cell at the cut side view of the capillary walls (Fig. 1b, c). The surface appearances of the GBMs observed from the endothelial side showed distinct coarse meshwork structures. Figure 1d shows a TEM image obtained from another AS biopsy sample. In this case, the GBM displayed a basket-weave appearance with partial thickening, splitting, or fragmenting of the lamina densa. The fibrous inclusions or coarse meshwork structures observed under LVSEM were thought to correspond to the lamina densa of the GBMs observed under TEM. Moreover, focal effacements of foot processes of podocytes were also noted.

Figure 2 shows LVSEM and TEM images of glom-
Discussion

In this study, we reported on two important findings of AS and TBMN specimens. First, we found that the GBMs of both AS and TBMN specimens were clearly visible under LVSEM through the overlying of other elements, by detecting the BSE signals from PAM-positive GBMs of both the subepithelial and subendothelial sides. Second, we demonstrated that the GBMs in AS specimens under LVSEM showed characteristic coarse meshwork appearances, whereas those in TBMN specimens had thin and sheet-like appearances; and found that the meshwork structures of the GBMs were not conspicuous in TBMN. These different findings of GBMs between AS and TBMN by LVSEM were obvious and easily demonstrated, and were thought to be useful compared to the eruli obtained from TBMN specimens. The PAM-positive GBMs and Bowman’s basement membrane were also recognized at low magnification (Fig. 2a). At higher magnification, widespread thinning of the sheet-like GBMs was noted (Fig. 2b). However, the meshwork structures of the GBMs observed in some parts of the capillary walls were not very conspicuous, even at higher magnifications (Fig. 2b, c), owing to the lower brightness of the GBMs; while the podocytes, endothelial cells, and mesangial cells of the glomeruli were generally obscure. Unlike AS specimens, almost all GBMs in the TBMN specimens exhibited thin linear patterns at the cut side view. The TEM image of the GBMs in TBMN showed widespread reduction in thickness, but no splitting or fragmenting, and the foot processes of the podocytes had normal appearances (Fig. 2d).
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for the differential diagnoses of these conditions.

The LVSEM revealed the three-dimensional structural features of the GBMs without removing podocytes or endothelial cells. Because the podocytes, endothelial cells, and mesangial cells of the glomeruli were not stained with PAM in the BSE mode of LVSEM, these cellular components showed dark appearances; and the contrasts of the BSE signals between the GBMs and cellular components (podocytes and endothelial cells) contributed to the clear observations of the GBMs in the BSE mode of LVSEM.

Although numerous cross-sectional findings of GBMs in AS under TEM have been reported, reports on the three-dimensional findings of GBMs in AS are rare. GBMs in AS under TEM typically show thickening, splitting, and fragmenting of the lamina densa, with several strands forming a basket-weave pattern (18). In the present study using LVSEM, we found that the GBMs, especially the lamina densa, stained with PAM in AS were distinctly observed in bright appearances, and showed coarse meshwork patterns similar to the TEM findings of the electron-dense lamina. Thus, our results suggest that genetic alterations of collagen type IV in AS lead to three-dimensional morphological changes of collagen type IV in the GBMs, which were shown as a basket-weave pattern under TEM. On the other hand, the three-dimensional morphologies of GBMs in TBMN specimens under LVSEM were distinctly different from those in the AS specimens. The GBMs in TBMN showed thin and sheet-like appearances, unlike the basket-weave pattern observed in AS. These morphological differences of the GBMs can likely be attributed to the genetic differences of collagen type IV between AS and TBMN. Because of the different genetic defects of collagen type IV in AS and TBMN, the GBMs consist of different collagen type IV alpha chains in these disorders, namely α1.α1.α2 (IV) in AS and α3.α4.α5 (IV) in TBMN (8). As different constitutions of alpha chains result in different alterations of the collagen type IV network in GBM (7), this may reflect the different three-dimensional structures of GBMs observed in AS and TBMN. Therefore, the results of the present study suggest that LVSEM is likely useful for the differential diagnoses of AS and TBMN.

However, this study had a number of limitations. Two main issues were that we were unable to assess the correlation between the LVSEM findings of the GBMs and the clinical features of the cases, and that we were unable to evaluate the changes of the glomerular cellular components. Regarding the former issue, analyses of the patient age, sex, hematurnia or proteinuria status, and presence of genetic disorders such as X-linked or autosomal recessive or autosomal dominant diseases, could not be performed because of the small sample sizes. Regarding the latter issue, the podocytes and endothelial cells were not estimated because they were PAM-negative elements. Fortunately, we have previously developed the Pt-blue staining method to reveal the morphological changes of glomerular cellular components under LVSEM (10, 16), and we hope that further studies will be performed in the future to address these issues.

In conclusion, the present evaluation method of three-dimensional structural alterations in GBMs by LVSEM using conventional paraffin sections may represent a novel approach to the histological diagnosis of AS and TBMN, including for retrospective investigations. If there are no glomeruli present in the dedicated biopsy sections for immunofluorescence and TEM analyses, the LVSEM method using paraffin sections with sufficient glomeruli could be essential for the diagnosis of AS and TBMN.

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