A novel approach to the histological diagnosis of pediatric nephrotic syndrome by low vacuum scanning electron microscopy

Shinichi O KADA1, Sumire INAGA2, Yasuo KAWABA1, Takuya HANADA1, Atsushi HAYASHI1, Hironobu NAKANE2, Tomonori NAGURO2, Toshiyuki KAIYOH2, and Susumu KANZAKI1

1 Division of Pediatrics and Perinatology and 2 Department of Anatomy, Faculty of Medicine, Tottori University

(Received 21 April 2014; and accepted 6 May 2014)

ABSTRACT

Despite intensive treatment, steroid-resistant nephrotic syndrome (NS) often progresses to end-stage renal disease. Therefore, a more accurate and quick histological diagnosis is required to properly treat such patients. The aim of this study was to introduce a novel approach to the histological diagnosis of pediatric NS by low vacuum scanning electron microscopy (LVSEM) and to describe the morphological differences in glomeruli between steroid-sensitive and steroid-resistant NS specimens. The subjects were three patients with steroid-sensitive NS and four patients with steroid-resistant NS. Conventional renal biopsy paraffin sections were stained with platinum-blue (Pt-blue) or periodic acid methenamine silver (PAM) and directly observed under LVSEM at magnifications between ×50 and ×10,000. The Pt-blue-stained sections showed three-dimensional structural alterations in glomerular podocytes and foot processes. PAM-stained sections showed changes in the structure and thickness of the glomerular basement membrane (GBM). Consequently, many round-shaped podocytes and elongated primary foot processes were exclusively recognized in steroid-resistant NS, although irregularities in foot process interdigitation, fusions, effacements, and microvillus transformations were observed in both steroid-sensitive and steroid-resistant NS. Irregularities in thickness and the wrinkling of GBMs were clearly detected in steroid-resistant NS. The evaluation by LVSEM is probably useful for the renal histological diagnosis of pediatric NS.

Nephrotic syndrome (NS) comprises proteinuria, hypoalbuminemia, hypercholesterolemia, and edema. Pediatric patients with NS are usually treated with corticosteroids (13). Patients who respond to steroids (steroid-sensitive NS) have good clinical outcomes, whereas patients who do not respond to steroids (steroid-resistant NS) are predisposed to the development of end-stage renal disease. Histopathologically, most pediatric patients with steroid-sensitive NS show minor glomerular abnormalities, and patients with steroid-resistant NS generally have focal segmental glomerulosclerosis (FSGS) (25). Under light microscopy (LM), minor glomerular abnormalities are defined as a slight or no increase in the mesangial matrix or cellularity without focal segmental glomerular collapse, scarring, endocapillary proliferation, or adhesions. FSGS shows focal and segmental glomerular collapse, scarring, endocapillary proliferation, or adhesions of glomeruli with minimal abnormalities (21). However, it is not clear whether the glomeruli with no obvious changes in FSGS are different from those in minor glomerular abnormalities. In addition, it is difficult to diagnose FSGS when the NS biopsy section does not include glomeruli with the typical alterations of FSGS described above, although the differential diagnosis between minor glomerular abnormalities and FSGS is very

Address correspondence to: Shinichi Okada, MD, PhD Division of Pediatrics and Perinatology, Faculty of Medicine, Tottori University, 36-1 Nishi-cho, Yonago City, Tottori, Japan
Tel: +81-859-38-6557, Fax: +81-859-38-6559
E-mail: sokada@med.tottori-u.ac.jp
important in pediatric cases of NS.

The typical renal histological findings of NS by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are podocyte foot process fusions, effacements, and microvillus transformations (23), although these features cannot be elucidated under LM due to resolution limitations. TEM is superior to investigate cross-sectional images not only of podocytes but also of other components in glomeruli; however, performing three-dimensional and whole-wide observations using ultra-thin sections is difficult due to the limitation of the observable size. Therefore, with TEM, focal glomerular abnormalities may be missed in some cases. SEM is suitable for the three-dimensional observation of surface alterations of podocytes as processing is easier and shorter with SEM than with TEM (1, 2, 11, 16, 19, 24, 28). In addition, SEM of acellular glomeruli in human glomerulonephritis was developed to visualize the surface structure of the glomerular basement membrane (GBM) that is present between podocytes and endothelial cells (4, 6). Using this technique, some researchers have investigated GBM alterations and gap formations in a three-dimensional manner (5, 7, 8, 20, 26). Despite such advantages, however, conventional SEM is not currently utilized for the pathological diagnosis of renal biopsy specimens, because dedicated preparation techniques and equipment are required. On the other hand, Phillips et al. (22) reviewed new methodologies such as two-photon microscopy that optimize three-dimensional, multicolor imaging and single-cell segmentation of glomerular components in renal biopsy specimens to facilitate exploration of glomeruli.

We previously demonstrated the usefulness of low vacuum scanning electron microscopy (LVSEM) for the rapid three-dimensional analysis of renal biopsy samples by using conventional paraffin sections to evaluate the histological findings of glomeruli (9, 17). Vacuum condition is set at tens to hundreds Pa in the LVSEM chamber, although it is 10−3 Pa or higher in the conventional SEM. The advantages of the LVSEM approach include the ability to easily conduct overviews of whole renal paraffin sections at low magnifications typical of LM observations and detailed three-dimensional analysis of every glomerulus in a section with a high resolution close to that of TEM. The observable area of a biopsy sample is not limited under LVSEM, unlike with conventional TEM. Accordingly, LVSEM allows detailed and efficient three-dimensional whole-wide observation of renal biopsy specimens more easily than LM, TEM, or conventional SEM. In the backscattered electron (BSE) mode of LVSEM, glomerular components such as podocytes, endothelium, mesangium, and GBM can be distinguished and investigated by staining with platinum-blue (Pt-blue) or periodic acid methenamine silver (PAM), because both stains contain a heavy metal salt for enhancing the BSE signal (10). Both the surface and the subsurface structures of renal glomeruli can be observed with a high resolution of up to ×10,000 (9).

In the present study, we introduce a novel approach to the histological diagnosis of pediatric NS by LVSEM and describe the three-dimensional morphological findings of glomeruli in pediatric steroid-sensitive NS and steroid-resistant NS, especially podocytes, foot processes, and GBM.

MATERIALS AND METHODS

We used renal biopsy tissue samples obtained from seven pediatric NS patients (three steroid-sensitive NS cases and four steroid-resistant NS cases) that had been already diagnosed at Tottori University Hospital as minor glomerular abnormalities or FSGS, under the approval of the Ethics Committee of Tottori University (Permission No. 1093). The samples were prepared using paraffin sections for the LVSEM, introduced by Inaga et al. (9), and the conventional TEM method.

Tissue preparation and light microscopy. The renal biopsy tissue samples were fixed with a 10% neutral buffered formalin solution and embedded in paraffin, using the standard method. Each paraffin block was cut into thin sections (5–10 μm) and mounted on standard-sized glass slides (76 mm × 26 mm). The sections on the slides were deparaffinized with xylol and transferred to distilled water after passing through a graded alcohol series. After deparaffinization, some of the renal sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS), using the conventional staining method for LM observation.

Low vacuum scanning electron microscopy. The deparaffinized renal sections in each case were stained with Pt-blue to observe the surface structure of glomeruli or with PAM to observe GBMs. A Pt-blue aqueous solution was adjusted to pH 9 (TI-blue staining kit: Nisshin EM Co. Ltd., Tokyo, Japan) and placed on the renal sections for 10–15 min at room temperature. After washing with distilled water for 1–2 min, renal sections on the slides were observed
LVSEM observation of nephrotic syndrome

by LVSEM without cover slips. Some of the Pt-blue-stained sections were coated with platinum in an ion sputter (E-1030; Hitachi Co. Ltd., Tokyo, Japan) to observe the surface structure of podocyte foot processes more clearly, after the first LVSEM observation. PAM staining was performed using the conventional method for LM observation without the final HE staining process. The stained sections on the slides were directly observed and photographed with LVSEM (Hitachi Miniscope TM-1000 and TM3000; Hitachi Co., Ltd., Tokyo) at 30 Pa and an acceleration voltage of 15 kV, using the charge-up reduction BSE mode.

Transmission electron microscopy. We prepared ultrathin epoxy resin sections of several biopsy samples obtained from the same steroid-sensitive NS and steroid-resistant NS cases using the conventional method. They were fixed with a 2.5% glutaraldehyde solution and a 1% OsO₄ solution, embedded in epoxy resin, ultrathin sectioned, stained with uranyl acetate and lead citrate, and observed under TEM (Hitachi H-7100; Hitachi Co., Ltd.) at an acceleration voltage of 70 kV.

RESULTS

We observed the morphological alterations of glomeruli in renal biopsy sections of pediatric patients with steroid-sensitive NS and steroid-resistant NS under LM, LVSEM, and TEM. Figure 1 shows LM images of glomeruli from steroid-sensitive NS (Fig. 1a) and steroid-resistant NS (Fig. 1b) specimens stained with PAS. Glomeruli in both specimens showed similar findings with regard to minor glomerular abnormalities. This steroid-resistant NS specimen showed no FSGS. Figure 2 shows LVSEM images of two serial biopsy sections of steroid-sensitive NS specimens stained with Pt-blue (Fig. 2a) or PAM (Fig. 2c) observed at low magnification (×50). All glomeruli in each section could be observed in a broad range of specimens. Each glomerulus appeared as a different image due to variations in BSE signal brightness that depended on the stain. A complete sectioned renal glomerulus is shown at a magnification of approximately ×1,000 under LVSEM (Fig. 2b, d). The cellular constituents of glomeruli were well distinguished by staining with Pt-blue (Fig. 2b), whereas the GBM and the mesangial matrix were distinctly detectable with PAM staining (Fig. 2d). Every glomeruli component, especially podocytes, endothelial cells, and GBM, was investigated at higher magnifications of up to ×10,000. Figures 3 and 4 show three-dimensional images of Pt-blue-stained glomeruli in steroid-sensitive NS and steroid-resistant NS, respectively. The cut surface view of a glomerulus in steroid-sensitive NS showed no hypercellularity, and the glomerular capillary lumens were fully patent (Fig. 2b, 3a). Podocytes, endothelial cells, and mesangial cells appeared bright, whereas the GBM and mesangial matrix appeared dark. In Bowman’s space, surface structures of podocytes and the interdigitation of foot processes were observed three-dimensionally (Fig. 3a–d). Podocytes from surface views had normal alignments of primary and secondary foot processes, but had disruptions of interdigitation of foot processes in part. Irregular width of secondary foot processes of podocytes, shortening changes of foot processes, and unclear margins of each secondary foot process

Fig. 1 Light microscopy images of renal glomeruli stained with PAS in steroid-sensitive NS (a) and steroid-resistant NS (b) specimens with a direct magnification of ×400. There were no significant pathological findings in glomeruli with minor abnormalities from both specimens. The section observed in this case of steroid-resistant NS (b) shows no focal segmental glomerulosclerosis. Bars: 50 μm
Figure 5c and 5d shows the parts of other glomeruli from steroid-resistant NS specimens. Protruded and round-shaped podocyte cell bodies and detachments of podocytes from the GBM were also noted. The changes of podocyte foot processes were elongated primary processes and retraction of secondary processes.

Figure 5 shows Pt-blue-stained glomeruli of steroid-resistant NS specimens that had been diagnosed as FSGS. Most of the glomerular capillary lumina were occluded in these sections. The characteristic findings in FSGS specimens were many round-shaped podocyte cell bodies (Fig. 5a, b: arrows), and an adhesion of glomerular tufts to Bowman’s capsule (Fig. 5a: arrowhead). Podocytes and their foot processes were clearly visible at a higher magnification. Figure 5c and 5d shows the parts of other glomeruli from steroid-resistant NS specimens. Protruded and round-shaped podocyte cell bodies and detachments of podocytes from the GBM were also noted. The changes of podocyte foot processes were elongated primary processes and retraction of secondary processes.

In PAM-stained specimens, positively stained GBMs and mesangial matrix of glomeruli were clearly observed under LVSEM in each steroid-sensitive NS (Fig. 6a, c) and steroid-resistant NS (Fig. 6b, d) section. PAM-negative cellular components such as podocytes, endothelial cells, and mesangial cells of glomeruli showed dark appearance, and podocyte foot processes were indistinguishable. LVSEM at a higher magnification allowed detailed investigation of the intact aspects of GBMs through the overlying of other elements by detecting the BSE signals from PAM-positive GBMs of both the subepithelial side and the subendothelial side. Irregularities in thickness and the wrinkling of GBMs were clearly detected in several steroid-resistant NS glomeruli (Fig. 6d), while steroid-sensitive NS GBMs had almost uniform and thinner appearances (Fig. 6c) under LVSEM.
Figure 7 shows the TEM images obtained from the same biopsy sample shown in Figure 6b and 6d from a steroid-resistant NS patient diagnosed as having FSGS. The fusions, effacements, and flattening of foot processes of podocytes and the detachments of podocytes from the GBM were observed in a glomerular capillary wall segment (×2,500).

DISCUSSION

We used LVSEM for the histological diagnosis of pediatric NS in this study. The novel approach using LVSEM enabled the investigation of three-dimensional structural alterations of glomerular podocytes and GBMs in conventional renal biopsy paraffin sections obtained from pediatric NS patients. The advantages of the present method are that the preparation is very simple and easy, and the differential stainability of glomerular components with Pt-blue or PAM enables the complementary observation of renal biopsy sections under LVSEM with a high resolution close to that of TEM. Because we placed slides with wet sections in the LVSEM chamber, non-dried sections were gradually dried even in the low vacuum condition. Nevertheless, we confirmed that there was no serious deformation of the glom-
In the present study, the LVSEM findings of glomeruli differed between the steroid-sensitive NS and steroid-resistant NS specimens, especially in cases with FSGS. At a magnification of $\times1,000$ under LVSEM, protruding round-shaped podocyte cell bodies were distinctly observed in steroid-resistant NS specimens, although changes of podocyte cell bodies were not conspicuous in steroid-sensitive NS specimens. The three-dimensional recognition of a...
Evaluation of the morphological changes of podocytes is important for understanding the pathogenesis of NS. Normal podocytes attached to GBMs have flat-shaped cell bodies. The effaced foot processes of podocytes seen in NS are associated with detachments of the podocytes themselves from the GBM (27). Once the podocytes are damaged for any reason, they become detached from the GBM without recovery. Damaged podocytes have shown morphological changes (18), and therefore, detachment of the damaged podocytes from the GBM may cause the podocyte cell body shape to change to a rounded shape. A uninephrectomy model which leads to FSGS showed enlargement and attenuation of podocyte cell bodies after local detachment of podocytes from the GBM in those areas (18). In the present study, the round-shaped podocyte cell bodies were observed using LVSEM, and the detachment of podocytes from the GBM was observed using TEM. Although our findings of round-shaped podocyte cell bodies seem to be different from those of the wide range of pathological findings of podocytes at a relatively lower magnification is one of the advantages of LVSEM (9). At a higher magnification, various morphological alterations of foot processes of podocytes, so called “foot process effacements or fusions” and “microvillus transformations” were detected in both steroid-sensitive NS and steroid-resistant NS specimens. These findings correspond to the previous TEM findings observed for minor glomerular abnormalities. In addition to these findings, widespread microvillus transformations over most glomerular surfaces, elongations of primary foot processes, and many round-shaped podocytes were protruded under LVSEM in steroid-resistant NS patients diagnosed as having FSGS. It is difficult to discriminate glomeruli with no obvious changes in minor glomerular abnormalities from those with no sclerosis in FSGS using LM. The present LVSEM findings suggest the morphological differences in minor glomerular abnormalities between steroid-sensitive NS and steroid-resistant NS specimens.
podocytes with pseudocysts which was found by using TEM and SEM in previous studies (14–16, 25), our results support the idea that damaged podocytes undergo cell-body morphological changes.

The present results on podocyte alterations observed by LVSEM are also similar to the findings of previous reports. Kuusniemi et al. (15) reported pathological findings in congenital NS of the Finnish type (NPHS1), which comprises extreme proteinuria and progresses to end-stage renal disease; SEM revealed protruded podocyte cell bodies with microvillus degeneration, elongated primary processes, and foot process effacement. In puromycin aminonucleoside (PAN) nephrosis, an experimental model of NS in which podocyte injury is caused by PAN administration, severe proteinuria and podocyte foot process effacements develop (27). Kriz et al. (14) described the various structural responses of podocytes to stress and injury, focusing on foot process effacement and detachment. In our clinical data, patients with steroid-resistant NS had more intense proteinuria and hypoalbuminemia than patients with steroid-sensitive NS. Accordingly, the present morphological findings determined by LVSEM suggest that many round-shaped podocytes and elongations of primary foot processes are possibly correlated with the clinical states of NS.

Changes in podocyte slit diaphragm proteins are also known to cause proteinuria. Nephrin deficiency leads to NPHS1 with massive proteinuria; podocin, CD2-associated protein, or α-actinin-4 deficiency also leads to proteinuria. Indeed, abnormalities in podocyte slit diaphragm proteins have been found in NS cases (29). Reductions in podocyte slit diaphragm proteins caused morphological changes in podocyte foot processes and result in proteinuria. Furthermore, abnormalities in slit diaphragm proteins in podocyte foot processes caused cytoskeletal changes in podocytes (12). A previous study showed that signals between slit diaphragm proteins have an important role in the regulation of podocyte function, survival, and actin remodeling (3). Morphological changes in the foot processes of podocytes have been correlated with changes in podocyte cell bod-
LVSEM observation of nephrotic syndrome

In conclusion, we considered that the evaluation method of three-dimensional structural alterations in glomerular podocytes and GBMs by LVSEM is probably useful as a novel approach to the histological diagnosis of renal biopsy paraffin sections in pediatric NS.

Acknowledgements

This work was supported by a grant from the Japan Society for the Promotion of Science (a Grant-in-Aid for Scientific Research Number 23591568).

REFERENCES


