Rapid three-dimensional analysis of renal biopsy sections by low vacuum scanning electron microscopy

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Summary. Renal biopsy paraffin sections were examined by low vacuum scanning electron microscopy (LVSEM) in the backscattered electron (BSE) mode, a novel method for rapid pathological analysis which allowed detailed and efficient three-dimensional observations of glomeruli. Renal samples that had been already diagnosed by light microscopy (LM) as exhibiting IgA nephropathy, minor glomerular abnormalities, and membranous glomerulonephritis (GN) were rapidly processed in the present study. Unstained paraffin sections of biopsy samples on glass slides were deparaffinized, stained with platinum blue (Pt-blue) or periodic acid silver-methenamine (PAM), and directly observed with a LVSEM. Overviews of whole sections and detailed observations of individual glomeruli were immediately performed at arbitrary magnifications between ×50 to ×18,000. Cut surface views and surface views of glomeruli were demonstrated at the same time. On Pt-blue-stained sections, podocytes, endothelia, mesangium, and glomerular basement membranes (GBMs) could be distinguished due to the different yields of BSE signals, and pathological features were investigated in every sample. The abnormal surface appearances of podocytes with foot processes and the varying thicknesses of GBM were revealed three-dimensionally, features difficult to observe under LM and transmission electron microscopy. PAM-positive GBM alterations in membranous GN were distinctly visualized through overlying cells without cell removal under LVSEM at high magnification. Not only prominent spike formation but also slight protrusions were clearly revealed in the side views of GBM. Crater-like or hole-like structures were shown in the en face views of GBM. Accordingly, LVSEM is expected to provide a novel approach to the pathological diagnosis of human glomerular diseases using conventional renal biopsy sections.

Introduction

The evaluation of renal biopsies for definitive diagnoses, especially in human glomerular disease, has been conventionally performed by light microscopy (LM) and transmission electron microscopy (TEM). However, disadvantages with the use of TEM include long processing times, sample size limitations, and difficulty in obtaining three-dimensional information. Although the rapid processing of TEM has been available for renal biopsy samples, special handling and extra costs have been required (Johannessen, 1973; Swain et al., 1982; Schrier, 2007). As for the three-dimensional morphological analysis of the human kidney by TEM,
a computer-aided reconstruction technique using serial sections has been employed (Wen and Yamanaka, 1993). This technique, however, requires a significant amount of labor. In contrast, scanning electron microscopy (SEM) has provided information about the three-dimensional morphology of the glomerulus—such as surface alterations of podocytes and endothelial cells—with processing that is easier and shorter than that of TEM (Arakawa, 1971, Arakawa and Tokunaga, 1972; Jones, 1977; Tarpley and Williams, 1980; Yorioka 1980; Ng et al., 1982; Lahdenkari et al., 2004). However, investigation of the glomerular basement membrane (GBM), which is altered in response to subepithelial deposits in GN, has been difficult using conventional SEM in the secondly electron (SE) mode due to overlying cells. In response, Bonsib (1984, 1985a-c) adapted cell extraction procedures (Carlson and Kenney, 1980, 1982; Carlson and Chatteyee, 1983) to visualize the actual three-dimensional ultrastructure of the GBM of acellular glomeruli in various human GN with SEM. Some investigators have demonstrated that acellular SEM observations are reliable for detecting GBM alterations occurring at various stages of subepithelial immune complex formations and in GBM gap formations (Bonsib, 1985b; Bonsib and Plattner, 1986; Weidner and Lorentz Jr, 1986; Nishimura et al., 1989; Hidaka et al., 1990). In spite of these advantages, however, SEM is not currently utilized for the pathological diagnosis of renal biopsies.

In contrast, by means of low vacuum scanning electron microscopy (LVSEM), we have improved the approach for observing biological specimens with the use of a platinum blue (Pt-blue) staining method in the backscattered electron (BSE) mode (Tanaka and Inagaki, 1993; Tanaka et al., 1997, 1998; Inaga et al., 2002). We demonstrated that not only the surface structures but also the subsurface structures of specimens can be studied using the BSE mode of LVSEM, if the subsurface structures are stained with heavy metals such as platinum (Pt), silver (Ag), and others. Pt-blue is a deep blue aqueous complex prepared from the reaction of cis-Pt with thymidine that has actually been available as an aqueous complex prepared from the reaction of cis-Pt with thymidine that has actually been available as an alternative to uranyl acetate for heavy metal staining in TEM (Inaga et al., 2007). Furthermore, we introduced a simple and useful method for direct observation of histological paraffin sections by LVSEM with high resolution up to ×10,000 by utilizing the differential stainability of cells and tissues with Pt-blue (Inaga et al., 2009a). Consequently, individual cells and tissues in paraffin sections obtained from the rat tongue were able to be clearly distinguished and investigated under LVSEM due to the different yields of BSE signals that vary with surface structure details and different affinities to Pt-blue. In renal biopsy samples, periodic acid silver-methenamine (PAM) staining has been routinely used for LM and TEM to obtain pathological information about the GBM. We anticipated that LVSEM would allow the three-dimensional detailed analysis of renal biopsy paraffin sections that were stained with Pt-blue and PAM because both stains contain a heavy metal salt for enhancing the BSE signal (Inaga et al., 2009b).

In the present study, we demonstrate that LVSEM is useful for the rapid three-dimensional pathological analysis of human glomerular disease, including not only the glomerular surface structure but also alterations of the GBM.

Materials and Methods

Renal biopsy samples that had been already diagnosed with glomerular disease using LM at Tottori University Hospital were prepared using the LVSEM method for paraffin sections introduced by Inaga et al. (2009a) under the approval of the Ethics Committee of Tottori University (Permission No. 1093). Biopsy samples obtained from nine cases that included three different types of glomerular disease (three each cases of IgA nephropathy, minor glomerular abnormalities, and membranous GN) were first fixed in a 10 % formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Tokyo) and embedded in paraffin for LM, after which each paraffin block was cut into thin sections (5–10 μm) and mounted on glass slides. The sections on the slides were deparaffinized with xylol and alcohol and transferred to distilled water. They were then stained with a Pt-blue solution—adjusted to pH 9 by adding a small volume of ammonia solution (TI-blue staining kit: Nishhin EM Co. Ltd., Tokyo)—for 10–15 min at room temperature and washed in distilled water for 1–2 min. Some other deparaffinized sections obtained from two cases of membranous GN samples were stained only with PAM for GBM observation according to the conventional PAM staining method. After washing in distilled water, each slide with a wet section was put on a sample stub using a piece of carbon tape and placed in the LVSEM chamber. The stained sections on the slides were directly observed in a drying state without metal coating and photographed with LVSEMs (Hitachi Miniscope TM-1000 and TM3000; Hitachi Co., Ltd., Tokyo) at 30 Pa and an acceleration voltage of 15 kV with the charge-up reduction BSE mode. To examine the artifact produced by drying in the LVSEM chamber, several sections were freeze-dried using t-butyl alcohol before observation. The LVSEM used in the present study had a wide low
LVSEM image of a whole section stained with Pt-blue. Every glomerulus (arrows) in the section can be observed under LVSEM as well as LM. b, c, d: Higher magnification of glomeruli obtained from membranous GN sections unstained (b), stained with Pt-blue (c), and stained with PAM (d). Each glomerulus appears as a differently contrasted BSE image depending on the stain. Although it is difficult to distinguish glomerular constituents in the unstained section (b) due to the low image contrast, the entire structure of the glomerulus is clearly revealed in the Pt-blue stained section (c) with moderate contrast. PAM-positive components such as GBM and collagen fibers are distinctly demonstrated with high contrast in the PAM-stained section (d). Bars = 500 μm (a), 50 μm (b–d).

vacuum chamber accommodating a whole glass slide of the standard size (76 mm × 26 mm) without requiring its cutting into smaller pieces. For a case of membranous GN, ultrathin epoxy resin sections of the biopsy sample were made, stained with uranyl acetate and lead citrate, and observed with a TEM (Hitachi H-7100; Hitachi Co., Ltd., Tokyo) at an acceleration voltage of 70 kV.

Results

Three-dimensional observation of renal biopsy paraffin sections containing several glomeruli was performed under LVSEM at arbitrary magnifications between ×50 to ×18,000. Figure 1 shows a low-magnification LVSEM image of a whole section stained with Pt-blue. Every glomerulus in a section could be individually investigated in detail under LVSEM. The higher magnification images of glomeruli stained with Pt-blue and PAM were compared with the unstained one (Fig. 1b). Each glomerulus appeared as a different image due to variations in the BSE signal brightness that depended on the stain. The cellular constituents of glomeruli were well-distinguished with Pt-blue, while the GBM and the mesangial matrix were distinctly detectable with PAM, although each constituent was difficult to distinguish in the unstained section. The structure of glomerular tufts observed in the current section was compared with that freeze-dried before LVSEM observation (Fig. 2). Erythrocytes and podocyte foot processes were clearly
observed, showing similar three-dimensional LVSEM images in both sections stained with Pt-blue (Fig. 2a, c). Also, PAM-positive GBM showed almost similar bright BSE images in both sections stained with PAM (Fig. 2b, d). There was essentially no appearance of any serious deformation of the glomerulus fine structure that might be produced by drying in the low vacuum chamber. Cut surface views and surface views of glomeruli were often observed at the same time in a paraffin section. The thickness of the GBM was determined on the cut surface image using the LVSEM measurement function. Pathological features of the external surface and the internal cut surface of glomerular tufts in each stained sections were investigated in detail.

**Pt-blue-stained sections**

In the sections stained with Pt-blue (Fig. 3–5), podocytes, endothelial cells, and mesangial cells were bright under LVSEM while GBM and the mesangial matrix were slightly dark. Alterations in a podocyte and the glomerular capillary wall could be investigated in all three types of glomerular disease. Figure 3 shows representative images obtained by the present method using an IgA nephropathy

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**Fig. 2.** Comparison of the fine structure of glomerular tufts between freeze-dried (a, b) and not dried specimens (c, d) before LVSEM observation. a, c: Pt-blue-stained sections of minor glomerular abnormalities. Note the erythrocytes (white arrows) and glomerular foot processes (black arrows). They display almost similar three-dimensional appearances in both sections. b, d: PAM-stained sections of membranous GN. PAM-positive GBM (arrows) shows a similar bright BSE image in both sections. Bars = 5 μm (a–d).
Fig. 3. LVSEM images of Pt-blue-stained glomeruli in IgA nephropathy. a: Cut surface view of a glomerulus. A slight lobulation of glomerular tufts is recognized. b: Surface view of a part of a glomerulus. Granular or protuberant structures (small black arrows) are observed on podocytes. Leaking red blood cells (white arrows) are occasionally found in the Bowman's space. c: Cut surface of glomerular tufts observed at a higher magnification. Mesangial regions are shown as slightly dark portions of the glomerulus. GBM (arrows) are shown as dark curved bands. Inset shows the GBM with an irregular thickness (57.6 nm, 206 nm and 232 nm). E: endothelial cell, L: capillary lumen, M: mesangial region, P: podocyte. Bars = 50 µm (a), 5 µm (b, c)
Fig. 4. LVSEM images of Pt-blue-stained glomeruli in minor glomerular abnormalities. a: Cut surface view of a glomerulus. The glomerular capillary lumens are fully patent. b: Higher magnification of the glomerulus. GBM (arrows) is recognized as dark curved bands with 129-213 nm in thickness. The surface of a podocyte (P) showing a rough appearance (asterisk) and the interdigitiation of foot processes (open arrow) are observed from the Bowman's space (Bs). An erythrocyte is seen inside the capillary lumen (L). c: Higher magnification of the podocyte surface indicated by the asterisk in b. Many small protrusions (arrowheads) are recognized. d: High magnification of the interdigitating foot processes of various thicknesses (direct magnification is \( \times 18,000 \)). Bars = 50 \( \mu m \) (a), 5 \( \mu m \) (b), 1 \( \mu m \) (c, d).

Fig. 5. Higher magnifications of the Pt-blue-stained glomerular tufts in membranous GN shown in Figuer 1c. a: The surface appearance of podocytes facing Bowman's space and the cut surface of glomerular tufts are observed three-dimensionally at the same time under LVSEM. The granular surface appearance of podocytes (open arrow) and entirely thickened capillary walls (arrows) are recognized in this section. b: Cut surface view of a thick glomerular capillary wall observed at high magnification (direct magnification is \( \times 10,000 \)). A thick capillary wall is recognized as consisting of three layers of different brightnesses, the result of different affinities to Pt-blue. The bright outer layer, the dark middle layer, and the bright inner layer correspond to foot processes of podocytes, the GBM, and endothelial cells, respectively. Inset shows the thicknesses of the GBM (868 nm) and capillary wall (2.10 \( \mu m \)). Subepithelial immune deposits (arrows) are barely recognized within the middle layer. c: TEM image of a thick glomerular capillary wall obtained from the same biopsy sample. The dense deposits (arrows) correspond to the subepithelial immune deposits in b. E: endothelial cell, L: capillary lumen, M: mesangial region. P: podocyte. Bars = 20 \( \mu m \) (a), 2 \( \mu m \) (b, c).
Fig. 5. Legend on the opposite page.
Fig. 6. LVSEM images of PAM-stained glomerular sections obtained from two different cases of membranous GN. PAM-positive GBM and collagen fibers around Bowman's capsule and urinary tubules appear brightly demonstrated in both sections. 

a: Case 1. GBM in the thickened capillary wall shows the prominent spike formation (arrows).

b: Case 2. Mildly thickened GBM shows no spike formation with smooth sheet-like appearances of en face views (arrows). Bars = 50 μm (a, b).
sample. Here, glomerular tufts were slightly lobulated judging from the cut surface view of a glomerulus (Fig. 3a). A moderate increase in the mesangial matrix and a moderate mesangial hypercellularity of glomeruli were revealed under LVSEM. From the Bowman’s space (Fig. 3b), granular or protuberant surface structures of podocytes and the interdigitation of foot processes were observed three-dimensionally. Leaking red blood cells were occasionally found in Bowman’s space. At higher magnification of the cut surface of glomerular tufts (Fig. 3c), the nuclei of mesangial cells were less intensely visible with Pt-blue than those of endothelial cells and podocytes. GBMs were recognized as dark curved bands of irregular thickness (57.6 to 232 nm in Fig. 3c: inset). The cytoplasm of mesangial cells was integrated with the surrounding mesangial matrix.

In the case of minor glomerular abnormalities (Fig. 4), the glomerular capillary lumens were fully patent and the GBM was not thickened (Fig. 4b: 129 to 213 nm in thickness) judging from the cut surface view of glomerular tufts. From the Bowman’s space, the rough surface appearance of podocytes with small protrusions (Fig. 4c) and the interdigitating foot processes in various thicknesses (Fig. 4d: direct magnification was $\times 18,000$) could be observed at higher magnification under LVSEM, although no significant ultrastructural abnormalities were detectable under LM.

In membranous GN (Fig. 5), the irregular surface appearance of podocytes facing Bowman’s space was shown three-dimensionally at higher magnification. The glomerular capillary walls were entirely thickened and had a rigid aspect on the cut surface of the tuft (Fig. 5a). The capillary walls exhibited a diffuse, chain-like thickening. At high magnification, the thick glomerular capillary wall was recognized as being composed of three layers due to variations in the BSE signal brightness, which depended on the different affinities to Pt-blue. The bright outer layer and the inner layer were intensely stained while the dark middle layer was weakly stained with Pt-blue. The outer layer was a cytoplasmic sheet of podocytes, the middle layer corresponded to the GBM with immune deposits, and the inner layer was a cytoplasmic sheet of the endothelial cell. The middle GBM layer between podocytes and endothelial cells was partially measured at 868 nm in thickness (Fig. 5b). The subepithelial immune deposits, which were shown as dense deposits in the TEM micrograph (Fig. 5c) obtained from the same biopsy sample, were barely recognizable in the middle layer under the LVSEM.

**PAM-stained sections**

PAM-stained sections obtained from two cases of membranous GN were examined under LVSEM. PAM-positive constituents, such as GBM, the mesangial matrix, and collagen fibers were distinctly visualized (Fig. 6). The BSE image of thickened GBM was extremely bright, which allowed for a detailed investigation of the intact aspects of the GBM through overlaying other elements from both the subepithelial side and the subendothelial side at higher magnification. Other elements such as podocytes, endothelia, and immune deposits were obscure because they were PAM-negative. The prominent spike formation and the ladder-like deformities of the GBM were clearly observed not only in the cut surface view but also in the en face view of the capillary wall in Case 1 (Fig. 6a, 7a-c). The appearance of the subepithelial structure of the thickened GBM was very rough and various crater-like structures of the GBM were found while the subendothelial structure of the GBM was relatively flat. In Case 2 (Fig. 6b, 7d, e), slight spike formations of less than 0.5 μm in height or irregular small protrusion of mildly thickened GBM were distinctly observed on the subepithelial face; these were obscure under LM. A pinhole-like structure was recognized from the GBM subendothelial side. These findings in Case 2 indicated an earlier phase in the alterations of the GBM in membranous GN.

**Discussion**

The advantage of the present LVSEM approach is the ability to easily conduct overviews of whole renal paraffin sections at low magnifications like LM observations together with a 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 conventional TEM. Additionally, the present preparations are very simple to perform because they require only staining with Pt-blue or PAM after deparaffinization. Pt-blue and PAM, both of which contain heavy metals (platinum and silver, respectively), are effective at enhancing the BSE signal and thus improving the performance of LVSEM in the observation of renal biopsy sections, as reported previously (Tanaka et al., 1998; Inaga et al., 2002, 2009a). Furthermore, it has been suggested that the renal tissues are hardened with Pt-blue and PAM. Because slides with wet sections were placed in the LVSEM chamber, it was thought that the sections were gradually dried even in the low vacuum condition. Nevertheless, the
Fig. 7. Legend on the opposite page.
erythrocytes, podocyte foot processes, and GBM in the present sections exhibited three-dimensional appearances almost similar to those in the freeze-dried sections (Fig. 2). The serious deformation of the glomerular structures produced by drying seems to be reduced by hardening them with Pt-blue and PAM, even though a slight shrinkage could not be avoided. Taking all this into account the present LVSEM method does allow the three-dimensional structural analysis of renal biopsy paraffin sections that were stained with Pt-blue and PAM.

It is important for pathological diagnosis of renal samples under LVSEM to observe both Pt-blue-stained sections and PAM-stained sections. The differential stainability of glomerular components with Pt-blue or PAM enabled the complementary observation of renal biopsy sections from a new perspective under LVSEM. Cellular elements and the electron dense deposits in glomeruli were positively stained with Pt-blue, even though they were PAM-negative. In contrast, GBM and mesangial matrix were intensely stained with PAM while being Pt-blue-negative. Thus, the external surface of glomeruli and the internal cut surface of tufts on renal paraffin sections obtained from different three types of glomerular disease could be investigated three-dimensionally in the present study.

Pathological findings on Pt-blue-stained sections under LVSEM included granular or multiple protuberant appearances of podocyte surface structures were shown in the cases of IgA nephropathy, membranous GN (Fig. 3, 5). The rough surface appearance of podocytes with small protrusions and the various interdigitating foot processes were observed in the case of minor glomerular abnormalities (Fig. 4). These ultrastructural findings are difficult to demonstrate under LM. The GBM was distinguishable as a dark band due to its weaker affinity to Pt-blue. The measurement of the GBM thickness with LVSEM enabled the detailed investigation of GBM abnormalities for each disease case. The subepithelial immune deposits thought to cause membranous GN, which were recognized as dense deposits in TEM images, were barely detected in sections stained with Pt-blue.

On the other hand, PAM staining routinely used for LM and TEM was excellent for GBM observations in membranous GN under LVSEM (Fig. 1d, 6, 7). Actually, both the cut surface view and the en face view of GBM within capillary walls were visualized without any cell removal using LVSEM. PAM-positive GBM was distinctly detected through overlying cellular elements under LVSEM, as PAM-negative cellular elements yielded little BSE signals and were obscure. We could indirectly recognize the existence of subepithelial immune deposits because of GBM deformities in PAM-stained sections. The present study proved that BSE imaging in LVSEM is useful for analyzing not only surface structures but also subsurface structures, which have been stained with heavy metal salts such as silver-methenamine in PAM. Consequently, the present LVSEM images represented the intact aspects of the GBM observed from the both subepithelial side and subendothelial side although previous images obtained using acellular SEM represented the naked GBM surface structure with holes and craters observed only from the subepithelial side (Bonsib, 1985; Bonsib and Plattner, 1986; Weidner and Lorentz Jr, 1986).

The exact stage of membranous GN is generally classified based on TEM observations of the GBM (Ehrenreich and Churg, 1968). In the present study, not only typical spike formation but also slight alterations of the epithelial side of the GBM were clearly recognized three-dimensionally in two different cases of membranous GN. Additionally, the en face view of the GBM under LVSEM showed crater-like deformities in Case 1 (Fig. 7b, c) and small protrusions in Case 2 (Fig. 7d, e). Nishimura et al. (1989) reported using an acellular SEM technique that the morphological changes in the urinary surface of the GBM varied from pinhole to craters and

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**Fig. 7.** Higher magnifications of the PAM-stained glomerular tufts shown in Figure 6: Case 1 (a, b, c) and Case 2 (d, e). a: Cut surface view of the capillary wall. PAM-positive prominent spike formation of the GBM, the newly formed GBM (arrows), and the mesangial matrix are clearly observed. PAM-negative cellular elements (E: endothelial cell, P: podocyte) and mesangial cell nuclei (white asterisks) are obscure. b: The en face view of the capillary wall. Crater-like structures (arrows) of GBM are distinctly found on the subepithelial side of the GBM. c: Hole-like structures (arrows) observed from the subendothelial side of the GBM showing a flat appearance. d: Higher magnification of the capillary wall in Case 2. Slight spike formation of less than 0.5 μm in height (arrows) or irregular small protrusions are distinctly observed on the GBM subepithelial side. These findings indicate an earlier phase of GBM alterations in membranous GN. e: Pinhole-like structures recognized on the subendothelial side of the GBM in Case 2. L: capillary lumen, M: mesangial region, asterisks: mesangial cell nuclei, star: the subepithelial side, open star: the subendothelial side. Bars = 5 μm (a–c).
reticula, according to the severity of the disease stage in various human GN. Our findings using LVSEM with PAM-stained sections suggested the possibility of efficiently investigating the three-dimensional morphologic features of the GBM and classifying the disease stage of membranous GN accordingly. Thus, we have introduced a new observation method for the pathological diagnosis of human glomerular diseases by LVSEM using renal biopsy sections.

In conclusion, the present study demonstrated the usefulness of LVSEM for rapid three-dimensional analyses of renal biopsy samples by the use of conventional paraffin sections. Paraffin sections can be routinely made and are easily available in any pathology laboratory. We expect that LVSEM will provide a novel approach for the pathological diagnosis of human glomerular diseases to compensate for deficiencies in conventional LM and TEM observations.

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