Ultrastructural study of human glomerular capillary loops with IgA nephropathy using quick-freezing and deep-etching method

E. Sawanobori1, N. Terada2, Y. Fujii2, K. Higashida1, S. Nakazawa1 and S. Ohno2
Departments of 1Pediatrics and 2Anatomy and Molecular Histology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo-shi, Yamanashi, Japan

Summary. Immunoglobulin A (IgA) nephropathy shows great variability regarding the histological features of the lesions of human renal glomeruli. In the present study, the quick-freezing and deep-etching (QF-DE) method was used to analyze the glomerular ultrastructure of biopsied kidney tissues from children with IgA nephropathy. Biopsied renal tissues were routinely prepared for light microscopy, immunofluorescence microscopy, conventional electron microscopy, and replica electron microscopy. The three-dimensional ultrastructure of glomeruli of the kidney was clearly observed by using the QF-DE method. Three layers of glomerular basement membranes, i.e., middle, inner and outer layers, were clearly detected in the replica electron micrographs. The middle layer was 343.0±24.2 nm (n=20) in width and formed polygonal meshwork structures. We also observed slit diaphragms, electron-dense mesangial deposits, and increased amounts of mesangial matrix and foot process effacement. Many delicate filaments were found to be distributed from the apical to the bottom portions between neighboring foot processes. The ultrastructural difference between the replica electron micrographs and conventional electron micrographs was found to be especially marked in the appearance of foot processes and connecting filaments between the neighboring foot processes. The examination of extracellular matrix changes, as revealed at high resolution by the QF-DE method, gave us some morphofunctional information relevant to the mechanism of proteinuria with IgA nephropathy.

Key words: IgA nephropathy, Slit diaphragm, Basement membrane, Quick-freezing, Replica electron micrograph

Introduction

Immunoglobulin A (IgA) nephropathy is a kidney disease which many children suffer from. Initial reports about IgA nephropathy described a predominantly focal and segmental type of glomerular lesions, and what was at first considered to be a benign kidney disease (Berger, 1969; Zimmerman and Burkholder, 1975). Later, other researches reported a great variety of glomerular lesions and clinical courses (Bogenschutz et al., 1990; Okada et al., 1990). Although the pathogenesis and pathophysiology of IgA nephropathy have been investigated for the past few decades, they remain unclear.

The quick-freezing and deep-etching (QF-DE) method for electron microscopy has often been used to examine the three-dimensional ultrastructure of cells and extracellular matrices in situ at high resolution (Heuser and Kirschner, 1980; Ohno, 1985; Ohno et al., 1996). This QF-DE method has also been used to examine some renal disease models in experimental animals (Namoto et al., 1991; Nakazawa et al., 1992; Duan et al., 1993; Moriya et al., 1993, 1996), normal human kidneys (Moriya et al., 1995), and patients with diabetic nephropathy (Tanaka et al., 1996). In the present study, we applied the QF-DE method to examining the renal glomerular ultrastructure of biopsied kidney specimens from patients with IgA nephropathy to get some morphofunctional insight into the kidney disease.

Materials and methods

Four children with IgA nephropathy were finally diagnosed by kidney biopsy at the Yamanashi University.
Hospital. The diagnosis of IgA nephropathy was histologically based on the presence of IgA as the predominant component of immunoglobulin deposits in the glomerular mesangium. The clinical features of the patients at the time of the biopsy are summarized in Table 1. Patients No.1 and No.2, were evaluated after they had received therapy for 2 years; the others had not received any therapy. All four patients showed positive reactions for hematuria with moderate proteinuria (>0.5 g/day) at the beginning of the clinical courses. No patient had petechiae or arthralgia, and serum creatinine of all patients was normal throughout the clinical course.

They were all normotensive. The urinalysis of patients No.1 and No.2 turned out to be negative at the time of the second biopsy after 2 years' treatment. Informed consent was strictly obtained from all patients under the guidelines for clinical experiments of the University of Yamanashi.

Biopsied renal tissues were divided into four portions that were used for routine light microscopy, immunofluorescence microscopy, conventional electron microscopy, or the QF-DE method. For light microscopy, one part of the specimen was fixed with 10% formalin, dehydrated in a graded series of ethanol and routinely embedded in paraffin wax. Thin sections were cut at 3-4 mm thickness and subjected to periodic acid-Schiff (PAS) and Masson trichrome (MT) staining. Were cut at 3-4 µm in a cryostat and immunostained with primary antisera against human IgG, IgA, IgM, C3c, C4c, C1q, and fibrinogen (DACO, Denmark), followed by secondary fluorescein-labeled antibody. The immunostained sections were observed with a Nikon fluorescence microscope.

For conventional electron microscopy, the third part of the fresh biopsied tissues was routinely prefixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (PB), pH7.4, for 1h and then postfixed with 1.5% osmium tetroxide in 0.1M PB for 1h. After routine dehydration with a graded series of ethanol, they were embedded in Quetol-812 (Nissin EM Co., Tokyo, Japan). Thin sections were cut using an ultramicrotome with a diamond knife, doubly stained with uranyl acetate and lead citrate, and examined using electron microscopes (Hitachi H-600 and H-7500).

For the QF-DE method, the last part of the fresh biopsied tissues was processed as described previously (Hora et al., 1990; Furukawa et al., 1991; Yu et al., 1998). Briefly, small tissue blocks were slightly fixed with 2% paraformaldehyde (PF) in 0.1M PB for 20-30 min and then cut into slices with razor blades. They were washed with 0.1M PB for 15min to remove soluble matrix components from the exposed tissue surface and postfixed with 0.25% glutaraldehyde in 0.1M PB for 30min. They were immersed in 10% methanol, and then quickly frozen by a contact freezing method with copper metal cooled in liquid nitrogen (-196°C) using a JFD-RFA freezing apparatus (JEOL, Tokyo, Japan). The surface areas of the frozen renal tissues were freeze-fractured with a scalpel in liquid nitrogen as reported before (Hora et al., 1990; Furukawa et al., 1991; Yu et al., 1998). The samples were deeply etched under high vacuum conditions of 2-3x10^{-7} Torr at -95°C in an Eiko FD-3AS freeze-etching device (Eiko Co., Ibaraki, Japan) for 20-30 min. After the deep-etching, they were rotary-shadowed with platinum up to 2 nm thickness at an angle of 30° and then carbon at an angle of 90°. The prepared replica membranes were coated with collodion in amyl acetate and treated with household bleach to dissolve tissue components. They were finally washed with distilled water and mounted on Formvar-filmed copper grids, and immersed in amyl acetate solution to dissolve the collodion. Finally, they were observed using electron microscopes (Hitachi H-600 and H-7500).

Results

Histopathology of glomeruli with light microscopy

The histological findings obtained from each renal biopsy are summarized in Table 2. Two patients, No.2 and No.4, were diagnosed with focal segmental proliferative glomerulonephritis. The other patients, No.1 and No.3, were diagnosed with diffuse mesangial proliferative glomerulonephritis with segmental lesions. Mesangial matrix areas were expanded due to increased amounts of the matrix and mesangial cell proliferation (Fig. 1a-d). Such pathological changes were found to be variable among the glomeruli of a biopsied specimen and also within each glomerulus. By immunofluorescence microscopy, all specimens were predominantly immunopositive for IgA in the mesangial areas (Fig. 1e-h), though the IgA immunoreaction was

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yo)</th>
<th>Sex</th>
<th>Duration (months)</th>
<th>Creatinine (mg/dl)</th>
<th>Total Protein (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>Proteinuria (g/day)</th>
<th>Hematuria</th>
<th>Urinalysis (sediment)</th>
<th>Treatment before biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.1</td>
<td>13</td>
<td>F</td>
<td>30</td>
<td>0.46</td>
<td>6.4</td>
<td>4.0</td>
<td>(-)</td>
<td>(-)</td>
<td>10-20 rbc/hpf</td>
<td>PSL+AZT+DP+W</td>
</tr>
<tr>
<td>No.2</td>
<td>17</td>
<td>M</td>
<td>72</td>
<td>0.93</td>
<td>6.9</td>
<td>4.3</td>
<td>(-)</td>
<td>(-)</td>
<td>20-50 rbc/hpf</td>
<td>DP+sai-rei-tou</td>
</tr>
<tr>
<td>No.3</td>
<td>9</td>
<td>F</td>
<td>2</td>
<td>0.50</td>
<td>5.6</td>
<td>3.4</td>
<td>1g</td>
<td>10-20 rbc/hpf</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td>No.4</td>
<td>13</td>
<td>M</td>
<td>2</td>
<td>0.67</td>
<td>6.8</td>
<td>4.1</td>
<td>0.7g</td>
<td>10-20 rbc/hpf</td>
<td>Not done</td>
<td></td>
</tr>
</tbody>
</table>

PSL: prednisolone; AZT: azathioprine; DP: dipyridamole; W: warfarin.
Fig. 1. Light and immunofluorescence micrographs showing characteristics of IgA nephropathy. a. There is moderate to severe mesangial proliferation with sclerosis as well as adhesion to Bowman’s capsule at 9 o’clock in the micrograph (patient No.1). MT-staining. x 200. b. Mesangial cells are slightly proliferated (patient No.2). MT-staining. x 200. c. Moderate mesangial cell and matrix proliferation is seen at 12 o’clock in the image (patient No.3). PAS-staining. x 200. d. Mild mesangial proliferation is seen (patient No.4). PAS-staining. x 200. e-h. Immunofluorescent reaction products of IgA in patients, No.1-4. They are mostly localized in mesangial areas and also partially along glomerular capillary walls.
Replica electron micrographs of IgA nephropathy

Table 2. Histological features of kidney biopsy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mesangial proliferation</th>
<th>Crescents (%)</th>
<th>Adhesions (%)</th>
<th>Segmental sclerosis (%)</th>
<th>T-I change (%)</th>
<th>Immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>Matrix</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgA IgG IgM C3</td>
</tr>
<tr>
<td>No.1</td>
<td>Diffuse ++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Fibrocellular</td>
<td>19</td>
<td>7</td>
<td>&lt;5</td>
<td>Mesangial</td>
</tr>
<tr>
<td>No.2</td>
<td>Focal +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>&lt;5</td>
<td>Mesangial</td>
</tr>
<tr>
<td>No.3</td>
<td>Diffuse +++</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>++</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>-</td>
<td>Mesangial</td>
</tr>
<tr>
<td>No.4</td>
<td>Focal ++</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0 &lt;5</td>
<td>+++ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Mesangial</td>
</tr>
</tbody>
</table>

T-I change: tubulo-interstitial change.

Fig. 2. Replica electron micrographs demonstrating three relatively well-ordered layers of the glomerular basement membrane (GBM) (patient No. 4).

a. Foot processes (F) closely cover the GBM, and cross-linking filaments (arrows) are seen between foot processes and middle layers (asterisk), corresponding to lamina rara externa in conventional ultrathin sections. The middle layer (asterisk) has fine polygonal meshworks. Other cross-linking filaments are also seen between endothelial cells (E) and the middle layer (arrowhead). Bar: 500 nm.

b. In slit spaces, connecting filaments (arrows) are seen between neighboring foot processes (F). Bar: 500 nm.

c. Filamentous meshworks appear to be loosened at subendothelial sites at other glomerular capillary loops (arrows). The middle layer is thinner at these areas (asterisks). F: foot processes; E: endothelial cells; L: blood capillary lumens. Bar: 500 nm.
also partially positive along the glomerular walls in patient No.3 (Fig. 1g). C1q and C4c were weakly immunopositive in mesangial areas in patients No.1 and No.2. Fibrinogen was immunopositive along the capillary walls and crescent in patient No.3.

Replica electron micrographs of glomeruli in children with IgA nephropathy

Next we examined the following ultrastructural features of the glomeruli which were obtained from biopsied kidney samples of patients with IgA nephropathy: i) glomerular basement membranes, ii) slit diaphragms, iii) mesangial areas, iv) endothelial cells in glomerular capillaries, and v) podocytes.

Glomerular basement membrane (GBM)

In the areas where GBM and foot processes were well preserved in some glomeruli, the GBM consisted of

![Replica electron micrograph of IgA nephropathy](image)

**Fig. 3.** Replica electron micrographs demonstrating structures of slit spaces and slit diaphragms between foot processes (patient No. 3). **a.** Slit spaces (arrows) and slit diaphragms (arrowhead), which were freeze-fractured almost in parallel to the GBM, show filamentous or sheet-like substructures, respectively. Some primary or secondary processes of podocytes (P) contain many intermediate filaments, and foot processes contain compact networks (asterisks). GBM: glomerular basement membrane. Bar: 200 nm. **b.** Glomerular capillary loops were freeze-fractured at an oblique angle to the GBM on the left side and also in parallel to the GBM on the right side. There are narrow slit spaces and slit diaphragms (arrows) between foot processes (F). The GBM shows tiny meshworks in the middle layer (asterisks). Some cytoplasmic sides of cell membranes are decorated with filamentous structures (arrowheads). E: endothelial cells; L: capillary lumens. Bar: 500 nm.
middle, inner and outer layers in the replica micrograph (Fig. 2a, patient No.4), corresponding to lamina densa, lamina rara interna and lamina rara externa, respectively, as reported before (Takami et al., 1991). The middle layer, which was 343.0±24.2 nm (n=20) in width, formed polygonal meshwork structures. In the outer layer, the cross-linking filaments perpendicularly connected epithelial cell membranes with meshworks of the middle layer. The similar filaments of the inner layer also connected endothelial cell membranes with the meshworks. The total width of the three layers was 452.4±39.3 nm (n=20). Other connecting filaments were also seen between neighboring foot processes (Fig.2b), and between foot processes and primary processes of podocytes (Fig.2c). Filamentous meshworks appeared to be loosened at subendothelial sites, where the middle layer became thinner (Fig.2c).

Slit diaphragms and their relationship to GBM

When slit diaphragms between foot processes were freeze-fractured almost in parallel to the outer layer surface of GBM, they showed filamentous or sheet-like substructures, not zipper-like ones (Fig.3a, patient No.3). In the areas which were freeze-fractured at an oblique angle to the GBM, they also showed cross-linking filamentous substructures. The mean value of the width of the slit diaphragms between cell membranes of the foot processes was about 39 nm (n=20). Some primary processes of podocytes contained many intermediate filaments, and other foot processes contained thin filament networks (Fig. 3a). In addition, at horizontally or obliquely freeze-fractured GBM (Fig. 3b), meshworks in the middle layer of the GBM were more widely seen to be localized between cell membranes of foot processes and endothelial cells, and some cytoplasmic sides of podocytes were decorated with filamentous structures (Fig. 3b, arrowheads).

Mesangial areas

Replica electron micrographs also showed mesangial areas of glomeruli in three-dimensional images (Fig. 4, patient No.1). The mesangial matrix had proliferated to form large areas of filamentous meshworks between mesangial cells. Some soluble materials were associated with basic filamentous structures, resulting in the formation of more compact meshworks. The

![Fig. 4. Replica electron micrograph showing mesangial areas (patient No. 1). The mesangial matrix (MM) is proliferated to form large areas of meshworks between mesangial cytoplasmic processes (MC). Large amounts of materials are associated with basic filamentous structures, resulting in the formation of compact meshworks. Bar: 1 µm. Inset; corresponding area in a conventional electron micrograph obtained from ultrathin sections. Bar: 500 nm.](image-url)
Fig. 5. Replica electron micrographs showing luminal sides of endothelial cells (E) in glomerular capillary loops (patient No. 1). a. Endothelial fenestrae (arrows) are seen to be heterogeneously scattered along the blood capillary wall. Bar: 1 µm. Inset; higher magnification of the area. Arrows: Freeze-fractured fenestrae; Arrowheads: Invaginating pits; L: lumen; GBM: glomerular basement membrane. Bar: 500 nm. b. Slit diaphragms (arrows), which were freeze-fractured almost in parallel to the GBM, show sheet-like substructures between foot processes (F). The middle layer (asterisks), corresponding to the conventional lamina densa, forms fine meshworks. Some cross-linking filaments (arrowheads) connect the middle layer (asterisks) with endothelial cells (E). OL: Outer layer between horizontally freeze-fractured foot processes (F). GBM: glomerular basement membrane. Bar: 500 nm.
Fig. 6. Replica electron micrograph of freeze-fractured cell body of podocyte (P) and foot processes (F) or primary processes (Pp) (patient No. 2). Many thick intermediate filaments are seen to be localized in the primary processes (Pp). Other thin filaments are localized in the foot processes (F). S: cell membrane surface of the podocyte; U: urinary space. Bars: 500 nm. Inset: higher magnification of the cell body of the podocyte. Thinner filaments (arrows) are bridging between the intermediate filaments.
conventional electron micrograph showed the corresponding area, which was obtained in ultrathin sections (Fig. 4, inset).

Endothelial cells in glomerular capillary loops

Luminal sides of endothelial cells in glomerular capillary loops were examined in the replica electron micrographs (Fig. 5a, patient No.1). The number of endothelial fenestrae decreased and they were heterogeneously arranged. In this patient, the slit diaphragms, which were freeze-fractured almost in parallel to the GBM (Fig. 5b), also showed sheet-like substructures between the foot processes. The middle layer, corresponding to the conventional lamina densa, formed fine meshworks. Some cross-linking filaments connected the middle layer with cell membranes of endothelial cells.

Podocytes and their cytoskeletons

Replica electron micrographs showed primary processes of freeze-fractured podocytes (Fig. 6, patient No.2). Many intermediate filaments were localized in the primary processes, where they might have acted to stabilize the structures. In addition, extending secondary processes contained not only intermediate filaments, but also thin filaments. Many such filaments were localized in the foot processes, where they were thought to function in dynamic movement.

Discussion

The QF-DE method is a useful electron microscopic technique that permits three-dimensional views of cells and tissues, including cell organelles, cytoskeletal elements and extracellular components. It has previously been used to examine some renal diseases of experimental animal models (Naramoto et al., 1991; Nakazawa et al., 1992), but its application to human renal diseases is still limited because of the difficulty and complexity of human tissue preparation. In the present study, we investigated IgA nephropathy of children by using the QF-DE method, and observed three well-ordered layers of GBM, which were composed mainly of meshwork filaments (Takami et al., 1991). The total width of the GBM was 452.4±39.3 nm, which was wider than that of 300-400 nm obtained by conventional electron microscopy in normal adults (Tiebosch et al., 1974). This difference of width was probably caused by shrinkage during the TGO fixation and dehydration procedures, which partially as a sheet-like substructure. The width of the slit diaphragm was 38±2 nm by the immuno-cryosection method, which is wider than that of 32±2 nm.
obtained by the resin-embedding section. Moreover, their figures obtained using the high-pressure freezing and freeze-substitution method look like sheet-like substructures because of their smaller pores, rather than zipper or ladder shapes (Wartiovaara et al., 2004).

As for the primary processes of podocytes, 10-nm intermediate filaments were localized in most of the central cytoplasm, whereas 7–9-nm actin microfilaments were more predominant in foot processes (Andrews, 1981; Vasman et al., 1984; Renneberg et al., 2003). The foot processes were firmly attached to the slit membrane and GBM by matrix filaments. We also found many delicate cross-linking filaments between neighboring foot processes from the apical to the bottom portions. These cross-linking filaments, which are not visible with conventional fixation, may contribute to the podocytes’s roles in protein filtration barriers and attachment between foot processes.

An increase of mesangial matrices was observed as an expansion of newly formed meshworks. These increased matrices were suggested to be composed of type IV collagen, laminin, fibronectin, perlecain and tenasin (Timpl, 1989; Miner, 1999), which exist in normal mesangium, with altered distributions according to mesangial proliferation and sclerosis (Oomura et al., 1989; Schnaper et al., 2003). In some reports, focal and segmental accumulation of type I, III, or V collagen was closely related to the pathological development of glomerular sclerosis (van den Born et al., 1993; Yang et al., 2001). The patients in the present study were young, and their duration of IgA nephropathy was not so long. In the light microscopic examination, only small amounts of sclerosis and fibrosis were found in their biopsied kidneys. In some glomerular areas (Fig. 4), huge homogeneous filaments were widely distributed, indicating that some growth factor such as PDGF or TGF-$\beta$ might locally regulate the mesangial matrix production (Schocklmann et al., 1999; Yang et al., 2001).

To clarify the variable histological changes in IgA nephropathy, we examined human biopsied kidneys by electron microscopy. The QF-DE method enabled us to determine the three-dimensional ultrastructure of meshwork matrices in detail, and to detect some pathological changes and cell-cell interactions in IgA nephropathy. In addition, we are planning to perform replica-immunostaining of IgA and various slit diaphragm-associated proteins in the replica membranes to clarify the localization of immune-complex formation and changes of component proteins in further studies. The detection of extracellular matrix changes, as seen by the QF-DE method, will provide useful information for clarifying the mechanism of IgA nephropathy.

**Acknowledgements.** The authors thank Miss Y. Kato in the Department of Anatomy, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, for her technical assistance in the present study.

**References**


Kestilä M., Kalluri R., Seth P., Patten A., Cameron J.D., Wieslander J. and Michael A.F.
Replica electron micrograph of IgA nephropathy


Accepted September 14, 2007