Podocytes Are Firmly Attached to Glomerular Basement Membrane in Kidneys with Heavy Proteinuria

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Abstract. Glomerular epithelial cells (podocytes) play an important role in the pathogenesis of proteinuria. Podocyte foot process effacement is characteristic for proteinuric kidneys, and genetic defects in podocyte slit diaphragm proteins may cause nephrotic syndrome. In this work, a systematic electron microscopic analysis was performed of the structural changes of podocytes in two important nephrotic kidney diseases, congenital nephrotic syndrome of the Finnish type and minimal-change nephrotic syndrome (MCNS). The results showed that (I) podocyte foot process effacement was present not only in proteinuric glomeruli but also in nonproteinuric MCNS kidneys; (2) podocytes in proteinuric glomeruli did not show detachment from the basement membrane or cell membrane ruptures; (3) the number of pinocytic membrane invaginations in the basal and apical parts of the podocytes was comparable in proteinuric and control kidneys; (4) in proteinuric kidneys, the podocyte slit pore density was decreased by 69 to 80% and up to half of the slits were so “tight” that no visible space between foot processes was seen; thus, the filtration surface area between podocytes was dramatically reduced; and (5) in the narrow MCNS slit pores, nephrin was located in the apical part of the podocyte foot process, indicating vertical transfer of the slit diaphragm complex in proteinuria. In conclusion, these results suggest that protein leakage in the two nephrotic syndromes studied occurs through defective podocyte slits, and the other structural alterations commonly seen in electron microscopy are secondary to, not a prerequisite for, the development of proteinuria.

The striking morphologic change in proteinuric kidneys is the replacement of discrete glomerular podocytic foot processes by large expanses of flattened epithelial cytoplasm. This abnormality was recognized in the earliest electron microscopic studies in the 1950s (1–3) and has constantly been described in human glomerular diseases as well as in animal models of proteinuria. The effacement of the foot processes has been regarded as an abnormal response of the epithelium either to direct injury or to alterations elsewhere in the glomerulus. The process is probably associated with changes in the actin cytoskeleton of the podocyte foot processes, but its molecular basis is still not known (4,5).

The loss of the complex interdigitation of podocytes does not explain protein leakage in nephrotic kidneys. On the contrary, the wide expanses of epithelial cytoplasm often cover the glomerular basement membrane (GBM) of the capillary wall so that only occasional interruptions are present. These residual sites of previous slit pores may also show close apposition of adjacent podocyte cell membranes, forming “tight” or “close” junctions (6–8). Thus, the total area available for the passage of the urinary ultrafiltrate in nephrotic kidney is probably much less than normal, and it is difficult to see how massive proteinuria occurs in such a situation.

Two explanations have been offered to connect the ultrastructural findings and proteinuria. The first hypothesis suggests that proteinuria actually results from the detachment of the epithelial cells from the GBM and formation of focal gaps in the epithelial covering. In these areas, increased water flux would cause plasma proteins to be dragged across the GBM filter to the urinary space. In animal models, the onset of proteinuria coincides well with the development of areas of denuded GBM (9,10), and such areas have also been described in two reports on human nephrotic kidneys (11,12). The second theory is that, in nephrotic kidneys, large amounts of protein would pass through the epithelial cells into urine via active endocytosis. This is supported by the fact that electron microscopic studies have revealed increased amounts of pinocytic vesicles, phagosomes, and lysosomes in epithelial cells in human proteinuric kidneys as well as in acute aminonucleoside nephrosis in rats (7,13,14).

The electron microscopic studies mainly were performed at
a time when little was known of the molecular basis of the glomerular filtration. Traditionally, the podocyte alterations were believed to be associated with increased leakiness of the GBM (4,15), although the possible role of the slit diaphragm connecting the podocyte foot processes was also suggested (16). During the past few years, the work on genetic diseases has indicated that the podocyte slit diaphragm is probably more important than the GBM in the protein sieving. Defects in many podocyte proteins, such as nephrin, Neph1, podocin, Fat, and CD2AP, result in nephrotic syndrome (17–21). How the classic electron microscopic observations on nephrotic kidneys and the new molecular findings fit together have not been studied.

In this study, we analyzed the ultrastructure of the podocytes and their attachment to the GBM in kidney samples from patients with congenital nephrotic syndrome of the Finnish type (NPHS1) and minimal-change nephrotic syndrome (MCNS). The NPHS1 kidneys are especially suitable for these studies because the molecular defect in this disorder is known, and the kidneys show massive proteinuria with little histologic lesions or renal failure. NPHS1 is caused by mutations in NPHS1 gene that encodes a major slit diaphragm protein, nephrin (17). Patients with severe mutations in NPHS1 do not express nephrin in the glomerulus and have a defective podocyte slit diaphragm (22). Conversely, MCNS is the most common nephrotic disease in children with unknown cause and a prototype of “acquired” nephrotic syndromes. Proteinuria is fluctuating in MCNS, which offers an opportunity to study kidneys in different phases of the disease.

Materials and Methods

Samples
Renal tissue blocks for electron microscopy (EM) were obtained by core needle biopsies taken on clinical indications from patients who were treated between 1969 and 2003 at the Hospital for Children and Adolescents, University of Helsinki. Some of the biopsies from NPHS1 children were taken at nephrectomy, when the kidneys still had normal blood flow. In total, 10 renal biopsies from patients with MCNS and eight biopsies from patients with NPHS1 were included. The diagnosis of MCNS was based on clinical data as well as renal histology. Five MCNS patients had proteinuria varying from 6 to 10.4 g/L, and five were in remission at the time of biopsy. The diagnosis of NPHS1 was verified by analysis of the NPHS1 gene in every case. As controls, we used 11 renal biopsy samples from patients with no proteinuria. These samples were obtained from patients who underwent control renal biopsy because of having a renal transplant (three cases), a liver transplant (three cases), or tubulointerstitial nephritis (TIN) (five cases). Cryo-EM and immunogold labeling were performed on four of the MCNS and three control samples. Two of the MCNS patients were in remission at the time of sampling.

Scanning EM

The glomeruli for scanning EM (SEM) were isolated under the dissection microscope immediately after the biopsies. The samples were fixed with 2.5% (wt/vol) phosphate-buffered glutaraldehyde for 2 h. Some samples were also treated with 1% (wt/vol) osmium tetroxide. After washing and dehydration in ethanol, critical-point drying was performed using the Bal-Tec CDP 030 critical-point drying unit. The samples were mounted on aluminum stubs with double-sided tape and silver glue and then sputter coated with platinum or chromium. The specimens were observed using both Zeiss scanning electron microscope DMS 962 and a Jeol field emission scanning electron microscope JSM 6335F.

Transmission EM

The preparation of the renal biopsies for transmission EM (TEM) was performed according to standard procedures. The tissue blocks were fixed by 2.5% glutaraldehyde and embedded in Epon. Each sample was sectioned in series so that 10 grids were collected from levels that were 1.2 µm apart from each other. Five to 10 different capillaries were studied from each glomerulus with an FEI Tecnai F12 electron microscope at 80 kV. The analysis of the podocyte pores was performed at a magnification of 8200. All areas that were suspected to show denuded GBM were photographed for further inspection.

Cryo-EM and Immunogold Labeling for Nephrin

For cryosectioning, biopsy samples from renal cortex were fixed in phosphate-buffered 3.5% paraformaldehyde and 0.02% glutaraldehyde. After fixation and washing, the samples were embedded in 10% gelatin, infiltrated with 2.3 M sucrose in PBS, and frozen in liquid nitrogen. Immunolabeling was done by the Tokuyasu method (23,24). Approximately 70-nm-thick sections were labeled for 60 min with antinephrin and then incubated with fresh protein A coupled to 10 nm of gold for 30 min. Polyclonal rabbit antinephrin antibody against the intracellular domain of nephrin was used (25). The second antibody (protein A–gold conjugate) was purchased from University of Utrecht, School of Medicine, Department of Cell Biology. Sections were observed on an electron microscope (Tecnai F12; FEI, Eindhoven, Holland) and photographed for detailed analysis.

Results

Podocyte Structure in SEM

Glomeruli were prepared for SEM analysis from nine control kidneys and from six proteinuric kidneys, including four patients with NPHS1 and two patients with MCNS. The control kidneys showed a well-preserved organization of the glomerular capillary wall with podocyte nuclear areas and primary, secondary, and tertiary processes (Figure 1A). With high magnification, one could see the slender foot processes with a rough surface and occasional filaments connecting them (Figure 1B).

In NPHS1 glomeruli, the podocyte cell bodies were prominent and had a balloon-like appearance (Figure 2, A and C). Often only one primary process connected the cell body to a flat cytoplasmic sheet with no branching (Figure 2, B and C). Thus, epithelial cytoplasmic plates with narrow cell borders usually covered the major part of the capillary wall (Figure 2, B and D). Variation in the appearance, however, was evident, and in some areas, primitive ramification (Figure 2E) or slender processes with filamentous connections were observed (Figure 2F). Especially in NPHS1, there were also occasional lumpy protrusions of the cytoplasm, which were aggregated on the surface of the podocyte foot processes. Most important, no areas of denuded GBM or holes in the epithelial cell covering were observed in the NPHS1 glomeruli.

The organization of podocyte processes in MCNS glomeruli was not, in general, as severely affected as in NPHS1. Branch-
ing of the primary processes was present in some areas (Figure 1, C and D). Also, foot processes could be recognized, but they were disorganized and had a thin and a flattened appearance (Figure 1, E and F). No areas of bare GBM were detected as was the case in NPHS1 kidneys.

**Podocyte Foot Processes in Transmission EM**

A systematic transmission EM (TEM) analysis of podocyte-GBM attachment, podocyte pinocytic invaginations, and the frequency and the quality of the podocyte slit pores was performed on seven MCNS, four control, and four NPHS1 kidneys. Four of the seven MCNS samples were obtained in relapse and three in the remission phase of the disease. A total of 1544 visual fields corresponding to 5250 μm of the GBM could be analyzed in serial sections cut at 1.2-μm intervals (Table 1).

**Podocyte Foot Process Effacement**

In contrast to controls, the effacement of the podocyte foot processes was evident in all proteinuric kidneys and also in samples from the three MCNS patients who had been in remission from 5 d to 4 mo (Figure 3). The podocyte pore density (number of slits per underlying GBM length) was decreased from 1542 pores/mm GBM in controls to 308 pores/mm in NPHS1 and to 478 pores/mm in proteinuric MCNS kidneys (Table 1). Pore density was reduced also in nonproteinuric patients with MCNS. One patient, who had been in remission for 4 mo, still had slit pores half of the normal (Table 1).

**Attachment of the Podocytes to the GBM**

Epithelial cells were seen to cover the GBM in all samples, and no areas of detachment were noticed even in NPHS1 glomeruli with massive proteinuria. NPHS1 glomeruli contained occasional balloon-like spaces, which, however, were located intracellularly and not between the podocyte and the GBM (Figure 4, A and B). Two MCNS glomeruli had a small
area of denuded GBM at the edge of the section, where capillaries were easily subjected to distension (Figure 4, D and E). The first patient was in remission, and the other had a relapse at the time of biopsy. The areas in the two samples presented 0.03 to 0.3% of the whole length of the GBM screened. The epithelial covering was seen to vary in thickness, which partly resulted from a different plane of a cross-section (Figure 4C).

Podocyte Invaginations

To assess the endocytic activity in the podocytes, we counted the number of membranous invaginations (coated pits) at the basal and apical surface. As shown in Table 1, no clear difference in their frequency was found between proteinuric and nonproteinuric kidneys. These invaginations were seen in all samples and both in areas with effacement and in the areas where podocytes showed normal architecture (Figure 5, A and B).

Podocyte Slit Pores in TEM and Immuno-EM

The glomeruli studied by TEM contained a total of 3445 podocyte slit pores, and the average width was 27 to 28 nm in control, NPHS1, and MCNS kidneys as measured from high-magnification micrographs. In proteinuric kidneys, however, the width of a slit pore showed a large variation and was occasionally increased up to 50 to 80 nm (Figure 5, C and F). However, more often, the slit pores looked narrow, and in highly proteinuric NPHS1 glomeruli, almost half (47%) of the pores were so “tight” that no visible slit between adjacent foot processes was seen (Figure 5G). Pores were classified as “tight” or “closed” when there was only a narrow space (5 to 10 nm) or no visible space between the membranes of the neighboring foot processes. This observation is partly dependent on the angle of the cross-section, because 20% of the pores looked tight also in control kidneys. In MCNS glomeruli, 19 to 66% of the slit pores gave a similar image (Table 1).

In glutaraldehyde-fixed samples, normal slit diaphragms give a filamentous image on TEM (Figure 3A). We have previously found that this filament is totally missing in the open slit pores in NPHS1 kidneys (with no nephrin expression) and greatly reduced (by 39%) in proteinuric MCNS kidneys (26). The analysis in this work revealed that 13.7% of the pores in NPHS1 glomeruli had diffuse filamentous material (Figure 5, D and E, Table 1). These pores represented 3.4% (relapse) and 5.6% (remission) of the slits in MCNS (Figure 5H), which is clearly more than in controls (0.9%). However, as a result of the dispersion, no statistical difference could be verified. Immuno-EM of the MCNS glomeruli showed that nephrin, which is a major component of a normal slit diaphragm, was located to the apical part of this filamentous material in narrow slit pores (Figure 6B). In wider pores, nephrin was seen at the level of the filamentous material either in the apical region of the foot process (Figure 6, C and D) or at the “right” level above the GBM (Figure 6A).

Discussion

In this study, we analyzed the structural changes in glomerular epithelial cells (podocytes) in two important nephrotic kidney diseases, NPHS1 and MCNS. The loss of the normal podocyte foot process organization (effacement, fusion) was...
evident in proteinuric NPHS1 and MCNS kidneys, as reported previously in nephrotic human and animal kidneys (3,9,27). On the average, the lesions were more severe in NPHS1 kidneys with constant massive proteinuria than in MCNS kidneys. Foot process broadening, however, was present also in nonproteinuric MCNS kidneys from patients who had been in remission from 5 to 4 mo, indicating that protein leakage is not directly associated with the epithelial lesions.

In SEM, the most dramatic change was observed in NPHS1 kidneys, where podocytes often gave a “tadpole” image instead of the normal “octopus”-like structure. The balloon-like cell bodies often hung from a filamentous primary process that was attached to a large cytoplasmic plate. This finding is different from that seen in rats (10) and from the two previous reports on the SEM findings in human nephrotic kidneys (28,29). In these studies, the podocyte nuclear portions were found to be attenuated, and spreading of the cell body area obliterated the cytoplasmic branching. It is interesting that the SEM images of the capillary wall in NPHS1 varied so that also more normal areas were observed. Primitive branching was focally evident, resembling the changes in MCNS kidneys. This favors the idea that the epithelial changes are associated with proteinuria as such and not caused by the defect in the podocyte slit diaphragm. In SEM, the other major finding was that the epithelial sheets in NPHS1 and MCNS kidneys did not show membrane ruptures, as has been reported in the animal models (30).

The major observation in our work was that epithelial cell detachment and formation of areas of denuded GBM were not found in the proteinuric glomeruli. The extensive search for such areas in SEM and TEM revealed only two small patches of bare GBM in two MCNS kidneys (one in relapse and the other in remission). This is clearly opposite to the results reported previously, especially in the aminonucleoside nephrosis in rats (9,14,30,31). In addition to the animal models, focal areas of externally denuded GBM have been observed in the glomeruli from nephrotic patients with focal glomerulosclerosis (11,31), amyloidosis (32), and diabetes (33). Also, Yoshikawa et al. (12) found small foci of epithelial detachment in 16 of the 33 patients with MCNS and nephrosis. The findings in human diseases, however, are less clear than in the rats.

Detachment of the epithelium suggests that the mechanism by which epithelial cells and the GBM normally adhere to one another is impaired. The other possibility is that foot process retraction leads to separation of neighboring podocytes. That we did not see areas of denuded GBM in NPHS1 is interesting in two ways. First, it shows that even massive proteinuria (up to 100 g/L) is possible without problems in podocyte-GBM adherence. Second, the NPHS1 kidneys lack the slit diaphragm and its major component, nephrin, and large defects in podo-
cyte contacts with separation of the foot processes would seem possible. This was clearly not the case, however, indicating that the epithelial cell connections and the basal anchoring were enough to prevent the formation of epithelial gaps. The tissue sections studied represented only a small portion of the whole kidney, and it is possible, especially in MCNS, that focal epithelial gaps were missed because they were not included in the analyzed material. For obtaining maximal coverage, the sections used in the analyses were taken 1 μm apart (every 20th microtome section), which, of course, did not completely solve the problem. Two pieces of information, however, speak against the “epithelial gap” theory. In animal models, the denuded areas have been numerous (one gap in every fifth section) (10), suggesting that the 200 sections analyzed from the 10 proteinuric MCNS glomeruli in our work should have contained ~40 gaps, instead of one small area in the edge of a section. Also, the kinetics of the foot process alterations seem “wrong.” In MCNS, proteinuria typically resolves within a few days after commencement of prednisone therapy, whereas the podocyte lesions seem to normalize very slowly (within months). Thus, the rapid response is more easily explained by molecular reorganization of podocyte proteins rather than healing of the possible epithelial gaps, representing a severe form of injury.

Podocytes can endocytose proteins, and it has been proposed that during nephrosis, large amounts of protein may pass through the epithelial cells into urinary space via cytoplasmic vesicles and vacuoles. Increased endocytic activity in podocytes was described already in the early EM studies of human proteinuric kidneys (1,2). Pinocytic invaginations (coated pits) have also been described repeatedly in aminonucleoside nephrosis in rats (6,7,14,30,34,35). The epithelial vacuoles have been suggested to communicate with the extracellular space overlying the GBM on one side and with urinary space on the other side of the cell. The flattening of foot processes in nephrosis may facilitate the uptake and transport of proteins by pinocytosis (36). There is also evidence that the epithelium functions as a monitor to recover proteins that leak through the basement membrane even in normal filtration (37,38). Recently, Kim et al. (39) found that mice that had defects in the podocytic endocytosis (CD2AP deficiency) had increased susceptibility to glomerular injury, suggesting that protein clearance by podocytes may be an important modulator of glomerular diseases. On the basis of these data, we tried to evaluate the endocytic activity of the podocytes in NPHS1 and MCNS kidneys by counting the pinocytic invaginations in the basal membrane as well as in the apical areas of the cell membrane. The analysis, however, showed no difference in the invagination frequency between proteinuric and nonproteinuric glomeruli, which suggests that endocytosis does not play a significant role in protein transport through the capillary wall in NPHS1 or MCNS.

The number of podocyte slit pores was dramatically reduced in proteinuric kidneys. They also showed qualitative changes in SEM and TEM, so that up to 50% of the residual slits were closed or tight. This phenomenon has previously been described in human nephrotic kidneys (6,7) as well as in amino-
nucleoside nephrosis in rats (8,35,40). It is probable that many of the tight slits restrict normal ultrafiltration (and protein leakage), and, on the basis of our survey, the area of “open” slit pores was reduced to 10% of normal in NPHS1 kidneys and to 23% in nephrotic MCNS kidneys. Because neither of these diseases is associated with impaired glomerular clearance of water and small solutes, it seems that the filtration surface of the capillary wall in normal kidneys is extensive.

Our knowledge of the normal podocyte slit pore and the slit diaphragm has greatly increased during the past few years (4,41,42). The slit diaphragm area has been reported to contain at least nephrin, Neph1, FAT, and P-cadherin, which interact with each other and also with the podocyte proteins, such as podocin, CD2AP, and ZO-1. The precise molecular architecture of slit diaphragm, however, needs further clarification. It is clear that EM is a crude method for studying such a complex protein structure as the slit diaphragm. However, previously, we observed that the normal slit diaphragm image in the open slit pores is completely lacking in NPHS1 kidneys (22) and reduced in MCNS kidneys (26). A new finding in this report was that one could see filaments between the neighboring foot processes in high-power SEM of normal glomeruli. In TEM, numerous filaments and a fuzzy material with no clear slit diaphragm layer was detected in proteinuric kidneys. Also, the immunogold labeling of the cryosections revealed nephrin only in the apical parts of the narrow slits in MCNS kidneys. This is in accordance with previous findings in rats indicating that the slit diaphragm may move upward during nephrosis and also fits the idea that the reverse changes in MCNS patients in remission resemble the normal differentiation process that occurs in the fetal period (9,35,43).

In conclusion, the results in the present work favor the idea that proteinuria in NPHS1 and MCNS is caused by molecular changes in the slit diaphragm structure, and the loss of the slit diaphragm area in nephrotic rats (8,35,40). It is probable that many of the tight slits restrict normal ultrafiltration (and protein leakage), and, on the basis of our survey, the area of “open” slit pores was reduced to 10% of normal in NPHS1 kidneys and to 23% in nephrotic MCNS kidneys. Because neither of these diseases is associated with impaired glomerular clearance of water and small solutes, it seems that the filtration surface of the capillary wall in normal kidneys is extensive.

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