Immune-mediated podocyte injury and the idiopathic nephrotic syndrome
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Chapter 6

Podocyte foot process effacement is not correlated with the level of proteinuria in human glomerulopathies

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Podocyte foot process effacement is not correlated with the level of proteinuria in human glomerulopathies

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Nephrotic syndromes result from increased glomerular permeability to proteins and are structurally believed to be associated with podocyte foot process effacement. Despite increasing knowledge of the molecular composition of the glomerular filtration barrier, the relationship between proteinuria and foot process effacement is unclear. We conducted a morphological study on the relationship between podocyte foot process effacement and proteinuria. Electron microscope (EM) pictures of glomerular capillaries were randomly taken from 27 cases in various stages of minimal change nephrotic syndrome (MCNS), from 6 cases of IgA nephropathy with high proteinuria (IgAN) and from 6 control kidneys. From each picture, the mean width of the foot processes (FPW) was quantitated. In normal kidney the mean FPW was 580 ± 40 nm. In biopsies from patients with MCNS without treatment, foot processes were diffusely effaced, reflected by a FPW of 1600 ± 440 nm. In biopsies from patients with MCNS relapsing under prednisolone treatment, foot processes were significantly less effaced than in untreated MCNS (FPW 920 ± 200 nm). In biopsies displaying IgAN, effacement was significantly more segmental than in untreated MCNS (FPW 800 ± 170 nm). Proteinuria did not differ significantly among the groups. Neither in MCNS nor in IgAN was the extent of foot process effacement correlated with the level of proteinuria. The differences in podocyte effacement between MCNS, MCNS relapsing under prednisolone treatment, and IgAN may point to different mechanisms of podocyte injury in these diseases.

Podocytes are highly specialized epithelial cells that cover the glomerular basement membrane with their numerous interdigitating foot processes. In combination with the glomerular basement membrane (GBM), podocytes constitute the filtration barrier of the glomerular capillary wall. Proteinuria is associated with striking changes in podocyte architecture, as detected by electron microscopy (EM). These changes comprise of loss or effacement of the podocyte foot processes. Podocyte foot process effacement can be the solitary hallmark in the renal biopsy specimen, as in minimal change nephrotic syndrome (MCNS), or can be accompanied by other abnormalities characteristic of the underlying disease, such as immune deposits, inflammation or fibrosis [1]. Whether a solitary lesion or not, podocyte foot process effacement seems a uniform response of the podocyte to injury. The etiology of this phenomenon is as yet unknown, despite the rapid increase in the knowledge of podocyte structure and function over the last few years [2]. Furthermore, how effacement of podocytes is related to proteinuria and vice versa, is a matter of debate.

To evaluate the relationship between proteinuria and foot process effacement in human glomerular disease, we conducted a morphological study on podocyte foot processes in renal biopsies obtained from 3 patient groups with proteinuric glomerulopathies and in normal human kidney. Our findings may provide new insight in the etiology of foot process effacement and proteinuria in human glomerulopathies.
Methods

Renal specimens and Electron Microscopy

Renal tissue was obtained for diagnostic purposes by needle biopsies from 31 patients. The cases were diagnosed as minimal change nephrotic syndrome (MCNS, n=27), proteinuric IgA nephropathy (IgAN, n=6) or normal 6-12 months after transplantation (control group, n=6). Additional control human renal tissue was taken from an apparently unaffected part of a kidney extirpated because of renal adenocarcinoma (n=1). At the time of the biopsy, 13 MCNS patients had not yet received any immunosuppressive drugs, and 10 had relapsed under treatment with methylprednisolone. Four MCNS patients had achieved remission under treatment with methylprednisolone. Since these patients experienced severe side effects, alternative treatment was considered and a control biopsy was performed to exclude FSGS lesions.

Renal specimens were fixed in Karnovsky’s fixative, post-fixed in osmium tetroxide and embedded in epon (LADD Research Industries Inc., Williston, VT). The EM sections were stained with uranyl acetate and lead citrate and examined with a CM10 electron microscope (Philips, Eindhoven, The Netherlands). For the present studies, 10 areas of 1 to 2 glomeruli in each patient were photographed in a random and unbiased fashion and printed on 23x23 cm paper, giving a final magnification of 11,000-fold. To study slit diaphragm morphology, random photographs were taken at a final magnification of 108,000-fold.

Measurement of podocyte foot process effacement and proteinuria

From each picture, the GBM was traced and measured in an image processing and analysis program (Scion Corporation, Frederick, MD, USA). The number of podocyte foot processes along the GBM was counted by hand. A foot process was defined as any connected epithelial segment butting on the basement membrane, between two neighboring filtration pores or slits. From each photograph, the arithmetic mean of the foot process width was calculated as follows:

\[
FPW = \frac{\pi}{4} \cdot \frac{\sum \text{GBM length}}{\sum \text{foot process}}
\]

where \( \Sigma \) foot process is the total number of foot processes counted in each picture, \( \Sigma \) GBM length is the total GBM length measured in each picture, and the correction factor of \( \pi/4 \) serves to correct for presumed random variation in the angle of section relative to the long axis of the podocyte [3]. For each patient, the mean FPW was calculated and that value was used to finally calculate a mean FPW for each patient group.

In all patients except the control group, proteinuria was determined by measuring 24-hrs excretion of total protein into the urine. Total protein was turbidimetrically determined using benzethonium chloride. Urine was collected within two days prior to the biopsy. In the group of patients treated with steroids at the time of biopsy, proteinuria was also determined within two days after the biopsy to ensure that proteinuria was persistent. In the control group, (absence of) albuminuria was determined using Albustix (Roche Diagnostics, Almere, The Netherlands).

Statistical analysis

Differences between groups were determined by Kruskal-Wallis tests, followed by Mann-Whitney tests to calculate statistical significance. Differences were considered as significant when \( P < 0.05 \). Correlation analysis was performed using the Spearman test.

Results

Representative pictures from each patient group are shown in Figure 1. From each case at least 400 \( \mu \)m GBM was measured. The mean foot process width (FPW) in the individual pictures ranged from minimally 430 nm to more than 6000 nm. In normal kidneys, podocyte foot processes were well conserved, yet segmental spreading of the processes was present. The FPW in normal kidneys was 580 \pm 40 nm (Figure 2A), which is in accordance with the normal range described by others [4-6]. Podocyte foot processes from MCNS patients in remission, \textit{i.e.} without proteinuria, were well conserved, resulting in a mean FPW of 660 \pm 110 nm. In biopsies from patients with active MCNS without treatment podocyte foot processes were diffusely effaced, reflected by a mean FPW of 1630 \pm 440 nm. In biopsies from patients with MCNS relapsing
CHAPTER 6

Figure 1. Representative EM pictures from the patient groups studied, used for morphometric analysis of podocyte foot process effacement. Scale 1:11000. CL: Capillary lumen.
A. Normal kidney, 6 months after transplantation. No proteinuria.
B. Minimal change nephrotic syndrome. Proteinuria 5 g/24hrs.
C: Minimal change nephrotic syndrome, relapsing during treatment with methylprednisolone. Proteinuria 4g/24 hrs.
D: IgA nephropathy. Proteinuria 6 g/24 hrs.

Figure 2. A. Morphometric analysis of podocyte foot process effacement in the control group and in the four patient groups, expressed as the mean width of the podocyte foot processes (FPW). MCNS: minimal change nephrotic syndrome. MCNS remission: patients previously diagnosed as having minimal change nephrotic syndrome; no proteinuria at the time of biopsy. MCNS prednisolone: patients with MCNS relapsing during treatment with methylprednisolone. IgAN: IgA nephropathy. The error bars represent standard deviation.
B. Proteinuria in the 3 proteinuric patient groups. In the control group and in the group of MCNS patients in remission at the time of biopsy, proteinuria was absent by definition. The error bars represent standard deviation.

under methylprednisolone treatment, podocyte foot processes were significantly more conserved than in untreated MCNS (FPW 920 ± 200 nm, \( P < 0.001 \) versus untreated MCNS). In biopsies displaying IgAN, effacement was moderate and segmental, with a mean FPW of 805 ± 170 nm (\( P = 0.001 \) versus untreated MCNS). Importantly, proteinuria did not differ significantly between the proteinuric groups (\( P = 0.38 \); Figure 2B). The individual FPW values of proteinuric patients are shown in Figure 3.

82
Neither in active MCNS nor in IgAN, FPW was correlated to proteinuria (r = 0.05 for untreated MCNS, P = 0.87.; r = 0.50 for MCNS under prednisolone treatment, P = 0.11; r = -0.23 for IgAN, P = 0.66). A correlation was also absent when all proteinuric MCNS patients were considered one group (r = 0.25, P = 0.25), or when all proteinuric patients were grouped together (r = 0.34, P = 0.07).

MCNS has been reported to be associated not only with podocyte foot process effacement, but also with distortion of the slit diaphragms. In accordance with reports by others [7, 8], we observed diffusely distorted filtration slits in our MCNS patients, with displacement of the deformed slits towards the apical cell membrane of the foot processes and the formation of occluding junctional complexes between neighboring foot processes elsewhere (Figure 4C). Also, the interpedical spaces were irregularly widened or even absent when junctions were formed. In contrast, the changes in slit diaphragm morphology in MCNS patients relapsing under prednisolone treatment were less extensive and limited to the effaced podocyte foot processes. The slit diaphragms that connected conserved foot processes appeared normal (Figure 4D and E). Also in IgAN patients, damage to the slit diaphragms was only focal and mainly, apparently normal slits could be observed (Figure 4F).

**Discussion**

In 1957, Farquhar et al. were the first to describe extensive effacement of podocyte foot processes in biopsies of patients with nephrotic syndrome [7]. Foot process effacement has since been documented by many authors and has been subject of extensive investigation in humans and in animal models. Despite this, the pathogenesis of podocyte foot process effacement and its relation to proteinuria are still not clear. Podocyte foot process effacement seems a stereotypic reaction of podocytes to damage [1]. In laboratory animals, there are various ways to induce foot process effacement and proteinuria by injuring podocytes. The velocity of development of foot process effacement and its severity differ between the models. Examples are immune-complex mediated injury by complement in the Heymann-nephritis model [9, 10], direct toxic podocyte injury by puromycin aminonucleoside [11, 12] or adriamycin [13, 14], and injury by injecting antibodies directed against distinct epitopes on podocytes, such as podoplanin [15] and aminopeptidase A [16]. Studies on knock-out mice have shown that also genetic disruption of genes encoding structural components of the podocyte foot process or slit diaphragm can lead to podocyte foot process effacement and knock-out phenotypes have also been identified in human forms of hereditary nephrotic syndromes (as reviewed in [17]). Although the initial cause leading to foot process...
Figur ee 4. Slit diaphragm morphology in the patient groups studied.

A: Normal kidney, 6 months after transplantation, with intact slit diaphragms.
B: Minimal change nephrotic syndrome in remission under treatment with methylprednisolone, with intact slit diaphragms.
C: Minimal change nephrotic syndrome. Proteinuria 11 g/24hrs. Note the ladder-like slit diaphragm that is displaced towards the apical pole of the cells (large arrow).
D and E: Minimal change nephrotic syndrome, relapsing during treatment with methylprednisolone. Proteinuria 5g/24 hrs (D) and 8 g/24 hrs (E) respectively. Slit diaphragms are largely normal (see text). In E the classical zipper-like morphology of the slit diaphragm as firstly reported by Rodewald and Karnovsky [33] is detected by tangential sectioning (arrowhead).
F: IgA nephropathy. Proteinuria 5 g/24 hrs. Slit diaphragms are largely intact.

Scale 1:108,000 (A-D, F) and 1:150,000 (E). The small arrows indicate slit diaphragms.

Effacement in these models and diseases may seem evident, its relation to the development of proteinuria is still not fully understood.

The pathogenic mechanism underlying podocyte foot process effacement in acquired nephrotic syndromes, such as MCNS, primary focal segmental glomerulosclerosis or nephrotic syndrome in the context of IgA nephropathy (IgAN) is unknown. In analogy to animal models, different pathogenic mechanisms may underlie proteinuria and foot process effacement also in acquired human glomerulopathies.

During routine examination of electron micrographs of human renal biopsy tissue, we were struck by differences in the degree of podocyte foot process effacement in biopsies from nephrotic patients with comparable levels of proteinuria. To validate these differences and to further evaluate the relationship of podocyte foot process effacement and proteinuria, this study was undertaken.

Morphometric analysis confirmed that there were significant differences in the degree of foot process effacement between patients with MCNS, MCNS relapsing under prednisolone treatment, and IgAN. These differences however were independent of the level of proteinuria. Although an abnormal foot process width was invariably associated with proteinuria, a rather large fraction of patients with relapsing MCNS and IgAN presenting with gross proteinuria had only segmental to moderate effacement of the podocyte foot processes. This is in line with other studies, both in animals and in humans, showing that proteinuria is not uniformly associated with podocyte foot process effacement.

After injection of monoclonal antibodies directed against slit diaphragm components nephrin or nep1, rats rapidly develop proteinuria in the presence of intact foot processes [18, 19]. Male MWF rats develop spontaneous proteinuria with age, without changes in the podocyte foot processes [20]. In humans, a familial form of nephrotic syndrome has been reported to occur in the absence of effacement of the podocyte foot processes [21] and in patients with glomerulonephritis, proteinuria is not always associated with foot process effacement [22].
We did not find a correlation between proteinuria and foot process effacement in nephrotic patients, in contrast to two previous studies that described weak but significant correlations between foot process effacement and proteinuria in MCNS patients [4, 6]. It should be stated that the correlations in both studies were calculated by considering all MCNS patients as one group, i.e. including a significant number of patients who were treated with prednisolone and patients achieving remission, thereby presenting with proteinuria less than 1 g/24 hrs. Only when we would also consider all MCNS patients as one group, including patients in remission (without proteinuria), we would indeed find a weak, yet significant correlation ($r = 0.52; P = 0.028$). To our opinion, however, this only shows that in patients who achieve remission, podocytes return to their normal shape and that abnormal foot process width is invariably associated with proteinuria.

In an attempt to explain the occurrence of proteinuria in the absence of extensive foot process effacement, we studied the morphology of the podocyte slit diaphragms. The slit diaphragm represents an intercellular junction and not only forms a functional filter, but also establishes the contact between two adjacent foot processes which are derived from two different podocytes. The critical role of the slit diaphragm in the maintenance of the glomerular filtration barrier is highlighted by the discovery that forms of familial nephrotic syndrome are caused by aberrant expression of the slit diaphragm constituents nephrin [23] and podocin [24]; genetic disruption of these and other slit diaphragm components and intracellular slit diaphragm linker proteins, such as nep1 and CD2AP, concordantly results in proteinuria in mice [25–28]. The pathogenic role of these slit diaphragm proteins in MCNS, a nonfamilial disease, is still not elucidated. Morphologically, the slit diaphragms in MCNS biopsies are dislocated, apparently absent or replaced by junctions ([7, 8], and Figure 4B). In contrast, we found that the slit diaphragms appeared largely preserved in the group of patients relapsing under prednisolone treatment and in IgAN patients. Intact slit diaphragms were observed in conjunction with intact foot processes; only focally, damaged slit diaphragms were present. Thus, in these patients, proteinuria could not be explained by overt damage to the podocyte slit diaphragms. We next examined glomerular expression of podocin and nephrin by immunofluorescence on cryostat sections that were available from the patients presented here. The expression of podocin appeared normal in all groups, showing a linear distribution along the peripheral capillary loops of the glomeruli (not shown). The distribution of nephrin showed also a linear glomerular pattern in most groups. Only in patients with untreated MCNS, the staining pattern of nephrin was altered into a more granular pattern (not shown), which is in line with earlier studies [6]. Thus, the differences in foot process effacement between the groups reported here cannot be explained by differences in podocin expression. Furthermore, nephrin protein expression appears to be correlated with the extent of podocyte foot process effacement.

In summary, our study confirms that proteinuria, foot process effacement and slit diaphragm damage are associated, yet neither foot process effacement nor slit diaphragm damage seem to be a prerequisite for proteinuria. The lack of correlation between proteinuria and foot process effacement renders an exclusive causal relationship between podocyte foot process effacement and proteinuria unlikely. A potential causal relationship may be present in the protein overload model, in which injection of rats with large doses of bovine serum albumin causes proteinuria associated with podocyte foot process effacement [29, 30]. The possibility of injury of podocytes by albumin itself cannot be excluded, yet our findings suggest that such a mechanism is not likely in the glomerulopathies studied here. The results support our hypothesis that the extent of foot process effacement is determined by the mechanism of injury, supposing a different nature of podocyte injury in MCNS and IgAN. The latter may not be unexpected, yet we also found striking differences between MCNS patients relapsing under prednisolone treatment and those not receiving prednisolone at the time of biopsy. Patients frequently relapsing under prednisolone treatment, often referred to as steroid-dependent, make up about 30% of the MCNS patients [31] and thus constitute an important clinical problem. To maintain remission these patients require high doses of steroids, resulting in toxic side-effects. It has recently been shown that not only familial cases, but also some sporadic cases of steroid-resistant nephrotic syndrome can be caused by mutations in the gene encoding podocin [32]. Those patients usually show no response to immunosuppressive
CHAPTER 6

treatment and progress to focal segmental glomerulosclerosis, which was not observed in our patients. Also, the renal expression of podocin was normal in our patients, as detected by immunofluorescence (not shown). Therefore, the steroid-dependent patients presented here seem to represent a distinct patient group, with a specific hallmark in the renal biopsy, i.e. limited foot process effacement and preservation of slit diaphragms, in the presence of proteinuria. In the future this feature might help to identify patients that require treatment different from steroids, such as cyclosporine, cyclophosphamide and levamisole, and thus deserves further investigation.

In conclusion, the severity of foot process effacement in biopsies of nephrotic patients is dependent on the nature of the underlying disease and is independent on the level of proteinuria. It appears that podocyte foot process effacement and proteinuria are independent sequelae of injury to the glomerular filtration barrier. For acquired NS, the nature of the initiating injury remains to be identified.

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