Proteinuria and function loss in native and transplanted kidneys

Koop, K.

Expression of Podocyte-Associated Molecules in Acquired Human Kidney Diseases

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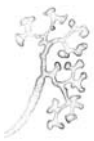
Abstract

Proteinuria is a poorly understood feature of many acquired renal diseases. Recent studies concerning congenital nephrotic syndromes and findings in genetically modified mice have demonstrated that podocyte molecules make a pivotal contribution to the maintenance of the selective filtration barrier of the normal glomerulus. However, it is unclear what role podocyte molecules play in proteinuria of acquired renal diseases. This study investigated the mRNA and protein expression of several podocyte-associated molecules in acquired renal diseases. Forty-eight patients with various renal diseases were studied, including minimal change nephropathy, focal segmental glomerulosclerosis, IgA nephropathy, lupus nephritis, and diabetic nephropathy, together with 13 kidneys with normal glomerular function. Protein levels of nephrin, podocin, CD2-associated protein, and podocalyxin were investigated using quantitative immunohistochemical assays. Real-time PCR was used to determine the mRNA levels of nephrin, podocin, and podoplanin in microdissected glomeruli. The obtained molecular data were related to electron microscopic ultrastructural changes, in particular foot process width, and to clinical parameters. In most acquired renal diseases, except in IgA nephropathy, a marked reduction was observed at the protein levels of nephrin, podocin, and podocalyxin, whereas an increase of the glomerular mRNA levels of nephrin, podocin, and podoplanin was found, compared with controls. The mean width of the podocyte foot processes was inversely correlated with the protein levels of nephrin ($r = -0.443$, $P < 0.05$), whereas it was positively correlated with podoplanin mRNA levels ($r = 0.468$, $P < 0.05$) and proteinuria ($r = 0.585$, $P = 0.001$). In the diseases studied, the decrease of slit diaphragm proteins was related to the effacement of foot processes and coincided with a rise of the levels of the corresponding mRNA transcripts. This suggests that the alterations in the expression of podocyte-associated molecules represent a compensatory reaction of the podocyte that results from damage associated with proteinuria.



Introduction

Proteinuria is an important risk factor for the progression of renal disease, but the pathophysiologic mechanisms underlying its development are unclear (1). Ultrafiltration in the glomerulus is accomplished by a fenestrated endothelial layer, the glomerular basement membrane (GBM), and the overlying podocytes, highly specialized cells with primary and secondary interdigitating branches (foot processes) that cover the external surface of the GBM (2). On the basis of its structural arrangement and isoporous substructure, the slit diaphragm, bridging the space between adjacent foot processes, was assumed to make an important contribution to the molecular sieve for glomerular filtration (3,4). However, this assumption remained a matter of debate (5). Only recently, increased insight in the molecular makeup of the podocyte foot processes and slit diaphragm led to the support for the pivotal role of these structures in the maintenance of permselectivity (6-8).

In kidney diseases accompanied by a nephrotic syndrome, the spatial organization of the podocyte is simplified, characterized by effacement of the foot processes and sporadic detachment of the podocyte from the GBM (9). In the development of the congenital nephrotic syndrome, several molecules have been pointed out to play an important role, including those that assemble and stabilize the slit diaphragm and those that anchor the foot process to the GBM. Mutations in NPHS1 and NPHS2, the genes encoding the slit diaphragm–associated proteins nephrin and podocin, respectively, lead to the development of a nephrotic syndrome (10,11). Similarly, injection of monoclonal antibody 5-1-6, directed against the extracellular part of nephrin (12), causes heavy proteinuria in rats (13). Absence of CD2-associated protein (CD2AP), normally linking nephrin and podocin to the actin cytoskeleton (14,15), also evokes proteinuria (16). Podoplanin is one of the molecules that possibly serve in the connection between foot process and GBM, and injection of antipodoplanin antibodies in rats gives rise to proteinuria and foot process effacement (17). The sialoprotein podocalyxin is normally located at the apical part of the foot process. Absence of this protein or changes in its spatial organization also has a detrimental effect on the glomerular filtration function (18,19). Thus, disruption of the normal gene regulation and protein distribution in the podocyte is related to effacement of foot processes and proteinuria. The study of molecules associated with defective glomerular ultrafiltration in acquired renal diseases might lead to a better understanding of its pathogenesis.

In the current study, we sought to examine whether glomerular expression levels of several podocyte-associated molecules differ between groups of patients who have various acquired renal diseases. We also investigated whether the expression levels were related to clinical parameters and ultrastructural changes. For this, we simultaneously quantified the expression of

several podocyte-associated molecules at the protein and the mRNA levels and combined this with clinical parameters and morphometric analysis of ultrastructural changes of the podocyte.

Materials and Methods

Patients and Controls

Forty-eight patients who had acquired renal diseases were included. From each patient, two core biopsies were taken on clinical indication. A small piece of cortex was removed from fresh biopsies, and glomeruli were microdissected for RNA extraction. One of the biopsies was snap-frozen and stored at -80°C, and the other was formalin fixed and embedded in paraffin. From 29 patients, an additional piece of cortex was available for electron microscopic analysis. Diagnostic groups were made on the basis of routine pathologic examinations. We defined the following groups: minimal change disease (MCD); focal segmental glomerulosclerosis (FSGS); IgA nephropathy; lupus nephritis; diabetic nephropathy; light-chain excretion nephropathy (LCEN); nephrotic syndrome as a result of other causes (membranoproliferative glomerulonephritis [n = 2] and membranous glomerulopathy [n = 1]); and nephritic syndrome as a result of other causes (pauci-immune glomerulonephritis [n = 3] and postinfectious glomerulonephritis [n = 1]). For controls, renal tissue was derived from cadaver donor kidneys unsuitable for transplantation for technical reasons (n = 5), autopsy kidneys (n = 1), tumor nephrectomy samples (n = 2), and biopsies without glomerular lesions (n = 5). In the last group, two showed interstitial nephritis; the biopsies of three other patients were indecisive concerning the diagnosis. However, in all biopsies,

Table 1. Patient characteristics

Diagnostic group	N	EM (b)	age (years ± SD)	gender (female)	proteinuria (g/24 h ± SD)	serum creatinine (µmol/l ± SD)
Controls (a)	13	3	51 ± 19	7 (53%)	0.4 ± 0.3	150 ± 104
MCD	10	8	31 ± 24	4 (40%)	4.4 ± 3.8	76 ± 28
FSGS	5	3	36 ± 8	1 (20%)	5.1 ± 2.1	113 ± 67
IgA nephropathy	10	6	37 ± 23	1 (10%)	2.4 ± 2.8	166 ± 150
Lupus nephritis	7	3	30 ± 12	7 (100%)	2.7 ± 2.0	147 ± 94
Diabetic nephropathy	6	1	50 ± 21	3 (50%)	1.1 ± 0.9	141 ± 85
LCEN	3	1	64 ± 6	1 (33%)	1.8 ± 1.3	354 ± 254
Nephrotic Syndrome – other causes	3	1	44 ± 8	2 (67%)	6.7 ± 3.9	92 ± 33
Nephritic syndrome – other causes	4	3	54 ± 22	2 (50%)	2.0 ± 1.4	214 ± 99
Total	61	29	44 ± 21	28 (46%)	2.7 ± 2.8	146 ± 118

a) The control group consisted of one autopsy kidney, five cadaveric donor kidneys, two tumor nephrectomy samples (taken from a location remote from the tumor), and five biopsy samples without glomerular lesions. b) Number of samples studied with electron microscopy.

the glomeruli were unaffected, (ie, they were normocellular, had a normally developed capillary network, and had a normal GBM and mesangium). The control samples were processed in the same way as the patient biopsies. Clinical characteristics, including age, gender, serum creatinine levels, and proteinuria, of the patients and controls were collected. The patient groups and their clinical characteristics are listed in Table 1.

Antibodies

The polyclonal antibody against human nephrin was generated in rabbits immunized with 1 mg of human nephrin peptide (ERDTQSSTVSTTEAEPYYRSLC, located in the cytoplasmic region) conjugated with KLH three times with an interval of 2 wk. Human podocalyxin-like protein was prepared with a WGA column according to the same method for the purification of rat podocalyxin described by Kerjaschki et al (20). Briefly, isolated glomeruli from normal human kidneys were extracted with 0.2% Triton X-100 in PBS containing protease inhibitors. The extract was then incubated with WGA-Sepharose 4B at 4°C overnight, and unbound material was removed by washing with PBS. The sialic-acid–rich material that bound to the WGA column was released with 120 mM N-acetyl-b-glucopyranoside in PBS. Rabbits were immunized with the sialic-acid–rich material three times with an interval of 2 wk. The rabbits were killed and bled 2 wk after the last immunization. Rabbit anti-human podocin antibody p35 raised against the C-terminal part of human podocin (21) was supplied by Dr. C. Antignac (Hôpital Necker, Paris, France). Rabbit anti-CD2AP was obtained from Santa Cruz Biotechnology (SC9137; Santa Cruz, CA). Horseradish peroxidase–conjugated anti-rabbit Envision was obtained from DAKO (Glostrup, Denmark). FITC-conjugated goat anti-rabbit IgG antibody was obtained from Sigma (St. Louis, MO).

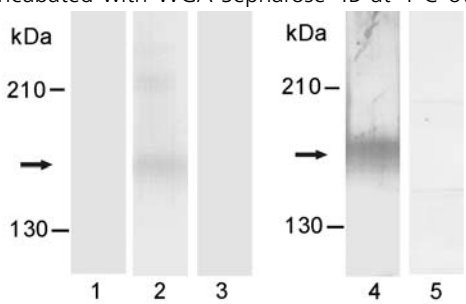


Figure 1. Characterization of the anti-nephrin and anti-podocalyxin antibodies. Western blot for anti-human nephrin antibody (lanes 1, 2 and 3) and anti-human podocalyxin antibody (lanes 4 and 5). A glomerular extract was incubated with preimmune rabbit serum (lanes 1 and 5), the anti-human nephrin antibody produced in rabbits immunized with a peptide of 21 amino acids of human nephrin sequence (lane 2), with the anti-human nephrin antibody preabsorbed with the peptide used for immunization (lane 3), or the anti-human podocalyxin antibody produced in rabbits immunized with a WGA-column purified glomerular lysate (lane 4). A clear band of approximately 180 kD was seen in lanes 2 and 4. No bands were observed in lanes 1, 3 or 5.

Western Blot Analysis

Western blot analysis of the anti-human nephrin antibody was performed with glomeruli isolated from normal human kidney by a standard sieving method with PBS containing protease inhibitors (PBS-PI; 1 mM each antipain, benzamidine, di-isopropylfluorophosphate, leupeptin, pepstatin A, and PMSF). The glomeruli were solubilized with RIPA buffer (consisting of 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 10 mM EDTA in 25 mM Tris-HCl [pH 7.2]) with protease inhibitors

described above. For Western blot analysis of the anti-human podocalyxin antibody, human glomerular lysate solubilized with 0.2% Triton X-100 in PBS-Pis was used. Insoluble material was removed by centrifugation at 15,000 x g for 10 min. Solubilized material was subjected to SDS-PAGE with 7.5% acrylamide gel according to the method of Laemmli (22) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by electrophoretic transblotting for 30 min using Trans-Blot SD (Bio-Rad). After blocking with BSA, strips of membranes were exposed to preimmune rabbit serum, anti-human nephrin, or anti-human nephrin preabsorbed with the peptide used for immunization, and anti-human podocalyxin or preimmune rabbit serum. The strips were then washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Bio Source International, Tago Immunologicals, Camarillo, CA). The reaction was developed with an alkaline phosphatase chromogen kit (Biomedica, Foster City, CA).

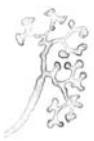
Immunohistochemistry and Immunofluorescence

Three-micrometer cryostat sections were cut, transferred to Starfrost slides, air dried, and stored at -20°C until use. For immunohistochemistry, the slides were washed in PBS and incubated for 1 h at room temperature with the primary antibody diluted in 1% BSA in PBS (rabbit anti-nephrin 1:1000; rabbit anti-podocalyxin 1:2000; rabbit anti-podocin 1:2000). The slides were then washed in PBS and incubated for 30 min with horseradish peroxidase–conjugated anti-rabbit Envision (1:1). The slides were again washed in PBS, and the staining was developed with diaminobenzidine. The color was enhanced by rinsing the slides in 0.5% $CuSO_4$ solution for 5 min. After counterstaining with hematoxylin, the slides were dehydrated and mounted.

For immunofluorescence, the slides were thawed in PBS, fixed in a mixture of 50% alcohol and 50% acetone for 5 min and subsequently in 100% alcohol for 10 min, and washed in PBS. The slides were then incubated overnight with the primary antibody at room temperature (rabbit anti-CD2AP 1:500) and thereafter washed in PBS. The slides were incubated with the FITC-conjugated anti-rabbit IgG antibody (1:200) for 30 min, washed in PBS, and covered with Vecta shield (Vector Laboratories, Burlingame, CA). For each antibody, all samples were stained in one session.

Digital Image Analysis

Of the immunohistochemically stained samples, images of all of the glomeruli in the section were taken at a x400 magnification using a Zeiss Axioplan microscope equipped with a Sony DXC-950P 3CCD color camera (Sony Corporation, Tokyo, Japan) and further analyzed using KS-400 image analysis software (Windows version 3.0; Carl Zeiss Vision, Oberkochen, Germany). The glomerular area stained was calculated by drawing a region of interest around the glomerulus in which the amount of staining within a color spectrum specific for the diaminobenzidine staining and above a fixed intensity threshold was determined, as described before (23,24).



Slides stained by immunofluorescent methods were evaluated with a Zeiss Axioplan 2 microscope, equipped with an AxioCam CCD color camera, connected to a computer equipped with AxioVision 3.0 software (Carl Zeiss Vision). In each section, five images of individual glomeruli were recorded at x400 magnification. The intensity of the staining was determined by drawing a region of interest around the glomerulus and measuring the mean luminosity value of the region with the histogram function of ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov/ij), as described in detail elsewhere (25). Recording and analysis of the digital images were performed with fixed settings.

mRNA Isolation, cDNA Synthesis and Real-Time PCR

Total RNA was extracted from microdissected glomeruli using the Trizol-method and used for cDNA synthesis with the aid of the sensiscript-RT kit (Qiagen, Westburg BV, Leusden, The Netherlands), as described previously (26). For nephrin, podocin, podoplanin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2 microglobulin (B2M), TATA box binding protein (TBP), hydroxymethyl-bilane synthase (HMBS), and hypoxanthine phosphoribosyltransferase 1 (HPRT1), forward and reverse primers (Isogen Bioscience BV, Maarsen, The Netherlands) and probes (Eurogentec Nederland BV, Maastricht, The Netherlands) were designed using Primer Express 1.5 software (PE Applied Biosystems, Foster City, CA). The sequences of the primers and probes are shown in Table 2. Real-time PCR was performed using the ABI PRISM 7700 sequence detector and software (PE Applied Biosystems). All measurements were performed in duplicate. Amplification cycles were 95°C for 10 min, followed by 50 cycles at 95°C for 30 s and at 60°C for 60 s. To correct for the amount of tissue used for RNA extraction and the efficiency of cDNA synthesis, we used the ratio between the mRNA levels of nephrin, podocin, and podocalyxin and the mRNA level of GAPDH, a constitutively expressed gene. The suitability of GAPDH as a housekeeping

Table 2. Primer and probe sequences

Molecule	Forward primer	Reverse primer	Probe
Nephrin	AGGACCGAGTCAGGAACGAAT	CTGTGAAACCTCGGGAATAAGACA	TCAGAGCTCCACGGTCAGCACAACAG
Podocin	GGCTGTGGAGGCTGAAGC	CTCAGAAGCAGCCTTTTCCG	CAGCAATCATCCGCACTTTGGCTTG
Podoplanin	CCAGGAACCAGCGAAGACC	GCGTGGACTGTGCTTTCTGA	TTGACACTTGTTGCCACCAGAGTTGTCAA
GAPDH	TGGTCACCAGGGCTGCTT	AGCTTCCCGTTCTCAGCCTT	TCAACTACATGGTTTACATGTTCCAATATGATTCCACCAA
B2M	TGCCGTGTGAACCATGTGA	CCAAATGCGGCATCTTCAA	TGATGCTGCTTACATGTCTCGATCCCACT
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC	TGTGCACAGGAGCCAAGAGTGAAGA
HMBS	CTGGTAACGGCAATGCGGCT	GCAGATGGCTCCGATGGTGA	CGAATCACTCTCATCTTTGGGCT
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	CTTGACCATCTTTGGATTATACTGCCTGACCA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; B2M, β2 microglobulin; TBP, TATA box binding protein; HMBS, hydroxymethyl-bilane synthase; HPRT1, hypoxanthine phosphoribosyl-transferase 1. Primers and probes are located within 1500 bp from the 3′ end of the mRNA. All 5′ ends of the probes were 6-carboxy-4,7,2′,7′-tetrachloro-fluorescein (TET)-labeled, except those for GAPDH and TBP, which were 6-carboxy fluorescein (FAM)-labeled. The quencher dye at the 3′ ends of the probes was 6-carboxy-tetramethyl-rhodamine (TAMRA).

gene for standardization of mRNA levels was confirmed by testing correlations between GAPDH mRNA for all samples (n = 61) and each of the other four housekeeping genes (B2M, TBP, HMBS, and HPRT1) measured.

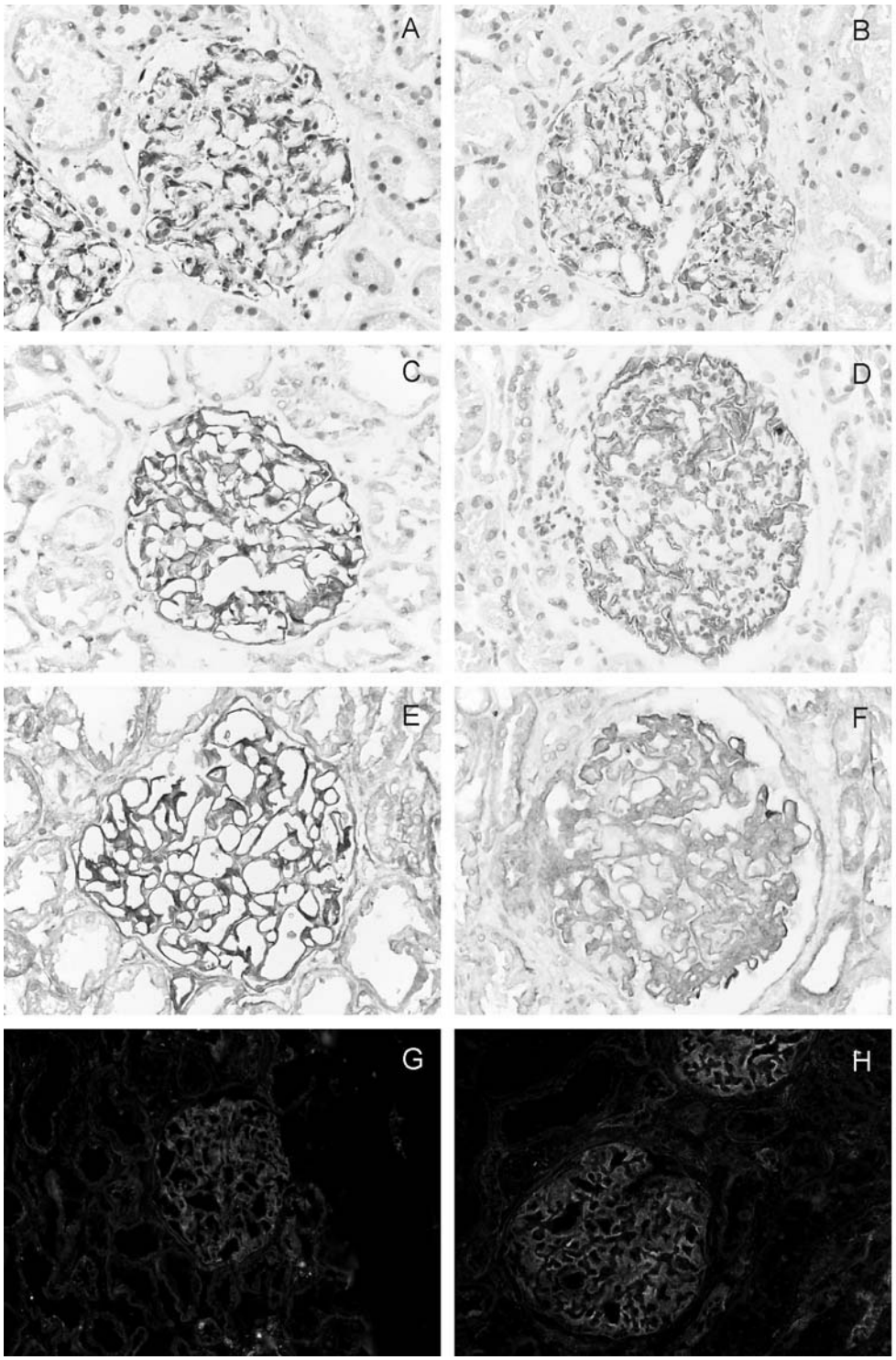
Transmission Electron Microscopy and Morphometry

Small pieces of cortex were fixed in 1.5% glutaraldehyde and 1% paraformaldehyde, dehydrated, and embedded in Spurr. In semithin sections stained with toluidine blue, nonsclerosed glomeruli were localized. Ultrathin sections were made of one or two glomeruli per tissue specimen and stained with lead citrate for transmission electron microscopy. Four to ten photographs, covering one or two glomerular cross-sections, were made with a Philips CM10 transmission electron microscope (Philips, Eindhoven, the Netherlands). A calibration grid with 2160 lines/mm was photographed to determine the exact magnification. Negatives were digitized, and images with a final magnification of approximately x17,500 were obtained. With the use of ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov/ij), the length of the peripheral GBM was measured and the number of slit pores overlying this GBM length was counted. The arithmetic mean of the foot process width (FPW) was calculated as follows: $\overline{W}_{FP} = \frac{\pi}{4} \cdot \frac{\sum \text{GBM length}}{\sum \text{slits}}$ where slits is the total number of slits counted, GBM length is the total GBM length measured in one glomerulus, and the correction factor $\pi/4$ serves to correct for the random orientation in which the foot processes are sectioned (27,28). A mean GBM length of 276 µm was evaluated in each glomerulus.

Statistical Analyses

Data are presented as means ± SD. One-way ANOVA combined with a least significant difference post hoc correction was used to test differences between groups. Correlations were calculated using Pearson correlation test. $P < 0.05$ was considered statistically significant.

Figure 2. Staining pattern of nephrin, podocin, podocalyxin, and CD2-associated protein (CD2AP) in normal and diseased human kidney sections. Nephrin (A), podocin (B), and podocalyxin (C) show a podocyte-like staining pattern in normal glomeruli as visualized by an immunohistochemical diaminobenzidine staining. The staining pattern of nephrin is more dispersed than that of podocin and podocalyxin, which show a fine glomerular basement membrane (GBM)-like line along the capillary loops of the glomerulus. CD2AP, visualized with immunofluorescence, shows a GBM-like staining pattern (D). In diseased situations, the staining for nephrin (E; minimal change disease), podocin (F; focal segmental glomerulosclerosis [FSGS]), and podocalyxin (G; FSGS) is less intense, and nephrin and podocin stainings show a more granular staining pattern. CD2AP staining shows no clear differences between control (D) and diseased tissue (H; diabetic nephropathy). Magnification x 400.


A
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Characterization of Antibodies

The results of the Western blot assays of nephrin and podocalyxin are shown in Figure 1. For both antibodies, the Western blot assay showed a clear band of approximately 180 kD in the lane incubated with the anti-human nephrin or anti-human podocalyxin antibodies, whereas no bands were seen in the lanes incubated with preimmune rabbit serum or with antibody preabsorbed with the peptide used for immunization. This confirmed the specificity of the anti-nephrin and anti-podocalyxin antibodies.

Immunohistochemistry and Immunofluorescence

In normal glomeruli, nephrin, podocin, and podocalyxin showed an intense epithelial staining along the peripheral capillary loops of the glomeruli. Nephrin showed a more dispersed pattern than podocin and podocalyxin. The GBM-like pattern for CD2AP was very subtle, and the staining, therefore, was analyzed using immunofluorescence. In glomeruli of diseased kidneys,

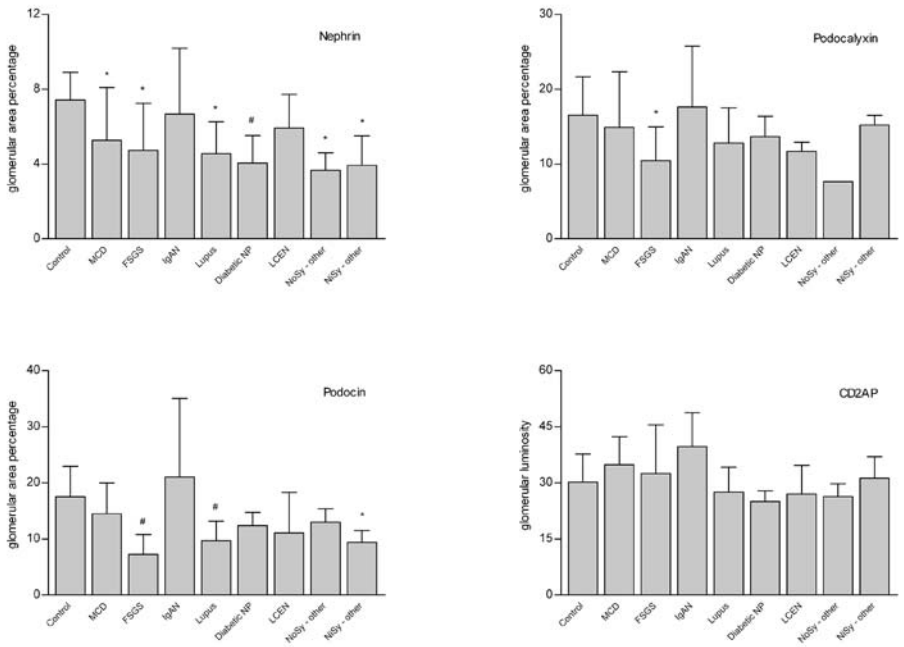

Figure 3. Glomerular expression of podocyte-associated proteins. Nephrin, podocin, and podocalyxin stainings were performed immunohistochemically. Images of the glomeruli in the sections were recorded and analyzed using digital image analysis as described in the Materials and Methods section. Nephrin, podocin, and podocalyxin show a downregulation in many disease categories compared with controls. CD2AP protein levels, as determined by measuring the mean luminosity in glomeruli stained with anti-CD2AP antibody, showed no significant differences between patient groups and controls. NoSy, other, nephrotic syndrome as a result of other causes; NiSy, other, nephritic syndromes as a result of other causes. *$P < 0.05$; ‡$P < 0.001$.

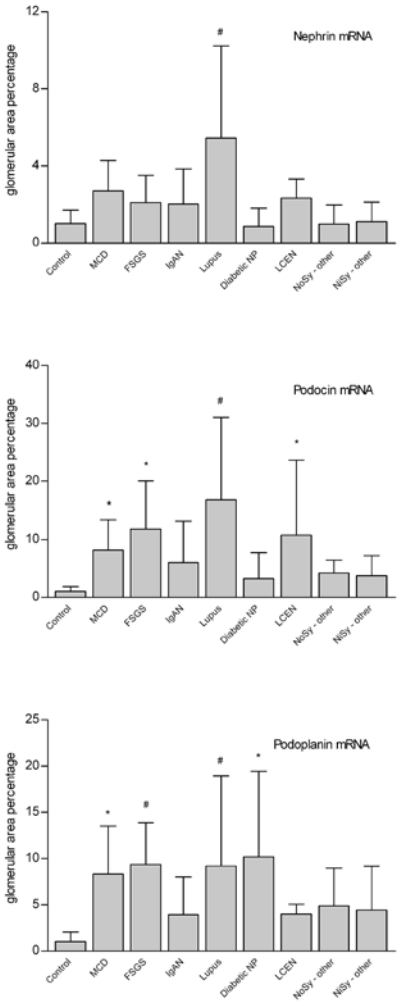

Figure 4. Glomerular mRNA levels of nephrin, podocin, and podoplanin. mRNA levels of nephrin, podocin, and podoplanin were measured in microdissected glomeruli using real-time PCR. Podocin and podoplanin mRNA levels are most prominently altered in diseased states. *P < 0.05; ‡P < 0.001.

the staining of nephrin, podocin, and podocalyxin was weaker, and sometimes the staining showed a more granular appearance. Figure 2 gives a graphical overview of the staining patterns for the different molecules in control and diseased kidneys. Quantification of the staining using digital image analysis showed that the stained glomerular surface for nephrin and podocin was significantly diminished in several disease categories, including FSGS, lupus nephritis, and the group of nephritic syndrome as a result of other causes. Podocalyxin also showed a decrease in stained glomerular surface, although in MCD and diabetic nephropathy, this was less prominent. Protein levels of all molecules were not significantly altered in IgA nephropathy compared with controls (Figure 3). The glomerular fluorescence intensity of the CD2AP staining did not differ significantly between controls and disease groups (Figure 3).

There was a strong correlation between protein levels of nephrin and podocin ($r = 0.576$, $P < 0.001$), nephrin and podocalyxin ($r = 0.477$, $P = 0.001$), and podocin and podocalyxin ($r = 0.693$, $P < 0.001$). There was no correlation between serum creatinine levels and protein levels of nephrin, podocin, or podocalyxin.

mRNA Quantification

mRNA levels for nephrin, podocin, and podoplanin were measured and corrected for the mRNA level of GAPDH. In all of the 61 samples used in this study, significant correlations ($P < 0.001$) were found between GAPDH mRNA and mRNA for each of the four other housekeeping genes, namely B2M ($r = 0.81$), TBP ($r = 0.78$), HMBS ($r = 0.82$), and HPRT1 ($r = 0.80$). This confirmed the suitability of GAPDH as a housekeeper gene in this case.

The corrected mRNA levels for the various molecules are depicted in Figure 4. In general, mRNA levels of nephrin, podocin, and podoplanin in isolated glomeruli were elevated in most disease

categories compared with controls. The mRNA levels of nephrin showed an upregulation in most disease categories, reaching a significant difference in lupus nephritis. The nephrin mRNA levels of patients who had diabetic nephropathy were slightly lower than in controls, although not significantly. The mRNA levels of podoplanin and podocin were most prominently upregulated, reaching significant difference in MCD, FSGS, and lupus nephritis for both podocin and podoplanin.

Electron Microscopy and Morphometry

Assessment of the mean FPW revealed various degrees of foot process effacement between patients, being most outspoken in patients who had MCD and FSGS (ie, up to 1900 nm compared with 640 nm in controls). The mean FPW correlated with proteinuria ($r = 0.585$, $P = 0.001$), protein levels of nephrin ($r = -0.443$, $P < 0.05$), and podoplanin mRNA levels ($r = 0.468$, $P < 0.05$). Correlation plots are shown in Figure 5.

Discussion

Resulting from the identification of the important role that podocyte-associated molecules play in maintaining the glomerular filtration barrier, the hypothesis emerged that these molecules might also be involved in acquired nephrotic syndromes (6,12). We investigated whether glomerular expression of podocyte-associated molecules is different between various groups of patients with acquired renal diseases and whether these expression levels are related to clinical parameters and ultrastructural changes.

The basolateral region of the podocyte foot process is the insertion place of the slit diaphragm (8). Nephrin and podocin are two crucial proteins in the complex of molecules that assemble and reinforce the slit diaphragm. These molecules are bound to the actin cytoskeleton via CD2AP, a general adapter molecule (14,15). The basal aspect of the podocyte foot processes is firmly attached to the GBM by several linkage proteins, including $\alpha 3\beta 1$-integrin and α- and β-dystroglycan (2,29). Podoplanin, a 43-kD molecule expressed in the glomerulus and in lymphatic tissue, is thought to have a similar function (8) and controls the shape of the podocyte foot processes (30). Proteins located at the apical part of the podocyte foot process play an important role in ultrafiltration, mainly by maintenance of the negative charge of the membrane domain. The sialoprotein podocalyxin is the most important of these molecules (20) and plays a crucial role in the maintenance of the normal morphology of the podocyte foot processes (19).

At the protein level, we observed in most patient groups a decrease of the examined podocyte-associated molecules compared with controls, except for CD2AP. We found that the glomerular amount of nephrin was significantly decreased in most proteinuric kidney diseases. Several studies concerning nephrin expression in human acquired renal diseases have recently been performed, but the various observations do not firmly support each other. Patrakka et al (31) did not find significant changes in the expression of nephrin at the protein level, whereas Doublier et al (32) and Wang et al (33) both reported a downregulation of nephrin protein expression in MCD, FSGS, and membranous glomerulonephritis. Our observations are in line with the latter findings. In a variety of experimental models, nephrin protein levels have been shown to decrease, suggesting a crucial role for nephrin in the development of proteinuria (25,34-36).

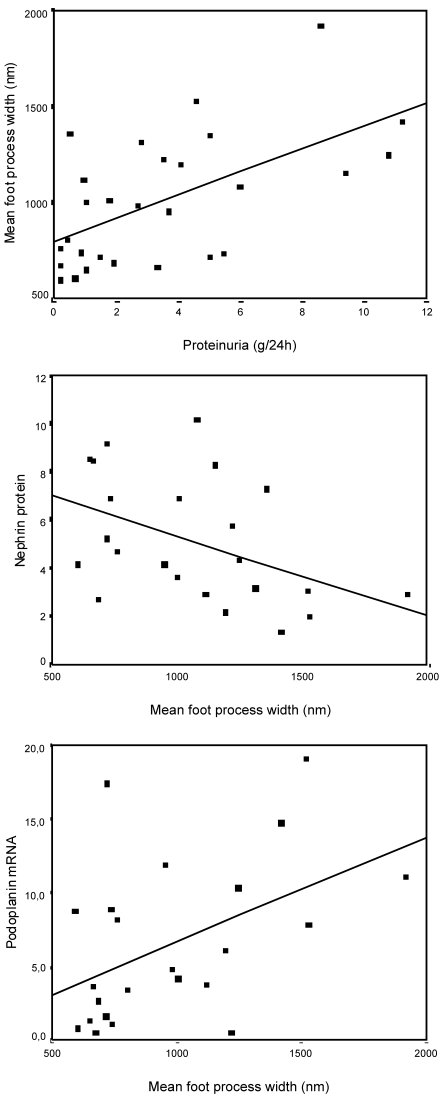

Figure 5. Correlation plots. (A) The amount of proteinuria was related to the mean foot process width (FPW; r = 0.585, P = 0.001). (B) The mean FPW of all patients was significantly correlated with the glomerular area percentage of nephrin (r = 0.443, P = 0.034). (C) Podoplanin mRNA levels were also correlated to the mean FPW (r = 0.430, P = 0.036).

Expression of podocin in human acquired proteinuric diseases has until now not been described. In our study, we found that the podocin protein levels were significantly decreased in most disease categories compared with controls. The expression patterns of podocin protein resemble those described for puromycin aminonucleoside (PAN) nephrosis in the rat, a model for MCD, which is characterized by heavy proteinuria (37).

In contrast to nephrin and podocin, the protein expression of CD2AP did not show major differences between patient groups and controls in our study. The same pattern has been described for the passive Heyman nephritis model in the rat, in which an overt decrease of nephrin protein expression was observed, whereas CD2AP protein expression remained unaltered (25). Still, it is remarkable that in proteinuric states, podocin and nephrin protein levels are severely decreased, whereas in the same cluster of molecules, CD2AP remains unimpaired. This might

suggest that upon damage to the podocyte, the binding of CD2AP with nephrin and podocin is disconnected, whereas CD2AP itself remains attached to the actin cytoskeleton (25). As observed in experimental models, the uncoupled proteins might be shed from the podocyte membrane and excreted in the urine (38,39), whereas CD2AP condenses in the scaffold of microfilaments in the effaced foot processes (25,40).

The glomerular podocalyxin protein expression in most proteinuric diseases was reduced compared with controls. This might suggest uncoupling of podocalyxin from the actin cytoskeleton as a result of rearrangements of the cytoskeleton. This phenomenon has been described in rat models of proteinuria, in which the complex linking podocalyxin to the actin cytoskeleton is disrupted (19).

In remarkable contrast to decreased protein levels in most patient groups, the mRNA levels in proteinuric states were generally increased compared with controls. Nephrin mRNA levels were significantly elevated in lupus nephritis, whereas only in diabetic nephropathy was there a slight decrease in nephrin mRNA levels. The latter finding is in line with other studies measuring nephrin mRNA expression in diabetic nephropathy by in situ hybridization (41). Our results are in sharp contrast with the study by Furness et al (42), which described a downregulation of nephrin mRNA in four patients with nephrotic syndrome. Patrakka et al (31) did not find significant changes in nephrin mRNA levels, as studied in proteinuric patients using in situ hybridization. In experimental studies, both up- and downregulation of nephrin mRNA in proteinuric states has been described (34,35,38,43). Following the same pattern as nephrin mRNA, the podocin mRNA levels showed an increase in most patient groups. Likewise, podoplanin mRNA levels were increased in most patient groups compared with controls. In PAN nephrosis of the rat, mRNA levels of molecules that bind the podocyte to the GBM are increased, suggesting an effort of the podocytes to remain attached to the GBM (37). Similarly, in human and experimental proteinuric states, the mRNA expression of α-actinin-4 is increased, probably reflecting a compensatory reaction of the podocyte (44,45). The rise in mRNA levels of nephrin, podocin, and podoplanin might likewise result from a compensatory reaction to the damage inflicted on the podocyte.

The pathogenic effects exerted on the glomerulus in the various renal disease entities studied differ in nature and severity. For example, putative circulating permeability factors might cause the loss of the glomerular permselectivity in MCD (46). In lupus nephritis and membranous nephropathy, subepithelial Ig deposits are formed, thereby inflicting damage on the podocyte, whereas in IgA nephropathy, more preferentially mesangial areas are affected (47). Diabetic nephropathy is thought to impair the charge selectivity of the glomerular filtration barrier and might thereby indirectly damage the podocyte (48). Damage to the podocyte leads, irrespective of its origin, to effacement of foot processes (40). Therefore, as a measure of podocyte damage, we examined the extent of foot process effacement by assessment of the mean FPW. The mean FPW was positively

correlated with proteinuria and podoplanin mRNA levels, whereas it was inversely correlated with nephrin protein levels. This suggests that the changes seen in protein expression are related to the severity of the damage inflicted on the podocyte. For instance, that IgA nephropathy is characterized primarily by damage to the mesangial areas and, to a lesser extent, to the podocytes might be reflected by the absence of significant changes in the podocyte-associated molecules at the protein level, as has been observed before (32). The observation that downregulation of nephrin protein is paralleled by foot process effacement is in line with results from a study by Huh et al (49) and Wernerson et al (50), which suggests that not the quantity of nephrin between adjacent foot processes is different but that merely the total glomerular amount of nephrin is decreased as a result of foot process effacement. The decreased protein staining of nephrin, podocin, and podocalyxin might also be explained by a reduction of podocytes, as has been observed in several diseases (51,52). Furthermore, the accessibility of the antigen might be diminished.

The development of a nephrotic syndrome and effacement of podocyte foot processes is nowadays known to occur upon mutations in a wide range of podocyte-associated molecules, including those investigated in this study. The molecular scaffold in the podocyte foot process therefore seems to be fragile, disassembling when one of its components is removed. Generally, in the current study, the protein expression of most podocyte-associated molecules was diminished in acquired proteinuric diseases, coinciding with an increase in mRNA levels. A divergence between mRNA and protein levels has been described for synaptopodin and α-actinin 4 in patients with nephrotic syndromes (24,44) and in podocyte-associated molecules in the PAN nephrosis rat model (37,45). There was no direct correlation between the level of proteinuria and the protein and mRNA expression of the molecules studied. However, nephrin protein levels and podoplanin mRNA levels were correlated with the effacement of podocyte foot processes, which might point in the direction of an association between the expression of podocyte-associated molecules and proteinuria. It is still unclear whether changes in expression of these podocyte-associated molecules are the underlying cause of the development of proteinuria. Taken together, there is a cluster of molecules, at both the protein and the mRNA levels, that seem to show a stereotypic reaction in proteinuric diseases. Besides taking into account the correlation between foot process effacement and protein and mRNA levels, the observed changes in expression levels merely seem to be the consequence of podocyte foot process effacement as a result of other causes and the subsequent reaction of the podocyte to this phenomenon.

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