Nephrin Redistribution on Podocytes Is a Potential Mechanism for Proteinuria in Patients with Primary Acquired Nephrotic Syndrome

Sophie Doublier,* Vesa Ruotsalainen,[†]
Gennaro Salvidio,[‡] Enrico Lupia,* Luigi Biancone,*
Pier Giulio Conaldi,[§] Paula Reponen,[†]
Karl Tryggvason,[¶] and Giovanni Camussi*

From the Dipartimento di Medicina Interna,* Università di Torino, Torino, Italy; the Dipartimento di Medicina Interna,[‡] Cattedra di Nefrologia, Università di Genova, Genova, Italy; the Dipartimento di Scienze Cliniche e Biologiche,[§] Università dell'Insubria, Varese, Italy; the Department of Biochemistry,[†] University of Oulu, Oulu, Finland; and the Department of Medical Biochemistry and Biophysics,[¶] Karolinska Institute, Stockholm, Sweden

We investigated the distribution of nephrin by immunofluorescence microscopy in renal biopsies of patients with nephrotic syndrome: 13 with membranous glomerulonephritis (GN), 10 with minimal change GN, and seven with focal segmental glomerulosclerosis. As control, six patients with IgA GN without nephrotic syndrome and 10 normal controls were studied. We found an extensive loss of staining for nephrin and a shift from a podocyte-staining pattern to a granular pattern in patients with nephrotic syndrome, irrespective of the primary disease. In membranous GN, nephrin was co-localized with IgG immune deposits. In the attempt to explain these results, we investigated *in vitro* whether stimuli acting on the cell cytoskeleton, known to be involved in the pathogenesis of GN, may induce redistribution of nephrin on the surface of human cultured podocytes. Aggregated but not disaggregated human IgG₄, plasma-malemmal insertion of membrane attack complex of complement, tumor necrosis factor- α , and puromycin, induced the shedding of nephrin with a loss of surface expression. This phenomenon was abrogated by cytochalasin and sodium azide. These results suggest that the activation of cell cytoskeleton may modify surface expression of nephrin allowing a dislocation from plasma membrane to an extracellular site. (*Am J Pathol* 2001, 158:1723–1731)

Nephrotic syndrome is a clinical disorder associated with several primary and secondary glomerulonephritis (GN) that may be caused by different pathogenetic mechanisms.¹ Several studies have addressed the mechanisms

involved in the loss of perm-selectivity of glomerular capillary walls. Most of these studies have focused on the role of glomerular basement membrane components as well as of glomerular anionic sites. Recently, the identification of nephrin has stressed the role of podocytes in maintaining glomerular permeability.² Nephrin is a 1,242-amino-acid residue transmembrane protein of the immunoglobulin superfamily, specifically expressed at the slit diaphragm located between the glomerular podocyte foot processes.³ Mutations in the nephrin gene have been found in both Finnish and non-Finnish patients with congenital nephrotic syndrome of the Finnish type,^{4,5} suggesting a general involvement of nephrin gene in hereditary nephrotic syndromes.⁵ In addition, mutations in the nephrin gene have been described in proteinuric patients who do not exhibit the classical severe Finnish-type congenital nephrotic syndrome.⁵ Because nephrin resides in the slit diaphragm and mutations in the gene cause massive nephrotic syndrome at birth, this protein may have a relevant role in the filtration mechanism of glomeruli and more generally in the pathogenesis of proteinuria. In experimental models of GN a correlation between changes in nephrin expression and proteinuria has been shown.^{6–8} The injection into rats of monoclonal antibody (mAb) 5-1-6, which recognized the extracellular domain of nephrin, induced severe proteinuria.⁶ Moreover, an altered distribution of nephrin was observed in puromycin aminonucleoside-induced nephrosis and in mercury chloride-treated rats.^{7,8} These experiments raise the possibility that, beside genetic mutations of nephrin, an acquired alteration of nephrin distribution at the level of slit diaphragm may also account for proteinuria.

The aim of the present study was to investigate whether the expression of nephrin was altered in biopsies from patients with primary acquired nephrotic syndrome. Because we found that in these patients the immunohistochemical-staining pattern of nephrin was severely altered, we also

Supported by the National Research Council, Targeted Project Biotechnology and by Ministero Università e Ricerca Scientifica e Tecnologica (MURST) ex60%. Dr. Sophie Doublier is recipient of a Marie Curie individual fellowship from the European Community.

Accepted for publication February 5, 2001.

Address reprint requests to Dr. G. Camussi, Cattedra di Nefrologia, Dipartimento di Medicina Interna, Ospedale Maggiore S. Giovanni Battista, Corso Dogliotti 14, 10126, Torino, Italy. E-mail: giovanni.camussi@unito.it.

Table 1. Clinical Features of Patients

	Membranous GN	Minimal change GN	FSGS	IgA GN
<i>n</i>	13	10	7	6
Age (years)	59 ± 16	40 ± 25	42 ± 10	50 ± 21
Sex (M/F)	4/7	6/4	2/5	4/2
UP (g/day)	5.9 ± 3.4	7.1 ± 2.4	4.4 ± 1.2	0.2 ± 0.2
NS (%)	85 (11/13)	100 (10/10)	86 (6/7)	0 (0/6)
Scr (mg/dL)	1.2 ± 0.8	0.9 ± 0.2	1.4 ± 0.8	1.8 ± 0.7

Abbreviations: UP, proteinuria; NS, nephrotic syndrome; Scr, serum creatinine.

studied the effect of various stimuli on the surface expression of nephrin on human cultured podocytes.

Materials and Methods

Reagents

The mouse anti-nephrin antibody (IgG₁) is a mAb specific for the extracellular fibronectin type III-like motif of the recombinant human nephrin produced in A293 cells (V. Ruotsalainen and K. Tryggvason, manuscript in preparation). In Western blots, nephrin antibody recognized the extracellular domain of recombinant human nephrin and a 180-kD protein in lysates of human glomeruli.³ An irrelevant IgG₁ isotypic control antibody was purchased from Cedarlane (Hornby, Ontario Canada). Rabbit antiserum to human-IgG was purchased from Dade Behring (Marburg, Germany). Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (adsorbed with human serum proteins); tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (adsorbed with human IgG); FITC-phalloidin; human IgG₄ kappa (IgG₄); human tumor necrosis factor- α (TNF- α); cytochalasin B; sodium azide; C5, C6, C7, C8, and C9 complement fragments; methionine; chloramine-T; and puromycin were obtained from Sigma Chemical Co. (Saint Louis, MO). Mouse mAb anti-human C9 neoantigen of the C5b-9 complex (MAC) was purchased from DAKO (Glostrup, Denmark). The antibody anti-E-selectin was obtained from Chemicon Int. (Temecula, CA). Vectashield mounting medium was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Patients

The study included 30 proteinuric patients (27 of 30 with nephrotic syndrome) and six patients with minimal proteinuria. Nephrotic syndrome was defined as proteinuria ≥ 3.5 g/day and serum albumin ≤ 3.0 g/dL. The histological diagnosis of proteinuric patients was membranous GN in 13 cases, minimal change GN in 10 cases, and focal segmental glomerulosclerosis (FSGS) in seven cases. Patients with minimal proteinuria selected for the study presented IgA GN. None of the patients had evidence of systemic disease on a clinical or laboratory basis. Table 1 depicts some of the clinical features of the patients included in the study. As control, 10 specimens

were obtained from normal kidney portions of patients undergoing surgery for cancer. Patients were selected for absence of proteinuria and lack of glomerular abnormalities detected by light and immunofluorescence microscopy.

For all patients, the protein content of 24-hour urinary samples was measured by the pyrogallol red method. The creatinine concentration in plasma was analyzed by kinetic Jaffé method with a Beckman Synchron CX3.

Culture of Glomerular Epithelial Cells

Decapsulated glomeruli were isolated by differential sieving from renal cortex fragments taken from surgically removed kidneys of five Caucasian patients.^{9,10} Primary cultures of glomerular epithelial cells (GECs) were established as previously described.¹¹ GECs were obtained by plating at high density glomeruli untreated with collagenase. After a 10-day incubation in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, the cultures were trypsinized and the outgrowing GECs were expanded. Phenotypic characterization was performed according to cell morphology (polyhedral cells with cobblestone-like appearance); positive staining for synaptopodin, podocalyxin, zonula occludens-1 (ZO-1), cytokeratin, vimentin, and laminin; negative staining for smooth muscle-type myosin, FVIII:Ag, and CD45; cytotoxicity in response to puromycin aminonucleoside (10 to 50 μ g/ml).^{10,12-14}

Established lines of differentiated GECs were obtained by infection of pure primary cultures with a hybrid Adeno5/SV40 virus as previously described.^{11,15} Individual foci of immortalized cells were subcultured and cloned. The selected clones were used between passages 25 and 40. The GEC line used in the present study was characterized as previously described according to the phenotype, following the criteria mentioned above.^{11,13}

Immunofluorescence Studies

Immunofluorescence (IF) studies were performed on kidney biopsies from the patients described above. The tissues were rapidly frozen in liquid nitrogen, and 2- μ m-thick cryostat sections were fixed in 3.5% paraformaldehyde for 15 minutes and washed in phosphate-buffered saline (PBS). The sections were incubated with anti-nephrin mAb at a concentration of 10 μ g/ml or with the irrelevant mouse IgG₁ isotypic control antibody, for 2 hours at room temperature, washed in PBS, and incubated with FITC-conjugated sheep anti-mouse IgG. Double staining was performed on biopsies from patients with membranous GN using a rabbit antiserum to human IgG and a TRITC-conjugated goat anti-rabbit IgG. A rabbit nonimmune serum was used as irrelevant control.

IF on cultured GECs was performed as previously described.⁹ Briefly, coverslip-attached GECs at subconfluent density were fixed in 3.5% paraformaldehyde containing 2% sucrose for 15 minutes at room temperature and washed in PBS. In experiments aimed to study intracellular localization of nephrin or cell cytoskeleton, cells

were made permeable to large molecules by soaking coverslips for 5 minutes at 0°C in HEPES-Triton X-100 buffer (20 mmol/L HEPES, pH 7.4, 300 mmol/L sucrose, 50 mmol/L NaCl, 3 mmol/L MgCl₂, and 0.5 Triton X-100). Cells were then incubated with antibodies as described for the biopsies. The slides were then washed, mounted with Vectashield mounting medium, and examined.

Control experiments included incubation of sections or cells with nonimmune isotypic control antibodies or the omission of primary antibodies followed by the appropriate labeled secondary antibodies. The specificity of anti-nephlin mAb was tested by pre-adsorption of the antibody (10 µg/ml) with purified recombinant extracellular nephlin (30 µg/ml).

The number of glomeruli available on each biopsy for analysis of nephlin expression ranged between 3 to 7. Three nonsequential sections were examined for each specimen. Nephlin expression was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows MicrolImage, version 3.4 CASTI Imaging, Venezia, Italy) of images obtained using a low-light video camera (Leica DC100, Wetzlar, Germany) on a 180-µm diameter field. The results were expressed as relative fluorescence intensity on a scale from 0 (fluorescence of background of tissue) to 255 (fluorescence of standard filter).

Detection of Nephlin mRNA Expression by Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

RT-PCR was performed using total RNA from GECs. Total RNA was extracted using Tri Reagent (Sigma) and precipitated with isopropanol. Complementary DNA was obtained by using oligo-p(dT)₁₅ primers (Boehringer Mannheim, Mannheim, Germany). Reverse transcription was performed at 42°C for 60 minutes; in addition to 1 µg of RNA, the reaction mixture (20 µl) contained 10 mmol/L Tris, 50 mmol/L KCl, pH 8.3, 5 mmol/L MgCl₂, 1 mmol/L dNTPs, 50 U RNase inhibitor and 20 U AMV reverse transcriptase (Boehringer Mannheim). For reverse transcriptase-negative controls, the enzyme was omitted. cDNA was then subjected to 35 cycles of amplification by the PCR in an automated DNA thermal cycler (Hybaid, Ashford, UK). For detection of human nephlin and human glyceraldehyde phosphate dehydrogenase (h-GAPDH), used as housekeeping gene, sequence-specific oligonucleotide primers (purchased from TIB Molbiol, Genova, Italy) were designed (nephlin: 3'reverse, TAC ACC AGA TGT CCC CTC AG; 5' forward, TCT TAT TCC CGA GGT TTC AC; GAPDH: 3'reverse, TCT AGA CGG CAG GTC AGG TCC ACC; 5' forward, CCA CCC ATG GCA AAT TCC ATG GCA). The PCR mixture (50 µl) contained 10 mmol/L Tris-HCl, pH 8.8, at 25°C, 50 mmol/L KCl, 0.1% Triton X-100, 1.25 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 25 pmol of (+) and (−) primers, and 1.25 U thermostable DNA polymerase (Finnzymes). Times and temperatures for denaturation, annealing, and extension were 1 minute at 95°C, 1 minute at 60°C, and 3 minutes at 72°C, respectively. Amplification products (257 bp) for nephlin

and (598 bp) for GAPDH were visualized by ethidium bromide staining after agarose gel electrophoresis.

Detection of Nephlin Expression by Western Blot Analysis

Protein concentration of GEC lysates obtained as previously described¹⁶ was determined by the Bradford technique, and the protein content of the samples was normalized to 100 32 µg/sample by appropriate dilution with Laemmli buffer. Proteins were directly subjected to 8% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose. The filters were incubated with blocking solution (10% low-fat milk in 20 mmol/L Tris/HCl, pH 7.6, and 17 mmol/L NaCl) for 60 minutes. The anti-nephlin mAb was then added at a concentration of 2.5 µg/ml, and the incubation was performed overnight at 4°C. For detection, the filters were washed four times (15 minutes each wash) with PBS and 0.5% Tween 20, and reacted for 60 minutes at room temperature with peroxidase-conjugated protein A (200 ng/ml; Amersham, Buckinghamshire, UK). The enzyme was removed by washing as above. The filters were incubated for 2 minutes with a chemiluminescence reagent (ECL, Amersham) and exposed to an autoradiography film for 1 to 5 minutes.

As negative control for the expression of nephlin, an immortalized cell line of human renal tubular epithelial cells was used.¹⁵

Experimental Design

The expression of nephlin by GECs was evaluated by indirect IF on cells fixed with paraformaldehyde followed or not by permeabilization with Triton X-100. In some experiments, living (unfixed) GECs were incubated at 37°C or 4°C for 60 minutes with anti-nephlin mAb to evaluate antibody-induced antigen redistribution.⁹ To study *in vitro* the role of immune complexes on the surface expression and redistribution of nephlin, GECs were incubated with human aggregated IgG₄ (agIgG₄) (1 µg/ml) in Dulbecco's modified Eagle's medium and 10% fetal bovine serum for 60 minutes at 37°C before fixation and staining with anti-nephlin mAb and rabbit anti-human-IgG. AgIgG₄ was obtained by heating at 63°C for 30 minutes, as described.¹⁷ As control, cells were incubated with human disaggregated IgG₄ in Dulbecco's modified Eagle's medium and 10% fetal bovine serum for 60 minutes at 37°C before staining with antibodies. Human IgG₄ were disaggregated after centrifugation for 4 hours at 100,000 × g at 4°C.¹⁸

Actin microfilament alterations in agIgG₄-stimulated GECs were evaluated by FITC-phalloidin staining after permeabilization of the cells. Other stimuli, such as TNF-α, puromycin, or MAC, known to be involved in the pathogenesis of GN and to affect the cell cytoskeleton, were used to evaluate the role of actin microfilament alterations on the expression and redistribution of nephlin. Cells were incubated with human TNF-α (10 ng/ml) or puromycin (5 µg/ml) in Dulbecco's modified Eagle's me-

dium and 10% fetal bovine serum for 1 hour at 37°C before staining with anti-nephrin mAb. Actin microfilament alterations in TNF- α or puromycin-stimulated GECs were evaluated as FITC-phalloidin staining. Assembly of the MAC on GECs was performed as previously described.¹⁹ Briefly, nonenzymatic formation of a C5b-like C5C6 complex was obtained by incubation of 10 μ g of C5 in 10 μ l of veronal buffer with 10 μ l of 0.32 mmol/L chloramine-T for 10 minutes at room temperature. After inactivation of chloramine-T with 10 μ l methionine (1 mmol/L), purified C6 (20 μ g) was added in 300 μ l of serum-free medium and incubated for 24 hours at 37°C. The assembly of the MAC into cell plasma membrane was obtained by 15 minutes of pre-incubation (37°C) of C7 (10 μ g/ml) with the C5b-C6 complex (5 μ g/ml). After two washings with serum-free medium, C8 (10 μ g/ml) and C9 (10 μ g/ml) complement components were added and incubated for 30 minutes at 37°C. In selected experiments heat inactivated (100°C for 30 minutes) or polymyxin B-treated (5 to 50 μ g/ml) complement components were used as control. After cell fixation, the expression of nephrin was evaluated by IF with anti-nephrin mAb. In parallel experiments the insertion of MAC into GEC plasma membrane was assessed by IF using a mAb anti-human C5b-9 reacting with a neoepitope exposed only in activated C9 in the solid-phase membrane form and in the fluid form of MAC, but not in native C9.²⁰

In some experiments, before incubation with the various stimuli, the GECs were pre-incubated for 15 minutes with drugs that interfere with cytoskeletal function such as cytochalasin B (5 and 10 μ g/ml) or with cell metabolism such as sodium azide (10^{-3} to 10^{-1} mol/L). The cell viability was assessed by trypan blue exclusion.

Statistical Analysis

All results are given as mean values $\pm$ SD. Differences between multiple groups were analyzed by one-way analysis of variance in combination with Tukey's multiple comparison test. Linear regression analysis was performed between relative fluorescence intensity for nephrin and extent of proteinuria. A *P* value of <0.05 was considered significant.

Results

Expression of Nephrin in Primary Acquired Glomerular Diseases

The expression of nephrin was evaluated by indirect IF in 30 proteinuric patients (27 with nephrotic syndrome) including 13 membranous GN, 10 minimal change GN, and seven FSGS, and in six patients with IgA GN and minimal proteinuria (Table 1) using mAb specific for the extracellular domain of nephrin. As control, 10 specimens of normal kidney portions obtained from patients undergoing surgery for renal tumors were used.

In normal controls, nephrin exhibited a glomerular epithelial pattern with a punctate/linear distribution along the peripheral capillary loops (Figure 1A). In glomeruli of

patients with membranous GN, a more granular pattern or a loss of staining of nephrin was observed (Figure 1B). Figure 1, C and D, shows the co-localization of nephrin and IgG evaluated by double IF in the same glomerulus of a patient with membranous GN. In Figure 1C, the green fluorescence depicts the distribution of nephrin. The red fluorescence observed in Figure 1D shows the granular distribution of IgG along the glomerular basement membrane. The overlap of staining for nephrin (green) and IgG (red) results in a yellow staining of granular deposits in the merge (Figure 1D). A granular pattern with aspects of apparent plasmalemmal dislocation from the normal expression sites of nephrin and a loss of staining were also observed in patients with minimal change GN (Figure 2, A and B) and FSGS (Figure 2C). In IgA GN, the staining pattern of nephrin showed an epithelial distribution, similar to that of controls (Figure 2D). As shown in Figure 3, relative fluorescence intensity was significantly reduced in all proteinuric patients irrespective of the primary disease. In contrast, the fluorescence intensity in glomeruli of patients with IgA GN with minimal proteinuria did not significantly differ from that of controls (Figure 3). The average variability in nephrin staining pattern from one glomerulus to the next within individual patients and controls was 20.6 ± 16 and 14.4 ± 4 , respectively.

As shown in Figure 4 an inverse correlation between relative fluorescence intensity and extent of proteinuria was detected when all patients with GN were analyzed. In contrast, when linear regression analysis was performed only on patients with nephrotic syndrome, no significant correlation was observed.

The staining for nephrin was completely abrogated by pre-adsorption of the antibody with the purified human recombinant extracellular nephrin, indicating staining specificity. Moreover, sections incubated with the nonimmune isotypic control antibodies or with the appropriate labeled secondary antibodies without the primary antibody were always negative (data not shown).

Expression of Nephrin by GECs

Primary and immortalized GECs showed expression of the nephrin mRNA and of the protein by RT-PCR (Figure 5A) and Western blot analysis (Figure 5B), respectively. When tested by indirect IF, antibody specific for the extracellular domain of nephrin bound to GECs unfixed or fixed with paraformaldehyde with a punctate granular pattern, suggesting a surface expression of nephrin (Figure 6A). In paraformaldehyde-fixed and permeabilized GECs a combination of fine granular and diffuse staining was observed suggesting the presence of an intracellular pool of nephrin. In permeabilized cells the staining was more evident around the nuclei (data not shown).

Modulation of Nephrin Expression on GECs

Experiments of nephrin redistribution were performed on immortalized GECs. When living (unfixed) GECs were incubated with anti-nephrin mAb for 60 minutes at 37°C, but not at 4°C, the binding was rapidly converted from

Figure 1. Immunofluorescence staining for nephrin in glomeruli of normal controls (**A**), and of patients with membranous GN (**B**, **C**, and **D**). Co-localization of nephrin (green) and IgG (red) was evaluated in glomeruli of membranous GN patients by double IF. **C**: Distribution of nephrin. The overlap of staining for nephrin and IgG results in a yellow staining of granular deposits in the merge (**D**). Original magnifications: $\times 400$ (**A** and **B**); $\times 600$ (**C** and **D**).

fine granular into a patchy pattern, suggesting antibody-induced antigen redistribution (data not shown). Therefore, we tested whether aglgG₄ were able to change the surface expression of nephrin. Incubation of living GECs at 4°C for 60 minutes with aglgG₄ resulted in diffuse surface binding of IgG₄ to GECs (data not shown). When incubated at 37°C for 60 minutes, aglgG₄ appeared redistributed in one or multiple aggregates (capping) (data not shown). When cells were fixed with paraformaldehyde after 60 minutes incubation with aglgG₄, nephrin appeared focally redistributed on the cell surface, leaving variable parts of the cell surface devoid of antigen (Figure 6B). Double staining of nephrin and IgG showed focal co-localization on the cell surface (Figure 6B, inset). In most GECs the disappearance of nephrin from the surface was predominant, as indicated by a significant decrease of IF staining (Figures 6C and 7A), suggesting shedding of the protein from the cell surface. AglgG₄ induced disruption of normal cellular organization of F-actin (Figure 6D) in GECs, with loss of stress fibers and peripheral actin localization (Figure 6, E and F). In contrast, disaggregated IgG₄ did not induce nephrin redistribution and disappearance (Figure 6G) or cytoskeletal

changes (data not shown). We therefore investigated whether the observed nephrin redistribution could be ascribed to cytoskeletal reorganization induced by aglgG₄. Plasma membrane insertion of MAC, TNF- α , or puromycin, stimuli known to affect the cytoskeleton of GECs,^{14,21,22} also induced a significant reduction of IF intensity (Figure 7A) and a staining pattern compatible with surface patching and shedding of nephrin (Figure 6, H and I). Within 24 hours of removal of TNF- α from the culture medium, nephrin was fully re-expressed on GECs, indicating that the effect of this cytokine was not to be ascribed to a cytotoxic effect (Figure 6J). Moreover, the process of nephrin redistribution was an active energy-requiring process, because sodium azide, an inhibitor of oxidative phosphorylation and of glycolysis, prevented patching and shedding induced by aglgG₄, TNF- α , and puromycin (Figures 6K and 7B). Similar results were obtained with cytochalasin B, a compound that affects the microfilaments of the microtubular system,²³ which prevented reduction of fluorescence intensity and morphological evidences of nephrin redistribution (Figures 6L and 7C).

Figure 2. Immunofluorescence staining for nephrin in glomeruli of minimal change GN (A and B), FSGS (C), and IgA GN (D). Original magnifications: $\times 400$ (A–D).

In all of the experiments of cytoskeleton activation, the reduction in cell viability ranged from 6 to 11%.

In control experiments, the staining for nephrin was completely abrogated by pre-adsorption of the antibody with the purified human recombinant extracellular nephrin. When the relevant antibodies were substituted with the nonimmune isotypic control antibodies or with the appropriate labeled secondary antibodies without the primary antibody the immunofluorescence was always negative (data not shown).

Figure 3. Semiquantitative analysis of nephrin expression in glomeruli of normal control; membranous GN; and minimal change GN, FSGS, and IgA GN patients. **, $P < 0.001$ versus control patients.

Discussion

The pivotal role of nephrin in the regulation of glomerular filter integrity has recently emerged from the recognition that mutations in the nephrin gene (NPHS1) underline the development of the congenital nephrotic syndrome of the Finnish type (CNF).⁴ Nephrin, a transmembrane protein of the immunoglobulin superfamily, is specifically located in the kidney at the slit diaphragm of glomerular podocytes.

Figure 4. Linear regression analysis between relative fluorescence intensity for nephrin staining and extent of proteinuria in all GN patients (membranous GN and minimal change GN, FSGS, and IgA GN).

Figure 5. **A:** Expression of the GAPDH mRNA (lane 2) and the nephrin mRNA (lane 3) in GECs. **Lane 1:** Molecular weight marker (range, 200 to 2,000 bp). **B:** Analysis of the expression of nephrin by Western blot in nonconfluent GECs (lane 2) and in confluent GECs (lane 3). **Lane 1** shows an irrelevant antibody (anti-E-selectin) in GECs. **Lane 4:** The absence of nephrin in a renal tubular epithelial cell line.

cytes.³ It has an extracellular portion containing eight Ig motifs and one type III-fibronectin domain. The intracellular domain contains eight tyrosine residues, suggesting that nephrin may behave as a signaling adhesion molecule.⁴ The observation that genetic mutations of nephrin are associated with massive nephrotic syndrome at birth suggested that nephrin has a relevant role in the functional and structural organization of slit diaphragm.² The fact that nephrin knockout mice also exhibit massive proteinuria and die within 24 hours after birth²⁴ supports this notion. Moreover, knock-out mice for CD2-associated protein, which is expressed in the slit diaphragm and strictly associated with nephrin, were shown to develop a congenital nephrotic syndrome, suggesting that not only nephrin but other proteins expressed at the level of the slit diaphragm may have a critical role in maintaining glomerular permeability.²⁵

Evidences for a critical role of nephrin in maintaining glomerular permeability in acquired proteinuric diseases was first derived from experiments in rats injected with mAb 5-1-6 that has been shown to be directed at the extracellular domains of nephrin.⁶ This treatment induced proteinuria and nephrotic syndrome.⁶ Moreover, a decrease in nephrin mRNA expression and redistribution of the protein were observed in several animal models such as puromycin aminonucleoside nephrosis and mercuric chloride GN.^{7,8}

The present results demonstrated that, in patients with acquired nephrotic syndrome, the IF staining for nephrin was significantly reduced in intensity with extensive loss and granular redistribution. In membranous GN, granular deposits of nephrin were co-localized with the extracellular immune deposits. Moreover, granular distribution of nephrin suggesting a plasmalemmal dislocation from the normal expression sites at the interpodocytes filtration slits was also observed in minimal change GN and FSGS.

Figure 6. Immunofluorescence staining for nephrin on GECs incubated 60 minutes with vehicle alone (**A**), aglgG₄ (1 μg/ml) (**B** and **C**), disaggregated IgG₄ (1 μg/ml) (**G**), TNF-α (10 ng/ml) (**H**), or after the plasma membrane insertion of MAC (**I**). Within 24 hours of removal of TNF-α from the culture medium, nephrin was fully re-expressed on GECs (**J**). Reduction of fluorescence intensity and morphological evidences of nephrin redistribution after incubation with TNF-α were prevented by pre-incubation with sodium azide (**K**) and cytochalasin B (**L**). FITC-phalloidin staining of actin microfilaments on permeabilized GECs was performed after incubation for 60 minutes with vehicle alone (**D**) or aglgG₄ (1 μg/ml) (**E** and **F**). Double staining for nephrin (green) and for IgG (red) on GECs incubated for 60 minutes with aglgG₄ (1 μg/ml) showed focal co-localization on the cell surface (**B**, inset). Nuclei were stained with ethidium bromide (dilution 1/1,000). Original magnifications: ×600 (**A-L**); ×240 (inset).

Similar results have been reported in experimental animal models such as puromycin aminonucleoside nephrosis,⁷ mercuric chloride-treated rats,⁸ and nephritis induced by injection of mAb 5-1-6,⁶ in which the pattern of nephrin IF staining shifted from epithelial/linear to granular. In the present study we did not observe significant differences in the reduction of glomerular staining for nephrin among membranous GN, minimal change GN, and FSGS. In patients with IgA GN with minimal proteinuria neither a significant loss of IF staining nor a redistribution of nephrin were observed. This suggests that the reduced expression of nephrin was not related to a specific glomerular disease but rather to the proteinuric state. A decrease in glomerular expression of nephrin mRNA has been recently reported in one case of membranous GN and three cases of minimal change GN.²⁶ These results suggested that nephrin may be a target of injury in acquired proteinuric diseases. However, it remains to be

Figure 7. **A:** Semiquantitative analysis of nephrin expression on GECs incubated for 60 minutes with vehicle alone (control), aIgG₄ (1 μ g/ml), TNF- α (10 ng/ml), puromycin (5 μ g/ml), or after the plasma membrane insertion of MAC. **, $P < 0.001$ versus control. **B:** Dose effect of sodium azide pre-incubation (10^{-3} mol/L to 10^{-1} mol/L) on immunofluorescence staining for nephrin on GECs incubated for 60 minutes with aIgG₄ (1 μ g/ml) or TNF- α (10 ng/ml). **C:** Dose effect of cytochalasin B pre-incubation (5 μ g/ml and 10 μ g/ml) on immunofluorescence staining for nephrin on GECs incubated for 60 minutes with aIgG₄ (1 μ g/ml) or TNF- α (10 ng/ml). Results are expressed as percent of control (GECs incubated for 60 minutes with vehicle alone).

determined whether this was the cause of proteinuria or the consequence of podocyte injury.

In the present study we investigated whether different stimuli may influence surface distribution of nephrin on

glomerular podocytes. The interaction of nephrin with specific antibodies induced a redistribution of immune complexes formed on the cell surface with patching and shedding of immune complexes containing nephrin. This led to temporary disappearance of the antigen from the cell surface. This process is reminiscent of antibody-induced redistribution of Heymann antigen of the surface of cultured podocytes.^{9,27} We therefore investigated whether addition to GECs of preformed immune complexes mimicked by aIgG₄ induced the modulation of surface expression of nephrin. IgG₄ was chosen because it is the most represented immunoglobulin subclass in membranous GN.²⁸ The results obtained indicate that aIgG₄ induced focal redistribution and extensive loss of nephrin on the cell surface in association with changes in the cytoskeleton organization. Because disaggregated IgG₄ did not stimulate nephrin redistribution, one can speculate that the altered distribution of nephrin is not because of a heterotopic association between nephrin and IgG₄, but rather to the interaction of aIgG₄ with the specific neonatal Fc receptor, expressed by GECs.²⁹ Fc receptor stimulation may induce cytoskeletal rearrangement and consequent redistribution of nephrin that seemed to be connected with actin.³⁰ Indeed, other stimuli affecting cytoskeleton organization, such as MAC, TNF- α , and puromycin, induced redistribution and loss of nephrin from the cell surface. The role of the cytoskeleton was also confirmed by the effect exerted by cytochalasin B, which disorganizes microfilaments,²³ preventing nephrin redistribution on the surface of podocytes. This result, together with the recent report on the role of nephrin in podocyte morphology,³⁰ suggests that stimuli affecting cytoskeleton organization may induce also redistribution and shedding of nephrin from the surface of podocytes. The inhibitory effect of sodium azide indicates that this process is energy-dependent. Sodium azide inhibits oxidative phosphorylation and glycolysis, and prevents capping and shedding in both lymphocytes³¹ and podocytes.⁹

In conclusion, although the observations made with cultured GECs must be interpreted cautiously because of the obvious difference between the organization of podocytes in glomeruli and in culture, one can speculate that the reduction of nephrin protein expression observed in glomeruli of patients with acquired primary nephrotic syndrome may be the consequence of a podocyte injury triggered by different stimuli, such as antibodies, immune complexes, terminal components of complement, and cytokines.

References

1. Eddy AA, Schnaper HW: The nephrotic syndrome: from the simple to the complex. *Semin Nephrol* 1998, 18:304–316
2. Tryggvason K: Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm. *J Am Soc Nephrol* 1999, 10:2440–2445
3. Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestila M, Jalanko H, Holmberg C, Tryggvason K: Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci USA* 1999, 96:7962–7967

4. Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan CE, Peltonen L, Holmberg C, Olsen A, Tryggvason K: Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol Cell* 1998, 1:575–582
5. Lenkkeri U, Mannikko M, McCready P, Lamerdin J, Gribouval O, Niaudet PM, Antignac CK, Kashtan CE, Homberg C, Olsen A, Kestila M, Tryggvason K: Structure of the gene for congenital nephrotic syndrome of the Finnish type (NPHS1) and characterization of mutations. *Am J Hum Genet* 1999, 64:51–61
6. Topham PS, Kawachi H, Haydar SA, Chugh S, Addona TA, Charron KB, Holzman LB, Shia M, Shimizu F, Salant DJ: Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin. *J Clin Invest* 1999, 104:1559–1566
7. Kawachi H, Koike H, Kurihara H, Yaoita E, Orikasa M, Shia MA, Sakai T, Yamamoto T, Salant DJ, Shimizu F: Cloning of rat nephrin: expression in developing glomeruli and in proteinuric states. *Kidney Int* 2000, 57:1949–1961
8. Luimula P, Ahola H, Wang SX, Solin ML, Aaltonen P, Tikkanen I, Kerjaschki D, Holthofer H: Nephrin in experimental glomerular disease. *Kidney Int* 2000, 58:1461–1468
9. Camussi G, Brentjens JR, Noble B, Kerjaschki D, Malavasi F, Roholt OA, Farquhar MG, Andres G: Antibody-induced redistribution of Heymann antigen on the surface of cultured glomerular visceral epithelial cells: possible role in the pathogenesis of Heymann glomerulonephritis. *J Immunol* 1985, 135:2409–2416
10. Striker GE, Striker LJ: Glomerular cell culture. *Lab Invest* 1985, 53:122–131
11. Conaldi PG, Biancone L, Bottelli A, De Martino A, Camussi G, Toniolo A: Distinct pathogenic effects of group B coxsackie viruses on human glomerular and tubular kidney cells. *J Virol* 1997, 71:9180–9187
12. Moutabarrik A, Nakanishi I, Zaid D, Namiki M, Kawaguchi N, Onishi S, Ishibashi M, Okuyama A: Interleukin-1-beta activation of cultured glomerular epithelial cells. *Exp Nephrol* 1994, 2:196–204
13. Delarue F, Virone A, Hagege J, Lacave R, Peraldi M-N, Adida C, Rondeau E, Feunteun J, Sraer J-D: Stable cell line of T-SV40 immortalized human glomerular visceral epithelial cells. *Kidney Int* 1991, 40:906–912
14. Coers W, Huitema S, van der Horst MLC, Weening JJ: Puromycin aminonucleoside and adriamycin disturb cytoskeletal and extracellular matrix protein organization but not protein synthesis of cultured glomerular epithelial cells. *Exp Nephrol* 1994, 2:40–50
15. Conaldi PG, Biancone L, Bottelli A, Wade-Evans, Racusen LC, Orlandi V, Serra C, Camussi G, Toniolo A: HIV-1 kills renal tubular cells by a mechanism that involves fas and caspase-3 activation. *J Clin Invest* 1998, 102:2041–2049
16. Brizzi MF, Aronica MG, Rosso A, Bagnara GP, Yarden Y, Pegoraro L: Granulocyte-macrophage colony-stimulating factor stimulates JAK2 signaling pathway and rapidly activates p93fcs, STAT1 p91, and STAT3 p92 in polymorphonuclear leukocytes. *J Biol Chem* 1996, 271:3562–3567
17. Mancilla-Jimenez R, Appay MD, Bellon B, Kuhn J, Bariety J, Druet P: IgG Fc membrane receptor on normal human glomerular visceral epithelial cells. *Virchows Arch A Pathol Anat Histopathol* 1984, 404:139–158
18. Camussi G, Caldwell PR, Andres G, Brentjens J: Lung injury mediated by antibodies to endothelium. II Study of the effect of repeated antigen-antibody interactions in rabbits tolerant to heterologous antibody. *Am J Pathol* 1987, 127:216–228
19. Kilgore KS, Schmid E, Shanley TP, Flory CM, Maheswari V, Tramontini NL, Cohen H, Ward PA, Friedl HP, Warren JS: Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. *Am J Pathol* 1997, 150:2019–2031
20. Bariety J, Hinglais N, Bhakdi S, Mandet C, Rouchon M, Kazatchkine MD: Immunohistochemical study of complement S protein (Vitronectin) in normal and diseased human kidneys: relationship to neoantigens of the C5b-9 terminal complex. *Clin Exp Immunol* 1989, 75:76–81
21. Camussi G, Mariano F, Biancone L, Montrucchio G, Vercellone A: Effect of cytokines on the cytoskeleton of resident glomerular cells. *Kidney Int* 1993, 39:S32–S36
22. Camussi G, Salvadio G, Biesecker G, Brentjens J, Andres G: Heymann antibodies induce complement-dependent injury of rat glomerular visceral epithelial cells. *J Immunol* 1987, 139:2906–2914
23. Wessells NK, Spooner BS, Ash JF, Bradley MO, Luduena MA, Taylor EL, Wrenn JT, Yamaa K: Microfilaments in cellular and developmental processes. *Science* 1971, 171:135–143
24. Putaala H, Soininen R, Kilpeläinen P, Wartiovaara J, Tryggvason K: The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Hum Mol Genet* 2001, 10:1–8
25. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH, Shaw AS: Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 1999, 286:312–315
26. Furness PN, Hall LL, Shaw JA, Pringle JH: Glomerular expression of nephrin is decreased in acquired human nephrotic syndrome. *Nephrol Dial Transplant* 1999, 14:1234–1237
27. Camussi G, Kerjaschki D, Gonda M, Nevins T, Rielle JC, Brentjens J, Andres G: Expression and modulation of surface antigens in cultured rat glomerular visceral epithelial cells. *J Histochem Cytochem* 1989, 37:1675–1689
28. Oliveira DB: Membranous nephropathy: an IgG₄-mediated disease. *Lancet* 1998, 351:670–671
29. Haymann JP, Levraud JP, Bouet S, Kappes V, Hagege J, Nguyen G, Xu Y, Rondeau E, Sraer JD: Characterization and localization of the neonatal Fc receptor in adult human kidney. *J Am Soc Nephrol* 2000, 11:632–639
30. Khoshnoodi J, Ruotsalainen V, Tryggvason K: The role of nephrin in cytoskeleton organization and podocyte morphology. *J Am Soc Nephrol* 2000, 11:A0280
31. Unanue ER, Karnovsky MJ, Engers HD: Ligand-induced movement of lymphocyte membrane macromolecules. III. Relationship between the formation and fate of anti-Ig-surface Ig complexes and cell metabolism. *J Exp Med* 1973, 137:675–689