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

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We investigated the distribution of nephrin by immuno-fluorescence microscopy in renal biopsies of patients with nephrotic nephropathy (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, plasmalemmal 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.⁶ 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

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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. An irrelevant IgG1 isotypic control antibody was purchased from Cederlane (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 IgG kappa (IgGκ); human tumor necrosis factor-α (TNF-α); cytokeratin, vimentin, and laminin; negative staining for smooth muscle-type myosin, FVIIIr:Ag, and CD45; cytotoxicity in response to puromycin aminonucleoside (10 to 50 μg/ml). Established lines of differentiated GECs were obtained by infection of pure primary cultures with a hybrid Adeno5/SV40 virus as previously described. 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.

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-μm-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 μg/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.

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. Primary cultures of glomerular epithelial cells (GECs) were established as previously described. 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, FVIIIr:Ag, and CD45; cytotoxicity in response to puromycin aminonucleoside (10 to 50 μg/ml). Established lines of differentiated GECs were obtained by infection of pure primary cultures with a hybrid Adeno5/SV40 virus as previously described. 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.

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.

Table 1. Clinical Features of Patients

<table>
<thead>
<tr>
<th></th>
<th>Membranous GN</th>
<th>Minimal change GN</th>
<th>FSGS</th>
<th>IgA GN</th>
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<tr>
<td>n</td>
<td>13</td>
<td>10</td>
<td>7</td>
<td>6</td>
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<tr>
<td>Age (years)</td>
<td>59 ± 16</td>
<td>40 ± 25</td>
<td>42 ± 10</td>
<td>50 ± 21</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>4/7</td>
<td>6/4</td>
<td>2/5</td>
<td>4/2</td>
</tr>
<tr>
<td>UP (g/day)</td>
<td>5.9</td>
<td>7.1 ± 2.4</td>
<td>4.4 ± 1.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>NS (%)</td>
<td>85 (11/13)</td>
<td>100 (10/10)</td>
<td>86 (6/7)</td>
<td>0 (0/6)</td>
</tr>
<tr>
<td>Scr (mg/dL)</td>
<td>1.2 ± 0.8</td>
<td>0.9 ± 0.2</td>
<td>1.4 ± 0.8</td>
<td>1.8 ± 0.7</td>
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Abbreviations: UP, proteinuria; NS, nephrotic syndrome; Scr, serum creatinine.
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-nephrin mAb was tested by pre-adsorption of the antibody (10 μg/ml) with purified recombinant extracellular nephrin (30 μg/ml).

The number of glomeruli available on each biopsy for analysis of nephrin expression ranged between 3 to 7. Three nonsequential sections were examined for each specimen. Nephrin expression was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows MicroImage, version 3.4 CASTI Imaging, Venezia, Italy) of images obtained using a low-light video camera (Leica DC 100, 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 Nephrin 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 nephrin and human glyceraldehyde phosphate dehydrogenase (h-GAPDH), used as housekeeping gene, sequence-specific oligonucleotide primers (purchased from TIB Molbiol, Genova, Italy) were designed (nephrin: 5' forward, CCA CCC ATG GCA AAT and (598 bp) for GAPDH were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Detection of Nephrin Expression by Western Blot Analysis**

Protein concentration of GEC lysates obtained as previously described was determined by the Bradford technique, and the protein content of the samples was normalized to 100 32 mg/sample by appropriate dilution with Laemml buffer. Proteins were directly subjected to 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-nephrin 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 nephrin, an immortalized cell line of human renal tubular epithelial cells was used.

**Experimental Design**

The expression of nephrin 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-nephrin mAb to evaluate antibody-induced antigen redistribution. To study in vitro the role of immune complexes on the surface expression and redistribution of nephrin, 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-nephrin mAb and rabbit anti-human-IgG. AgIgG₄ was obtained by heating at 63°C for 30 minutes, as described. 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. 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 nephrin. 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. Briefly, nonenzymatic formation of a C5b-like C5C6 complex was obtained by incubation of 10 μg of C5 in 10 μl of serum-free buffer with 10 μl of 0.32 mmol/L chloramidine-T for 10 minutes at room temperature. After inactivation of chloramidine-T with 10 μl methionine (1 mmol/L), purified C6 (20 μg) was added in 300 μl 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 μg/ml) with the C5b-C6 complex (5 μg/ml). After two washings with serum-free medium, C8 (10 μg/ml) and C9 (10 μg/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 μg/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 on 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
fine granular into a patchy pattern, suggesting antibody-induced antigen redistribution (data not shown). Therefore, we tested whether agIgG4 were able to change the surface expression of nephrin. Incubation of living GECs at 4°C for 60 minutes with agIgG4 resulted in diffuse surface binding of IgG4 to GECs (data not shown). When incubated at 37°C for 60 minutes, agIgG4 appeared re-distributed in one or multiple aggregates (capping) (data not shown). When cells were fixed with paraformaldehyde after 60 minutes incubation with agIgG4, 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. AgIgG4 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 agIgG4. 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 reduction of fluorescence intensity and morphological evidences of nephrin redistribution (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 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: ×400 (A and B), ×600 (C and 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).

**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) underlie 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 podo-
cytes. 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. 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. The fact that nephrin knockout mice also exhibit massive proteinuria and die within 24 hours after birth 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.

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. This treatment induced proteinuria and nephrotic syndrome. Moreover, 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.

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. Similar results have been reported in experimental animal models such as puromycin aminonucleoside nephrosis, mercuric chloride-treated rats, and nephritis induced by injection of mAb 5-1-6, 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. These results suggested that nephrin may be a target of injury in acquired proteinuric diseases. However, it remains to be

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.

Figure 6. Immunofluorescence staining for nephrin on GECs incubated 60 minutes with vehicle alone (A), agIgG4 (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 agIgG4 (1 µg/ml) (E and F). Double staining for nephrin (green) and for IgG (red) on GECs incubated for 60 minutes with agIgG4 (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).
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. We therefore investigated whether addition to GECs of preformed immune complexes mimicked by agIgG4 induced the modulation of surface expression of nephrin. IgG4 was chosen because it is the most represented immunoglobulin subclass in membranous GN. The results obtained indicate that agIgG4 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 agIgG4 with the specific neonatal Fc receptor, expressed by GECs. Fc receptor stimulation may induce cytoskeletal rearrangement and consequent redistribution of nephrin that seemed to be connected with actin. 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, preventing nephrin redistribution on the surface of podocytes. This result, together with the recent report on the role of nephrin in podocyte morphology, 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 lymphocytes and podocytes.

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