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Complement mediates nephrin redistribution and actin dissociation in experimental membranous nephropathy[1]

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Complement mediates nephrin redistribution and actin dissociation in experimental membranous nephropathy.

Background. The onset of proteinuria in passive Heymann nephritis, (PHN), a rat model of human membranous nephropathy (MN), is complement-dependent and is associated with altered podocyte slit diaphragm integrity and dissociation of nephrin from the actin cytoskeleton. These studies examined if complement is responsible for these podocyte changes.

Methods. PHN was induced with sheep anti-Fx1A. Controls were injected with normal sheep globulin. A third group was injected with anti-Fx1A and depleted of complement with cobra venom factor. Four days later, proteinuria was measured, slit diaphragm integrity was examined by electron microscopy, nephrin distribution was studied by immunofluorescence, and the glomerular content of nephrin and its association with actin were assessed by sequential extraction of isolated glomeruli and Western blotting.

Results. Four days after immunization, seven out of eight PHN rats were proteinuric, whereas none of the complement depleted group had proteinuria despite similar levels of antibody deposition. Complement depletion preserved slit diaphragm morphology. Immunofluorescence microscopy with an antibody to the extracellular domain of nephrin showed a normal staining pattern in the rats depleted of complement and a shift to a more dispersed and clustered pattern in the PHN group. Western blot analysis of the glomerular extracts showed a significant reduction in the total amount of nephrin and in the fraction of actin-associated nephrin in the PHN group, whereas the amounts in the complement-depleted rats were similar to normal controls.

Conclusion. The onset of proteinuria in the PHN model of MN is coincident with complement-dependent alterations in the association of nephrin with the actin cytoskeleton and loss of podocyte slit diaphragm integrity.

[1]**See Editorial by Quigg, p. 2318.**

Key words: passive Heymann nephritis, cytoskeleton, proteinuria, podocyte, slit diaphragm.

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Membranous nephropathy (MN) is an autoimmune disease in which the primary clinical manifestation is proteinuria. Although the target antigen/s responsible for idiopathic MN are unknown, they are likely to be constituents of the basal plasma membrane of podocyte foot processes, as in the Heymann nephritis model of MN [1, 2] and as documented in a case of neonatal MN induced by the transplacental transfer of alloantibodies to neutral endopeptidase [3]. The onset of proteinuria in the passive Heymann nephritis (PHN) model of MN in the rat is the result of podocyte injury induced by antibody-mediated assembly of complement C5b-9 [4, 5], and a similar process appears to occur in active cases of human MN [6].

Although podocyte foot process effacement and expansion of the glomerular basement membrane (GBM) are characteristic features of advanced MN and PHN, the earliest pathologic changes are subtler. We recently observed that the podocyte slit diaphragm in several filtration slits was displaced and sometimes disrupted at the onset of proteinuria in PHN before any appreciable changes in the foot processes or GBM were evident [7]. Accompanying these changes, we found that the slit diaphragm protein, nephrin, partly dissociated from the actin cytoskeleton and its expression in the slit diaphragm and foot processes was altered and reduced [7]. Considering that the podocyte actin cytoskeleton undergoes condensation in PHN [8], and that sublethal injury of glomerular epithelial cells in culture by C5b-9 initiates a series of signaling events and disrupts the actin microfilaments [9–11], we proposed that the insertion of C5b-9 into the podocyte plasma membrane triggers processes that cause nephrin to dissociate from the cytoskeleton and thereby affect its stability within the cell membrane [7].

In the studies reported here, we sought to determine if, indeed, complement is responsible for the dissociation of nephrin from the actin cytoskeleton and its altered distribution in experimental MN.

METHODS

Experimental protocol

Sheep anti-Fx1A was prepared as described [12]. PHN was induced in 180 to 200 g adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) by two intravenous injections of anti-Fx1A, 0.25 and 0.6 mL, on successive days. Controls were injected with an equal amount of normal sheep globulin. A third group was injected with anti-Fx1A (as above) and depleted of complement (C-depleted PHN) as follows. Starting 24 hours before the first dose of anti-Fx1A, rats were injected intraperitoneally with 300 U/kg of purified cobra venom factor (Quidel, San Diego, CA, USA) divided into two equal doses 12 hours apart. Thereafter, they received a daily dose of 100 U/kg until the end of the study. The control and untreated PHN rats received equivalent intraperitoneal injections of saline on the same schedule.

Serum samples were obtained before the experiment and on days 2 and 4 after the first injection to measure C3 levels by radial immunodiffusion. Urine was collected from all rats overnight in individual metabolism cages on days 3 and 4 after the first injection and analyzed for protein by a sulfosalicylic acid method. On day 4, the kidneys were removed under anesthesia and the animals were sacrificed. Two 3 to 4 mm coronal slices of cortex were embedded in Tissue-Tek® O.C.T. Compound (Sakura, Torrance, CA, USA) and snap-frozen at −80°C without prior fixation for immunofluorescence. One millimeter cubes of tissue were fixed in 2% glutaraldehyde and stored at 4°C in 0.1 mol/L cacodylate buffer (pH 7.4) for electron microscopy. The rest of the tissue was frozen immediately and stored at −80°C until used for glomerular isolation (vide infra).

Antibodies and reagents

A rabbit antinephrin antibody was produced by immunization with a 21 amino acid peptide from the C terminus of rat nephrin [13]. This antibody was used for Western blots. A monoclonal antibody that identifies an epitope in the extracellular domain of rat nephrin, mAb 5-1-6 [14], was kindly provided by Dr. Hiroshi Kawachi and Dr. Fujio Shimizu (Niigata University, Niigata, Japan) and was used for immunofluorescence studies. Rabbit antiactin (A2066), goat antirabbit IgG-horseradish peroxidase (A8275) and fluorescein-conjugated goat antirabbit IgG (F0382) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CY3-conjugated goat antirabbit IgG (AP132C) and donkey antimouse IgG (AP129C) were from Chemicon (Temecula, CA, USA). Goat antirat C3-fluorescein isothiocyanate (FITC) (ICN55751) was from Cappel (Aurora, OH, USA). Chemicals and reagents were from Sigma-Aldrich unless stated otherwise.

Measurement of serum levels of complement C3

Serum C3 levels were measured by radial immunodiffusion with rabbit antirat C3 prepared and incorporated into 1% agarose in veronal buffer (3.41 g/L barbital, 18.95 g/L barbital sodium, and 0.01% sodium azide, pH 8.6). The precipitation rings were stained for 1 hour with 1% tannic acid, scanned into Adobe Photoshop® 5.0 (Adobe Systems, Mountainview, CA, USA) and the area measured with Image software (version 1.61, National Institutes of Health, Bethesda, MD, USA). A standard curve was generated from the area of the rings produced by serial dilutions of pooled normal rat serum. The results of the C-depleted PHN group are expressed as a percentage of undiluted normal rat serum.

Isolation of glomeruli

Glomeruli were isolated from the kidneys of individual rats by differential sieving [12] using phosphate-buffered saline (PBS) (10 mmol/L phosphate buffer, pH 7.4, and 100 mmol/L NaCl) with a cocktail of protein inhibitors (PI) (1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL soybean trypsin inhibitor, 4 mmol/L N-ethylmaleimide, and 5 mmol/L benzamidine hydrochloride). Phase-contrast microscopy indicated that >75% of the isolated glomeruli were decapsulated.

Sequential extraction of glomeruli and depolymerization of actin

Glomeruli from individual rats were extracted sequentially with Triton X-100 into a detergent-soluble fraction followed by extraction of the detergent-insoluble fraction in the presence of potassium iodide (KI) to depolymerize actin as previously described [7]. In brief, glomeruli were extracted on ice for 40 minutes with extraction buffer composed of 1% Triton X-100 in 50 mmol/L Tris buffer (pH 7.4), 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$ and PI. The Triton X-100–insoluble material was removed by centrifugation at 14,000 rpm in a microcentrifuge for 10 minutes at 4°C and then incubated in 1% Triton X-100 in extraction buffer with the addition of 1 mol/L KI on ice for 40 minutes after sonication with a Sonifier Cell Disrupter (S250A, Branson Ultrasonics Corp., Danbury, CT, USA) at output 6 and duty cycle 50% for 2×5 bursts with 10-second intervals. The soluble proteins were separated from the insoluble proteins by centrifugation at 14,000 rpm for 10 minutes at 4°C and the insoluble proteins were resuspended in 1% Triton X-100 and KI buffer by sonication. The protein concentration of the Triton X-100 extracts was measured by the BCA protein assay (Pierce, Inc., Rockford, IL, USA). The protein concentration of the Triton X-100 extracts was measured and the volume of each sample was adjusted so that 15 μg of

each Triton X-100 extract and equivalent volumes of the KI-soluble fractions and insoluble pellets were analyzed by Western blotting.

Western blot analysis

Samples were boiled for 5 minutes in sodium dodecyl sulfate (SDS) sample buffer containing 2% dithiothreitolol, centrifuged, and loaded onto 4% to 20% SDS polyacrylamide gels (Ready Gel Tris-HCl, Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes (Osmonics, Inc., Westborough MA, USA), blocked with 8% skim milk in Tris-buffered saline with Tween 20 (TBST) (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, and 0.2% Tween 20) and immunoblotted with rabbit antimouse nephrin (1:1000) and goat antirabbit IgG-horseradish peroxidase (1:5000) with TBST washes between antibodies. The same membranes were subsequently immunoblotted with antiactin (1:500). Immunoreactive proteins were identified by enhanced chemiluminescence (Supersignal) (Pierce, Inc.) and autoradiography. Each immuoreactive band was scanned from the autoradiographs into Adobe PhotoShop 5.0 and the densities were measured with NIH Image 1.61. The nephrin band densities were normalized with the corresponding actin bands.

Immunofluorescence microscopy

Four micron cryosections of kidneys from the control, PHN, and C-depleted PHN groups were transferred to Superfrost/Plus slides (Fisher Scientific, Pittsburgh PA, USA) and fixed with acetone at –20°C for 5 minutes. They were then washed with cold PBS, blocked with 1% bovine serum albumin (BSA)/1% goat serum in PBS and stained with mAb 5-1-6 (1:100) followed by donkey antimouse IgG-CY3 (1:800), as well as rabbit antisheep IgG-CY3 (1:500) and goat antirat C3-FITC (1:300). Sections stained with donkey antimouse IgG-CY3 alone were negative. The sections were examined by epifluorescent microscopy with a Nikon 40× Plan Apo oil-immersion lens. The images were captured with a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and exported into Adobe Photoshop 5.0. All exposure settings were kept constant for each group of kidneys. Fluorescence intensity was measured by outlining the perimeter of five glomeruli in each section and reading the luminosity from the histogram command in the "image" pull-down menu in Adobe Photoshop. A qualitative analysis of the distribution of nephrin in the three experimental groups was performed by confocal microscopy using a Perkin-Elmer UltraView confocal laser microscope equipped with a Hamamatsu ORCA ER Firewire cooled CCD detector (Perkin-Elmer Life Sciences, Boston, MA, USA).

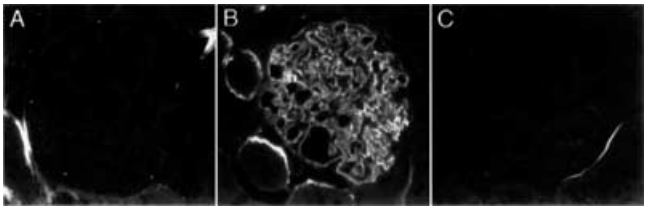

Fig. 1. Representative immunofluorescent micrographs of glomeruli from control, passive Heymann's nephritis (PHN), and complement-depleted (C-depleted) PHN rats on day 4. Cryosections were stained with goat anti-rat C3-fluorescein isothiocyanate (FITC). The glomeruli from control (*A*) and C-depleted PHN (*C*) rats are entirely negative, whereas the glomeruli from a saline-treated PHN rat (*B*) shows intense, granular capillary wall staining (original magnification ×400).

Transmission electron microscopy

The cortex of kidneys from the PHN, C-depleted PHN, and control groups was cut into 1 mm^3 blocks, immersion-fixed with 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer at 4°C overnight, washed in 0.1 mol/L cacodylate buffer, postfixed with 1% osmium in 0.1 mol/L cacodylate buffer for 1 hour, dehydrated in graded ethanols, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate for transmission electron microscopy. Ultrathin sections were examined and photographed with a Philips CM10 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ, USA).

Statistical analysis

Analysis of variance (ANOVA) and Dunnett's or Bonferroni's post hoc test analyses were performed with KaleidaGraph (Synergy Software, Reading, PA, USA).

RESULTS

Complement depletion and proteinuria

At the time of sacrifice on day 4, all rats in the C-depleted PHN group had serum C3 levels less than 10% of baseline as determined by radial immunodiffusion. Immunofluorescence studies for C3 revealed intense granular glomerular capillary wall staining in all of the saline-treated PHN rats. In contrast, there was no staining for C3 in the glomeruli of the C-depleted PHN or control rats (Fig. 1). As determined by quantitative fluorescence microscopy, sheep IgG deposition was the same in the PHN and C-depleted PHN groups (PHN, 5.1 ± 1.19, $N = 20$ glomeruli from four rats; C-depleted PHN, 5.1 ± 1.55, $N = 15$ glomeruli from three rats; luminosity units). Overnight urine protein excretion was measured between days 3 and 4. None of the animals in the C-depleted PHN group had urine protein excretion above the level of control animals, whereas seven of eight rats in the PHN group had levels that exceeded the upper limit of controls (Fig. 2).

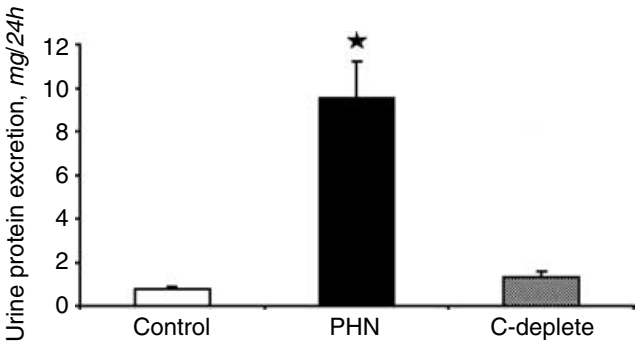

Fig. 2. Urine protein excretion in control, passive Heymann's nephritis (PHN), and complement-depleted (C-depleted) PHN rats on day 4. Proteinuria greater than the upper limit of controls (1.3 mg/24 hours) was present in seven of eight PHN rats on day 4. It was mild in five (3.4 to 8.3 mg/24 hours) and more severe in two (19.0 and 31.8 mg/24 hours). None of the C-depleted PHN group was proteinuric. Values are mean ± SEM. $N = 8$/group; analysis of variance (ANOVA), $P = 0.005$. *The mean value of the PHN group was significantly greater than the controls and C-depleted PHN groups, $P < 0.02$ (Bonferroni's all pairs comparison).

Podocyte slit diaphragm morphology

The ultrastructural findings in the PHN group on day 4 were consistent with our previous observations [13] and revealed that slit diaphragms in the vicinity of subepithelial deposits were often displaced or absent, and that podocyte foot processes were generally well-preserved at this early stage. In contrast, the slit diaphragms and foot processes in the C-depleted PHN group appeared to be similar to controls injected with normal sheep IgG despite the presence of subepithelial deposits in the C-depleted PHN group (Fig. 3). To determine if these findings in PHN were (*1*) quantitatively different from normal controls; (*2*) the result of complement-mediated injury; and (*3*) reflected in the severity of proteinuria, we performed an analysis of slit diaphragm integrity in the various groups, including PHN rats with lower and higher levels of proteinuria. The numbers of normal and abnormal (absent or displaced) slit diaphragms and subepithelial electron-dense deposits were counted and expressed as a function of the number of filtration slits. As shown in Table 1, slit diaphragm integrity was similar in the control and C-depleted PHN (nonproteinuric) rats, and in PHN with low proteinuria. However, in the presence of high proteinuria, slit diaphragm integrity was significantly altered in PHN despite a similar number of immune deposits (Table 1). As previously noted [13], occluding-type junctions were seen to have developed in 1% to 3% of filtration slits in the PHN rats regardless of the level of proteinuria and were observed also in the C-depleted PHN group.

Distribution of nephrin

Immunofluorescent staining and confocal microscopy with mAb 5-1-6, a monoclonal antibody to the extracellular domain of rat nephrin, revealed the normal interrupted linear staining pattern in control glomeruli (Fig. 4A). As previously described [13], an alteration in the distribution of nephrin in the PHN group to a granular and clustered pattern was already evident on day 4 (Fig. 4B). In contrast, the pattern of nephrin staining in the C-depleted PHN group (Fig. 4C) was not appreciably different to the controls.

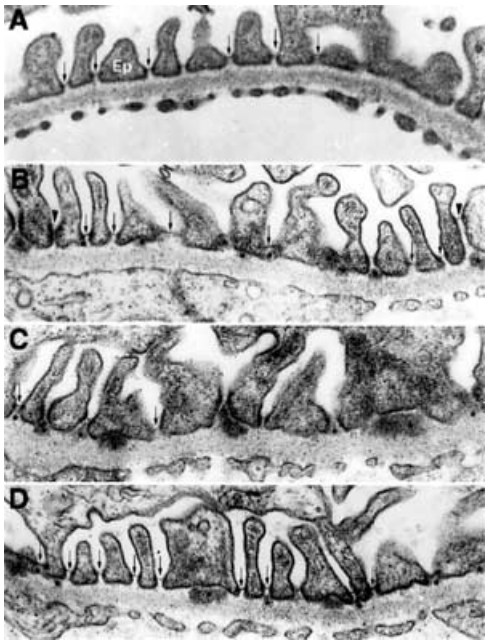

Fig. 3. Representative transmission electron micrographs of glomeruli from passive Heymann's nephritis (PHN), and complement-depleted (C-depleted) PHN rats on day 4. (*A*) Glomerulus from a normal control shows normal podocyte foot process (Ep) morphology with slit diaphragms visible in most filtration slits (arrows). (*B*) Glomerulus from a mildly proteinuric PHN rat shows normal podocyte foot process morphology with slit diaphragms visible in many filtration slits (arrows) and subepithelial electron dense deposits (∗). In some places, the filtration slits are narrowed and replaced by occluding type junctions or "ladder-like" structures (arrowheads). (*C*) Glomerulus from a more heavily proteinuric rat demonstrates subepithelial electron dense deposits (∗), more widespread loss and displacement of slit diaphragms (arrows) and distortion of foot processes. (*D*) Glomerulus from a C-depleted PHN rat shows subepithelial electron dense deposits (∗) and preserved slit diaphragms in most filtration slits (arrows). Similar observations were made in the glomeruli of another rat in the C-depleted PHN group (original magnification ×11,500).

Quantitation of nephrin and its association with actin

To measure the amount of glomerular nephrin and its association with actin immunochemically, glomeruli were isolated individually from the kidneys of all eight rats in each group and sequentially extracted with 1% Triton X-100 at 4°C followed by Triton X-100 plus KI to depolymerize F-actin and release the actin-associated nephrin. The Triton X-100, Triton X-100 plus KI and residual pellet fractions from one rat in each group were selected at random and analyzed by Western blotting for nephrin and

Table 1. Quantitative analysis of the slit diaphragm frequency of control and complement-depleted (C-depleted) passive Heymann nephritis (PHN) glomeruli, and of glomeruli from PHN rats with low and high levels of proteinuria

Group	Normally situated diaphragms[a]	Subepithelial deposits[a]	Proteinuria
	% filtration slits	*% filtration slits*	*mg/24 hours*
Control	49 ± 5.2	0	1.0
C-depleted	39 ± 5.3	57 ± 15	0.2
PHN low	49 ± 4.5	63 ± 35	8.8
PHN high	16 ± 7.0[b]	57 ± 15	38.0

Normal or abnormal slit diaphragms and all subepithelial electron deposits were counted on 7 to 12 separate electron micrographs from each rat and expressed as a percentage of filtration slits (106 to 284/rat). Slit diaphragms are clearly visible in about 50 of filtration slits in normal controls.

[a]Values are mean ± SEM. Analysis of variance (ANOVA), $P = 0.0013$; [b]$P < 0.05$ vs. all other groups (Bonferroni's all pairs comparison).

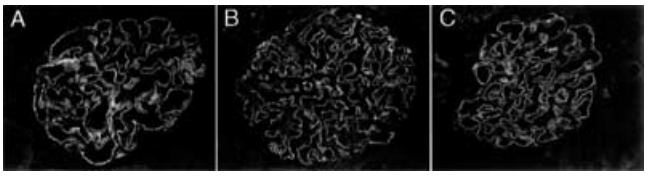

Fig. 4. Representative confocal laser micrographs of glomeruli from control, passive Heymann's nephritis (PHN), and complement-depleted (C-depleted) PHN rats on day 4. Cryosections (4 microns) were stained with mAb 5-1-6, which detects an epitope on the external domain of nephrin, and 0.2 micron equatorial sections were selected. A control glomerulus (*A*) illustrates the normal interrupted linear staining pattern of the slit diaphragms with this antibody. The staining of the PHN glomerulus (*B*) is more dispersed and granular, whereas the pattern in the C-depleted PHN glomerulus (*C*) resembles the normal control (original magnification ×400).

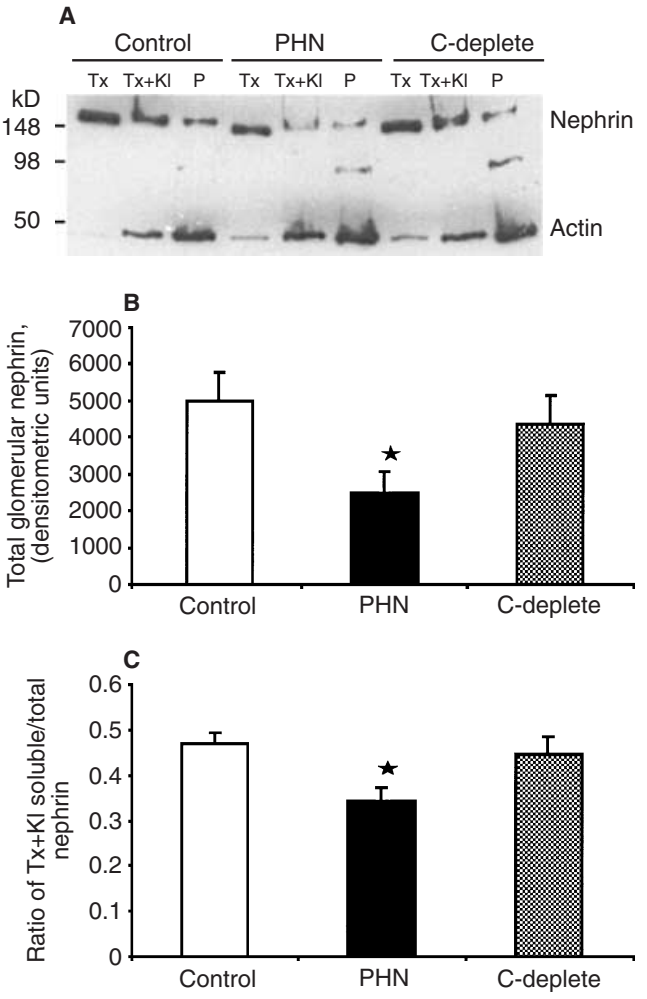

Fig. 5. Quantitaive analysis of total glomerular nephrin and actin-associated [potassium iodide (KI)-soluble] nephrin in control, passive Heymann's nephritis (PHN), and complement-depleted (C-depleted) PHN glomeruli on day 4. Glomeruli from individual rats were isolated and sequentially extracted with Triton X-100 (Tx) followed by Triton X-100 plus KI (Tx + KI). The Triton X-100- and Triton X-100 + KI-soluble fractions and residual pellet (P) from each isolate was resolved on 4% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to nitrocellulose membranes, and immunoblotted sequentially with rabbit antibodies to nephrin and actin. Representative Western blot of glomeruli from one rat in each group (*A*). Note the reduced density of the Triton X-100 + KI and P bands of nephrin in the PHN glomeruli as compared to the control and C-deplete PHN glomeruli despite similar actin density. Eight similar membranes from eight rats in each group were analyzed in parallel. Densitometry of the individual bands indicates that total glomerular nephrin (*B*) and the actin-associated fraction of cell membrane nephrin (*C*) are significantly reduced in the PHN group as compared to the control and C-depleted PHN groups [analysis of variance (ANOVA) total nephrin $P = 0.0003$; Triton X-100 + KI/total nephrin $P = 0.0157$; $^*P < 0.02$, Dunnett's post hoc test for multiple comparisons]. Values are mean ± SEM; $N = 8$/group.

actin on the same membrane as shown in Figure 5A. A total of eight such membranes, representing a different rat from each group, were analyzed and the densities of the resulting bands were compared. As shown in Figure 5B, total nephrin was significantly reduced in the PHN group as compared to the control group, whereas the C-depleted PHN group was not significantly different to the controls. Similarly, the fraction of nephrin that is associated with actin was significantly reduced in the PHN group when compared to the control group but the C-depleted PHN group did not differ from the controls (Fig. 5C).

DISCUSSION

The role of the slit diaphragm as the final barrier to protein permeation is now well established and its molecular structure is rapidly being elucidated [15]. Nephrin, an Ig-like transmembrane protein, is an essential, if not the major, component of the slit diaphragm [16–18]. Recent studies have shown that the cytoplasmic tail of nephrin is linked to the actin cytoskeleton, possibly via adapter molecules, including CD2AP and podocin [7, 19–21], and that this complex may serve a signaling function [22, 23]. Podocin, a member of the stomatin family of membrane-associated proteins, appears to reside in lipid rafts in the cholesterol-rich apical plasma membrane above the slit diaphragm and may also serve to anchor nephrin and the slit diaphragm in the lateral plasma membrane. In

addition, ZO-1, P-cadherin, the protocadherin FAT, and the nephrin homologue Neph1 have all been identified in the vicinity of the slit diaphragm [24–27], with potential homo- and heterophilic interactions and signaling functions. Together, these findings suggest that normal podocyte foot process morphology and slit diaphragm integrity may be regulated by a dynamic interaction between the actin cytoskeleton and the protein complexes that facilitate cell-matrix and cell-cell adhesion as well as barrier function.

Past studies have documented that the onset of proteinuria in the heterologous phase PHN is due to podocyte injury induced by antibody-mediated activation of complement and assembly of C5b-9 on the cell membrane [4, 5, 28]. Although podocyte foot process effacement and basement membrane expansion develop over time, ultrastructural studies at the onset of proteinuria show well-preserved foot processes but focal displacement or disruption of slit diaphragms. Recently, we found these changes in the slit diaphragms were associated with a redistribution and loss of nephrin and its dissociation from actin [13]. However, those studies did not establish if the changes in nephrin and the slit diaphragms were simply the result of antibodies reacting with cell surface proteins and the formation of subepithelial immune deposits, or were the consequence of C-mediated podocyte injury. We have previously shown that C5b-9-mediated sublethal injury of glomerular epithelial cells in culture causes disruption of actin microfilaments [11], which suggests that complement might alter the association of nephrin with the actin cytoskeleton, and thereby disrupt the slit diaphragms and allow for the development of proteinuria. To test this possibility, we compared slit diaphragm integrity and the distribution of nephrin and its association with actin at or just before the onset of proteinuria in PHN rats and in PHN rats that were protected from developing proteinuria by depleting them of complement.

Our results showed that there was significant disruption or displacement of slit diaphragms in proteinuric PHN rats accompanied by an alteration in the distribution of nephrin and its association with actin, as previously described [13]. In contrast, slit diaphragm integrity and nephrin distribution were maintained in C-depleted PHN rats, despite the presence of similar amounts of sheep antibody deposition and subepithelial immune deposits. Furthermore, complement depletion prevented the loss and the dissociation of nephrin from actin that was seen in C-replete PHN rats.

In the experiments reported here, we used whole glomeruli rather than glomerular cell membranes as in our previous report [13] for extraction with Triton X-100 $\pm$ KI. Nonetheless, the results in the PHN group on day 4 are remarkably consistent between the two studies with a reduction in total nephrin of about 45% and in the actin-associated fraction of about 20%. It is likely that these losses would have been more severe at later time points [13] but we chose to perform our analyses at 4 days for two reasons. First, cobra venom factor ceases to be effective in depleting complement after 4 to 5 days due to immune neutralization and we wished ensure that there was no complement-mediated injury. The total absence of glomerular C3 on immunofluorescence in the C-depleted PHN group confirmed that this goal was attained. Second, our goal was to study the earliest events that lead to proteinuria rather than its consequences. Proteinuria in the PHN group ranged from normal levels to 20 times the upper limit of normal yet the reduction in total nephrin and actin-associated nephrin was found throughout this group ($P < 0.02$) regardless of the level of proteinuria. This and the fact that we found no correlation between the amount of proteinuria and the reduction in nephrin attests to the likelihood that the changes in nephrin were the cause rather than the result of proteinuria. At the same time, it appears that the heterogeneity of proteinuria between animals in the PHN group does have a morphologic correlate in that the number of filtration slits with altered slit diaphragms was greater in the presence of more severe proteinuria. Our inability to measure an increase in the number of altered slit diaphragms in the presence of less severe proteinuria despite the reduction in total nephrin and actin-associated nephrin is probably because the methods are too insensitive to detect subtle changes. In contrast to the PHN group that differed from the controls as noted above, the C-depleted PHN rats were virtually indistinguishable from the controls except for the presence of subepithelial immune deposits containing injected sheep IgG antibody. Thus, it would appear that the onset of proteinuria results from complement-mediated alterations in the podocyte slit diaphragms.

An additional consistent, but infrequent, finding on electron microscopy in this and our previous study [13] was the development of occluding-type junctions in some filtration slits of the PHN rats. Here, we found that these changes occurred regardless of the level of proteinuria or the presence or absence of complement-mediated podocyte injury. This loss of patency of filtration slits in several models of foot process effacement has been attributed to uncoupling of the sialoglycoprotein podocalyxin from the actin cytoskeleton due to disruption of a cytosolic complex of ezrin and Na^+/H^+-exchanger regulatory factor 2 and loss of the repulsive action of podocalyxin from the cell surfaces of opposing foot processes [29]. Whereas this is an attractive explanation for the occlusion of filtration slits, the mechanisms appear to be distinct from those that alter slit diaphragm integrity and lead to proteinuria, at least in the PHN model.

The mechanisms by which complement alters the nephrin-actin association are presently unknown. However, knowledge of some of the consequences of complement-mediated cell injury and characteristics of

nephrin, its associated proteins and their possible interactions raises several possibilities. Our previous studies indicated that complement-mediated glomerular epithelial cell injury causes substantial adenosine triphosphate (ATP) depletion, dephosphorylation of focal adhesion proteins, and dissolution of actin microfilaments [11]. Considering that nephrin has several putative phosphorylation sites and is known to be tyrosine phosphorylated upon injection of a nephritogenic antibody that recognizes a podocyte-specific 9-O-acetylated GD3 ganglioside [22], it is possible that alterations in the phosphorylation state of nephrin might affect its interaction with actin or with adaptor molecules such as CD2AP or podocin. Another possibility is that the primary abnormality lies with actin itself. Ultrastructural studies in experimental and human membranous nephropathy (as well as in other proteinuric diseases) frequently show condensed actin in the base of effaced foot process. Cellular ATP depletion may cause actin depolymerization and we have found that regenerating ATP preserves actin microfilaments in complement-injured glomerular epithelial cells (unpublished observations). Thus, it is conceivable that complement-mediated podocyte injury in PHN may disrupt the actin cytoskeleton and thereby cause foot process effacement through loss of anchorage to the GBM as well as slit diaphragm disruption through loss of the nephrin-actin connection. These possibilities will be amenable to study once a stable cell line is established that expresses cell surface nephrin and antigens that can serve as targets for antibody-directed, complement-mediated injury.

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