Nephrotic Plasma Alters Slit Diaphragm–Dependent Signaling and Translocates Nephrin, Podocin, and CD2 Associated Protein in Cultured Human Podocytes

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Podocytes are critical in maintaining the filtration barrier of the glomerulus and are dependent on the slit diaphragm (SD) proteins nephrin, podocin, and CD2-associated protein (CD2AP) to function optimally. The effects of normal human plasma and nephrotic plasma on podocytes were tested, focusing particularly on the SD complex. With the use of a conditionally immortalized human podocyte cell line, it first was shown that exposure to normal and non-nephrotic human plasma leads to a concentration of nephrin, podocin, CD2AP, and actin at the cell surface. Next, the effects of plasma from patients with nephrotic conditions to non-nephrotic conditions were compared. When exposed to all nephrotic plasma samples (and a non-human serum control), nephrin podocin and CD2AP assumed a cytoplasmic distribution; nephrin and synaptopodin were selectively downregulated, and the relocation of nephrin induced by nephrotic plasma could be rescued back to the plasma membrane by co-incubation with non-nephrotic plasma. Furthermore, intracellular calcium signaling was altered by nephrotic plasma, which was mediated by tyrosine kinase phosphorylation. With the use of nephrin mutant human cell lines, it was shown that this signaling and translocation response to normal plasma is nephrin dependent. This work demonstrates that nephrotic plasma seems to be deficient in factors that act via the podocyte SD complex, which are essential in maintaining its physiologic function.

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In recent years, the podocyte slit diaphragm (SD) proteins have been shown to be central to the development of congenital and early childhood-onset human nephrotic syndromes through the seminal work of Kestila and Boute (1,2). Furthermore, the importance of CD2-associated protein (CD2AP) is critical in maintaining the integrity of the slit diaphragm (3) and has been demonstrated to be related closely to nephrin, podocin, and actin in human podocytes in culture (4). Of great interest is the role of these proteins in the much more common acquired nephrotic conditions, but *in vitro* work has been hampered by the lack of a representative human podocyte cell line. The podocyte displays a highly developed architectural phenotype, and in particular the SD is a unique type of cell junction (5), the permeability characteristics of which are determined by the unique proteins located there. Of these proteins, nephrin has been shown to be capable of signaling in combination with podocin (6), and these molecules are known to be intimately related to the actin cytoskeleton and to CD2AP in the podocyte (4,7). We hypothesized that these critical SD proteins are maintained in their structural conformation by factors in normal plasma that signal through this molecular complex. We studied the properties of the SD in a conditionally immortalized human cell line (8) when cultured in plasma from patients with no renal disease and compared these properties with plasma from patients with nephrotic conditions (three patients with focal segmental glomerulosclerosis [FSGS] in relapse and the same patients in remission) and a patient with Lupus nephritis. These were compared and contrasted with standard fetal calf serum (FCS) culture conditions.

We further examined how nephrin-deficient podocyte cell lines behave in conditions that mimic the normal and nephrotic podocyte milieu to ascertain the importance of nephrin for the function of the SD complex. To determine whether these nephrin-mediated effects alter cellular physiology, we also measured the intracellular calcium handling of wild-type (WT) and nephrin mutant cells when exposed to plasma. We present evidence for the presence of factors in normal human plasma that are essential for maintenance of the mature SD complex.

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Materials and Methods

Human Podocyte Cell Culture

Conditionally immortalized WT human podocyte cells were developed by the use of the temperature-sensitive large T antigen-SV40 transgene as described previously (8). These cells have been shown to differentiate fully by 14 d after switching from 33 to 37°C. Experiments

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to compare normal plasma with disease plasma were performed after day 14. Cell passages between 5 and 20 were used in all experiments. Podocytes were routinely cultured in RPMI-1640 medium with glutamine (R-8758; Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Grand Island, NY) and insulin transferrin sodium selenite (Sigma I-1184; 1 ml/100 ml). FCS was substituted with human plasma at the same concentration (10%) for the experiments. A second human podocyte cell line, developed in the same way in our laboratory, derived from the normal pole of a kidney with Wilm's tumor, was also used in some experiments to confirm further immunofluorescence (IF) findings. This was to exclude clonal variation as a potential explanation for differences seen.

Patient Samples

Plasma was obtained from patients who had undergone therapeutic plasma exchange for biopsy-proven FSGS (two boys aged 14 and 12 yr; one girl aged 11 yr) in relapse and the same patients in remission at the end of their plasma exchange course, together with a 14-yr-old girl who required plasma exchange for systemic lupus erythematosus (SLE) and a control patient with no renal disease but on prophylactic plasma exchange for cryoglobulinemia. Normal human plasma (and serum) was examined from an AB Rhesus-negative donor (Sigma) and from healthy adult volunteers. The profiles of the plasma samples are shown in Table 1.

Rescue Experiments

With rescue experiments, day 14 podocytes were initially exposed to 48 h of nephrotic or non-nephrotic plasma, and then the medium was changed with the initial plasma replaced at the same concentration (10%) and 10% of nephrotic or non-nephrotic plasma added for an additional 48 h.

Nephrin Mutant Podocyte Development

A kidney was removed for therapeutic reasons from a Finnish infant (male, 0.9 yr) who had congenital nephrotic syndrome secondary to the homozygous Fin_{major} mutation (nt121 del 2), which results in a two-nucleotide deletion in exon 2 of chromosome 19 and subsequently a truncation of the protein (1). Another nephrin mutant kidney (female, 1.8 yr) that was removed because of severe early-onset nephrotic syndrome as a result of a homozygous missense exon 11 mutation was also studied. Full informed consent from the family and ethical committee approval were obtained for use of the discarded kidneys. Primary culture, subcloning, and propagation were carried out as described previously (8). These cell lines were transfected with both SV40 large T antigen gene as described previously and a telomerase construct (9), to prevent senescence. This allows the cell line to replicate *ad infinitum* at 33°C, while the SV40 large T antigen was switched on, but at 37°C, the large T antigen is silenced and the cell line can differentiate. The cell lines when transformed were characterized and expressed WT1, synaptopodin, podocin, and CD2AP on IF and/or Western blotting. No nephrin was detectable on IF of the kidney sections and on IF or Western blotting on the podocyte cell line of the Fin major kidney (Figure 1). Cell passage between 7 and 15 were used for all experiments. Culture conditions were identical to the WT podocytes described above. All experiments on mutant cells were carried out in the Fin major cell line, and the second mutant cell line was used for additional data in the calcium flux experiments.

Antibodies

Primary nephrin antibodies used included a rabbit polyclonal antibody (10) that recognizes the intracellular fragment of the molecule and a panel of both rabbit polyclonal and mouse monoclonal antibodies against the extracellular domain of nephrin as described previously (4). These all were gifts from Karl Tryggvason (Karolinska Institute, Stockholm, Sweden). A rabbit polyclonal podocin c-terminal antibody was used for both Western blotting and IF (gift from Corinne Antignac, Paris, France) (8). Other antibodies used included synaptopodin (Progen, Heidelberg, Germany) and polyclonal rabbit antibodies raised against CD2AP and WT1 (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary species-specific horseradish peroxidase–associated antibodies (Sigma) and FITC- and TRITC-labeled antibodies (Jackson Immunoresearch, Philadelphia, PA) were also used. The F-actin cytoskeleton was imaged using a directly conjugated Texas red–Phalloidin stain (Molecular Probes, Eugene OR) and Western blotted with a monoclonal antibody (Sigma).

Table 1. Patient and plasma details

Patient/Disease	Age; Gender	Serum Albumin (g/L)	24-Hour Urine Protein: Creatinine Ratio (μg/mg)	Total Cholesterol (mmol/L)	Serum Creatinine (μmol/L)	Urea (mmol/L)	Immunosuppression Changes during Plasma Exchange Course
Transplant FSGS, relapse	14 y; male	25	918	3.2	146	12.7	Relapse sample = day 0, remission sample = day 11
Transplant FSGS, remission		33	19	1.5	160	12.5	500 mg/m^2 intravenous methylprednisolone on day 4
Transplant FSGS, relapse	16 y; male	28	1640	9.5	191	17.2	Relapse sample = day 0, remission sample = day 9
Transplant FSGS, remission		46	20	1.6	202	15.3	Cyclosporine dose changed from 175 to 200 mg twice a day on day 6
Native FSGS, relapse	11 y; female	17	280	4.8	45	8.1	Relapse sample = day 0, remission sample = day 16
Native FSGS, remission		41	10	2.4	51	5.3	Prednisolone changed from 40 mg on alternate days to 30 mg daily on day 1
Lupus nephritis	14 y; female	22	860	2.87	69	14.3	
Control non-nephrotic patient	62 y; female	40	<10	2.4	82	6.2	
FCS	NA	NA	NA	<1.2	NA	NA	

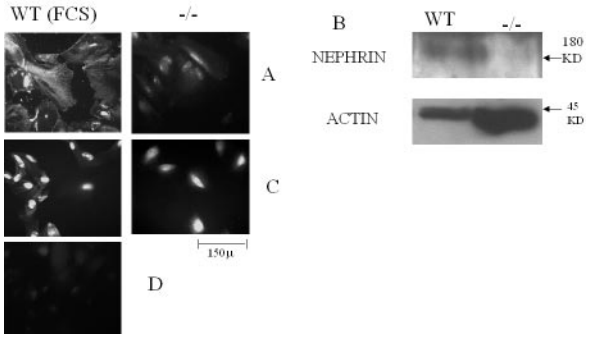

Figure 1. Characterization of the Fin major human podocyte cell line. Immunofluorescence (IF) staining and Western blotting of differentiated cells, either Fin major nephrin–deficient (−/−) or wild-type (WT) podocytes. (A) Nephrin IF using a polyclonal rabbit antibody K2966. Expression in WT podocyte but not nephrin knockouts. WT podocyte cultured exclusively in FCS giving cytoplasmic distribution of nephrin. (B) Nephrin Western blot. No expression in Fin major cells. Actin loading shown. (C) WT1 expressed in the nuclei of Fin major and WT podocytes. (D) Non-immunized rabbit Ig control. All above antibodies of rabbit origin.

Immunofluorescence

Immunolabeling was done as described previously (4). Images were obtained by using a Leica photomicroscope attached to a Spot 2 slider digital camera (Diagnostic Instruments, Sterling Heights, MI) and processed with Adobe Photoshop 5.0 software. All images were analyzed by an investigator who was blinded to the identity of the samples and ranked from cortical to cytoplasmic distribution of the molecules on a scale of 0 to 3. At least 20 cells per coverslip were ranked. Each blinded experiment was carried out on three independent occasions. Images were taken at ×400 magnification.

Environmental Scanning Electron Microscopy

Environmental scanning electron microscopy was performed as described previously on these cells (8). The images were categorized by two blinded investigators and ranked according to surface blebbing and fine process formation on a scale from 0 to 4. For each condition, at least five images were obtained and ranked, with four to 15 cells seen per image. Representative images are shown.

Western Blotting and Protein Extraction

The exact techniques as described previously were used for protein extraction and Western blotting (8). Protein was quantified using a modified Bradford assay (11) to ensure equal loading of lanes in comparative studies and confirmed by actin loading.

Lipid Raft Fraction Preparation

For preparation of low-density Triton X-100–insoluble membrane domains, cells were treated for 48 h as before with either normal plasma or FSGS relapse plasma. The protocol was followed exactly as described previously (12). Fractions then were probed with anti-nephrin (48E11) or anti-actin antibody as above.

Intracellular Calcium Studies

Cells were grown on coverslips and then incubated with Fura 2-AM, excited, and assayed using ratiometric fluorescence measurement as described before (13). Undiluted samples of relapse and remission plasma from one patient with FSGS were perfused in random order on the same coverslip with a 20-min wash period of HBSS media (Life Technologies BRL) in between. HBSS was also used as a baseline control. A 10-min preincubation of 70 μM genistein (Sigma) was used to block tyrosine kinase. Control cells of immortalized proximal tubular cells (HK2) (14) and human vein embryonic endothelial cells (HUVEC) (ATCC, Manassas, VA) were treated in exactly the same manner as the podocytes, and their response to plasma was recorded. There was a replicate of at least four in all sets of experiments.

Results

Nephrin, CD2AP, Podocin, and Actin

After 48 h of exposure, there were differences in the distribution of nephrin, podocin, and CD2AP in differentiated podocytes that were exposed to nephrotic compared with non-nephrotic patient plasma. Nephrotic plasma–exposed podocytes demonstrated a cytoplasmic distribution of all three molecules. Nephrin was observed in a diffuse cytoplasmic and filamentous pattern (average score, 0.4; Figure 2), podocin distribution was predominantly diffusely cytoplasmic (average score, 1.0), and CD2AP was seen in unevenly distributed "spots" in a submembranous and cytoplasmic location (average score, 0.2; Figure 3), as described previously (15). Plasma from all patients with nephrotic syndromes resulted in the same protein distribution, regardless of the underlying nephrotic cause. In contrast, samples from non-nephrotic individuals and healthy volunteers (data not shown) resulted in a highly enriched cortical, plasma membrane, location of nephrin (average score, 2.3), podocin (2.0), and CD2AP (2.6) in the differentiated podocyte (Figures 2 and 3). These enriched areas were not present continuously around the plasma membrane or indeed specifically at cell–cell junctions but seemed prominent at the leading edge of softly rounded cell protrusions, suggestive of a subtle alteration of cell shape to coincide with this distribution. A similar distribution was seen using samples from normal volunteers, patients with no previous renal disease, and patients who were in remission from established FSGS. Intriguing, human plasma (Figure 2) and serum (data not shown) also affected the location of nephrin when compared with FCS in podocytes of the same passage and age, with increased cortically located nephrin in the human compared with bovine samples. Prolonged culture in serum-free medium resulted in podocyte detachment, granulation, and necrosis, so these conditions were not included in the control experiments. Cellular morphology was disrupted in the presence of plasma from nephrotic patients, which occurred as early as 6 h, with retraction of fine process formation (average score, 1.2) and blebbing (average score, 2.9) on the cell surface noted (Figure 4) when viewed by environmental scanning electron microscopy. This appearance was also seen in the FCS-treated cells, although notably process retraction was not as widespread (average score, 2.3). In contrast, in remission and normal plasma–treated samples, there were many long processes (average score, 3.3 and 3.8, respectively) and very few surface blebs (1.9 and 1.4, respectively). Paired t test for either observation, between relapse/FCS and normal/remission, was significant at <0.01. In conjunction with this morphologic change, actin patterning at 48 h was also altered between ne-

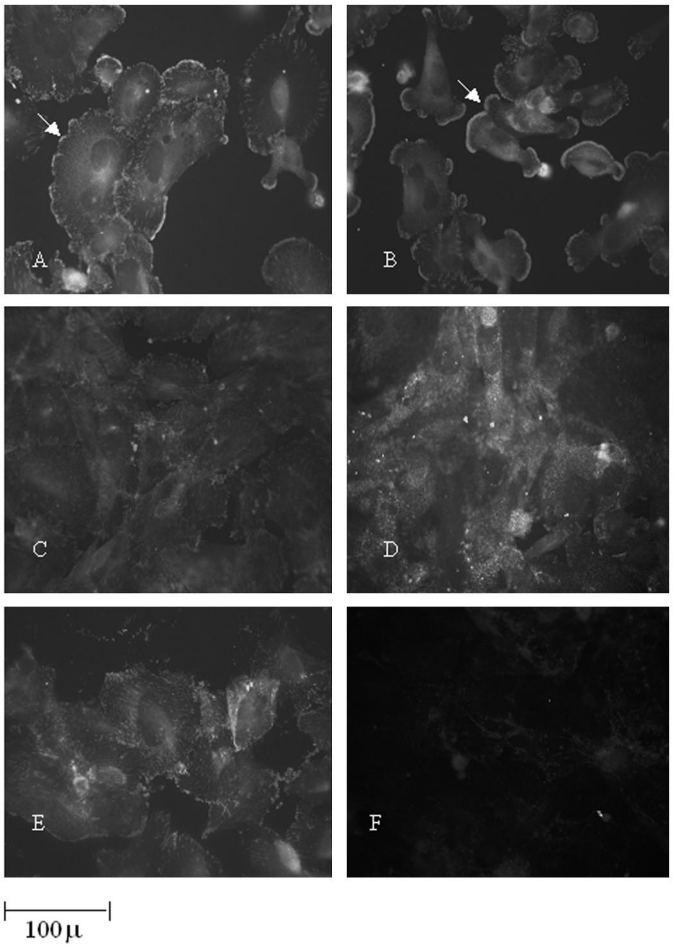

Figure 2. Nephrin immunolocalization in WT human podocytes incubated with various human plasma samples. Differentiated cells incubated with various plasma samples. Ten percent plasma applied to the cells for 48 h. IF with monoclonal 48E11 antibody. Peripheral nephrin shown by arrows. Representative of three independent, blinded experiments. (A) Normal human plasma. (B) Plasma from patient with focal segmental glomerulosclerosis (FSGS) in remission. (C) Plasma from FSGS patient in relapse. (D) Plasma from patient with systemic lupus erythematosus. (E) FCS alone. (F) Control of nephrin 48E11 antibody applied to proximal tubular HK2 cells. Negative staining.

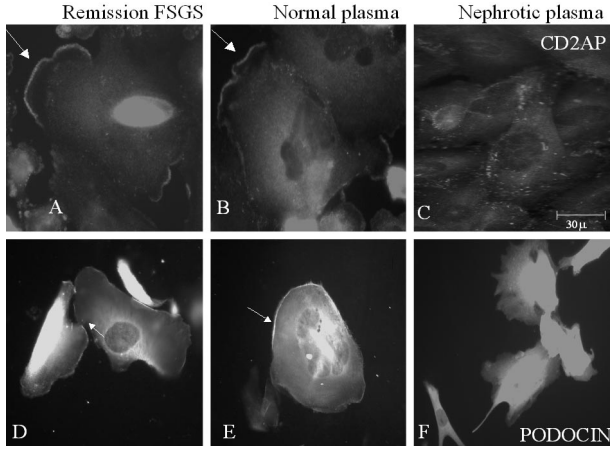

Figure 3. Effects of nephrotic and non-nephrotic plasma on CD2 associated protein (CD2AP) and podocin in WT human podocytes. Differentiated cells incubated with different plasma samples. Ten percent plasma applied to the cells for 48 h. Representative panels from three independent experiments. Peripheral location of the molecules arrowed. (A) CD2AP from patient in remission from FSGS. (B) CD2AP from normal patient plasma. (C) CD2AP from nephrotic patient (FSGS relapse). (D) Podocin from patient in remission from FSGS. (E) Podocin from normal patient plasma. (F) Podocin from nephrotic patient (FSGS relapse).

phrotic/FCS and non-nephrotic plasma. In cells that were treated with normal or remission plasma, actin filaments in the cytoplasm were weak or absent and cortical actin distribution was strong (average score, 2.1), whereas in cells that were treated with nephrotic plasma or FCS, cytoplasmic actin filaments were strongly expressed throughout, and cortical distribution was relatively weaker (average score, 1.0; paired *t* test $P < 0.05$; Figure 4).

Quantitatively, Western blotting revealed that nephrin was downregulated by 48 h when podocytes were exposed to nephrotic plasma, but the other SD proteins (podocin and CD2AP) were not affected (Figure 5, A and B). In addition, we used a protocol that isolates lipid rafts on the basis of their insolubility in TX-100 and low buoyant density in sucrose density gradients (16). Nephrin was seen in both the heavy (cytoskeletal bound) fraction and raft fractions in normal plasma–treated cells, which correlates with its distribution in glomerular preparations (16), compared with just being present in the heavy fraction in FSGS relapse plasma–treated cells (Figure 5C). It is interesting that of the molecules studied, the only one that was downregulated early, at 24 h, in response to nephrotic plasma was synaptopodin, an actin associated podocyte protein, suggesting that the podocytes were becoming dedifferentiated (Figure 5A). This observation is consistent with studies of biopsy specimens showing downregulation of synaptopodin in affected podocytes in nephrotic diseases (17–19).

By analyzing podocytes from a patient with Fin major congenital nephrotic syndrome, we showed that under control (FCS) conditions, CD2AP was present in its subplasma membrane and cytoplasmic location as before (average score, 0.3), but after 48 h of incubation in normal plasma, there was no translocation to the cell surface (average score, 0.6; $P < 0.01$; Figure 6). In addition, the cortical concentration of actin seen in response to normal plasma with WT cells was less marked in the mutant cells, with no change to the cytoplasmic filamentous stress fibers (average score, 1.5 *versus* 1.9; $P < 0.1$). This demonstrated that CD2AP is present in nephrin-deficient cells but that nephrin needs to be present to allow the plasma membrane translocation of CD2AP and actin reorganization at the cell surface to occur.

Rescue of Nephrin Translocation

We examined whether the nephrotic plasma was responsible for the cytoplasmic relocation of nephrin directly via putative

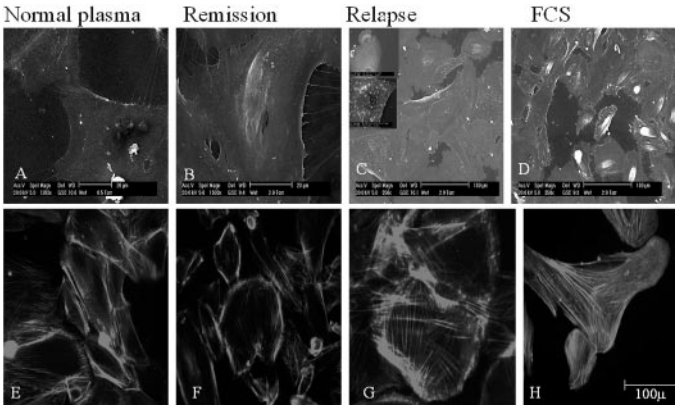

Figure 4. Effects on the actin cytoskeleton by human plasma samples and FCS on WT human podocytes. Differentiated cells incubated with different plasma samples. Ten percent plasma applied to the cells for 6 h. (A through D) Environmental scanning electron micrographs of the podocytes, of same passage and age, exposed to the following. (A) Normal human plasma. Process formation illustrated. (B) FSGS patient in remission. Again, process formation demonstrated by arrow. (C) Nephrotic (FSGS relapse). Lack of foot processes and blebs noted. Insets show expanded images of affected areas. (D) FCS. Shows similar blebs to C. (E through H) The actin cytoskeleton by phalloidin staining at 48 h after exposure to the following plasma samples. (E) Normal human plasma. Predominantly cortical distribution. (F) FSGS patient in remission, similar to E. (G) FSGS patient in relapse. Cytoplasmic actin stress fibers more prominent. (H) FCS. Cytoplasmic actin stress fibers more prominent.

circulating factor(s) or was due to factors that were missing from nephrotic plasma and normally found in human plasma that were important. We found that nephrotic plasma induced a cytoplasmic distribution of nephrin, but this could be rescued and directed to the plasma membrane by subsequently adding an equal amount of non-nephrotic to nephrotic plasma (Figure 7). Importantly, this was not disease-specific, *i.e.*, non-nephrotic plasma co-incubation could rescue FSGS and SLE nephropathic states, which would argue against a specific inhibitory factor present in normal plasma that may be antagonizing a "circulating factor" in FSGS (20).

Intracellular Calcium Signaling

We studied the effect of plasma from a nephrotic patient in relapse and in remission on both WT and on two different nephrin mutant podocyte cell lines. We found in WT cells, with remission plasma, that there was no [Ca^{2+}] response (peak/baseline ratio = 1), whereas relapse plasma caused a statistically significant increase in peak [Ca^{2+}], which was inhibited by the tyrosine kinase inhibitor genistein. The effect on nephrin mutant cells was revealing: With remission plasma, there was a considerable increase in peak [Ca^{2+}] in both mutant cell lines, compared with WT cells, suggesting that the presence of nephrin in the correct conformation and cellular location prevents an increase in [Ca^{2+}] brought about by this plasma. With relapse plasma, there was a constant peak [Ca^{2+}] response that was

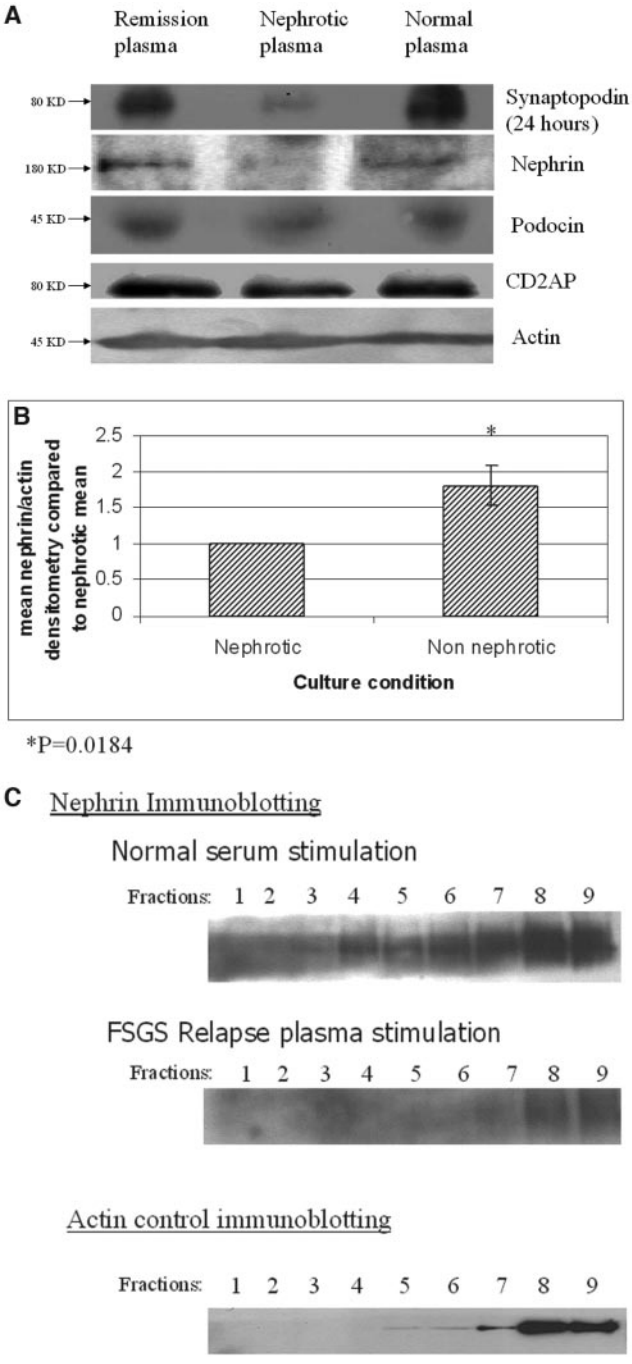

Figure 5. Western blot of podocyte proteins and their modulation by nephrotic plasma. Differentiated cells incubated with various plasma samples. Ten percent plasma applied to the cells for 24 or 48 h. Cells were lysed, and the lysates were probed with the following antibodies. (A) This shows representative blots of synaptopodin (at 24 h), and nephrin, podocin, CD2AP, and actin at 48 h ($n = 4$). (B) Densitometry on nephrin to actin ratio after 48 h of exposure. Nephrotic groups compared with non-nephrotic groups; four independent experiments. SEM shown. Data analyzed by a two-tailed nonparametric Wilcoxon test. (C) Sucrose density centrifugation of cells that were treated with either FSGS relapse plasma or normal human plasma. Fractions were probed with nephrin monoclonal Ab 48E11. Lighter fractions (1–4) correspond to those that normally contain lipid rafts (16). Control fractions were probed with actin to demonstrate efficiency of the gradient.

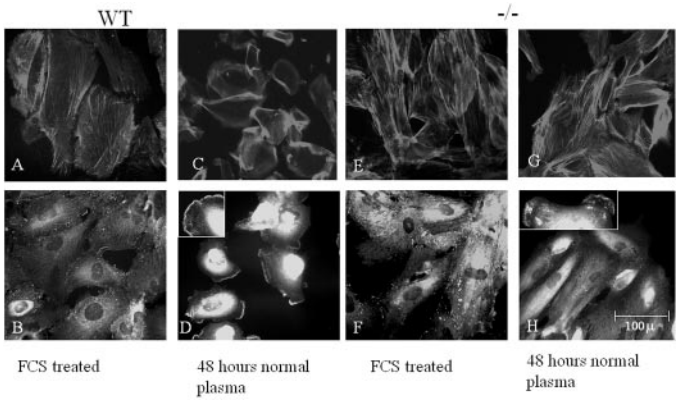

Figure 6. Peripheral translocation of CD2AP is dependent on nephrin. WT podocytes and Fin major podocytes were initially cultured in FCS and then exposed for 48 h to normal human plasma. Cells then were immunostained as follows. For wild types: (A) The actin cytoskeleton shows cytoplasmic stress fibers when cultured in FCS, with weak cortical distribution. (B) CD2AP is diffusely distributed with intracytoplasmic spots in FCS culture. (C) On exposure to normal human plasma, the actin cytoskeleton assumes a thick cortical distribution, with almost complete loss of cytoplasmic filaments. (D) CD2AP translocates to the plasma membrane (high magnification view on inset panel). For the Fin major (−/−) podocytes: (E) The actin cytoskeleton is similar to WT podocytes with FCS. (F) CD2AP is diffuse and intracellular in distribution with FCS. (G) With normal human plasma, the actin cytoskeleton does not become cortically distributed but preserves the cytoplasmic stress fibers. (H) CD2AP remains intracellular, diffuse, and in spots. In areas where CD2AP seems to have reached the plasma membrane, the pattern is disrupted and remains submembranous (inset). Magnification, ×400.

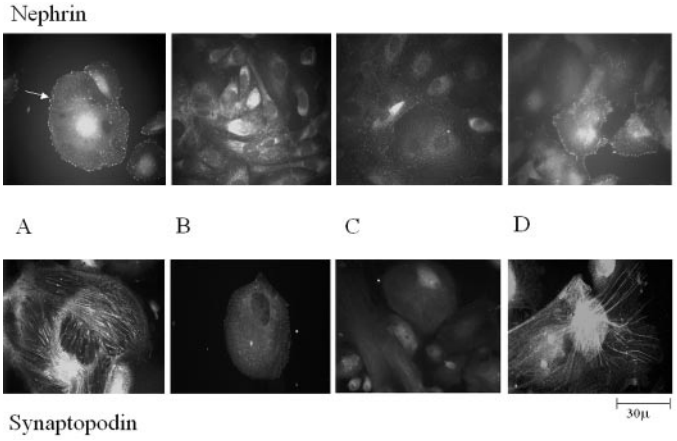

Figure 7. Peripheral nephrin and synaptopodin rescue of nephrotic plasma exposed cells by non-nephrotic plasma in WT human podocytes. IF performed using the mouse monoclonal antibody 48E11 (top) or synaptopodin (bottom). Each experiment shows day 14 thermoswitched podocytes exposed to 48 h of initial plasma then fresh co-incubation of original plasma sample with another plasma sample. All samples applied at 10% concentration. The same passage number and culture time of podocyte were used in each experiment. Representative experiment shown. (A) Normal plasma (10%) followed by normal plasma–normal plasma (20%). Peripheral nephrin localization arrowed. (B) Nephrotic plasma for 48 h (10%) followed by nephrotic–nephrotic plasma (20%). Cytoplasmic nephrin distribution. (C) FCS 10% followed by FCS (20%). Cytoplasmic nephrin distribution. (D) Nephrotic plasma (10%) for 48 h followed by nephrotic plasma (10%) and normal plasma (10%) for 48 h. Nephrin again peripherally located (synaptodin up-regulated with normal plasma, Panel A and D). Magnification, ×400.

consistent between podocyte cell types (Figure 8, A and B). It is interesting that genistein had *no* effect on the nephrin mutant cells' response to remission plasma (Figure 8C). This shows that the effect of remission plasma on intracellular calcium is independent of tyrosine kinase activation in the absence of nephrin expression. This therefore supports the suggestion that nephrin plays a role in the calcium response in WT cells and that the response seen in nephrin knockout cells is a result of perturbation of this normal pathway. The effect of plasma seemed to be podocyte specific in that neither relapse nor remission plasma caused any increase in $[Ca^{2+}]$ in either proximal tubular or human endothelial cells (Figure 8D).

Discussion

Key podocyte SD molecules are critical in maintaining the filtration barrier of the kidney and preventing protein loss into the urine. This study shows that human plasma seems to contain factors that are crucial to the mature distribution of the SD proteins nephrin, podocin, and CD2AP and that are absent from nephrotic plasma. In conjunction with this mature distribution of SD proteins, we showed that actin is cortically reorganized. Furthermore, we have demonstrated using a human nephrin "knockout" cell line that nephrin is crucial for mature distribution of the whole SD complex, with or without plasma factors. Finally, our calcium flux data strongly suggest that this mature SD complex is involved in mediating intracellular signaling, which is influenced by these plasma factors.

Previous authors have studied the distribution of nephrin and podocin in health and nephrosis, and some have found that they are localized away from the SD in nephrotic syndromes (21,22). However, others have not demonstrated this (23,24). It has been noted in several human biopsy studies (25,26) and animal models (27,28) that nephrin, podocin, and CD2AP distribution in nephrotic disease changes from a linear capillary loop pattern to a granular pattern, supportive of our observation of intracellular translocation. Previously, it has been impossible to study their location *in vitro*, as cell lines have not convincingly expressed these proteins. We have shown that, *in vitro*, these molecules are located in a characteristic peripheral location in the presence of normal human plasma, which intuitively would seem to be the correct targeting of these proteins and be analogous with the known human SD location in health *in vivo* (10). Furthermore, our finding that nephrin is vital to allow CD2AP to locate at the plasma membrane of the cell and does not occur when nephrin mutants are subjected to normal plasma suggests that the molecules are interdependent on each other to find their normal location. Nephrin, via CD2AP, inter-

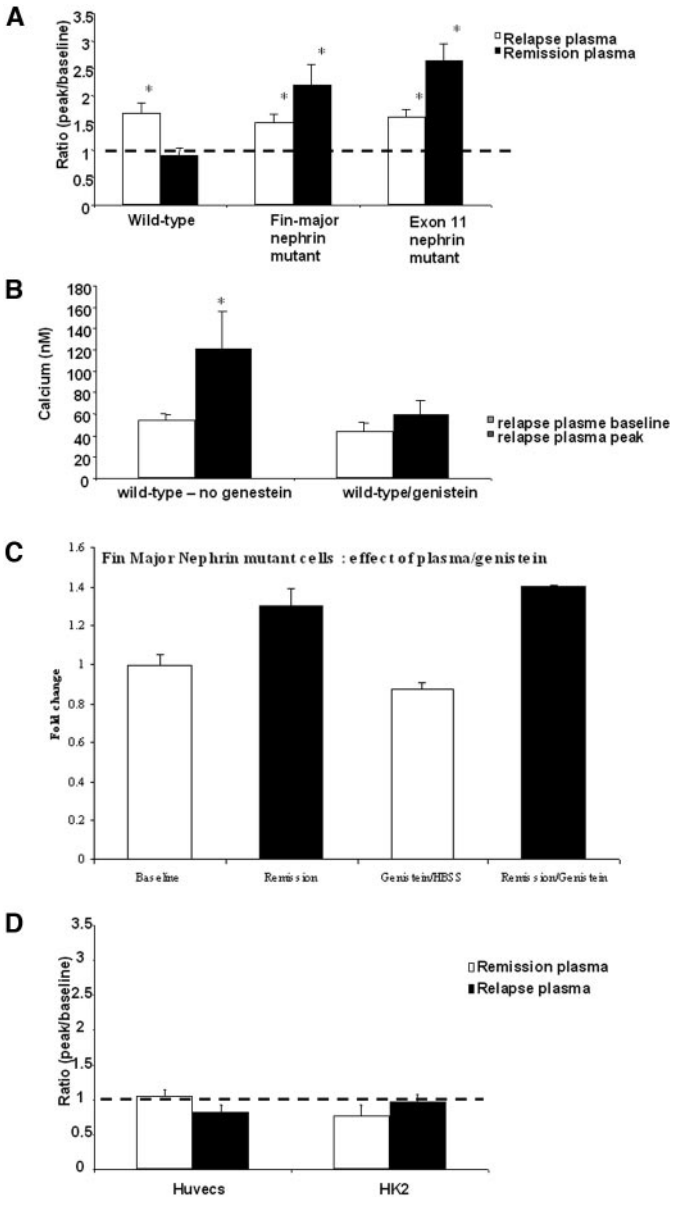

Figure 8. Podocyte calcium flux data. Cells were grown on coverslips and then incubated with Fura 2-AM, excited, and assayed using ratiometric fluorescence measurement. Undiluted samples of relapse and remission plasma from one patient with FSGS were perfused in random order on the same coverslip with a 20-min wash period of HBSS medium in between. HBSS was also used as a baseline control. Data expressed as ratio of peak/baseline of $[Ca^{2+}]_{Intensity}$. (A) Relapse plasma significantly increases $[Ca^{2+}]_i$ levels in differentiated WT podocytes (1.67 ± 0.19; paired *t* test $P < 0.001$; $n = 9$), exon 11 nephrin mutant podocytes (relapse, 1.5 ± 0.15; paired *t* test $P < 0.001$; $n = 7$) and Fin major podocytes (relapse, 1.6 ± 0.12; paired *t* test $P < 0.001$; $n = 7$) compared with baseline. Remission plasma does not significantly increase $[Ca^{2+}]_I$ levels in WT podocytes (0.9 ± 0.146; *t* test $P < 0.001$; $n = 9$); however, it does in both exon 11 mutant podocytes (remission, 2.6 ± 0.3; paired *t* test $P < 0.001$; $n = 7$) and Fin major podocytes (remission, 2.2 ± 0.37; paired *t* test $P < 0.005$; $n = 6$) compared with baseline. (B) Preincubation of WT podocytes with 70 mM genistein attenuates the $[Ca^{2+}]_i$ response stimulated by relapse plasma (relapse, 121.5 ± 34.7 nM $[Ca^{2+}]$ *versus* 54.8 ± 4.9 $[Ca^{2+}]$; ANOVA Bonferroni multiple comparison test, $P < 0.05$, $n = 5$; genistein/relapse, 59.8 ± 12.9 nM $[Ca^{2+}]$ *versus* 44.3 ± 7.9 nM $[Ca^{2+}]$, NS). *$P < 0.05$. (C) Preincubation of Fin major podocytes with 70 mM genistein shows no significant abrogation of the increased $[Ca^{2+}]$ response to remission plasma (seen in A; $n = 4$; paired *t* test $P < 0.004$, remission *versus* remission/genistein). (D) Effect of the plasma is podocyte specific. The data are expressed as a ratio of peak/baseline $[Ca^{2+}]$. In HK2 cells (a proximal tubular epithelial cell line) and human vein embryonic endothelial cells (HUVEC), neither remission nor relapse plasma has a significant effect on $[Ca^{2+}]$ flux (HK2 cell: remission 0.77 ± 0.14, relapse: 0.96 ± 0.11; HUVEC: remission 1 ± 0.11, relapse 0.8 ± 0.1 *versus* baseline; ANOVA Bonferroni multiple comparison test; $n = 7$).

acts directly with the actin cytoskeleton (which is the structural backbone of the foot process), and we showed previously that disruption of nephrin results in concomitant disruption of actin (4). Thus, the cytoplasmic relocalization of the SD complex under nephrotic conditions could be related directly to foot process effacement.

The identification of a circulating factor as the pathogenic molecule in nephrotic syndromes, particularly minimal-change nephrosis and FSGS, has long been a holy grail for researchers in this field (29). A number of *in vitro* studies have been based on studying albumin permeability of isolated glomeruli, which follow the trail of a putative circulating factor, although to date with inconsistent results. Our study for the first time addresses the question by the direct examination of the *in vitro* podocyte and, more specifically, the effect on SD proteins. The role of additional putative circulating factors in the pathogenesis of conditions such as FSGS (29) have been challenged recently (30,31). It has been suggested tentatively that nephrosis could result from the lack of a factor that is normally present in human plasma and not the addition of a circulating factor (30,31). In fact, there is tantalizing evidence to support the loss of vital factor(s) in nephrosis from a study by Carraro *et al.* (30), in which plasma derived from proteinuric patients with NPHS2 gene mutations (congenital nephrotic patients) induced albumin permeability of isolated glomeruli *in vitro*, whereas urine from these patients (and not normal urine controls) blocks this increased permeability, hence suggesting the loss of crucial factors into the urine of nephrotic patients. Our findings provide direct evidence for this hypothesis. The observation that FCS as well as nephrotic plasma results in loss of peripheral nephrin-podocin-CD2AP localization suggested to us that there is something missing in the medium that promotes mature SD complex distribution and cell signaling, and accordingly we found that normal plasma is able to rescue the cytoplasmic nephrin and move it to the periphery of the cell. This was not specific to FSGS and seems, in relation to nephrin, to be associated with the nephrotic syndrome *per se*. Clearly, nephrotic syndromes are a heterogeneous group of diseases, and it may be that in disease such as FSGS, there is a primary loss or imbalance of important plasma factors that disrupt SD integrity and subsequently foot process morphology, whereas in other nephroses, such as those caused by SLE nephritis, the disrup-

tion is secondary to a generalized loss of proteins. There is a broad literature documenting that a proportion of cases of FSGS is treatable by plasma exchange, suggesting removal of a pathogenic factor, and also by protein A immunoadsorption. However, there are many potential anomalies/variables in interpreting these data, beyond the scope of this brief discussion. For example, in our schema, it is possible that plasma exchange (often being replaced by fresh plasma) is in fact reestablishing a natural balance of circulating cytokines, lipoproteins, etc. that in certain (although perhaps not all) circumstances allows restoration of the mature SD. Also, immunoadsorption has been shown to be effective in non-FSGS nephrotic syndromes (32), favoring a nonspecific beneficial effect. The details of which unique factors are present in human plasma are clearly areas for future investigation, and this *in vitro* system would be a valuable resource to study these questions.

Nephrin, podocin, and CD2AP are known to be associated, together with actin in the podocyte in a lipid raft complex at the SD (4,7). One of the key functions postulated for lipid rafts is to facilitate rapid signaling events (33), and nephrin and podocin have been shown to be interdependent in initiating an intracellular signaling cascade (6), although the downstream consequences of this signaling pathway remain to be determined. It is now known that membrane-associated nephrin is tyrosine phosphorylated, with the Src family kinases Fin and Yes present within the membrane complex (34). *In vivo* deletion of Yes resulted in dramatically increased nephrin phosphorylation, whereas deletion of Fyn results in reduced phosphorylation and coarsening of foot process morphology, suggesting complex regulation of nephrin signaling. We have shown within minutes of exposure to nephrotic plasma that there is enhanced calcium signaling relative to remission plasma. Moreover, there is a dramatic increase in calcium signaling by remission plasma in nephrin-deficient cells. This strongly suggests that the intact SD complex at the plasma membrane, in response to steady-state signals from normal plasma, suppresses a calcium-mediated signaling cascade. Whether activation of this cascade is involved in the subsequent SD mislocalization is yet to be determined. Together, these data lead us to postulate an updated concept for the pathogenesis of nephrotic syndromes. In this model, the podocyte in its mature form (*i.e.*, intact nephrin-podocin-CD2AP complex at the SD, interdigitating foot processes) is constantly maintained by a milieu of factors, some from the circulation and probably some from local autocrine/paracrine production (our own unpublished data). Disruption or loss of this balance, be it primary or secondary, results in disruption of nephrin-mediated signaling, leading to loss of SD stability and hence reorganization of associated actin filaments, causing intracellular relocalization of the SD complex and foot process effacement.

Nephrotic syndromes are a heterogeneous group of conditions, and this study addresses the cell biology of the podocyte in relation to a subset of patients. Our results were consistent across the samples, although it is certainly possible that in other nephrotic patients there may be alternative mechanisms at play. The observations that we describe, however, do fit comfortably with the emerging knowledge of podocyte biology, in particular a role for the SD complex in signaling and actin regulation.

In conclusion, we have demonstrated that podocytes are affected directly by nephrotic plasma with early nephrin-dependent signaling events, followed by actin reorganization and interdependent translocation of the proteins nephrin, podocin, and CD2AP away from the plasma membrane into the cytoplasm of the cell. This process can be rescued by normal plasma, suggesting the loss of a factor in nephrosis. At this stage, our understanding of the factors that are responsible for maintaining podocyte differentiation *in vivo* is simplistic, and we hope that this study will be a step toward a more detailed study of these in health and disease.

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