

Serum Glomerular Permeability Activity in Patients with Podocin Mutations (NPHS2) and Steroid-Resistant Nephrotic Syndrome

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Abstract. A plasma factor displaying permeability activity *in vitro* and possibly determining proteinuria has been hypothesized in idiopathic focal segmental glomerulosclerosis (FSGS). *In vitro* permeability activity (P_{alb}) was determined in sera of five patients with autosomal recessive steroid-resistant nephrotic syndrome (NPHS2), an inherited condition indistinguishable from idiopathic FSGS on clinical grounds, but in which proteinuria is determined by homozygous mutations of podocin, a key component of the glomerular podocyte. All patients had presented intractable proteinuria with nephrotic syndrome; four developed renal failure and received a renal allograft. For comparison, sera from 31 children with nephrotic syndrome were tested. Pretransplant P_{alb} was high in all cases (mean 0.81 ± 0.06), equivalent to levels observed in idiopathic FSGS. Overall, P_{alb} did not correlate with proteinuria. The posttransplant outcome was complicated in two patients by recurrence of proteinuria after 10 and 300 d, respectively, that responded to plasmapheresis plus cyclophosphamide. P_{alb} lev-

els were high at the time of the recurrence episodes and steadily decreased after plasmapheresis, to reach normal levels in the absence of proteinuria after the seventh cycle. In an attempt to explain high P_{alb} in these patients, putative inhibitors of the permeability activity were studied. Coincubation of serum with homologous nephrotic urine reduced P_{alb} to 0, whereas normal urine did not determine any change, which suggests loss of inhibitory substances in nephrotic urine. The urinary levels of the serum P_{alb} inhibitors apo J and apo E were negligible in all cases, thus suggesting that other urinary inhibitors were responsible for the neutralizing effect. These data indicate that P_{alb} is high in NPHS2, probably resulting from loss of inhibitors in urine. Lack of correlation of P_{alb} with proteinuria suggests a selective loss of inhibitors. As in idiopathic FSGS, proteinuria may also recur after renal transplantation in NPHS2 patients, and post-transplant proteinuria is associated with high P_{alb} . The relationship between elevated P_{alb} and proteinuria in NPHS2 remains to be determined.

Serum from patients with focal segmental glomerulosclerosis (FSGS) increases glomerular permeability to albumin when incubated with rat glomeruli *in vitro* (1,2). Indirect evidence supports the concept that permeability activity (P_{alb}) is mediated by a circulating factor that may survive over time in patients with FSGS and induce posttransplant recurrence of the disease. In fact, high P_{alb} levels are strongly predictive of posttransplant recurrence of FSGS (2,3), and permeability activity is removed with *ex vivo* techniques such as plasmapheresis and immunoabsorption (3–5). Permeability activity may

also be transmitted during gestation from mother to fetus (6). It has recently been suggested that the putative plasma factor responsible for the permeability activity is neutralized by normal serum components such as apolipoproteins (7,8), which raises the question of whether inhibitors loss in urine may in some way mediate the effect. Loss of inhibitors may play a central role in the process, leading to recurrence of FSGS after transplantation and possibly to proteinuria also in the original disease. A first step is the definition of the permeability activity specificity; if loss of inhibitors is relevant, it could also be found in other proteinuric conditions resembling idiopathic FSGS. We studied children carrying mutations of podocin, which is a structural component of podocytes responsible for the glomerular barrier to filtration of proteins. For its strategic function, molecular defects of podocin should directly cause proteinuria. We determined serum P_{alb} in five children with autosomal recessive podocin mutations and have followed the posttransplant outcome in the four who received a renal allograft.

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Table 1. Clinical features in five FSGS patients with mutations of the NPHS2 gene and altered P_{alb} serum levels

Patient	Gender	Age (yr)	Age at Onset of Proteinuria	Therapy ^a	Proteinuria/day at Presentation	Proteinuria/day after Therapy	Dialysis (Age)	Transplant (Age)	Posttransplant Outcome	NPH2 mutation (nt change)
1	F	10	8.0	Ste/Cyc/Csa	6 to 7	unchanged				538G>A / 413G>A
2	M	20	1.5	Ste	6 to 7	unchanged	12 yr	15 yr	good	419delG / 419delG
3	M	19	14.0	Ste/Csa	5 to 6	unchanged	17 yr	19 yr	good	467/8insT/ 538G>A
4	M	12	1.0	Ste/Cyc/Csa	8 to 9	unchanged	7 yr	9 yr	Proteinuria after 10 d	413G>A / 413G>A
5	M	10	2.0	Ste/Cyc	8 to 9	unchanged	4 yr	4.5 yr	Proteinuria after 300 d	413G>A / 413G>A

^a Ste, steroid; Cyc, cyclophosphamide; Csa, cyclosporin.

Materials and Methods

Patients

After podocin had been identified as a cause of proteinuria in families with recessive inheritance by Boute *et al.* (9), we screened for podocin mutations in 44 sporadic patients with steroid-resistant nephrotic syndrome who had been previously classified with idiopathic FSGS on the bases of clinical and pathologic findings. Nine patients who presented homozygous or composite heterozygous mutations have been exhaustively described elsewhere (10). Five patients of this group had their serum tested for permeability activity according to a diagnostic protocol utilized at our institution. Serum was not available for the remaining four, who were therefore excluded from the study. All patients were enrolled in Italy, had an age ≤ 17 yr at diagnosis, had presented the full-blown picture of steroid-resistant nephrotic syndrome, and had been treated at the onset of the disease with steroids, cyclophosphamide, and in some cases with cyclosporin according to standardized protocols. Resistance to steroids was defined as the persistence of proteinuria after a protracted (at least 2 mo) regimen with 2 mg/kg prednisolone and 6 methylprednisolone pulses (10 mg/kg). At this point, cyclophosphamide was given at 2 mg/kg for 2 mo followed by cyclosporin at an initial dose of 5 mg/kg. When corticoreistance was ascertained, all patients had a renal biopsy showing at least one area of segmental/global glomerulosclerosis with mesangial expansion of both the cellular and extracellular matrix components with deposition of immunoglobulins. These clinical details are reported in Table 1, which also shows the outcome in respect to progression of renal failure and renal transplantation. Four patients (patients 2 to 5) had developed end-stage renal failure at an age between 4 and 17 yr and received a renal allograft within 2 yr of beginning a dialysis program. One girl had normal renal function at the age of 10 yr. In patients who developed renal failure, the evaluation of P_{alb} was done before the renal transplant and urine was collected in parallel according to a program active at our institution on FSGS allograft. Serum was obtained in the morning after an overnight fast, immediately centrifuged at $1500 \times g$ for 15 min and stored at -80°C .

Normal Controls and Controls of Other Proteinuric States

The control group for P_{alb}, apo J, and apo E in serum and in urine consisted in 20 subjects of the hospital staff and their children (mean age, 18 yr; range, 3 to 36). The control group for comparison of P_{alb} in other proteinuric states consisted of 31 children with nephrotic syndrome who were subdivided into the following three groups according to the response to steroids: (1) six patients with good response to steroids or with frequent relapses; (2) five patients with strict

corticoredependence who were taking cyclosporin; and (3) 20 patients with corticoreistance, most of whom had also a histologic diagnosis of FSGS. They had variable age between 4 and 66 yr and a prevalence of children under 6 yr. Steroid resistance was diagnosed according to the criteria defined above. Molecular analysis of podocin excluded in all cases an implication of inherited defects of this protein. Clinical details, including the level of proteinuria and therapeutic approach, are given in Table 2.

Molecular Analyses of Podocin

DNA was isolated from fresh peripheral blood samples. Molecular analyses of podocin were performed as described previously (9,10). Exons were amplified by PCR using flanking intron primers. All samples were subjected to automated sequence analysis by dye-terminator reactions (Automated Sequencer ABI 377; Applied-Bio-system, Milan, Italy).

Measurement of Albumin Permeability Activity

Frozen sera were available from the five NPHS2 children and urine from three. Spot morning urine specimens were collected in sterile conditions and dialyzed against water for 48 h using membranes with a molecular weight cutoff of 8000 kD. The protein content was measured, and urine volumes equivalent to 100 μg were lyophilized.

The method for measuring permeability activity in sera of patients with NPHS2 follows the original description by Savin *et al.* (1) with minor variations as already described (8). Glomeruli were isolated by sieving in isotonic phosphate buffer solution from the renal cortex of healthy male Sprague-Dawley rats weighing 200 to 300 g. The pH had been titrated to 7.4. The medium also contained 5 g/dl bovine serum albumin (BSA) as an oncotic agent. The isolated glomeruli were then washed in 1 ml of fresh medium, and an aliquot of 0.1 ml was incubated at 37°C for 10 min in 0.9 ml of medium, which included 2 to 4% vol/vol patient serum. For experiments concerning coinubation of urine with serum, homologous and nonhomologous urine (including normal urine) were preincubated with an equal volume of serum at 37°C for 5 min; 50 μl of the mixture was then added to the medium containing the isolated glomeruli according to the standard procedure. Pooled normal human serum served as the control. The glomeruli were then plated onto a glass coverslip, coated with poly-L-lysine as an adherent, and covered with fresh medium. The samples were masked to eliminate operator bias.

The rationale and methodology for the determination of albumin permeability has been described in detail in the literature (1,2). In brief, each of 10 to 16 glomeruli per test serum were videotaped through an inverted microscope before and after a medium exchange to one containing BSA 1 g/dl. The medium exchange created an

oncotic gradient across the basement membrane, resulting in a glomerular volume change ($\Delta V = [V_{\text{final}} - V_{\text{initial}}]/V_{\text{initial}}$), which was measured off-line by a video-based image analysis program (MCID; Imaging Research Inc., St. Catharines, Ontario). The computer program determines the average radius of the glomerulus in two-dimensional space, and the volume is derived from the formula $V = 4/3\pi r^3$ (3). The magnitude of ΔV was related to the albumin reflection coefficient, σ_{alb} , by the following equation:

$$(\sigma_{\text{alb}})_{\text{experimental}} = (\Delta V)_{\text{experimental}}/(\Delta V)_{\text{control}}$$

the σ_{alb} of the control glomeruli was assumed to be equal to 1. P_{alb} is defined as $(1 - \sigma_{\text{alb}})$ and describes the movement of albumin subsequent to water flux. When σ_{alb} is zero, albumin moves across the membrane with the same velocity as water, and P_{alb} is 1.0. Con-

versely, when σ_{alb} is 1.0, albumin cannot cross the membrane with water, and P_{alb} is zero. It should be stressed, however, that differences in P_{alb} are derived strictly from alterations of σ_{alb} ; an elevated P_{alb} does not imply that albumin has physically leaked out of the glomerular capillary. In other words, a diminished reflection coefficient (increased P_{alb}) fails to *prevent* the albumin from leaking; however, at the time of the measurement, nearly all the albumin remains in the capillary.

Determination of Apolipoproteins J and E

Serum and urinary levels of both apo J and apo E were determined by dot blot, using peroxidase-labeled anti-apo J polyclonal antibodies (Chemicon, Temecula, CA) and anti-apo E monoclonal antibodies

Table 2. P_{alb} and proteinuria in 31 children with idiopathic nephrotic syndrome subdivided according to responsiveness to steroids^a

Patients	Gender	Age (yr)	Pathology	Proteinuria (gd)	P_{alb}
Responders					
1	M	6	nd	3.9	0.39
2	M	9	nd	6.9	0.73
3	M	4	nd	0.01	0.96
4	M	5	nd	0.3	0.41
5	M	9	nd	0.87	0.68
6	M	11	nd	15	0.67
Corticodependent					
7	M	7	IgM	2	0.67
8	F	17	IgM	2.7	0.79
9	M	10	IgM	nd	0.64
10	M	5	nd	4.4	0.88
11	M	5	nd	nd	0.65
Corticoresistant					
12	M	46	FSGS	0.2	0
13	F	47	FSGS	3	0.14
14	M	31	IgM	18	0.45
15	M	6	nd	10	0.65
16	F	7	IgM	2	0.64
17	F	21	FSGS	nd	0.98
18	F	77	FSGS	6.5	1
19	M	16	FSGS	1	0.68
20	F	6	FSGS	nd	0.72
21	M	16	FSGS	2.4	0.75
22	?	37	FSGS	0.4	0.19
23	M	23	FSGS	0.37	0.79
24	M	17	IgM	16.5	0
25	F	30	FSGS	1.2	0.47
26	M	18	FSGS	0.4	0.92
27	M	8	IgM	0.01	0.22
28	M	42	FSGS	2.9	0.71
29	M	66	FSGS	1.2	0.08
30	F	7	FSGS	1.4	0.76
31	M	16	FSGS	1	0.74

^a Responders of frequent relapsers ($n = 6$); corticodependent ($n = 5$); corticoresistant ($n = 20$); FSGS, focal segmental glomerulosclerosis; IgM, mesangial proliferation with IgM deposition; nd, no biopsy.

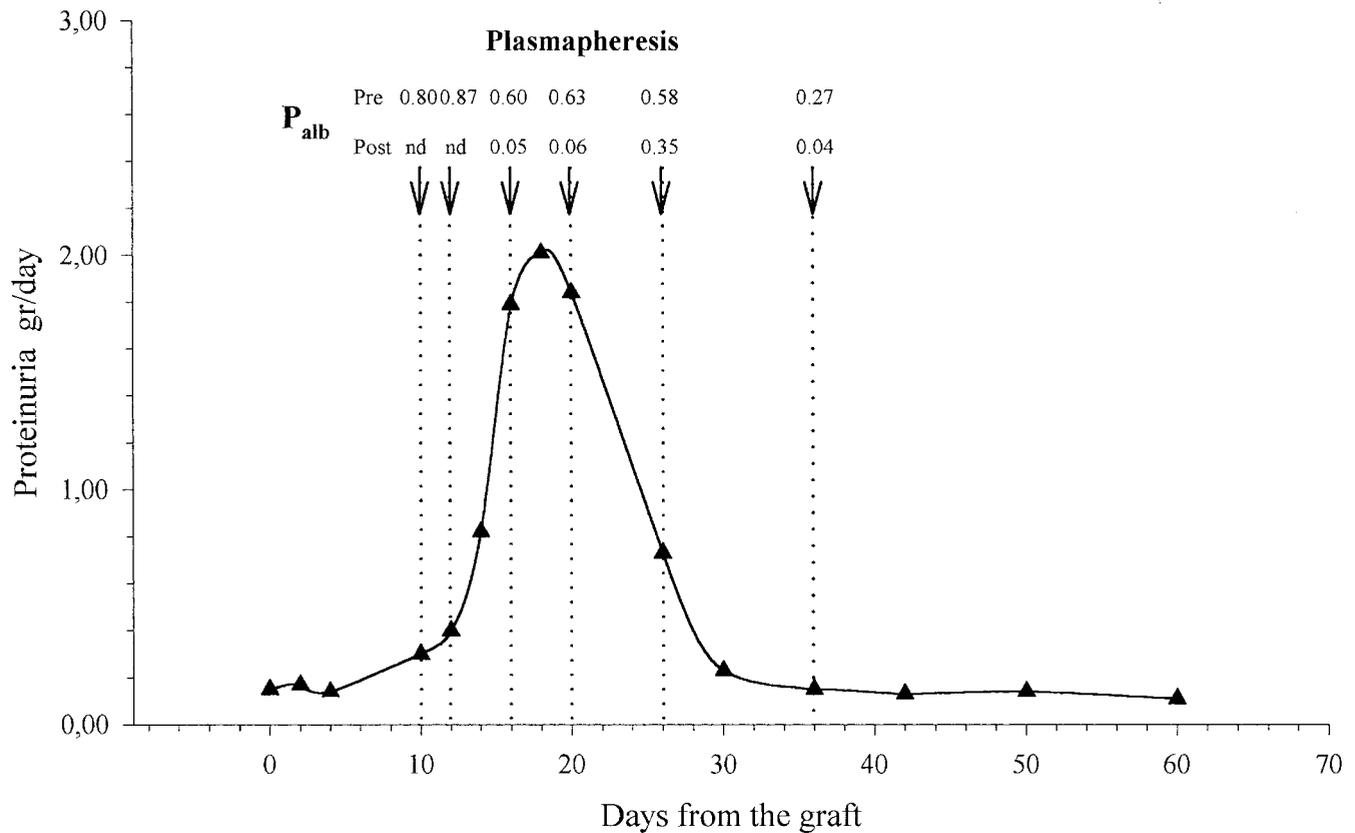


Figure 1. Outcome of proteinuria and P_{alb} in patient 4, who presented recurrence after 10 d from the renal graft. He was treated with six plasmapheresis associated with cyclophosphamide, and proteinuria relapsed. P_{alb} steadily decreased during the treatment and was normalized in concomitance with proteinuria. Pre, P_{alb} before plasmapheresis; Post, P_{alb} after plasmapheresis; nd, not determined.

(Cell clone 2E11; Roche, Monza, Italy). Apolipoproteins were first adsorbed under vacuum to Hybond C super nitrocellulose (Amersham-Pharmacia, Little Chalfont, UK). Specific antibodies were incubated overnight at room temperature, and visualization was achieved with ECL Plus (Amersham-Pharmacia Biotech). Apo J for the standard titration curve was purified according to Calero *et al.* (11); standards for apo E were purchased from Daichi Pure Chemicals (Tokyo, Japan).

The luminescent signal was acquired with a STORM 860 laser scanner (Amersham Pharmacia Biotech, Milan Italy) using 420 nm and 460 nm as excitation and emission waves, respectively.

Apo E Genotyping

Apolipoprotein E genotypes were determined in all patients by multiplex amplification refractory mutation system PCR (ARMS-PCR) as described by Donohoe *et al.* (12)

Statistical Analyses

Data are presented as mean \pm SD. One-way ANOVA was used to test differences in apolipoproteins levels.

Results

Molecular and Clinical Characterization of NPHS2 Patients

The molecular features of the five enrolled patients are reported in Table 1, which also reports the age of onset of

proteinuria, the original response to the treatment used at the onset of proteinuria, and the outcome. Four children had developed end-stage renal failure by the mean age of 10 (range, 4 to 17) yr and had received a renal allograft within 2 yr of the beginning of a dialytic program (see below).

P_{alb} and Posttransplant Outcome

Pretransplant P_{alb} in the five patients who had their serum tested is reported in Table 3. P_{alb} was elevated in all cases (mean, 0.81; range, 0.73 to 0.88), resembling the typical values of idiopathic FSGS (2,3). The posttransplant outcome was good in two cases (patients 2 and 3), whereas two children presented a recurrence of proteinuria after 10 d (patient 4) and 300 d (patient 5), respectively. Both patients had been treated with plasmapheresis (six to ten treatments) and with cyclophosphamide (2 mg/kg for 60 d) with prompt response. Figure 1 shows the outcome of proteinuria in one patient (patient 4) and its relationship with P_{alb} evaluated during the follow-up. Post-plasmapheresis P_{alb} was high but steadily decreased after four plasmapheresis sessions to reach normal values after six treatments; at which time proteinuria was reduced to normal levels.

Patients with Idiopathic Nephrotic Syndrome

A cohort of 31 patients with idiopathic nephrotic syndrome and for whom podocin sequence excluded NPHS2 were en-

Table 3. Prerenal transplant clinical and laboratory data in five children with FSGS and podocin mutations

Patients	P _{alb}			Apo J		Apo E	
	Serum Alone	Serum + Urine	Serum + Normal Urine	Serum (μg/ml)	Urine (μg/ml)	Serum (μg/ml)	Urine (μg/ml)
NPHS2							
1	0.77 ± 0.01	0.1 ± 0.03	0.75 ± 0.02	0.21	0.001	0.10	0.0001
2	0.88 ± 0.04	0.1 ± 0.01	0.88 ± 0.01	0.18	0.0005	0.20	0.00012
3	0.87 ± 0.08			0.20	0.0007	0.15	0.0002
4	0.73 ± 0.02			0.20	0.0012	0.14	0.00018
5	0.80 ± 0.04	0.11 ± 0.02	0.79 ± 0.02	0.15	0.001	0.18	0.0001
Mean ± SD	0.81 ± 0.06	0.07 ± 0.06	0.80 ± 0.07	0.19 ± 0.02 ^b	0.0009 ± 0.0002	0.17 ± 0.03 ^b	0.0001 ± 0.00004
Controls (20)	<0.2	<0.2	<0.2	0.35 ± 0.07	nd	0.08 ± 0.02 ^a	0.0015 ± 0.0001

^a nd, not detectable.

^b P < 0.01 versus normal controls.

rolled for comparison of P_{alb}. They consisted of patients with good steroid responsiveness, corticoid dependence, or corticoid resistance, in which most presented a pathologic picture of FSGS. Proteinuria was variable in all subgroups, depending on the class and the time of enrollment, and this allowed an analysis of possible correlation with P_{alb}. As shown in Table 2, P_{alb} was also variable in these patients, with more than 50% of patients with a good response to steroids presenting P_{alb} >0.5 and patients of the steroid-resistant group with a P_{alb} <0.5. Figure 2 shows the lack of correlation between P_{alb} and proteinuria.

P_{alb} Inhibitors

As shown in Table 3, when the sera from three patients of our cohort (patients 1, 2, and 5) were pre-incubated with the homologous nephrotic urine, P_{alb} values decreased to 0.1 showing neutralization of the activity. Coincubation of serum with normal urine did not modify the original high P_{alb}. After this observation we looked at inhibitors of permeability activity (8) in serum and urine and determined levels of apo J and apo E that are two molecules recognized to display strong inhibitory activity *in vitro*. As shown in Table 3, the serum levels of apo J were 50% lower than in controls (0.19 ± 0.02 versus 0.35 ± 0.07, P < 0.01), and the urinary levels were negligible. Serum levels of apo E were increased in nephrotic patients compared with normal controls, whereas apo E levels very low in urine. The genotype of apo E was in all cases E3/E3 encoding apo E₃, the most frequent genotype (13).

Discussion

Podocin is a structural protein of podocytes that interacts with other podocyte components to form the glomerular barrier to filtration of proteins. Homozygous mutations of the podocin gene are considered causative of proteinuria in a recessive condition known as NPHS2 (9). Familial kindreds of NPHS2 and sporadic cases have been reported (9,10), representing the most frequent genetic cause of nephrotic syndrome in children (20 to 30% of cases with FSGS). The pathologic result of NPHS2 is focal segmental glomerulosclerosis, a picture indistinguishable from idiopathic FSGS, which also presents the same clinical features of intractable proteinuria and evolution toward renal failure. Idiopathic FSGS is also characterized by recurrence after renal transplant in 30% of cases, with often rapid onset of proteinuria after the renal graft and good response to plasmapheresis (14–16). The post-transplant recurrence of FSGS and the resolution upon removal of plasma substances by plasmapheresis led to the hypothesis that a circulating factor was causing recurrent proteinuria in such a condition. Savin *et al.* (2) developed an *in vitro* bioassay that evaluates the volume changes of isolated rat glomeruli upon exposure to FSGS serum and gives an indirect measure of permeability activity (P_{alb}) in which P_{alb} of 1 indicates maximal effects. Following its description, high P_{alb} was associated with high risk of recurrence: practically 100% with values greater than 0.9. In our experience, a P_{alb} >0.6 in children is highly predictive of recurrence with an odds ratio of 10.

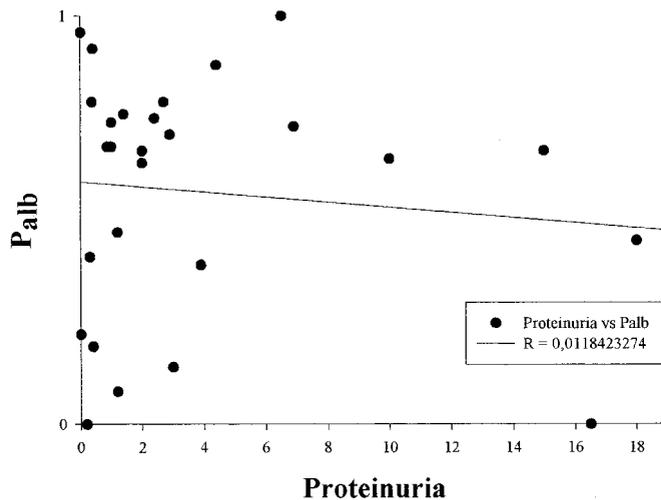


Figure 2. Lack of correlations between P_{alb} in 31 patients with idiopathic nephrotic syndrome.

Our initial interest in measuring P_{alb} in patients with NPHS2 was that, as already stated, proteinuria is likely to be induced by the genetic defect in these patients, and high P_{alb} values should not have a pathogenetic role. In fact, the high P_{alb} values observed in the five patients studied cast doubts on the overall specificity of the test. We also found that P_{alb} evaluated in patients with idiopathic nephrotic syndrome did not correlate with the degree of steroid responsiveness. Other data from the literature (17) indicate that P_{alb} may also be high in patients with membranous glomerulonephritis, suggesting that the nephrotic milieu may influence the bioassay to some extent. However, recurrence of proteinuria after renal transplantation, which is the prime evidence supporting a role of humoral factors in the genesis of FSGS, occurred in two of four patients of our small sample. Proteinuria in these cases was observed a few to several days after a period of normal urinalysis and responded to a brief cycle of plasmapheresis. Although the prompt response precluded renal biopsy, for all the features above, these were considered effective recurrence episodes that could not be explained on the basis of the genetic defect. On the other hand, the rapid onset of proteinuria excluded, at least in one case, an implication of anti-podocin antibodies, and a rough estimate with indirect immunofluorescence of their presence was negative (Ghiggeri, personal observation). In one patient who presented recurrence of proteinuria 10 d after transplantation, we performed a careful day by day evaluation of P_{alb} and were able to show a strict correlation between plasma activity and proteinuria. Accordingly, P_{alb} decreased progressively after plasmapheresis and was paralleled by normalization of proteinuria. It seems reasonable, therefore, to propose a relationship between P_{alb} and posttransplant recurrence in this case and possibly to extend this concept to the pretransplant condition. Given the possibility of recurrence after transplantation in patients with molecular defects of the filtration barrier, it is clear that we need to improve our knowledge of the mechanisms of glomerular protein perme-

ability before attempting an explanation of proteinuria in NPHS2, and we must be cautious in extending the same concept in the pathogenesis of proteinuria in idiopathic nephrotic syndrome. We have characterized a few proteins in serum of FSGS patients that demonstrate permeability activity *in vitro* after their purification (18), and the characterization of other candidates is in progress. A clear differentiation between FSGS and normal serum is essential to substantiate their role in proteinuria; in this light, NPHS2 must also be carefully studied.

We (8) and other investigators (7) have recently demonstrated that permeability activity may be due to a balance between plasma factors that enhance and others that inhibit permeability. Five normal constituents of normal serum that belong to the HDL lipoprotein complex—apo AIV, apo L, apo E2, apo E4, and apo J—were shown to inhibit permeability induced by FSGS serum *in vitro* (8). Accordingly, normal serum coincubated with FSGS serum inhibits permeability activity; however, when the same protective serum was depleted of apo J and apo E by specific antibodies, it failed to exert protection, demonstrating the pivotal role of these components. In the present study, we looked at inhibitors in two ways: (1) coincubating serum from the three NPHS2 patients from whom urine was collected at the time of proteinuria and (2) determining the serum and urinary levels of apo J and apo E and characterizing the genotype of apo E, because the *in vitro* studies showed apo E3 not to be protective. The results demonstrated that homologous urine neutralized the original permeability plasma activity, but the urinary levels of both apo J and apo E were too low to imply a role in the protective mechanism. Other inhibitors present in nephrotic urine exert an inhibitory effect, and their characterization is now in progress.

In summary, we found abnormally high permeability plasma activity in NPHS2 patients, a feature more directly associated with idiopathic FSGS. As in FSGS, proteinuria recurred in two children with NPHS2 who had been transplanted and promptly responded to a short treatment with plasmapheresis. P_{alb} steadily decreased after plasmapheresis and normalized after 30 d. Finally, loss of inhibitors in urine is a determinant of high P_{alb} , but the exact mechanism and the substances are still to be characterized. Whether proteinuria in general is linked with the urinary loss of inhibitors is currently unclear, but lack of correlation between P_{alb} and proteinuria would suggest a selective loss.

Acknowledgments

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