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Recurrence of Nephrotic Syndrome after Transplantation in CNF Is due to Autoantibodies to Nephrin

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Key Words

Nephrin · Congenital nephrotic syndrome, Finnish type · Nephrotic syndrome, recurrence

Abstract

The novel gene NPHS1 is defective in the patients with congenital nephrotic syndrome of the Finnish type (CNF) leading to abnormal expression of the respective protein product nephrin in glomerular cells. CNF patients are treated with early nephrectomy and renal transplantation, but about 20% show recurrence of nephrotic syndrome (NS). We used indirect immunofluorescence microscopy and immunoblotting and an ELISA assay to search for circulating autoantibodies to nephrin, the protein defect in CNF patient kidneys. In serial serum samples gathered before and after recurrence of NS, we show an increased antibody titer to nephrin prior to the NS episode and a subsequent drop in antibody level after its successful treatment and reactivity of the high titer sera with glomeruli in indirect immunofluorescence microscopy as well. The results show that the transplantation treatment introduces a neoantigen inducing production of autoantibodies, which may be pathogenic for perturbation of the function of the glomerular filtration barrier.

Introduction

Congenital nephrotic syndromes (CNS) are pediatric kidney diseases presenting with massive proteinuria at or soon after birth [1, 2]. One of the best characterized CNSs includes the congenital nephrotic syndrome of the Finnish type (CNF) with early onset treatment-resistant proteinuria but no symptoms from other tissues. The current treatment of CNF with nephrectomy and final renal transplantation appears to cure all symptoms [3]. However, we have earlier reported that about 20% of CNF patients with this treatment will develop recurrence of the nephrotic syndrome (NS) [4]. After successful treatment, a repeated phase of nephrosis has been found in some patients.

Kestilä et al. [5] recently identified the causative gene NPHS1 of CNF, whose putative protein product, nephrin, is a transmembrane protein with eight immunoglobulin-like domains. In situ hybridization and Northern blot results have pointed that nephrin gene is expressed exclusively in podocytes [6] and our results have further shown that nephrin protein appears early during glomerulogenesis [7]. Nephrin is found preferentially in the filtration slit area of the podocytes [6–8]. The results of Kestilä et al. [5] have indicated that nephrin gene is not expressed in other tissues.

Here we used nephrin-specific peptides in an ELISA assay to search for putative autoantibodies to the possible

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1 MALGTTLRAS LLLLGLLTEG LAQLAIPA .....

1029 TQ LPITTPGL**HQ PSGEPEDQLP TEPPSG**PS

1059 GL PLLPVLFALG GLLLLSNASC VGGVLW .....

1101 **TEAGSEEDRV RNEYEESQWT GERDTQ** .....

1131 STTEAEPYYR SLRDFSPQLP PTQEEVSYSR .....

1221 AGDLDTLEPD SLPFELRGHL V

Fig. 1. The amino acid sequence of nephrin. The nephrin-specific areas selected are given in bold. The underlined domain shows the transmembrane area.

new antigens introduced with the transplantation treatment. The high level of circulating antinephrin antibodies most likely explained the transient episode of NS in these patients.

Patients and Methods

CNF Patients

Diagnosis of CNF (n = 8) was made on the typical clinical features at birth (placental weight > 40% of the weight of the newborn, edema, massive proteinuria), exclusion of other types of congenital nephroses and by the typical renal pathology at nephrectomy [2, 3, 9]. The patients were treated by nephrectomy, temporary dialysis and final renal transplantation according to an established treatment protocol [3]. Five of the patients developed recurrence of NS during the follow-up period; serial serum samples from the patients were collected at the repeated hospital visit during the disease course. All procedures were approved by the Ethical Committee of the Helsinki University Central Hospital.

Indirect Immunofluorescence Microscopy

The presence of autoantibodies in the patient sera was screened on normal human kidney tissue by indirect immunofluorescence microscopy. For this purpose, normal human kidney was obtained from nephrectomies due to renal malignancy at the opposite pole of the kidney as previously described [9, 10]. Frozen kidney sections were cut at 4 µm and incubated with the patient sera (30 min at +20°C) and, after rinsing thoroughly, stained for fluorescein isothiocyanate (FITC)-labeled rabbit anti-human IgG (Dako, Glostrup, Denmark). A fluorescence microscope equipped with appropriate filters was used for microscopy (Olympus, BX 50, Japan).

Immunoblotting

For immunoblotting, human kidney glomeruli were solubilized in RIPA buffer (150 m*M* NaCl, 1% NP-40, 0.5% sodium deoxylate, 0.1% SDS, 50 m*M* Tris, pH 7.6) including proteinase inhibitors (10 m*M* pH 8.0 EDTA, antipain 4.5 µg/ml, pepstatin 4.5 µg/ml, 10 m*M* PMSF), boiled for 10 min, briefly sonicated, centrifuged 10,000 *g* 10 min at +4°C. The supernatants were collected, incubated on ice 10 min and centrifuged again as above. The supernatants were run in reducing Laemmli buffer (62.5 m*M* pH 6.8 Tris-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue) through 8% polyacrylamide gels in Protean Mini-gel electrophoresis system (Bio-Rad Laboratories, Richmond, Calif., USA). After electrophoresis, proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) by using Novablot semidry blotting system (LKB, Bromma, Sweden). After blocking with 5% fat-free milk powder in PBS, one filter was incubated overnight at +4°C with rabbit antinephrin antibodies (4 µg/ml) raised against nephrin-specific domain (see below). As a control the other filter was incubated with an irrelevant rabbit IgG (4 µg/ml). The filters were washed with 5% fat-free milk powder in PBS-0.1% Tween 20 twice 15 min, further incubated with swine anti-rabbit immunoglobulins conjugated with HRP (1:2,000) (Dako, Glostrup, Denmark). After thorough washing with PBS, the bound antibodies were detected with the ECL™ blotting kit (10; Amersham Life-Science, Amersham International, Bucks., UK) according to the manufacturer's instructions.

Design of Synthetic Peptides

Sequence specificity for intracellular (aa 1101–1126) and extracellular (aa 1039–1056) oligopeptides (fig. 1) was selected over the human nephrin sequence (Gene bank accession No. AF035835) using the PredictProtein program via Internet at European Molecular Biology Laboratory (Heidelberg, Germany). These peptides showed no homology to other known protein sequences and were synthesized and purified at a local peptide synthesis unit (Haartman Institute, University of Helsinki) and coupled to a high-density multiple antigenic peptide-polylysine matrix [11] to raise polyclonal antibodies [7].

Measurement of Antibodies

For measurement of antinephrin antibodies, an ELISA analysis for the circulating intracellular and extracellular antibodies was performed. For this purpose, 100 ml of the respective intracellular (PAM 243) or extracellular (PAM 376) peptide (10 mg/ml, in 0.1 *M* $NaHCO_3$ in 150 m*M* NaCl, pH 8.8 at +20°C) was first bound to the 96-well microtiter plates (DNA-Bind, Corning Costar Corp., Mass., USA) for 2 h. The optimal concentration (1 mg/ml) of peptide was selected after testing 100, 10 and 1 mg/ml respectively for coating. After thorough washing in PBS, 2% bovine serum albumin (BSA, Fraction V, Boehringer Mannheim, Mannheim, Germany, 150 ml/well) in PBS was used for blocking overnight at +4°C. After thorough washing, 1:50 and 1:200 dilutions of patient sera (in 10% fetal calf serum (FCS)-PBS, 100 ml/well) were incubated for 2 h. After washing, peroxidase-conjugated swine anti-rabbit IgG (Dako; 1:2,000 in 10% FCS-PBS) for 1 h followed by 0.1 *M* citrate buffer (pH 5.0) containing *o*-phenylenediamine (OPD, 0.4 mg/ml) in 0.04% H_2O_2 and absorbance measured at 450 nm with an ELISA reader (Labsystems, Helsinki, Finland). For controls, a pool from 50 normal sera of consecutive healthy blood donors was used.

Results

Detailed characteristics of CNF patients with recurrence of NS including HLA matching, cytomegalovirus and Epstein-Barr virus infection history, clinical course before and after recurrence of nephrotic syndrome, histo-

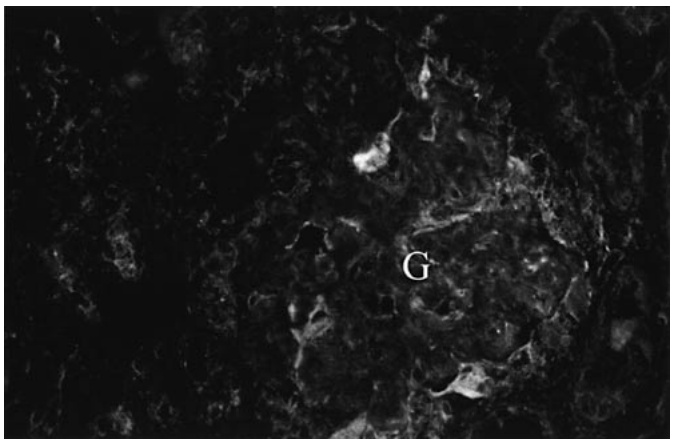

Fig. 2. Section of normal human kidney stained with high titer patient serum shows reactivity in glomeruli. G = glomerulus. × 280.

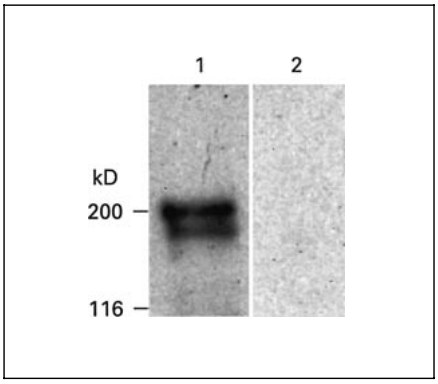

Fig. 3. Immunoblotting with anti-intracellular nephrin antibodies of human glomerular lysate showing a double band at the 200-kDa area.

logic analysis, description of the clinical pre- and post-transplantation and nephrosis treatments as well as analysis of the kidney biopsies have been reported earlier in detail [4]. Briefly, there was no overrepresentation in male/female ratio, donor source, acute rejection or septic infections or significant HLA-A and -B mismatches in the NS recurrence patients, and blood cyclosporine concentration was within target limit. Serum creatinine concentration had increased slightly since the previous hospital visit. Serum albumin and protein concentrations were characteristically low and all patients had an elevated proteinuria level (table 1; for details, see Laine et al. [4]). The complete laboratory findings of urine and serum of the patients with recurrence of NS have been reported earlier [4].

In indirect immunofluorescence microscopy, a faint, patchy glomerular reactivity of the patients with high titer antibodies was seen on sections of normal human kidney (fig. 2).

Optimization of the ELISA assay was achieved by using different concentrations of the coating peptide and by preincubation of the patient sera with the competitive oligopeptide, respectively. Also the controls of the second and irrelevant antibody reactivities with or without coating peptide were negative. Optimization of the ELISA also included the reactivity with antibodies to synthetic nephrin-specific sequences. Elevated antibody titers for nephrin (OD values > 0.3) were found in 4 out of 8 CNF patients (fig. 4), while sera from 1 CNF patient with recurrence of NS failed to show such an elevation. After successful treatment of the nephrosis episode with steroids, cyclophosphamide and cyclosporin (for the regimen, see Laine et al. [4]), the antibody titers of the individual patients decreased within 1–3 months for both the intracellular and extracellular antibodies.

Table 1. Clinical characteristics of the CNF patients studied

	CNF patients No.				
	1	2	3	4	5
Sex	M	M	M	M	M
Age at Tx, years	1.3	7.3	2.3	2.7	1.8
At the onset of recurrence					
Time from Tx, months	7	14	5	1	33
S_{cr}, μmol/l	92	70	59	105	178
S_{alb}, g/l	17	23	21	23	18
U_{prot}, g/l	17	6	46	59	3

Tx = Transplantation; S_{cr} = serum creatinine concentration; S_{alb} = serum albumin concentration; U_{prot} = urine protein concentration.

Discussion

Here we show that recurrence of NS of CNF patients treated with renal transplantation according to the established treatment protocol [3] associates with preceding elevation of specific antibodies to nephrin as measured by ELISA method. ELISA analysis of antibodies for both intracellular and extracellular nephrin-specific domains

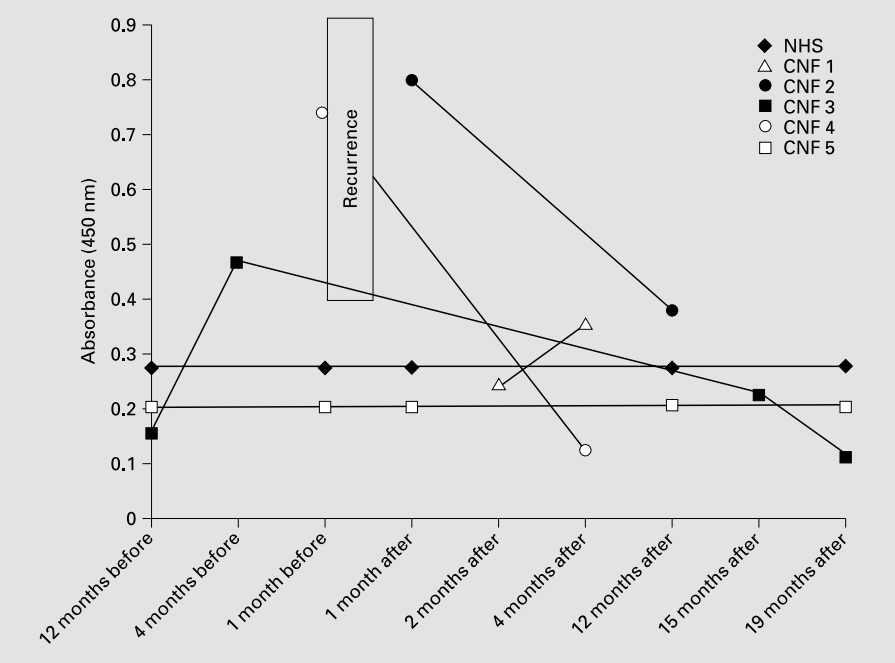

Fig. 4. Five CNF patient sera before and after recurrence of the NS together with normal human sera (NHS). In patient 3, the antinephrin antibodies peaked 4 months before recurrence but thereafter dropped quickly and 15 months later were close to the level of normal human serum.

showed similar results. The successful treatment of the recurrence of NS and subsequent maintenance of an effective antirejection therapy resulted in decrease of the antibody level within 3–6 months. This decrease follows a normal half-life kinetics of circulating antibodies.

The characteristics massive proteinuria of the CNF has been considered a unique human single gene model disease of the perturbed glomerular function [12–14]. Thus, the recent identification of the NPHS1 gene [5] responsible for the CNF phenotype has suggested that this gene and the respective protein product nephrin play a key role in the maintenance of the glomerular filtration barrier. Kestilä et al. [5] showed that NPHS1 encodes a putative transmembrane protein with sequence similarity to cell adhesion molecules of the immunoglobulin superfamily and with expression in the kidney glomeruli, most likely in podocytes but not in other tissues. Nephrin preferentially associates with the filtration slit area in normal kidney [6, 7]. The podocyte is located beyond the endothelial cell and glomerular basement membrane layer in a seemingly immunoprotected area. However, results from the widely used Heymann nephritis model of membranous glomerulonephritis show that immunoglobulins rapidly get access beyond the glomerular basement membrane to reaching their target epitopes on podocytes and form local deposits [15]. It is interesting that circulating antibodies can reach epitopes at this site and that sufficient antibodies in general are formed in patients with immunosuppressive therapy as in CNF patients after kidney transplantation. Basically the same pattern can be found in Alport syndrome patients but with antibodies developing against collagen IV of the glomerular basement membranes [16]. Thus CNF appears as the first human disease in which functionally important circulating antibodies develop against podocyte epitopes. Prominently, the patients with recurrence of the NS regularly presented with a prior infection with Epstein-Barr or cytomegalovirus before the new nephrosis episode [4]. Whether this infection was the trigger by inducing a major generalized immunoactivation can only be speculated. Our results have shown a major splicing variant of human nephrin lacking an exon spanning the transmembrane domain [7]. This most likely results in a secreted soluble form participating in local regulation of the transmembrane protein. Such a mechanism is well known for other membrane proteins including the interleukin-6 receptor [17], angiotensin II receptor [18] and T-cell receptor [19]. Thus, a secreted nephrin variant could also participate in the antibody production. The regulation of the secreted form is currently being studied in detail.

Two major NPHS1 gene defects responsible for CNF in the Finnish population have been reported [20]. The

Fin_{major} causes an early stop codon in exon 2 resulting in a total lack of protein, while Fin_{minor} results in a premature stop codon at the terminus of the intracellular domain in exon 28. Most obviously our patients developing recurrence do not express nephrin at all on their podocytes. In confirmation of this, our mRNA expression data show that the patients with recurrence are not the Fin_{minor} phenotype [7].

Acknowledgments

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References

1 Rapola J: Congenital nephrotic syndrome. Pediatr Nephrol 1987;1:441–446.
2 Hallman N, Norio R, Kouvalainen K: Main features of the congenital nephrotic syndrome. Acta Pediatr Fenn 1967;172:75–78.
3 Holmberg C, Antikainen M, Rönnholm K, Ala-Houhala M, Jalanko H: Management of congenital nephrotic syndrome of the Finnish type. Pediatr Nephrol 1995;9:87–93.
4 Laine J, Jalanko H, Holthöfer H, Krogerus L, Rapola J, von Willebrandt E, Lautenschlager I, Salmela K, Holmberg C: Posttransplantation nephrosis in congenital nephrotic syndrome of the Finnish type. Kidney Int 1993;44:867–874.
5 Kestilä M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan CE, Peltonen L, Holmberg C, Olsen A, Tryggvason K: Positionally cloned gene for a novel glomerular protein – nephrin – is mutated in congenital nephrotic syndrome. Mol Cell 1998;1:575–582.
6 Holzman LB, St Johns PL, Kovari IA, Verma R, Holthöfer H, Abrahamson DR: Nephrin localizes to the slit pore of the glomerular epithelial cell. Kidney Int 1999;56:1481–1491.
7 Holthöfer H, Ahola H, Solin ML, Wang SX, Palmén T, Luimula P, Miettinen A, Kerjaschki D: Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. Am J Pathol 1999;155:1681–1687.
8 Ruotsalainen V, Ljungsberg P, Wartiovaara J, Lenkkeri U, Kestilä M, Jalanko H, Holmberg C, Tryggvason K: Nephrin is specifically located at the slit diaphragm of glomerular podocytes. Proc Natl Acad Sci USA 1999;96:7962–7967.
9 Haltia A, Solin ML, Jalanko H, Holmberg C, Miettinen A, Holthöfer H: Mechanisms of proteinuria: Vascular permeability factor in congenital nephrotic syndrome of the Finnish type. Pediatr Res 1996;40:652–657.
10 Holthöfer H, Reivinen J, Miettinen A: Decrease of glomerular disialogangliosides in puromycin nephrosis of the rat. Am J Pathol 1996;149:1009–1015.
11 Tam JP: Synthetic peptide vaccine design, synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci USA 1988;85:5409–5413.
12 Ahola H, Wang SX, Luimula P, Solin ML, Holzman LB, Holthöfer H: Cloning and expression of the rat nephrin homologue. Am J Pathol 1999;155:907–913.
13 Kanwar YS, Liu ZZ, Kashihara N, Wallner EI: Current status of the structural and functional basis of glomerular filtration and proteinuria. Semin Nephrol 1991;11:390–413.
14 Kerjaschki D: Dysfunctions of cell biological mechanisms of visceral epithelial cells (podocytes) in glomerular diseases. Kidney Int 1994;45:300–313.
15 Kerjaschki D, Miettinen A, Farquhar MG: Initial events in the formation of immune deposits in passive Heymann nephritis. gp330-anti-gp330 immune complexes form in epithelial coated pits and rapidly become attached to the glomerular basement membrane. J Exp Med 1987;166:109–128.
16 Brainwood D, Kashtan C, Gubler MC, Turner AN: Targets of alloantibodies in Alport antiglomerular basement membrane disease after renal transplantation. Kidney Int 1998;53:762–766.
17 Säily M, Koistinen P, Pulkki K, Zheng A, Savolainen ER: Acute myoblastic leukemia cells produce soluble interleukin-6 receptor by mechanism of alternative splicing. Cytokine 1998;10:860–867.
18 Sugimura K, Tian XI, Hoffmen S, Ganten D, Bader M: Alternative splicing of mRNA coding for the human endothelial angiotensin-converting enzyme: A new mechanism for solubilization. Biochem Biophys Res Commun 1998;247:466–472.
19 Takase K, Okazaki Y, Wakizaka K, Shevchenko A, Mann M, Saito T: Molecular cloning of pTAC12: An alternative splicing product of the CD3gamma chain as a component of the pre-T cell antigen receptor complex. J Biol Chem 1998;273:30675–30679.
20 Lenkkeri U, Männikkö M, McCready P, Lamerdin J, Gribouval O, Niaudet P, Antignac C, Kashtan C, Olsen A, Kestilä M, Tryggvason K: Structure of the gene for congenital nephrotic syndrome of the Finnish type (NPHS1) and characterization of mutations. Am J Hum Genet 1999;64:51–61.