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In situ evaluation of podocin in normal and glomerular diseases

IZUMI HORINOUCHI, HITOSHI NAKAZATO, TOMOYASU KAWANO, KEN-ICHI IYAMA, AKIO FURUSE, KENJI ARIZONO, JIRO MACHIDA, TAMAMI SAKAMOTO, FUMIO ENDO, and SHINZABURO HATTORI

Department of Pediatrics and Department of Surgical Pathology, Kumamoto University School of Medicine, Kumamoto, Japan; Department of Pediatrics and Department of Nephrology, Kumamoto Central Hospital, Kumamoto, Japan; Department of Urology, Kumamoto Saiseikai Hospital, Kumamoto, Japan; Trans Genic, Inc., Kumamoto Immunochemical Laboratory, Kumamoto, Japan; and Department of Pediatrics, Kumamoto University College of Medical Science, Kumamoto, Japan

In situ evaluation of podocin in normal and glomerular diseases.

Background. Mutations of the NPHS2 gene are responsible for autosomal-recessive steroid-resistant nephrotic syndrome. Its product, podocin, faces the slit diaphragm area with its two ends in the cytoplasm of foot processes.

Methods. We generated rabbit polyclonal antibodies against conjugated peptides from human podocin N- and C-termini, and studied podocin and synaptopodin using kidney tissues of normal humans and those with glomerular diseases.

Results. Antipodocin antibodies detected the original 42 kD fragment and an extra smaller fragment by Western blot analysis using human isolated mature glomeruli. RNA analysis showed two bands, the original and the other of a decreased length. Immunohistochemically, podocin was detected in a linear pattern along the glomerular capillary loop. Antipodocin antibody (C-terminal) stained the smooth muscles of renal arterioles and aorta. Among 42 patients, podocin was normally expressed in glomeruli in purpura nephritis, IgA nephropathy (IgAN), and minimal-change disease (MCD), while it was either decreased or absent in most subjects with focal segmental glomerulosclerosis (FSGS). The expression of synaptopodin was similar to that of podocin, although some discrepancy existed.

Conclusion. Although indirect, our data suggest the existence of a vascular isoform of podocin with a different molecular mass. We propose that examination of podocin expression may help differentiate MCD from FSGS.

Plasma ultrafiltration during primary urine formation in the glomerulus is a central function of the kidney. The glomerular barrier is comprised of a fenestrated endothelium, glomerular basement membrane (GBM), and highly specialized visceral epithelial podocytes, the foot processes that cover the outer surface of the GBM. Dysfunction of the glomerular filter causes proteinuria and nephrotic syndrome ensues.

Key words: podocin, NPHS2, focal segmental glomerulosclerosis, synaptopodin, nephrotic syndrome.

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Several genes expressed by glomerular podocytes have been identified [1–3]. Nephrin, a major component of the slit diaphragm, is a transmembrane protein encoded by the *NPHS1* gene, which is mutated in congenital nephrotic syndrome of the Finnish type [1]. Glomerular expression of nephrin has been noted in kidney diseases in humans [4, 5]. Targeted deletion of CD2-associated protein (CD2AP) in mice developed nephrotic syndrome [6]. The *NPHS2* gene encodes podocin, another podocyte protein [2]. Mutations of *NPHS2* are associated with autosomal-recessive steroid-resistant nephrotic syndrome, which is characterized by early childhood onset of proteinuria, focal segmental glomerulosclerosis (FSGS), and rapid progression to end-stage renal disease (ESRD). Podocin is structurally similar to stomatins and is predicted to be an integral membrane protein of 383 amino acids. It has a single membrane domain forming a hairpin-like structure and with both N- and C-terminal domains in the cytosol [2, 7]. Podocin supposedly interacts with nephrin and CD2AP [8, 9]. This complex is a functional unit that anchors the slit diaphragm to the actin cytoskeleton and establishes its location in the lateral plasma membrane of the podocyte foot processes. It plays an important role in the molecular structure of the slit diaphragm and filtration barrier.

Synaptopodin is an actin-associated protein of podocyte and dendric spines in a subpopulation of exclusively telencephalic synapses. Synaptopodin was found to be associated with the actin microfilaments of podocyte foot processes and may be associated with remodeling of cell processes in the kidney [10].

Nephrotic syndrome can be caused by several glomerular diseases. Podocin is expressed in normal glomerular podocytes [7], but its expression in glomerular diseases is unknown. We generated rabbit antipodocin antibodies and studied the expression of podocin and synaptopodin in kidney tissues from normal subjects and those with glomerular diseases.

Table 1. Samples included in this study and clinical parameters at the time of biopsy

Diagnosis	Samples number					Urinary protein g/day			Hematuria[a]	Serum creatinine		Immunosuppressive therapy
	Total	Age years		Gender								
		<20	>20	Male	Female	<0.3	0.3–1.0	>1.0		Normal	Elevated	
HSPN	6	6	0	3	3	1	1	4	6	6	0	4
IgAN	6	6	0	4	2	2	3	1	5	5	1	0
MCD	6	3	3	3	3	1	1	4	0	6	0	4
MN	5	0	5	3	2	0	0	5	5	5	0	0
FSGS	19	4	15	11	8	0	1	18	2	12	7	6
Total	42	19	23	24	18	4	6	32	18	34	8	14

Abbreviations are: HSPN, Henoch-Schönlein purpura nephritis; IgAN, IgA nephropathy; MCD, minimal-change disease; MN, membranous nephropathy; FSGS, focal segmental glomerulosclerosis.
[a]More than five erythrocytes per high-power field.

METHODS

Patients

This study included 42 Japanese patients treated in the Department of Pediatrics and Third Department of Internal Medicine, Kumamoto University School of Medicine, and Department of Pediatrics and Department of Nephrology, Kumamoto Central Hospital (24 men and 18 women; 19 patients <20 years of age). Glomerular diseases included Henoch-Schönlein purpura nephritis (HSPN) ($N = 6$), IgA nephropathy (IgAN) ($N = 6$), minimal-change disease (MCD) ($N = 6$), membranous nephropathy (MN) ($N = 5$), and FSGS ($N = 19$) (Table 1). Patients with MN were in stage 1 ($N = 1$), stage 2 ($N = 3$), and stage 3 ($N = 1$). Renal tissue samples were obtained by percutaneous needle or by open biopsies. Diagnoses were established based on clinical symptoms, laboratory investigations, and kidney histopathology. Kidney biopsy specimens were examined under a light, an electron. and a fluorescent microscope. Data on proteinuria, hematuria, serum creatinine, and albumin were collected at the time of renal biopsy. Proteinuria was divided into three grades (<0.3 g/day, 0.3 to 1.0 g/day, and >1.0 g/day). Hematuria was defined as more than five erythrocytes per high-power field. Thirty-two patients had severe proteinuria (>1.0 g/day) and six had moderate proteinuria (0.3 to 1.0 g/day) at the time of biopsy. Eighteen patients were hematuric. Eight patients had elevated serum creatinine levels and 14 had been treated with steroids or other immunosuppressive therapy prior to renal biopsy.

We obtained informed consent from patients or their family members for analyzing podocin expression.

Antipeptide antibody

Oligopeptides (MERRARSSSRESRGR) corresponding to amino acid residues 1-15 (N-terminal) and (SKPVEPLNPKKKDS) to amino acid residues 367-380 (C-terminal) of human podocin were synthesized [2]. Neither N- nor C-terminal fragments of podocin had any homology with known protein sequences [2]. The peptides were conjugated with keyhole limpet hemocyanin (KLH). Rabbits were immunized with conjugated peptides four times every 2 weeks. For Western blot and immunohistochemical analyses, antisera were purified on immunoaffinity columns coupled with peptides used for immunization.

Western blot analysis

Normal adult human kidney samples were obtained from patients with renal cancer who underwent nephrectomy. Glomeruli were isolated by sieving, as described elsewhere [11]. Proteins were extracted from isolated glomeruli into lysis buffer [0.05 mol/L sodium phosphate/0.15 mol/L NaCl/1% Triton X-100/0.1 mmol/L phenylmethylsulphate/10 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.5]. After vortexing, protein samples were incubated on ice for 30 minutes, then centrifuged to clear the supernatant. The lysates were diluted in Laemmli sample buffer in the presence or absence of 5% 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred electrophoretically onto a polyvinylidene difluoride transfer membrane (Millipore, Bedford, MA, USA). The membrane was treated with 5% dry fat milk for blocking, and then treated with diluted antipodocin antisera (1:100) or normal rabbit IgG (1:100). Primary antibodies were detected using horseradish peroxidase-conjugated antirabbit IgG (Rockland, Gilbertsville, PA, USA) as the secondary antibody. Signals were detected using a chemiluminescent reagent ECL Plus System (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RNA analysis

Total RNA was extracted from normal adult human whole kidney using an acid guanidinium thiocyanate/phenol/chloroform extraction procedure [12]. First-strand cDNA was generated from total RNA and was reverse transcribed using an RNA polymerase chain reaction (PCR) kit (Takara, Otsu, Japan). The *NPHS2*

gene has eight exons. cDNA was amplified with an LA PCR kit (Takara) with NPHS2 primers 1A and 8B. Sequences of oligonucleotide primers were as follows [2]: 1A, 5′-GCAGCGACTCCACAGGGACT-3′ (exon 1, sense); 8B, 5′-TTCTATGGCAGGCCCCTTTA-3′ (exon 8, antisense). The amplified DNAs were electrophoresed on a 1.5% agarose gel.

Immunohistochemistry in glomeruli

Immunohistochemical analysis was done for human normal mature kidney and 42 patients' kidneys. For immunostaining, serial sections (4 μm) were fixed in acetone for 10 minutes at room temperature then incubated in 5% goat serum for 30 minutes at room temperature to block nonspecific binding. The sections were then incubated overnight at 4°C with affinity-purified antipodocin antibody (Podo-N) (1:100), or antisynaptopodin antibody (1:100). We used a mouse monoclonal antisynaptopodin antibody (Progen, Heidelberg, Germany). Thereafter, primary antibodies were detected using fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH, USA) or FITC-conjugated rabbit antimouse IgG (MBL) as secondary antibodies, respectively. Sections were examined under a fluorescent microscope. The expression of podocin was recorded by two independent observers using an arbitrary scale of (−), negative; (+), faintly positive; (2+), positive; and (3+), strongly positive.

To check the condition of normal kidney samples, we repeatedly froze and thawed samples ten times. Podocin was normally detected after ten times of freezing and thawing, while signals of synaptopodin decreased when the repetition exceeded five times. Therefore, we used samples subjected to less than five times of freezing and thawing.

For dual fluorochrome labeling, sample slides were simultaneously incubated overnight at 4°C with rabbit antipodocin antibody, mouse antisynaptopodin or rat anti-α5 chain of type IV collagen [α5(IV) chain] antibody. After washing with phosphate-buffered saline (PBS), the slides were simultaneously incubated with rhodamine-conjugated goat antirabbit IgG, FITC-conjugated rabbit antimouse IgG or FITC-conjugated goat antirat IgG, respectively, as secondary antibodies for 1 hour at room temperature. Sections were examined under fluorescent and confocal microscopes (Fluoview FV-500, Olympus, Tokyo, Japan).

To ascertain the presence of renal glomerular blood vessels in patients, we studied dual fluorochrome labeling of podocin and ZO-1, a component of cytoplasmic face of the slit diaphragm. Sample slides were stained as above with Podo-N antibody or mouse anti-ZO-1 antibody (Zymed, South San Francisco, CA, USA) as primary antibodies, and with FITC-conjugated goat antirabbit IgG or rhodamine-conjugated goat antimouse IgG, respectively, as secondary antibodies.

Immunohistochemistry in extraglomeruli

We studied dual fluorochrome labeling of smooth muscle actin (SMA) and podocin in human renal arterioles. Sample slides were stained as above with mouse anti-SMA antibody or Podo-C antibody as primary antibodies, and with FITC-conjugated rabbit antimouse IgG or rhodamine-conjugated goat antirabbit IgG, respectively, as secondary antibodies.

Immunohistochemical analysis was done for human normal mature kidney and aorta using serial paraffin sections to exclude the possibility of autoimmunofluorescence of the vascular elastic laminas. The sections were incubated with 0.1% trypsin at 37°C for 30 minutes, then incubated in 5% goat serum for 30 minutes at room temperature to block nonspecific binding. They were incubated overnight at 4°C with Podo-C antibody (1:10) as a primary antibody and peroxidase-conjugated goat antirabbit IgG (ICN Pharmaceuticals, Inc.) as a secondary antibody. Sections were stained with diaminobenzidine (DAB) substrate kit with nickel (Vector Laboratories, Inc., Burlingame, CA, USA), then examined under a light microscope.

RESULTS

Characterization of polyclonal antipodocin antibodies

Polyclonal rabbit antisera were raised against conjugated proteins derived from two regions of human podocin and then purified by immunoaffinity columns. Podo-N and Podo-C were N- and C-terminal antibodies, respectively. The Podo-N antibody did not react with a nonassociated peptide but was reactive with an oligopeptide of the N-terminal of podocin, and antibodies against the bridge region, including KLH, were removed by an immunoaffinity column (Fig. 1A). Likewise the Podo-C antibody reacted with an oligopeptide of the C-terminal of podocin (data not shown). The specificity of these antibodies was tested by Western blot analysis with protein derived from mature isolated glomeruli from humans (Fig. 1B). Both Podo-N and Podo-C antibodies recognized the original 42 kD band and the extra 32 kD band, while these bands were not detected by normal rabbit IgG (Fig. 1B). This result was confirmed by other independent Western blots using five normal controls. The 42 kD fragment is estimated to be intact podocin [7].

RNA analysis

Reverse transcription (RT)-PCR using total RNA from normal adult human kidney with primers 1A and 8B showed an original length (1275 bp) and another decreased length of approximately 200 bp in NPHS2 mRNA

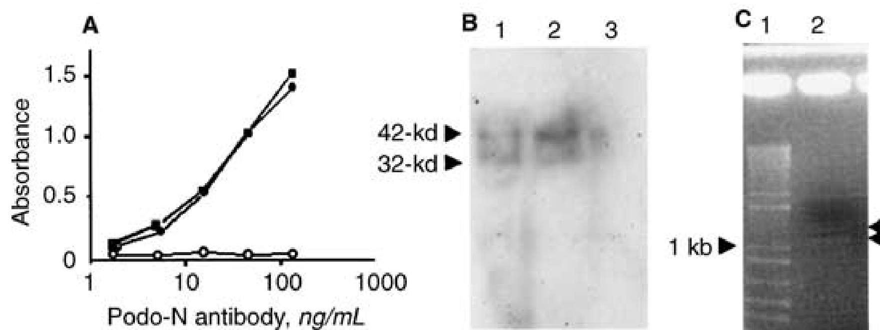

Fig. 1. (*A*) Reactivity of a rabbit polyclonal antibody directed against the N-terminal region of podocin (Podo-N) with three peptides by enzyme-linked immunosorbent assay (ELISA). Symbols are (●) oligopeptide (N-terminal); (■), keyhole limpet hemocyanin (KLH)-conjugated oligopeptide (N-terminal); (○), KLH-conjugated nonassociated peptide. Absorbance at 490 nm. The concentration of Podo-N antibody is indicated by ng/mL. The Podo-N antibody did not react with a nonassociated peptide but was reactive with the oligopeptide of the N-terminal of podocin, and antibodies against the bridge region, including KLH, were removed by an immunoaffinity column. (*B*) Western blot analysis of normal human glomerular extract using Podo-N (lane 1) and Podo-C (C-terminal region) (lane 2) antibodies. Bands at 42 kD and 32 kD can be seen by both antipodocin antibodies. The protein of 42 kD fragment was estimated to be intact podocin. Normal rabbit IgG (lane 3) did not show any immunoreactivity. (*C*) Reverse transcription-polymerase chain reaction (RT-PCR) using total RNA from normal adult human kidney with *NPHS2* primers 1A and 8B, and electrophoresis on a 1.5% agarose gel. Lane 1 is 1 kb marker. RT-PCR showed an original length (1275 bp) and a decreased length of approximately 200 bp in *NPHS2* mRNA, indicated by arrowheads (lane 2).

(Fig. 1C). This result indicates that NPHS2 mRNA is alternatively spliced and supports the Western blot analysis above.

Podocin expression in normal glomeruli

In immunofluorescent staining of normal mature glomeruli, a similar staining pattern was observed with the two antipodocin antibodies (Fig. 2A and B) but with no staining with normal rabbit IgG (Fig. 2C). Polyclonal antibodies Podo-N and Podo-C gave a linear labeling along the GBM, somewhat granular as described by Roselli et al [7].

To examine the subcellular localization of podocin, double immunofluorescent labeling was performed on human mature glomeruli, using the Podo-N antibody (in red) and antibodies directed against synaptopodin (in green) and the α5(IV) chain (in green) (Fig. 2D to J). Dual labeling with podocin and synaptopodin showed a tight colocalization of both markers as evidenced by yellow labeling outlining the GBM (Fig. 2D to F). The anti-α5(IV) chain antibody gave a strong linear labeling of the GBM. Dual stained sections seen at low magnification showed a nearly complete colocalization of red and green labeling (Fig. 2G to I). However, high magnification and confocal microscopy examination disclosed a distinct green linear labeling of the GBM with the anti-α5(IV) chain antibody from the red labeling of the base of the podocytes with the anti-podocin antibody (Fig. 2J).

Podocin expression in normal extraglomeruli

The Podo-N antibody stained glomeruli only, while the Podo-C antibody stained renal arteriole walls as well as glomeruli (Fig. 3A). Double immunofluorescent labeling of human mature kidneys using Podo-C and anti-SMA antibodies showed that a protein with the C-terminal region of podocin colocalized with the smooth muscles of renal arteriole walls (Fig. 3A to C). To exclude the possibility of autofluorescence of the vascular elastic laminas, we examined renal arteriole and aorta by immunoperoxidase staining. The Podo-C antibody stained not only the smooth muscle of renal arteriole (Fig. 3D), but also that of aorta (Fig. 3E). Tubuli and Bowman's capsules were not stained.

Podocin expression in glomerular diseases

The Podo-N antibody was used for immunohistochemistry studies as below. Podocin was normally detected in all patients with HSPN, IgAN and MCD (Fig. 4A, C, and E). Although podocin expression in MCD may appear high in the figure, the degree of immunostaining in MCD was in fact the same as that in HSPN and IgAN through a microscope. In 19 patients with FSGS, the expression of podocin was either decreased (1+) (7/19) or completely absent (−) (7/19) (Fig. 4I), and in only five patients was it present by more than 2+ (Tables 2 and 3). In FSGS, podocin was absent in the sclerotic regions.

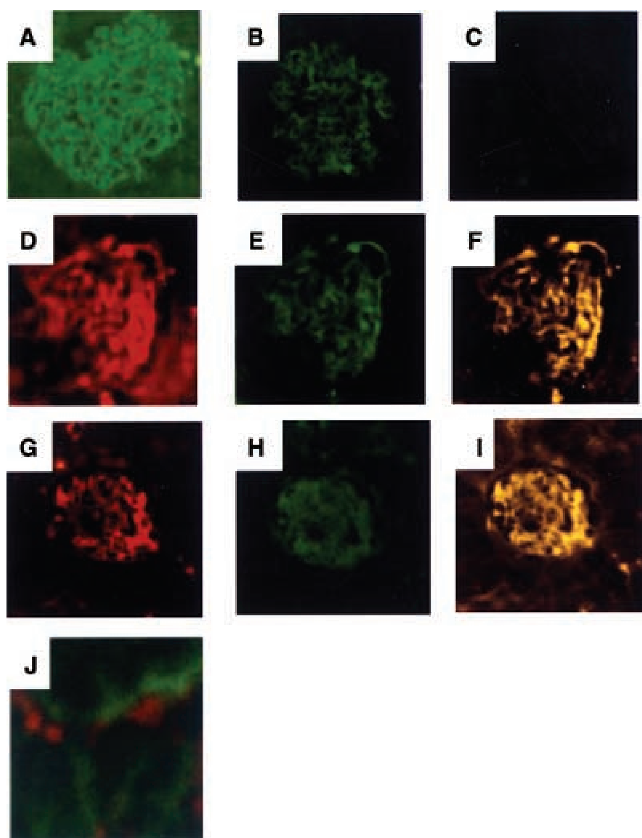

Fig. 2. Immunohistochemistry of podocin in normal human mature glomeruli. Immunostaining of podocin using anti-N-terminal region of podocin (Podo-N) (*A*) and anti-C-terminal region of podocin (Podo-C) (*B*) antibodies and using normal rabbit IgG (*C*). Podo-N and Podo-C gave the linear labeling along the glomerular basement membrane (GBM) (A and B), with no staining with normal rabbit IgG (C). Dual immunofluorescence with Podo-N (red) (*D* and *G*) and antibodies (green) against synaptopodin (*E*) or the α5 chain of type IV collagen (α5(IV) chain) (*H*), and merge (yellow) (*F* and *I*) in a human mature kidney. The red podocin labeling of podocytes is colocalized with synaptopodin labeling (F), and appears to be colocalized with the α5(IV) chain at low magnification (I), but follows the external aspect of the α5(IV) chain of the green GBM with high magnification and a confocal microscope (*J*). Tubuli and Bowman's capsule were not stained (A to I ×500, J ×1400).

In some cases, the staining pattern of podocin changed from linear to segmental. The reduction or absence in staining of podocin was seen in all examined glomeruli in patients with FSGS. In four of five patients with MN, it was present by more than 2+ (Fig. 4G). One case of MN with a decrease (1+) in signal of podocin was in stage 2.

Synaptopodin signals were similar to podocins (Fig. 4B, D, F, H, and J). In 11 of 19 patients with FSGS, synaptopodin was absent (Fig. 4J) (Tables 2 and 3), while in MCD it was normally present (Fig. 4F). Fibrous area could not be stained with either antipodocin or antisynaptopodin antibodies. There was some discrepancy between staining levels of podocin and synaptopodin in patients with FSGS (Table 3). In FSGS, both expression patterns were similar in glomeruli, and we could not point out any difference in staining areas.

Signals of ZO-1 were stable in all of these glomeruli (Fig. 4K), even when staining of podocin was absent or reduced in the three nephrotic diseases. We can therefore conclude that the tissue was not damaged and capillary loop was present.

Podocin expression and clinical parameters

We examined the correlation of podocin expression with clinical parameters in 19 patients with FSGS. We compared seven patients with no podocin expression with five patients who had podocin expression of more than 2+. Proteinuria was 4.7 +/− 3.4 g/day (mean +/− SD) vs. 3.6 +/− 0.5 g/day, serum creatinine was 2.6 +/− 3.6 mg/dL vs. 0.7 +/− 0.1 mg/dL, serum albumin was 3.0 +/− 0.9 g/dL vs. 2.7 +/− 0.9 g/dL, respectively. There was no statistical significance between podocin expression and the three clinical parameters in patients with FSGS.

DISCUSSION

We generated two antipodocin antibodies. Both antibodies detected the same original band of 42 kD fragment, which is consistent with the molecular weight of podocin [2]. Roselli et al [7] also generated rabbit antipodocin polyclonal antibodies, which detected a single band of 49 kD by Western blot analysis. They used transfected HEK293 cell lysates as a protein for Western blot analysis, while we used a protein derived from human isolated mature glomeruli. The mouse and rat podocin has been recently identified and is predicted to be a protein of 385 and 383 amino acids, respectively [13, 14]. The amino acids sequences of murine podocin show more than 80% identity to human podocin. Molecular weight of both murine podocins are 42 kD by Western blot analysis using normal murine isolated glomeruli [13–15]. The difference in band size of human podocin may be caused by the nature of the original cells used, or be related to post-translational modifications. Furthermore, immunoblot analysis showed that both our antibodies recognized an extra band of 32 kD fragment in mature glomeruli. This result was confirmed by other independent Western blot analyses using five normal controls. Forty to sixty percent of human genes have alternative splice forms [16]. RT-PCR using RNA from human mature kidney showed two bands, one with an original length and the other with a decreased length of approximately 200 bp in NPHS2 cDNA. RT-PCR with other primers amplifying exons 2 to 8 showed two bands, one with a putative length and the other with a decreased length of approximately 200 bp (data not shown). The deletion may be between exons 3 and 7. Strausberg reported a clone similar to the *NPHS2* gene. This clone deletes exon 5 (204 bp) in-frame and encodes a smaller podocin of 315 amino acids including both the N- and C- termini of podocin

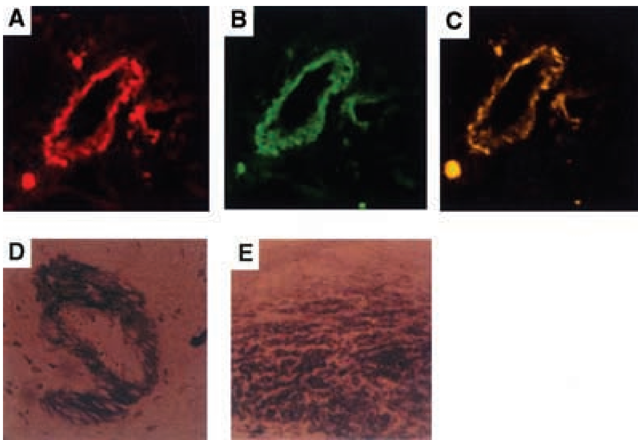

Fig. 3. Podocin expression in the normal extraglomeruli. Dual immunofluorescence with anti-C-terminal region of podocin (Podo-C) antibody (red) (*A*) and antismooth muscle actin (SMA) antibody (green) (*B*), and merge (yellow) (*C*) in normal human mature kidney. A Podo-C antibody stained renal arteriole. Podocin colocalized with smooth muscle of renal arteriole walls (A to C). Immunoperoxidase staining showed that Podo-C antibody stained not only smooth muscle of renal arteriole (*D*), but also that of aorta (*E*). Tubuli and Bowman's capsules were not stained (A to E ×500).

Table 2. Expression of podocin and synaptopodin in glomerular diseases

Diagnosis	Samples number	Degree of expression of podocin 3+	2+	1+	—	Degree of expression of synaptopodin 3+	2+	1+	—
HSPN	6	6				4		2	
IgAN	6	6				5	1		
MCD	6	6				5	1		
MN	5	3	1	1		3	1	1	
FSGS	19	1	4	7	7	4	2	2	11
Total	42	22	5	8	7	21	5	5	11

Abbreviations are: HSPN, Henoch-Schönlein purpura nephritis; IgAN, IgA nephropathy; MCD, minimal-change disease; MN, membranous nephropathy; FSGS, focal segmental glomerulosclerosis.

(GenBank accession # BC029141). The smaller podocin in the present study may be derived from an alternatively spliced mRNA of podocin.

In glomeruli, both antipodocin antibodies Podo-N and Podo-C showed the same staining patterns as reported [7]. The Podo-C antibody did stain the smooth muscles in renal arteriole and aortic walls, while Podo-N antibody did not. Since vascular elastic laminas have a green autofluorescence, we confirmed it by immunoperoxidase staining. Although indirect, our data suggest the existence of a vascular isoform of podocin with a different molecular mass. We thought of two possibilities to explain how an isoform of podocin may lose antigenecity for the Podo-N antibody in the vessels immunohistochemically and still be reserved by immunoblot analysis. First, proteins change structure to a straight form in Western blot analysis, while in tissue the area that the Podo-N antibody recognizes maintains a three-dimensional structure. This may lead to a loss in antigenecity in tissue.

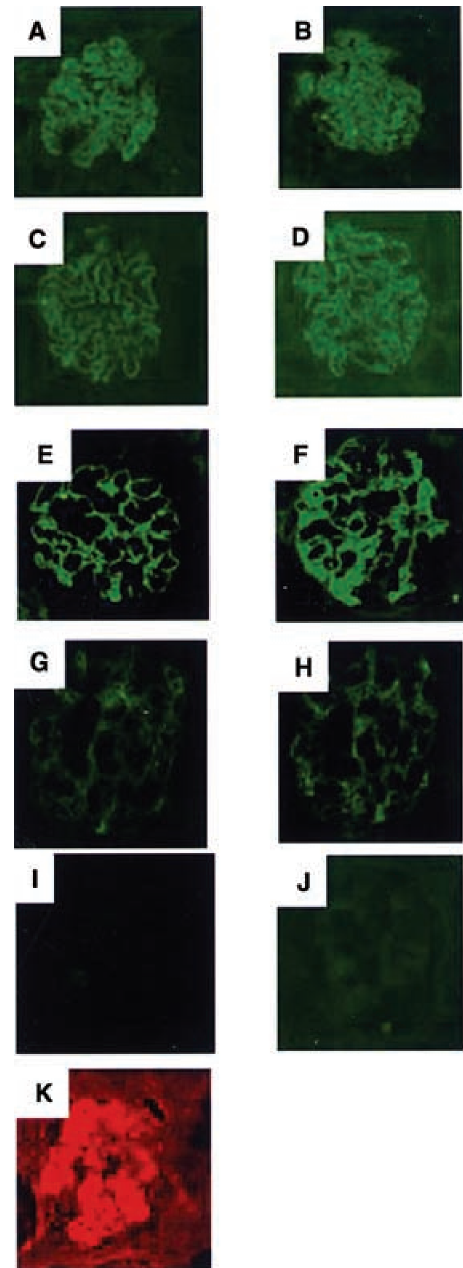

Fig. 4. Immunofluorescent study for podocin (*A, C, E, G,* and *I*), synaptopodin (*B, D, F, H,* and *J*) and ZO-1 (*K*) in biopsy samples of glomerular diseases which are comprised of Henoch-Schönlein purpura nephritis (HSPN) (A and B), IgA nephropathy (IgAN) (C and D), minimal-change disease (MCD) (E and F), membranous nephropathy (MN) (G and H), and focal segmental glomerulosclerosis (FSGS) (I to K). Podocin was normally present in all patients with HSPN, IgAN, and MCD (A, C, and E). In contrast, podocin expression decreased or was absent in fourteen of 19 patients with FSGS (I). In four of five patients with MN, podocin was normal (G). In some cases, podocin changed from a linear to a segmental pattern. The signal of synaptopodin was similar to that of podocin (B, D, F, H, and J). In eleven of 19 patients with FSGS, the expression of synaptopodin was absent (J), while in MCD it was normally present (F). The signal of ZO-1 was stable in patients with FSGS (K) (A to K ×500).

Table 3. Clinical course of patients with minimal-change disease (MCD) and focal segmental glomerulosclerosis (FSGS)

			Staining[c]		Serum		Urine		Treatment[d]		
Cases	Age[a] years	Gender[b]	PN	Sy	Creatinine mg/dL	Albumin mg/dL	Protein g/day	Hematuria	Prebiopsy	Postbiopsy	Prognosis[e]
MCD											
1	3	M	3+	3+	0.3	3.5	1.3	–	PSL	PSL	CR
2	10	F	3+	3+	0.5	3.8	0.03	–	PSL	PSL	CR
3	18	M	3+	3+	0.8	1.6	7.6	–	PSL+CsA	PSL	CR
4	21	M	3+	2+	0.7	1.3	5.0	–	PSL	PSL	CR
5	39	F	3+	3+	0.6	2.5	2.1	–	—	PSL	CR
6	59	F	3+	3+	0.5	3.4	0.7	–	—	—	CR
FSGS											
1	19	M	3+	3+	0.8	3.5	3.4	–	Steroid-pulse, HD	PSL + CsA	Relapse, CR
2	16	F	2+	3+	0.5	1.6	3.6	–	—	PSL + CsA	CR
3	30	M	2+	1+	0.8	3.0	4.4	–	—	PSL	IR
4	37	F	2+	—	0.7	3.6	3.0	–	Steroid-pulse	—	IR
5	63	F	2+	3+	0.7	1.9	3.7	–	—	PSL + CsA	CR
6	2	M	1+	—	0.3	3.2	0.3	+	PSL	PSL + CsA	IR
7	29	M	1+	3+	1.2	1.6	9.0	–	PSL	PSL + LDL apheresis	IR
8	32	F	1+	1+	0.7	4.6	1.6	–	—	—	Denial of treatment
9	43	F	1+	2+	0.7	2.4	6.8	–	—	PSL + CsA	CR
10	55	M	1+	—	1.1	3.8	2.5	–	—	—	CR
11	61	F	1+	2+	4.4	1.6	12.0	+	—	PSL + CsA	CR
12	71	F	1+	—	0.7	2.0	7.0	–	—	LDL apheresis	CR
13	6	M	—	—	0.8	2.6	6.2	–	PSL	PSL	ESRD (CAPD/HD)
14	6	M	—	—	0.6	3.7	1.8	–	PSL	PSL	ESRD (CAPD/HD)
15	44	M	—	—	1.9	3.8	2.5	–	—	PSL + CsA	IR
16	51	M	—	—	11.0	3.4	3.0	–	—	PSL + CsA	Denial of HD, IR
17	56	M	—	—	1.0	4.0	2.5	–	—	PSL	IR
18	59	F	—	—	0.6	1.8	12.0	–	—	PSL + CsA	CR
19	66	M	—	—	2.4	1.8	5.5	–	—	PSL + CsA	Relapse, CR

[a] Age at biopsy; [b] F, female; M, Male; [c] PN, staining of podocin-N; Sy, staining of synaptopodin; [d] PSL, prednisolone; CsA, cyclosporin; [e] CR, complete remission; IR, incomplete remission; ESRD, end-stage renal disease; CAPD: continuous ambulatory peritoneal dialysis; HD, hemodialysis.

Second, the deletion of NPHS2 exon 5 may change the structure of podocin, so that the Podo-N antibody would not stain immunohistochemically. The mRNA of rat podocin is expressed not only in podocytes but also in nerve tissues such as the cerebrum, cerebellum, and the medulla oblongata [13]. Rat podocin is localized in the outer area of astrocyte-facing vessels. Podocin may play an important role in the vessels such as renal arteriole and aortic walls as well as maintain the barrier function in the glomerular capillary wall and blood-brain barrier [13].

The expression of nephrin has been reported controversially in proteinuric diseases [17–19]. This is the first report of podocin expression in human glomerular diseases, especially in nephrotic syndrome such as MCD, FSGS and MN. The amount of podocin did not decrease in any of the six patients with MCD. Four of them were treated with immunosuppressive therapy (Table 3). One was in partial remission, three had proteinuria, and two were nephrotic at the time of renal biopsy. Podocin expression in MCD seems to be maintained whether a patient is in an active or a remission phase, and whether treated or not. In MCD, five patients responded to steroid and one patient received no therapy; all are currently in complete remission. In contrast, podocin expression seems to be either decreased or absent in 74% cases of FSGS. Examination of podocin expression will be useful to differentiate MCD from FSGS.

In FSGS, cases with sufficient podocin expression tended to have good prognosis. Case 1 of FSGS, whose podocin was normally expressed, experienced some relapse, but responded to steroid and is now in complete remission. On the other hand, of the seven FSGS patients with no podocin expression, two (29%) progressed to ESRD (Table 3), indicating possible poor prognosis. Cases 13 and 14 of FSGS had steroid-resistant nephrotic syndrome at an infant stage, and dialysis was introduced. Case 13 had a familial history. After obtaining informed consent for gene analysis in the case 13 from his parents, we analyzed the *NPHS2* gene, but did not find any mutation. There are several causes of FSGS. Although it is not clear what the variation of podocin expression means, our view is that a decrease or absence of podocin means damage of the filtration barrier either primarily or secondarily, and possible resistance to immunosuppressive therapy.

In MN, podocin was normal in most cases, and there was no correlation between the staining pattern of podocin and the stage of the disease.

Podocin was normally present in HSPN and IgAN. The principal region responsible for the two diseases is not the slit diaphragm but the mesangial region. Therefore, podocin would not be primarily involved in these diseases. Amounts of podocin detected by immunochemistry are likely to be as follows: HSPN, IgAN, MCD > MN > FSGS, although further examination is needed in many more cases with glomerular diseases.

We compared the expression of podocin with synaptopodin in glomerular diseases. Their stainings of them were similar, although some discrepancy existed (Table 3). Synaptopodin is not only a marker of the podocyte but also a marker of the differentiation of the podocyte [20]. Reduction or absence of podocin and synaptopodin stainings should mean injury of podocyte structure and/or function.

We examined the correlation of podocin expression with clinical parameters in patients with FSGS, but there was no significance, which may be due to the small number of patients. In this study, the expression of podocin was examined only once in each individual case. It would be ideal to examine chronological changes of podocin and clinical parameters in cases of repeated biopsies.

CONCLUSION

Although indirect, our data suggest the existence of a vascular isoform of podocin with a different molecular mass. This isoform may play an important role in the vessels. Podocin was normally present in glomeruli in HSPN, IgAN, MCD, and most cases of MN, while it was either decreased or absent in most cases (74%) of FSGS. Podocin in MCD is maintained even in an active phase. Examination of podocin expression should be useful to differentiate MCD from FSGS.

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Reprint requests to Hitoshi Nakazato, M.D., Ph.D., Department of Pediatrics, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan.
E-mail: hnakazat@fc.kuh.kumamoto-u.ac.jp

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