Dissociation of NEPH1 from nephrin is involved in development of a rat model of focal segmental glomerulosclerosis

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Otaki Y, Miyauchi N, Higa M, Takada A, Kuroda T, Gejyo F, Shimizu F, Kawachi H. Dissociation of NEPH1 from nephrin is involved in development of a rat model of focal segmental glomerulosclerosis. Am J Physiol Renal Physiol 295: F1376–F1387, 2008.—Focal segmental glomerulosclerosis (FSGS) is a disease showing severe proteinuria, and the disease progresses to end-stage kidney failure in many cases. However, the pathogenic mechanism of FSGS is not well understood. The slit diaphragm (SD), which bridges the neighboring foot processes of glomerular epithelial cells, is understood to function as a barrier of the glomerular capillary wall. To investigate the role of SD dysfunction in the development of FSGS, we analyzed the expression of SD-associated molecules in rat adriamycin-induced nephropathy, a mimic of FSGS. The staining of the SD molecules nephrin, podocin, and NEPH1 had already shifted to a discontinuous dotlike pattern at the initiation phase of the disease, when neither proteinuria nor any morphological alterations were detected yet. The alteration of NEPH1 expression was the most evident among the molecules examined, and NEPH1 was dissociated from nephrin at the initiation phase. On day 28, when severe proteinuria was detected and sclerotic changes were already observed, alteration of the expressions of nephrin, podocin, and NEPH1 worsened, but no alteration in the expression of other SD-associated molecules or other podocyte molecules was detected. It is postulated that the dissociation of NEPH1 from nephrin initiates proteinuria and that the SD alteration restricted in these molecules plays a critical role in the development of sclerotic changes in FSGS.

proteinuria; glomerular epithelial cells; podocyte; slit diaphragm; adriamycin

FOCAL SEGMENTAL GLOMERULOSCLEROSIS (FSGS) is a diagnostic term for a clinical-pathological syndrome showing proteinuria and the pathological findings of focal segmental glomerular consolidation and scarring. The most important clinical features of FSGS are persistent nephrotic-range proteinuria and, in many cases, progressive deterioration to end-stage kidney failure (43). Although clarification of the pathogenesis and establishment of a novel effective therapy for FSGS are awaited, its precise pathogenic mechanism is not yet fully understood. FSGS is understood to have multiple etiologies and pathogenic mechanisms. However, because the principal pathological feature of FSGS is a flattening of the foot processes of the glomerular epithelial cells (podocytes) followed by glomerulosclerosis, FSGS is thought to be a podocyte disease.

The podocytes are highly specialized cells, and their specific shape is characterized by the interdigitating foot processes. Neighboring foot processes are derived from different cell bodies and are bridged by a specific intercellular junction called the “slit diaphragm” (SD). The distance between neighboring foot processes is ~40 nm (42), and this wide interval permits the high hydraulic permeability of the glomerular capillary wall. On the other hand, the glomerular filtration barrier maintains low permeability to plasma proteins. It is now becoming accepted that the SD also plays a critical role as a barrier. Some constituents of the SD, such as nephrin (24), podocin (3), NEPH1 (6), CD2-associated protein (CD2AP) (46), and α-actinin 4 (15), have been identified during the past decade.

We have reported (19, 37, 52) that monoclonal anti-nephrin antibody, if injected into rats, causes massive proteinuria. These studies indicate that these SD molecules are important for the maintenance of glomerular filter integrity. Recently, some experimental and clinical studies have shown that SD dysfunction might be involved in the development of proteinuria in several types of glomerular disease, such as minimal change nephrotic syndrome (MCNS) (57), membranous nephropathy (13, 33), and diabetic nephropathy (22, 53). However, no study specifically analyzing the contribution of SD dysfunction to the development of proteinuria in FSGS has yet been reported.

It is considered that another important role of the interdigitating foot processes and the SD connecting them is maintenance of the glomerular architecture. The specific shape of the podocyte contributes to counteraction of the high transmural distending force (26). It is known that, in several types of glomerular disease showing proteinuria, the foot processes of the podocytes retract into the cell bodies and the SD is replaced by a tight junction (23, 48). If these changes in the podocyte are not reversed, they ultimately lead to glomerulosclerosis and end-stage renal failure (25, 26, 50). It is plausible to assume that the SD-associated molecules CD2AP, podocin, and ZO-1 connect the extracellular molecules of the SD to the cytoskeleton. Shih et al. have reported (46) that CD2AP-deficient mice show not only massive proteinuria but also glomerular sclerotic changes. We have previously demonstrated (32) that the minor podocyte damage detected as a decreased expression of the SD

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MATERIALS AND METHODS

that NEPH1 is dissociated from nephrin at the initiation phase. Among the SD-associated molecules, we also demonstrated yet. The alteration of NEPH1 staining was the most evident proteinuria nor any morphological alterations were detected and NEPH1 were already shifted to a discontinuous dotlike pattern at the initiation phase of the disease, when neither proteinuria nor any morphological alterations were detected yet. The alteration of NEPH1 staining was the most evident among the SD-associated molecules. We also demonstrated that NEPH1 is dissociated from nephrin at the initiation phase. On day 28, when severe proteinuria was detected and sclerotic changes were already observed, the expression of NEPH1, nephrin, and podocin was clearly decreased but no alteration in the expression of other SD-associated molecules, such as CD2AP and ZO-1, or other podocyte molecules was detected. These results suggest that the dissociation of NEPH1 from nephrin is involved in the initiation of proteinuria, and that the SD alteration restricted in these molecules plays a role in the development of sclerotic changes in FSGS.

NEPH1-NEPHRIN DISSOCIATION IN RAT FSGS MODEL

Antibodies. Rabbit anti-rat NEPH1 polyclonal antibody was prepared according to the method described previously (17). In brief, the rabbits were immunized with the rat NEPH1 peptide of KLDKVKETVNRPLT, a 100% match to human NEPH1. The peptide was purchased from GenScript (Scotch Plains, NJ). The rabbits were immunized with 2.0 mg of the peptide conjugated with the carrier protein KLH and boosted twice with 1.0 mg of each antigen. The rabbits were bled 2 wk after the last immunization. The specificity of the anti-NEPH1 antibody was confirmed by IF and Western blot analysis. Murine monoclonal antibodies to rat nephrin (MAb 5-1-6) (16, 37) and rat podocalyxin (MAb 4D5) (11) and rabbit polyclonal antibody to podocin (17), CD2AP (33), and fibronectin (51) were prepared as described previously. Rabbit anti-rat β-actin and mouse anti-rat α-smooth muscle actin (α-SMA) antibodies were purchased from Sigma BioSciences (St. Louis, MO).

Rabbit anti-rat desmin was purchased from Chemicon. Mouse anti-rat ZO-1, mouse anti-rat α-integrin, and rabbit anti-rat collagen type I antibodies were purchased from Zymed Laboratories (San Francisco, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and Cosmo Bio (Tokyo, Japan), respectively.

Morphological and immunohistochemical studies in ADR-induced nephropathy. Light microscopy (LM) and electron microscopy (EM) studies were performed basically according to methods described previously (11, 17, 33). The sections were stained with periodic acid-Schiff (PAS) reagent. For the semiquantitative evaluation for glomerular lesions, 50 randomly selected full-sized glomeruli from each rat were used. The sections were analyzed in a blind manner, and the degree of glomerular mesangial matrix expansion was scored from 0 to 4 according to the percentage of glomerular involvement, as described previously (14). Crescent formation rate was also determined with the same sections.

IF studies were performed basically according to the method described previously (21). Renal tissue was quickly frozen in n-hexane cooled at −70°C. Three-micrometer-thick cryostat sections were fixed, incubated with the primary antibodies described above, and stained with FITC-conjugated anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) (for anti-nephrin MAb 5-1-6, anti-ZO-1, and anti-α-integrin), FITC-conjugated anti-mouse IgG2a (Southern Biotechnology Associates) (for anti-podocalyxin MAb 4D5 and anti-α-SMA), or FITC-conjugated anti-rabbit immunoglobulins (Dako) (for anti-nephrin, anti-podocin, anti-NEPH1, anti-CD2AP, anti-desmin, anti-fibronectin, anti-collagen type I). For dual-labeling IF analysis, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG1 (Southern Biotechnology Associates) was used to detect anti-nephrin MAb 5-1-6. The species were observed with IF microscopy (BX50; Olympus, Tokyo, Japan). For the semiquantitative analysis, the extent of glomerular expression of nephrin, podocin, NEPH1, CD2AP, ZO-1, podocalyxin, and integrin was evaluated by a blind observer. Scores were assigned to individual glomeruli of each section as follows: score 4, the area in which the continuous staining was strikingly disrupted and the staining intensity clearly decreased covered 0–25% of the glomerular tuft area; score 3, 25–50%; score 2, 50–75%; score 1, 75–100%.

The final score of each rat was then calculated as the weighted mean

$$S_{\text{Nephrin}} = \left( \frac{1}{2} N_{i} + 1 \times \frac{1}{2} N_{i} + 1 \times \frac{1}{2} N_{i} + 1 \times \frac{1}{2} N_{i} \right) N_{i} + N_{i} + N_{i} + N_{i}$$

where $N_{i} (i = 1–4)$ is the number of glomeruli in each category. One hundred full-sized glomeruli from each rat were randomly chosen and examined as described previously (30).

Reverse transcription-polymerase chain reaction. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) with glomerular RNA was performed basically according to the method described previously (17, 18). In brief, glomeruli were isolated from pooled kidneys in each group by differential sieving through mesh brass sieves (nos. 60, 150, and 200). Total RNA was extracted from the isolated glomeruli with TRIzol (Life Technologies, Gaithersburg, MD). For the quantification of PCR product, all samples including control were run on the same gel, and the band intensity was

Materials. All in vivo experiments were performed in specific pathogen-free female Wistar rats weighing ~150 g at the age of 6 wk. All rats used in this study were purchased from Charles River Japan (Atsugi, Japan). All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures for the present study were approved by the Animal Committee of Niigata University School of Medicine, and all animals were treated according to the guidelines for animal experimentation of Niigata University. For the analysis with developing kidneys, kidneys were removed from rats on embryonic day 20.5. The human kidney specimen used for immunofluorescence (IF) study was isolated from the normal part of the kidney that had been obtained from nephrectomy.

Induction of adriamycin nephropathy. A total of 15 rats were injected intravenously with ADR (6.0 mg/kg body wt, dissolved in saline) under anesthesia as described previously (39), and 5 rats each were killed on day 7 and day 28 and at 24 wk after the injection. As controls, five rats were injected with the same volume of saline and killed on day 7. The pieces of the kidney removed were used for IF and other morphological analyses. The remaining kidney pieces were pooled in each group and used for isolation of glomeruli. The glomeruli isolated were used for the purification of RNA. For the preparation of glomerular lysate, another five rats were injected with ADR, and their kidneys were removed on day 7 and used for the isolation of glomeruli. The glomeruli isolated were solubilized with SDS-PAGE sample buffer for Western blot analysis or with RIPA buffer for the interaction assay with sequential immunoprecipitation and Western blot analysis. As controls, another five rats were injected with saline and killed on day 7.

Urine samples were collected for 24 h in the metabolic cages. The protein concentration of the urine was measured by colorimetric assay with a Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA) and BSA as a standard.
determined by image analysis with the BioDoc-It System (UVP, Upland, CA). As primers for rat NEPH1, we employed sense primer CGG TGA TTC TGC TGC AAG CAG and antisense primer GAG CGA GAG GTA CCG CTT TAT. The sequences of primers for rat nephrin, podocin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (17, 18). The ratio of the densitometric signal of the molecules examined to that of GAPDH was determined. The data are shown as ratios relative to control findings and expressed as means ± SD of the results of three independent experiments.

**Western blot analysis.** Western blot analysis was performed basically according to the method described previously (17). Isolated glomeruli from normal and ADR nephropathy rats were solubilized with SDS-PAGE sample buffer [2% SDS, 10% glycerol, 5% 2-mercaptoethanol in 62.5 mmol/l Tris-HCl (pH 6.8)] with protease inhibitors. Equal amounts of these glomerular lysates were subjected to SDS-PAGE with 7.5% acrylamide gel according to the method of Laemmli (27) and transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Pall, Pensacola, FL) by electrophoretic blotting for 45 min with Trans Blot SD (Bio-Rad). After blocking with skim milk, the strips of membrane were exposed to each primary antibody. They were then washed and incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulins. The reaction was developed with an alkaline phosphatase chromogen kit (Biomedica, Foster City, CA). The density of the positive bands was quantitated by image analysis with the BioDoc-It system (UVP). This procedure was carried out three times. The ratio of the densitometric signal of the molecules examined to that of β-actin was determined. The data are shown as ratios relative to control findings and expressed as means ± SD of the results of three independent experiments.

**Interaction assays.** For immunoprecipitation, normal rat glomeruli were solubilized with RIPA buffer [0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 150 mmol/l NaCl, and 10 mmol/l EDTA in 25 mmol/l Tris-HCl (pH 7.2)] with protease inhibitors. Anti-nephrin antibody, anti-NEPH1 antibody, or preimmune normal rabbit serum (NRS) was coupled with AminoLink Coupling Gel (Pierce, Rockford, IL) according to the manufacturer's protocol. The glomerular lysate was incubated with the gel coupled with each of the antibodies at 4°C overnight, followed by centrifugation for 15 min at 15,000 rpm. The pellet was washed three times with 1% Triton X-100 and then eluted with SDS-PAGE sample buffer. The elution fractions were separated by SDS-PAGE followed by immunoblotting with anti-nephrin antibody, anti-NEPH1 antibody, or preimmune NRS.

**Cloning of rat NEPH1.** To clone a rat homolog of NEPH1, we employed PCR with the primers designed from the mouse NEPH1 sequence. PCR cloning was performed basically according to the method described previously (18).

**Statistical analysis.** Statistical significance (defined as P < 0.05) was evaluated with the Mann-Whitney U-test. All values are expressed as means ± SD. Data were analyzed with StatView for Windows (Abacus Concepts, Berkeley, CA).

### Results

**Parameters for renal function in ADR-induced nephropathy.** No abnormal proteinuria (>20 mg/day) or abnormal albuminuria (>5 mg/day) was detected in any rats on day 7 after ADR injection, and abnormal proteinuria was first detected on day 8 after the ADR injection. The amount of proteinuria increased with time. The rate of proteinuria increase peaked on day 28, and the amount of proteinuria reached 775 ± 23.1 mg/day at 24 wk. The kinetics of the proteinuria observed in this study is basically consistent with that published previously (31, 35). The time courses of body weight, kidney weight, the parameters for renal function, and the amount of proteinuria (albuminuria) are shown in Table 1. No increase in the values of serum creatinine and blood urea nitrogen (BUN) was detected in rats on day 7. On day 28, increase in the values of serum creatinine and BUN was not yet detected, although the amount of proteinuria reached 294 ± 171 mg/day. At 24 wk, the values of serum creatinine and BUN evidently increased.

**Characterization of anti-NEPH1 antibody.** Western blot findings of anti-NEPH1 antibody with rat glomerular lysate solubilized with SDS-PAGE sample buffer are shown in Fig. 1A. An ~110-kDa band was detected by anti-NEPH1 antibody. No band was detected by preimmune NRS or anti-NEPH1 antibody preabsorbed with its immunizing peptide. The size of the band (~110 kDa) coincided with that in previous reports (29). The IF finding of anti-NEPH1 antibody in a normal rat kidney section is shown in Fig. 1B. Positive signals for NEPH1 were observed as a linear pattern along the glomerular capillary loop. No specific signal was detected with NRS or the preabsorbed anti-NEPH1 antibody. Positive staining of NEPH1 was observed in a human kidney section in a pattern similar to that in the rat section (data not shown).

**Morphological studies of glomeruli and tubulointerstitium in ADR-induced nephropathy.** To evaluate the kinetics of the morphological alteration in ADR nephropathy, LM, EM, and immunohistochemistry of fibronectin, collagen type I, -SMA, and desmin were performed. The representative LM findings of glomeruli and the EM findings showing the basal area of the podocyte are shown in Fig. 2A. The structure of the SD was found to be preserved on day 7 in EM. On day 28, mesangial matrix expansion was observed in LM and effacement of the podocyte foot process was observed in EM. Severe glomerulosclerosis with mesangial matrix expansion and a crescent were observed in LM, and effacement of the podocyte foot process and the thickness of glomerular basement membrane (GBM) were broadly observed in EM at 24 wk. The results of semiquantitative analyses for mesangial matrix score and crescent formation rate are shown in Fig. 2, B and C, respectively. The LM and IF findings showing the tubulointerstitial area as well as the glomeruli are shown in Fig. 2D. Obvious changes were not detected in the tubulointerstitial area or in the glomeruli in LM on day 7. No increased deposition of fibronectin or -SMA in the mesangial area of glomeruli was observed on day 7. Increased deposition of fibronectin and -SMA in the mesangial area was observed on day 28, whereas the deposi-

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Values are means ± SD. ADR, adriamycin; BW, body weight; KW, kidney weight; BUN, blood urea nitrogen.
and NEPH1 were observed as a linear pattern in normal rats, and were evaluated as score 4. The scores of these molecules in ADR nephropathy were decreased on day 7 (nephrin, 3.66 ± 0.04; podocin, 3.84 ± 0.10; NEPH1, 3.45 ± 0.10). The semiquantitative data showed that the reduction of NEPH1 staining was more evident than that of other molecules. The results of the semiquantitative analyses are shown in Fig. 4A. We could detect that NEPH1 alteration was more global than the evaluation by the scale adopted in this study, because not striking but clear disruption of the staining of NEPH1 was observed in almost all glomerular tufts in some of the glomeruli, staining of which were given score 3 on the scale used in this study (Fig. 4B).

Analyses of protein amounts of nephrin, podocin, and NEPH1 in ADR-induced nephropathy. The amounts of protein of nephrin, podocin, and NEPH1 on day 7 of ADR nephropathy were analyzed by Western blot using the glomerular lysate solubilized with SDS-PAGE sample buffer. The results are shown in Fig. 5A. The intensities of the nephrin and NEPH1 bands in the glomeruli clearly decreased on day 7, but reduction of podocin was not detected. Nephrin was detected as double bands in glomerular lysate by Western blot analysis. The intensities of both bands decreased in ADR nephropathy, but the intensity of the lower band was decreased more evidently than that of the upper band (upper band, 70.0 ± 9.1% of normal; lower band, 40.2 ± 3.5%). The rate of the decrease of the lower band of nephrin is quite similar to that of NEPH1 (42.0 ± 24.4%).

Analyses of mRNA expression of nephrin, podocin, and NEPH1 in ADR-induced nephropathy. mRNA expression of nephrin, podocin, and NEPH1 was analyzed by RT-PCR with glomerular RNA. No decrease in the mRNA expression of nephrin, podocin, or NEPH1 was detected on day 7. A decrease in the mRNA expression of these molecules was first detected on day 28. The results are shown in Fig. 5B.

Analyses of interaction of nephrin, podocin, and NEPH1. To analyze the interaction of nephrin, podocin, and NEPH1, dual-labeling IF studies were performed in normal and ADR nephropathy rats. Representative dual-labeling IF findings of NEPH1 and podocin with nephrin are shown in Fig. 6. The merged staining of NEPH1 and podocin with nephrin in normal rat sections was detected as a continuous yellow staining pattern along the glomerular capillary wall, indicating that NEPH1 and podocin were colocalized with nephrin. Staining of nephrin, NEPH1, and podocin shifted to a discontinuous dotlike pattern on day 7 of ADR nephropathy. The shifts in the staining of NEPH1 and nephrin were not parallel, and some portions of the nephrin staining were clearly apart from the NEPH1 staining. By contrast, the shifts in the staining of podocin and nephrin were parallel.

To analyze the interaction of nephrin and NEPH1 in glomerular lysate, sequential immunoprecipitation and Western blot analysis were performed with normal rat glomerular lysate solubilized with RIPA buffer. A positive band of NEPH1 was detected in the precipitate with anti-nephrin antibody. Nephrin was detected as double bands in the RIPA-solubilized glomerular lysate. Although the lower band of nephrin was clearly detected in the precipitate with anti-NEPH1 antibody, the upper band of nephrin was very faint. The results of the interaction assay are shown in Fig. 7A. We then analyzed whether or not the interaction of nephrin and NEPH1 was

Fig. 1. Characterization of anti-NEPH1 antibody. A: Western blot finding of anti-NEPH1 antibody with rat glomerular lysate solubilized with SDS-PAGE sample buffer. A band of ~110 kDa was detected by anti-NEPH1 antibody (arrow). No band was detected by preimmune rabbit serum (normal rabbit serum, NRS) or anti-NEPH1 antibody preabsorbed with the peptide used for immunization (preabsorbed). B: immunofluorescence (IF) findings of anti-NEPH1 antibody in a normal rat kidney section. Normal rat kidney sections were stained with anti-NEPH1 antibody, NRS, or anti-NEPH1 antibody preabsorbed with NEPH1 peptide. No specific signal was detected with NRS or the preabsorbed anti-NEPH1 antibody.
altered in glomerular lysate of rats on day 7 of ADR nephropathy. The NEPH1 band detected in the nephrin-rich fraction precipitated with anti-nephrin antibody was faint, indicating that major parts of the remaining nephrin on day 7 have lost the interaction with NEPH1.

Analyses of expression of NEPH1 and nephrin in developing glomeruli. The expression of NEPH1 at the presumptive podocyte was analyzed with the kidney section of the rat embryo (embryonic day 20.5). NEPH1 staining was first detected in the presumptive podocyte at the early capillary loop stage, but major parts of the staining did not overlap with the nephrin staining. Almost all NEPH1 staining signals in the glomeruli were detected at day 7. Severe interstitial fibrosis was observed by LM, and increased deposition of fibronectin and collagen type I in the interstitial area was detected at 24 wk.

Cloning of rat NEPH1 cDNA. Nucleotide sequencing analysis revealed an open reading frame of 2,370 nucleotides coding for a predicted protein of 789 amino acids. The deduced amino acid sequence of rat NEPH1 showed 97.85% identity with the sequence of mouse NEPH1 and 72.66% identity with...
Fig. 3. Kinetics of the IF findings of SD-associated molecules in ADR nephropathy. IF findings of nephrin, podocin, NEPH1, CD-associated protein (CD2AP), ZO-1, podocalyxin, and integrin are shown. Staining of nephrin, podocin, NEPH1, CD2AP, ZO-1, and α5-integrin presented a continuous linear-like pattern along the glomerular capillary loop, and podocalyxin staining was observed as a regularly arranged granular pattern along the capillary loop in normal rats. The staining patterns of nephrin, podocin, and NEPH1 shifted to a discontinuous dotlike pattern (arrowheads) on day 7. Bottom right, enlarged image of the areas indicated by boxes. Alteration of NEPH1 staining was more definite than that of nephrin or podocin. Staining of CD2AP, ZO-1, podocalyxin, and α5-integrin were not altered on day 7. Staining intensities of nephrin, podocin, and NEPH1 were clearly reduced, and their staining patterns became more discontinuous on day 28. No striking changes in CD2AP, ZO-1, podocalyxin, or α5-integrin were detected on day 28. The area showing the continuous staining of nephrin, podocin, and NEPH1 was clearly reduced at 24 wk. Staining of CD2AP, ZO-1, podocalyxin, and integrin also shifted to a discontinuous pattern at 24 wk. Magnification: ×200, left and ×400, right for each molecule.
the sequence of human NEPH1. The sequence data are available from GenBank under accession number AY249056.

DISCUSSION

FSGS is one of the most common diseases showing nephrotic-range proteinuria in children and adults, and in many cases the patients show severe glomerulosclerosis that progresses to end-stage kidney failure. To elucidate its pathogenic mechanism, several experimental models of FSGS, such as ADR-induced nephropathy (2, 34, 35), the model induced by repeated injections of puromycin aminonucleoside (PAN) (5, 10), and partial nephrectomy (9, 36), are used. In this study, we adopted the rat model caused by a single administration of ADR, because this model shows the most important clinical feature of FSGS, namely, increasing proteinuria followed by severe sclerotic changes in the glomeruli.
Recently, it has been accepted that the SD, which bridges the neighboring foot processes of the podocytes, functions as a critical barrier of the glomerular capillary wall (20). FSGS patients in many cases show a “low-selective” proteinuria, in which large-size molecules are allowed to pass into the urine from the early phase of the disease. We have confirmed, by means of SDS-PAGE analysis, that there were large-size molecules, including immunoglobulin, in the urine samples of rats with ADR nephropathy (data not shown), suggesting that the size barrier of the glomerular filter is damaged in this model. Although the selective roles of the charge and size barrier structures of the glomerular capillary wall are still controversial, some reports have proposed that the negative charge of the GBM functions as a charge-selective barrier (8, 41), while the SD functions as a size-selective barrier (18, 20, 54). It is also postulated that the unique shape of the podocyte, which is characterized by interdigitating foot processes interconnected by the SD, contributes to the maintenance of the glomerular architecture (49). Thus we hypothesized that SD dysfunction is involved in the initiation of proteinuria and the progression of glomerulosclerosis in FSGS.

Fig. 5. Analyses of the protein amounts and mRNA expressions of nephrin, podocin, and NEPH1 in ADR nephropathy. A: top left: Western blot analyses with glomerular lysate. Intensity of the nephrin and NEPH1 bands in the lysate decreased on day 7. Nephrin was detected as double bands in the glomerular lysate, and the decrease in the intensity of the lower band was more definite than that of the upper band. Top right: CBB staining of the PVDF membrane, where equal amounts of lysates were loaded. Bottom: signal of each band to that of actin. Data are shown as ratios relative to the data for normal rats and expressed as means ± SD of the results of 3 independent experiments. The rate of decrease of the lower nephrin band is 40.2 ± 3.48% of the normal rat material, and the rate is quite similar to that of NEPH1 (42.0 ± 24.39%). B: semiquantitative RT-PCR analyses with glomerular RNA. Top: characteristic agarose-gel electrophoretic patterns. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Bottom: ratios of the densitometric signals of the SD-associated molecules to that of GAPDH. Data are shown as ratios relative to the normal group and expressed as means ± SD of the results of 3 independent experiments.

Fig. 6. Dual-labeling IF findings of NEPH1 and podocin with nephrin on day 7 of ADR nephropathy. NEPH1 and podocin were stained in green (A, G), and nephrin was stained in red (C, I). The merged staining of NEPH1 and podocin with nephrin in normal rat sections was detected as a continuous yellow staining pattern along the glomerular capillary wall (B, H), indicating that NEPH1 and podocin were colocalized with nephrin. On day 7 of ADR nephropathy, staining of NEPH1, podocin, and nephrin shifted to a discontinuous pattern (D, F, J, L). The merged staining of NEPH1 and nephrin revealed that the shifts in the staining of these molecules were not parallel, and that some portions of the nephrin staining were clearly apart from the NEPH1 staining (seen as red staining; arrow). NEPH1 staining not accompanied by nephrin, seen as green staining, was also detectable (arrowhead). By contrast, no isolated staining of podocin or nephrin was detectable, and the shifts in the staining of these molecules were parallel.

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Recently, it has been accepted that the SD, which bridges the neighboring foot processes of the podocytes, functions as a critical barrier of the glomerular capillary wall (20). FSGS patients in many cases show a “low-selective” proteinuria, in which large-size molecules are allowed to pass into the urine from the early phase of the disease. We have confirmed, by means of SDS-PAGE analysis, that there were large-size molecules, including immunoglobulin, in the urine samples of rats with ADR nephropathy (data not shown), suggesting that the size barrier of the glomerular filter is damaged in this model. Although the selective roles of the charge and size barrier structures of the glomerular capillary wall are still controversial, some reports have proposed that the negative charge of the GBM functions as a charge-selective barrier (8, 41), while the SD functions as a size-selective barrier (18, 20, 54). It is also postulated that the unique shape of the podocyte, which is characterized by interdigitating foot processes interconnected by the SD, contributes to the maintenance of the glomerular architecture (49). Thus we hypothesized that SD dysfunction is involved in the initiation of proteinuria and the progression of glomerulosclerosis in FSGS.
that plays a major role in connecting the podocyte to the GBM. of the podocyte, podocalyxin, and a basal molecule, integrin, also analyzed the expression of an apical cell surface molecule components to the actin cytoskeleton (7, 28). In this study, we and ZO-1 are understood to play a role in connecting SD molecules to the extracellular site of the SD (1, 29, 45). CD2AP recognized as an important scaffolding molecule that interacts with nephrin (44). NEPH1 is understood to be another core molecule of the SD (38), and podocin is nephrin, NEPH1, CD2AP, and ZO-1 in rat ADR nephropathy. The kinetics of the expression of SD constituents nephrin, podocin, NEPH1, and ZO-1 and CD2AP. The alteration of NEPH1 staining was more evident than that of nephrin and podocin (Fig. 4). We also observed that the staining pattern of NEPH1 had already shifted in some glomeruli on day 5, when no changes in the staining of nephrin or podocin were detected (data not shown). These observations suggest that, in this model, NEPH1 is the most sensitive site of the SD and that NEPH1 could be an early marker of podocyte injury.

We then investigated whether or not these alterations of the staining patterns of SD molecules are accompanied by a reduction in the amount of protein and mRNA expression. We previously reported (17, 18) that nephrin was detected by Western blot analysis as clear double bands around 180 kDa in whole glomerular lysate completely solubilized with SDS-PAGE sample buffer. In an analysis using sequentially solubilized materials, we also showed (17) that the upper band is more resistant to solubilization than the lower band. We showed here that the intensity of the lower band of nephrin clearly decreased in glomerular lysates of rats on day 7 of ADR nephropathy (Fig. 5A). The intensity of the NEPH1 band decreased to 42.0% of the normal rat material, and, interestingly, the rate is similar to that of the lower band of nephrin (40.2%). Because a reduction in the mRNA expression of NEPH1 or nephrin was not detected by RT-PCR analysis, the decrease in the protein level of these molecules is considered to be a posttranslational change (Fig. 5B). Although the meanings of the alterations of these molecules are not fully understood, these observations suggest that NEPH1 and the lower-band nephrin were actively degraded or excreted into the urine at the very early phase of this disease. Because the decrease of podocin was not detected by Western blot analysis, the alteration of the podocin staining was due to the dislocalization caused by the decrease of other SD-associated molecules nephrin and NEPH1. Although the precise mechanisms of the alterations of these molecules have not yet been clarified, the alterations of nephrin, NEPH1, and podocin are involved in the initiation event of this disease.

To verify this hypothesis, in the present study we analyzed the kinetics of the expression of SD constituents nephrin, podocin, NEPH1, CD2AP, and ZO-1 in rat ADR nephropathy. Nephrin is a core molecule of the SD (38), and podocin is recognized as an important scaffolding molecule that interacts with nephrin (44). NEPH1 is understood to be another core molecule of the extracellular site of the SD (1, 29, 45). CD2AP and ZO-1 are understood to play a role in connecting SD components to the actin cytoskeleton (7, 28). In this study, we also analyzed the expression of an apical cell surface molecule of the podocyte, podocalyxin, and a basal molecule, integrin, that plays a major role in connecting the podocyte to the GBM.

We observed that abnormal proteinuria occurred on day 8 after ADR injection and that the proteinuria increased with time. The rate of proteinuria increase peaked on day 28, but the amount of proteinuria continued to increase gradually up to the 24th week. Therefore, we prepared kidney materials on day 7 just before proteinuria occurred, on day 28, and 24 wk after ADR injection.

We first analyzed the expression of SD-associated molecules on day 7 of ADR nephropathy. The staining patterns of nephrin, podocin, and NEPH1 clearly shifted from a linear pattern to a discontinuous dotlike pattern, and their staining brilliance decreased on day 7 (Fig. 3), although no alteration was detected in the expression of the other SD molecules, ZO-1 and CD2AP. The alteration of NEPH1 staining was more evident than that of nephrin and podocin (Fig. 4). We also observed that the staining pattern of NEPH1 had already

**Fig. 7. Interaction assay of nephrin and NEPH1.** A: interaction assay using normal rat glomerular lysate. To analyze the interaction between nephrin and NEPH1, sequential precipitation (IP) and Western blot analysis were performed with glomerular lysate solubilized with RIPA buffer. A positive band of NEPH1 was detected in the precipitate with anti-nephrin antibody. Nephrin was detected as double bands in the RIPA-solubilized glomerular lysate (c). Although the lower band of nephrin was clearly detected in the precipitate with anti-NEPH1 antibody, the upper band of nephrin was very faint (g). B: interaction assay using glomerular lysate from rats on day 7 of ADR nephropathy. A nephrin-rich fraction was prepared by immunoprecipitation with anti-nephrin antibody (n). The NEPH1 band detected in the anti-nephrin antibody-precipitated material was very faint (j), whereas the nephrin band (lower band) detected in the anti-NEPH1 antibody-precipitated material was clear (o).

**Fig. 8.** Dual-labeling IF findings of NEPH1 with nephrin in developing glomeruli. Dual-labeling IF findings of anti-NEPH1 antibody (green) and anti-nephrin antibody (red) in glomeruli from the early capillary-loop stage (D–F) and the late capillary-loop stage (A–C) are shown. NEPH1 staining was already detected in the presumptive podocytes at the early capillary loop stage, but major parts of the NEPH1 staining did not overlap with the nephrin staining (A–C). Almost all NEPH1 staining signals in the glomeruli from the late capillary loop stage were colocalized with the nephrin staining signals (D–F). Magnification: ×400.
To further elucidate the molecular events at the early phase of disease, we analyzed the interactions of these SD molecules, using dual-labeling IF and immunoprecipitation assay with glomerular lysate. On day 7 of ADR nephropathy, the staining patterns of NEPH1 and nephrin shifted to a discontinuous pattern. The shift of NEPH1 staining was more evident than that of nephrin, and some nephrin staining was not colocalized with that of NEPH1. By contrast, the shift in podocin staining paralleled that of nephrin. In the Western blot analysis we could detect a clear band of NEPH1 in the anti-nephrin antibody precipitate and a clear band of nephrin in the anti-NEPH1 antibody precipitate. It should be noted that nephrin was detected in the glomerular lysate as clear double bands by Western blot analysis. However, the upper band was very faint in the precipitate with anti-NEPH1 antibody, although the lower band of nephrin was clearly detected. These observations suggest that NEPH1 had a high affinity for the fraction of nephrin that was detected as the lower band. Next, we analyzed the interaction of these molecules in glomerular lysate of rats on day 7 of ADR nephropathy. Because the amounts of nephrin and NEPH1 in glomerular lysate of rats on day 7 of ADR nephropathy decreased, nephrin and NEPH1 were concentrated with an affinity column coupled with anti-nephrin antibody or anti-NEPH1 antibody. The NEPH1 band detected in anti-nephrin antibody-precipitated material was very faint (Fig. 7), which means that major parts of the nephrin remaining on day 7 had lost the interaction with NEPH1. On the other hand, a clear band of nephrin was detected in the anti-NEPH1 antibody-precipitated material, which means that some parts of NEPH1 remaining on day 7 still had the interaction with nephrin. These observations showed that the dissociation of NEPH1 from nephrin is a critical event in the initiation phase of ADR nephropathy.

Although several molecules have been identified as constituents of the SD in the past decade, the precise molecular nature and interactions of these SD constituents are not fully understood yet. Nephrin is understood to be a critical molecule for maintaining the barrier function of the SD. Recently, it has been postulated that nephrin plays multiple roles, including that of a filtration barrier (38). However, the molecular nature of nephrin is not precisely understood yet. As we and other groups have previously reported, nephrin was detected in glomerular lysate as double bands by Western blot analysis. Although the selective molecular nature of the nephrin detected as an upper and a lower band was uncertain, we previously reported (18) that the lower band of nephrin dominantly decreased in PAN nephropathy. In this study, we showed that the lower band of nephrin dominantly decreased in ADR nephropathy as well. We also demonstrated here that NEPH1 had a high affinity with the fraction of nephrin that was detected as the lower band. These findings may suggest that the fraction that was detected as the lower band is the active form of nephrin.

Although the proteinuria in ADR nephropathy continued to worsen with time, the increase rate of the proteinuria peaked on day 28. Therefore, we next analyzed the expression of the SD-associated molecules on day 28. Although fibrosis in the interstitium was not evident yet, severe mesangial matrix expansion was already observed on day 28 (Fig. 2B). At this stage no evident alteration in the staining pattern of integrin was detected, although decrease in the staining intensity of integrin and effacement of the foot processes were observed. Any alteration in the staining of podocalyxin, a podocyte apical cell surface molecule, or of the SD molecules CD2AP and ZO-1 was not detected on day 28. By contrast, the alterations of the expressions of NEPH1, nephrin, and podocin worsened, and their staining patterns changed to very patchy discontinuous patterns (Fig. 3). The causal relationship between this restricted alteration in the SD and the mesangial alteration is not clear. The important finding we would like to emphasize is that the alteration of the podocyte was still limited in these SD molecules when mesangial alteration was actively progressing. Some previous reports have shown that the podocyte damage was responsible for the expansion of the mesangial matrix (32, 46). In light of these previous reports, it is conceivable that this very restricted alteration in the SD is linked with the development of mesangial matrix expansion in ADR nephropathy.

Although an early start of the proper treatment for FSGS is important for preventing its progressive deterioration to renal failure, it is sometimes not easy to discriminate FSGS from other diseases with transient proteinuria, such as MCNS in its early stages. Detachment of the podocytes from the GBM is understood to be one of the most important pathological characteristics of FSGS, and it could be the specific characteristic that distinguishes FSGS from MCNS. However, this characteristic is not so frequently observed, and when it is it is usually seen in the late phase of disease. It is understood that integrin, which is located at the basal surface of the podocyte, is the principal molecule anchoring the podocyte to the GBM and that dysfunction of integrin results in detachment of the podocyte. However, in the present study on ADR nephropathy, neither detachment of the podocytes nor evident alteration of the integrin staining pattern was detected yet in the active phase of the disease (day 28), when severe proteinuria and mesangial matrix expansion were detected. Chen et al. (4) investigated the glomerular injury occurring in ADR nephropathy in α1β1-integrin-knockout mice and showed that there was no difference in the severity of proteinuria in the initiation phase between these mice and wild-type mice. Regele et al. (40) reported that expression of dystroglycan, another important molecule that connects the podocyte to the GBM, is reduced in MCNS but not in FSGS. It is plausible to assume that these findings suggest that detachment of the podocyte from the GBM is not an essential alteration causing the severe proteinuria and the glomerulosclerosis at the early stage of FSGS. In this study, we postulated that NEPH1 is the most sensitive molecule in ADR nephropathy, and that alteration in expression of this molecule may lead to severe progressive proteinuria and mesangial alterations in this disease. It is conceivable that alteration of NEPH1 staining could be an early marker of podocyte injury leading to glomerulosclerosis. NEPH1 was identified in mouse embryonic cells as a molecule that belongs to the nephrinlike immunoglobulin superfamily. It is reported that NEPH1-knockout mice show severe proteinuria at birth (6). Liu et al. (29) reported that the combined administration of antibodies against nephrin and NEPH1 caused proteinuria in mice, whereas the exclusive administration of anti-nephrin antibody failed to induce proteinuria. These reports indicate that NEPH1 is one of the critical SD components whose function is to maintain the SD’s barrier function. Recently, we have cloned a rat homolog of NEPH1 (GenBank AY249056) and revealed that rat NEPH1 has >90% identity...
with human and mouse NEPH1 at the amino acid sequence level. The high homology among these species also supports the idea that NEPH1 is a functional molecule of the SD. Although the precise role of NEPH1 in the SD is not fully clarified yet, the findings of this study suggest that NEPH1 could be included in the design of a novel FSGS therapy.

To better understand the role of nephrin-NEPH1 interaction in formation of the SD, we also examined the expression of nephrin and NEPH1 and their interaction in the presumptive podocyte of developing glomeruli. NEPH1 was first detected at the early capillary loop stage, but the NEPH1 staining at this stage was absent from the nephrin staining. Colocalization of NEPH1 and nephrin was observed at the later capillary loop stage, when the foot processes were in the process of interdigitating. This observation suggests that the interaction between nephrin and NEPH1 is one of the final important events in the formation of the SD and the podocyte-specific cytoskeletal organization of the interdigitating foot processes. Some studies have shown that NEPH1 interacts with ZO-1 (12, 29). Because ZO-1 interacts with the actin cytoskeleton, NEPH1 is considered to play a role in connecting the SD structure to the cytoskeleton through ZO-1. It should be mentioned that in this study we did not detect any alteration in the staining pattern of ZO-1, even in the active phase (day 28) of ADR nephropathy. Although it could not be well explained why the decrease in NEPH1 expression was not accompanied by that in ZO-1 expression in ADR nephropathy, one possible explanation is that because ZO-1 is not an SD-specific molecule and is also expressed at the tight junction newly formed in the injured podocyte, detection of a decrease in ZO-1 expression is difficult. Another explanation would be that NEPH1 has a route for connecting the SD with the cytoskeleton other than via ZO-1. Although the role of the interaction between NEPH1 and ZO-1 remains uncertain, it is conceivable that NEPH1-nephrin interaction in the SD is essential to maintenance of the specific cytoskeletal organization of the podocyte.

In conclusion, we showed that NEPH1 interacts with the fraction of nephrin detected as the lower band in Western blot analysis, and that the decreased expression and dissociation of NEPH1 and nephrin play a role not only in the development of proteinuria but also in mesangial matrix expansion in ADR nephropathy. We suggest that NEPH1 is the most sensitive molecule in this disease and that it could be an early marker of FSGS. We propose NEPH1 as one of the candidates for the design of a novel FSGS therapy. Although FSGS is considered to have multiple etiologies, this study on ADR nephropathy contributes to better understanding of the pathogenic mechanism of one type of FSGS.

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REFERENCES

NEPH1–NEPHRIN DISSOCIATION IN RAT FSGS MODEL


