Original Contribution Kitasato Med J 2011; 41: 115-122

Injection of rabbit polyclonal antibodies induced by genetic immunization with rat nephrin cDNA causes massive proteinuria in the rat: a new model of nephrotic syndrome

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Objectives: The protein nephrin is responsible for the Finnish-type nephrotic syndrome. Anti-nephrin antibodies produced in a patient with this syndrome after renal transplantation caused massive proteinuria. Here, we report the establishment of a rat experimental model of nephrotic syndrome using rabbit IgG generated by immunization with full-length rat nephrin cDNA.
Methods: Using a genetic immunization method, 8 female New Zealand white rabbits were administered full-length nephrin cDNA, a truncated nephrin cDNA containing its eight immunoglobulin-like motifs or an empty control vector. Polyclonal rabbit anti-rat nephrin IgG was administered to Wister Kyoto rats.
Results: Thirteen of 18 rats injected with rabbit anti-full-length-rat nephrin IgG showed significant proteinuria, which maximally reached more than 500 mg/day. None of the 44 rats injected with rabbit anti-truncated nephrin IgG or anti-empty vector IgG showed proteinuria. All 15 rats with proteinuria showed minor glomerular abnormalities with podocyte foot process effacement.
Conclusions: We successfully established a new rat model of minimal change nephrotic syndrome by genetic immunization.

Key words: nephrotic syndrome, nephrin, genetic immunization, rat model

Introduction

Nephrotic syndrome is characterized by a massive leakage of plasma proteins into urine through the glomerular filtration barrier consisting of endothelial cells, basement membrane and podocytes. Recent advances have revealed some of the proteins located in the glomerular podocyte that are responsible for nephrotic syndrome, such as nephrin, podocin, CD2-associated protein (CD2-AP), transient receptor potential cation channel 6 (TRPC6) and α actinin 4.[1-6] However, the molecular mechanisms of the nephrotic syndrome in glomerular podocytes and the filtration barrier have not yet been elucidated. The nephrin gene was identified as responsible for the Finnish-type of the congenital nephrotic syndrome in 1998.[7] Nephrin protein is a major component of the slit diaphragm between the podocytes.[8] It has eight extracellular immunoglobulin (Ig)-like domains, a fibronectin type III domain, a transmembrane domain and a cytoplasmic C-terminal domain.[9] Mature nephrin exhibits N-linked glycosylation but not O-linked glycosylation,[10,11] and it is located on the cell surface of glomerular podocytes. Anti-nephrin antibodies produced in a patient with Finnish-type nephrotic syndrome after renal transplantation caused massive proteinuria through the transplanted kidney.[12] We previously reported the production of polyclonal anti-nephrin antibodies induced by genetic immunization with human nephrin cDNA. These antibodies bind the nephrin protein located in the slit diaphragm between the foot processes of the glomerular podocytes.[13,14] Here, we describe a rat experimental model of nephrotic syndrome generated by injection of IgG from rabbits immunized with rat nephrin cDNA. This model may contribute to the elucidation of the molecular mechanisms of the Finnish-type nephrotic syndrome and the pathophysiology of the nephrotic

Received 29 October 2010, accepted 9 November 2010
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syndrome.

Materials and Methods

Experimental animals

Eight female New Zealand white rabbits weighing 2-3 kg were purchased from the Charles River Breeding Laboratory (Atsugi) and kept in our breeding laboratory for 2 weeks. Eight-week-old female Wister Kyoto (WKY) rats weighing 150-170 g were obtained from the Charles River Breeding Laboratory. The rats were kept on a constant 12-hour dark and light cycle and fed standard laboratory chow (SLC Japan, Shizuoka) with free access to water. All animal experiments were performed under an experimental protocol approved by the Ethical Review Committee for Animal Experiments of Kitasato University School of Medicine.

Immunization plasmids

Full-length cDNA encoding rat nephrin[15] was synthesized and purified by TaKaRa Custom Services (Ohtsu). An extracellular domain, truncated nephrin cDNA including the Ig-like motifs 1-8 (amino acids 1-957; termed nephrin$_{1\text{-}957}$) was generated by PCR using full-length rat nephrin cDNA as a template. The 3' primers were designed to contain a stop codon in-frame with a *Not* I site, and the 5' primers were designed with an upstream *Eco* RI site. PCR-amplified cDNA fragments were cleaved using *Not* I and *Eco* RI, then purified by gel filtration. The full-length and nephrin$_{1\text{-}957}$ cDNAs were inserted into the pAP3neo™ mammalian expression vector containing an SV40 promoter (TaKaRa, Shiga). The two expression vectors were transformed into *E. coli* JM 109 competent cells (TaKaRa) according to the manufacturer's protocol. After culturing for 2 days, plasmids were harvested using a QIAGEN Plasmid Maxi Kit (QIAGEN, Tokyo). The authenticity of the cDNA constructs was confirmed by sequencing using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences AB, Uppsala, Sweden).

Administration of plasmid DNA into rabbits and purification of rabbit IgG

The expression vectors were bound to gold particles of 1-μm diameter (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Gold particles covered with plasmid vectors were injected into the subcutaneous tissue of the bilateral inner thighs of 2-3 rabbits in each group. Thirty micrograms of plasmid DNA was administered 4 times, once every 2 weeks. An empty expression vector, without nephrin cDNA, was similarly administered to 3 rabbits in the control group. Rabbit sera were taken at week 8 (2 weeks after the last administration of plasmid DNA). Serum samples from all rabbits were processed by ammonium sulfate precipitation as follows. Fifty milliliters of serum were stirred and an equal volume of saturated ammonium sulfate solution was slowly added, then stored at 4℃ overnight. Samples were centrifuged at 3,000 ×g for 20 minutes, and the precipitates were dissolved in Melon™ Gel IgG Purification Buffer (Thermo-Scientific, Waltham, MA, USA). They were then dialyzed with Melon™ Gel IgG Purification buffer, using a volume 300-fold greater than that of the samples. The samples were purified through a column of the Melon™ Gel IgG purification kit. The eluates were collected and concentrated using Amicon 15 filter units (Millipore Corporation, Billerica, MA, USA). Finally, samples were dialyzed with 0.2 M sodium phosphate buffer (pH 7.4). The purities of rabbit IgG were more than 99% when analyzed by SDS-polyacrylamide gel electrophoresis.

Administration of rabbit anti-rat nephrin IgG to WKY rats and urine sampling

Rabbit anti-rat nephrin IgG (2, 4, 8, or 16 mg) in 1.0 ml phosphate-buffered saline (PBS) was administered to WKY rats intra-arterially through the tail artery. Urine samples were collected every 24 hours using metabolic cages, from day 1 to day 15, for the determination of urinary protein excretion by the biuret method. Urine obtained 0.5, 1, 2, 3, and 8 hours after IgG injection was tested semi-quantitatively for proteinuria using a dipstick test. Blood samples and kidney specimens were taken at 0.5, 1, 2, 3, and 8 hours, and at 3, 9, and 15 days after IgG injection.

Light microscopy

Renal cortices were fixed in buffered formalin (pH 7.2). Specimens were dehydrated through an ethanol-xylol series and embedded in paraffin. The renal cortices were cut into 3-4-μg sections and stained with hematoxylin and eosin or periodic acid-Schiff and then examined.

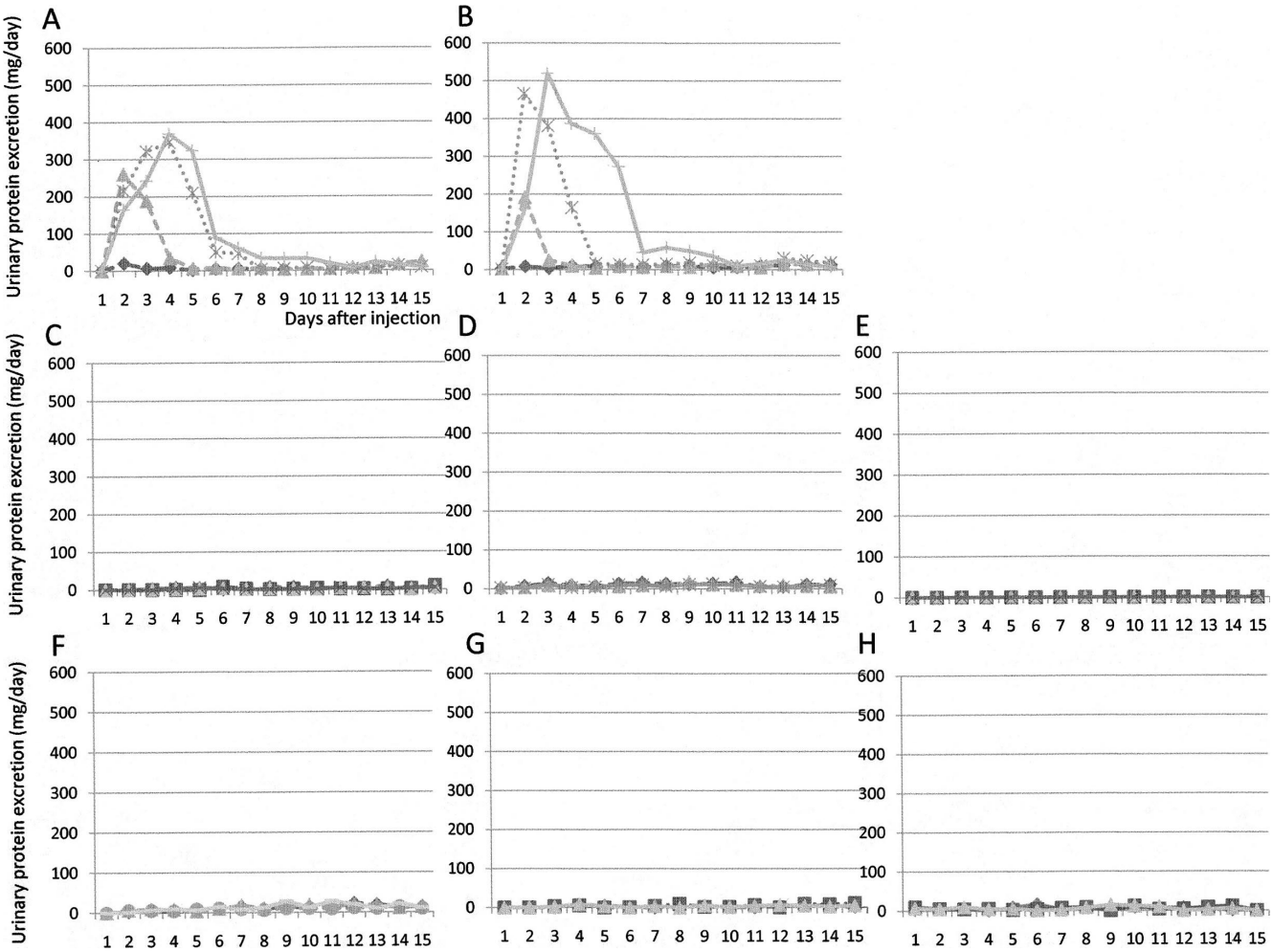
Immunofluorescence

Small blocks of renal cortex were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. The frozen samples were sliced into 3-μm sections then fixed with ice-cold acetone for 5 minutes. After washing with PBS for 5 minutes, the cryostat sections were reacted with fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz,

CA, USA) for 40 minutes at room temperature. Then, after washing 3 times with PBS, they were evaluated using a fluorescence microscope equipped with appropriate filters (Olympus, BX 51, Tokyo).

Electron microscopy

The renal cortex was cut into small pieces and prefixed with 2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) for several days at 4℃. They were then washed in 0.1 M-cacodylate buffer (pH 7.4) and postfixed in 2% OsO_4 in 0.1-M-cacodylate buffer (pH 7.4) for 1 hour. After the material was dehydrated with ethanol, the buffer was substituted with QY-1, and the samples were embedded in Quetol 812 resin mixture. Ultrathin sections (80 nm) were prepared and then stained with 3% uranyl acetate and lead citrate (the Reynold's method). Ultrathin sections of renal cortex were evaluated under an electron microscope (model JEX-1200EX; JEOL Ltd., Tokyo).



A, B. Urinary protein excretions in rats administered anti-full-length rat nephrin IgG. (**A**) Data generated using IgG from rabbit no. 5. Protein excretion started immediately following injection of IgG and attained peak levels of 288 ± 58 mg/day (n = 3) on day 2 with 4 mg injected, 346 ± 104 mg/day (n = 3) on day 4 with 8 mg injected and 369 mg/day (n = 1) on day 4 with 16 mg injected. Urinary protein levels returned to normal by day 15. Administration of 2 mg of IgG did not induce significant proteinuria (n = 2) in rats. (**B**) Data generated using IgG from rabbit no. 6. Urinary protein excretion reached peak levels of 191 ± 107 mg/day (n = 3) on day 2 with 4 mg, 466 ± 38 mg/day (n = 2) on day 2 with 8 mg injected and 520 mg/day (n = 1) on day 3 with 16 mg injected. Administration of 2 mg rabbit IgG did not induce proteinuria (n = 3).

C-E. Urinary protein excretion in rats administered rabbit anti-rat nephrin$_{1\text{-}957}$ IgG. n = 3 for 2, 4, or 8 mg injected (except for n = 2 for rabbit no. 2) and n = 1 for 16 mg injected. (**C**) IgG from rabbit no. 1. (**D**) IgG from rabbit no. 2. (**E**) IgG from rabbit no. 3. No abnormal proteinuria was observed.

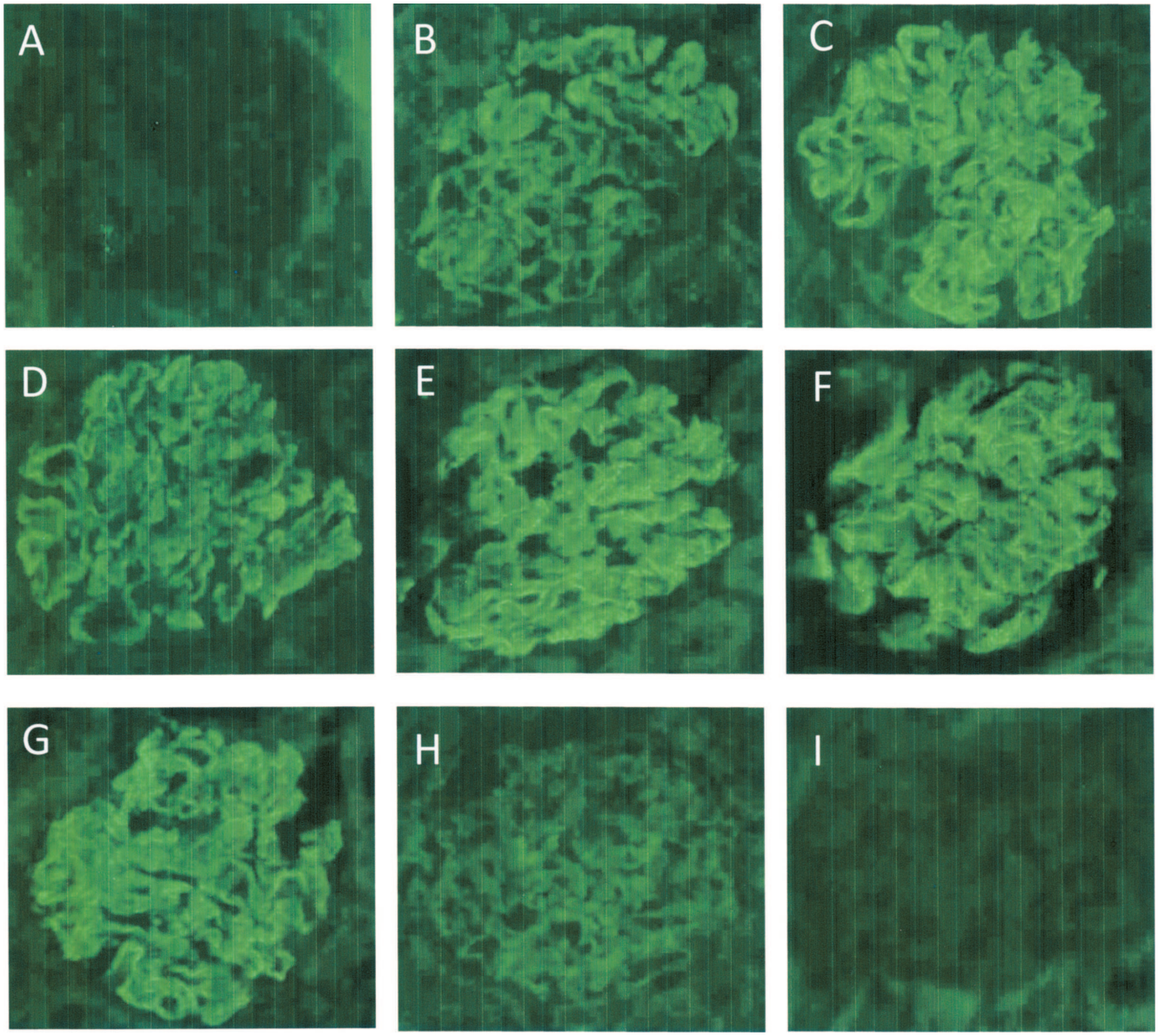
F-H. Urinary protein excretion in rats administered rabbit anti-empty vector IgG. n = 2 for 4 mg injected, n = 2 for 8 mg injected, n = 1 for 16 mg injected. (**F**) IgG from rabbit no. 7. (**G**) IgG from rabbit no. 8. (**H**) IgG from rabbit no. 9. No abnormal proteinuria was observed.

Figure 1. Urinary protein excretion in WKY rats administered purified rabbit IgG induced by rat nephrin cDNA immunization

Results

Proteinuria in WKY rats

The time course of urinary protein excretion in rats administered rabbit IgG is shown in Figure 1. As shown in panels 1A and 1B, 4, 8, and 16 mg of two different anti-full-length nephrin IgG samples caused a dose-dependent, massive proteinuria in rats. Significant proteinuria was detected 2 hours after intra-arterial administration of 4 mg IgG and thereafter. When 16 mg of IgG was administered, the maximum proteinuria reached more than 500 mg per day on day 3, before it gradually decreased. In contrast, 2 mg or less of two different rabbit anti-full-length nephrin IgG samples did not induce proteinuria during this time course (data not shown). Similarly, no proteinuria was induced by any dose (2, 4, 8, or 16 mg) of three different anti-nephrin$_{1\text{-}957}$ IgG samples (Figure 1C-E). Three different rabbit IgG samples generated using the empty plasmid also induced no proteinuria (Figure 1F-H).



Kidney specimens were stained with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (×400). Kidney specimens were obtained before (**A**), 30 minutes (**B**), 1 hour (**C**), 2 hours (**D**), 3 hours (**E**) and 8 hours (**F**), and on days 3 (**G**), 9 (**H**), and 15 (**I**) after rabbit IgG administration. Rabbit IgG bound to rat glomeruli 30 minutes (**B**) after *in vivo* injection of rabbit IgG was maintained until day 3 (**G**) and had disappeared by day 15 (**I**).

Figure 2. Immunofluorescence findings in kidney specimens obtained at various times after intra-arterial administration of 4 mg rabbit IgG induced by immunization with full-length rat nephrin cDNA

Glomerular histology

Light microscopic findings (Table 1): Glomerular histology of kidney specimen obtained on day 15 after intra-arterial injection of rabbit anti-nephrin IgG is shown in Table 1. No focal glomerular sclerosis lesions were observed in any kidney glomeruli on day 15 after injection of anti-rat full-length nephrin IgG, anti-rat nephrin$_{1\text{-}957}$ IgG or anti-empty vector IgG. Also, there was no significant difference in the number of glomerular adhesions among the 3 groups of rats.

Immunofluorescence findings (Figure 2): Thirty minutes after injection, the kidneys of rats administered rabbit anti-full-length nephrin IgG demonstrated IgG bound to the glomeruli along the basement membrane (Figure 2B). Glomerular binding of rabbit IgG was sustained until day 3, but had decreased on day 9 (Figure 2H) and disappeared by day 15 (Figure 2I).

Electron microscopic findings (Figure 3): Abnormalities of the glomerular epithelial cell foot process were seen only in the rats injected with rabbit anti-full-length nephrin IgG. Effacement of the glomerular epithelial cell foot processes was initially shown 1 hour after rabbit IgG injection (Figure 3C). Foot process abnormalities were evident at 2, 3, and 8 hours and on day 3 after IgG administration (Figure 2E-G, respectively). Foot process abnormalities had partially recovered by day 9 (Figure 2H) and completely recovered by day 15 (Figure 2I). Abnormalities of glomerular epithelial cell foot processes were not seen throughout the course of the experiment in the rats injected with rabbit anti-nephrin$_{1\text{-}957}$ IgG or anti-empty vector IgG (data not shown).

Discussion

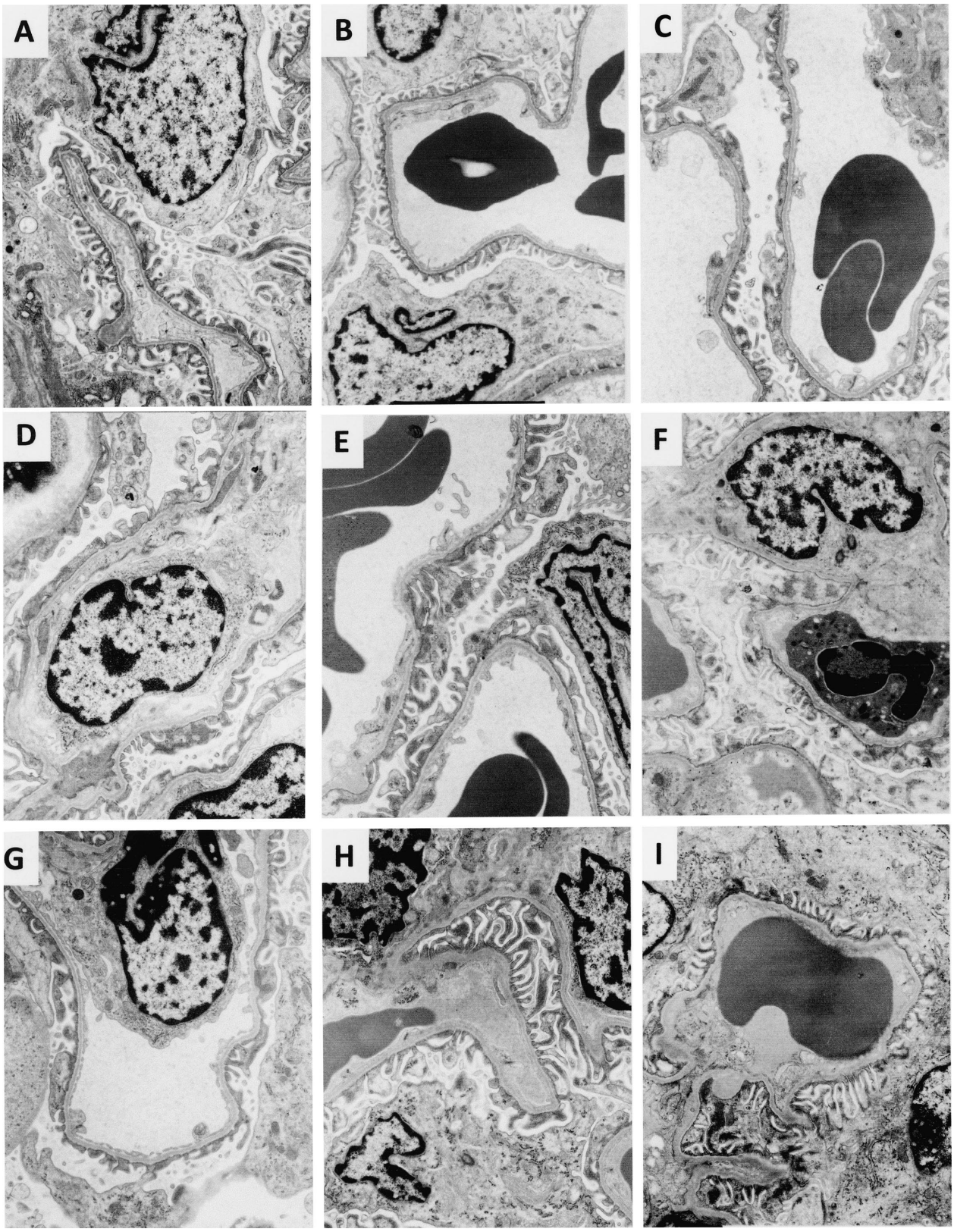
We successfully established a new model of rat nephrotic syndrome using a genetic immunization method. This rat nephrotic model is easily reproducible by the injection of polyclonal rabbit anti-rat nephrin antibodies produced by genetic-immunization with full-length rat nephrin cDNA. This method circumvents the time-consuming steps required to obtain pure nephrin protein or recombinant nephrin protein for immunization.

Previous studies have shown that rabbit polyclonal antibodies induced by genetic-immunization with full-length rat nephrin cDNA recognize multiple antigenic epitopes of the rat nephrin protein but do not recognize any other molecules.[13,14] After *in vivo* administration in WKY rats, these polyclonal anti-nephrin antibodies bind to the glomerular capillary walls and cause massive proteinuria. The rabbit anti-rat nephrin antibodies may react with the nephrin protein on the plasma membrane but do not directly injure the glomerular podocytes. The massive proteinuria is induced by changes to the nephrin protein in the slit diaphragm between the foot processes of the glomerular podocytes. Immunohistological examination of the kidneys in this rat model showed minor glomerular abnormalities with foot process effacement of the glomerular podocytes. Accordingly, this rat model is a counterpart of the minimal change nephrotic syndrome in humans.

In 1988, Orikasa et al. reported that a single intravenous injection of the monoclonal anti-nephrin antibody 5-1-6 into rats caused a massive, though

Table 1. Glomerular histology of kidney specimens

Rabbit anti-nephrin IgG antibody induced by:	No. of rabbits	Amount of rabbit IgG injected (No. of rats tested)	No. of glomeruli with FSGS lesion/ No. of glomeruli examined	Mean number of adhesion in a glomerulus (No. of rats tested, No. of glomeruli examinied in each kidney)
Full-length nephrin cDNA	2	2 mg (n = 5)	0/50	3.46 ± 0.31 (5, 50)
		4 mg (n = 6)	0/50	3.39 ± 0.44 (6, 50)
		8 mg (n = 5)	0/50	3.50 ± 0.34 (5, 50)
		16 mg (n = 2)	0/50	3.78 ± 0.28 (2, 50)
cDNA with Ig-like motifs 1-8	3	2 mg (n = 9)	0/50	3.56 ± 0.21 (9, 50)
		4 mg (n = 9)	0/50	3.46 ± 0.28 (9, 50)
		8 mg (n = 8)	0/50	3.54 ± 0.58 (8, 50)
		16 mg (n = 3)	0/50	3.77 ± 0.16 (3, 50)
Plasmid without nephrin cDNA	3	4 mg (n = 6)	0/50	3.37 ± 0.48 (6, 50)
		8 mg (n = 6)	0/50	3.60 ± 0.18 (6, 50)
		16 mg (n = 3)	0/50	3.44 ± 0.48 (3, 50)



Magnification ×6,500. Kidney specimens were obtained before (**A**), 30 minutes (**B**), 1 hour (**C**), 2 hours (**D**), 3 hours (**E**), and 8 hours (**F**), and on days 3 (**G**), 9 (**H**), and 15 (**I**) after rabbit IgG administration. There were no morphological abnormalities in the foot processes of glomerular podocytes before (**A**) and 30 minutes after (**B**) rabbit IgG administration. Glomerular epithelial cell foot process fusions were initially seen 1 hour after rabbit IgG administration (**C**). Partial foot process effacement was maintained until day 3 (**G**) and had disappeared by day 15 (**I**).

Figure 3. Electron microscopic findings in rat kidney glomeruli obtained at various times after *in vivo* administration of 4 mg rabbit IgG induced by immunization with full-length rat nephrin cDNA

transient, proteinuria.[16] Eleven years later, the extracellular domain of the rat nephrin protein was reported to be the binding site of monoclonal antibody 5-1-6.[17] Two milligrams of this antibody 5-1-6 induced a maximum proteinuria of approximately 150 mg/day on day 8, and the proteinuria had disappeared by day 18. In our experiment, 16 mg of cDNA-induced polyclonal rabbit anti-rat nephrin antibodies produced a maximum proteinuria of approximately 500 mg/day on day 3, and the proteinuria had disappeared by day 12. The difference in the scale of the proteinuria may depend on the number of antigenic epitopes in the nephrin protein, because the monoclonal antibody 5-1-6 recognizes only one epitope, while the rabbit polyclonal antibody binds multiple epitopes of the nephrin protein. The specific locations of the epitopes may also affect the proteinuria; these are not yet known for four of our rabbit polyclonal antibodies. Despite the differences in scale, the polyclonal and monoclonal anti-nephrin antibodies showed almost the same time course of proteinuria. This may indicate the same processing system for the two different immune complexes in the plasma membrane of podocytes. The most important difference between these 2 antibodies is the reproducibility of the antibody. As a particular monoclonal antibody can only be generated by chance, the rat nephrotic syndrome model using monoclonal antibody 5-1-6 has not been widely used. On the other hand, our nephrotic syndrome model using a nephrin cDNA-induced polyclonal antibody is easily reproducible and may be commonly used.

In the former models of rat nephrotic syndrome, such as puromycin aminonucleoside nephrosis and adriamycin-induced nephrotic syndrome, massive proteinuria was induced by damage to the glomerular podocytes. In these models, the molecular mechanisms of the nephrotic syndrome cannot be studied because of widespread damage to the metabolic pathways in glomerular podocytes. In the latter model, however, study of the molecular mechanisms may be possible because only the nephrin protein on glomerular podocytes is affected, and there is no large-scale damage to the glomerular podocytes.

In conclusion, we have successfully established a new model of the rat nephrotic syndrome that resembles the minimal change nephrotic syndrome in humans.

Acknowledgments

We thank Mrs. Naoko Ishigaki for technical assistance.

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