A new mouse experimental model of focal segmental glomerulosclerosis produced by the administration of polyclonal anti-mouse nephrin antibody

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Objectives: Nephrin is a component of the slit diaphragm between podocyte foot processes. Deficiencies or mutations of nephrin protein result in the nephrotic syndrome in humans. This study was designed to establish a mouse model of nephrotic syndrome using rabbit IgG against mouse nephrin cDNA.

Methods: Female New Zealand White rabbits were immunized 4 times every 2 weeks with 30 μg expression vector containing mouse nephrin cDNA encoding full-length, the immunoglobulin (Ig)-like 1-8 motifs or the fibronectin motif. After 8 weeks, rabbit IgG was purified, and 4 mg was administered to C57BL/6N mice. Urinary protein, serum albumin, serum total cholesterol, and kidney specimens were evaluated.

Results: Administration of rabbit IgG induced by full-length mouse nephrin cDNA resulted in massive proteinuria in 18 C57BL/6N mice from days 1 to 14. Mouse serum albumin decreased on days 3 and 7, whereas serum total cholesterol increased on days 3, 7, and 14. Focal segmental glomerulosclerosis was observed in 9 mouse kidney specimens on days 7 to 28. Control rabbit IgG did not induce proteinuria or kidney disease in mice.

Conclusion: We successfully established a new mouse model of focal segmental glomerulosclerosis with nephrotic syndrome.

Key words: focal segmental glomerulosclerosis, nephrin, genetic immunization, C57BL/6N mice

Introduction

Glomerular capillary walls, consisting of endothelial cells, glomerular basement membrane and podocytes, constitute the barrier that filters plasma proteins into urinary space. The slit diaphragm bridging foot processes of podocytes have a porous proteinaceous membrane, composed of nephrin, NEPH 1 and 2, P-cadherin, filtrin and FAT 1 and 2, and play a major role in this glomerular filtration barrier. Nephrin is a protein consisting of a large extracellular domain with 8 immunoglobulin (Ig)-like motifs and 1 fibronectin type-III motif, a single transmembrane region and an intracellular domain. Nephrin is linked to the intracellular actin-based cytoskeleton through adaptor proteins, including CD2-associated protein (CD2-AP), zonula occludens (ZO)-1, podocin, β-catenin, and Nck.

Mutations of the NPHS1 gene, which encodes nephrin protein, have been found to cause various types of the congenital nephrotic syndrome, including the nephrotic syndrome of the Finnish type (CNF); congenital, childhood and adulthood focal segmental glomerulosclerosis (FSGS); and minimal change nephrotic syndrome (MCNS). Recurrence of kidney diseases after kidney transplantation in patients with FSGS or CNF is partially due to autoantibodies to nephrin protein.

Inducible non-diabetic experimental models of FSGS and MCNS include models of puromycin aminonucleoside nephrosis and adriamycin nephrosis, as well as anti-glomerular protein antibody, lipopolysaccharide, podocyte injury by immunotoxin,
protamine sulfate, and protein overload models, all of which result in extensive damage to glomerular podocytes.10,11 MCNS models of rat can be generated by administering mouse monoclonal antibody 5-1-6 against rat nephrin protein12,13 or rabbit polyclonal antibody produced by genetic immunization with rat nephrin cDNA.14 In these models, glomerular podocytes had a limited effect through the nephrin protein with a binding of monoclonal or polyclonal anti-nephrin antibody. Following nephrin-dependent signaling,15 constituents of glomerular podocytes may change, inducing proteinuria, with a spontaneous recovery of podocyte constituents resulting in the disappearance of proteinuria.

Antibodies produced by genetic immunization with full-length or fragmented human nephrin cDNA showed site-specific reactivity with human nephrin protein. Rat antibodies induced by full-length or fragmented human nephrin cDNA encoding Ig-like motifs 1-2 and 1-8 also caused clustering of human nephrin protein on plasma membranes of human embryonic kidney (HEK) 293 cells.15 This cluster formation is induced by tyrosine phosphorylation of the intracellular domain of nephrin protein by Src family protein kinase p59fyn (Fyn).16 These anti-nephrin antibodies may signal the intracellular components of glomerular podocytes. This study describes a new mouse experimental model of nephrotic syndrome, induced by administration of rabbit IgG against mouse nephrin cDNA. This experimental model may contribute to understanding of the molecular mechanisms of proteinuria and podocyte injury that develop into FSGS.

Materials and Methods

Animals

Fourteen female New Zealand white rabbits weighing 2–3 kg and 10-week-old male C57BL/6N mice weighing 19–24 g were purchased from the Charles River Breeding Laboratory (Atsugi) and kept in our breeding laboratory for 2 weeks. Animals 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 mouse nephrin cDNA with signal sequence (Gene ID: 54631; Protein ID: NP_062332.2; amino acid: aa 1-1256) and 2 mouse nephrin cDNA fragments, encoding Ig-like motifs 1-8 plus signal sequence (amino acid: aa 1-953) and the fibronectin type III, single-span transmembrane region and intracellular motifs plus signal sequence cDNA (aa 1-35; aa 955-1256), were synthesized and inserted into the pAP3neo mammalian expression vector containing a SV40 promoter by Takara Custom Services (Takara, Ohtsu). The control vector consisted of pAP3neo alone without nephrin cDNA. Each expression vector was used to transform Escherichia coli (E. coli) JM109 competent cells (Takara). After culturing for 2 days, plasmids were harvested using QIAGEN Plasmid Maxi Kits (QIAGEN, Tokyo) according to the manufacturer's protocol. The authenticity of the cDNA constructs was confirmed by sequencing with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences AB, Uppsala, Sweden).

Immunization with expression vectors containing mouse nephrin cDNA

The plasmids were coated onto gold particles 1 μm in diameter according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). These particles were administered into the subcutaneous tissue of both inner thighs of rabbits using the gene gun method (Bio-Rad). Each rabbit was given 30 μg plasmid DNA 4 times every 2 weeks. Rabbit serum was obtained 2 weeks after the last administration of plasmid DNA.

Purification of rabbit IgG

Rabbit sera were processed by ammonium sulfate precipitation. Precipitated IgG was resuspended and dialyzed 3 times against a 300-fold volume of Melon Gel IgG Purification Buffer (Thermo Fisher Scientific, Rockford, IL, USA). Rabbit IgG were purified by loading onto a column of the Melon Gel IgG purification Kit. The eluates were collected, concentrated using Amicon Ultra-15 filters (Millipore, Billerica, MA, USA), and dialyzed against 0.2M sodium phosphate buffer (pH 7.4). The purity of each rabbit IgG sample was almost 80% when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Administration of rabbit anti-mouse nephrin IgG to C57BL/6N mice

Each C57BL/6N mouse was injected in the tail vein with 4 mg rabbit IgG, produced by injection of cDNA encoding full-length mouse nephrin, its Ig-like motifs 1-8 or its fibronectin motif, in 0.3 ml of 0.2 M sodium phosphate buffer (pH 7.4). As a control, mice were injected with 4 mg rabbit IgG produced by the expression vector in the absence of mouse nephrin cDNA.
Collections of 24-hour urine samples, blood samples, and kidney specimens

Mice were housed in metabolic cages and 24-hour urine samples were obtained each day from day 0 to day 7 and on days 9, 14, 17, 21, 24, and 28 after administration of rabbit IgG. Mean urinary protein excretion was evaluated in 2 mice administered with IgG. The normal range of proteinuria in C57BL/6N mice was determined by assaying 84 urine samples of mice administered with 4 mg of IgG produced in rabbit vector DNA alone. The upper limit of the normal range of proteinuria was calculated as 29.7 mg/mg Cr. Blood samples were obtained from mice on days 0, 3, 7, 14, and 28, and kidney samples on days 2, 7, and 28, after administration of rabbit IgG.

Blood and urine chemistry

Urinary creatinine and protein concentrations were measured using an enzymatic method and the pyrogallol red method, respectively. The amount of urinary protein was expressed as mg protein per mg creatinine. Serum albumin and total cholesterol were measured using DRI-CHEM 7000V (Fuji Film, Tokyo).

Light microscopy

Small blocks of renal cortices were fixed in buffered formalin (pH 7.2), dehydrated through an ethanol-xylol series and embedded in paraffin. Renal cortices were cut into 3-4 μm sections and stained with hematoxylin and eosin or periodic acid-Schiff.

Direct and indirect 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 2-3 μm sections, which were fixed with ice-cold acetone for 5 minutes.

For indirect immunofluorescence, rabbit sera before and after cDNA immunization were diluted 50-fold with PBS and incubated with kidney sections of normal C57BL/6N mice for 1 hour at room temperature and overnight at 4°C. After 3 washes with PBS, the samples were incubated with a 1:200 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) for 40 minutes at room temperature and washed with PBS 3 times.

For direct immunofluorescence, fixed cryostat sections of mouse kidneys obtained after systemic administration of rabbit IgG were incubated with a 1:200 dilution of FITC-labeled goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) for 40 minutes at room temperature. After 3 washes with PBS, these kidney sections were evaluated using a fluorescence microscope equipped with appropriate filters (Olympus, BX51, Tokyo).

Electron microscopy

Small blocks of renal cortex were prefixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for several days at 4°C. The tissue samples were washed in 0.1M sodium cacodylate buffer (pH 7.4) and postfixed in 2% OsO4 in 0.1M sodium cacodylate buffer (pH 7.4) for 1 hour. Following dehydration with ethanol, the buffer was replaced with QY-1, and the samples were embedded in a Quetol 812 resin mixture. Eighty-nanometer ultrathin sections of renal cortex were prepared and stained with 3% uranyl acetate and lead citrate using the Reynolds method. Ultrathin sections of renal cortex were evaluated by electron microscopy (JEX-1200EX; JEOL, Tokyo).

Statistical analyses

Values are expressed as mean ± 1 SEM throughout this text. Mann-Whitney U tests were used to compare the 2 groups, whereas the Kruskal-Wallis test and the Dunn method were used for multiple comparisons. A P value of <0.05 was considered statistically significant.

Results

Binding to mouse nephrin protein by rabbit antisera immunized with mouse nephrin cDNA

The ability of rabbit antisera obtained at 8 weeks after mouse nephrin cDNA immunization to bind to normal C57BL/6N mouse kidney specimens was evaluated by indirect immunofluorescence (Table 1, Figure 1).

<table>
<thead>
<tr>
<th>Sera from rabbits immunized with the cDNA of</th>
<th>No. sera</th>
<th>No. sera graded as</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector only</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Ig-like 1-8 motifs</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Fibronectin motif</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Full-length nephrin</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

Intensity of immunofluorescence (IF) was evaluated as negative (-), weakly positive (+), moderately positive (#) or strongly positive (##)
Figure 1. Indirect immunofluorescence of rabbit sera immunized with fragmented or full-length nephrin cDNA (magnification ×800)

Rabbit sera obtained 8 weeks after immunization with (A) the vector alone or cDNAs encoding (B) nephrin Ig-like motifs 1-8 or (C) nephrin fibronectin III motif, or (D) full-length nephrin was overlaid onto normal kidney specimens from C57BL/6N mice. After washing, the specimens were incubated with FITC-labeled goat anti-rabbit IgG.

Figure 2. Clinical courses of urinary protein excretion by C57BL/6N mice injected with IgG from rabbits immunized with mouse nephrin cDNA

Mice were injected with 4 mg of purified IgG from rabbit sera obtained 8 weeks after immunization with (A) the vector alone or cDNAs encoding (B) nephrin Ig-like motifs 1-8 or (C) nephrin fibronectin III motif, or (D) full-length nephrin, and the course of proteinuria was assessed over time. Each point represents the mean level of proteinuria in 2 mice.

The hatched area indicates a normal range of proteinuria, defined as the mean ± 3 SD of urinary proteins in mice administered with 4 mg IgG from rabbits injected with the vector alone.
Antisera from 4 of 5 rabbits, immunized with full-length mouse nephrin cDNA, showed a granular staining pattern along the glomerular basement membrane (GBM) (Figure 1D), whereas the fifth serum sample did not react with normal glomeruli of C57BL/6N mice. Serum from 1 rabbit immunized with cDNA encoding the nephrin Ig-like motifs 1-8 showed weakly positive binding to normal mouse glomeruli (Figure 1B), whereas the other 2 sera were negative. Sera from all 3 rabbits immunized with cDNA encoding fibronectin type III and from all 3 rabbits injected with empty vector were negative for binding to mouse glomeruli.

Urinary protein excretion by mice administered with rabbit antibody to mouse nephrin cDNAs

Clinical courses of urinary protein excretions by C57BL/6N mice before and after a single intravenous administration of 4 mg purified rabbit IgG are shown in Figure 2. IgG from 4 of 5 rabbits immunized with full-length mouse nephrin cDNA induced significant proteinuria in mice, beginning on day 1, maximizing on day 4 at 100–150 mg/mg•Cr and gradually decreasing, but still remaining high, through day 14 and continuing at a lower level thereafter (Figure 2D). Among mice with systemic edema and ascites, 1 died on day 6, and 2 died on day 17. IgG from the fifth rabbit immunized with full-length mouse nephrin cDNA was unable to induce significant proteinuria, as was the IgG from the 3 rabbits immunized with cDNA encoding Ig-like motifs 1-8, the 3 rabbits immunized with cDNA encoding the fibronectin type III motif, and the 3 rabbits injected with the vector alone.

Clinical courses of serum albumin and serum total cholesterol

Serum albumin levels in C57BL/6N mice administered with IgG from rabbits immunized with full-length mouse nephrin cDNA decreased significantly, to 1.29 ± 0.04 g/dl on day 3, gradually returning to normal levels thereafter. Serum albumin levels were unaffected through day 28, however, in mice administered with IgG from rabbits injected with cDNAs encoding Ig-like motifs 1-8 or fibronectin type III motif or the vector alone (Figure 3A).

Administration of IgG from rabbits immunized with full-length mouse nephrin cDNA markedly increased total serum cholesterol concentrations, to 509 ± 27 mg/dl on day 3, with these levels remaining high through day 14. Serum total cholesterol concentrations, however, were unchanged in mice administered with IgG from rabbits injected with cDNAs encoding Ig-like motifs 1-8 or fibronectin type III motif or the vector alone (Figure 3B).

Renal histology

Examination of kidney specimens showed FSGS in all 5 C57BL/6N mice euthanized 7 days after administration

![Figure 3](image-url)

**Figure 3.** Clinical courses of (A) serum albumin and (B) total cholesterol in C57BL/6N mice injected with IgG from rabbits immunized with mouse nephrin cDNA

- ○: Mice injected with IgG from rabbits immunized with vector alone, ●: Mice injected with IgG from rabbits immunized with full-length mouse nephrin cDNA, ■: Mice injected with IgG from rabbits immunized with Ig-like motifs 1-8 of mouse nephrin cDNA, ▲: Mice injected with IgG from rabbits immunized with fibronectin type III motif of mouse nephrin cDNA.

* Mean ± 1 SE * P < 0.05 vs. vector alone
of IgG from rabbits immunized with full-length mouse nephrin cDNA, as well as in 1 mouse euthanized on day 28 (Table 2). Similar findings were observed in the 1 mouse in this group that died on day 6 and the 2 mice that died on day 17 from systemic edema. FSGS lesions on day 7 showed focal segmental cell proliferation and focal segmental degeneration (Figures 4E, 4F). Glomerular lesions in the mice that died on day 17 and those euthanized on day 28 showed typical focal segmental sclerosis considered FSGS (Figures 4G, 4H). All kidney specimens from the 5 mice in this group euthanized on day 2 showed minor glomerular abnormalities (Figure 4D) with the clustering of nephrin protein in mouse glomeruli (Figure 5D). Electron microscopic examination of these mouse kidney glomeruli showed diffuse effacement of glomerular podocytes (Figure 6D).

Table 2. Glomerular histology of mouse kidney specimens following administration of IgG from rabbits immunized with mouse nephrin cDNA

<table>
<thead>
<tr>
<th>IgG from rabbits immunized with the cDNA of:</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 28</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. of mice</td>
<td>No. of mice with Minor glomerular abnormality</td>
<td>No. of mice</td>
</tr>
<tr>
<td>Vector only</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Ig-like 1-8 motifs</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Fibronectin motif</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Full-length nephrin</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

( ), numbers of mice that died before day 28. One mouse died on day 6, and 2 mice died on day 17. Glomerular lesions of these 3 mice showed focal segmental glomerulosclerosis.

Figure 4. Glomerular lesions of mice injected with IgG from rabbits immunized with mouse nephrin cDNA (periodic acid-Schiff staining, magnification ×800)

(A-D) Glomerular lesions of mouse kidneys obtained 2 days after administration of IgG from rabbits injected with (A) vector alone, (B) Ig-like motifs 1-8 of mouse nephrin cDNA, (C) fibronectin type III motif of mouse nephrin cDNA, or (D) full-length mouse nephrin cDNA. Glomerular lesions in all 4 panels showed minor abnormalities. (E-H) Photographs of lesions from mice injected with IgG from rabbits immunized with full-length mouse nephrin cDNA. (E) A glomerulus on day 7, showing focal segmental degeneration with adhesion to Bowman's capsule. (F) A glomerulus on day 7, showing focal segmental degeneration and focal segmental cell proliferation. (G) The kidney from one of the mice that died on day 17; a glomerulus showed a lesion typical of focal segmental glomerulosclerosis. (H) A glomerulus on day 28, also showing a lesion typical of focal segmental glomerulosclerosis.
Kidney specimens of C57BL/6N mice administered with IgG from rabbits injected with cDNAs encoding Ig-like motifs 1-8 or fibronectin type III motif also showed minor glomerular abnormalities, in 3 mice each on day 2 (Figure 4B and 4C) and in 5 and 6 mice, respectively, on day 28. All kidney specimens from mice administered with IgG from rabbits injected with cDNA encoding Ig-like motifs 1-8 showed diffuse binding of rabbit IgG along the GBM but no evidence of clustering formation on day 2 (Figure 5B). In contrast, none of the 3 kidney specimens from mice administered with IgG from rabbits injected with cDNA encoding the fibronectin III motif showed binding of rabbit IgG to glomeruli on day 2 (Figure 5C). All 11 kidney specimens from mice administered with IgG from rabbits injected with the vector alone showed minor glomerular abnormalities and no glomerular depositions of rabbit IgG on days 2, 7, and 28. Electron microscopic examination of the glomeruli

Figure 5. Direct immunofluorescence of mouse kidney specimens 2 days after administration of IgG from rabbits immunized with mouse nephrin cDNA (magnification ×800)

Frozen sections of mouse kidneys obtained 2 days after administration of IgG from rabbits injected with (A) the vector alone, (B) Ig-like motifs 1-8 of mouse nephrin cDNA, (C) fibronectin type III motif of mouse nephrin cDNA, or (D) full-length mouse nephrin cDNA, were incubated with FITC-labeled goat anti-rabbit IgG.

Figure 6. Electron microscopy of kidney glomeruli obtained 2 days after administration of IgG from rabbits immunized with mouse nephrin cDNA (magnification ×3,000)

Electron microscopic examination of mouse kidney glomeruli obtained 2 days after administration of IgG from rabbits injected with (A) the vector alone, (B) Ig-like motifs 1-8 of mouse nephrin cDNA, (C) fibronectin type III motif of mouse nephrin cDNA, or (D) full-length mouse nephrin cDNA.
of mice euthanized 2 days after administration of IgG from rabbits injected with the vector alone (Figure 6A) or cDNAs encoding Ig-like motifs 1-8 (Figure 6B) or fibronectin III motif (Figure 6C) showed no abnormalities.

Discussion

We have successfully established a mouse model of FSGS with nephrotic syndrome. Light microscopic examination showed that the glomerular lesions in this model were similar to those of human FSGS. Moreover, the glomerular lesions observed on day 2 were similar to those of human MCNS. Thus, the administration to C57BL/6N mice of polyclonal anti-nephrin antibody induced by the immunization of rabbits with full-length mouse nephrin cDNA resulted in MCNS followed by FSGS.

Although indirect immunofluorescence showed that nephrin protein was distributed in a fine granular pattern along the GBM in a normal glomerulus of C57BL/6N mice, mice euthanized 2 days after injection of IgG from rabbits immunized with full-length mouse nephrin cDNA, with massive proteinuria, showed a lumpy pattern of nephrin distribution along the GBM, consistent with cluster formation. Clustering of nephrin protein has been observed on the plasma membranes of HEK 293 cells by adding antibodies to full-length or fragmented human nephrin cDNA, including cDNAs encoding Ig-like motifs 1-2 and 1-8. Binding of these antibodies to the extracellular domain of nephrin transmits an intracellular signaling, resulting in the tyrosine phosphorylation of the intracellular domain of nephrin by the Src family protein kinase p59\(^{59}\). Though the exact mechanism linking clustering to proteinuria remains obscure, the in vivo change in the glomerular distribution of nephrin, from a fine granular to a lumpy pattern may provide clues for understanding this mechanism. This new mouse model may, therefore, be useful for analyzing the molecular mechanism of proteinuria.

The mechanism leading to the emergence of human FSGS is also unclear, although FSGS in most patients is caused by congenital minor mutations of podocyte proteins, including nephrin, podocin, CD2-AP, and alpha-actinin-4.\(^{6,7}\) Our mouse model of acquired FSGS, induced only by alterations in nephrin, can be used to assess the histological development of FSGS and to analyze its molecular mechanism.

Two experimental models of MCNS in rats involve anti-nephrin antibodies, monoclonal antibody 5-1-6\(^{12,13}\) and anti-rat nephrin antibody produced by the administration of full-length rat nephrin cDNA.\(^{14}\) To date, no rat in either model has developed FSGS. In contrast, mice in our model developed FSGS, possibly because the polyclonal anti-nephrin antibody could bind to multiple sites on nephrin and have a greater impact on its activity than a monoclonal antibody that binds to a single site on this protein. Moreover, the amount of antibody per kidney weight was relatively large in our mouse model when compared with the rat model using anti-nephrin antibody. In conclusion, although there are some experimental models of FSGS, to our knowledge, this is the first model in which FSGS was induced by antibody binding to nephrin protein alone.

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References


