Passive Heymann nephritis induced by genetic immunization using megalin cDNA fragments

Hiromi Tazaki,1 Kouju Kamata,1 Shokichi Naito,1 Tomoko Okamoto,1 Kei Kobayashi,1 Nozomu Yamanaka,2 Togo Aoyama1

1Department of Internal Medicine, Kitasato University Graduate School of Medical Sciences and Department of Nephrology in Internal Medicine, Kitasato University School of Medicine
2Department of Surgery, National Defense Medical College

Objectives: Heymann nephritis is a rat experimental model of membranous glomerulonephritis. Megalin has been cloned as an authentic pathogenic antigen for Heymann nephritis. In the present study, we aimed to establish passive Heymann nephritis using genetic immunization.

Methods: Three different rabbit anti-rat megalin antibodies were produced by genetic immunization with rat megalin cDNA encoding amino acids 1-236 (L1-6), 1-156 (L1-4), or 157-236 (L5-6). Purified rabbit IgG was injected into the tail artery of Lewis rats.

Results: Three of 6 rats in the L1-6 group and 1 of 9 rats in the L5-6 group injected with rabbit IgG showed significant proteinuria on day 21. The remaining 22 rats had no significant proteinuria. In the kidneys, on day 21 after IgG injection, binding of rabbit IgG to rat glomeruli was not observed among the groups. Rat IgG binding to rat glomeruli was observed in only 4 rats with significant proteinuria. Glomerular subepithelial electron dense deposits consistent with Heymann nephritis were found in only 4 rats with significant proteinuria.

Conclusions: This is the first report of passive Heymann nephritis induced by genetic immunization with megalin cDNA fragments. Further investigation is needed to establish whether or not there is an increase in the incidence of disease by genetic immunization.

Key words: passive Heymann nephritis, megalin, genetic immunization, proteinuria, membranous glomerulonephritis

Introduction

Heymann nephritis (HN), an experimental model of membranous glomerulonephritis in rats,1 has been studied extensively because of its close resemblance to human membranous glomerulonephritis.2 Active HN induced in rats by immunization with a crude fraction of a renal brush border protein, named Fx1A, or glomerular protein,3 shows massive proteinuria and membranous glomerulonephritis. Passive HN induced in rats by the injection of a pathogenic antibody that is produced in rabbits by immunization with Fx1A also shows typical disease similar to active HN.

Many studies have been carried out to define the pathogenic antigen for HN. Megalin, receptor associated protein (RAP) and dipeptidyl peptidase-4 (DPP-4) were found to be antigens that induced HN.4 Megalin has a C-terminal cytoplasmic domain (213 amino acid residues), a single transmembrane domain (22 amino acid residues), and an extremely large extracellular domain (4,400 amino acid residues); moreover, it is a member of the low-density, lipoprotein receptor family. Megalin is known as a multiligand endocytic receptor expressed that is in a number of epithelial cells. In the kidney, megalin is localized on the plasma membrane of the brush border in proximal renal tubules and functions as an endocytic receptor for albumin. Megalin is also localized at clathrin-coated pits on the basis of glomerular visceral epithelial cells, and it is the target antigen of HN.5

It has been reported that a 60 kD N-terminal fragment (1-563 amino residues) of gp 600 (megalin protein) digested and purified from rat frozen kidney can induced full-blown active HN with massive proteinuria and glomerular deposits of IgG, C3, and C5b-9.5 Furthermore,
smaller protein fragments of amino acid residues 1-236 and 1-195 generated in a baculovirus-insect cell system can induce full-blown disease similar to that induced by the 60 kD N-terminal fragment, whereas the protein fragments of amino acid residues 1-156 and 1-120 produce only mild disease.

Genetic immunization is a simple method for eliciting an immune response. The polyclonal antibody produced by genetic immunization with a single cDNA reacts with the sole protein that is encoded in plasmid cDNA for genetic immunization.

We previously reported production and characterization of anti-human nephrin antibody induced by genetic immunization with human nephrin cDNA, and established a new model of rat nephrotic syndrome by genetic immunization with rat nephrin cDNA. Using this genetic immunization technique, we designed the current series of experiments to establish a passive HN model with 3 different sizes of megalin cDNA encoding amino acids 1-236, 1-156, and 157-236.

Materials and Methods

Experimental animals
Nine female New Zealand white rabbits weighing 2-2.5 kg were purchased from the Charles River Breeding Laboratory (Atsugi), and kept in our breeding laboratory for 2 weeks. Eight-week-old female Lewis rats weighing 150-200 g were also obtained from the Charles River Breeding Laboratory. The rats were kept in a constant dark and light cycle of 12 hours 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
The cDNA encoding rat megalin protein of amino acid residues 1-293 with a signal peptide sequence was synthesized and purified by TaKaRa Custom Service (Ohtsu). Rat megalin cDNA fragments encoding amino acid residues 1-236 (L1-6), 1-156 (L1-4), and 157-236 (L5-6) with a signal peptide sequence (25 amino acid residues) were generated by PCR using rat megalin cDNA as a template. Fragmented megalin cDNAs were subsequently constructed and inserted into pTARGET™ mammalian expression vectors using a CMV promoter (Promega, Madison, WI, USA). Expression vectors including the cDNA fragments were inserted into Escherichia coli JM-109 competent cells (TaKaRa) according to the manufacturer's protocol. After being cultured for 1 day, each plasmid was collected using a QIAGEN Plasmid Maxi Kit (QIAGEN, Tokyo). The authenticity of the cDNA construct was confirmed by sequencing with a Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Framingham, MA, USA).

Administration of plasmid DNA into rabbits and purification of rabbit IgG
The expression vectors were bound to gold particles that were 1 μm in diameter (Bio-Rad Hercules, CA, USA) according to the manufacturer’s protocol. The gold particles coated with plasmid vectors were shot into the subcutaneous tissue of both inner thighs using a gene-gun (Bio-Rad). Three female New Zealand white rabbits were administered with expression vector containing a rat megalin cDNA fragment of L1-6, L1-4, L5-6, or control expression vector. Thirty micrograms of plasmid DNA were administered 4 times every 2 weeks. Blood samples were taken at 8 weeks and sera were processed by ammonium sulfate precipitation as follows. The total volume of 50 ml of serum was stirred, to which an equal volume of saturated ammonium sulfate solution was slowly added, and then the mixture was left standing at 4°C overnight. After the samples were centrifuged at 3,000 g for 20 minutes, the precipitate was dissolved in Melon™ Gel IgG Purification Buffer (Thermo Scientific, USA). They were dialyzed with Melon™ Gel IgG Purification buffer with a 300-fold greater volume than that of the samples. Samples were purified through the column of the Melon™ Gel IgG Purification Kit. The eluates were collected and concentrated using Amicon-15 (Millipore Corporation, Billerica, MA, USA). Finally, samples were dialyzed with 0.2 M sodium phosphate buffer (pH 7.4). Purities of rabbit IgG were more than 99% as analyzed by SDS-PAGE.

All of the rabbit serum from the L1-6, L1-4, or L5-6 groups showed obvious binding to the brush border of proximal tubules in normal Lewis rat kidneys; however, none of the serum from control rabbits showed binding.

Induction of passive HN by rabbit IgG and collection of samples
Purified rabbit anti-rat megalin IgG in 1 ml of phosphate buffered saline (PBS) was injected into the tail artery. Six rats were injected with rabbit IgG induced by the administration of cDNA of L1-6 or L1-4. Nine rats were injected with rabbit IgG produced by cDNA of L5-6. Five rats were injected with rabbit IgG produced by cDNA of a control vector. Twenty-four-hour urine samples
were collected using metabolic cages on days 0, 3, 7, 10, 14, and 21. Urinary protein excretions were quantitated by the biuret method. Renal tissues were obtained on day 21 when the rats were euthanized.

Light microscopy
Renal cortices were fixed in buffered formalin (pH 7.2). Specimens were dehydrated through an ethanol-xylol series and embedded in paraffin. One to 1.5 μm sections of renal cortices were stained with periodic acid-methenamine silver.

Direct 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 and then fixed with ice-cold acetone for 5 minutes. After blocking with 1% BSA (bovine serum albumen)/PBS for 1 hour, cryostat sections were reacted with fluorescein isothiocyanate

Figure 1. Urinary protein excretion of rats injected with purified polyclonal anti-rat megalin IgG produced in rabbits by genetic immunization with rat megalin cDNA fragments.

Panel A: Five of 5 rats injected with control group rabbit IgG had a urinary protein excretion of less than 20 mg/day throughout the experiment. A urinary protein excretion of the mean ± 2 SD was used as the normal range of proteinuria each week (hatched area of panels B-D).

Panel B: Urinary protein in rats injected with L1-6 group rabbit IgG is shown. Three of 6 rats (administered with 2, 2, and 4 mg of rabbit IgG) showed a significantly high level of proteinuria on day 21. The remaining 3 rats (administered with 4, 8, and 8 mg of rabbit IgG) had a normal range of proteinuria throughout the experiment. Rats with 25-50 mg/day proteinuria from days 0-14 had bilateral hydronephrosis when euthanized on day 21.

Panel C: Urinary protein in rats injected with L1-4 group rabbit IgG is shown. All rats showed almost the normal range of proteinuria throughout the experiment.

Panel D: Urinary protein in rats injected with L5-6 group rabbit IgG is shown. Only 1 rat administered with 4 mg of rabbit IgG showed a significantly high amount of proteinuria on day 21.
Figure 2. Direct immunofluorescence findings of rat glomeruli obtained on day 21

Frozen sections of kidney tissue were stained with fluorescein-isothiocyanate-conjugated antibody specific for rat IgG. Photographs of glomeruli in rats with a normal range of proteinuria are shown in panels A-D. Photographs of glomeruli in rats with significant proteinuria are shown in panels E-H.

**Panel A** (rat no. 1): control rabbit IgG was administered.
**Panel B** (rat no. 8): L1-6 group rabbit IgG was administered.
**Panel C** (rat no. 12): L1-4 group rabbit IgG was administered.
**Panel D** (rat no. 26): L5-6 group rabbit IgG was administered.
**Panels E** (rat no. 6), **F** (rat no. 7) and **G** (rat no. 9): L1-6 group rabbit IgG was administered.
**Panel H** (rat no. 19): L5-6 group rabbit IgG was administered. Negative staining for rat IgG is shown in panels A-D, and positive staining for rat IgG is shown in panels E-H.

Figure 3. An electron micrograph of a glomerulus taken on day 21 from rat no. 9 injected with L1-6 group rabbit IgG

↓: Subepithelial electron dense deposits, #: glomerular basement membrane (GBM), *: red blood cells in glomerular capillary loops. Subepithelial electron dense deposits along the GBM consistent with membranous glomerulonephritis are shown, ×10,000.
(FITC)-labeled goat anti-rabbit IgG or FITC-labeled goat anti-rat IgG (Santa Cruz Biotechnology, Inc., CA, USA) for 1 hour at room temperature. Then after washing the sections 3 times with PBS, they were evaluated using a fluorescence microscope equipped with the appropriate filters (Olympus, BX51, 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°C. After being washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 2% OsO4 in 0.1 M cacodylate buffer (pH 7.4) for 1 hour, the material was then dehydrated with ethanol substituted with QY-1 and embedded in Quetol 812 resin mixture. Ultrathin sections of 80 nm were prepared and then stained with 3% uranyl acetate and lead citrate (Reynold’s method). Renal tissue was evaluated by electron microscopy (model JEX-1200EX; JEOL Ltd., Tokyo).

**Results**

**Proteinuria**

Urinary protein excretion of rats injected with 4 different rabbit IgG antibodies are shown in Figure 1. All rats injected with 3 different doses (2, 4, and 8 mg) of control group rabbit IgG had urinary protein excretions of less than 20 mg/day through the course of the experiment (Figure 1, panel A). Three of 6 rats injected with L1-6 group rabbit IgG showed a significantly high level of proteinuria on day 21 (Figure 1, panel B). Rats with significant proteinuria were administered with a small amount (2, 2, or 4 mg) of rabbit IgG compared with the other rats (administered with 4, 8, or 8 mg of IgG) with a normal range of proteinuria. Rat with 25-50 mg/day proteinuria from days 0 to 14 had bilateral hydronephrosis when euthanized on day 21. All of the 6 rats administered with L1-4 group rabbit IgG had a normal range of proteinuria throughout the experiment (Figure 1, panel C). Only one of 9 rats injected with L5-6 group rabbit IgG showed a high level of proteinuria on day 21 (Figure 1, panel D).

**Renal histology**

We examined the kidneys obtained on day 21. Under light microscopic evaluation, none of the 21 kidneys from rats injected with L1-6, L1-4, or L5-6 group rabbit IgG showed vacuolization, spike formation, or thickening in the glomerular basement membrane, similar to that shown in kidneys of rats injected with control group rabbit IgG (data not shown). Immunofluorescence showed that glomeruli had negative staining for rabbit IgG in all the rats injected with control, L1-6, L1-4, or L5-6 group rabbit IgG. Glomerular staining for rat IgG was positive in only 4 rats with a high amount of proteinuria on day 21 (Figure 2, panels E-H). The remaining 22 rat kidneys showed negative or equivocal staining for rat IgG on day 21 (Figure 2, panels A-D). Electron microscopy showed that the 4 rats with significant proteinuria on day 21 had electron dense deposits in the subepithelial space along the glomerular basement membrane (Figure 3). The 22 rats with a normal range of proteinuria on day 21 had no subepithelial electron dense deposits along the glomerular basement membrane.

**Discussion**

We successfully established passive HN by genetic immunization with megalin cDNA fragments. This molecular-based new model of passive HN is simply reproducible without isolation and purification of megalin protein.

HN has been induced by the administration of brush border protein of rat renal proximal tubules for more than 40 years. Brush border protein contains proteins that induce glomerular subepithelial deposits. Megalin, RAP, and DPP-IV have been identified as the antigens that induce HN. Although megalin is known as the major antigen of HN, induction of HN with overt proteinuria by only using megalin protein is uncommon. Oleinikov et al. successfully induced distinctive HN with massive proteinuria by the active immunization of a small N-terminal 60 kD fragment of megalin protein. They also reported that active immunization of smaller N-terminal megalin protein fragments of amino acid residues 1-236 (L6) or 1-195 (L5), which are produced by the baculovirus-insect cell expression system, developed distinctive HN in rats. Smaller megalin N-terminal protein fragments with less than amino acid residues 1-146 only produce mild disease. According to their findings, we used megalin cDNA fragments encoding amino acid residues 1-236, 1-156, and 157-236 for our genetic immunization experiment.

Tramontano et al. reported that one or more molecular determinants inducing a full-blown form of active HN are localized in amino acid residues 157-256 of megalin protein; however, our results were not consistent with the previous study. Only 1 (11%) of 9 rats injected with rabbit IgG antibodies produced by the megalin cDNA fragment encoding amino acid residues 157-256 developed distinctive HN with significant proteinuria. However, 3 (50%) out of 6 rats injected with the rabbit
IgG antibody produced by the megalin cDNA fragment encoding amino acid residues 1-236 developed HN. These data suggested that putative antigenic determinants may be localized in amino acid residues 157-236 of megalin protein, but longer amino acid residues may be needed for the induction of a pathogenic antibody for a full-blown form of HN.

In passive HN with injection of a rabbit antibody that is induced by Fx1A, rats have an initial peak of proteinuria of approximately 40 mg/day on day 8 and a second peak of proteinuria greater than 100 mg/day from day 12. The rat produces antibodies against rabbit IgG from days 7-8 after rabbit IgG injection. In the present study, rats had no proteinuria on days 7 and 10, whereas there was a significant proteinuria on days 14 and 21. The proteinuria may have been induced by both rabbit anti-rat megalin antibody and rat anti-rabbit IgG antibody. Glomerular binding of rat IgG was only found in rats with significant proteinuria on day 21. We did not observe glomerular binding of rabbit IgG in the kidney in rats with a significant proteinuria. However, our results showed that a tiny amount of rabbit anti-rat megalin IgG antibody was localized on glomerular subepithelial deposits because rat IgG binds glomerular subepithelial deposits mediated by rabbit anti-megalin IgG antibody.

Only 4 of 21 rats with injections of rabbit IgG antibody produced by megalin cDNA fragments encoding amino acid residues 1-236, 1-156, or 157-236 developed HN. The induction rate of passive HN was only 19% in the present study. Some possible reasons for this low expression of HN are: (1) there was a small number of antigenic epitope, (2) there was a low titer of rabbit anti-rat megalin antibody, and (3) there was no production or a low titer of rat anti-rabbit IgG antibody. Further studies are required to obtain an increase in the incidence of passive HN by genetic immunization.

In conclusion, we successfully induced passive HN by genetic immunization with megalin cDNA fragments. This simple model may provide new insight into the molecular mechanism of membranous glomerulonephritis.

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References


