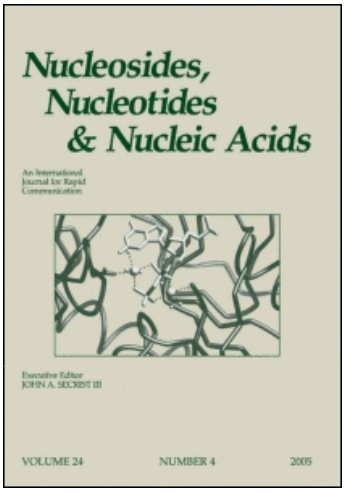

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PRODUCTION AND CHARACTERIZATION OF TRANSGENIC MICE HARBORING MUTANT HUMAN *UMOD* GENE

Yuichi Takiue,[1] Makoto Hosoyamada,[1] Takuya Yokoo,[1] Masaki Kimura,[1] Manami Ochiai,[2] Kiyoko Kaneko,[2] Kimiyoshi Ichida,[3] Tatsuo Hosoya,[3] and Toshiaki Shibasaki[1,3]

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□ *Familial juvenile hyperuricemic nephropathy is caused by mutations in the UMOD gene encoding uromodulin. A transgenic mouse model was developed by introducing a human mutant UMOD (C148W) cDNA under control of the mouse umod promoter. Uromodulin accumulation was observed in the thick ascending limb cells in the kidney of transgenic mice. However, the urinary excretion of uromodulin in transgenic mice did not decrease and LC-MS/MS analysis indicated it was of mouse origin. Moreover, the creatinine clearance was not different between wildtype and transgenic animals. Consequently, the onset of the disease was not observed in transgenic mice until 24 weeks of age.*

Keywords Familial juvenile hyperuricemic nephropathy; transgenic mice; uromodulin; LC-MS/MS; creatinine clearance

INTRODUCTION

Familial juvenile hyperuricemic nephropathy (FJHN) is an autosomal dominant hereditary renal disease related to hyperuricemia of the underexcretion type.[1] Renal insufficiency in FJHN patients is usually recognized between the ages of 20 and 40 years and leads to progressive renal failure.[2]

FJHN is caused by mutations in the UMOD gene, which encodes the most abundant glycoprotein in normal human urine, uromodulin.[3] Uromodulin is distributed in epithelial cells of the thick ascending limb (TAL) of Henle's loop and the early distal convoluted tubule except the macula densa.[4] The clinical features of FJHN patients reveal diffuse intracellular

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and marked aggregation of uromodulin in TAL cells and a decreased urinary excretion irrespective of gender, age, or renal function.[5–8]

However, the relationship between the accumulation of uromodulin and the symptoms of FJHN such as hyperuricemia of the underexcretion type and renal insufficiency remains to be determined. In this study, transgenic mice were produced harboring a human *UMOD* mutant gene. The clinical features of FJHN, that is, decreased urinary uromodulin excretion and renal insufficiency, were investigated to confirm the validity of using the hemizygotic transgenic mice as an animal model for the study of FJHN.

MATERIALS AND METHODS

Animal

Human UMOD cDNA was amplified by polymerase chain reaction (PCR) from the reverse transcription product of human kidney poly $(A)^+$ RNA (Takara-Clontech, Japan). A T444G mutation was introduced by QuikChange II Site-Directed Mutagenesis kit (Stratagene, USA). The DNA fragment of the mouse *umod* promoter amplified from mouse-tail genomic DNA by PCR was ligated upstream of the T444G mutated human UMOD cDNA. The authenticity of the 5587bp mouse UMOD promoter-enhanced T444G human UMOD chimeric gene was verified by DNA sequencing and microinjected into fertilized eggs of C57/BL6 inbred mice for transgenic (Tg) mouse production by the YS Institute Inc. (Tochigi, Japan). Tg mice harboring human mutant *UMOD* and wildtype (WT) mice were genotyped using a pair of primers corresponding to UMOD cDNA. Twelve and 24 week-old male WT and Tg mice were placed into metabolic cages to obtain daily urine samples. Animals were anesthetized with 50 mg/kg pentobarbital i.p. and Serum samples and kidneys were obtained. All animal experiments were carried out in accordance with Kyoritsu University of Pharmacy Guide for the Care and Use of Laboratory Animals.

Western Blotting

Urinary proteins of mice corresponding to 1/500 of daily excretion were prepared as described previously,[9] separated on 10% polyacrylamide minigels by the Laemmli method, and semi-dry blotted onto a nitrocellulose filter. After incubation for 1 hour at room temperature in PBS blocking solution with 0.02% Tween 20, the filter was stained overnight at 4°C using rabbit anti-human uromodulin polyclonal antibody (Santa Cruz Biotechnology, Inc., USA) at 1:200 dilution in the PBS containing 0.1% Tween 20 and 2% bovine serum albumin (BSA). Immunoreactive bands were detected using an ECL kit according to the manufacturer's instructions (GE Healthcare

Bio-Science Corp., USA). Band densities were analyzed using Quantity One soft ver. 4.3.0 (Bio-Rad Laboratories, Inc., USA).

Immunohistochemistry

The kidneys of WT and Tg mice were fixed by perfusion with 4% paraformaldehyde and embedded in paraffin. The paraffin sections were cut at a thickness of 3 μm and blocked with PBS containing 2% BSA (BSA-PBS) for 1 hour at room temperature. These sections were stained overnight at 4°C with anti-Tamm Horsefall Glycoprotein antibody (Biogenesis Ltd, UK) at 1:200 dilution in BSA-PBS, followed by treatment with Envision kit (DakoJapan Inc., Japan).

HPLC

Serum samples and acetonitrile (1:4) were filtered and centrifuged at 12,000 rpm for 30 minutes using Ultrafree-MC (Durapore PVDF 0.22 μm, Millipore Corp., MA, USA). Urine samples in the mobile phase were filtered and centrifuged at 6,600 rpm for 30 minutes using Ultrafree-MC (Biomax-5 membrane, Millipore Corp.). Filtered samples were evaporated by a vacuum evaporator centrifuge. The precipitates were resuspended in the solvent and used for HPLC analysis. The analytical conditions were as follows: column: Phenomenex Synergi Fusion-RP 4μ 80Å, 2.0 mm ID × 250 mm; solvent: 50 mM ammonium acetate, pH 5.0; flow rate: 0.200 mL; UV detection: 230 nm.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Urinary uromodulin separated by SDS-PAGE was excised from the CBB-stained gel and digested with trypsin according to published procedures (10). A portion of the tryptic peptide solution was analyzed by LC-MS. The HPLC system and mass spectrometer employed was a Magic 2002 and ThermoQuest LCQ_{DACA} equipped with an ion trap, and with a nano ESI interface from Michrom BioResources, Inc. (USA). The HPLC conditions were as follows; column: Magic C_{18}, 0.2 mm ID × 50 mm; solvent: 0.1 M formic acid with acetonitrile gradient (5% to 65% in 20 minutes); flow rate: 2 μL/minute. The electrospray voltage was 1.5 kV and the temperature of the heated capillary was 170°C. The experiment produced a full-scan MS spectrum and full-scan MS/MS spectrum based on a maximum intensity threshold. The MS/MS spectra were subjected to a database search using SEQUEST (X calibur 1.3, Bioworks 3.0).[11]

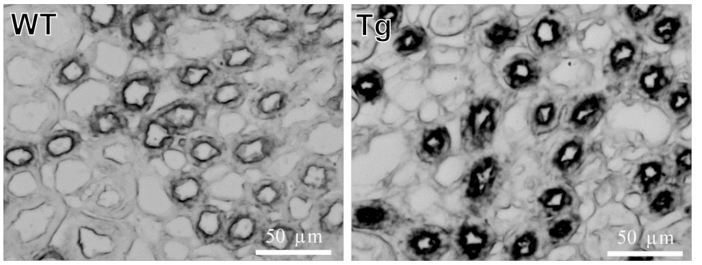

FIGURE 1 Expression and localization of human uromodulin in the kidney of 12 week-old Tg mice. Immunohistochemistry of uromodulin in the kidneys of WT (left panel) and Tg (right panel) mice. These paraffin sections were stained overnight at 4°C with anti-human uromodulin antibody, followed by treatment with diaminobenzidine. The staining of uromodulin was localized in the TAL cells. Uromodulin staining in the kidney of Tg mouse was overexpressed when compared to the WT mouse. Magnification: ×400.

RESULTS AND DISCUSSION

The breeding ability of Tg mice was normal and one strain was selected based on immunofluorescence staining of human uromodulin in kidney, which was relatively greater than a WT mouse (Figure 1). Moreover, uromodulin in the Tg mouse kidney appeared to be in an aggregated form. The C148W uromodulin mutant is delayed for export to the plasma membrane by being retained in the endoplasmic reticulum in transiently transfected HEK293 cells.[12] Therefore, mutant human uromodulin expressed in Tg mouse may be retained in the ER.

Broad bands of 110 kDa were detected by Western blot analysis as urinary uromodulin in WT and Tg mice. There was no statistical difference in

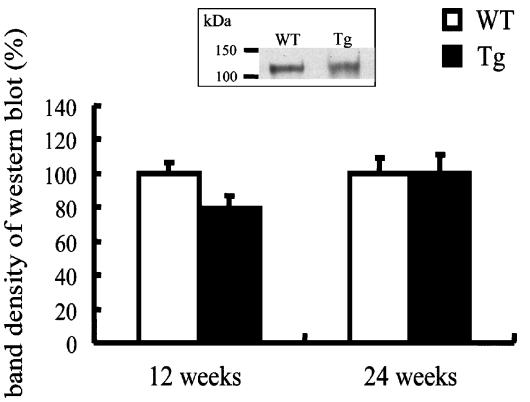

FIGURE 2 Quantification and characterization of uromodulin in the urine of WT and Tg mice. The amount of uromodulin in the urines of 12- and 24-week-old mice. Open and closed bars represent the percentages of urinary uromodulin in WT and Tg mice, respectively. The amount of uromodulin in the urine of WT mice was fixed to 100%. Mean ± S.E.M., n = 3. There was no statistical difference in the amounts of urinary uromodulin between WT and Tg mice. Western blot analysis of urinary uromodulin at 12 weeks using anti-human uromodulin antibody is shown in the inset. Urinary proteins were 1/500 of the daily amount. The 110 kDa protein corresponds to mouse uromodulin.

the amounts of urinary uromodulin between WT and Tg mice (Figure 2). In addition, the human uromodulin specific peptide was not detected in the urine of Tg mice by LC-MS/MS. Thus, the 110 kDa bands detected in urine samples of WT and Tg mice were considered to be mouse uromodulin. Conversely, human uromodulin in Tg mice was not excreted in the urine, but did accumulate in the kidney. There was no statistical difference in the creatinine clearance between WT and Tg mice (12 week-old Tg mice was 87.7 ± 9.9 mL/day/body in Tg mice versus 98.9 ± 17.0 mL/day/body in WT mice at 12 weeks**;** and 76.3 ± 16.2 mL/day/body in Tg mice versus 119.7 ± 15.9 mL/day/body in WT mice at 24 weeks). Therefore, renal insufficiency was not observed in Tg mice until 24 weeks.

In summary, transgenic mice harboring mutant human *UMOD* accumulated of uromodulin in their kidney. However, the decrease of urinary uromodulin excretion and renal impairment wasn't observed until 24 weeks. Further investigation is needed using mice older than 24 weeks.

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