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A transgenic rat with the human ATTR V30M: A novel tool for analyses of ATTR metabolisms

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Abstract

Amyloidogenic transthyretin (ATTR) is the pathogenic protein of familial amyloidotic polyneuropathy (FAP). To establish a tool for analyses of ATTR metabolisms including after liver transplantations, we developed a transgenic rat model expressing human ATTR V30M and confirmed expressions of human ATTR V30M in various tissues. Mass spectrometry for purified TTR revealed that rat intrinsic TTR and human ATTR V30M formed tetramers. Congo red staining and immunohistochemistry revealed that nonfibrillar deposits of human ATTR V30M, but not amyloid deposits, were detected in the gastrointestinal tracts of the transgenic rats. At 24 h after liver transplantation, serum human ATTR V30M levels in transgenic rats that received livers from normal rats became lower than detectable levels. These results thus suggest that this transgenic rat may be a useful animal model which analyzes the metabolism of human ATTR V30M including liver transplantation studies.

Keywords: Amyloid; Familial amyloidotic polyneuropathy; Transthyretin; Liver transplantation; Transgenic rat; Gender difference

Familial amyloidotic polyneuropathy (FAP) is a fatal hereditary amyloidosis, with the amyloidogenic proteins being the mutated amyloidogenic transthyretin, apolipoprotein A-I, and gelsolin [1,2]. Of these proteins, ATTR is the most common amyloidogenic protein in the world [1]. In 1952, Andrade first reported a large focus of FAP patients with ATTR in Portugal [3]; additional foci of these patients were discovered in Japan and Sweden [4,5]. As a result of progress in biochemical and molecular genetic methods, this disease is now believed to occur in all over the world [6]. Today, more than 100 different points of single or double mutations or a deletion in TTR have been reported [7], the majority of which are found in small kindreds or show no family history.

In addition to sensorimotor polyneuropathy, disorders of the gastrointestinal tract, heart, and kidney failure, autonomic nervous system dysfunction, and ocular disorders have been documented in patients with FAP ATTR V30M [1,8]. Despite many investigations, the precise mechanism of amyloid formation remains to be elucidated [9], with the result that the optimal therapy for FAP, except for liver transplantation, has not yet been established [10].

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Because both normal TTR and variant TTR are predominantly synthesized by the liver, liver transplantation is now considered to be a promising therapy for FAP patients [10]. The positive outcome of such transplantation has stimulated research and use of more complex procedures, such as sequential (domino) liver transplantation [11], in which a resected liver from a patient with FAP is transplanted into a patient with a severe liver disorder or cancer. However, ocular manifestations induced by amyloid deposition occur even after liver transplantation because variant TTR continues to be produced by the retina [12]. Recently, we reported one patient, who underwent a sequential liver transplantation using an FAP patient's liver, started to show both amyloid deposits and clinical manifestations of FAP, 7 years after transplantation [13]. However, we do not know whether all of the second recipients eventually show the symptoms of FAP.

To establish a tool for analyses of ATTR metabolisms including after liver transplantations, we recently developed using the albumin promoter transgenic rats possessing a human ATTR V30M gene which can be used for experiments of liver transplantation. In this study, we investigated as follows: (1) we determined the sites of production of human ATTR V30M in tissues. (2) We also examined the levels and forms of human ATTR V30M by means of ELISA and matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry (MALDI/TOF-MS), respectively. (3) In addition, we performed liver transplantation so that we measured changes in serum human ATTR V30M levels in the transgenic rats receiving the liver from normal rats.

Materials and methods

Reagents and antibodies. William's Medium E, Dulbecco's modified Eagle's medium, and fetal bovine serum were purchased from Life Technologies (Rockville, MD). A polyclonal rabbit anti-human TTR antibody and a horseradish peroxidase-coupled goat anti-rabbit IgG antibody were obtained from Dako (Dakopatts, Glosttrup, Denmark). A polyclonal rabbit anti-rat TTR was produced in the previous study [14]. Other chemicals used in the studies were of analytical grade.

Plasmid construction and generation of the transgenic rat. The plasmid expression vector pTK3 was constructed by using a human TTR cDNA fragment, a 0.45-kb fragment obtained by use of *Eco*RI and *Nar*I, and a mouse albumin enhancer/promoter coding sequence (2.3 kb), with the pBluescript II SK(+) plasmid (Stratagene, La Jolla, CA). The 2.75-kb *Not*I–*Xho*I fragment was isolated by electrophoresis of digested pTK3 on 0.8% agarose gel, electroeluted, purified via the QIAEX II Gel Extraction Kit (Qiagen, Tokyo, Japan), and diluted to a concentration of 1–2 ng/ml in 5 mM Tris–HCl and 0.1 mM EDTA, pH 7.4. Fertilized rat eggs were recovered from supervaluated DA rat females mated with DA males (Charles River, Kanagawa, Japan). After microinjection of the male pronucleus with the DNA solution, eggs were transferred into both oviducts of day 0 pseudopregnant DA females, as previously described [15].

DNA analysis. Genomic DNA was extracted from tail samples of transgenic rats by using the QIAamp DNA Mini Kit (Qiagen). To screen for transgenic rats with human ATTR V30M cDNA, E2-S (5′-GGCACCGGTGAATCCAAGTGT-3′) and E4-AS (5′-TTCCTTGGG ATTGGTGACGAC-3′) were used as the forward and reverse primers, respectively. Polymerase chain reaction (PCR) was performed in 35 cycles with a final volume of 50 μl containing 0.5 μg of genomic DNA, primer pairs (25 pmol), dNTPs (200 mM each), and Gene Amp PCR reagents including *Taq* polymerase. Each cycle consisted of denaturation for 1 min at 94 °C, primer annealing for 1 min at 60 °C, and polymerization for 1 min at 72 °C. The PCR products were electrophoresed through 3% NuSieve GTG agarose gel, stained with ethidium bromide, and photographed under UV light. Each primer exists in a different exon of the human TTR gene, and the 372-bp band was detected only in the transgenic rat that had the human ATTR V30M cDNA but not the rat TTR gene.

Quantitative RT-PCR. Total RNA was isolated by use of the PURESCRIPT RNA Isolation Kit (Gentra, Minneapolis, MN). External standards, consisting of serial dilutions of human ATTR V30M cDNA (10^7, 10^5, and 10^3 copies), were constructed by means of RT-PCR. To evaluate the human ATTR V30M mRNA copy, the upstream and downstream primer sequences used were 5′-GGCCCTACGGGCACCGGT-3′ and 5′-CCTTCTACAAATTCCTCCTCA-3′, respectively. The hybridization probe sequences were 5′-TGTGGCCGTGCATGTGT-3′-FITC and LC Red 640-5 5′-CAGAAAGGCTGCTGATGACACCTGGGAGCCATTT GCCTCTGGG-3′-OH. The primers, hybridization probes, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) external standards (4×10^4, 4×10^3, and 4×10^2 copies) for evaluating the rat GAPDH mRNA copy were obtained from Nihon Gene Research Laboratories (Sendai, Japan). The reaction mixture consisted of 3.25 mM $Mn(OAc)_2$, primers (0.3 μM each), hybridization probes (0.2 μM), 7.5 μl of RNA LightCycler RNA Master Hybridization probe mixture (Roche Molecular Biochemicals, Tokyo, Japan), 50 ng cDNA samples or external standards, and MilliQ water up to a final volume of 20 μl. The crossing point values of these standards were used to generate an external standard curve to provide accurate quantification. The ratio of human ATTR V30M mRNA copies to rat GAPDH mRNA copies was estimated.

Examination of serum TTR levels. To determine rat TTR levels and human variant TTR levels in rats, the peroxidase–antiperoxidase method for sandwich ELISA was employed as described previously [14].

Western blotting. To detect the human ATTR V30M in transgenic rats, Western blotting was employed using the polyclonal rabbit anti-human TTR antibody. Each of tissues was homogenized with five volumes of Phosphate-buffered saline (PBS) centrifuged at 9000*g* for 5 min. And these supernatants were collected and used for analyses.

TTR isolation. Rat serum (50 μl) was mixed with 20 μl of anti-human TTR antibody or anti-rat TTR antibody. The precipitate was centrifuged at 9000*g* for 5 min and washed with 100 μl of saline and 100 μl of water twice, respectively, at 4 μl. The precipitate was dissolved in 50 μl of 4% acetic acid and 4% acetonitrile in water, and the solution was passed through a 1000-kDa centrifugal concentrator (Pall Filtron Co., Northborough, MA) to obtain the TTR dissociated from the antibody in the pass-through fraction. The centrifugal devices were washed three times with 100 μl of the same solution.

MALDI/TOF-MS. Purified rat TTR and human variant TTR were analyzed by use of a Bruker Reflex mass spectrometer (Bruker Franzen Analytik GmbH, Bremen, Germany) operated at a wavelength of 337 nm. The best TTR spectra were obtained at an ion-accelerating voltage of 27.5 kV and a reflectron voltage of 30 kV. The spectra were calculated by using external calibration with $[M+H]^-$ ions produced from horse cytochrome *c* (*m*/*z* 12,360.08) and horse myoglobin (*m*/*z* 16,951.46). The matrix was a saturated solution of sinapinic acid in acetonitrile plus water (1:2, v/v) containing 0.1% trifluoroacetic acid. The samples were deposited onto the sample probe assembly.

Congo red staining. To examine the presence of amyloid deposits in the tissues of transgenic rats, sections of the heart, kidney, liver, stomach, small intestine, large intestine, skin, brain, and peripheral nerve of 6–24 month-old rats (male 6, female 6) were stained with alkaline Congo red plus hematoxylin–eosin (H–E). The Congo red-stained materials were examined under polarized light.

Immunohistochemistry. For immunohistochemistry using a polyclonal rabbit anti-human TTR antibody and a polyclonal rabbit anti-rat TTR antibody, the same tissues as examined by Congo red staining were deparaffinated, dehydrated in a modified alcohol series, and incubated in blocking buffer (1% bovine serum albumin (BSA) and 5% goat serum in