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.

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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].
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, Glostrup, Denmark). 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 EcoRI and NarI, 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 NotI–XhoI fragment was isolated by electrophoresis of digested pTK3 on 0.8% agarose gel, electroduted, 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 superovulated 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 human ATTR V30M cDNA, E2-S (5’- GAGCAGGGGTCTATGCATGT-3’) and E4-AS (5’-TTCCCTGCGG ATTTGTCGACG-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 μM 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 (Genta, Minneapolis, MN). External standards, consisting of serial dilutions of human ATTR V30M cDNA (105, 104, and 103 copies), were generated 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’-CCCTCTACAAATTCTCTCTA-3’, respectively. The hybridization probe sequences were 5’-TGTTGGCCGTGCAAGTGT-3’-FITC and LC Red 640 5’-CAGAAGGCTCAGTGACCACCTGAGGCACGATGG-3’-OH. The primers, hybridization probes, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) external standards (4 × 105, 4 × 104, and 4 × 103 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 10 μl water up to a concentration 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 9000g 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 10000g for 5 min and washed with 100 μl of saline and 100 μl of water twice, respectively, at 4°C. 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 Fransen 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 reflection 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 16,951.40). 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.

Congenital goiter. 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 deparaffinized, dehydrated in a modified alcohol series, and incubated in blocking buffer (1% bovine serum albumin (BSA) and 5% goat serum in
Results and discussion

Human ATTR V30M gene expression in tissues

The generation of transgenic rats with human ATTR V30M gene was confirmed using PCR analysis (Fig. 1A). To examine human ATTR V30M gene expression, mRNA levels in the liver, kidney, testis, lung, brain, heart, muscle, and eye were examined by quantitative reverse transcription (RT)-PCR. Fig. 1B shows that human ATTR V30M mRNA was strongly expressed in liver and brain, and results were weakly positive in the lung, eye, and kidney. In the heart, testis, and muscle, ATTR V30M mRNA was below detectable levels. The transgenic mouse models of FAP developed previously showed the human variant TTR gene expressions in the liver and choroid plexus, but no human variant TTR mRNA in the ocular tissues [18,19]. For FAP patients, ocular manifestations induced by amyloid deposition still continue even after liver transplantation, because variant TTR produced by the retina forms amyloid fibrils in transplanted patients [20]. Our transgenic rat is the first animal model which shows the expression of human ATTR V30M in the ocular tissues and may be a useful model to analyze TTR metabolisms in the ocular tissues before and after liver transplantation.

To confirm the presence of human ATTR V30M, Western blotting was performed in serum, CSF, liver, brain, and eye using an anti-human TTR antibody. As shown in Fig. 1C, presence of human ATTR V30M was confirmed in these tissues. In normal DA rats, anti-human TTR antibody did not react with any bands (data not shown).

Serum levels of human ATTR V30M and forms of TTR in transgenic rats

Serum levels of human ATTR V30M and levels of human ATTR V30M mRNA in livers were higher in male transgenic rats than in female transgenic rats (Fig. 2A and B). To clarify the effect of sex hormones on human ATTR V30M synthesis, we measured serum levels of human ATTR V30M before and after surgical castration in male transgenic rats. As demonstrated in Fig. 2C, serum human ATTR V30M levels of male rats that received surgical castration did not become similar levels as those of female rats, but were significantly decreased, compared with those before surgery. One week after castration, serum human ATTR V30M levels became the minimum and slightly elevated after two weeks from the castration. In a sham operation group, serum ATTR V30M levels did not change. Serum intrinsic rat TTR levels did not show the significant change after castration (data not shown). The reason for the higher human ATTRV30M levels in the male transgenic rats may derive from the albumin promoter with which the human ATTR V30M cDNA construct was conjugated. Those results suggest that male sex hormones partially play a role in regulating serum human ATTR V30M levels in...
the transgenic rats. In fact, in transgenic rats possessing the Alb-DS Red2 gene that were previously developed, the inserted gene was expressed only in adult male rats, not in female rats [21].

TTR exists in tetrameric form in the blood circulation. It is widely accepted that instability of the tertiary structure is induced by a combination of mutated and wild-type TTR [32]. MALDI/TOF-MS of the transgenic rat serum revealed that human ATTR V30M and rat TTR formed tetramers (Fig. 3A). However, the anti-rat TTR antibody reacted predominantly with tetramers formed by only rat intrinsic TTR (Fig. 3B). Serum levels of rat intrinsic TTR (32.5 ± 3.8 mg/dl) was much higher than that of human ATTR V30M (3.1 ± 1.9 mg/dl). The results suggest that most of TTR tetramer in the transgenic rats may be formed from only rat intrinsic TTR. This form of rat intrinsic TTR may be more difficult to form amyloid fibrils than human ATTR V30M in the transgenic rats. Anti-human TTR antibody did not react with normal DA rat serum (data not shown).

Nonfibrillar human TTR deposits in transgenic rats

In 6-months-old rats and 6–24-month-old female rats, neither Congo red positive nor human ATTR V30M positive lesions were observed. In contrast, in 10–24-month-old male rats, human ATTR V30M deposits were observed in the submucosal lesions of the large intestine (Fig. 4A), while Congo red positive lesions could not be found even in 24-month-old rats (data not shown). In other tissues, including heart, kidney, liver, stomach, small intestine, skin, brain, and peripheral nerve, human ATTR V30M

![Graph A: Serum levels of human ATTR V30M in transgenic rats.](image)

![Graph B: Levels of ATTR V30M mRNA in the livers of transgenic rats.](image)

![Graph C: Changes in serum ATTR V30M levels after surgical castration.](image)

Fig. 2. (A) Serum levels of human ATTR V30M in transgenic rats. In this part of the study, 33 male and 25 female transgenic rats, aged 7–20 weeks, were used. The P-value difference between female rats and male rats (**P < 0.0001). (B) Levels of ATTR V30M mRNA in the livers of transgenic rats. Liver mRNA levels of male and female transgenic rats were compared. Six and seven livers obtained from male and female rats, respectively, were used for this study. GAPDH mRNA copies were used as an internal control, *P < 0.02. (C) Changes in serum ATTR V30M levels after surgical castration. Five-month-old male transgenic rats (n = 3) were surgically castrated and serum ATTR V30M levels were measured by ELISA as described in the text. Open triangles: sham-operated rats, and closed squares: castrated rats. *P < 0.05: ATTR V30M levels before castration vs. after castration.
deposits were not detected. Using an anti-rat TTR antibody, no rat TTR deposition was observed in the same tissues (data not shown). In a previous study, before amyloid fibril formation, nonfibrillar TTR deposits were detected in the peripheral nerves of FAP patients [22] and transgenic mouse models [18,19]. From these observations, this transgenic rat may become an animal model in which we can analyze ATTR V30M metabolism and amyloid formation mechanisms in the tissue.

Liver transplantation in transgenic rats

Livers from normal rats were transplanted into transgenic rats with human ATTR V30M. Analysis of serum human ATTR V30M levels revealed that at 24 h after the transplantation serum levels of human ATTR V30M had decreased to below detectable levels (Fig. 4B). Liver transplantation is now thought to be a promising therapy for FAP patients, to save their lives; in addition, a shortage of donor livers has led to the use of sequential transplantation of livers from FAP patients to patients with serious liver diseases as one way to save lives [10,11,13]. However, metabolism of ATTR after liver transplantation, affected by TTR expressions of the choroid plexus and ocular tissues, has not been fully studied. Moreover, the optimal age of the patient to undergo sequential liver transplantation is still unclear: FAP is an adult-onset disease, and sufficient duration may be needed for developing the disease after the pathologic protein begins to be produced by the liver [6]. The transgenic rat may be the first useful model

Fig. 3. TTR forms in serum of the transgenic rats. (A) TTR forms analyzed by use of MALDI/TOF-MS with an anti-human TTR antibody: a, free form of rat TTR (13,597 Da); b, Cys-conjugated form of rat TTR (13,717 Da); c, free form of human ATTR V30M (13,793 Da); and d, Cys-conjugated form of human ATTR V30M (13,912 Da). (B) TTR forms analyzed by use of MALDI/TOF-MS with an anti-rat TTR antibody: a, free form of rat TTR (13,597 Da); b, sulfonated form of rat TTR (13,677 Da); c, Cys-conjugated form of rat TTR (13,793 Da); and d, glutathione-conjugated form of rat TTR (13,904 Da).

Fig. 4. (A) Immunohistochemistry for human ATTR V30M in the large intestine of a 10-month-old male transgenic rat. Nonfibrillar human ATTR V30M deposition (arrows) was observed in the transgenic rat. Original magnification: 100×. (B) Changes in serum ATTR V30M levels after liver transplantation. Three transgenic rats underwent liver transplantation. They lived until day 5 after the surgery; blood samples were taken on days 0, 1, and 3.
animal to answer these questions. As seen in Fig. 4B, the human ATTR levels in rats that received a normal liver decreased to below detectable levels at 24 h after liver transplantation. Our preliminary experiment revealed that normal rats receiving the transgenic rat liver had significant human ATTR levels in the blood at 24 h after the surgery. These results also suggest that serum human ATTR V30M is mainly secreted from the liver in the transgenic rat. From above mentioned reasons, these rats become a useful tool for the studies of TTR metabolisms before and after the liver transplantations.

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