Generation and characterization of transgenic mice expressing a human mutant α-galactosidase with an R301Q substitution causing a variant form of Fabry disease

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Abstract Transgenic mice expressing a human mutant α-galactosidase with an R301Q substitution, which was found in a patient with a variant form of Fabry disease, were established. The mice transcribed a sufficient amount of α-galactosidase mRNA, but the steady-state levels of the enzyme protein were decreased in liver, kidney and heart, only residual activity being detected in these tissues. The mice will be useful for the clarification of the defective regulation of the structurally altered enzyme protein expressed by the mutant gene at the organ or individual level as well as for the evaluation of drugs that stabilize and/or activate the mutant α-galactosidase.

Key words: Fabry disease; α-Galactosidase; Transgenic mouse; Gene mutation

1. Introduction

Fabry disease is an X-linked genetic disorder resulting from decreased activity of α-galactosidase (EC 3.2.1.22) [1]. In classical Fabry disease, deficient α-galactosidase activity results in the progressive accumulation of ceramide trihexoside and causes systemic manifestations, including renal and cardiovascular disorders. Recently, atypical Fabry variants with residual enzyme activity and cardiomyopathy of late onset were reported [2,3], and a screening study revealed that these variants were found in 3% of unrelated male patients with left ventricular hypertrophy referred to a cardiology clinic in Japan [4]. The incidence of the variant Fabry disease seems to be unexpectedly high, and clarification of the pathogenesis underlying the variant Fabry disease is very important.

The R301Q (CGA→CAA) substitution is one of the gene mutations found in patients with variant Fabry disease [2]. A male patient carrying the R301Q mutation developed chest pain at the age of 52 years, and hypertrophy of both the ventricular septum and the left ventricle was found by electrocardiographic and echocardiographic analysis. Biopsy of the left ventricular endocardium revealed numerous lamellar inclusions in myocardium electron microscopically. The leukocyte α-galactosidase activity of the patient was 4% of the normal control level.

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In this report, we generated and characterized transgenic mice expressing a human mutant α-galactosidase with the R301Q substitution.

2. Materials and methods

2.1. Generation of transgenic mice

α-Galactosidase cDNA was obtained from cultured lymphoblast poly(A)$^+$ RNA by means of reverse transcription/polymerase chain reaction (PCR) [5]. cDNA encoding human normal α-galactosidase or mutant α-galactosidase with the R301Q substitution was inserted into vector pCXN2 (supplied by Dr. J. Miyazaki, Tohoku University, Japan), as described previously [6]. The constructs were digested with *Nde*I (New England BioLabs, Beverly, MA, USA), and a 3-kbp DNA fragment including the chicken β-actin promoter and human α-galactosidase cDNAs was obtained, and purified by means of the cesium chloride method. The standard microinjection method [7] was used to introduce the transgenic constructs into pronuclei of fertilized eggs from superovulated C57BL/6CrSlc female mice. The zygotes were implanted in pseudo-pregnant female Slc:ICR mice. Animal experiments were performed according to local institutional guidelines for the care and use of laboratory animals.

2.2. Screening of transgenic mice expressing human α-galactosidase

The resulting mice were screened for the presence of the transgene by means of PCR [8] of DNA. Genomic DNA was prepared from punched ear tissue by the standard method [9], and PCR was performed using a pair of primers (5′-TGAGAATTCGTATCTTGGACTGGACATCT-3′ and 5′-GCCGAATTCTTAAAGTAAGTCTTTTAATGAC-3′) to amplify a DNA fragment including a partial human α-galactosidase cDNA sequence (cDNA #722–1290) in mouse genomic DNA. The PCR comprised 30 cycles, each cycle consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 75°C for 1 min.

2.3. Preparation of genomic DNA and poly(A)$^+$ RNA

Genomic DNA was isolated from liver tissue, as described above. Total RNA was isolated from liver, kidney and heart by the guanidine isothiocyanate method [9], and poly(A)$^+$ RNA was purified with oligo(dT)-Latex beads (Nippon Roche, Tokyo, Japan).

2.4. Southern blot analysis

Genomic DNA (10 μg) was digested with *Pvu*II (New England BioLabs), electrophoresed on a 1% agarose gel, transferred to a nitrocellulose membrane, and then hybridization was performed. A *Pvu*II fragment of human α-galactosidase cDNA (cDNA #7–827) was ^{32}P-labeled, as described previously [2], and used as a probe.

2.5. Northern blot analysis

One microgram of poly(A)$^+$ RNA from liver, kidney and heart was separated by electrophoresis on a 1% agarose gel containing formaldehyde, transferred to a nitrocellulose membrane, and then hybridized with ^{32}P-labeled full-length human α-galactosidase cDNA as a probe, as described previously [2]. The filter was rehybridized with ^{32}P-labeled human β-actin cDNA (Nippon Gene, Tokyo, Japan) to determine the quality and quantity of samples.

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2.6. Western blot analysis

Tissue homogenates of liver, kidney and heart were centrifuged at 6000×*g* for 10 min at 4°C, and the resultant supernatants were used as samples. The samples (liver, 3 μg protein; kidney, 22 μg protein; and heart, 9 μg protein) were subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis on a 5–20% gradient gel under reducing conditions, using Laemmli's system [10]. Then, the proteins were transferred to an Immobilon membrane (Millipore, Bedford, MA, USA). The membrane was reacted with a rabbit anti-human α-galactosidase antibody [11] for 1 h at room temperature, and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antiserum (Amersham, Buckinghamshire, UK). The detection of the reacted protein was performed using enhanced chemiluminescence (Amersham) according to the manufacturer's method.

2.7. Assaying of α-galactosidase activity

α-Galactosidase activity was determined using the supernatants of tissue homogenates with 4-methylumbelliferyl α-D-galactopyranoside (Nacalai Tesque, Kyoto, Japan) as a substrate [12]. The amount of protein was determined by means of a Bio-Rad dye-binding assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a standard.

3. Results

In this study, we have screened and assessed the presence of a transgene as a 587-bp PCR product (data not shown). Two mouse lines were obtained, one with a transgene of mutant α-galactosidase and the other with one of normal α-galactosidase. They were designated C57BL/6CrSlc-TgN (GLAR301Q)1962Rin and C57BL/6CrSlc-TgN(GLA)1953-Rin, respectively (abbreviated as TgN(GLAR301Q)1962 and TgN(GLA)1953, respectively).

The transgene carrier status was also confirmed by Southern blotting using liver DNAs from TgN(GLAR301Q)1962 and TgN(GLA)1953 (Fig. 1). In this analysis, we used mouse genomic DNAs digested with *Pvu*II as samples, and the transgene was detected by hybridization with a labeled *Pvu*II fragment of human α-galactosidase cDNA as a probe. Therefore, the existence of the transgene was determined as a 0.8-kb band on a film, and the dose of the transgene integrated was roughly estimated from the density of the corresponding band. The results showed that almost the same amount of the transgene was integrated into both TgN(GLAR301Q)1962 and TgN(GLA)1953.

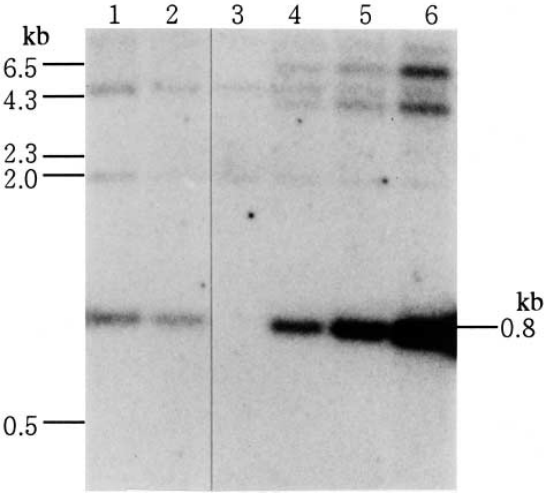

Fig. 1. Southern blot analysis of transgenic mice. 10 μg of genomic DNA from transgenic mice, TgN(GLA)1953 (lane 1) and TgN(GLAR301Q) 1962 (lane 2), was digested with *Pvu*II and then analyzed. Non-transgenic mouse genomic DNA digested with *Pvu*II was used as a negative control (lane 3). One, two or five copies (15 pg, 30 pg and 75 pg; lanes 4, 5 and 6) of plasmid pCXN2 DNA, which contained normal α-galactosidase cDNA, were mixed with non-transgenic mouse genomic DNA before *Pvu*II digestion and used as positive controls. The existence of the transgene is detected as a 0.8-kb band, and a few non-specific bands of larger molecular sizes are also found.

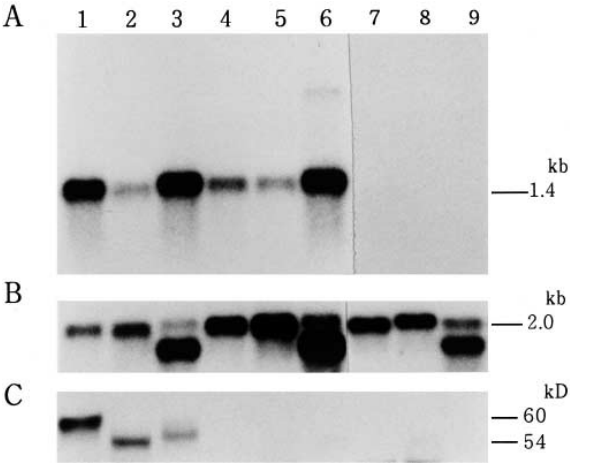

Fig. 2. Northern and Western blot analyses of transgenic mice. A: Detection of human α-galactosidase mRNA transcribed in mice on Northern blotting with human α-galactosidase cDNA as a probe. B: Detection of β-actin mRNA in mice on Northern blotting with human β-actin cDNA as a probe. C: Detection of the human α-galactosidase protein expressed in mice on Western blotting with an anti-human α-galactosidase antibody. Lanes 1–3, TgN(GLA)1953; lanes 4–6, TgN(GLAR301Q)1962; lanes 7–9, non-transgenic mice. Liver (lanes 1, 4 and 7), kidney (lanes 2, 5 and 8), and heart (lanes 3, 6 and 9). In Northern blotting of β-actin, a doublet signal was found in the heart tissue. The reason is not clear. It might be due to the tissue specificity or the assay system with the probe of human origin used here, but not RNA degradation. In Western blotting, the expressed human α-galactosidase proteins were detected as 54–60-kDa bands. The differences in molecular size of the expressed α-galactosidase may be due to the heterogeneity of the sugar chains posttranslationally modified in tissues, because glycopeptidase F treatment showed an identical molecular mass for the peptide moiety of the enzyme (data not shown).

Northern blot analysis revealed that the transgene was highly transcribed in liver and heart, and to a lesser amount in kidney (Fig. 2A). The transcript was clearly detected as a 1.4-kb band on a film, and the size of the transcript was the same as that found in cultured human lymphoblasts (data not shown). A sufficient amount of human α-galactosidase mRNA was transcribed in TgN(GLAR301Q)1962, although it was slightly decreased as compared with that in TgN(GLA)1953, when estimated from the amount of β-actin mRNA as an internal control (Fig. 2B).

α-Galactosidase activity in serum, liver, kidney and heart is summarized in Table 1. High enzyme activity was found in tissues of TgN(GLA)1953 (about 75–510-fold higher than in non-transgenic mice). However, the enzyme activity was very low in tissues of TgN(GLAR301Q)1962 (only about 2-fold higher than in non-transgenic mice, and the enzyme activity might be due to the expressed human mutant enzyme besides the internal enzyme of the mouse), and there were no differences in distribution. Western blot analysis showed expressed human α-galactosidase proteins, detected as 54–60-kDa bands, in tissues of TgN(GLA)1953 (Fig. 2C). However, we could not detect any band representing α-galactosidase in

Table 1
α-Galactosidase activity in transgenic mice

	Serum[a]	Liver[b]	Kidney[b]	Heart[b]
TgN(GLA)1953	7082	6100	900	2200
TgN(GLAR301Q)1962	30	58	22	28
Non-transgenic mouse	14	26	12	12

α-Galactosidase activity: [a]nmol/h/ml, [b]nmol/h/mg protein.

tissues of TgN(GLAR301Q)1962 on Western blotting under the analytical conditions described here (Fig. 2C), although there might be a small amount of an expressed product, considering the results of enzyme assaying. These data suggested that the reduced activity of α-galactosidase in tissues of TgN(GLAR301Q)1962 mainly resulted from a decreased amount of the expressed enzyme protein.

4. Discussion

The human α-galactosidase gene is approximately 12 kb long and consists of seven exons, and its cDNA encodes a precursor peptide of 429 amino acids including a 31-residue signal peptide [1]. The mutation, R301Q, was found in the α-galactosidase gene of a 52-year-old Japanese male Fabry patient with mild clinical manifestations and residual α-galactosidase activity.

We prepared transgenic mice expressing a human mutant α-galactosidase with the R301Q substitution and analyzed them biochemically. The transgenic mice transcribed a sufficient amount of α-galactosidase mRNA in liver, kidney and heart. But the steady-state level of the enzyme protein decreased in all of these tissues, although a small amount of the mutant α-galactosidase with enzymatic activity might exist in the tissues. So, a defect of translation or posttranslational abnormality might occur in the mouse.

Previously we tried to characterize the mutation using cultured cells as samples. The α-galactosidase activity in cultured lymphoblasts from the patient carrying the R301Q mutation was 4% of the normal control level [2,13]. Transient expression of the mutant α-galactosidase cDNA in COS-1 cells revealed that a sufficient amount of α-galactosidase mRNA was transcribed, and low residual enzyme activity was expressed [9]. Furthermore, the addition of galactose to the culture medium of lymphoblasts derived from the patient increased the α-galactosidase activity significantly [13]. The same effect was also found in a mutant, Q279E (CAG→GAG), found in an unrelated patient with the variant form of Fabry disease who expressed α-galactosidase with normal affinity toward a substrate and with decreased molecular stability [6]. Considering the results, such mutant enzymes might be posttranslationally degraded, and the development of drugs that stabilize and/or activate such enzyme proteins may allow the curing of some patients with Fabry disease.

We conclude that transgenic mice expressing human mutant α-galactosidase with the R301Q substitution were established. These mice will be useful for research to clarify the defective regulation of the structurally altered enzyme protein resulting from the mutant gene at the organ or individual level, and furthermore for evaluation of drugs that stabilize and/or activate the mutant enzymes.

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