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α-Galactosidase transgenic mouse: Heterogeneous gene expression and posttranslational glycosylation in tissues

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We produced six transgenic mouse lines expressing human α-galactosidase (α-Gal) in order to evaluate its posttranslational modification. Among them, serum α-Gal activity increased 3000-fold in two transgenic mouse lines (TgN2 and TgN51), as compared to that in non-transgenic lines. The heart and liver of the TgN2 mouse expressed a high amount of transcript as well as high α-Gal activity. Its gene products in the heart and kidney were sensitive to endoglycosidase H digestion, but those in the spleen and liver were largely resistant. Glycopeptidase F treatment confirmed an identical molecular mass for the peptide moiety of the enzyme. We concluded that heterogeneous molecular mass of the gene products was caused by different degrees of posttranslational glycosylation in murine tissues.

***Keywords*: α-galactosidase; Fabry disease; transgenic mouse; glycosylation**

Introduction

α-Galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22; α-Gal) is a lysosomal enzyme catalyzing catabolism of glycoconjugates with terminal α-galactosidic moiety. Hereditary deficiency of this enzyme in humans results in a genetic disease, causing generalized vasculopathy with various clinical manifestations involving the skin, kidney, heart, and nervous system (Fabry disease) [1]. Recent gene analysis revealed various mutations such as coding region insertions and deletions [2–6], splicing defects [6–8], nonsense mutations [6, 9] and missense mutations [5, 6, 8–12].

Some clinical trials of enzyme replacement have been reported [13–15]. The half-life of the exogenous enzyme is short, however, and the enzyme activity rapidly disappears from circulation. In addition, the half-life was different between the enzymes purified from spleen and plasma, probably because of different contents of sialic acid and/or phosphate [15]. Intracellular metabolic turnover of α-Gal is still poorly understood for the reason of low expression and its lack of accessibility in human tissues.

In this study, we produced transgenic (Tg) mice expressing human α-Gal for the purpose of further characterization of the gene product in various tissues.

Materials and methods

Construction of transgene and production of transgenic mice

A DNA fragment comprising chicken β-actin promoter and human α-Gal cDNA was prepared by digestion of pCXN2Gal [16] with *Nde*I and *Apa*LI (New England BioLabs, Beverly, MA, USA). An approximately 3 kb fragment was isolated by agarose gel electrophoresis, recovered on the NA45 DEAE membrane (Schleicher & Schuell, Keene, NH, USA), and purified by cesium chloride centrifugation. The purified DNA fragment was suspended in the injection buffer, containing 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA.

Tg mice were generated by injecting the DNA fragment into the pronuclei of fertilized mouse ova taken from superovulated C57BL/6CrSlc female mice, and the embryos

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were implanted into pseudopregnant Slc : ICR mice [17]. Tg founder mice were identified by polymerase chain reaction (PCR) with a primer set (primers 5 and 6) described in our previous report [4]. A transgene fragment amplified by PCR was confirmed as a single 600 bp band on agarose gel electrophoresis.

Southern and Northern blot analyses

Murine genomic DNA was extracted from liver [18], and digested with *Pvu*II (New England BioLabs). Aliquots were electrophoresed in 1% agarose gel, and transferred to a nylon membrane with the TurboBlotter rapid downward transfer system (Schleicher & Schuell). Total RNA was prepared from murine tissues by acid guanidinium thiocyanate-phenol-chloroform extraction [19]. Poly(A)$^+$RNA was separated by oligo(dT)-cellulose column chromatography. Four micrograms of poly(A)$^+$RNA were electrophoretically separated in a formaldehyde-agarose gel, and transferred to a nylon membrane with the TurboBlotter system. Transferred DNA and RNA were fixed by baking at 80 °C for 2 h. Hybridization and gene detection were performed with the Fluorescein Gene Images labeling and detection system (Amersham International, Buckinghamshire, UK), using plasmid DNA pCXN2Gal and human α-Gal cDNA as probes for Southern and Northern blot analyses, respectively.

Enzyme assay and protein determination

A tissue sample (approximately 0.1 g wet weight) was homogenized in 0.3 ml water with Physcotron (Niti-on, Chiba, Japan), and centrifuged at 10 000 × **g** for 5 min. The water-soluble extract was used as the enzyme source. α-Gal activity was determined with an artificial substrate, 4-methylumbelliferyl α-D-galactopyranoside (Nacalai Tesque, Kyoto, Japan) as described previously [20]. Protein content was measured by the method of Bradford [21].

Endoglycosidase treatment and immunoblotting

A sample aliquot was digested at 37 °C for 16 h, with 2 mU endoglycosidase H (Endo H; New England BioLabs) in 30 μl of 50 mM sodium citrate buffer (pH 5.5), or with 0.3 U of glycopeptidase F (GPase F; Boehringer Mannheim Biochemicals, Tokyo, Japan) in 30 μl of 0.25 M sodium phosphate buffer (pH 7.4), containing 10 mM EDTA and 10 mM 2-mercaptoethanol. Samples before or after endoglycosidase treatment were applied to 7.5–15% polyacrylamide-sodium dodecyl sulfate (SDS) gel, and electrophoresed by the method of Laemmli [22]. Immunoblotting was performed as described previously [16].

Results

α-Gal activity in serum and tissues

Six Tg founder mouse lines were obtained from 80 newborn mice that had been injected with the DNA fragment. They were designated as C57BL/6CrSlc-TgN(aGal)2Rin, C57BL/6CrSlc-TgN(aGal)5Rin, C57BL/6CrSlc-TgN(aGal)44Rin C57BL/6CrSlc-TgN(aGal)45Rin, C57BL/6CrSlc-TgN(aGal)51Rin, and C57BL/6CrSlc-TgN(aGal)53Rin; or TgN2, TgN5, TgN44, TgN45, TgN51, and TgN53, respectively, in abbreviation. All lines showed a marked increase in α-Gal activity in serum (Table 1). The highest α-Gal activity was observed in TgN2 and TgN51, 3000-fold in serum, as compared to those in a non-transgenic (Non-Tg) mouse. Tissue distribution of the enzyme activity was similar in TgN2 and TgN51. A high copy number of transgene was detected in TgN2 and TgN51 by Southern blot analysis (data not shown).

We used the TgN2 line for characterization of the gene product. A marked increase in α-Gal activity was observed in all tissues examined (Table 2); 28 800-, 990-, 410- and 2600-fold inceases in heart, kidney, spleen and liver, respectively.

α-Gal gene expression in TgN2

Northern blot analysis demonstrated a high expression of α-Gal mRNA in the heart and liver of a TgN2 mouse, and less in kidney and spleen (Figure 1).

Characterization of human α-Gal expressed in mouse tissues

Western blot analysis was performed for the gene product expressed in the TgN2 tissues (Figure 2A). The enzyme

Table 1. α-Gal activity in serum from Tg mice.

Mouse	*α-Gal activity (nmol h^{-1} ml^{-1})*
Non-Tg	10
TgN2	30 400
TgN5	3060
TgN44	2170
TgN45	830
TgN51	28 900
TgN53	4570

The second generation mice were used for enzyme assay.

Table 2. α-Gal activity in transgenic mouse tissues.

Mouse	*Heart*	*Kidney*	*Spleen*	*Liver*
Non-Tg	3 ± 1	9 ± 1	39 ± 3	32 ± 5
TgN2	86 500 ± 6800	8900 ± 1600	16 000 ± 2300	83 700 ± 11 500

Enzyme activity: nmol h^{-1} mg^{-1} protein (mean ± SD; n = 6). Non-Tg, non-transgenic; Tg, transgenic.

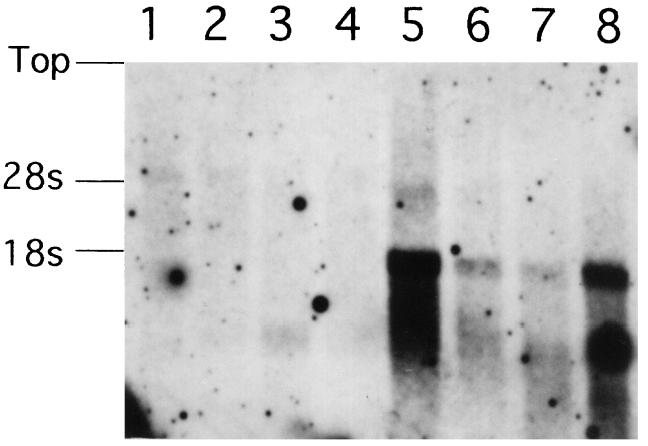

Figure 1. Northern blot analysis of TgN2 mouse tissues. Sample: 4 μg of poly(A)$^+$RNA prepared from heart (lanes 1 and 5), kidney (lanes 2 and 6), spleen (lanes 3 and 7) or liver (lanes 4 and 8) from Non-Tg (lanes 1–4) or TgN2 (lanes 5–8).

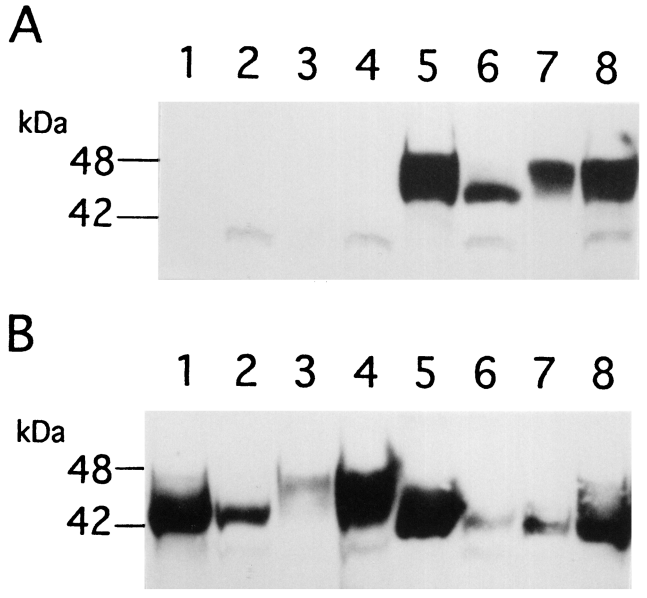

Figure 2. Heterogeneity of human α-Gal in Tg mouse tissues. Tissue extracts (10 μg protein) were electrophoresed on 7.5–15% acrylamide-SDS gels, Western-transferred and immunoblotted with anti-human α-Gal antibody. Lanes 1 and 5: heart; lanes 2 and 6: kidney; lanes 3 and 7: spleen; lanes 4 and 8: liver. Panel A: immunoblotting of human α-Gal from Non-Tg (lanes 1–4) or TgN2 (lanes 5–8) without endoglycosidase treatment. Panel B: immunoblotting of human α-Gal after digestion with Endo H (lanes 1–4) or GPase F (lanes 5–8).

protein appeared as a broad band at 44–48 kDa in the heart, spleen and liver, and as a relatively narrow band at 45 kDa in the kidney. The kidney and liver showed an additional band at 40 kDa in both Tg and Non-Tg mice. No other cross-reacting material was observed against the anti-human α-Gal antibody used in this study.

Human α-Gal expressed in the heart and kidney was Endo H-sensitive, but the expression product in the spleen and liver was largely Endo H-resistant (Figure 2B). Digestion with Endo H resulted in heterogeneous molecular size in the four tissues examined. However, the enzyme after GPase F digestion appeared uniformly as a 42 kDa protein.

Discussion

This is the first report describing production of the human α-Gal Tg mouse and results of the gene product analysis in tissues. Overexpression of human α-Gal apparently did not cause phenotypic abnormalities, and no adverse effect has been observed up to 18 months after birth. Pathological or biochemical investigations have not yet been completed.

All six Tg mouse lines studied in this report expressed human α-Gal stably at remarkably high levels with chicken β-actin promoter in the transgene. Among them, two Tg lines (TgN2 and TgN51) expressed sufficiently high catalytic activity to allow us to analyze the human enzyme in murine tissues. We used the TgN2 mouse for evaluating posttranslational modification of human α-Gal in murine tissues. Overexpression of the human enzyme was sufficient to overshadow the endogenous mouse enzyme.

Tissue expression of human α-Gal was heterogeneous in glycosylation in the glycoprotein enzyme molecule. A complete deglycosylation after treatment with GPase F resulted in a uniform molecular mass at 42 kDa in the polypeptide moiety of the enzyme in all tisues examined in this study. This size is in accordance with that in our previous report using another gene expression system [20]. Bishop and Desnick [23] reported that α-Gal proteins from human spleen and placenta were highly sialylated, and the degree of sialylation was different between the two organs. The α-Gal expressed in mouse spleen and liver was Endo H-resistant in this study. This result suggested that the enzyme in our mice contained a high amount of complex-form oligosaccharides.

At present, the fifth generation of the TgN2 line has been confirmed to express the enzyme activity at the same level as its founder. We will utilize this recombinant mouse for investigations on the tissue-specific posttranslational modification of the human α-Gal enzyme, and for therapeutic animal experiments for Fabry disease in humans.

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