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RESEARCH

Naked Plasmid DNA-Based α-Galactosidase A Gene Transfer Partially Reduces Systemic Accumulation of Globotriaosylceramide in Fabry Mice

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Abstract Fabry disease is an X-linked recessive inborn metabolic disorder in which a deficiency in lysosomal enzyme α-galactosidase A (Gal A) causes the systemic accumulation of globotriaosylceramide (Gb3). Although many investigators have attempted to treat α-Gal A knock-out mice (Fabry mice) with gene therapy, no report has demonstrated therapeutic effects by the retrograde renal vein injection of naked DNA. We recently developed a naked plasmid vector-mediated kidney-targeted gene transfer technique. A solution containing naked plasmid DNA encoding human α-Gal A (pKSCX-α-Gal A) was rapidly injected into the left kidney of Fabry mice (pKSCX-α-Gal A mice). pKSCX was used for mock transfections (pKSCX mice). We confirmed that vector-derived human α-Gal A mRNA was present in the left kidney but not in other tissues, by reverse transcriptase polymerase chain reaction. Compared with the pKSCX mice, the pKSCX-α-Gal A mice showed partial therapeutic effects: increased α-Gal A activity in the injected kidney and in the liver, heart, and plasma, and decreased Gb3 in the injected kidney, contralateral kidney, liver, heart, and spleen. Our results demonstrated that, although further studies are needed to improve the outcome, this method has promise as a potential treatment option for Fabry disease.

Keywords Naked plasmid DNA · Fabry disease · Catheter-based gene transfer · Renal vein injection · CAG promoter · Hydrodynamics-based transfection

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Introduction

Fabry disease is a pan-ethnic, X-linked, lysosomal storage disorder (LSD) with an estimated frequency of 1 in 40,000 male births [1]. It is caused by a deficiency in the lysosomal enzyme α-galactosidase A (Gal A) [2]. Undigested glycosphingolipids with terminal α-galactosyl moieties, mainly globotriaosylceramide (Gb3), progressively accumulate in most organs. Patients with classical Fabry disease have little or no detectable Gal A enzyme activity and suffer from angiokeratoma, hypohidrosis, and episodic pain crises in the extremities during childhood or adolescence. With advancing age there is increased morbidity and mortality due to renal failure, cardiac disease, and early stroke. Heterozygous female Fabry patients manifest a wide spectrum

of disease severity, ranging from a virtually symptom-free course [3] to a course comparable to that in males [4].

The rationale behind the gene therapy for Fabry disease is as follows. Overexpressed hydrolytic enzymes implicated in LSDs, including α-Gal A, are secreted from the transduced cells, and the circulating enzymes are then transferred to other cells by mannose-6-phosphate receptor-mediated endocytosis, a phenomenon called metabolic cooperativity. In gene therapy, expressed α-Gal A could be transferred into uncorrected cells by the same mechanism. Relatively low levels of enzyme correction may suffice to reduce the Gb3 storage in a number of clinically relevant organs [5, 6]. A tight regulation of enzyme production levels is unlikely to be necessary, in part because the acidic pH optimum for enzymatic activity probably means the enzyme is inactive in the circulation at neutral pH [7].

In gene therapy studies using α-Gal A knock-out mice (Fabry mice), several organs have been used as depots for the production of α-Gal A by a variety of vectors [5, 8–16]. The liver was targeted by injecting adenoviral vector via the tail vein [8] or by injecting adeno-associated vectors via the portal vein [9], or the tail vein [10, 14]; the lungs were targeted by the pulmonary instillation of an adenoviral vector [11] or by injecting lipoplex via the tail vein [12]; the muscle was targeted by injecting adeno-associated vector [5] and naked plasmid vector [16]; and systemic organs were targeted by injecting a lentiviral [13] or adenoviral [15] vector.

The kidney is the one of the main organs affected by Fabry disease [17, 18]. We recently developed a novel kidney-targeted gene transfer method using hydrodynamics-based transfection in rats [19] and mice [20]. We demonstrated that this method can transfer naked plasmid DNA into renal interstitial fibroblasts near the peritubular capillaries (PTC) and that the kidney can serve as a depot organ for the production of therapeutic proteins such as rat erythropoietin [19] and mouse interleukin 10 with the Fc protein of mouse IgG2a, IL10/Fc [20]. Here, to decrease the Gb3 level in the kidneys, with an expected concomitant reduction of Gb3 in other organs, we introduced the human α-Gal A gene directly into the left kidney of Fabry mice.

Materials and Methods

Plasmid DNA

Plasmid pKSCX-α-Gal A was constructed by inserting human α-Gal A cDNA into the unique *Eco*RI site between the CAG promoter (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) and the 3′-flanking sequence of the rabbit β-globin gene of the pKSCX expression vector [21], which is based on pCAGGS but with a kanamycin-resistance gene as the selective marker. Plasmids were grown in *Escherichia coli* JM109 (Toyobo, Osaka, Japan) and prepared as described previously [22]. The empty pKSCX plasmid was used for mock transfections.

Transient α-Gal A Expression in COS7 Cells

COS7 cells (RIKEN BRC Cell Bank No. 0539, Ibaraki, Japan) were cultured as described previously [21]. The COS7 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. About 24 hlater, the cells were harvested by trypsin treatment, rinsed with phosphate-buffered saline, and homogenized in 0.2 ml water with a Physcotron (NS-310E, Niti-on, Chiba, Japan). The supernatant was obtained by centrifugation at 10,000 g and used as the enzyme source.

Western Blotting

Supernatants of cell homogenates were used for Western blotting with an anti-α-Gal A antibody raised in rabbits [23] and a horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ, USA). The protein concentration was determined using the DC Protein Assay kit (Bio-Rad Hercules, CA, USA) with bovine serum albumin as the standard. Samples were separated by electrophoresis on 10% SDS-polyacrylamide gels (Ready Gel-J; Bio-Rad). Following electrophoresis, the proteins were electrotransferred to nitrocellulose (Hybond-P; Amersham), and the signals were visualized using enhanced chemiluminescence (Amersham).

Mice

Fabry mice [24] were bred at Niigata University. This experiment was reviewed by the Committee on the Ethics of Animal Experimentation at Niigata University. Hemizygous affected males were bred to homozygous affected females, yielding only affected offspring. Genotypes were determined by PCR analyses [25]. In this study we used only female mice because of possible sex-related differences in the levels of testosterone-induced Gb3 (see Fig. 6, below). About 9-week-old C57BL/6J mice were purchased from Charles River Japan (Tokyo, Japan).

DNA Injection

The plasmid DNA was diluted in Ringer's solution (Ohtsuka, Tokushima, Japan). The mice were anesthetized, and

an incision was made in the median section of the abdomen. Immediately before injection of the DNA solution, the left renal vein and artery were clamped with angled-type Diethrich bulldog clamps (Muromachi Kikai, Tokyo, Japan). It is technically difficult to perform renal vein injection in mice, because their renal vein is thin and short. To overcome this problem, a 1-ml-capacity Myjector syringe with a 29-gauge needle was used (Terumo, Tokyo, Japan). The naked plasmid DNA solution was injected into the vein within 2 sec, and blood flow was re-established immediately afterwards. To avoid narrowing the internal diameter of the renal vein, care was taken not to pull on the kidney as the needle was inserted. Hemostasis was seen at the infected site after applying pressure for 5 sec.

Total Tissue RNA Extraction and RT-PCR Analysis

At 1, 2, 4, and 8 weeks after gene transfer, the mice were killed and their injected kidneys were harvested. Total RNA of the tissue samples was obtained with Isogen (Nippon Gene, Tokyo, Japan). The sequences of the primers were: human α-Gal A gene, backward primer (α-Gal A backward primer), 5′-TCCATTGTCCAGTGCT CTAG-3′; and forward primer (α-Gal A forward primer), 5′-TCTGACTGACCGCGTTACTC-3′. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was also detected by RT-PCR [20]. The α-Gal A primer set encompassed the intron between the CAG promoter and the human α-Gal A cDNA, allowing us to distinguish PCR products from contaminating plasmid DNA. The length of the expected product was 201 bp for α-Gal A mRNA and 452 bp for G3PDH mRNA. The RT-PCR conditions were 60°C for 30 min, 94°C for 2 min, then 40 cycles of 94°C for 1 min, 60°C for 1.5 min, and 60°C, for 7 min.

Quantitative Real-Time PCR Analysis for α-Gal A mRNA in the Injected Kidneys

The total RNA was used for the synthesis of first-strand cDNA with Moloney Murine Leukemia Virus reverse transcriptase (RT; Gibco BRL, Rockville, MD, USA) and random hexamers (Promega, Madison, WI, USA). The RT product was amplified by PCR with *Taq* DNA polymerase (Promega) and specific primers for the human α-Gal A gene, which were identical to the α-Gal A backward and forward primers, and for the mouse housekeeping gene, G6PDH: backward primer, 5′-TTCTTGGTCATCATCTT GGTGTAT-3′; forward primer, 5′-TATCTCAGAGGTGG AAACTGACAA-3′. The primer set for the detection of G6PDH mRNA was also designed to span introns. The length of the expected product was 342 bp for the G6PDH mRNA. These PCR products were directly inserted into the pGEM-T Easy Vector (Promega), to create pGEM-T-α-Gal A or pGEM-T-G6PDH, respectively. These plasmids were grown in *E. coli* DH5α cells and prepared with a QIAprep Spin Miniprep kit (Qiagen) for use as the external standard.

To determine the levels of transgene-derived α-Gal A mRNA in the injected kidney, we performed quantitative real-time PCR analysis using the LightCycler Quick system 330 (Roche Diagnostics, Mannheim, Germany). PCR was performed with LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), according to the manufacturer's protocol. The PCR reaction consisted of 95°C for 10 min, then a total of 40 cycles of 95°C briefly, 62°C for 10 sec, and 72°C for 13 sec with a transition rate of 20°C/sec between temperature plateaus. The data were quantified by the LightCycler analysis software (version 3, Roche Diagnostic). The results were expressed initially as the number of target molecules/2 μl cDNA. To standardize the results for variability in RNA and cDNA quantity and quality, we quantified the total number of G6PDH transcripts in each sample as an internal control. The normalized levels of the α-Gal A transcripts were calculated as the ratio of the number of α-Gal A transcripts to G6PDH transcripts. We confirmed the RT-PCR products by 4% agarose gel electrophoresis.

Enzyme Assay

Tissue samples (approximately 0.1 gwet weight) were homogenized in 0.3 ml water using a Physcotron and spun at 10,000 gfor 5 min. The protein concentration was determined as above. The α-Gal A activity was assayed with an artificial substrate, 4-methylumbelliferyl α-D-galactoside (4MU-α-Gal), as described by Fan et al. [26].

Detection of Neutral Glycosphingolipids by Thin-Layer Chromatography (TLC) Analysis

Tissues from Fabry or wild-type mice were minced with scissors and then homogenized in water. After the protein content was measured, the crude lipids were extracted from homogenates containing 5–10 mg protein with 20 volumes of chloroform–methanol (2:1, v/v). The lipids were dried under a stream of nitrogen and subjected to mild alkaline treatment with 1 ml of 0.2 N NaOH in methanol at 40̊C for 2 h. The solution was neutralized with glacial acetic acid, and the glycosphingolipids were subjected to Folch's partition (chloroform–methanol–H_2O, 8:4:3 in v/v/v), recovered in the lower phase and applied to Thin-Layer Chromatography (TLC) plates. TLC analyses were performed quantitatively with high-performance

TLC (HPTLC)-Silica gel 60 plates (Merck & Co., Inc., Whitehouse Station, NJ, USA) and a solvent system of chloroform–methanol–water (60:35:8, v/v/v). Glycosphingolipids were visualized by spraying the plates with orcinol–sulfuric acid reagent, and their band intensities were determined using Scion Image software. Standard Gb3 from porcine erythrocytes was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Histology

For light microscopy, 1 week after the injection, the mice were sacrificed, and the harvested kidneys were immersion fixed for 4 days in 10% phosphate-buffered formalin, embedded in paraffin, and 10-μm tissue sections were cut and stained with Methylene Blue. For electron microscopy, the mice were placed under general anesthesia, and perfused through the aorta with 10 ml of PBS followed by 10 ml periodate-lysine-paraformaldehyde using a gravity-feed system. The tissue blocks were fixed in 4% formaldehyde/1% glutaraldehyde in phosphate buffer at 4̊C for 24 h. The samples were washed three times in 0.1 M cacodylate buffer with 0.2 M sucrose and postfixed in 1% osmium tetroxide buffered with symcollidine (pH 7.2) at 4̊C for 1 h. After dehydration in serial alcohol and propylene oxide solutions, the samples were infiltrated with and embedded in Epok 812 (Oken, Tokyo, Japan), which is equivalent to Epon. Ultrathin (100 nm) sections were stained with uranyl acetate and lead citrate, and examined with an H-600A electron microscope (Hitachi, Ibaragi, Japan).

Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Anti-Human-α-Gal A IgG Antibodies

Enzyme-Linked Immunosorbent Assay (ELISA) plates (96-well; Nunc, Maxisorb, Roskilde, Denmark) were coated with 1 μg/μl agalsidase beta (Fabrazyme, Genzyme Corp., Cambridge, USA). Serial dilutions of plasma were added to the coated wells. Bound anti-human-α-Gal A IgG antibodies were detected using horseradish peroxidase-labeled goat antihuman IgG anti-body (Cappel, Eschwege, Germany), then incubating with a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 (TMB microwell peroxidase substrate system, KPL, Gaithersburg, MD, USA) for 10 min for color development. Optical density was measured at 450 nm on an ELISA plate reader (Ultrospec Visible Plate Reader; Amersham Pharmacia Biotech, Wals/Satzburg, Austria). The titer was determined by the maximum dilution at which the plasma of the pKSCX-α-Gal A mouse had an absorbance at least twice that of an age-matched pKSCX mouse at the same dilution.

Statistical Analysis

The data are presented as mean values ± standard deviation. All data were analyzed using the StatView statistical program for Macintosh (SAS, Cary, NC, USA). Statistical significance was evaluated using Student's *t*-test. *P* values of < 0.05 were considered to be statistically significant.

Results

In Vitro Expression of α-Gal A

We first tested the expression of human α-Gal A protein in COS7 cells by Western blot analysis. A robust signal of 46 kD, the expected molecular mass, was obtained (Fig. 1). The biological activity of pKSCX-α-Gal A was also examined by measuring the α-Gal A activity in COS7 cells and in their medium (secreted α-Gal A) before and after transfection. The intracellular and secreted α-Gal A activity of COS7 cells significantly increased after the introduction of pKSCX-α-Gal A (Fig. 2). These in vitro results confirmed that this construct delivered functional transgene to the cells and that the overexpressed enzyme was secreted.

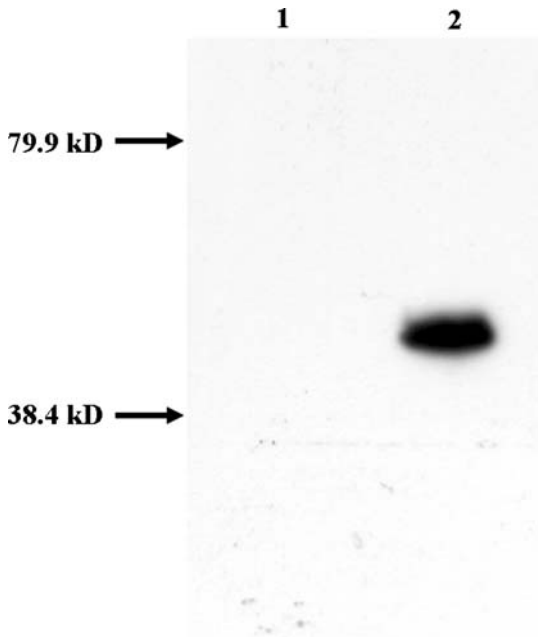

Fig. 1 Western blot of α-Gal A expressed in COS7 cells. Lane 1, empty pKSCX transfection. Lane 2, pKSCX-α-Gal A transfection

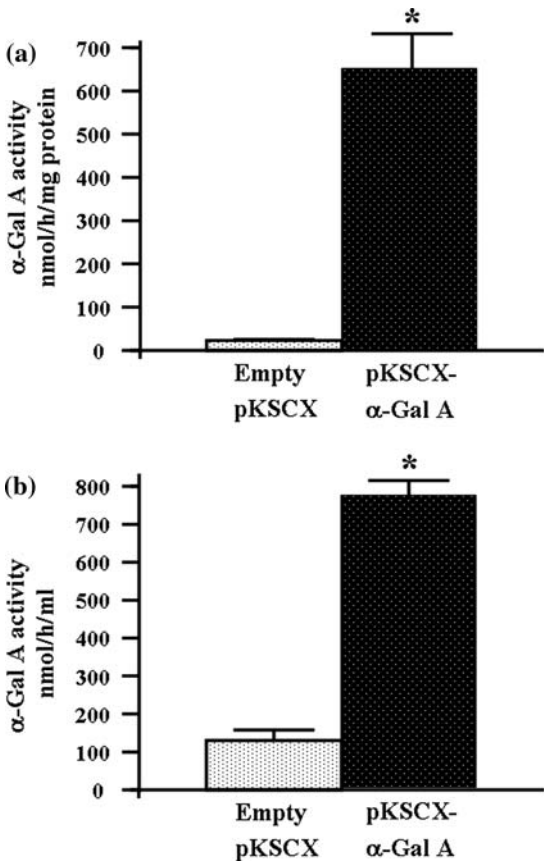

Fig. 2 α-Gal A activity in COS7 cells transfected with pKSCX-α-Gal A (**a**) and in their culture medium (secreted α-Gal A) (**b**). $n = 5$ in each group. *, $P < 10^{-5}$ compared with empty pKSCX

RT-PCR Analysis for α-Gal A mRNA in Major Organs

We detected the transgene-derived α-Gal A mRNA by RT-PCR only in the left kidney on day 1 after the injection (Fig. 3a). We did not detect the transgene-derived α-Gal A mRNA in the kidneys of the pKSCX mice (data not shown), although the control G3PDH mRNA was detected in all the injected kidneys. The expression of transgenic α-Gal A mRNA was sustained in the injected kidney for at least 8 weeks (Fig. 3b).

Quantitative Real-Time PCR Analysis for α-Gal A mRNA in the Injected Kidneys

Quantitative real-time PCR analysis was performed at different time points following the injection of pKSCX-α-Gal A to determine the quantity of transgene-derived human α-Gal A mRNA in the injected kidneys. The quantity of the expressed gene was highest at 2 weeks and then declined over time, but there were no statistical differences between the α-Gal A levels at each time point

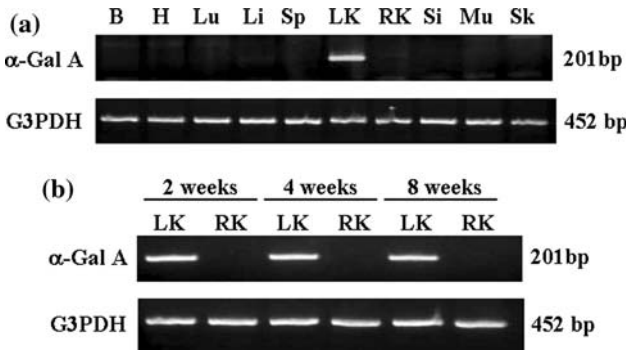

Fig. 3 Brain, heart, lungs, liver, spleen, kidneys, small intestine, muscle, and skin harvested 1 day, and 2, 4, and 8 weeks after transfection RT-PCR analysis for α-Gal A mRNA and control G3PDH mRNA 1 day after pKSCX-α-Gal A injection in major organs (**a**) and 2, 4, and 8 weeks after injection in bilateral kidneys (**b**). B, brain; H, heart; Lu, lungs; Li, liver; Sp, spleen; LK, left kidney; RK, right kidney; Si, small intestine; Mu, muscle; Sk, skin

(Fig. 4). This finding confirmed that an approximately constant level of α-Gal A expression continued for 8 weeks in the injected kidneys of the treated mice.

α-Gal A Levels

We evaluated the time course of α-Gal A activity in the kidneys, heart, liver, spleen, small intestine, and plasma in the treated mice. The pKSCX mice exhibited only low

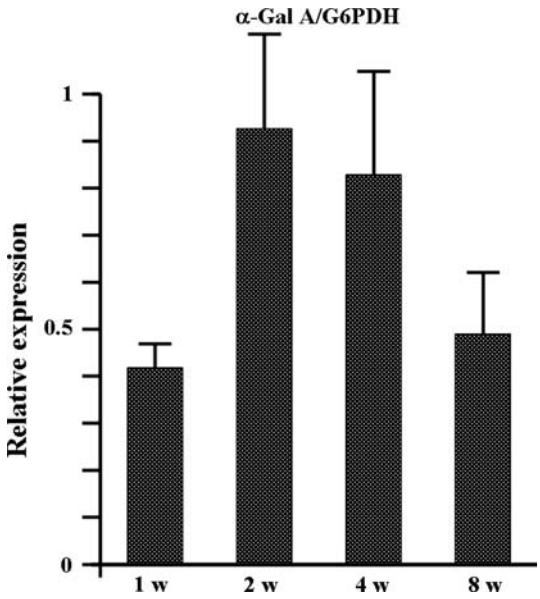

Fig. 4 The levels of α-Gal A mRNA and G6PDH mRNA in the injected kidneys of pKSCX-α-Gal A mice were determined by real-time PCR analysis at 1, 2, 4, and 8 weeks after transfection. Normalized levels of the α-Gal A transcripts were calculated as the ratio of the number of α-Gal A transcripts to G6PDH transcripts. $n = 3$ in each group

levels of α-Gal A activity in the tissues assayed (Fig. 5). This residual enzyme activity was probably attributable to α-galactosidase B [27]. The level of this residual activity in the spleen and small intestine of the pKSCX mice varied by approximately twofold at the three time points shown, and this difference was significant for the small intestine for the values obtained at 2 weeks and at 4 weeks. The reason for these differences, and whether the significant Gb3 reduction in the spleen at the 1- and 2-week time points (see Fig. 7, below) has any bearing on this observation, are unclear at present.

In the pKSCX-α-Gal A mice at 1 week post-injection, the injected kidney showed the highest levels of α-Gal A expression (Fig. 5). Much lower α-Gal A levels were detected in the heart, liver, and plasma, but they were significantly greater than in the pKSCX mice. In the other organs, no significant difference in enzyme activity was found between the pKSCX-α-Gal A and pKSCX mice. About 2 weeks after the injection, there was no significant difference between the pKSCX-α-Gal A and pKSCX mice, even in the injected kidney. Thus, low and transient levels of α-Gal A were expressed in the injected kidney, the secreted enzyme entered the bloodstream, and detectable amounts of enzyme were taken up by the heart and liver.

Gb3 Analysis

We observed an intense signal corresponding to ceramide disaccharide in the kidneys of male C57BL/6J and Fabry mice (Fig. 6a, b). In male C57BL/6J kidneys, a relatively intense signal corresponding to Gb3, compared with the female kidneys, was also observed (Fig. 6a). The male C57BL/6J kidney contained about three times as much Gb3 as the female kidney (Fig. 6c). This abundant Gb3 in the male kidney was probably induced by testosterone [28]. The ceramide disaccharides in the male C57BL/6J kidney were mainly galabiglycosylceramide, which is also induced by testosterone [28], and a small amount of lactosylceramide. The Fabry mouse [24] is a chimera resulting from

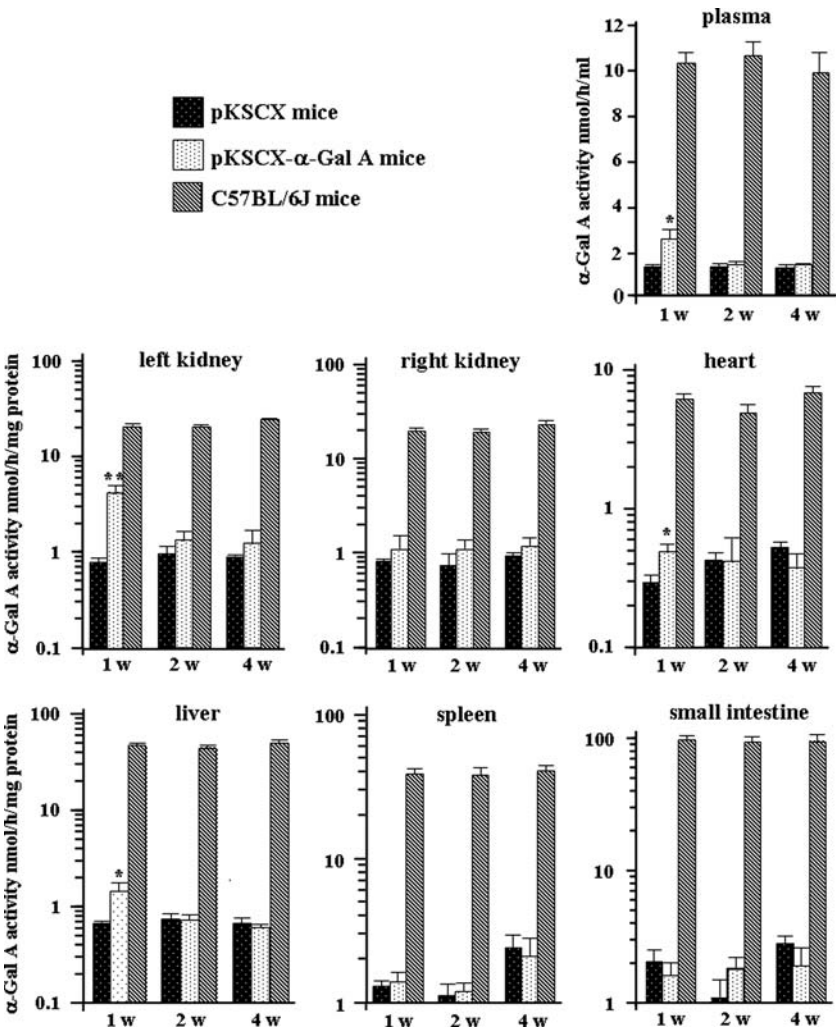

Fig. 5 α-Gal A activity in tissues from pKSCX, pKSCX-α-Gal A, and age-matched C57BL/6J mice. $n = 5$ in each group. *, $P < 0.05$, and **, $P < 10^{-2}$ compared with pKSCX mice

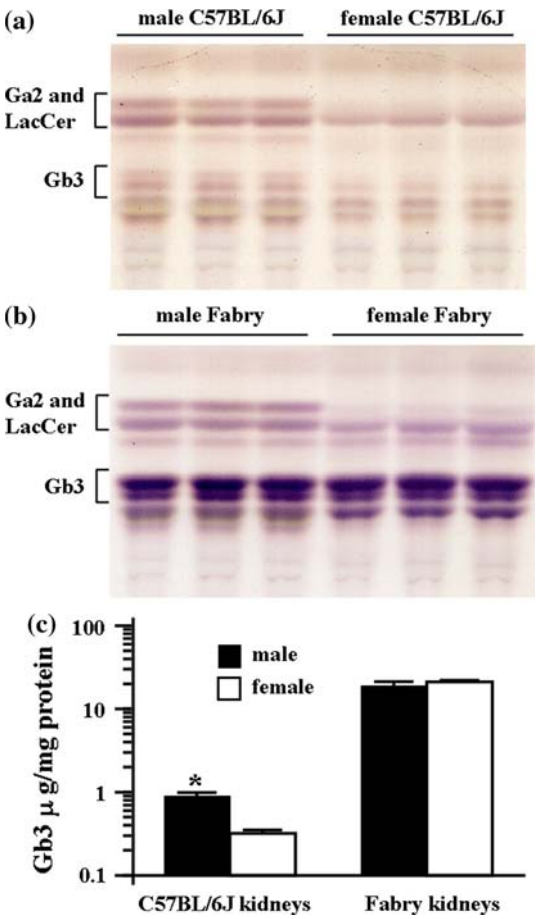

Fig. 6 TLC of neutral glycosphingolipids of C57BL/6J (**a**) and Fabry (**b**) kidneys. Ga2, galabiglycosylceramide. LacCer, lactosylceramide. Gb3 content of C57BL/6J and Fabry (**c**) kidneys. $n = 3$ in each group. *, $P < 10^{-2}$ for males compared with females

modified J1 cells injected into a C57BL/6 background. Therefore, to avoid the possibility of skewing our data with testosterone-induced glycosphingolipids, we used only female mice in all of our gene therapy experiments. We observed no testosterone-induced glycosphingolipids in other organs.

Figure 7 shows the effect of the retrograde injection of pKSCX-α-Gal A into the left renal vein on the Gb3 levels in various organs. A statistically significant reduction in Gb3 levels was observed at 1 week in the injected kidney. A significant reduction in Gb3 was also seen in the spleen, with small but significant reductions in the liver and uninjected kidney, even though no α-Gal A activity was detected in the spleen or uninjected kidney at any time point. The observed reduction in Gb3 in organs that had no detectable enzyme activity may have been owing to a transient, undetectable enzyme activity that reduced the Gb3 level. Subsequently, the reaccumulation of Gb3 in these organs was observed. The Gb3 reduction was especially striking in the spleen at 1 week, being of a similar order of magnitude to that seen in the injected kidney at the same time point. Although the half-life of α-Gal A in the kidney is 0.73 days that of α-Gal A in the spleen is 5.62 days [29]. We therefore assume that the Gb3 level in the spleen was reduced because of the prolonged enzyme half-life in this organ, even though the amount of enzyme was undetectable in the present study. In the injected kidney, and in the liver and spleen, the reductions in Gb3 were sustained for 2 weeks; by 4 weeks, Gb3 had reaccumulated to some extent. In the heart, there was some reduction in Gb3 levels at 4 weeks. The basis for this delayed effect is unclear at present. The levels of Gb3 in the pKSCX tissues remained unchanged or increased slightly over the 4-week interval, except for an initial drop in and recovery of Gb3 content in the spleen 2 weeks after the transfection. The reductions in Gb3 levels in the different pKSCX-α-Gal A tissues were closely correlated with the levels of α-Gal A in these organs.

Histology

To investigate which cell compartments were involved in reducing the Gb3 content and to evaluate histologically the therapeutic effects in the kidney, we analyzed renal specimens of pKSCX-α-Gal A and pKSCX mice by light and electron microscopy. As described elsewhere [24], lipid inclusions with electron-dense concentric lamellar structures in the lysosomes were observed only in the renal tubular cells of Fabry mice. These inclusions appeared not to be affected by our treatment (data not shown), so we could not assess its subcellular effects.

Anti-Human-α-Gal A Antibody

Plasma samples were collected at various time points and analyzed for the presence of anti-human-α-Gal A antibodies by ELISA. pKSCX-α-Gal A mice ($n = 5$) 1-week post-transfection showed no antibody titer. However, both the number of mice that developed antibodies and the antibody titer increased over time (Fig. 8).

Body Weight and Growth

Compared with C57BL/6J females (mean 21.5 g, range 20.2–22.2 g), the female Fabry mice had a low body weight (mean 20.6 g, range 19.4–21.0 g) ($p < 0.05$), but otherwise appeared normal. Although the treated and untreated Fabry mice grew and gained weight during the 8-week duration of the experiment, the pKSCX-α-Gal A

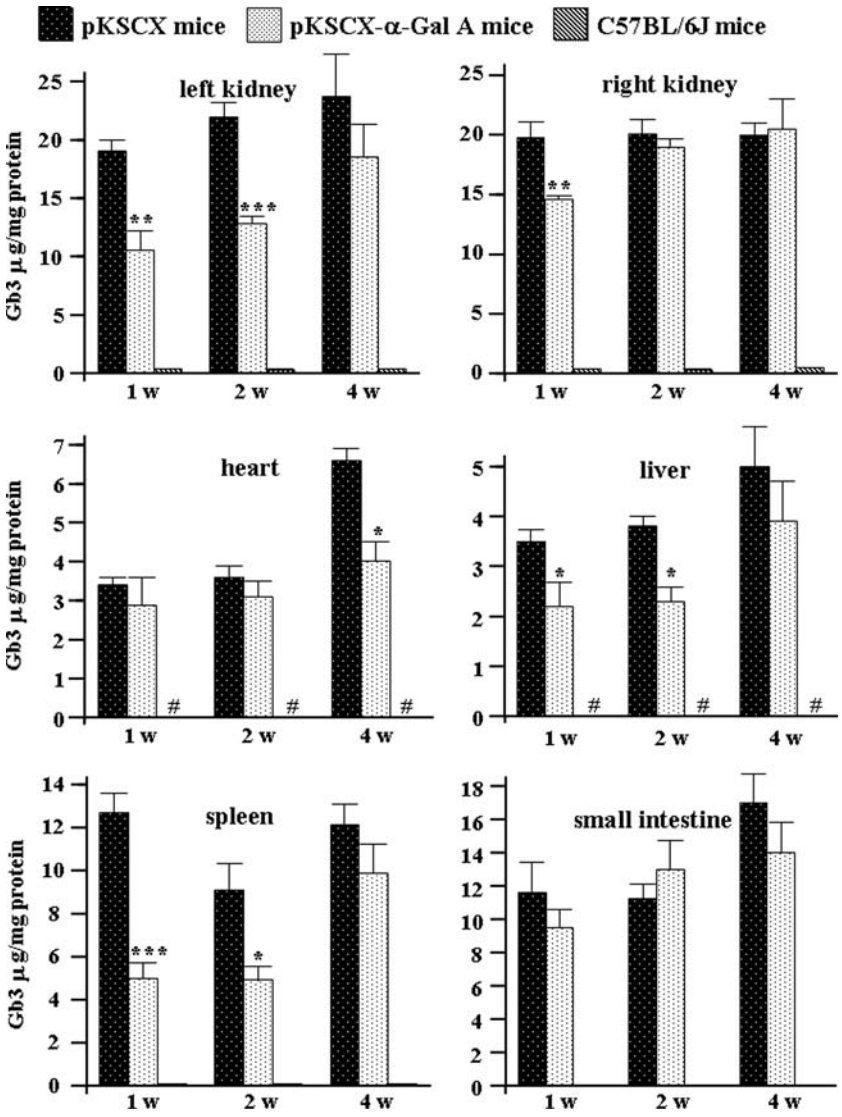

Fig. 7 Gb3 levels in tissues of pKSCX, pKSCX-α-Gal A, and age-matched C57BL/6J mice. $n = 5$ in each group. *, $P < 0.05$, and **, $P < 10^{-2}$, and ***, $P < 10^{-3}$ compared with pKSCX mice. #, not detected. Columns are not shown for some data points because of the low Gb3 levels in the C57BL/6J mice

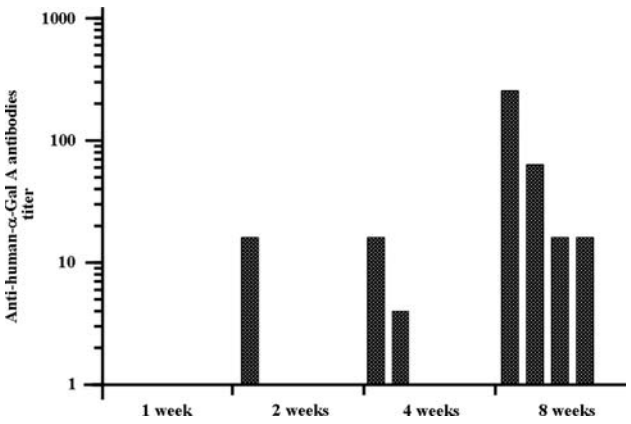

Fig. 8 Anti-human-α-Gal A IgG antibodies were detected by ELISA. Five animals were used for each time point. About 1 week after transfection, no antibodies were detected. At 2 weeks, one of five mice developed a low antibody titer. At 4 weeks,two of five mice showed a low antibody titer. At 8 weeks, four of five mice had developed antibodies, and the titers were relatively high. No antibodies were detected in the pKSCX mice (data not shown)

mice did not gain as much weight as the same-age wild-type mice.

Discussion

The present results demonstrate that a pKSCX-α-Gal A gene that was transferred into the kidney by hydrodynamics-based transfection expressed α-Gal A exclusively in the injected kidney of Fabry mice. Although only a small amount of α-Gal A was expressed, the pKSCX-α-Gal A mice showed partial therapeutic effects in the injected kidney, and therapeutic effects to varying extents in other organs through metabolic cooperativity.

Since kidney involvement is an important cause of morbidity and mortality in patients with Fabry disease [17, 18], the kidneys should be one of the main organs targeted for treatment. However, the Gb3 in the Fabry mouse kidney is relatively resistant to enzyme replacement therapy

(ERT) by systemic intravenous injection [30, 31]. In ERT, most (65 ~ 70%) of the injected enzyme is recovered in the liver via the hepatic asialoglycoprotein and mannose receptors, and less than roughly 1% of the injected dose is detected in the Fabry mouse kidney [32].

The effect of gene therapy in the Fabry mouse kidney seems to depend on the transgene method and/or transduced cells: some studies [11–13] reported no benefit, some [8–10] showed partial therapeutic effects (60 ~ 74% of the Gb3 levels in the untreated Fabry mouse kidney), and some [5, 14, 15] demonstrated a significant reduction in Gb3 levels (3 ~ 25% of the untreated Fabry mouse kidney). Although Takahashi et al. [5] found that the intramuscular injection of a recombinant adeno-associated vector construct led to sustained circulating levels of α-Gal A and reduced Gb3 in the kidney, Park et al. [14], using a similar but not identical method, obtained no systemic α-Gal A activity. Clearly, additional experiments are required to clarify why completely different results were obtained in these two studies.

Although a significant therapeutic effect in the kidney can be obtained using viral vectors, potential problems include the humoral immunological reaction against viral vectors [33] and the possible presence of pre-existing antiviral antibodies, which could influence the transduction efficiency [34]. Plasmid vectors have several advantages over viral vectors. A large quantity of highly purified plasmid DNA can be obtained easily and inexpensively, and gene transfer can be repeated without any apparent immune response to the DNA vector. The central problem with plasmid DNA transfection is efficiency, as most of the transferred DNA does not get into the target cells or is quickly degraded. Also, because the plasmid DNA does not integrate with genomic DNA, expression of the transgene is transient. However, since low enzyme activity is sufficient to clear the Gb3 deposits in various organs [5, 6], direct gene transfer to the kidney by naked plasmid vector, to provide a direct therapeutic effect in the transfected kidney and in other visceral organs through metabolic cooperativity, appears to be a rational strategy for treating Fabry disease.

Replagal® (agalsidase alfa, Transkaryotic Therapies, Cambridge, MA, USA) and Fabrazyme® (agalsidase beta, Genzyme, Cambridge, MA, USA) are genetically engineered forms of α-Gal A. These two glycoproteins have identical amino acid sequences but are produced in different cell lines (Replagal® in a genetically engineered human cell line, and Fabrazyme® in a Chinese hamster ovary cell line), resulting in different glycosylations at the N-linked carbohydrate attachment sites. Fabrazyme® contains a higher fraction of sialylated and phosphorylated carbohydrate; Replagal® contains a greater amount of complex carbohydrate [32]. Interestingly, following a single intravenous bolus injection of 3 mg/kg of Replagal® or Fabrazyme® into the Fabry mouse, approximately twice as much Fabrazyme® is detected in the kidney as Replagal® [32]. In Fabrazyme®, the carbohydrate at N215, mostly biphosphorylated oligomannose, extends away from the protein, in an ideal position to bind the mannose-6-phosphate receptor, but in Replagal® the carbohydrate at this site is mostly singly phosphorylated oligomannose [35]. Since these two polypeptides are derived from the same human α-Gal A gene, these different post-translational modifications, especially the carbohydrate composition, are probably responsible for their differences in tissue distribution and dose response. We have shown that a minimal expression of α-Gal A reduced the Gb3 levels in the injected kidney, and that secreted α-Gal A reduced Gb3 in the contralateral kidney (where no detectable α-Gal A activity was observed). Our present results are different from those of previous studies, in which no therapeutic effects in the Fabry mouse kidney were observed using other transgene methods [11–13]. Different post-translational modifications in the transgene-expressing cells could explain these discrepancies.

A decrease in glycolipid inclusions is observed in the renal vascular endothelium, but not in other renal cell compartments, in response to ERT [36]. An in vitro study using immortalized human renal tubular epithelial cells demonstrated that these cells have a relatively low affinity for α-Gal A [37]. Consistent with these findings, we did not observe a reduced number of inclusions in the renal tubular cells of the α-Gal A-treated Fabry mice. However, Thurberg and colleagues observed a significant reduction in Gb3 levels in mesangial cells and a modest one in podocytes within 5 months of initiating ERT [38]. Theoretically, in humans, circulating α-Gal A cannot pass through glomerular capillary walls because this barrier normally traps molecules greater than 50 Å [39], and α-Gal A is 75 Å × 75 Å × 50 Å [34]. It is possible that the injured glomerular endothelium allowed some protein to pass through the barrier in Thurberg's study [38]. Nevertheless, the finding that circulating α-Gal A cannot be efficiently taken up by renal cells supports the strategy of transducing the α-Gal A gene and expressing α-Gal A in the Fabry kidney.

Although we confirmed that a relatively constant level of transgene expression was maintained for at least 8 weeks, α-Gal A activity could be detected for only 1 week after the transfection, and Gb3 had reaccumulated in the kidneys 4 weeks after the transfection. We detected some anti-human-α-Gal A antibodies in the pKSCX-α-Gal A mice, probably because Fabry mice do not express endogenous α-Gal A. We speculate that the deactivation of α-Gal A was mainly due to the anti-human-α-Gal A antibodies, as is the case with anti-human factor VIII antibodies in Hemophilia A model mice [40].

In this study, we could not reduce the Gb3 to the levels in C57BL/6J mice, even in the injected kidney, and new methods are needed to improve the outcomes. However, although the safety of this approach needs to be further investigated in large animals, we demonstrated that the pKSCX-α-Gal A gene transferred by hydrodynamics-based transfection has the potential to be an alternative treatment option for Fabry disease, especially targeting the kidney, alone or in combination with ERT.

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