Conditional expression of human acid β-glucosidase improves the visceral phenotype in a Gaucher disease mouse model

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Abstract The reversibility and regression of histological and biochemical findings in a mouse model of Gaucher disease (4L/PS-NA) was evaluated using a liver-enriched activator protein promoter control of a tetracycline-controlled transcriptional activation-responsive human acid β-glucosidase (hGCase) transgenic system. 4L/PS-NA has the acid β-glucosidase (GCase) V394L/V394L (4L) point mutation combined with hypomorphic (~6% wild-type) expression of the mouse prosaposin transgene (PS-NA). The hGCase/4L/PS-NA had exclusive liver expression of hGCase controlled by doxycycline (DOX). In the absence of DOX, hGCase was secreted from liver at levels of ~120 μg/ml serum with only ~8% of full activity, following exposure to pH 7.4 in serum. The hGCase activity and protein were detected in cells of the liver (massive), lung, and spleen, but not the brain. The visceral tissue storage cells and glucosylceramide (GC) accumulation in hGCase/4L/PS-NA were decreased from that in 4L/PS-NA mice. Turning off hGCase expression with dietary DOX led to reaccumulation of storage cells and of GC in liver, lung, and spleen, and macrophage activation in those tissues. This study demonstrates that conditionally expressed hGCase supplemented the existing mutant mouse GCase to control visceral substrate accumulation in vivo.—Sun, Y., B. Quinn, Y-H. Xu, T. Leonova, D. P. Witte, and G. A. Grabowski. **Conditional expression of human acid β-glucosidase improves the visceral phenotype in a Gaucher disease mouse model.** ***J. Lipid Res.*** **2006.** 47: **2161–2170.**

The defective lysosomal hydrolysis of glucosylceramide (GC) in Gaucher disease is caused by mutations in the gene (*GBA*) that encodes acid β-glucosidase (GCase) (EC3.2.1.45) (1). About 300 mutations in the *GBA* have been identified in Gaucher disease patients with variable phenotypes (1, 2). The resultant accumulation of GC leads to the major phenotypic manifestations, which include hepatosplenomegaly, bony lesions, and in selected variants, central nervous system (CNS) involvement (1, 3–5). In visceral tissues, cells of the monocyte/macrophage lineage are the principle cells containing the accumulated glycolipid (1). Regular intravenous administrations of GCase, i.e., enzyme therapy, have improved visceral outcomes and quality of life in patients with Gaucher disease (6–8).

Saposins (A, B, C, and D) are small (80 amino acids) sphingolipid activator proteins that are proteolytically derived from a common precursor, prosaposin, and each enhances the activity of specific lysosomal hydrolases (9–11). GCase requires saposin C for optimal in vitro and in vivo activity (12–14). The deficiency of prosaposin/saposins ($PS^{-/-}$) leads to a decrease of GCase activity in selected tissues of affected humans and mice due to the loss of the proteolytic protective effects of saposin C (15–19).

Recently, mouse models with point mutations in *gba* were developed (20) and bred into a hypomorphic prosaposin (PS-NA) mouse with ~6% of wild-type (WT) levels of saposins in liver (19). The *gba* mutant that encoded a V394L substitution was bred into PS-NA, and was termed 4L/PS-NA. In humans, the V394L allele has been observed in heteroallele state (3). Mice homozygous for V394L(4L) displayed minor phenotypic abnormalities and normal life span (20). The PS-NA mice showed survival to 8 months with progressive CNS deterioration, but little GC accumulation in the CNS or visceral organs. In comparison, the 4L/PS-NA mice showed numerous engorged macrophages and large amounts of GC accumulation in visceral tissues, including liver, lung, spleen, and thymus (19). The partial deficiency of saposins led to decreased stability of the mutant GCase to proteolysis and subsequent additional decreases in V394L GCase activity. This model mimics a more severe Gaucher disease phenotype and is a

Abbreviations: CNS, central nervous system; CRIM, cross-reacting immunological material; DOX, doxycycline; GC, glucosylceramide; GCase, acid β-glucosidase; hGCase, human acid β-glucosidase; LAP, liver-enriched activator protein; SA, specific activity; tTA, tetracycline-controlled transcriptional activation; WT, wild type.

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useful tool for testing therapeutic approaches and studying pathophysiological mechanisms of Gaucher disease.

Tetracycline-controlled transcriptional activation (tTA) systems have been widely used for the generation of cell lines and animals for the conditional expression of genes (21). This system has set up a "gene switch" to control the gene expression in response to tetracycline (22–24). The applications of this system have provided invaluable insights into the temporal or spatial regulation of the genes in physiological and pathological conditions (22, 24). Here, a tTA system was used to test the organoid approach for the treatment of glycosphingolipid storage diseases. A tTA-responding human acid β-glucosidase (hGCase) transgene was introduced into 4L/PS-NA mice for controllable hGCase expression in liver in response to doxycycline (DOX). The effects of hGCase on the pathological lesions in 4L/PS-NA mice showed temporal and tissue-specific effects. These data also provide insight into the ability of an intravenous continuous supply of hGCase to affect CNS accumulation, the amounts of prosaposin/saposins needed for GCase activity, and assessments of therapeutic interventions and substrate accumulation. This model provides a system to evaluate a variety of effects on tissues of endogenous enzyme therapy for Gaucher disease and assessment of GC reaccumulation following periods off enzyme therapy.

MATERIALS AND METHODS

Materials

The following were from commercial sources: Trizol™ reagent, Superscript™ First-Strand Synthesis System for RT-PCR Kit, NuPAGE 4–12% Bis-Tris gel, and NuPAGE MES SDS running buffer (Invitrogen, Carlsband, CA); 4-methylumbelliferyl-β-D-glucopyranoside (4 MU-Glc; Biosynth AG, Switzerland); sodium taurocholate (Calbiochem, La Jolla, CA); rat anti-mouse F4/80 and CD68 monoclonal antibodies (Serotec, Oxford, UK); mammalian protein extraction reagent and bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL); Molecular Dynamics Storm 860 scanner; Hybond™-ECL™ nitrocellulose membrane and an ECL detection reagent (Amersham Biosciences, Piscataway, NJ); antifade/4,6-diamidino-2-phenylindole (DAPI); ABC Vectastain and Alkaline Phosphatase Kit II (Black) (Vector Laboratory, Burlingame, CA); and FITC-conjugated goat anti-rabbit antibody and rhodamine-conjugated goat anti-rat antibodies (ICN Biomedicals, Inc., Aurora, OH).

Methods

Generating hG/4L/PS-NA mice. hG/4L/PS-NA mice were generated by breeding 4L/PS-NA (PS-NA; V394L/V394L) (19) mice with mice containing a 2.9 kb transgene fragment of the liver-enriched activator protein (LAP) promoter and tTA gene (22). The 4L/PS-NA mice have a low-level-expressing (hypomorphic) prosaposin transgene in the background of the prosaposin knockout ($PS^{-/-}$) (25). Additionally, these mice are homozygous for the V394L point mutation at the *gba* locus (20). The LAP-tTA transgene expresses specifically in hepatocytes (22), and tetracycline derivatives, i.e., DOX, turn off expression of responsive genes. The TetO-hGCase transgenic line contains the Tet operator element fused with hGCase cDNA (1.6 kb) that is tetracycline-responsive. The TetO-GCase transgene construct also contains the cytomegalovirus(CMV) minimal promoter, an intron of the β-globulin gene, and the CMV polyA (21). Only one of several founders contained a TetO-hGCase that was significantly responsive to expressed LAP-tTA and was used for all experiments. The strain background for hG/4L/PS-NA mice was 1/2:1/4:1/4, FVB/C57BL6/129SvEvbrd. The mice were maintained in a microisolator pathogen-free environment and in accordance with institutional guidelines of the institutional animal care and use committee at Cincinnati Children's Hospital Research Foundation.

PCR genotyping

After mutation validation of *gba* by sequencing, the lox-P site was used as a marker for V394L genotyping (20). The prosaposin knock-out allele was genotyped as previously described (25). LAP-tTA and TetO-hGCase transgenes were genotyped by multiplex PCR with 64°C as the annealing temperature. The primers for LAP-tTA (forward, 5′CGCTGTGGGGCATTTTACTTTAG-3′ and reverse, 5′CATGTCCAGATCGAAATCGTC-3′) generated a 500 bp PCR product. For TetO-hGCase, primers (forward, 5′GACTTCTCCATCCGCACCTA-3′ and reverse, 5′CCACAACAGCAGAGCCATCG-3′) were used in PCR to generate a 993 bp band.

RT-PCR

Total RNA was extracted from mouse tissues using TriZol™ reagent (Invitrogen). Reverse transcription of total RNA (1 μg) for each tissue was carried out using Invitrogen Superscript™ First-Strand Synthesis System for RT-PCR Kit and oligo (dt) 12–18 (0.5 μg) and random hexamers primers (50 ng). The resulting cDNA was amplified using 10 pmoles of the hGC forward (5′CCACGCTGTTTTGACCTCCATAGA-3′) and hGC reverse (5′GTTGAGAGCAGCAGCATCTGTCAT-3′) primers to generate a 400 bp band from the hGCase transgene. The primers were from the TetO-CMV promoter and 5′ of hGCase cDNA regions. GAPDH was the internal control for RT-PCR. The PCR reaction of each RNA sample without adding reverse transcriptase in the RT reaction was carried out as negative control.

Histological studies

Routinely, tissues were collected, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H and E). Karnovsky's fixative was used for ultrastructural studies. For CD68 monoclonal antibody staining, the 4% paraformaldehyde-fixed tissue sections were treated with acetone for 10 min at −20°C and then with autozyme for 10 min at 37°C. The samples were blocked in 2.5% normal rabbit serum diluted in PBS containing 5% BSA for 20 min and then incubated with rat anti-mouse CD68 (1/200 in PBS containing 5% BSA) overnight at 4°C. Detection was performed using ABC Vectastain and Alkaline Phosphatase Kit II (Black) according to the manufacturer's instructions. The slides were counterstained with methylene green for nuclei. For hGCase and F4/80 immunofluorescence staining, the frozen tissue sections were incubated in rabbit anti-hGCase antiserum (1/200 diluted in PBS with 1% normal goat serum) and rat anti-mouse F4/80 monoclonal antibody (1/20). FITC-conjugated goat anti-rabbit antibody (1/100 in PBS) and rhodamine-conjugated goat anti-rat antibody (1/50) were applied to the sections, respectively. The sections were counterstained with antifade/DAPI. The signals were visualized by a Zeiss Axiovert 200M microscope equipped with an ApoTome.

Enzyme activity assay

Tissues collected from saline-perfused mice were homogenized, and GCase activities were determined fluorometrically

with 4 MU-Glc in 0.25% sodium taurocholate and 0.25% Triton X-100. Assay mixtures were preincubated in the presence and absence of the GCase irreversible inhibitor, conduritol B epoxide (1 mM), a specific inhibitor of GCase, for 40 min at 37°C to ensure that only GCase activities, and not nonspecific glucosidase activities, were being assessed. The substrate (4 MU-Glc) was added, and the reactions were stopped after an additional 30 min of incubation (37°C). WT control tissues were run in parallel for all assays.

GCase activities in plasma were determined as above. The amount of active hGCase in plasma samples was determined using matched activity samples from plasma and purified recombinant hGCase (imiglucerase) subjected to immunoblotting. From the standard curve of the pure enzyme, the amount of plasma hGCase protein that provided a particular level of activity could be quantified and compared with the immunoreactive signal standard curve for imiglucerase developed on the same gels. These measurements are termed CRIM (cross-reacting immunological material) specific activity (SA).

Tissue lipid analyses

The lipids in tissue samples (~100 mg wet weight) were extracted as described previously (19). Relative proportions of lipids from the tissue samples were determined by thin-layer chromatography (TLC) with borate-impregnated plates (10 cm^2, Merck HPTLC silica gel 60, 200 μm). Plates were developed in chloroform-methanol-water (65:25:4; v/v/v). Lipids were visualized with primulin spray (100 mg/l in 80% acetone) and quantified by blue fluorescence scanning (Storm 860, Amersham Pharmacia Biotech). The amount of GC in each sample was quantitated by fluorescence intensity scanning relative to the GC duplicate standards for each mouse. The intensities were within the linear ranges of the instrument. GC values are from more than three mice for each genotype, and at least two experiments were used in the lipids quantitation.

Immunoblots

Tissue extracts were separated on NuPAGE 4–12% Bis-Tris gel with NuPAGE MES SDS running buffer, and electro-blotted on Hybond™-ECL™ nitrocellulose membranes. The membranes were blocked in 3% BSA for 1 h, followed by incubation overnight with the rabbit anti-hGCase antibody (1/1,500 in 0.15% milk and 1% BSA) to detect hGCase. The signal was developed using an ECL detection reagent according to the manufacturer's instructions. Protein concentrations were determined using BCA protein assay reagent.

RESULTS

Generating hG/4L/PS-NA mice

4L/PS-NA mice were generated by crossing 4L homozygous mice with those bearing a hypomorphic prosaposin transgene (PS-NA) (19). The tetracycline-mediated expression of the hGCase transgene driven by the LAP promoter was introduced into the 4L/PS-NA mice by crossing 4L/PS-NA with either TetO-hGCase or LAP-tTA transgenic mice. The resulting LAP-tTA/4L/PS-NA or TetO-hGCase/4L/PS-NA mice were cross-bred to obtain the LAP-tTA/TetO-hGCase/4L/PS-NA mice, designated hG/4L/PS-NA.

Male and female hG/4L/PS-NA mice were fertile for ~3 months and able to produce one to two litters (**Table 1**).

TABLE 1. Mouse Models

Genotype	Strains	%	Life Span	Visceral Tissues	Fertility	GCase Activity in Liver	GC Level in Liver	Saposin Level in Liver	References
						% WT	*μg/mg Tissue*	*% WT*	
GCase mutant mice									
V394L	C57BL/6 (50)	129SvEvBrd (50)	~2 Years	Slight GC accumulation by TLC; very few storage cells	Normal	7	ND	100	(20)
Prosaposin-deficient mice									
PS-NA	FVB		32 Weeks	Slight GC accumulation by TLC; few storage cells	Male and female <3 months	60	0.5	6	(19, 25)
V394L and PS-NA mice									
4L/PS-NA	FVB (50)	C57BL/6 (25) 129SvEvBrd (25)	22 Weeks	Large GC accumulation by TLC; engorged macrophages	Male <3 months	3	1.5	6	(19)
TetO-h GCase in 4L/PS-NA									
hG/4L/PS-NA	FVB (50)	C57BL/6 (25) 129SvEvBrd (25)	35 Weeks	Slight GC accumulation by TLC; few storage macrophages	Male and female <3 months	⩾300	0.4	6	Current

ND, not done.

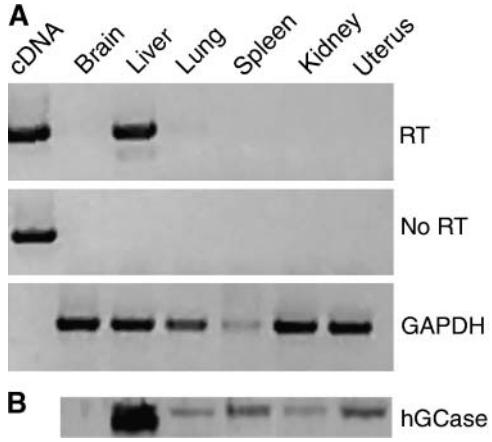

Fig. 1. Expression of human acid β-glucosidase (hGCase) in hG/4L/PS-NA mice. A: RT-PCR analysis of hGCase mRNA in tissues showed that hGCase was expressed only in liver [top panel (RT)]. The middle panel (No RT) shows the negative control, i.e., the RT reaction without reverse transcriptase. The bottom panel is the GAPDH internal control. cDNA is TetO-hGC plasmid DNA as positive control in the PCR reaction. B: Western blot analysis using anti-hGCase antibody that reacts only with the human protein (26) showed hGCase protein in perfused tissues, including liver (large amounts) and lung, spleen, kidney, and uterus (lower levels). No hGCase was detected in brain. Tissue lysate (50 μg) was loaded in each lane. Because anti-hGCase antiserum detects only human proteins, no GCase can be detected in mouse tissues, including 4L/PS-NA and wild type (WT).

The hG/4L/PS-NA mice survived for up to 35 weeks, whereas the 4L/PS-NA mice lived for up to 22 weeks. The males of 4L/PS-NA mice are fertile, but females rarely produced litters. The neurological signs and neuropathology in hG/4L/PS-NA mice developed at ~3 months of age and were similar to those of the 4L/PS-NA mice (19). This aspect was unaltered and will not be evaluated here.

Expression of hGCase in hG/4L/PS-NA mice not on DOX

Tissues were collected from hG/4L/PS-NA mice after saline perfusion. By RT-PCR analyses, hGCase mRNA was expressed in liver only (**Fig. 1A**). Using anti-hGCase antiserum that detects only the human enzyme, Western blot analyses showed that hGCase protein was present in liver (Fig. 1B) in large amounts, and in spleen, lung, kidney, and uterus at lower levels. No hGCase protein was detected in the hG/4L/PS-NA brain of 4L/PS-NA or WT mice (data not shown).

To determine whether hGCase was secreted from liver, serum from mice not on DOX (DOX[−]) (DOX[−] hG/4L/PS-NA mice) was analyzed for GCase activity and protein. High levels of GCase activity (~7,000 nmoles/h/ml) were detectable in serum of hG/4L/PS-NA mice. No GCase activity was detected in sera from either WT or 4L/PS-NA mice. The CRIM SA of hGCase in mouse serum was assessed (see Methods). The active enzyme in serum accounted for ~8% of the total hGCase (~120 μg/ml) present in serum. This result indicates that the majority of secreted hGCase was partially denatured, probably by exposure to the pH 7.4 environment in the bloodstream (26), before being taken up by other tissues.

The cellular localization of hGCase in tissues was evaluated by immunofluorescence using rabbit anti-hGCase antiserum. Markedly positive signals were found in hepatocytes, and these varied somewhat between hepatocytes. hGCase was also detected in the hepatic interstitial spaces and sinusoidal lining cells (endothelial cells and Kupffer cells) (**Fig. 2**). Staining with F4/80, a macrophage-specific membrane antigen, documented hGCase colocalization within F4/80-positive cells, i.e., Kupffer cells (Fig. 2). This antibody does not react with mouse GCase. No hGCase signal was found in WT mouse liver (negative control). In lung and spleen, cellular hGCase was not found at detectable levels by immunofluorescence, although it was detected by immunoblots (Fig. 1). GCase activity in the CNS was the same as in PS-NA mice, and no immunoreactive hGCase was detected in the CNS by immunofluorescence (data not shown) or immunoblots (Fig. 1). Thus, hGCase expression was driven by the LAP-tTA system in hG/4L/PS-NA mice to high levels in hepatocytes. hGCase was secreted into the peripheral circulation, where it was taken up by sinusoidal lining cells in the liver and other visceral tissues, but it did not cross the blood-brain barrier.

hGCase corrected storage in visceral tissues in DOX[−] hG/4L/PS-NA mice

4L/PS-NA mice had GC accumulation and storage macrophages in liver, lung, and spleen (19) (**Fig. 3**). The

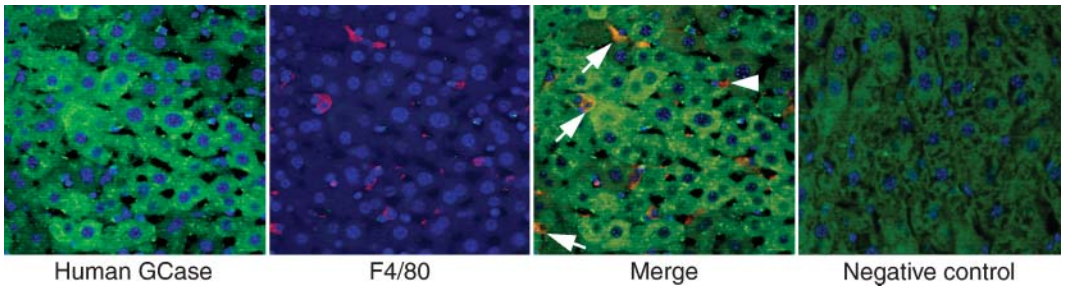

Fig. 2. Cellular localization of hGCase. hGCase protein is the green signal (FITC) in hepatocyte, interstitial spaces, and sinusoidal lining cells of an hG/4L/PS-NA mouse. Most Kupffer cells (red) were labeled by F4/80 colocalized with hGCase (arrow). Some Kupffer cells had no detectable hGCase (arrowhead). The negative control shows no hGCase signal in a WT mouse liver. 4,6-Diamidino-2-phenylindole (blue) stains nuclei.

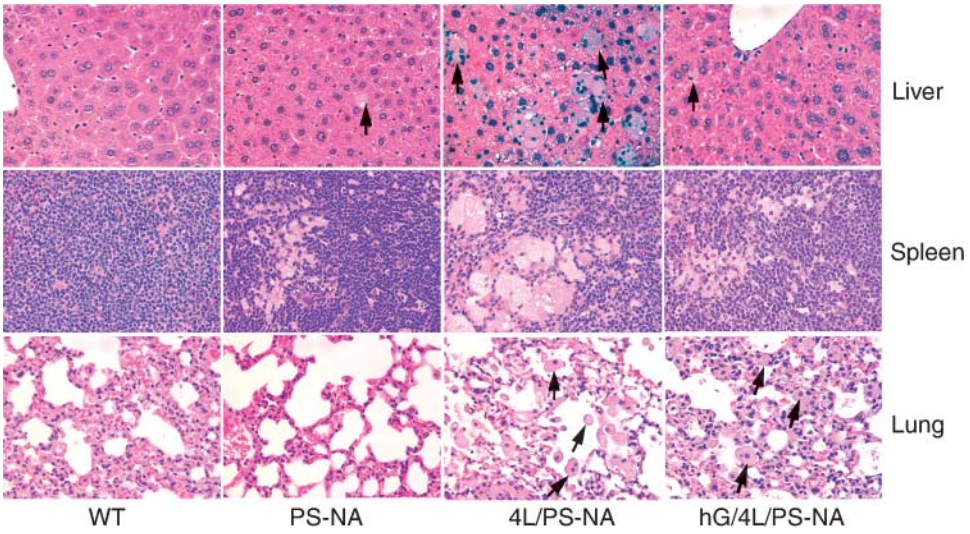

Fig. 3. Storage cells in visceral tissues. Paraffin tissue sections of WT, PS-NA, 4L/PS-NA, and hG/4L/PS-NA mice (21 to 22 weeks) were stained with hematoxylin and eosin (H and E). The liver, spleen, and lung of WT mice had a normal appearance. Rare storage cells were present in spleen and liver of PS-NA mice (arrows). Clusters of lipid storage cells (arrows) were in liver, spleen, and lung of 4L/PS-NA mice. In hG/4L/PS-NA mice, the expression of hGCase reduced storage cells markedly in liver and less in spleen and lung. Magnifications are 400×.

storage cells in hG/4L/PS-NA mice were substantially cleared from the liver, and moderately reduced in spleen and lung at 22 weeks (Fig. 3). TLC analyses showed that dual GC bands were present in visceral tissues. They arise from variable lengths of fatty acid chains on GC. Both GC bands were reduced in hG/4L/PS-NA mice. GC levels were decreased in liver (>75%), spleen (75%), and lung (40%) of hG/4L/PS-NA mice relative to 4L/PS-NA mice (**Figs. 4** and 7B). The GC levels in the CNS of hG/4L/PS-NA mice were unchanged compared with 4L/PS-NA mice. No difference in GC levels were found between male and female mice.

Ultrastructural examination of liver specimens showed that the expression of hGCase cleared most storage inclusions. A few sinusoid lining cells in liver of hG/4L/PS-NA mice had small numbers of inclusions, compared with 4L/PS-NA mice, which had more cells containing large clusters of inclusions (**Fig. 5**).

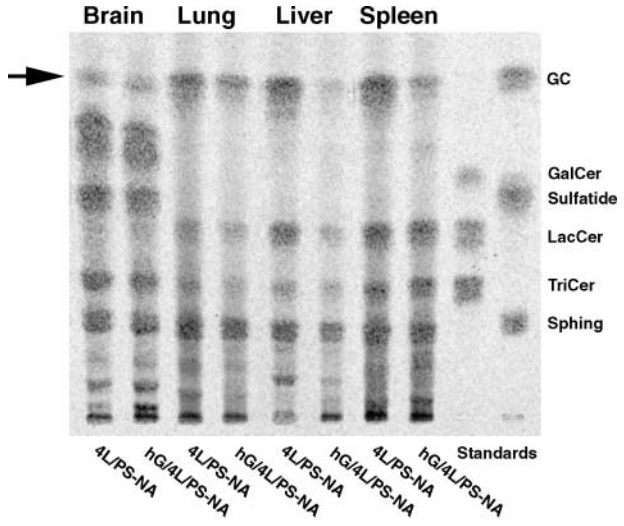

Fig. 4. TLC of glycosphingolipid analysis. In hG/4L/PS-NA mice, GC (arrow) was reduced in lung, liver, and spleen compared with 4L/PS-NA mice. No change of GC accumulation was seen in the brain of hG/4L/PS-NA relative to 4L/PS-NA mice. The amount of GC from multiple mice (n = 3–5) was quantitated relative to GC standards. Only one representative photo is shown.

hGCase expression is regulated by DOX

hGCase expression was consistently expressed at high levels in DOX[−] hG/4L/PS-NA mice (**Fig. 6**). Administration of DOX (2–3 mg/day) (DOX[+]) in food turned off hGCase expression by 24 h in hG/4L/PS-NA mice

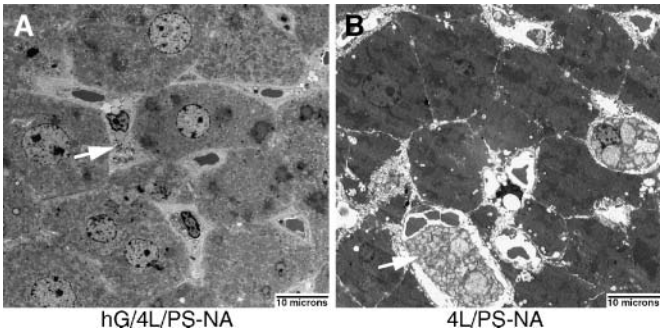

Fig. 5. Electron micrographs of inclusions in liver. Liver sections of hG/4L/PS-NA (A) and 4L/PS-NA (B) mice at 10 weeks. Reduction in storage inclusions was evident in hG/4L/PS-NA mice, compared with the large inclusions in sinusoidal lining cells of 4L/PS-NA (arrows). Magnifications are 2,500×.

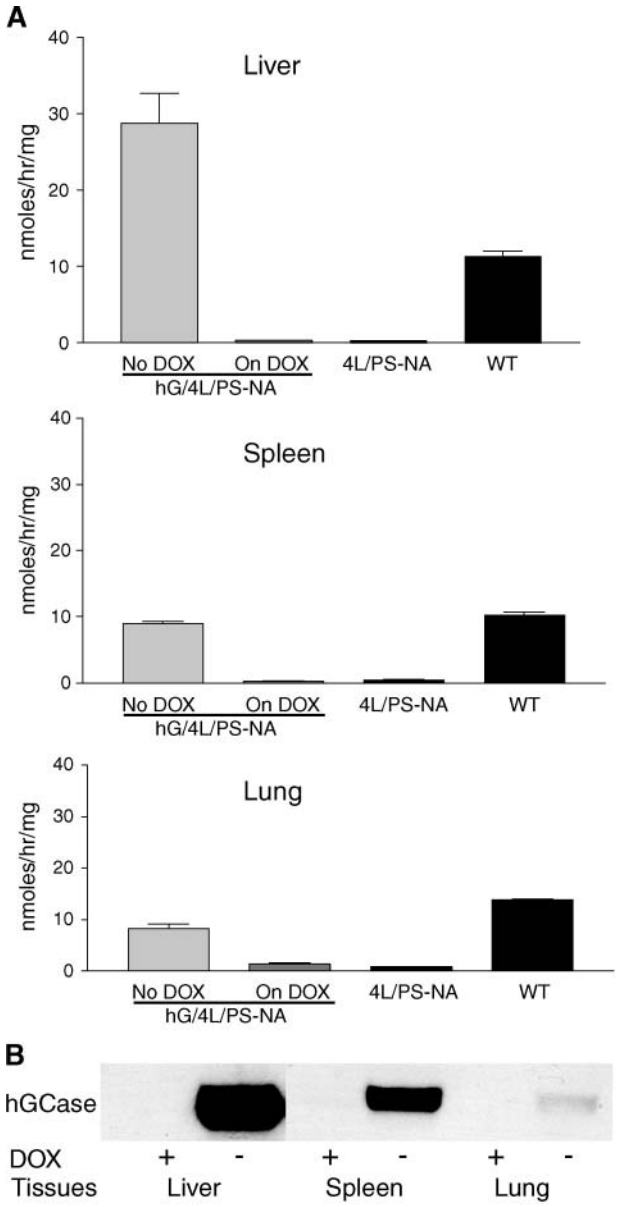

Fig. 6. DOX mediating hGCase expression. A: GCase activity in tissue lysates from hG/4L/PS-NA mice with (On DOX) or without DOX (No DOX). In hG/4L/PS-NA liver, GCase activity was ~3-fold over WT levels. The activities in lung and spleen of hG/4L/PS-NA mice were less than the WT level. On DOX for 4 weeks, hGCase activity decreased to 4L/PS-NA levels. B: Western blot analyses of hGCase protein in hG/4L/PS-NA with or without DOX using rabbit anti-hGCase antibody. The tissue lysates were from hG/4L/PS-NA mice (perfused saline) and were subjected to SDS-PAGE gel. In the presence of DOX for 1 month, no hGCase was detected in liver, spleen, and lung. Tissue lysate (50 μg) was loaded in each lane. Error bars represent SE from at least three determinations.

(data not shown). To study the effect of DOX-mediated hGCase expression, GCase activity and protein, tissue pathology, and GC levels were analyzed in three separate sets of mice; DOX[+] mice at 5 weeks of age were placed on food containing DOX. Two groups were on DOX for 4 weeks and 8 weeks, respectively. One group was on DOX for 4 weeks and then off DOX for 8 weeks.

Compared with WT, GCase activity in 4L/PS-NA mice was only 3% in liver, 4% in spleen, and 6% in lung. Expression of hGCase increased total GCase in vitro activity in liver of hG/4L/PS-NA mice by up to 3-fold over that in WT or 125-fold greater than that in 4L/PS-NA mice (Fig. 6A). Total GCase activity also was increased in spleen and lung of hG/4L/PS-NA mice, 21- and 9-fold greater than in 4L/PS-NA mice, respectively, but did not reach full WT levels (Fig. 6A). DOX[+] for 4 weeks resulted in GCase activity and protein equal to that in 4L/PS-NA livers, spleens, lungs (Fig. 6A, B), and sera (i.e., zero) (data not shown). Withdrawal of DOX for 8 weeks (following 4 weeks DOX[+]) returned GCase activity and protein levels to the same level as in mice that had never been exposed to DOX (data not shown). No difference of GCase levels was found between male and female mice. The results demonstrated that hGCase expression can be controlled by administering DOX.

Lipid analyses of liver tissues showed that GC migrated as dual bands in the mice homozygous for the V394L (4L) mutation (4L/PS-NA and hG/4L/PS-NA) (**Fig. 7**). In DOX[−] mice (i.e., hGCase on), GC (both bands) accumulation was significantly decreased compared with 4L/PS-NA (Fig. 7A; compare lanes 4–6 with lanes 7–9). The slower-migrating GC species were more significantly reduced than the faster band. DOX[+] turned off GCase expression (for 4 weeks), and GC accumulation increased in liver (1.6 μg/mg) to about the same level that 4L/PS-NA mice (1.4 μg/mg) had attained by 4 weeks (Fig. 7A, lanes 1–3; Fig. 7B). The stored GC in hG/4L/PS-NA liver (0.4 μg/mg) was cleared not to WT levels (no GC detected in WT; data not shown), but to levels similar to those in PS-NA mice (Fig. 7A, lanes 10–12; Fig. 7B). It should be noted that PS-NA mice express ~6% of normal saposin levels in liver (Table 1). These data suggest that the amount of saposins, most likely saposin C, was a limiting factor to full GCase activity in situ once internalized.

At the age of 5 weeks, hG/4L/PS-NA mice were placed on DOX for 4 to 8 weeks. Increased numbers of storage cells developed in the liver during this 1 to 2 month period (**Fig. 8**). Withdrawing DOX for 8 weeks following 4 weeks of feeding DOX led to improved pathological lesions in liver compared with the mice continually on DOX for 4 to 8 weeks (Fig. 8). GC accumulation in liver was also reduced after DOX was stopped (**Fig. 9A**). However, GC levels in lung (Fig. 9B) and spleen (data not shown) were not reduced to the same levels as those in mice that had never received DOX. One month of hGCase exposure reversed the pathological manifestations in liver, but this effect was less apparent at 1 month in spleen and lung (data not shown).

The activation of macrophages

Macrophage activation in hG/4L/PS-NA mice was assessed by immunohistochemistry using an anti-CD68 antibody. CD68 is an intracellular membrane glycoprotein that is expressed in quiescent and activated tissue macrophages (27). The CD68-positive macrophages in DOX[−]

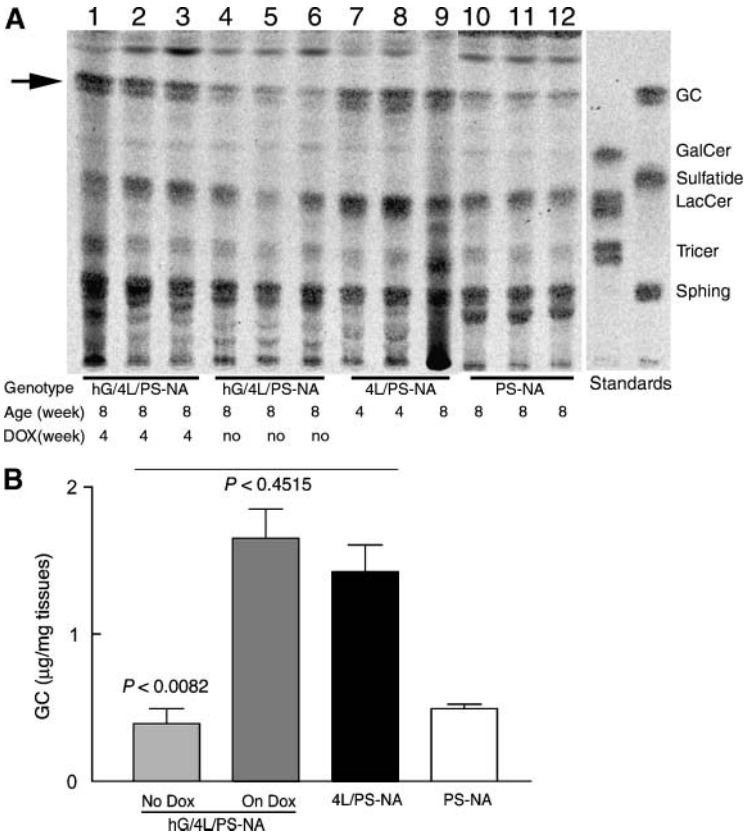

Fig. 7. Turning off hGCase expression led to GC accumulation. A: TLC of liver glycosphingolipids. GC (arrow) was reduced in hG/4L/PS-NA mice (lanes 4–6) compared with 4L/PS-NA mice (lanes 7–9). Feeding mice with DOX for 4 weeks (lanes 1–3), GC accumulated to the same levels as in 4L/PS-NA mice (lanes 7–9). B: Quantitation of GC level in liver. GC level in hG/4L/PS-NA (0.4 μg/mg tissue) is similar to that in PS-NA mice (0.5 μg/mg tissue) (Fig. 8A, lanes 10–12), and is significantly ($P < 0.0082$) reduced relative to 4L/PS-NA liver (1.4 μg/mg tissue). GC was accumulated (1.6 μg/mg tissue) in the mice on DOX. The amount of GC in each sample from multiple mice (n = 3) was quantitated relative to the GC standards. Error bars represent SE from at least three determinations.

hG/4L/PS-NA mice were reduced more in liver than in spleen and lung, when compared with those in 4L/PS-NA mice (**Fig. 10**). In the mice on DOX for 4 to 8 weeks, more engorged CD68-positive macrophage cells were present in liver, lung, and spleen compared with the DOX[−] mice. After withdrawal of DOX, CD68-positive storage cells were decreased significantly in liver (Fig. 10). The changes in spleen and lung were less obvious.

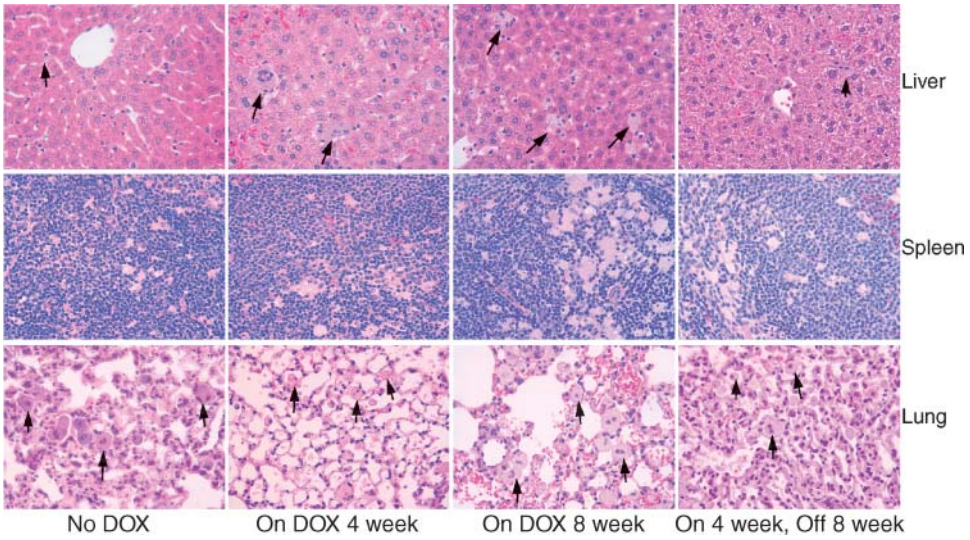

Fig. 8. Turning off GCase expression by DOX leads to pathological manifestations in hG/4L/PS-NA mice. Paraffin tissue sections of those mice were stained with H and E. A few storage cells showed in the liver of hG/4L/PS-NA mice (No DOX). On DOX for 4 or 8 weeks, clusters of lipid storage cells (arrow) were present in liver and spleen (On DOX 4 week, On DOX 8 week). When DOX was withdrawn for 8 weeks, the storage cells were reduced in the liver (On 4 week, Off 8 week). The patterns of storage cells in the lung were not corrected by DOX withdrawal. In hG/4L/PS-NA mice, the expression of hGCase reduced storage markedly in liver, and the effect is less in spleen and minimal in lung. Magnifications are 400×.

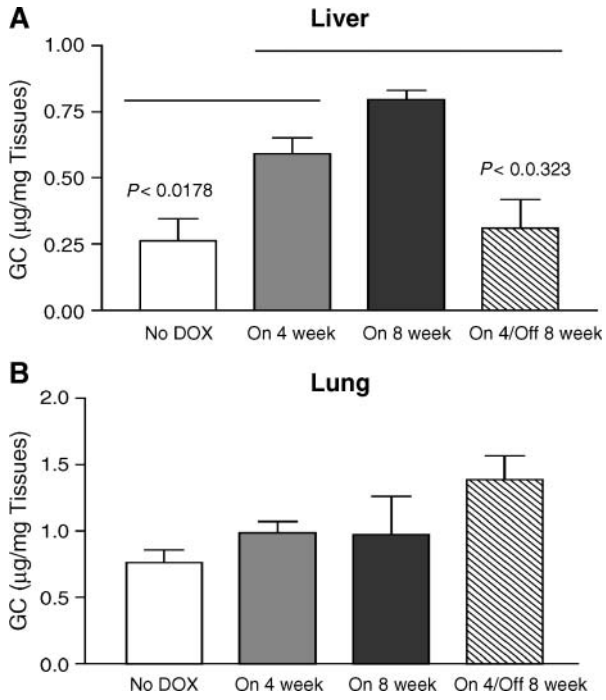

Fig. 9. DOX-mediated hGCase expression changes GC level. TLC lipid analyses of GC in liver (A) and lung (B) from DOX-treated mice. A: Liver GC accumulated in hG/4L/PS-NA mice on DOX for 4 or 8 weeks (On 4 week, On 8 week). In comparison to mice on DOX for 4 weeks (On 4 week), GC in hG/4L/PS-NA mice was significantly ($P < 0.0178$) reduced without DOX (No DOX). GC was reduced ($P < 0.0323$) in hG/4L/PS-NA mice withdrawn from DOX for 8 weeks following 4 weeks on DOX (On 4/Off 8 week). No GC was detected in WT control (data not shown). B: Lung of hG/4L/PS-NA mice on DOX for 4 or 8 weeks (On 4 week, On 8 week) showed GC accumulation. Moderately reduced GC was shown in hG/4L/PS-NA mice without DOX (No DOX). GC was not reduced in hG/4L/PS-NA mice upon withdrawing DOX for 8 weeks following 4 weeks on DOX (On 4/Off 8 week). No GC was detected in WT control (data not shown). The amount of GC in each sample (n = 2–4) was quantitated relative to the GC standards. Error bars represent SE from at least three determinations.

DISCUSSION

A controllable system for the expression of GCase was created to evaluate the organoid approach to therapy of Gaucher disease. For this system, the liver was used as a production factory for the secretion of large amounts of GCase into the serum for delivery to peripheral tissue. A mouse model, 4L/PS-NA, was used because it shows substantial storage of GC in all tissues, including brain (19). This model could be used to simulate potential approaches using hepatic-directed gene therapy to exogenously deliver viral vectors and to evaluate the intermittent use of enzyme therapy for Gaucher disease. For the latter, the use of DOX to turn off the expression of hGCase in liver provides the ability to evaluate the reaccumulation kinetics of GC in various tissues following various periods of intensive enzyme therapy intravenously by endogenous routes. The current system used the LAP-tTA controllable system and showed extensive reversal of GC storage and storage cell accumulation in a variety of tissues in the 4L/PS-NA model. This system provided insight into the use of endogenous enzyme therapy for Gaucher disease, including the effects of exposure of hGCase to in vivo plasma environments and the delivery of enzyme that has not been modified for macrophage uptake, i.e., terminal mannosyl exposure, to peripheral tissues. In addition, the level of saposins required for enzymatic activity can be evaluated in this system, because saposin C appears to be important for normal GCase in situ survival (19) and optimal functioning. Finally, the system provides for an assessment of a variety of effects of endogenous enzyme therapy on tissues and of the timing of the reaccumulation of GC in peripheral tissues, as well as insights into any direct toxicities of very high levels of hGCase.

hGCase is normally an intracellular membrane-bound lysosomal protein that is not secreted in significant amounts (2). However, in highly overexpressed systems in a variety of cells, including Chinese hamster ovary cells, fibroblasts (28), and myocytes (29), the enzyme can be secreted into the surrounding media in large amounts. Similarly, in the present tTA system, hGCase was expressed at very high levels in the liver, and large quantities of GCase were present in serum. GCase is known to be irreversibly denatured by exposure to neutral-to-slightly alkaline pHs in vitro (26). Here, evaluation of the hGCase in serum showed that compared with fully active purified human enzyme, the hGCase at high steady-state levels in plasma had substantially less than full catalytic activity (~8% of WT hGCase) by CRIM SA estimates. Such a denaturation was suggested previously in studies of intravenous bolus injections of hGCase into mice (26). In that system, the CRIM SA of the enzyme recovered from tissues was significantly lower (~50%) than that of the injected enzyme. Thus, it appears that although massive amounts of GCase, i.e., ~120 μg/ml, were present in the sera from hG/4L/PS-NA mice, only a fraction of the catalytic activity is retained and can be delivered to peripheral tissues for therapeutic effect. Clearly, continued presence in the circulation of oligosaccharide unmodified hGCase in mouse serum leads to significant losses of intrinsic activity. This implies that the more prolonged survival or exposure of GCase to plasma pH may lead to a diminished therapeutic effect and effectiveness of the administered enzyme.

Irrespective of the mechanism by which such denaturation could occur, substantial amounts of enzyme are delivered from the circulation to peripheral tissues and are taken up as an active enzyme form. This is evident in the decreased GC and storage cells in liver, spleen, and lung of the hG/4L/PS-NA mice exposed to the LAP-produced enzyme from serum. This effect is significant and leads to a 40 to >75% reduction in GC storage in various tissues. Importantly, the enzyme was secreted from liver, contained oligosaccharides with terminal sialic acid residues, was taken up by peripheral tissue, particularly macrophages, and had therapeutic effects. Thus, enzymes without exposed terminal mannosyl residues were taken up by some alternative receptors and exert their therapeutic effect intra-cellularly (30). Such mechanisms were suggested by the recent liver expression during gene ther-

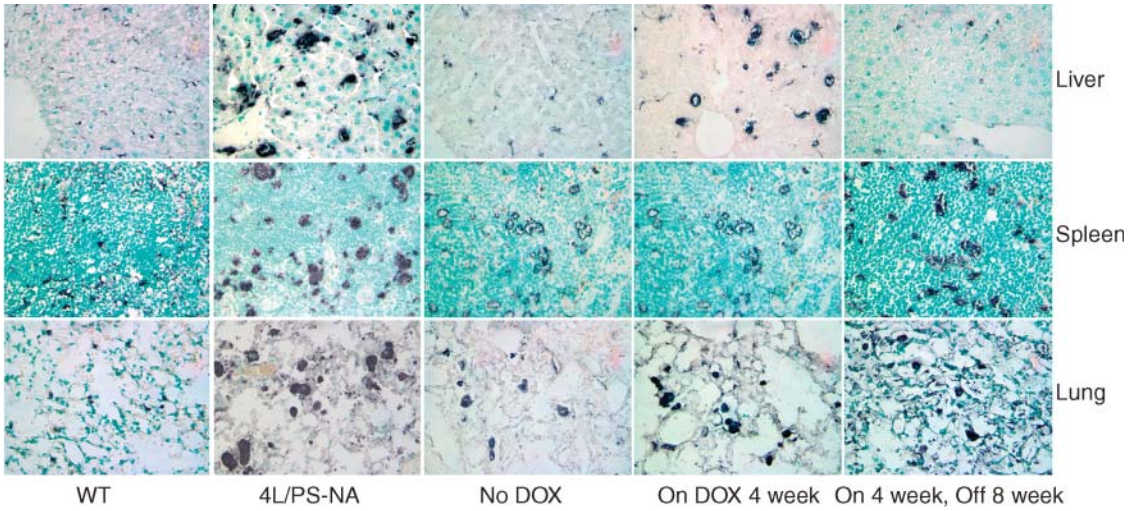

Fig. 10. Macrophage activation. Frozen tissue sections from various mice were stained with anti-CD68 monoclonal antibody (Black) and counterstained with methylene green for cell nuclei. The quiescent macrophages in WT mice are positive for CD68 in liver, spleen, and lung (WT). Compared with 4L/PS-NA tissues, without DOX, CD68-positive cells in liver of hG/4L/PS-NA mice were slightly increased in size and clusters of stained cells were present in spleen and lung (No DOX). On DOX for 4 weeks, the engorged CD68-positive cells were present in liver, spleen, and lung of hG/4L/PS-NA mice (On DOX 4 week). After withdrawal of DOX for 8 weeks, the CD68-positive cells were markedly decreased in liver, but not in spleen and lung (On 4 week, Off 8 week). Magnifications are 400×.

apy studies (31) and the therapeutic effect of lysosomal acid lipase in the mannose receptor-deficient mice (30). This therapeutic effect was due to the uptake of secreted enzyme from the liver, because 4L/PS-NA DOX[+] mice showed GC and macrophage storage cells reaccumulation in the tissues to levels similar to those of untreated mice. Thus, the visceral disease reemerges and is controllable by the expression of GCase in liver.

The effect of liver-produced and -secreted hGCase on visceral tissues was partial in this particular model. As indicated above, pH denaturation of the secreted enzyme could account for part of this incomplete effect on peripheral tissues, but there are also other potential causes including: *1*) the ineffective delivery to macrophages, because the secreted enzyme from liver does not contain large amounts of terminal mannose residues, and *2*) the amount of enzyme delivered to the target cell, peripheral macrophages, may not be sufficient or sufficiently active to lead to complete reversal and degradation of the stored GC in those tissues. In addition, the 4L/PS-NA mouse has a diminished level of prosaposin expression and production of saposins, including saposin C. The level was ~6% of WT in a variety of tissues evaluated here, and this level appeared to be sufficient to support substantial but not complete recovery of the tissues. This was also suggested in patients who received large amounts of enzyme intravenously but had normal amounts of prosaposin. The effectiveness of the administered GCase is apparently not diminished by radically altering the ratios of saposin C and GCase present in cells.

Importantly, the effects of diminished saposin are at least 2-fold. These include direct effects of saposin C on activation of GCase and the proteolytic protective effect of saposin C on GCase delivered to the lysosome (12, 18). Both could have an impact because of the smaller amounts of saposin C present in this particular model. Irrespective of these concerns, the histological and pathological changes in the visceral tissues were substantial and clearly due to the delivery of enzyme to those tissues. One cannot exclude the possibility that the large amounts of hGCase delivered to the tissues could be active in the absence of saposin C and have sufficiently high activity for function.

This model system with the conditional expression of hGCase in mice provides the ability to assess interruptions of treatment, lower levels of treatment, and potential toxic effects of large amounts of enzyme produced either in mice or by intravenous administration. The ability to administer DOX and turn off the enzyme allows for a timed assessment of the reaccumulation of the stored lipids and assessment of the redevelopment of the alterations of activated macrophages prior to or concomitant with lipid accumulation. DOX[+] hG/4L/PS-NA mice in whom DOX was stopped did not show a return to pre-DOX treatment GC or storage cell levels within a 4–8 week period. These data suggest that residual disease present before institution of enzyme therapy or additional progression of disease within tissues may make recovery to levels achieved during continuous treatment difficult and/or slow. This has significant implications for treatment holidays in human Gaucher disease.

Finally, the model system here shows significant GC accumulation in the CNS. This accumulation was much higher than that occurring in the prosaposin knockout mouse. The defective mouse GCase (4L) present in the hypomorphic prosaposin background appears to be sufficient to accelerate the accumulation of GC in the brain of this model. Indeed, the level of prosaposin expression in brain in this model is ~45% of WT (19). Interestingly, even

in the presence of large amounts of hGCase activity from at least postnatal day 1 (Sun et al., unpublished observation), constant exposure to high levels of plasma GCase activity showed no effect on CNS disease progression, nor was there evidence for delivery of hGCase to the CNS for potential therapeutic effect. The implication is that enzyme therapy by intravenous routes at any time during development, either continuously or intermittently, will be ineffective in the treatment of GC accumulation in the CNS. This has extensive implications for the treatment of Gaucher disease variants with CNS involvement.

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