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Evasion of Immune Responses to Introduced Human Acid α-Glucosidase by Liver-Restricted Expression in Glycogen Storage Disease Type II

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Glycogen storage disease type II (GSD-II; Pompe disease) is caused by a deficiency of acid α-glucosidase (GAA; acid maltase) and manifests as muscle weakness, hypertrophic cardiomyopathy, and respiratory failure. Adeno-associated virus vectors containing either a liver-specific promoter (LSP) (AAV-LSPhGAApA) or a hybrid CB promoter (AAV-CBhGAApA) to drive human GAA expression were pseudotyped as AAV8 and administered to immunocompetent GAA-knockout mice. Secreted hGAA was detectable in plasma between 1 day and 12 weeks postadministration with AAV-LSPhGAApA and only from 1 to 8 days postadministration for AAV-CBGAApA. No anti-GAA antibodies were detected in response to AAV-LSPhGAApA (<1:200), whereas AAV-CBhGAApA provoked an escalating antibody response starting 2 weeks postadministration. The LSP drove approximately 60-fold higher GAA expression than the CB promoter in the liver by 12 weeks following vector administration. Furthermore, the detected cellular immunity was provoked by AAV-CBhGAApA, as detected by ELISpot and $CD4^+/CD8^+$ lymphocyte immunodetection. GAA activity was increased to higher than normal and glycogen content was reduced to essentially normal levels in the heart and skeletal muscle following administration of AAV-LSPhGAApA. Therefore, liver-restricted GAA expression with an AAV vector evaded immunity and enhanced efficacy in GSD-II mice.

INTRODUCTION

Infantile-onset glycogen storage disease type II (GSD-II; Pompe disease; MIM 232300) causes death early in childhood from cardiorespiratory failure related to an underlying hypertrophic cardiomyopathy [1]. The deficiency of acid α-glucosidase (GAA; acid maltase; EC 3.2.1.20) in GSD-II affects the heart and skeletal muscle primarily, and infants with Pompe disease develop profound weakness and hypotonia. Milder juvenile- and adult-onset forms of GSD-II feature progressive weakness without cardiomyopathy, and patients with juvenile-onset GSD-II typically become ventilator-dependent due to respiratory muscle involvement. The cause of death in juvenile- and adult-onset GSD-II is usually from cardiorespiratory failure, which can be precipitous. The histopathology of GSD-II includes marked lysosomal accumulation of glycogen in cardiac and skeletal muscle, which culminates with the rupture of lysosomes and cytoplasmic pooling of glycogen. GAA is normally widely expressed from a promoter with resemblance to a "housekeeping" gene [2–4], and GAA deficiency causes lysosomal glycogen accumulation in virtually all tissues. Despite marked accumulation of glycogen in the spinal cord and brain stem, it remains unclear whether central nervous system involvement manifests clinically [1].

Enzyme replacement therapy (ERT) has been developed in Pompe disease and other lysosomal storage disorders, and the uptake of recombinant GAA is facilitated by mannose-6-phosphate receptor-mediated uptake [5–7]. Humoral immunity has complicated the develop-

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ment of ERT in GAA-knockout (GAA-KO) mouse models [8–10] and in a Phase I–II clinical trial of ERT in Pompe disease [11]. The complications of anti-GAA antibody formation could include decreased efficacy and life-threatening allergic responses [11,12]. The impact of neutralizing antibodies has been demonstrated by the lack of long-term efficacy from ERT in nontolerant GAA-KO mice [8,10] and in CRIM-negative Pompe disease patients [11].

Intravenous administration of adenovirus vectors encoding GAA previously demonstrated generalized correction of glycogen storage in the GAA-KO mouse model [13,14], although glycogen gradually reaccumulated in the months following vector administration [15]. The appearance of anti-GAA antibodies correlated with the disappearance of secreted hGAA precursor from the plasma [15]. The use of a liver-specific promoter (LSP) in an [E1$^-$, polymerase$^-$]Ad vector encoding hGAA reduced the formation of anti-GAA antibodies in GAA-KO mice; however, the level of hGAA secretion with that vector was insufficient to achieve efficacious glycogen clearance [16]. Experiments in immunodeficient GAA-KO mice further emphasized the relevance of immune responses to introduced hGAA, because long-term efficacy was achieved with an [E1$^-$, polymerase$^-$]Ad vector encoding hGAA in that model [17].

Adeno-associated virus (AAV) vectors have several advantages for gene therapy in genetic disease, including persistent gene expression, the lack of immune response to transduced cells, and the lack of any association of AAV with human disease [18]. An AAV2/1 vector corrected glycogen storage when injected intramuscularly into immunocompetent GAA-KO mice; however, the effect was observed only in the injected muscle [19]. We previously demonstrated the secretion and uptake of hGAA at low levels in immunodeficient, GAA-KO/SCID mice with AAV2/2 and AAV2/6 vectors [20]. Similarly, GAA was transiently detected in plasma following intravenous injection of the Ad–AAV vector in immunocompetent GAA-KO mice prior to the formation of anti-GAA antibodies [20,21]. Finally, AAV2/5 and AAV2/8 vectors containing a duck hepatitis B viral promoter to drive GAA expression provoked anti-GAA antibodies that prevented the reduction of glycogen storage in nontolerant GAA-KO mice [22].

Tissue-restricted expression of GAA through AAV vector-mediated gene therapy could ameliorate the formation of neutralizing antibodies in GAA-KO mice, as has been seen in hemophilia A and B mice [23–25] and Fabry mice [26]. We have developed an AAV vector containing an LSP to drive liver-restricted expression of hGAA [25] and cross-packaged this vector as AAV8 (AAV2/8) to increase liver transduction [27]. The impact of immune responses on the efficacy of this AAV2/8 vector was evaluated in immunocompetent GAA-KO mice.

RESULTS

Sustained, High-Level Expression of hGAA and Correction of GSD-II with an AAV2/8 Vector Containing a Liver-Specific Promoter

Liver-restricted expression of an introduced therapeutic protein has completely prevented the formation of neutralizing antibodies in other mouse models for genetic disease, although this has not been achieved with an AAV vector in GSD-II mice. Consequently, we administered an AAV2/8 vector containing a liver-specific promoter [25] to drive GAA expression (AAV-LSPhGAApA; 1×10^{11} or 5×10^{11} particles intravenously) to 3-month-old GAA-KO mice. We injected a second group of age-matched GAA-KO mice with 1×10^{11} particles of an AAV2/8 vector containing hGAA under the control of the CMV enhancer/chicken β-actin (CB) promoter for comparison (AAV-CBhGAApA [20]). High-level hGAA was detectable by Western blot in plasma at 6 weeks postinjection with AAV-LSPhGAApA in immunocompetent GAA-KO mice (Fig. 1A), in contrast to AAV-CBhGAApA, which produced low-level hGAA in the plasma only during the first week (not shown). The latter vector has produced sustained hGAA in plasma in immunodeficient GAA-KO/SCID mice (approximately 6 ng/μl), indicating the significance of the immune response to hGAA secretion [28]. The level of hGAA

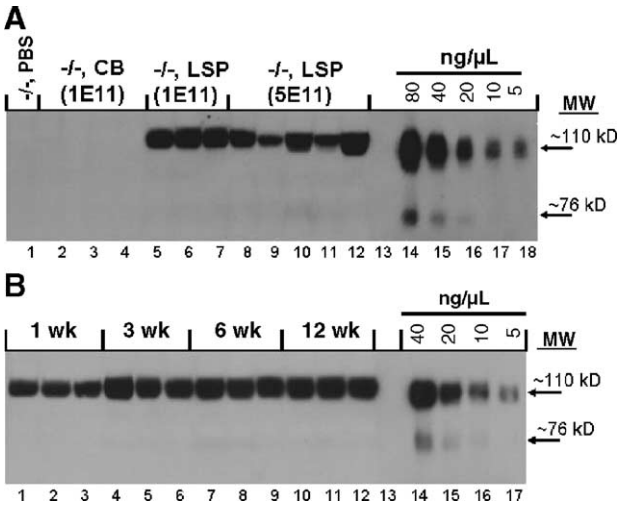

FIG. 1. Detection of hGAA following intravenous administration of an AAV2/8 vector in GAA-KO mice. (A) Western blot analysis of plasma from GAA-KO mice following AAV-CBhGAApA or AAV-LSPhGAApA administration. Samples were obtained 6 weeks following vector administration. Each lane represents one mouse. Recombinant hGAA (rhGAA) standards are shown for quantitation, and the concentration for each standard is indicated. (B) Western blot analysis of plasma following AAV-LSPhGAApA administration. Samples were obtained from male GAA-KO/SCID (n = 4) mice at 1, 3, 6, and 12 weeks following vector administration and loaded in the same order for each time point. rhGAA standards are shown for quantitation, and the concentration for each standard is indicated.

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110-kDa precursor was approximately 40 ng/μl 1 week following AAV-LSPhGAApA administration and was maintained at that level for 12 weeks (Fig. 1B).

ELISA performed at 1, 3, and 6 weeks following vector administration demonstrated no detectable anti-GAA antibodies in response to liver-specific hGAA expression with AAV-LSPhGAApA in GAA-KO mice (<1:200) (Fig. 2A), whereas constitutive hGAA expression with AAV-CBhGAApA provoked a vigorous antibody response (1:6400) at 6 and 12 weeks postinjection (Fig. 2B).

We performed semiquantitation of vector DNA to investigate the basis for lower hGAA expression with AAV-CBhGAApA compared to AAV-LSPhGAApA in GAA-KO mice (Fig. 3). Semiquantitation of vector DNA in the liver revealed approximately 1 to 10 vector genomes/cell in the liver for both vectors 12 weeks following vector administration (Fig. 3A). Therefore, decreased efficiency of transduction in the liver did not seem to be the only reason for lower GAA expression with AAV-CBhGAApA compared to AAV-LSPhGAApA. Interestingly, the copy number for vector DNA in heart and skeletal muscle was higher for AAV-LSPhGAApA (approximately 1 copy/cell) compared to AAV-CBhGAApA (approximately 0.1 copy/cell) (Fig. 3B). The reduced vector DNA copy number with AAV-CBhGAApA in striated muscle is consistent with a cellular immune response and clearance of vector DNA, as observed following intramuscular administration of AAV-CBhGAApA as AAV2/6 [29].

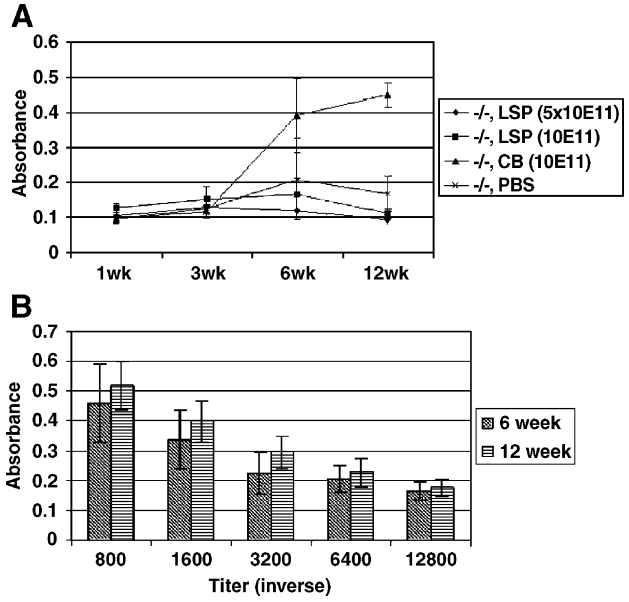

FIG. 2. ELISA for GAA-KO mice following AAV2/8 vector administration. The mean and standard deviation are shown. (A) Absorbance for 1:200 dilution of plasma from the indicated groups of mice. Mice received PBS or the indicated number of vector particles of AAV-LSPGAApA (LSP) or AAV-CBGAApA (CB). (B) Absorbance for the time points following AAV-CBGAApA administration at the indicated titers. The titers were deemed positive if absorbance exceeded 0.2.

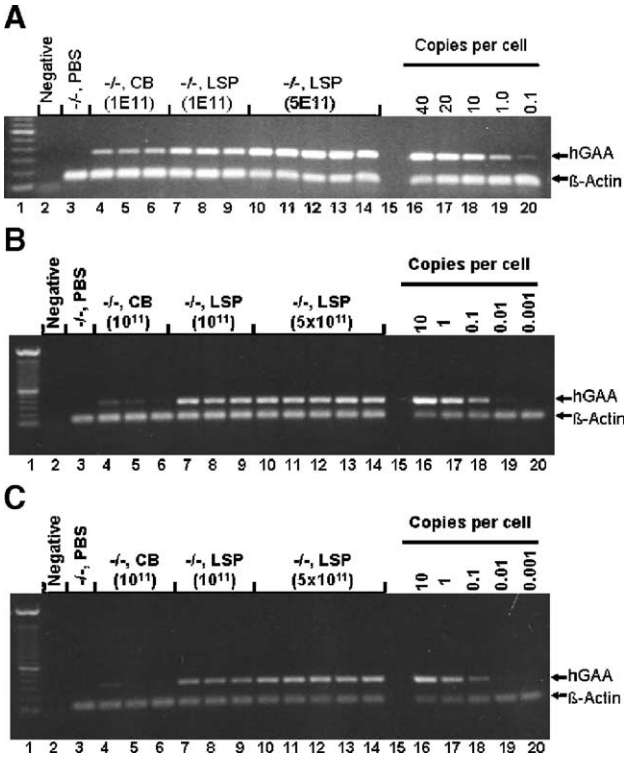

FIG. 3. Vector DNA analysis by semiquantitative PCR. Semiquantitation of vector DNA for (A) liver DNA, (B) heart DNA, and (C) quadriceps DNA at 12 weeks following AAV2/8 vector administration. Mice received PBS or the indicated number of vector particles of AAV-LSPhGAApA (LSP) or AAV-CBhGAApA (CB). Each agarose gel represents PCR semiquantitation for treated mice (lanes 4–14) and for a PBS-injected, age-matched GAA-KO control (lane 3). Each lane represents an individual mouse. Lane 1 shows a 100 bp ladder molecular weight marker. The negative control consisted of no input DNA (lane 2). The control samples for quantitation consisted of added AAV vector plasmid DNA in liver DNA from an untreated, GAA-KO mouse (lanes 16–20).

GAA was significantly elevated to near-normal levels in striated muscle at 12 weeks following administration of AAV-LSPhGAApA, and glycogen content was correspondingly reduced in the heart and skeletal muscle (Table 1). Liver GAA levels were elevated approximately 100-fold above normal levels following AAV-LSPhGAApA administration; although GAA activity was slightly increased in the group of GAA-KO mice that received a higher number of particles; the glycogen content was similarly reduced in heart and skeletal muscles in both treatment groups (Table 1). Administration of AAV-CBhGAApA failed to increase GAA activity above background levels observed for GAA-KO mice in heart and skeletal muscle, and liver GAA levels were only slightly elevated compared to those for normal mice (Table 1). As expected, AAV-CBGAApA failed to lower glycogen content in the heart and skeletal muscle of GAA-KO mice (Table 1).

TABLE 1: GAA activity and glycogen content 12 weeks following AAV2/8 vector administration

	Group[a]	GAA activity		Glycogen content	
		GAA[b] (mean ± SD)	Fold[c]	Glycogen[d] (mean ± SD)	Decrease[e]
Liver	–/–, LSP (5×10^{11})	20,000 ± 11,000**	140	—	—
	–/–, LSP (1×10^{11})	11,000 ± 2,200***	79	—	—
	–/–, CB (1×10^{11})	180 ± 120	1.3	—	—
	–/–, PBS	20 ± 7	—	—	—
	Normal	140 ± 34***	—	—	—
Heart	–/–, LSP (5×10^{11})	54 ± 31*	8.7	0.033 ± 0.038****	99
	–/–, LSP (1×10^{11})	18 ± 8*	2.9	0.0 ± 0.0****	100
	–/–, CB (1×10^{11})	3.6 ± 1.6	0.6	2.9 ± 2.1	—
	–/–, PBS	3.1 ± 1.3	—	2.5 ± 0.2	—
	Normal	6.2 ± 2.7	—	0.041 ± 0/034****	98
Diaphragm	–/–, LSP (5×10^{11})	26 ± 14*	4.2	0.13 ± 0.15**	82
	–/–, LSP (1×10^{11})	18 ± 7*	2.9	0.0 ± 0.0**	100
	–/–, CB (1×10^{11})	9.0 ± 5.0*	1.5	0.80 ± 0.11	0
	–/–, PBS	2.8 ± 0.4	—	0.72 ± 0.15	—
	Normal	6.2 ± 2.7	—	0.011 ± 0.082****	98
Quadriceps	–/–, LSP (5×10^{11})	25 ± 25	2.0	0.29 ± 0.22**	76
	–/–, LSP (1×10^{11})	9.8 ± 3.3*	0.8	0.25 ± 0.25	79
	–/–, CB (1×10^{11})	3.2 ± 1.7	0.3	1.2 ± 0.1	0
	–/–, PBS	1.8 ± 0.7	—	1.2 ± 0.4	—
	Normal	12.6 ± 1.2****	—	0.016 ± 0.010***	99
Gastrocnemius	–/–, LSP (5×10^{11})	6.9 ± 3.7	0.7	0.33 ± 0.16**	83
	–/–, LSP (1×10^{11})	12 ± 5.0*	1.2	0.13 ± 0.08**	93
	–/–, CB (1×10^{11})	8.9 ± 3.4*	0.9	1.7 ± 0.2	—
	–/–, PBS	1.9 ± 0.1	—	1.9 ± 0.6	—
	Normal	10 ± 2***	—	0 ± 0***	100

[a] Groups as follows: –/–, LSP (5×10^{11}), high dose AAV-LSPGAApA-treated GAA-KO mice ($n = 5$, except for GAA in diaphragm $n = 3$); –/–, LSP (1×10^{11}), low dose AAV-LSPGAApA-treated GAA-KO mice ($n = 3$); –/–, CB (1×10^{11}), low dose AAV-CBGAApA-treated GAA-KO mice ($n = 3$); control, untreated, age-matched male GAA-KO/SCID mice ($n = 3$); normal, C57BL/6 mice ($n = 5$).

[b] nmol/h/mg protein. *P* values are indicated as follows: *<0.05, **<0.01, ***<0.001, ****<0.0001.

[c] Group mean/Normal group mean.

[d] mmol glucose/g protein. *P* values are indicated as follows: *<0.05, **<0.01, ***<0.001, ****<0.0001.

[e] Reduction compared to Control group (%).

Liver-Restricted hGAA Expression and Uptake of Secreted hGAA Reduced Glycogen Storage in Striated Muscles

To elucidate further the basis for high-level GAA activity in liver and skeletal muscle with AAV-LSPhGAApA, we performed RT-PCR to detect vector RNA (Fig. 4). In support of the hypothesis that GAA was secreted by the liver and taken up by skeletal muscle through receptor-mediated endocytosis, vector RNA was detected only in liver at 12 weeks following AAV-LSPhGAApA administration (Fig. 4A), not in heart (Fig. 4B). This analysis was consistent with liver-restricted activity of the promoter in AAV-LSPhGAApA. For GAA-KO mice that received AAV-CBhGAApA, vector RNA was detected at low levels in the liver (Fig. 4A), consistent with the lower, near-normal level of GAA activity in the liver with that vector (Table 1).

Light microscopy of periodic acid–Schiff (PAS)-stained histologic sections confirmed the reduction of glycogen and restoration of normal myofiber morphology in heart and diaphragm 12 weeks following AAV-LSPhGAApA administration (Fig. 5A). Glycogen vacuolation persisted in some myofibers in quadriceps following AAV-LSPhGAApA administration (Fig. 5A), consistent with the slight residual elevation of glycogen content in the quadriceps (Table 1). Lysosomal glycogen accumulation was very marked in GAA-KO mice following AAV-CBhGAApA administration (not shown), similar to that in phosphate-buffered saline (PBS)-injected, age-matched GAA-KO mice (Fig. 5A) in heart and skeletal muscle. Multiple lymphocytic infiltrates were present in the liver of GAA-KO mice following AAV-CBhGAApA administration and were absent in mice that received AAV-LSPhGAApA (Fig. 5B). These data together imply that a cellular immune response reduced the level of transduction in the liver and striated muscle with AAV-CBhGAApA, as described for AAV-CBhGAApA packaged as AAV2/6 following intramuscular injection in GAA-KO mice [29].

We analyzed a biomarker for GSD-II previously shown to be elevated in Pompe disease, Glcα1–6Glcα1–4Glcα1–4Glc (Glc_4) [30–33], for GAA-KO mice following AAV vector administration. Urinary Glc_4 was significantly

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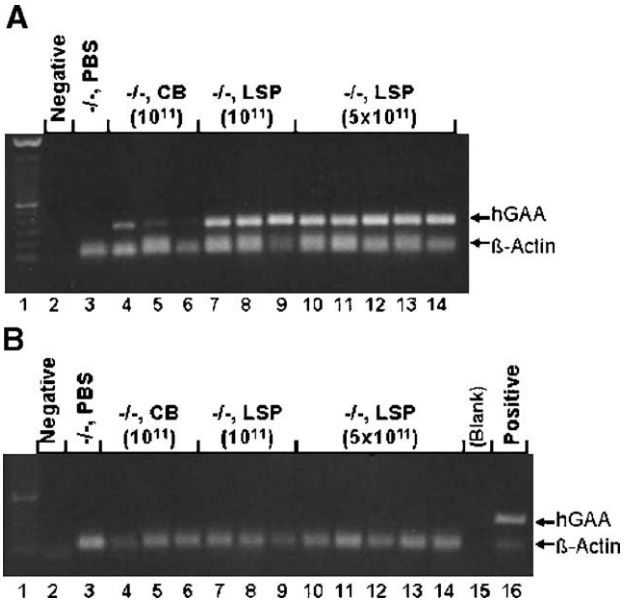

FIG. 4. RT-PCR analysis of vector RNA at 12 weeks following AAV2/8 vector administration. Mice received PBS or the indicated number of vector particles of AAV-LSPhGAApA (LSP) or AAV-CBhGAApA (CB). Each agarose gel shows RT-PCR samples for treated GAA-KO mice (lanes 4–14) and for a PBS-injected, age-matched GAA-KO control (lane 3). Each lane represents an individual mouse. Lane 1 shows a 100 bp ladder molecular weight marker. The negative control consisted of no input DNA (lane 2). Mouse β-actin RNA was amplified as an internal control for each sample. (A) Liver RNA. (B) Heart RNA. The positive control was liver RNA from a GAA-KO mouse that received AAV-LSPhGAApA (lane 16).

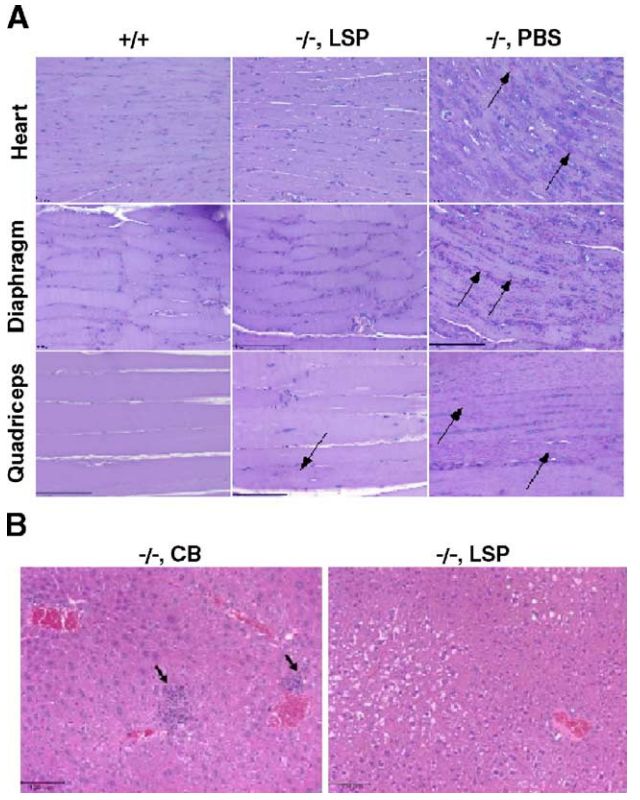

FIG. 5. Histological analyses following AAV 2/8 vector administration. (A) Glycogen staining in muscle following AAV-LSPhGAApA vector administration. PAS staining of glutaraldehyde-fixed, paraffin-embedded sections of gastrocnemius, diaphragm, and heart from a male GAA-KO mouse 12 weeks following administration of 5×10^{11} particles of AAV-LSPhGAApA (−/−, LSP) and from an age-matched, PBS-injected GAA-KO mouse (−/−, PBS). Vacuoles of glycogen are indicated (arrows). (B) Hematoxylin–eosin staining of glutaraldehyde-fixed, paraffin-embedded sections of liver from GAA-KO mice following AAV-CBhGAApA (−/−, CB) or AAV-LSPhGAApA (−/−, LSP) administration. Lymphocytic infiltrates are indicated by arrows.

reduced in GAA-KO mice that received AAV-LSPhGAApA (Fig. 6) and was associated with reduction in glycogen content in cardiac and skeletal muscle in these GSD-II mice (Table 1).

Evasion of Humoral and Cellular Immunity by Liver-Restricted hGAA Expression

Low hGAA expression driven by the CB promoter and the accompanying lack of efficacy could be attributed either to lower transduction or to immune responses directed toward hGAA. We evaluated these possibilities through the analysis of immune responses following administration of either AAV-CBhGAApA or AAV-LSPhGAApA (5×10^{11} particles intravenously) to immunocompetent GAA-KO mice. Increasing plasma hGAA was detected between 1 and 8 days postadministration for both vectors, but hGAA disappeared from plasma by 14 days following AAV-CB-hGAApA administration (Fig. 7A). In contrast, hGAA was detected in plasma from 1 day to 12 weeks following AAV-LSPhGAApA administration (Figs. 1B and 7A). Anti-GAA antibodies were present on day 14 in response to AAV-CBhGAApA ($P < 0.0001$; Fig. 7B), indicating a humoral response that cleared secreted precursor hGAA from the blood.

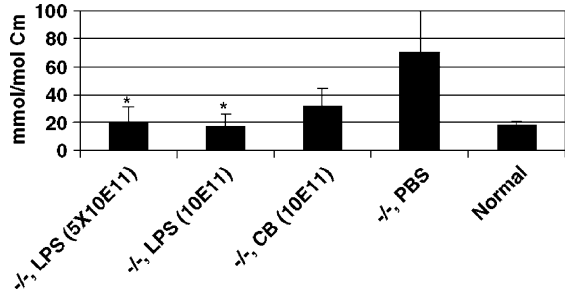

FIG. 6. Urinary Glc_4 following AAV2/8 vector administration. The mean [Glc_4] is shown for the groups of GAA-KO mice 12 weeks following AAV2/8 vector administration and for age-matched, untreated C57BL/6 (Normal) mice. GAA-KO mice (−/−) received PBS or the indicated number of vector particles of AAV-LSPhGAApA (LSP) or AAV-CBhGAApA (CB). Asterisks indicate $P < 0.05$ versus PBS-treated GAA-KO mice.

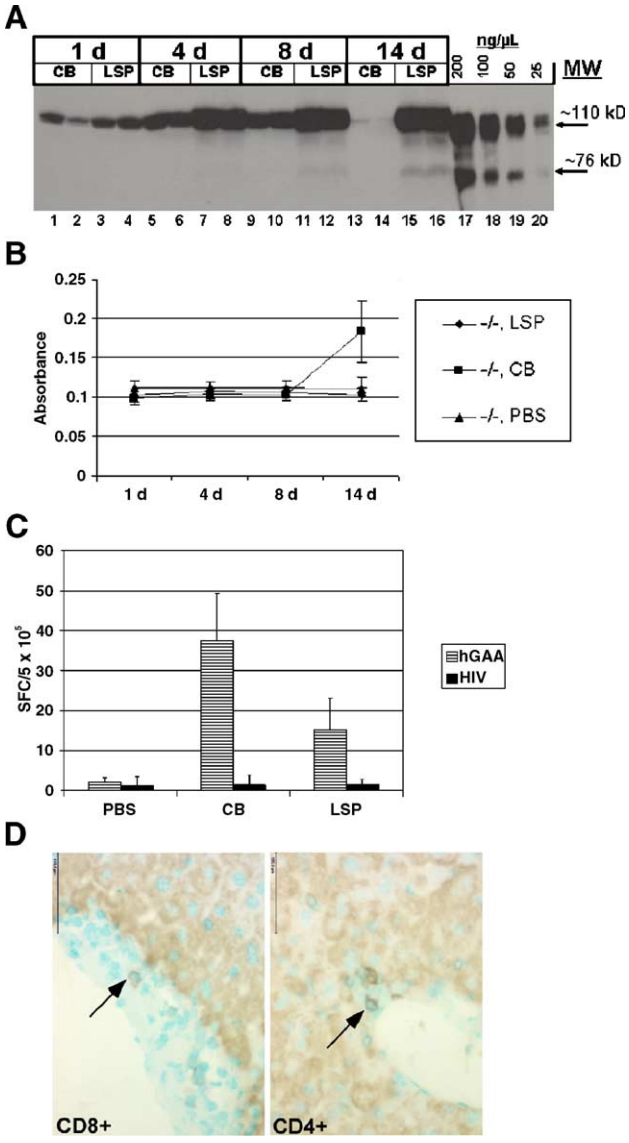

FIG. 7. Comparison of early hGAA expression and immunogenicity with the CB promoter or LSP. GAA-KO mice ($n = 4$ for each group) received 5×10^{11} particles of AAV-CBhGAApA (CB) or AAV-LSPhGAApA (LSP) or PBS alone. (A) Western blot analysis of plasma. Recombinant hGAA standards are shown for quantitation, and the concentration for each standard is indicated. (B) ELISA for anti-hGAA antibodies. Absorbance for 1:200 dilution of plasma (mean and standard deviation). (C) ELISpot for hGAA-specific T cell responses. The number of spot-forming cells (SFC) per 5×10^5 spleen cells in response to recombinant hGAA (100 μg) or the HIV Gag negative control (mean and standard deviation). (D) $CD8^+$ and $CD4^+$ lymphocyte immunodetection in the liver 2 weeks following AAV-CBhGAApA administration. Foci of lymphocytes are indicated (arrows). Original magnification: ×730.

The presence of cellular immunity against introduced hGAA was suspected, because humoral immunity did not explain the very low GAA activity observed in liver with AAV-CBhGAApA at 12 weeks postadministration (Table 1). Liver GAA was essentially similar for AAV-CBhGAApA and AAV-LSPhGAApA at 2 weeks postadministration (1400 ± 500 vs 3400 ± 700 nm · h · mg, respectively); however, the CB promoter produced 60-fold lower GAA activity than the LSP at the 12-week time point (Table 1). An ELISpot analysis revealed significantly increased antigen-specific T cell responses to administration of AAV-CBhGAApA, compared to AAV-LSPhGAApA ($P < 0.0001$; Fig. 7C). The weakly positive ELISpot in response to AAV-LSPhGAApA did not affect hGAA expression, because GAA activity in liver increased approximately 6-fold by 12 weeks following vector administration (Table 1). The presence of $CD4^+$ and $CD8^+$ lymphocytic infiltrates in liver occurred in response to only AAV-CBhGAApA, further demonstrating cytotoxicity related to universal hGAA expression (Figs. 7D and 7E). In summary, cellular immunity against hGAA expressed from the CB promoter was associated with waning GAA levels in the liver.

Discussion

Gene therapy in GSD-II will require sustained production of hGAA to achieve efficacy, and immune responses remain an important obstacle to gene therapy for GSD-II and other lysosomal storage disorders. Anti-GAA antibody formation has previously complicated gene therapy in nontolerant, immunocompetent GSD-II mice. No anti-GAA antibodies were formed in response to liver-restricted expression of hGAA driven by the LSP utilized here, and glycogen storage was corrected in the heart and skeletal muscle of nontolerant GSD-II mice. Recently, when GAA-KO mice were tolerized to hGAA by neonatal administration of the recombinant enzyme, a subset of those mice did not form anti-GAA antibodies in response to administration of an AAV2/8 vector encoding hGAA [22]. The absence of anti-GAA antibodies allowed peripheral uptake of liver-expressed hGAA and partial correction of GAA deficiency in striated muscle, accompanied by partial clearance of vacuolated glycogen. Currently, liver-restricted, supraphysiologic, efficacious hGAA expression was achieved in all treated GSD-II mice without pretreatment to induce tolerance to introduced hGAA.

Sustained expression of therapeutic proteins with AAV vectors containing a liver-specific promoter has been reported, including liver-targeted gene therapy with AAV vectors that failed to elicit neutralizing antibodies against coagulation factor IX (FIX) in hemophilia B mice [25,35–37]. Acquisition of tolerance to FIX required induction of regulatory $CD4^+$ T cells that suppressed neutralizing antibody formation, and higher levels of FIX expression increased the likelihood of tolerance [37]. The sinusoidal endothelial cells in liver are hypothesized to initiate tolerance through activation of naive T cells that do not differentiate into effector cells, and the liver is unique in its role in the induction of tolerance [38]. Thus, the enhanced efficacy of AAV vectors containing a liver-specific promoter seems to rely upon the induction of

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tolerance by liver-restricted, high-level expression of the introduced therapeutic protein.

Conversely, AAV vectors containing a universal promoter such as the hybrid CB promoter provoked cellular immunity in transduced myofibers. Dystrophin-deficient *mdx* mice had increased immune responses to β-galactosidase expression driven by a CMV promoter, compared to an analogous vector containing a muscle-specific creatine kinase (MCK) promoter/enhancer [39]. Similarly, the CMV promoter/enhancer in an AAV vector encoding γ-sarcoglycan increased the immune response and decreased levels of transgene expression in γ-sarcoglycan-deficient mice, compared to the analogous vector containing an MCK promoter enhancer [40,41]. The intramuscular administration of AAV-CBhGAApA as AAV2/6 provoked cellular immunity that cleared vector DNA from the injected muscle by 6 weeks postinjection [29], and in this study AAV-CBhGAApA transduced striated muscle less efficiently than AAV-LSPhGAApA. In concordance with these earlier results, expression of hGAA driven by the CB promoter in AAV-CBhGAApA provoked an hGAA-specific cytotoxic response to hGAA expression in the liver.

Efforts to develop gene therapy in GSD-II are justified by the limitations of ERT for GSD-II, which include the possibility of humoral immunity and the need for high levels of GAA replacement to achieve efficacy. The formation of anti-GAA antibodies and associated infusion reactions prevented continuation of ERT beyond 3 weeks in GAA-KO mice [10]. Only by the generation of tolerant GAA-KO mice by insertion of a low-expressing liver-specific transgene could long-term ERT be tested in a GSD-II mouse, and a reduction in the glycogen content of skeletal muscle required administration of 100 mg/kg recombinant hGAA (a high dose compared to other forms of ERT). Similarly, Pompe disease patients who lacked any residual GAA protein produced anti-hGAA antibodies and demonstrated markedly reduced efficacy from ERT [11,42]. In the two clinical trials of ERT in Pompe disease with published results, only two of six patients had marked clinical improvement and both were CRIM-positive [11,42]. The similarity with regard to neutralizing antibody response in GAA-KO mice and in a subset of CRIM-negative Pompe disease patients could be linked to the lack of residual GAA protein expression [10,11,43,44]. If GAA deficiency is caused by an underlying null mutation(s), the immune system is likely to react to ERT by forming neutralizing antibodies.

The combined approach to liver-targeted gene therapy of GSD-II with a liver-directed AAV serotype and a liver-specific promoter provides a substantially greater and more sustained level of hGAA expression *in vivo* than has previously been reported [22,28]. Thus, high-level, liver-restricted expression of hGAA evaded humoral and cellular immunity in GSD-II mice and will enhance the development of gene therapy in Pompe disease.

MATERIALS AND METHODS

Cell culture. 293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U penicillin per milliliter, and 100 μg streptomycin per milliliter at 37°C in a 5% CO_2–air atmosphere.

Construction of pAAV-LSPhGAApA vector plasmid. The ~816-bp LSP contains a thyroid hormone-binding globulin promoter sequence, two copies of an α1-microglobulin/bikunin enhancer sequence, and a leader sequence [34]. The LSP was amplified from pAAV-LSP-cFIX (a kind gift from Dr. Inder Verma, Salk Institute, La Jolla, CA, USA) by PCR with primers that created an upstream *Ase*I site and a downstream *Kpn*I site (sense, 5′-CCCGCCATGCTAATTAATTACGTAGCC-3′, and antisense, 5′-AAGAAGGGGAAGGTACCATCAAGGG-3′). The *Avr*II–*Kpn*I fragment containing the LSP was cloned into pAAV-CBhGAApA [21] to replace the hybrid CB promoter and to generate pAAV-LSPhGAApA.

Preparation of AAV vectors. Briefly, 293 cells were transfected with an AAV vector plasmid, the AAV packaging plasmid p5E18-VD 2/8 [27] (courtesy of Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA, USA), and pAdHelper (Stratagene, La Jolla, CA, USA). Cell lysate was harvested 48 h following infection and freeze–thawed three times and isolated by sucrose cushion pelleting followed by two cesium chloride gradient centrifugation steps. AAV stocks were dialyzed against three changes of Hanks' buffer, and aliquots were stored at −80°C. The number of vector DNA-containing-particles was determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the NIH.

In vivo administration of AAV vector stocks. The AAV vector stocks were administered intravenously (via the retroorbital sinus) to 12-week-old GAA-KO mice [45]. At the indicated time points postinjection, plasma or tissue samples were obtained and processed as described below. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Determination of hGAA activity and glycogen content. hGAA activity was measured following removal of tissues from control or treated mice, flash-freezing on dry ice, homogenization and sonication in distilled water, and pelleting of insoluble membranes/proteins by centrifugation. The protein concentrations of the clarified suspensions were quantified via the Bradford assay. hGAA activity tissues were determined as described [13].

Glycogen content of tissues was measured using the *Aspergillus niger* assay system, as described [46]. A two-tailed homoscedastic Student *t* test was used to determine significant differences in hGAA levels, glycogen content, and other measurements between GAA-KO mice with or without administration of the vector encoding hGAA.

Western blotting analysis of hGAA. For direct detection of hGAA in tissues and plasma, 50-μg samples of homogenate (or 5 μl undiluted plasma) were electrophoresed overnight in a 6% polyacrylamide gel to separate proteins and transferred to a nitrocellulose membrane. The blots were blocked with 5% nonfat milk solution, incubated with primary and secondary antibodies, and visualized via the enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ, USA) [16].

ELISA detection of plasma anti-hGAA. An ELISA was performed as described [16]. Briefly, recombinant hGAA (5 μg) in carbonate buffer was coated onto each well of a 96-well plate at 4°C overnight. After a wash with PBS containing 0.05% Tween 20, 1:200 dilutions of plasma samples were added to the wells and incubated for 1 h at room temperature. The wells were washed with 0.05% Tween 20 + PBS, incubated with a 1:2500 dilution of alkaline phosphatase-conjugated sheep anti-mouse IgG (H + L) at room temperature for 1 h, and washed, and alkaline phosphatase substrate (*p*-nitrophenyl phosphate) was added. The absorbance at 405 nm was measured with a Bio-Rad microplate reader. All samples yielded absorbance values that were within the linear range of the assay at this dilution.

Semiquantitation of AAV vector DNA by PCR. Genomic DNA was extracted from GAA-KO/SCID mouse tissues, and PCR was performed in a 50-μl reaction containing 500 ng of mouse DNA as described [20].

RT-PCR. Three micrograms of total RNA isolated from liver or heart was DNase I treated and subsequently reverse transcribed with 300 units of M-MLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA) and 300 ng of random hexamer primers in a 40-μl reaction. Four microliters of cDNA was subjected to PCR as described above. Samples were denatured at 94°C for 3 min and then followed by 32 cycles (27 cycles for β-actin, internal control) of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. Primers for RT-PCR were identical to those used in semiquantitation of AAV vector DNA [20].

Urinary Glc_4. Urinary Glc_4 concentrations were determined relative to creatinine by stable isotope–dilution electrospray tandem mass spectrometry as previously described [47].

ELISpot assay. Briefly, the ELISpot assay to detect hGAA-specific T cell responses was done as described [48] with modifications. Mouse splenocytes were harvested from freshly sacrificed mice and assayed for interferon-γ (IFN-γ) production, using an ELISpot for Mouse Interferon-γ kit (Mabtech, Inc, Mariemont, OH, USA) and following the manufacturer's instructions. Multiscreen-HA 96-well plates (Millipore, Bedford, MA, USA) were coated with 100 μl/well of 15 μg/ml anti-mouse IFN-γ monoclonal antibody AN18. A total of 5×10^5 spleen cells were tested in the presence of 1.25 μg/ml PMA and 0.25 μg/ml ionomycin (Sigma); 1.3 μg/ml HIV Gag mix (Becton–Dickinson, San Jose, CA, USA); 100, 50, or 25 μg/ml recombinant hGAA; or medium in a total volume of 200 μl/well in duplicate of six for 18 to 20 h at 37°C in 5% CO_2. The plates were washed, incubated with 100 μl of biotinylated monoclonal detection antibody (R4-6A2-biotin) at 1 μg/ml, and subsequently incubated with streptavidin–horseradish peroxidase. Color was developed using 100 μl 3-amino-9-ethyl-carbazole (Sigma), which reconstitutes in an acetate buffer for 4 min at room temperature in the dark. The membranes were dried and IFN-γ spot-forming cells were counted using the KS ELISpot automated reader system with KS ELISpot 4.2 software (Carl Zeiss, Inc., Thornwood, NY, USA) and expressed as the number of spots per well.

$CD4^+$ and $CD8^+$ lymphocyte immunodetection. Flash-frozen sections of gastrocnemius were fixed in cold acetone for 20 min and air dried prior to being washed in PBS (pH 7.4–7.6) and incubated with normal goat serum for 20 min at room temperature. Subsequently, slides were incubated with an anti-CD4 or anti-CD8 primary antibody (Pharmingen, San Diego, CA, USA) for 1 h at room temperature, followed by three washes in PBS, and incubated with secondary biotinylated goat anti-rat antibody (Pharmingen) for 30 min at room temperature followed by three washes PBS. Incubation with streptavidin-conjugated alkaline phosphatase (Biogenix, Bogota, DC, Colombia) for 20 min at room temperature followed by three washes with PBS preceded development with fresh fuchsin substrate (Biogenix) for 4–5 min to effect. Sections were counterstained with methyl green for 1 min and allowed to air dry to preserve substrate, prior to being coated with xylene and covered with a coverslip with permanent mounting medium.

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