Correction of Multiple Striated Muscles in Murine Pompe Disease Through Adeno-associated Virus–mediated Gene Therapy

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Glycogen storage disease type II (Pompe disease; MIM 232300) stems from the deficiency of acid α-glucosidase (GAA; acid maltase; EC 3.2.1.20), which primarily involves cardiac and skeletal muscles. An adeno-associated virus 2/8 (AAV2/8) vector containing the muscle creatine kinase (MCK) (CK1) reduced glycogen content by ~50% in the heart and quadriceps in GAA-knockout (GAA-KO) mice; furthermore, an AAV2/8 vector containing the hybrid α-myosin heavy chain enhancer-/MCK enhancer-promoter (MHCK7) cassette reduced glycogen content by >95% in heart and >75% in the diaphragm and quadriceps. Transduction with an AAV2/8 vector was higher in the quadriceps than in the gastrocnemius. An AAV2/9 vector containing the MHCK7 cassette corrected GAA deficiency in the distal hindlimb, and glycogen accumulations were substantially cleared by human GAA (hGAA) expression therein; however, the analogous AAV2/7 vector achieved much lower efficacy. Administration of the MHCK7-containing vectors significantly increased striated muscle function as assessed by increased Rotarod times at 18 weeks after injection, whereas the CK1-containing vector did not increase Rotarod performance. Importantly, type IIb myofibers in the extensor digitalis longus (EDL) were transduced, thereby correcting a myofiber type that is unresponsive to enzyme replacement therapy. In summary, AAV8 and AAV9-pseudotyped vectors containing the MHCK7 regulatory cassette achieved enhanced efficacy in Pompe disease mice.

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INTRODUCTION

Glycogen storage disease type II (Pompe disease; MIM 232300) is a classical lysosomal storage disease that causes death in infancy as a result of cardiomyopathy and cardiorespiratory failure. The single gene deficiency in acid α-glucosidase (GAA; acid maltase; EC 3.2.1.20) results in lysosomal accumulation of glycogen in various tissues, primarily in heart and skeletal muscle. Pompe disease could be effectively treated by the correction of GAA deficiency in striated muscle. GAA expression with pseudotyped adeno-associated virus (AAV) vectors[1–5] or with a helper-dependent adenovirus vector has achieved prolonged efficacy[6] in Pompe disease mice. None of the aforementioned vectors completely corrected the glycogen content of all skeletal muscles, and none of these studies evaluated the distal hindlimb muscles.

The clinical presentation of Pompe disease resembles that of the muscular dystrophies, featuring weakness of the proximal leg muscles that generalizes to all skeletal muscles.[7] Gene therapy in the muscular dystrophies represents a unique challenge, because humoral and cytotoxic immune responses occur frequently in response to introduced proteins.[8,9] Cytotoxic T lymphocyte responses against gene therapy vectors have been reduced by substituting muscle-specific regulatory cassettes for ubiquitously active viral promoter/enhancers. An AAV2 vector containing a muscle-specific creatine kinase (MCK) regulatory cassette evoked an attenuated immune response in *mdx* mice, in comparison with an analogous AAV vector containing the cytomegalovirus (CMV) promoter.[10] An AAV2/6 vector containing the MCK CK6 cassette[11] transduced skeletal muscle with lower efficiency, in comparison with an analogous vector containing a CMV promoter/enhancer; however, the MCK-driven β-galactosidase expression persisted longer than CMV-driven expression.[12] An AAV2/6 vector containing another MCK regulatory cassette [CK1 (refs. 13,14)] produced high-level human GAA (hGAA) expression and glycogen clearance in the injected gastrocnemius muscle, and the analogous AAV2/7 vector partially cleared glycogen storage in multiple muscle groups of GAA-knockout (GAA-KO) mice following intravenous administration.[2]

AAV2/8 vectors have efficiently transduced striated muscle following systemic delivery in mice.[15] As few as 3×10^{11} vector particles transduced the majority of cardiomyocytes.[15,16] More relevant to Pompe disease, an AAV2/1 vector encoding GAA

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reduced glycogen storage following intravenous administration to neonatal GAA-KO mice.[4] Thus, current data from Pompe and muscular dystrophy mice endorse the further investigation of AAV pseudotypes with enhanced muscle tropism in these models.[2,4,17,18]

Salva *et al.* designed a series of highly active muscle-specific regulatory cassettes that were evaluated in striated muscle following systemic delivery via tail vein injections of AAV6 vectors.[14] The most active cassette in a variety of anatomical muscles combined a 190-bp enhancer from the murine α-myosin heavy chain gene with a 570 bp abbreviated MCK regulatory cassette, termed the MHCK7. The latter expressed human placental alkaline phosphatase at very high levels in murine heart and skeletal muscle, exceeding levels achieved with the CMV promoter/enhancer in the heart, and expressed high levels of microdystrophin in skeletal and cardiac muscles of *mdx* mice.[14]

We hypothesized that systemic administration of an AAV vector containing a muscle-specific regulatory cassette could achieve long-term correction of multiple muscles in GAA-KO mice. An AAV vector containing the MHCK7 cassette was pseudotyped with AAV serotypes 7, 8, and 9, and the efficacy of each serotype was evaluated in GAA-KO mice.

RESULTS

Inclusion of an α-myosin heavy chain enhancer markedly activated an MCK regulatory cassette

The transduction of striated muscle with AAV2/8 vectors containing either an abbreviated MCK regulatory cassette (AAV2/8-CK1hGAApA[2]) or a hybrid α-myosin heavy chain enhancer/MCK cassette (AAV2/8-MHCK7hGAApA) were evaluated, following intravenous administration in adult GAA-KO mice (1×10^{11} or 1×10^{12} vector particles/mouse). The former vector contains the CK1 promoter[13] and the latter contains MHCK7.[14] GAA activity and glycogen content in the heart, liver, and skeletal muscles were analyzed 18 weeks following vector administration (**Figure 1**). GAA activity and glycogen content were significantly affected by both dosage and vector type for all tissues examined ($P < 0.05$ with a two-way ANOVA). Bonferroni post-test comparisons of the two vectors showed that GAA levels were significantly greater in all tissues treated with the higher dose of AAV2/8-MHCK7hGAApA compared with the equivalent dose of AAV2/8-CK1hGAApA(**Figure 1**). At the low dose the difference between the two vectors was not significant for any of the tissues, although the mean GAA levels were greater in each tissue for the AAV2/8-MHCK7hGAApA group. The lower GAA activity in the liver following AAV2/8-CK1hGAApA administration suggested a more stringent muscle-restricted expression with the CK1 cassette relative to the MHCK7 cassette (**Figure 1a**).[14] Real-time reverse transcriptase–PCR revealed that liver expression of GAA was significantly increased with AAV2/8-MHCK7hGAApA, in comparison with AAV2/8-CK1hGAApA, when each was administered at the higher dose (2.2 ± 1.2% of β-actin RNA versus 0.20 ± 0.05%, respectively; $P = 0.006$). However, secretion of hGAA from transduced muscles and uptake by the liver also could have contributed to the higher GAA activity in the liver following AAV2/8-MHCK7hGAApA administration.

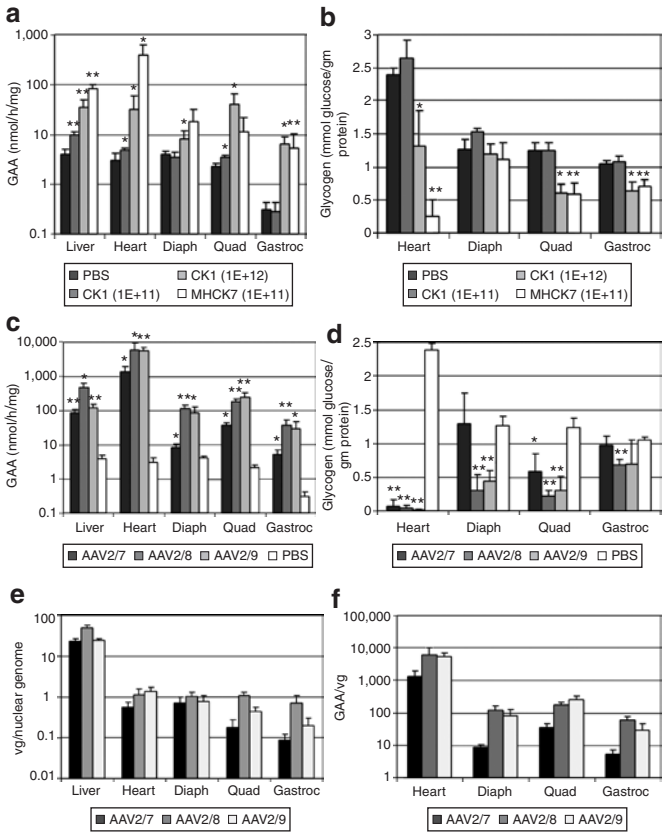

Figure 1 Transduction of striated muscle in adult acid α-glucosidase-knockout (GAA-KO) mice with intravenously administered adeno-associated virus (AAV) vectors containing the CK1 or α-myosin heavy chain enhancer-/MCK enhancer-promoter (MHCK7) regulatory cassettes. (**a**) GAA activity in the indicated striated muscles, 18 weeks following vector administration. AAV2/8-CK1hGAApA (CK1), either 1×10^{11} (1E+11; $n = 3$) or 1×10^{12} (1E+12; $n = 3$) vector particles (vp)/mouse, and AAV2/8-MHCK7hGAApA (MHCK7), 1×10^{11} (1E+11; $n = 4$) vp/mouse were injected intravenously at 3 months of age. Mock-treated GAA-KO mice were negative controls [phosphate-buffered saline (PBS); $n = 4$]. (**b**) Glycogen content for GAA-KO mice in **a**. (**c**) GAA activity in the indicated striated muscles, 18 weeks following administration of AAV-MHCK7hGAApA pseudotyped as AAV2/7 (5×10^{11} vp; $n = 5$), AAV2/8 (1×10^{12} vp; $n = 5$) vp/mouse, and AAV2/9 (5×10^{11} vp; $n = 5$). Mock-treated GAA-KO mice were negative controls (PBS; $n = 4$). (**d**) Glycogen content for GAA-KO mice in **c**. (**e**) Vector genome quantitation with real-time PCR for GAA-KO mice in **c**. (**f**) Ratio of GAA activity to vector genome quantity for GAA-KO mice in **c**. Mean ± SD shown. *$P < 0.05$ and **$P < 0.001$ indicated for the values for groups of GAA-KO mice following vector injection, in comparison with mock-treated GAA-KO mice.

Correction of multiple striated muscles with an AAV2/8 or AAV2/9 vector

Glycogen was significantly lower in all muscles treated with AAV2/8-MHCK7hGAApA at both low and high doses, in comparison with the equivalent dose of AAV2/8-CK1hGAApA (**Figure 1b**).

The presence of residual glycogen accumulation in hindlimb muscles, particularly the gastrocnemius, following AAV2/8 vector administration prompted a comparison with the AAV2/7 and AAV2/9 pseudotypes of AAV-MCHK7hGAApA. The GAA activities of the heart and skeletal muscles were significantly increased following AAV2/8-MHCK7hGAApA or

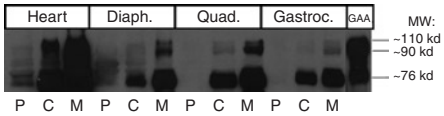

Figure 2 Western blot detection of human GAA (hGAA) in striated muscle 18 weeks following intravenous administration of adeno-associated virus 2/8 (AAV2/8) vectors in acid α-glucosidase-knockout (GAA-KO) mice. Lanes labeled as follows: P, control mouse injected with phosphate-buffered saline; C, AAV2/8-CK1hGAApA [1 × 10^{12} vector particles (vp)]; and M, AAV2/8-MHCK7hGAApA (1 × 10^{12} vp).

AAV2/9- MHCK7hGAApA administration ($P < 0.05$), in comparison with AAV2/7-MHCK7hGAApA (**Figure 1c**). The glycogen contents of the heart and skeletal muscles were significantly decreased following AAV2/8-MHCK7hGAApA or AAV2/9-MHCK7hGAApA administration ($P < 0.05$), in comparison with AAV2/7-MHCK7hGAApA (**Figure 1d**).

Vector genomes were quantified to allow a comparison of the transduction efficiency with each pseudotype of AAV-MHCK7hGAApA (**Figure 1e**). Long-term hGAA expression in heart and skeletal muscles was associated with the persistence of up to ~1 vector genome per nuclear genome in the heart, gastrocnemius, and quadriceps, whereas more than tenfold higher amounts of vector DNA were present in the liver (**Figure 1e**). The enhanced correction with the AAV2/8 and AAV2/9 vectors, in comparison with AAV2/7, correlated with slightly increased numbers of vector genomes in the heart, quadriceps, and gastrocnemius (**Figure 1e**); moreover, the AAV2/8 and AAV2/9 vectors demonstrated significantly increased normalized GAA expression in striated muscle and heart (**Figure 1f**) ($P < 0.05$). Taken together, these data indicated that the AAV2/8 and AAV2/9 vectors transduced striated muscle more efficiently and produced higher GAA activity in transduced myofibers, in comparison with the AAV2/7 vector.

Western blot analysis revealed the ~76 kd processed form of GAA in the heart, quadriceps, gastrocnemius, and liver of GAA-KO mice following administration of the higher number of particles of AAV2/8-CK1hGAApA and AAV2/8-MHCK6hGAApA, whereas a very low signal was detected in the diaphragm following transduction with AAV2/8-CK1hGAApA (**Figure 2**, liver GAA not shown). GAA activity and glycogen content were not significantly changed in diaphragm following administration of AAV2/8-CK1hGAApA (**Figure 1**), consistent with the low activity for the CK1 cassette in the diaphragm.[12–14]

Transduction of distal hindlimb muscles with AAV2/8 and AAV2/9 vectors

Glycogen staining revealed the basis for incomplete correction of skeletal gastrocnemius with AAV2/8-MHCK7hGAApA (**Figure 1b**). Multiple glycogen laden myofibers were detected in the gastrocnemius, consistent with the lack of correction of GAA deficiency within individual myofibers (**Figure 3a**). The quadriceps contained a higher number of normal-appearing myofibers (**Figure 3a**, arrows), consistent with the lower glycogen content in the quadriceps (**Figure 1b**). Thus, the relatively higher residual glycogen in the gastrocnemius could be attributed to the greater prevalence of untransduced myofibers. Lymphocytic infiltrates were absent in skeletal muscle, indicating a lack of cytotoxic T-cell

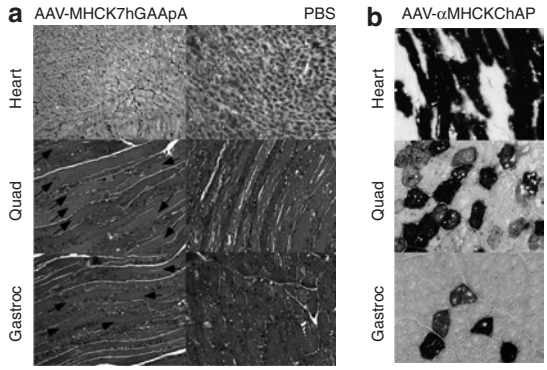

Figure 3 Transduction of striated muscle with an adeno-associated virus 2/8 (AAV2/8) vector. (**a**) Periodic acid/Schiff staining following intravenous AAV-MHCK7hGAApA vector administration to acid α-glucosidase-knockout (GAA-KO) mice. Control mice were sham-treated, age-matched GAA-KO mice [phosphate-buffered saline (PBS)]. Original magnification ×200. (**b**) Alkaline phosphatase staining following intravenous AAV-αMHCKChAP administration to GAA-KO mice. Sham-treated GAA-KO mice stained negatively for alkaline phosphatase in striated muscle (data not shown; see ref. 14, **Figures 3** and **5**). Original magnification ×400.

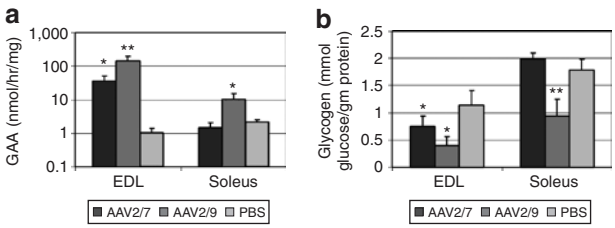

Figure 4 Transduction of hindlimb muscles in adult acid α-glucosidase-knockout (GAA-KO) mice with intravenously administered adeno-associated virus 2/7 (AAV2/7) or AAV2/9 vectors containing the α-myosin heavy chain enhancer-/MCK enhancer-promoter (MHCK7) regulatory cassette. (**a**) GAA activity in the indicated striated muscles, 18 weeks following administration of AAV-MHCK7hGAApA (5 × 10^{11} vector particles) pseudotyped as AAV2/7 ($n = 5$) and AAV2/9 ($n = 5$). Mock-treated GAA-KO mice were negative controls [phosphate-buffered saline (PBS); $n = 4$]. (**b**) Glycogen content for GAA-KO mice in **a**. Mean ± SD shown. *$P < 0.05$ and **$P < 0.001$ indicated for GAA-KO mice following vector injection, in comparison with mock-treated GAA-KO mice. EDL, extensor digitalis longus.

responses in transduced muscle (data not shown). To evaluate the distribution of functionally transduced myofibers, mice were injected with 1 × 10^{12} vector particles of an AAV2/8 vector carrying a human placental alkaline phosphatase reporter cDNA driven by the MHCK7 regulatory cassette. The highest level of functional transduction was detected in the heart, followed by the quadriceps, whereas fewer transduced myofibers were present in the gastrocnemius (**Figure 3b**). The soleus and extensor digitalis longus (EDL) were transduced at even lower frequency than the gastrocnemius (less than three positive myofibers/field; data not shown). The low transduction of the soleus and EDL demonstrated a clear limitation for the AAV2/8 pseudotype.

The GAA activities of the EDL and soleus were significantly increased following AAV2/9-MHCK7hGAApA administration ($P < 0.05$), in comparison with AAV2/7-MHCK7hGAApA (**Figure 4a**). As expected, the glycogen contents of the EDL and soleus were

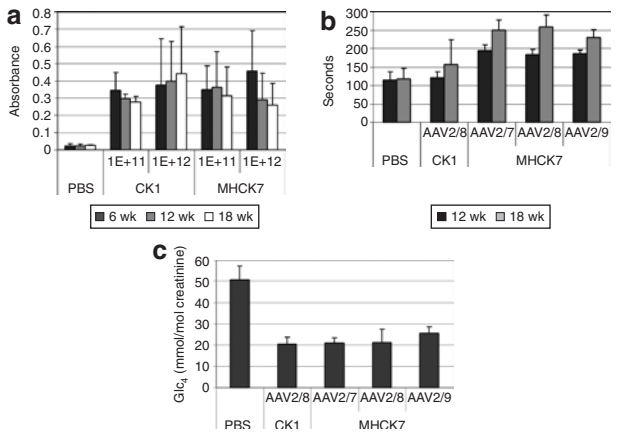

Figure 5 Humoral response, biomarker reduction, and Rotarod testing following systemic adeno-associated virus (AAV) vector administration in acid α-glucosidase-knockout (GAA-KO) mice. Testing done at the indicated times following AAV vector administration. Mean and SD are shown. (**a**) Enzyme-linked immunosorbent assay of GAA-KO mouse plasma (1:200 dilution) following AAV vector administration. The absorbance for anti-hGAA antibodies at different times following AAV vector administration are shown. The number of mice was as follows: control [phosphate-buffered saline (PBS), $n = 4$]; AAV2/8-CK1hGAApA (CK1), low dose (1×10^{11}, $n = 3$), high dose (1×10^{12}, $n = 3$); and AAV2/8-MHCK7hGAApA [α-myosin heavy chain enhancer-/MCK enhancer-promoter (MHCK7)], low dose (1×10^{11}, $n = 4$), high dose (1×10^{12}, $n = 5$). (**b**) Rotarod testing of GAA-KO mice in **a**, and additional groups following administration of AAV-MHCK7hGAApA (5×10^{11} vp) pseudotyped as AAV2/7 ($n = 5$) and AAV2/9 ($n = 5$). (**c**) Urinary Glc_4 concentrations for the groups of GAA-KO mice in **b**, 18 weeks following vector administration, and for age-matched, untreated GAA-KO controls ($n = 4$).

significantly decreased following AAV2/9- MHCK7hGAApA administration (**Figure 4b**). Thus, the transduction of the small hindlimb muscles was superior for the AAV9-pseudotyped vector.

Antibodies against hGAA have prevented efficient cross-correction of GAA deficiency through receptor-mediated uptake, despite the presence of secreted hGAA in the blood.[2,3,19] Anti-hGAA antibodies were significantly elevated by 6 weeks following administration of both low and high doses of AAV2/8 vector ($P < 0.05$), regardless of the regulatory cassette type (**Figure 5a**). Despite the presence of anti-GAA antibodies, Rotarod times were significantly increased ($P < 0.05$) at 12 and 18 weeks following administration of AAV-MHCK7hGAApA-injected mice for the AAV2/7, AAV2/8, and AAV2/9 pseudotypes (**Figure 5b**). However, Rotarod times were not significantly increased at 12 or 18 weeks for AAV-CK1hGAApA-treated mice. A glucotetrasaccharide biomarker for increased glycogen storage, Glcα1-6Glcα1-4Glcα1-4Glc, (Glc_4), was significantly reduced ($P < 0.05$) at 18 weeks for the high-dose group of all vector-injected GAA-KO mice, regardless of the regulatory cassette or pseudotype in comparison with phosphate-buffered saline–injected mice (**Figure 5c**). There was no difference in mean Glc_4 levels between the different vector-treated groups. Hence, a significant increase in Rotarod time and reduction of Glc_4 biomarker were achieved, despite the presence of an antibody response against hGAA.

Wide-spread clearance of glycogen in individual myofibers was demonstrated following AAV2/7 and AAV2/9 vector administration (**Figure 6**). Fiber typing confirmed the high prevalence of type I, slow-twitch fibers in the soleus of GAA-KO

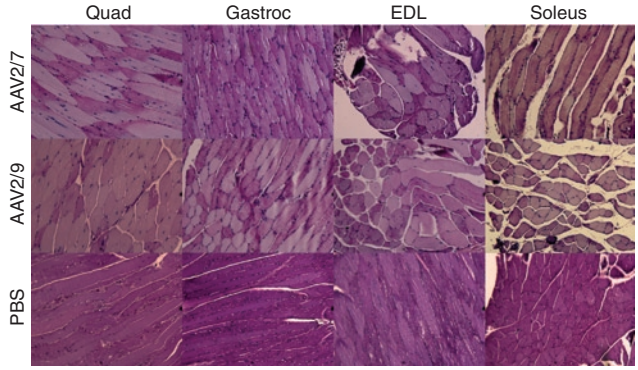

Figure 6 Transduction of striated muscle with adeno-associated virus 2/7 (AAV2/7) and AAV2/9 vectors. Periodic acid/Schiff staining following intravenous administration of AAV-MHCK7hGAApA, pseudotyped as AAV2/7 or AAV2/9, to acid α-glucosidase-knockout (GAA-KO) mice. Controls were sham-treated, age-matched GAA-KO mice [phosphate-buffered saline (PBS)]. Original magnification ×200. EDL, extensor digitalis longus.

mice, whereas the EDL was comprised mainly of type IIb myofibers (data not shown). The clearance of glycogen vacuolation within multiple myofibers in the EDL and soleus indicated that both type I and IIb myofibers were transduced by the AAV2/9 vector (**Figure 6**).

DISCUSSION

Gene therapy in the muscular dystrophies will likely require therapeutic gene expression in striated muscle generally, particularly for lethal muscular dystrophies such as Duchenne muscular dystrophy. Pompe disease uniquely responds to infused or secreted therapeutic protein, because unlike other muscular dystrophies Pompe disease is a lysosomal storage disorder amenable to enzyme replacement therapy. Owing to the high enzyme level requirements in enzyme replacement therapy and complicating antibody responses to GAA, muscle-targeted gene therapy is under development in Pompe disease.[2,20,21] Systemic delivery of an AAV2/8 vector encoding muscle-restricted hGAA achieved significant efficacy; however, the transduction of myofibers was partial, preventing the complete correction of glycogen storage in the gastrocnemius. The analogous AAV2/9 vector demonstrated significantly increased transduction of the small hindlimb muscles, in direct comparison with the AAV2/7 vector. Transduction of the EDL and soleus were inefficient with an AAV2/8 vector encoding human placental alkaline phosphatase, implying that the AAV2/9 pseudotype might have significant advantages with regard to transduction of the distal hindlimb muscles.

Type I fibers were more easily cleared of glycogen during enzyme replacement therapy, indicating the need to focus on the transduction of type II fibers in Pompe disease.[22,23] Type I myofibers were transduced less efficiently with an AAV2 vector than with an AAV2/6 vector, reflecting the potential advantage of newer serotypes over AAV2.[24,25] Currently, the AAV2/9 serotype efficiently transduced both type I and IIb myofibers, as well as cardiomyocytes, following intravenous administration. This study further endorses the role of AAV2/9 vectors for gene therapy in Pompe disease, demonstrating significant correction of not only the heart but all skeletal muscles examined. Previously AAV2/9

vectors have transduced striated muscle more efficiently than either an AAV2/1 vector in neonatal mice[26] or an AAV2/8 vector in adult mice.[27] Neither of these earlier studies demonstrated significant correction of multiple skeletal muscles in adult mice.[26,27] The improved efficacy of this new AAV2/9 vector further strengthens preclinical data in favor of clinical trials of gene therapy in Pompe disease.

The successful development of gene therapy in GAA-KO mice indicates that curative therapy for Pompe disease may become available in the foreseeable future; however, the efficacy of gene therapy in these experiments was inversely related to the presence of immune responses. Immunocompetent GAA-KO mice produced high titer anti-hGAA immunoglobulin G in response to an AAV vector containing the ubiquitously active CMV promoter/chicken β-actin (CB) promoter, packaged as either AAV2/6 or AAV2/8.[2,3] The CB-containing vector failed to secrete detectable hGAA in the plasma for >2 weeks or to reduce glycogen storage in the muscle of GAA-KO mice.[3] Immune responses to ubiquitously expressed hGAA included lymphocytic infiltrates and activation of $CD4^+$ and $CD8^+$ lymphocytes in injected skeletal muscle, and in the liver following intravenous injection.[2,3] Substitution of the CK1 regulatory cassette in place of the CB promoter prevented $CD8^+$ lymphocytic responses in the injected muscle. Therefore, both antibody production and cytotoxic T lymphocytes were elicited in response to hGAA production from a ubiquitously active CB promoter; however, vectors containing muscle-specific cassettes did not provoke lymphocytic infiltrates in transduced muscles and expressed hGAA for >18 weeks.[2] The latter experiment also demonstrated the imperviousness of muscle-specific hGAA expression to circulating anti-GAA antibodies. The presence of anti-GAA antibodies has prevented cross-correction of untransduced muscle cells,[1–3,19] implicating the transduction of individual muscle cells as the source of glycogen clearance in the current study.

The MHCK7 regulatory cassette has driven highly efficacious hGAA expression in the Pompe disease mouse model in this study. A comparison of AAV2/6 vectors containing either the MHCK7 or the CK1 cassette demonstrated high transgene expression within cardiac muscle and skeletal muscles,[14] which has now been replicated in Pompe disease mice for AAV2/7, AAV2/8, and AAV2/9 vectors. Virtually all myofibers were transduced in the muscles examined with the aforementioned AAV2/6 vector, although transgene expression was reduced in the diaphragm; moreover, transduction was enhanced by the co-administration of the vascular permeabilizing agent vascular endothelial growth factor at lower vector doses.[12] The current AAV2/8 vector achieved partial clearance of glycogen from the diaphragm in Pompe mice through the administration of reasonably low particle numbers, increasing the likelihood of translation to clinical applications in Pompe disease. The activity of the MCHK7 regulatory cassette was higher than that for the CK1 cassette in heart, quadriceps, gastrocnemius, and diaphragm, all critical targets for gene therapy in Pompe disease and other forms of muscular dystrophy. The combination of highly active muscle-specific regulatory cassettes and novel AAV serotypes promises to advance gene therapy for muscular dystrophy by providing curative therapy for these devastating disorders.

MATERIALS AND METHODS

Preparation of pseudotyped AAV vectors. AAV-MHCK7hGAApA contains the MHCK7 regulatory cassette,[14] the hGAA cDNA, and a human growth hormone polyadenylation sequence. The vector plasmid, pAAV-MHCK7hGAApA, was derived from pAAV-CBhGAApA.[28] The pAAV-MHCK7hGAApA was digested with *Kpn*I, blunt-ended with the Klenow fragment of DNA polymerase, then digested with *Xba*I; subsequently, the 5.7-kb blunt/*Xba*I fragment from pAAV-MHCK7hGAApA was ligated with a 0.8-kb *Xba*I/blunt *Sal*I fragment containing MHCK7 cassette from αMHCKChAP[14] (provided by Dr. Stephen Hauschka, University of Washington, Seattle, WA). AAV-CK1hGAApA has been described (formerly AAV-MCKhGAApA).[2] Briefly, 293 cells were transfected with an AAV vector, the AAV packaging plasmid[29] (courtesy of Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA), and pAdHelper (Stratagene, La Jolla, CA). Cell lysate was harvested 48 hours 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 in viral stocks was determined by DNase I digestion, DNA extraction, and Southern blot analysis. The Southern blot signal for vector genomes was quantified by comparison with the signals from standards consisting of *Ahd*I-digested vector plasmid. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the National Institutes of Health.

In vivo *analysis of AAV vector.* The AAV vector stocks were administered intravenously (via the retroorbital sinus) in 3-month-old GAA-KO mice.[30] At the indicated time points after injection, plasma or tissue samples were obtained and processed as described below. All animal procedures were performed in accordance with Duke University Institutional Animal Care and Use Committee–approved guidelines.

Rotarod testing was performed as previously described.[1] GAA activity and glycogen content were analyzed as described, and real-time RT-PCR was performed with standard methods using primers previously described.[31] Western blotting of hGAA was performed as previously described[1] using the hGAA monoclonal antibody (courtesy of Genzyme, Framingham, MA). Alkaline phosphatase staining was performed as previously described.[14] The enzyme-linked immunosorbent assay was performed as previously described.[3] All samples yielded absorbance values that were within the linear range of the assay at this dilution. Urinary Glc_4 concentrations were determined relative to creatinine by stable isotope-dilution electrospray tandem mass spectrometry as previously described.[32]

Statistical analyses. Two-way ANOVAs were performed using the independent variables of vector type and dose of vector, with Bonferroni post-tests for specific comparison between groups (Prism 3.0, GraphPad, San Diego, CA). Comparison of two groups was assessed by a homoscedastic Student's *t*-test. A P value of <0.05 indicated a significant difference between the observed values for each group.

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