Systemic correction of the muscle disorder glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid-α-glucosidase

(Pompe/gene therapy)


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ABSTRACT This report demonstrates that a single intravenous administration of a gene therapy vector can potentially result in the correction of all affected muscles in a mouse model of a human genetic muscle disease. These results were achieved by capitalizing both on the positive attributes of modified adenovirus-based vectoring systems and receptor-mediated lysosomal targeting of enzymes. The muscle disease treated, glycogen storage disease type II, is a lysosomal storage disorder that manifests as a progressive myopathy, secondary to massive glycogen accumulations in the skeletal and/or cardiac muscles of affected individuals. We demonstrated that a single intravenous administration of a modified Ad vector encoding human acid-α-glucosidase (GAA) resulted in efficient hepatic transduction and secretion of high levels of the precursor GAA proenzyme into the plasma of treated animals. Subsequently, systemic distribution and uptake of the proenzyme into the skeletal and cardiac muscles of the GAA-knockout mouse was confirmed. As a result, systemic decreases (and correction) of the glycogen accumulations in a variety of muscle tissues was demonstrated. This model can potentially be expanded to include the treatment of other lysosomal enzyme disorders. Lessons learned from systemic genetic therapy of muscle disorders also should have implications for other muscle diseases, such as the muscular dystrophies.

Although gene therapy has the potential to treat a number of disorders, several obstacles must be overcome before a specific "genetic therapy" will have any chance of being clinically successful. Some genetic diseases may be more amenable to gene therapy approaches than others, based on the biological aspects of the respective disorders. Unfortunately, the various classes of gene transfer vector currently available also will have specific limitations. Matching the attributes/limitations of the respective vector with the nuances of the disease to be treated will maximize the potential of any gene therapy protocol. In the studies reported here, we capitalized on the benefits of a modified adenovirus (Ad) vector to attempt gene therapy of a systemic muscle disorder, glycogen storage disease type II (GSD-II).

GSD-II is a lysosomal storage disorder that is caused by the deficiency of acid-α-glucosidase (GAA) activity. Lack of sufficient GAA activity results in the massive accumulation of glycogen in the lysosomes, with a subsequent disruption of cellular functions (1). In its most severe presentation (neonatal GSD-II, or Pompe disease), infants are affected by a hypertrophic cardiomyopathy, respiratory failure, and hypotonia secondary to the extensive glycogen accumulation in their cardiac and skeletal muscles (1). There are also juvenile and adult onset forms of this disorder (characterized by a lack of cardiac involvement, a later age of onset, and a slower progression), but eventual respiratory or limb muscle involvement results in significant morbidity and mortality for these individuals as well (1). In the milder forms of GSD-II, the amount of residual GAA activity present generally correlates with the severity of the disease phenotype.

Presently, only symptomatic support is available to GSD-II patients because there is no specific therapy. Research efforts have focused on exogenous enzyme replacement therapy as a treatment option, in a manner similar to that used for another lysosomal disorder, Gaucher disease (2–4). These strategies use receptor-mediated transport mechanisms to direct uptake of intravenously injected recombinant lysosomal enzymes into affected tissues. However, enzyme replacement therapies can be limited by large-scale production issues and the need for frequent treatments. Permanent correction of GSD-II, however, will only be possible after the introduction of a functional copy of the human GAA gene into those tissues predominantly affected in GSD-II patients.

There are three animal models of GSD-II: a bovine model, the acid maltase-deficient quail, and recently described GAA knockout mice (GAA-KO) (1, 5–7). The phenotypes of these models include widespread glycogen accumulation in the cardiac and skeletal muscles combined with clinical symptoms of a myopathy. Importantly, the extensive storage of glycogen noted in the muscles of the acid maltase-deficient quail or the GAA-KO mouse can be reduced after the repeated intravenous administration of recombinant human GAA (hGAA) (2, 3, 8). The mannose-6-phosphate receptor-mediated pathway likely facilitates uptake of recombinant hGAA into the affected muscle tissues.

Viral-mediated administration of the hGAA gene into the fibroblasts of affected patients, the muscles of normal mice, and the muscles of the acid maltase-deficient quail has been demonstrated, but GAA enzyme activity was only localized to the site of injection in the latter experiments (9–11). Because GSD-II is a systemic disorder of the cardiac and skeletal muscles, these strategies would likely require multiple injections directly into each of the affected muscle groups throughout the body, a potentially impractical approach. Unfortunately, a gene transfer vector has yet to be described that can successfully deliver a gene into every muscle cell of a living animal. For example, only a few muscle fibers can be primarily transduced after a single intravenous administration of Ad

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This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: Ad, adenovirus; GSD-II, glycogen storage disease type II; GAA, acid-α-glucosidase; KO, knockout; hGAA, human GAA.

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vectors (12, 13). However, 100% of the hepatocytes in a mouse can be successfully transduced after a single intravenous injection of high titer Ad vector preparations (13–15).

Based on these considerations, we hypothesized that, after Ad-mediated transduction of the hGAA gene, the biosynthetic capacity of the entire liver could be harnessed to produce high levels of the hGAA precursor enzyme. Over-expression of hGAA would result in saturation of the receptor-mediated lysosomal targeting mechanisms present within the liver (possibly mannose-6-phosphate mediated), resulting in secretion of the enzyme precursor into the circulatory system. The circulatory system then would distribute the precursor form of hGAA to the cardiac and skeletal muscles of the animal, allowing for uptake and receptor-mediated targeting of the precursor hGAA to the muscle lysosomes. If significant amounts of the enzyme were delivered to the muscles, reduction and/or correction of glycogen accumulations throughout the animal may be demonstrated. Specifically, in this manuscript, we now have confirmed that Ad-mediated delivery of the hGAA gene into the livers of GAA-KO mice can result in the reduction of glycogen accumulation in multiple muscles of the GAA-KO mouse.

MATERIALS AND METHODS

Construction of the AdhGAAΔpol Vector. The cytomegalovirus enhancer/promoter, human GAA cDNA, and the bovine growth hormone-derived polyadenylation signal were removed as a single, ~5.3-kilobase subfragment of DNA after XmnI digestion of the plasmid pcDNA3-hGAA [previously described (16)]. The XmnI subfragment was end-filled with T4 derived DNA polymerase and was ligated to the end-filled AscI site of the shuttling plasmid pAdAscLΔpol (17) to generate pAdAscLhGAAΔpol. Twenty micrograms of NheI-linearized pAdAscLhGAAΔpol shuttling plasmid was cotransfected with two micrograms of XbaI-, ClaI-, and ScaI-digested dl7001-TP virion DNA (E3 deleted) into the B-6 cell line (expressing both the Ad E1 and polymerase proteins) via the calcium phosphate technique (Fig. 1) (17, 18). Recombinant vector clones were expanded and confirmed to contain the hGAA gene by restriction enzyme digestion of vector-derived DNA as well as by multiple functional analyses (see Results) (17). Cesium chloride-purified AdhGAAΔpol vector was produced after infection (multiplicity of infection was ~10) of 60 150-mm tissue culture plates containing ~2.0 × 10^7 C-7 cells [express E1, polymerase, and preterminal protein (19)]. All vector titers were confirmed by plaque-forming unit assay of serial dilutions of the vector preparations on C7 cells.

In Vivo Administration of AdhGAAΔpol. 1 × 10^9 plaque-forming units of AdhGAAΔpol vector were intravenously administered (via the retro-orbital sinus) into C57BL/6 (wild-type) mice or 2-month-old GAA-KO mice (6 neo) (7). At the respective time points post-injection, plasma or tissue samples were obtained and processed as described below. All animal procedures were done in accordance with the Duke University Animal Care and Use Committee guidelines.

Measurement of Tissue/Plasma GAA Activity and Glycogen Accumulation. Tissues were snap-frozen in liquid nitrogen and were homogenized in water, and insoluble proteins were removed by centrifugation. The protein content of the resultant lysate was quantified via the Bradford assay. For plasma GAA activity detection, blood samples were collected by retro-orbital sampling into heparinized capillary tubes, followed by plasma isolation. GAA activity in each of the tissues or plasma was assessed by measurement of 4-methylumbelliferyl-α-D-glucoside cleavage at pH 4.3, as described (2). Acid-β-galactosidase was similarly assayed with a 4-methylumbelliferyl derivative as described (2). Glycogen content was determined by treatment of tissue extracts with Aspergillus niger amylglucosidase and measurement of glucose released. All extracts also were assessed for background glucose release, i.e., in the absence of the A. niger amylglucosidase. Final glycogen content values then were determined after untreated glucose levels were subtracted from the glucose content of each of the amylglucosidase-treated tissue extracts. n = 4 for C57BL/6 mice (age 6.5 months), n = 3 for GAA-KO mice (age 2 months), and n = 3 for Ad-treated GAA-KO mice (age 2 months at time of injection).

Morphological Assessment of Tissues. Sections were placed in embedding compound and were snap-frozen in liquid nitrogen-cooled isopentane. Ten-micrometer sections of the tissues were collected with a Leica (Deerfield, IL) cryomicrotome, were periodic acid-Schiff-stained, and were visualized with a Leica microscope and digital camera.

RNA Analysis. Total RNA was isolated from portions of mouse tissues after homogenization in 4.0 M guanidinium thiocyanate and cesium chloride purification. Total RNA (12.5 µg) from each tissue was electrophoretically separated in a 1% formaldehyde-agarose gel, was blotted onto a nylon membrane, was UV crosslinked, and was probed with a 32P-labeled 3.3-kilobase fragment containing the hGAA cDNA. The probed membranes were washed, exposed to autoradiographic film, and photographed.

Immunoblot Detection of GAA. Respective mouse tissues were frozen, homogenized, and centrifuged to remove insoluble proteins, and protein content of the supernatants was measured by the Bradford assay. Equivalent amounts of protein were electrophoretically separated in a 10% polyacrylamide-SDS gel, were transferred to a nylon membrane, and were probed with a rabbit anti-human GAA polyclonal antibody. Detection of the bound anti-GAA antibody was visualized via the ECL detection system (Amersham Pharmacia). For direct detection of GAA precursor in plasma, 2.5 µl of each sample was electrophoretically separated, followed by anti-GAA antibody probing of the immunoblot, as described above.

RESULTS

Construction of the AdhGAAΔpol Vector. We have previously described the production and clonal purification of modified Ad vectors deleted for both the Ad E1 and polymer-
ase genes (17). This class of modified Ad vector has been demonstrated to persist in hepatocytes, despite neontigen gene transfer into immunocompetent animals (15). The construction of the AdhGAAΔpol vector was carried out as described in Materials and Methods. Restriction enzyme mapping of DNA derived from the purified vector confirmed that it contained the cytomegalovirus–hGAA transgene cassette (data not shown). Further analysis demonstrated that the AdhGAAΔpol vector was capable of high level expression of the hGAA enzyme activity (as assessed by cleavage of 4-methylumbelliferyl-α-D glucoside) after infection of cultured human 293 cells (Fig. 2). Importantly, the supernatant overlying the infected human cells accumulated increasing amounts of hGAA activity with time, suggesting that AdhGAAΔpol infection resulted in both high level expression and secretion of an active GAA enzyme.

**Hepatic Targeting of the AdhGAAΔpol Vector and GAA Secretion.** Successful production of high levels of GAA in the plasma was demonstrated after the intravenous injection of 1 × 10^9 plaque-forming units of AdhGAAΔpol into wild-type mice (Fig. 3). Extremely high levels of GAA activity were detected in the plasma as early as 2 days after infection, although the levels diminished with time. We and others have previously demonstrated that a rapid down-regulation of cytomegalovirus enhancer activity occurs in hepatocytes and may have contributed to the decreased GAA activities detected in the plasma, although production of murine antibodies to the human enzyme also may have been present at the later time points (15, 20, 21).

Intravenous injections of the AdhGAAΔpol vector into the GAA-KO mouse also were done to ascertain the effectiveness of the secreted GAA to reduce glycogen accumulation in affected skeletal and cardiac muscles. The GAA-KO mouse was previously generated by targeted knockout of the murine GAA gene and has a phenotype that includes systemic glycogen accumulation in the cardiac and skeletal muscles, as well as a progressive clinical myopathy (7). After intravenous injection of the AdhGAAΔpol vector, high levels of enzyme activity were demonstrated in the plasma of the GAA-KO mice (Fig. 3). Importantly, protein immunoblot analysis of plasma from the treated animals demonstrated that the predominant form of GAA detected had a molecular mass of ~110 kDa, a size equivalent to the precursor (unprocessed) form of GAA (Fig. 4) (16). In addition, a significant amount of the processed form of the enzyme (~77 kDa) also was detected in the plasma. This may be the result of proteolysis of the mature GAA in the plasma or secondary to nonspecific release of the mature form of hGAA from infected hepatocytes. In contrast, acid β-galactosidase plasma activities (another lysosomal enzyme) in the AdGAAΔpol injected mice were not significantly different from the levels present in control mice. Specifically, control mice had 74.7 ± 9.04 nmol/hr/ml of acid β-galactosidase activity in their plasma samples whereas the 3-, 7-, and 12-day post-AdhGAAΔpol injected mice, respectively, had plasma acid β-galactosidase activities of 84.91 ± 12.6, 122.6 ± 22.2, and 99.03 ± 0.97 nmol/hr/ml. These results demonstrated that overexpression of the GAA enzyme did not result in the abnormal secretion of other lysosomal enzymes.

**Assessment of GAA Distribution in the GAA-KO Mouse.** After treatment with the AdhGAAΔpol vector, GAA-KO animals were killed, and tissues were analyzed for the presence of GAA activity. A pilot study demonstrated that, at 4 days post infection, GAA activity was detected in multiple muscle tissues but glycogen levels had only minimally begun to decrease (data not shown). By 12 days post-infection, GAA activity levels in the quadriceps, gastrocnemius, diaphragm, and cardiac muscles of the Ad-treated GAA-KO animals were
all significantly elevated when compared with the GAA levels detected in untreated GAA-KO mice and were higher than the GAA levels detected in wild-type mice (Fig. 5). To confirm that the GAA activity detected in the muscles of treated animals was not caused by transcription of GAA mRNA in the muscles, total RNA derived from various tissues were analyzed (Fig. 6). GAA-specific RNA transcripts were detected only in the liver and not in either the heart or quadriceps muscles of AdhGAAΔpol-treated GAA-KO mice, despite the fact that the latter tissues contained high levels of GAA activity. Note that, in a wild-type mouse, GAA mRNA was readily detected in these muscle tissues, despite the fact that the enzyme activities in these tissues were lower than that detected in the AdhGAAΔpol-treated animal. Immunoblot analysis of protein extracts derived from the liver, cardiac, and quadriceps muscles of the Ad-treated GAA-KO mice also demonstrated that only the liver had high levels of the precursor form of GAA present whereas the cardiac and quadriceps muscles did not (data not shown). Based on these observations, we concluded that most of the GAA activity detected in the muscle tissues of the AdhGAAΔpol-treated animals was derived exogenously; i.e., via uptake of precursor GAA secreted by the liver.

Systemic Reversal of Muscle Glycogen Accumulation in GAA-KO Mice After AdhGAAΔpol Therapy. We wished to confirm that the GAA enzyme activities detected in the muscles of the Ad-treated GAA-KO mouse resulted in correct lysosomal targeting of the enzyme. Previous studies have demonstrated that exogenously administered precursor GAA can target to lysosomes and act to reduce intralysosomal glycogen accumulation in both the acid maltase-deficient quail and the GAA-KO mouse (2, 3). Because lysosomal glycogen accumulation is the primary correlate of disease severity in the GAA-KO mouse, correct lysosomal targeting of the hepatocyte-secreted GAA should result in a reduction of glycogen accumulation in a number of muscles in the GAA-KO model. In situ periodic acid-Schiff staining for glycogen in multiple muscles of untreated and AdhGAAΔpol vector-treated GAA-KO mice were therefore evaluated (Fig. 7). In each of the respective muscle tissues, both granular and diffuse forms of glycogen accumulation (these staining patterns represented accumulation of glycogen in lysosomes and cytoplasm, respectively) were significantly reduced in the AdhGAAΔpol vector-treated GAA-KO mice in comparison to the staining observed in untreated GAA-KO mice. This result was confirmed after quantification of total intracellular glycogen levels in a variety of tissues in the treated mice (Table 1). The heart and diaphragm muscles appeared to be especially responsive to the AdhGAAΔpol treatment, an important observation considering that cardiac and/or respiratory muscle involvement is the primary cause of mortality in the various forms of GSD-II. Of note, exogenous administration of a breast milk-derived form of GAA did not reduce glycogen accumulation in the heart muscle (as well as other muscle groups) until 6 months after initiation of therapy (8). Based on these results, we confirmed that the increased GAA activities noted in the multiple muscles of AdhGAAΔpol vector-treated GAA-KO mice resulted in lysosomal targeting of the enzyme and a rapid phenotypic correction of the primary defect in GSD-II: namely, significant reductions in muscle cell glycogen accumulation.

**DISCUSSION**

GSD-II is a lysosomal storage disorder that manifests as a progressive myopathy secondary to massive amounts of glycogen accumulation in the skeletal and/or cardiac muscles of affected individuals. Because GSD-II is a systemic muscle disorder, intramuscular gene transfer directly into each of the affected muscles has been forwarded as a possible modality for treatment. Unfortunately, this method has been demonstrated to only allow for localized GAA expression (10, 11). Clinical efficacy of gene therapy for GSD-II will only be realized if the simultaneous correction of the multiple muscle groups affected by GSD-II can be attained. In this report, we demonstrated that the latter can potentially be achieved by capitalizing both on the normal attributes of Ad-based vectoring systems and the lysosomal targeting of recombinant lysosomal enzymes.

Specifically, hepatic transduction with an [E1-,polymerase-, E3-] Ad-based vector encoding hGAA resulted in the high level secretion of the precursor form of the GAA enzyme into the circulatory system. Subsequently, peripheral uptake of the precursor GAA enzyme by multiple muscle groups resulted in
decreased glycogen accumulation throughout the affected muscles of the GAA-KO mouse. This therefore demonstrates that a simple, single intravenous administration of an Ad vector can result in the systemic correction of a muscle disorder. Future studies should determine whether the disappearance of muscle glycogen also will result in improved overall muscle function. Lessons learned from the treatment of a muscle disorder such as GSD-II also may have direct implications for other muscle disorders, such as the various muscular dystrophies.

This model can potentially be expanded to include the treatment of other disorders involving other lysosomal enzymes as well. We have not, however, evaluated whether hepatically secreted lysosomal enzymes can penetrate the blood–brain barrier. This possibility also will need to be evaluated, considering that many lysosomal storage disorders (including GSD-II) result in abnormal substrate accumulation in the central nervous system.

The Ad vectoring systems offer several key advantages that makes their use in the treatment of GSD-II highly desirable. Specifically, Ad vectors can infect terminally differentiated, nondividing tissues and can allow for long-term expression of a number of genes in a variety of tissues (including liver, skeletal, and cardiac muscle) for periods of up to 1 year, even in immune competent animals. Uniquely, high titer Ad vector preparations can transduce 100% of the liver hepatocytes after a single intravenous injection, an advantage that cannot be approximated by any other vectoring system currently being investigated, including adeno-associated or lenti-virus-based vectoring systems.

Unfortunately, the major drawback of Ad-based vectors is their inability to sustain long term transgene expression after successful gene delivery into immune-competent animals. This limitation has been determined to be caused by a number of variables, including (i) what class of Ad vector is specifically used, (ii) the immunogenicity of the Ad encoded transgene,

### Table 1. Glycogen reduction in muscle tissues of AdhGAAΔpol-treated GAA-KO mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Glycogen content, μmol/mg</th>
<th>Wild-type*</th>
<th>GAA-KO†</th>
<th>AdhGAAΔpol‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.0078 ± 0.0130</td>
<td>2.4100 ± 0.9200</td>
<td>0.0032 ± 0.0045</td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0.0390 ± 0.0350</td>
<td>0.9300 ± 0.3300</td>
<td>0.1100 ± 0.1600</td>
<td></td>
</tr>
<tr>
<td>Quadriceps</td>
<td>0 ± 0</td>
<td>0.8800 ± 0.1200</td>
<td>0.1290 ± 0.0400</td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.0365 ± 0.0260</td>
<td>0.9300 ± 0.0700</td>
<td>0.1170 ± 0.1380</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.4600 ± 0.3800</td>
<td>0.2300 ± 0.0170</td>
<td>0.0206 ± 0.2000</td>
<td></td>
</tr>
</tbody>
</table>

*ₙₙ = 6
†ₙₙ = 3.
‡ₙₙ = 3.

FIG. 7. Periodic acid-Schiff detection of glycogen in situ. Cryosections from 2.5-month-old mouse tissues were periodic acid-Schiff stained for glycogen as described in Materials and Methods. Source of the tissue sections are as follows: (a) GAA-KO, gastrocnemius, untreated. (b) GAA-KO, gastrocnemius, 12 days post-AdhGAAΔpol. (c) GAA-KO, quadriceps, untreated. (d) GAA-KO, quadriceps, 12 days post-AdhGAAΔpol. (e) GAA-KO, diaphragm, untreated. (f) GAA-KO, diaphragm, 12 days post-AdhGAAΔpol. (g) GAA-KO, heart, untreated. (h) GAA-KO, heart, 4 days post-AdhGAAΔpol. (i) GAA-KO, heart, 12 days post-AdhGAAΔpol. (j) GAA-KO, heart, untreated. (k) Wild-type, heart, untreated.
(iii) the promoter used to drive transgene expression, and (iv) the animal model being tested. In this study, decreased amounts of GAA expression were likely caused by cytomegalovirus enhancer/promoter down-regulation events, a phenomenon previously noted to occur when Ad vectors persist in hepatocytes (15). Long term studies will be required to determine whether immune responses to the vector or the secreted protein also occur. These responses will likely be directed to immunogenic stimuli derived both from the epitopes encoded by the transgene and the genome of the Ad vector (22, 23).

Our group has addressed the latter drawback by construction of modified Ad vectors (used in this study) that can persist in vivo despite transduction of neoantigenic transgenes into immune-competent hosts (15). Several reports suggest that the utilization of modified Ad vectors may be mandatory for clinical use because traditional [E1-]-deleted Ad vectors can induce potent immune responses, even to self-antigens (22–24).

The demonstration that a single administration of an Ad vector can result in a systemic impact on the phenotype of the GAA KO mouse now opens the door to further explorations into the feasibility of gene therapy for this disorder. Future studies will evaluate questions that must be explored before this mode of therapy becomes clinical reality. These questions include (i) What is the duration of time that glycogen correction can be sustained? (ii) What types of immune responses are directed to either the vector and/or transgene product? And (iii) what are the direct effects of glycogen reduction on muscle functions in the animal models of GSD-II? Current research is focusing on these aspects of GSD-II gene therapy.

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