Deficiency of glucose-6-phosphatase (G6Pase), a key enzyme in glucose homeostasis, causes glycogen storage disease type Ia (GSD-Ia), an autosomal recessive disorder characterized by growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia. G6Pase is an endoplasmic reticulum-associated transmembrane protein expressed primarily in the liver and the kidney. Therefore, enzyme replacement therapy is not feasible using current strategies, but somatic gene therapy, targeting G6Pase to the liver and the kidney, is an attractive possibility. Previously, we reported the development of a mouse model of G6Pase deficiency that closely mimics human GSD-Ia. Using neonatal GSD-Ia mice, we now demonstrate that a combined adeno virus and adeno-associated virus vector-mediated gene transfer leads to sustained G6Pase expression in both the liver and the kidney and corrects the murine GSD-Ia disease for at least 12 months. Our results suggest that human GSD-Ia would be treatable by gene therapy.

INTRODUCTION

The most prevalent form of glycogen storage disease is type Ia (GSD-Ia), an autosomal recessive metabolic disorder with an incidence of 1 in 100,000 live births (reviewed in 1–3). This genetic disorder is caused by a deficiency in glucose-6-phosphatase (G6Pase), which is normally expressed at high levels in the liver and the kidney (4). G6Pase is a key enzyme in glucose homeostasis, catalyzing the hydrolysis of glucose 6-phosphate (G6P) to glucose and phosphate in the terminal steps of gluconeogenesis and glycogenolysis (4). Patients affected with GSD-Ia are unable to maintain glucose homeostasis and present with hypoglycemia, hepatomegaly, nephromegaly, growth retardation, hyperlipidemia, hyperuricemia, and lactic acidemia (1–3). Long-term complications include short stature, osteoporosis, gout, renal disease, pulmonary hypertension and hepatic adenomas that may undergo malignant transformation. In the past, many unrecognized and untreated GSD-Ia patients died in early infancy from profound hypoglycemia and acidosis (2). The current treatment of GSD-Ia is a dietary therapy designed to control symptomatic hypoglycemia. Patients receive continuous nasogastric infusion of glucose (5) or frequent oral administration of uncooked cornstarch (6). However, this treatment is intrusive, and the efficacy of dietary treatment is frequently limited due to poor compliance. While the dietary therapy does improve growth and metabolic abnormalities manifested by these patients and delays the development of renal diseases (7), the underlying pathological process remains untreated. As a result, long-term complications develop in adult patients. Almost all GSD-Ia patients who are more than 20 years of age manifest kidney complications, including proteinuria (8–10). Many also have hypertension, renal stones, nephrocalcinosis, altered creatinine clearance and, eventually, renal failure.

G6Pase is a highly hydrophobic protein anchored to the endoplasmic reticulum (ER) by nine transmembrane helices (11,12). The protein cannot be expressed in a soluble form and must embed correctly in the ER membrane and couple with other proteins to be functional (1–3). Therefore, enzyme
replacement therapy is not an option, but somatic gene therapy, targeting G6Pase to the liver to the kidney, is an attractive possibility. To develop novel therapies for GSD-Ia, we had previously generated G6Pase-deficient (G6Pase−/−) mice that manifest a metabolic profile and phenotype virtually identical to that of human GSD-Ia patients (13). If left untreated, the mice rarely survive weaning and live no more than 3 months, mimicking the lethality of the untreated human disorder. Indeed, the only significant difference is the absence of hyperlactacidemia in the G6Pase−/− mice (13). Previous studies evaluating the feasibility of gene replacement therapy for GSD-Ia using an adeno-associated virus carrying the mouse G6Pase cDNA (Ad-mG6Pase) (14). A single intravenous infusion of Ad-mG6Pase into the G6Pase−/− mice at 2 weeks of age restored 19% of normal hepatic G6Pase activity, improved survival and growth of GSD-Ia mice, and transiently corrected the metabolic abnormalities manifested by these mice. In most systems, however, adenovirus-mediated transgene expression is transient, and the recipient develops inflammation in the organ expressing the transgene (15, 16). Moreover, viral antigen synthesis triggers cytotoxic lymphocyte-mediated clearance of the recipient cells and leads to neutralizing antibodies that block efficient re-administration of the recombinant virus.

Recombinant adeno-associated virus (rAAV) has several unique characteristics that make it a more promising choice for gene therapy than adenovirus (17–20). First, wild-type AAV causes no known disease in humans. Second, the rAAV vectors lack the viral coding sequences and cannot produce potentially antigenic viral proteins. Third, rAAV efficiently transduces non-dividing cells and leads to minimal immune response to the vector capsid or the transduced cell. Finally, rAAV-mediated gene transfer directs efficient, stable transgene expression in the liver, and is associated with little inflammation or cellular immune response (21–25).

In this study, we show that an intravenous co-infusion of AAV-mG6Pase and Ad-mG6Pase into neonatal G6Pase−/− mice effectively delivers the G6Pase transgene to the liver and the kidney, and produces a functional G6Pase system that normalizes metabolic abnormalities and achieves long-term correction of the GSD-Ia disorder in mice.

RESULTS

Neonatal infusion of Ad-mG6Pase improves the survival of G6Pase−/− mice

The most critical clinical presentation in GSD-Ia is the life-threatening hypoglycemia. Using glucose therapy, we have improved the 20 day survival rate of G6Pase−/− mice from <1% to ~60% (14). However, glucose therapy cannot sustain the life of these mice much beyond weaning (21 days), and less than 15% of G6Pase−/− mice live past 4 weeks. Even when maintained on glucose therapy, the weaned G6Pase−/− mice continue to suffer from frequent hypoglycemic seizures, and few live to 3 months of age. Because of this, age-matched G6Pase−/− mouse controls are not always available for comparison with the G6Pase−/− mice treated by gene therapy.

In a previous study, using the recombinant adeno-viral vector Ad-mG6Pase, which expresses mouse G6Pase from a constitutive Rous Sarcoma virus (RSV) promoter, we showed that all 2-week-old G6Pase−/− mice infused with 2 × 10⁵ plaque-forming units (p.f.u.) of Ad-mG6Pase survived weaning and lived to at least 4 weeks of age (14). The transgene was expressed in the liver and transiently corrected the metabolic abnormalities manifested by the GSD-Ia mice. However, the Ad-mG6Pase did not transduce the kidney.

We have now extended that study to examine the efficacy of a neonatal low-dose Ad-mG6Pase infusion. All of the G6Pase−/− mice that received 4 × 10⁵ p.f.u./animal of Ad-mG6Pase survived weaning. Following weaning, however, they suffered frequent hypoglycemic seizures, even with supplemental glucose therapy. Of the 10 weaned mice, five died at 5–6 weeks, three died at 8–12 weeks, and two died at 14 weeks.

Three days post-infusion, hepatic microsomal G6Pase activity in the neonatally Ad-mG6Pase-infused G6Pase−/− mice had risen to 15.8% of normal (G6Pase+/+) levels (Table 1) but subsequently declined rapidly to near-background levels by 4 weeks post-infusion. Again, no G6Pase expression was detected in the kidneys of the infused animals (Table 1).

In the untreated G6Pase−/− mice, the lack of G6Pase activity is reflected by hypoglycemia (Table 1). In contrast, neonatal infusion of G6Pase−/− mice with Ad-mG6Pase normalizes

<table>
<thead>
<tr>
<th>Mice</th>
<th>Post-infusion (days)</th>
<th>Liver G6Pase activity (nmol/min/mg)</th>
<th>Kidney G6Pase activity (nmol/min/mg)</th>
<th>Glucose (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Uric acid (mg/dl)</th>
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<tr>
<td>+/- and +/-</td>
<td>3</td>
<td>303.9 ± 29 (100)</td>
<td>98.4 ± 2.2</td>
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<td>+/-</td>
<td>3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>58 ± 4</td>
<td>271 ± 61</td>
<td>349 ± 76</td>
<td>8.0 ± 0.6</td>
</tr>
<tr>
<td>+/-/Ad-mG6Pase</td>
<td>3</td>
<td>48.1 ± 0.7 (15.8)</td>
<td>&lt;0.1</td>
<td>190 ± 6</td>
<td>268 ± 38</td>
<td>223 ± 14</td>
<td>5.6 ± 0.3</td>
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<td>7–28</td>
<td>175.1 ± 8.9 (100)</td>
<td>145.6 ± 17</td>
<td>&lt;0.1</td>
<td>71 ± 5</td>
<td>301 ± 47</td>
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<td>&lt;0.1</td>
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<td>+/-</td>
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<td>&lt;0.1</td>
<td>58 ± 7</td>
<td>94 ± 13</td>
<td>62 ± 7</td>
<td>2.5 ± 0.2</td>
</tr>
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</table>

The background G6Pase activity (nmol/min/mg) in liver (5.1 ± 1.1) or kidney (6.6 ± 0.61) microsomes of untreated G6Pase−/− (−/−) mice has been subtracted from the activity reported here. Data are presented as mean ± SEM. Each value represents the mean of 4 animals, except the values of 7–28-day-old G6Pase+/+ and G6Pase−/− (+/+) and (−/−) mice, which represent the mean of 12 animals. Numbers in parentheses represent percentage of G6Pase+/+ and G6Pase−/− activity.
plasma glucose levels from day 3 to day 14 post-infusion (Table 1). Interestingly, by day 14, while only 3.9% of the normal G6Pase activity is present in the liver, the mice retain normoglycemia. However, when G6Pase activity in the infused liver decreases to the background level at 4 weeks post-infusion, the treated G6Pase−/− mice become hypoglycemic.

Both the Ad-mG6Pase-treated and untreated GSD-Ia mice have elevated levels of plasma cholesterol, triglyceride and uric acid during the first 2 weeks of life. These profiles improve for both groups between the ages of 2 and 4 weeks. By 4 weeks, the plasma profiles of the infused mice are near normal, while those of the untreated G6Pase−/− mice remain elevated.

**Ad/AAV-mG6Pase mediates stable G6Pase expression in the liver and the kidney**

An alternative, highly promising gene delivery vector is rAAV. However, neonatal infusion of an rAAV carrying the murine G6Pase cDNA under the control of a β-actin promoter (AAV-mG6Pase) fails to improve the survival of G6Pase-deficient mice during weaning. This failure is most likely due to the delayed kinetics of rAAV-mediated transgene expression, which typically peaks 5–7 weeks post-infusion (26,27). To compensate for this, we coinjected Ad-mG6Pase with AAV-mG6Pase with the intention that the Ad-mG6Pase would provide survival through weaning. After this, it was anticipated that the AAV-mG6Pase-mediated G6Pase expression levels would be sufficiently high to support longer-term survival. Because the size of the neonate restricts the dose volume, the viruses were given as two doses—10⁹ infectious units (IU) of AAV-mG6Pase and 4 × 10⁷ p.f.u. of Ad-mG6Pase, via the temporal vein, at birth, and an additional intravenous infusion of 5 × 10⁹ IU of AAV-mG6Pase at 2 weeks of age. None of the 10 infused animals died prematurely. All six mice not sacrificed for early analysis lived for at least 5 months and until sacrificed deliberately. This is in marked contrast to the survival rates of G6Pase−/− mice on glucose therapy alone. Since the generation of the G6Pase−/− mouse strain in 1995, only five among the 150–200 such animals have lived to 3 months and only one has survived for 4 months.

G6Pase−/− mice under glucose therapy are growth retarded, and by 2 weeks of age their average body weight is ~60% of that of their wild-type or heterozygote littermates (14). After neonatal Ad/AAV-mG6Pase infusion, the G6Pase−/− mice have a markedly improved growth rate, and the body weights of the infused animals are comparable to those of G6Pase+/+ and G6Pase+/− littermates at 6 weeks post-infusion (Fig. 1).

The expression of G6Pase was evaluated in the liver and the kidney of Ad/AAV-G6Pase-infused animals and compared to that of age-matched wild-type or heterozygote littermates and 4- to 6-week-old G6Pase−/− mice. The G6Pase−/− mice supported only by glucose therapy have no G6Pase activity.

Figure 1. Postnatal development of G6Pase+/+ and G6Pase−/− mice as well as Ad/AAV-mG6Pase-infused G6Pase−/− mice. Each point represents a minimum of four animals, except for the data between 8.5 and 12 months for the Ad/AAV-mG6Pase-infused G6Pase−/− mice, which represent the mean of two animals. Data are presented as mean ± SEM. Open circle, G6Pase+/+ and G6Pase+/− mice; closed circle, Ad/AAV-mG6Pase-infused G6Pase−/− mice.
In contrast, at 5 months post-infusion, the Ad/AAV-directed G6Pase activity in the liver was 33.1% of normal activity (Table 2). The high levels of hepatic G6Pase activity persisted for 8.5 months before declining to 22.7% of normal levels by 12 months post-infusion.

In contrast to Ad-mG6Pase-mediated gene transfer, Ad/AAV-mG6Pase also delivered a functional G6Pase transgene to the kidney (Table 2). Renal G6P phosphohydrolase activity in the infused animals mirrored that in the liver, reaching as high as 36.2% of normal levels at 5 months post-infusion and persisting for 8.5 months (Table 2). However, between 8.5 and 12 months, the G6Pase activity declined more rapidly in the kidney than in the liver, decreasing to 13.8% of normal levels at 12 months post-infusion (Table 2).

The distribution of the G6Pase expression in the liver and kidney was also investigated. In G6Pase+/+ and G6Pase−/− mice, G6Pase mRNA is distributed uniformly throughout the liver hepatocytes, but restricted to the cortex of the kidneys (28). This pattern of expression was confirmed by enzyme histochemical analyses (Fig. 2A). As expected, there was no stainable G6Pase activity in liver or kidney sections of untreated G6Pase−/− mice (Fig. 2A). For the Ad/AAV-mG6Pase-treated G6Pase−/− mice, the entire liver and kidney stained positive 8.5 months post-infusion. Consistent with the quantitative phosphohydrolase assays (Table 2), the staining was less intense than that of the G6Pase+/+ mice (Fig. 2A). This indicates that the Ad/AAV delivers the G6Pase transgene to the entire liver and kidney, and that non-cortical areas of the kidney are contributing to the total phosphohydrolase activities being measured.

Effective G6Pase activity in vivo is dependent upon coupling of the G6Pase enzyme to the membrane-anchored G6P transporter (G6PT) (2,3), which translocates G6P from the cytoplasm into the lumen of the ER for hydrolysis. We have shown previously that the transport of G6P into the hepatic and renal microsomes is markedly reduced in G6Pase−/− mice (13). We therefore evaluated the microsomal G6P uptake activity in the liver and kidney of the infused G6Pase−/− mice. Both the liver and kidney microsomes from G6Pase+/+ mice transport G6P efficiently (Table 2). In contrast, there is no significant G6P uptake by the liver or kidney microsomes of the untreated G6Pase−/− mice. In the liver of the Ad/AAV-mG6Pase-infused animals, microsomal G6P uptake activity was restored to 42.2–47.5% of normal levels for the duration of the 12-month study (Table 2). In the kidney, microsomal G6P uptake activity increased to ~48% of G6Pase+/+ and G6Pase+/− levels over 8.5 months, but declined, in parallel with the G6Pase activity, to 16.8% of the normal level by 12 months post-infusion (Table 2).

**Ad/AAV-mG6Pase infusion corrects pathological manifestations of GSD-Ia**

Under glucose therapy, G6Pase−/− mice manifest hypoglycemia, hyperlipidemia, and hyperuricemia (13). Although Ad/AAV-mG6Pase-infused G6Pase−/− mice were hypoglycemic at 1 month of age, their plasma glucose profiles normalized soon after (Fig. 2B). This normoglycemia was maintained in the Ad/AAV-mG6Pase-infused animals throughout their postnatal development. None of the mice suffered the frequent hypoglycemic seizures typical of the untreated G6Pase−/− mice and the weaned Ad-mG6Pase-infused G6Pase−/− mice. Moreover, like G6Pase+/+ and G6Pase+/− mice, the Ad/AAV-mG6Pase-infused G6Pase−/− mice maintained normoglycemia, even when deliberately starved for 2 h. While the Ad/AAV-mG6Pase-infused animals manifested hyperlipidemia and hyperuricemia at 2 weeks of age, their plasma cholesterol, triglyceride, and uric acid profiles were completely normal by 1 month of age, and this normality persisted for the duration of the 12-month study (Fig. 2B). The extent to which this normalization was mediated by the Ad/AAV-mG6Pase is not completely clear. Over the first 4 weeks, when age-matched controls were still available, there was some natural improvement in the plasma cholesterol, triglyceride, and uric acid profiles of the untreated G6Pase−/− mice (Table 1), even though they still remained significantly higher than the levels of the G6Pase+/+ and G6Pase+/− as well as the infused G6Pase−/− mice. Despite improvements in these metabolic markers, however, the untreated G6Pase−/− mice died prematurely, providing further evidence that these defects are secondary to the hypoglycemia.

Hepatomegaly is another clinical presentation in GSD-Ia mice, resulting from excessive glycogen accumulation in the liver. At 5 months (data not shown) and 8.5 months (Fig. 3) post-Ad/AAV-mG6Pase infusion, the transduced liver tissue sections show no histological abnormality, except for mild glycogen accumulation slightly less than glycogen deposition found in the liver of 1-week-old untreated G6Pase−/− mice (Fig. 3). Interestingly, 12 months post-infusion, hepatic glycogen storage in the infused G6Pase−/− mice is almost
indistinguishable from that of wild-type mice (Fig. 3). Since the hepatic G6Pase activity in the 12-month-old infused animals is only 22.7% of normal (Table 2), this suggests that complete restoration of hepatic G6Pase activity is not necessary to maintain glucose homeostasis in the body.

Nephromegaly is also a clinical manifestation of human and mouse GSD-Ia. There is marked glycogen deposition in the kidney tubular epithelial cells of 1-week-old untreated G6Pase−/− mice, resulting in their enlargement and compression of the glomeruli (Fig. 4). In contrast, both 5 months (data not shown) and 8.5 months (Fig. 4) post-Ad/AAV-mG6Pase infusion, the transduced kidney tissues of G6Pase−/− mice showed no histological abnormality, except for glycogen accumulation, which is less than that observed in the kidney of the 1-week-old untreated GSD-Ia mice (Fig. 4).

In the longer term, it appears that the 13.8% of normal renal G6Pase activity remaining (Table 2) cannot prevent the advancement of kidney disease. Twelve months post-infusion, glycogen accumulation increased in both the cortex and the medulla of the kidney, and was accompanied by the appearance of histological abnormalities in the kidney, including glomerular sclerosis and tubular dilatation (Fig. 4). On the other hand, plasma creatinine, an index of altered renal function (29), remained normal in Ad/AAV-mG6Pase-infused animals even at 12 months of age (Table 2).

**Absence of immune response against murine G6Pase**

To determine whether a humoral response directed against murine G6Pase is generated in the infused G6Pase−/− mice, we performed western blot analysis using sera from mice treated with Ad/AAV-mG6Pase. As a positive control, we used a rabbit anti-G6Pase antiserum raised against human G6Pase. This antiserum reacts well with both human (Fig. 5A, lane 1) and...
murine (Fig. 5A, lane 2; Fig. 5B, lane 1) G6Pase. Western blot analysis showed that anti-murine G6Pase antibodies were not detected in Ad/AAV-mG6Pase-infused G6Pase−/− mice 5 (Fig. 5B, lanes 5 and 6), 8.5 (lanes 8 and 9) and 12 (lanes 11 and 12) months post-infusion. Furthermore, there was no endogenous anti-murine G6Pase antibody present in the sera of G6Pase+/+ and G6Pase+/−/+ littermates (lanes 4, 7 and 10) or untreated G6Pase−/− mice (lanes 2 and 3).

DISCUSSION

G6Pase is a key enzyme in glucose homeostasis expressed primarily in the gluconeogenic organs—the liver and the kidney (4). The enzyme is a highly hydrophobic glycoprotein anchored to the ER by nine transmembrane helices (11,12). The catalytic center of G6Pase lies within the lumen of the ER (11). For G6Pase catalysis, the G6P substrate present in the cytoplasm must be translocated into the lumen of the ER by another transmembrane protein, G6PT (2,3). Together, G6Pase and G6PT comprise the G6Pase system. Both proteins are coupled functionally. In the GSD-Ia mice, the homozygous destruction of the G6Pase gene leads not only to the loss of G6Pase activity, but also to the marked reduction in hepatic and renal microsomal G6P uptake activity (13). Therefore, to cure the GSD-Ia disorder, a functional, coupled, G6Pase system must be restored in both the liver and kidney. In this study, we examine the effects of a gene therapy consisting of a neonatal co-infusion with AAV-mG6Pase and Ad-mG6Pase, followed by a second infusion of AAV-mG6Pase at 2 weeks of age. This strategy corrected the murine GSD-Ia disorder by delivering the transgene to both the liver and kidney, resulting in the sustained expression of a complete, functional, G6Pase system. Moreover, the Ad/AAV-mG6Pase-infused animals grow normally, with only mild glycogen accumulation in the liver and the kidney. They live to at least 12 months of age, and exhibit normal plasma glucose, cholesterol, triglyceride and uric acid profiles. In contrast, the untreated G6Pase-deficient mice manifest growth retardation, hepatomegaly, nephromegaly, hyperlipidemia and hyperuricemia, and rarely survive longer than 2–3 months because of their inability to maintain glucose homeostasis.

It is known that rAAV-mediated transgene expression increases slowly, typically peaking 5–7 weeks post-infusion (26,27). Therefore, it is not surprising that neonatal infusion of AAV-mG6Pase alone failed to normalize hypoglycemia or sustain the life of G6Pase−/− mice beyond weaning. Since adenovirus-mediated transgene expression from a double-stranded template is more rapid in vivo, and mice inoculated neonatally with adenovirus by the intravenous route do not mount typical humoral or cellular immune responses to the viral proteins (30), we tried co-infusion of neonatal G6Pase−/− mice with AAV-mG6Pase and a low dose of Ad-mG6Pase. The co-infused mice not only survived weaning but also tolerated a second AAV-mG6Pase infusion. Another recent study of neonate infusion with a recombinant adenovirus carrying the argininosuccinate synthetase cDNA also reported amelioration of neonatal crisis in argininosuccinate synthetase-deficient mice without immune complications (31). In this case, a second viral infusion was also tolerated. Humoral immune responses to the transgene product represent another important concern for any gene therapy. The lack of production of the anti-mG6Pase antibodies in the neonatal-infused animals was a promising result.

Figure 3. Glycogen content and histological analyses of the liver from the Ad/AAV-mG6Pase-infused G6Pase−/− mice. Plates show hematoxylin and eosin-stained liver sections at magnifications of 100× (upper panels) and 200× (lower panels). Glycogen contents, expressed as µmol glucosyl residues per gram wet weight of tissue, are shown below each plate. Note the decrease in glycogen deposition in G6Pase−/− mice 12 months post-Ad/AAV-mG6Pase infusion.
While the successful strategy involved a co-infusion of AAV-mG6Pase with Ad-mG6Pase, several lines of evidence suggest that the correction of GSD-Ia in mice is mediated primarily by AAV-mG6Pase, not Ad-mG6Pase. First, neonatal Ad-mG6Pase infusion does provide sufficient hepatic G6Pase activity to allow the infused G6Pase−/− mice to survive weaning. However, the Ad-mG6Pase-infused G6Pase−/− mice typically survive only 5–6 weeks, and none have survived beyond 14 weeks, even with additional glucose therapy. Second, the Ad-mG6Pase-mediated G6Pase expression is short-lived, and by 4 weeks post-infusion neither hepatic nor renal G6Pase expression is observed in Ad-mG6Pase-infused animals. Third, sustained G6Pase expression in both the liver and the kidney is achieved only in the Ad/AAV-mG6Pase-infused G6Pase−/− mice. Only the Ad/AAV-mG6Pase-infused G6Pase−/− mice have shown the ability to survive for 12 months without premature deaths. Therefore, AAV-mG6Pase seems essential for long-term correction of G6Pase deficiency. At this stage, because of the premature lethality of the G6Pase−/− phenotype, and the need for a G6Pase activity to survive weaning, it is not possible to test the efficacy of AAV-mG6Pase alone. The initial intent was to use Ad-mG6Pase as a short-term source of G6Pase activity to support weaning. However, it is possible that the efficacy of the Ad/AAV-mG6Pase-mediated gene therapy arises from a unique expression system created by a recombination/interaction between the Ad and AAV vectors. These aspects remain to be examined further.

To correct the GSD-Ia deficiency, liver and kidney expression of G6Pase is required. Liver expression of rAAV-directed genes is well established (21–25). There is one report showing that rAAV can mediate reporter gene expression in kidney tubular epithelial cells injected in vivo via the intraparenchymal route (32). However, there is no report of an rAAV vector delivering a functional gene into the kidney. A particularly interesting finding in this study is the successful delivery of the G6Pase gene to both the liver and kidney and the subsequent reconstitution of a complete G6Pase system in both tissues. Sustained hepatic and renal G6Pase expression was maintained over 8.5 months. The reason for the decline in G6Pase expression 12-months post-Ad/AAV-mG6Pase infusion is not known, but the elimination of transduced cells by a cell-mediated immune response to the viral proteins is one possible explanation.

We observed no histological abnormality in the transduced liver tissues throughout the 12-month study. The Ad/AAV-mG6Pase-infused animals had normal plasma glucose, cholesterol, triglyceride and uric acid profiles, indicating that glucose homeostasis could be maintained by restoring ~23% of normal G6Pase activity in the liver. In the kidney, the gene initially restored normal histology. This was slowly replaced by an abnormal accumulation of glycogen, and kidney disease did start to develop 12 months post-infusion, even with 14% of normal renal G6Pase activity remaining. In wild-type mice, G6Pase activity is restricted to the kidney cortex. In the Ad/AAV-mG6Pase-infused mice, the activity was uniformly distributed throughout the kidney. These results suggest that normal kidney function requires more than 14% of wild-type G6Pase activity, or at least more activity within the cortex than provided in the current study. Our findings do explain, however, the previously perplexing observation that most adult human GSD-Ia patients with good glucose homeostasis, maintained by dietary therapy, develop kidney complications (1).
The histological abnormalities identified in the kidney of the infused animals, if untreated, will undoubtedly compromise kidney function, leading to severe renal dysfunction in the longer term. Several strategies could be considered to prevent or delay the development of kidney disease. One strategy would be repeated AAV-mG6Pase infusions. This is promising, because so far there have been no signs of humoral immune responses to the transgene in the infused mice. Indeed, we have observed that the mice can tolerate at least three AAV-mG6Pase infusions without an immune consequence. A second strategy would be to elevate the level of G6Pase transgene expression in the liver and the kidney. This can be accomplished either by increasing the dosage of AAV-mG6Pase or by using AAV of a different serotype (33,34). Co-infusion with Ad-mG6Pase might be unnecessary if sufficient G6Pase expression can be restored in the liver as early as 3 weeks post-infusion. Another strategy would be to combine the two mentioned above—repeated infusions with higher doses or different serotypes.

In human gene therapy, the early-expression issues may be less problematic. In mice, a strategy to survive weaning is critical. In the human disease, dietary therapy consisting of nasogastric infusion of glucose or frequent oral administration of uncooked cornstarch has achieved a near 100% survival rate for GSD-Ia patients into the early teens. Thus, a practical approach may be a combination of AAV-mG6Pase infusion, supported by dietary therapy, until expression of the transgene is established.

In summary, using a murine model of GSD-Ia, we have demonstrated an Ad/AAV-mG6Pase-mediated gene therapy that results in stable, sustained expression of G6Pase. The strategy delivers the G6Pase transgene to both the liver and kidney and corrects the underlying metabolic disorder. Our results suggest that human GSD-Ia may be amenable to G6Pase gene therapy. The observation of viral kidney transduction may also offer an approach to gene therapy of renal diseases.

MATERIALS AND METHODS

Construction of AAV-mG6Pase

An rAAV vector, UF11-mG6Pase, containing murine G6Pase driven by the chicken β-actin promoter and the cytomegalovirus (CMV) enhancer (25), was generated by replacing the green fluorescent protein (GFP) cDNA in the rAAV vector, UF11-GFP, with the entire coding region of murine G6Pase cDNA. Recombinant virus (AAV-mG6Pase) was packaged by cotransfection of 293 cells with UF11-mG6Pase and a packaging/helper plasmid, pDG (35). The AAV-mG6Pase was obtained by freeze–thaw lysis of the transfected cells and virions purified by iodixanol gradient ultracentrifugation followed by heparin–agarose column chromatography (36). The vector preparations were quantified by dot–blot analysis for vector genome number (physical titer) and infectious center assay (functional or infectious titer). The ratio of physical particles to infectious particles was between 50 and 100 for the preparations used in this study.

Glucose therapy for G6Pase−/− mice

All animal studies were conducted under an animal protocol approved by the NICHD Animal Care and Use Committee. Starting within the first postnatal day, glucose therapy consisting of intraperitoneal injection of 25–100 µl of 10% glucose was administered every 12 h to the G6Pase−/− (+/+) littermates at age 5 months (lane 4), 8.5 months (lane 7) and 12 months (lane 10).
given unrestricted access to Mouse Chow (Zeigler Bros., Inc., Gardners, PA, USA).

**Infusion of G6Pase−/− mice with Ad-mG6Pase**

Neonatal (1- or 2-day-old) G6Pase−/− mice were infused via the temporal vein with 30 μl of a virus stock containing 4 × 10⁷ p.f.u. of Ad-mG6Pase. The construct is a second-generation recombinant adenovirus containing the viral E1 deletion and temperature-sensitive E2A/C0 mutations with the murine G6Pase cDNA under the control of the RSV promoter (14). Age-matched G6Pase−/− as well as G6Pase+/+ and G6Pase−/+ mice were used as controls.

**Infusion of G6Pase−/− mice with Ad/AAV-mG6Pase**

The volume of the virus that can be administered to a neonatal mouse is restricted by the size of the mouse. It was anticipated that ~3–5 × 10⁶ IU of AAV-mG6Pase per neonate was required to obtain an effective level of G6Pase in the liver. Currently, it is not feasible to generate a viral stock of sufficiently high titer to infuse this number of virions, in a single dose, to a neonate. Instead, we chose to infuse two doses—one in the neonatal period and a second infusion when the animals reached age 2 weeks.

Neonatal (1- or 2-day-old) G6Pase−/− mice were infused via the temporal vein with 30 μl of recombinant viruses containing 10⁷ IU of AAV-mG6Pase and 4 × 10⁷ p.f.u. of Ad-mG6Pase. A second infusion of 5 × 10⁶ IU of AAV-mG6Pase was administered via the retro-orbital vein when the Ad/AAV-mG6Pase-infused animals reached age 2 weeks. Age-matched G6Pase−/− and G6Pase+/+ as well as 4-6-week-old G6Pase−/− mice were used as controls. For the infused mice, glucose therapy was terminated at weaning but Mouse Chow was available ad libitum.

**Phosphohydrolase and G6P uptake assays**

Microsomal preparations and phosphohydrolase assays were performed as described previously (13). Appropriate amounts of microsomal proteins were incubated at 30°C for 10 min in reaction mixtures (100 μl) containing 50 mM sodium cacodylate buffer, pH 6.5, 10 mM G6P, and 2 mM EDTA. Disrupted microsomal membranes were prepared by incubating intact membranes in 0.2% deoxycholate for 20 min at 0°C. Non-specific phosphatase activity was estimated by preincubating microsomal preparations at pH 5 for 10 min at 37°C, a condition that inactivates the thermolabile G6Pase (15).

Enzyme histochemistry of G6Pase (37) was carried out by incubating tissue sections (10 μm) for 10 min at room temperature in a solution containing 40 mM Tris-maleate, pH 6.5, 10 mM G6P, 300 mM sucrose, and 3.6 mM lead nitrate. The trapped lead phosphate was visualized following conversion to the brown-colored lead sulfide.

G6P uptake measurements were performed as previously described (13). Briefly, microsomes (40 μg) were incubated at 30°C for 3 min in a reaction mixture (100 μl) containing 50 mM sodium cacodylate buffer, pH 6.5, 250 mM sucrose, and 0.2 mM [U-14C]G6P (50 μCi/μmol). The reaction was stopped by filtering immediately through a nitrocellulose filter (BA85, Schleicher & Schuell, Keene, NH, USA). Microsomes permeabilized with 0.2% deoxycholate, to abolish G6P uptake, were used as negative controls.

**Phenotype analysis**

Mouse plasma samples were collected by orbital bleeding as previously described (13). Glucose levels were determined using a glucometer, and plasma triglycerides, total cholesterol, uric acid and creatinine levels were analyzed using kits obtained from Sigma Diagnostics (St. Louis, MO, USA).

For hematoxylin and eosin staining, tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4–6 μm.

To determine the glycogen content of the liver and kidney, tissue was homogenized with HCl, boiled for 10 min, and neutralized with sodium acetate to a final pH of 4.5 (38). The hydrolyzed tissue was then digested with amylo-α-1,4-α-1,6-glucosidase (38), and the released glucose was measured enzymatically using the Glucose No. 115 kit from Sigma Diagnostics. Glycogen content is reported as μmol glucosyl residues per gram wet weight of tissue.

**Antibody assays**

Antibodies against murine G6Pase were detected by western blot analysis. Microsomal proteins from Ad-mG6Pase-infected CRE8 cells were resolved by electrophoresis through a 12% polyacrylamide–SDS gel and trans-blotted onto polyvinylidene fluoride membranes (Millipore Co., Bedford, MA, USA). The membrane was placed in a Multiscreen Apparatus (Bio-Rad, Hercules, CA, USA) containing multiple channels. The membrane strip under each channel was incubated with either a rabbit anti-human G6Pase antiserum at 1 : 3000 dilution or serum samples from Ad/AAV-mG6Pase-infused animals at 1 : 200 dilution. Serum samples from untreated G6Pase−/− mice and G6Pase+/+ littermates at 1 : 200 dilution were used as controls. After overnight incubation, the membrane strips were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The immunocomplex was visualized by the chemiluminescent system using the SuperSignal West Pico Chemiluminescent substrate from Pierce (Rockford, IL, USA).

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**REFERENCES**


