Glycogen storage disease type 1a (GSD-1a), characterized by hypoglycemia, liver and kidney enlargement, growth retardation, hyperlipidemia, and hyperuricemia, is caused by a deficiency in glucose-6-phosphatase (G6Pase), a key enzyme in glucose homeostasis. To evaluate the feasibility of gene replacement therapy for GSD-1a, we have infused adenoviral vector containing the murine G6Pase gene (Ad-mG6Pase) into G6Pase-deficient (G6Pase−/−) mice that manifest symptoms characteristic of human GSD-1a. Whereas <15% of G6Pase−/− mice under glucose therapy survived weaning, a 100% survival rate was achieved when G6Pase−/− mice were infused with Ad-mG6Pase, 90% of which lived to 3 months of age. Hepatic G6Pase activity in Ad-mG6Pase-infused mice was restored to 19% of that in G6Pase+/+ mice at 7–14 days post-infusion; the activity persisted for at least 70 days. Ad-mG6Pase infusion also greatly improved growth of G6Pase−/− mice and normalized plasma glucose, cholesterol, triglyceride, and uric acid profiles. Furthermore, liver and kidney enlargement was less pronounced with near-normal levels of glycogen depositions in both organs. Our data demonstrate that a single administration of a recombinant adenoviral vector can alleviate the pathological manifestations of GSD-1a in mice, suggesting that this disorder in humans can potentially be corrected by gene therapy.

Glycogen storage disease type 1a (GSD-1a)1 is the most prevalent form of GSD-1, with an overall frequency of ~1 in 100,000 live births (1). It is an autosomal recessive disorder characterized by hypoglycemia, liver and kidney enlargement, growth retardation, hyperlipidemia, hyperuricemia, and lactic academia (1). These symptoms are caused by a deficiency in glucose-6-phosphatase (G6Pase), which catalyzes the terminal steps in gluconeogenesis and glycogenolysis by converting Glu-6-P to glucose and phosphate (2). G6Pase, a key enzyme in glucose homeostasis, is an endoplasmic reticulum (ER) membrane-spanning protein with its active site facing the lumen (2, 3). Therefore, the Glu-6-P substrate originating in the cytoplasm must be translocated across the ER membrane by the Glu-6-P transporter (G6PT) before hydrolysis can occur (4). To understand the biology and pathophysiology of GSD-1a and to develop gene therapy for this disorder, we have generated G6Pase-deficient (G6Pase−/−) mice by gene targeting (5). The GSD-1a mice manifest essentially the same clinical and pathological symptoms as human patients. The extensive similarities between homozygous mutant animals and human GSD-1a patients indicate that the G6Pase−/− mouse is a valid animal model for GSD-1a. Using these mice, we have shown that Glu-6-P transport and hydrolysis are tightly coupled events (5). Furthermore, we demonstrated that Glu-6-P uptake is mediated by the G6PT and that G6Pase activity facilitates Glu-6-P transport into the ER lumen by the G6PT protein (6).

Current treatments of GSD-1a are directed toward controlling symptomatic hypoglycemia (1). Patients receive continuous nasogastric infusion of glucose (1) or frequent oral administration of uncooked cornstarch (1, 7). Both treatments significantly alleviate the metabolic abnormalities of GSD-1a and greatly improve their prognosis. However, the underlying pathological process remains untreated, and as a result, long-term complications develop in adult patients. Moreover, the efficacy of dietary treatment is frequently limited due to poor compliance. One attractive alternative to dietary therapy for GSD-1a is somatic gene therapy, whereby a functional recombinant G6Pase gene is targeted to the affected organ in vivo.

Glucose homeostasis is primarily maintained by the balance between glycogenolysis and gluconeogenesis in the liver and to a lesser degree in the kidney, the two organs that primarily express the G6Pase gene (2). Therefore, the hepatocyte is the preferred target for G6Pase gene replacement therapies. Clinical experience supports this assessment. Kidney transplantation into GSD-1a patients, intended to correct renal failure (8, 9), was unsuccessful in alleviating hypoglycemia or hyperlipidemia. On the other hand, liver transplantation into GSD-1a patients has improved growth and corrected hypoglycemia and other biochemical abnormalities associated with the G6Pase deficiency (10, 11).

In this study, we used the G6Pase−/− mouse model to evaluate the feasibility of using a second-generation recombinant adenovirus (12, 13) to deliver the G6Pase gene to the liver. Our results indicate that the G6Pase gene can be successfully delivered to the liver, producing a functional G6Pase system that normalizes the metabolic abnormalities associated with GSD-1a and that greatly increases the survival of G6Pase−/− mice.

MATERIALS AND METHODS

Construction of Ad-mG6Pase—A mouse G6Pase gene-bearing recombinant adenovirus (Ad-mG6Pase) carrying the viral E1-deleted and temperature-sensitive tsA mutations was generated through homologous recombination in 293 cells between a cotransfected linearized transfer vector (pAV6-mG6Pase) and the large ClaI fragment of the
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adenoviral Ad5-ts125 genome (tsE2A) (12, 13). pAV6-mG6Pase transfer vector (14) contained the entire coding region (nucleotides 12–1814) of the murine G6Pase cDNA (15) under the control of the Rous sarcoma virus promoter. Ad-mG6Pase was grown and purified by CaCl₂ density ultracentrifugation as described (14).

**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 postnatal day 1, glucose therapy consisting of intraperitoneal injection of 25–100 μl of 10% glucose every 8 h was administered to the G6Pase⁻/⁻ mice. Mice that passed weaning were given glucose injections and unrestricted access to water containing polycose (5%) and mouse chow (Zeigler Bros., Inc., Gardners, PA) containing primarily ground corn, ground wheat, soybean meal, wheat middlings, vitamins, and minerals.

**Infusion of Mice with Ad-mG6Pase—** Fourteen-day-old G6Pase⁻/⁻ mice (5) were infused with 0.1 ml of Ad-mG6Pase containing 2 × 10⁹ plaque-forming units via the retro-orbital vein. As controls, we used G6Pase⁻/⁻ mice that received buffer alone and wild-type (G6Pase⁺/⁺) and heterozygous (G6Pase⁻/+ ) littermates. Glucose therapy was terminated immediately in Ad-mG6Pase-infused G6Pase⁻/⁻ mice, and the weaning mice were given mouse chow ad libitum.

**Phosphohydrolase and Glu-6-P Uptake Assays—** Microsomal preparations and phosphohydrolase assays were performed as described (6).

**Enzyme histochemistry of G6Pase (16) was carried out by incubating** polycose (5%) and mouse chow (Zeigler Bros., Inc., Gardners, PA) containing primarily ground corn, ground wheat, soybean meal, wheat middlings, vitamins, and minerals.

**RESULTS**

**Infusion of Ad-mG6Pase Markedly Improves the Survival of G6Pase⁻/⁻ Mice—** The most important initial clinical presentation of both human and mouse GSD-1a is life-threatening hypoglycemia. As a result, most of our G6Pase⁻/⁻ mice died within the first 14 days of life in the absence of any form of therapy. To improve the survival rate of the G6Pase-deficient mice, we administered 10% glucose intraperitoneally every 8 h, starting within postnatal day 1, and included polycose in the drinking water. The survival rate of these mice greatly improved, and ~60% of the G6Pase⁻/⁻ mice lived up to 20–21 days of age. Unfortunately, this mode of therapy cannot sustain the life of G6Pase⁻/⁻ mice beyond weaning (21 days), and only ~15% of the G6Pase-deficient mice lived to be 28 days old. We therefore used 14-day-old G6Pase⁻/⁻ mice in this study.

G6Pase⁻/⁻ mice were infused via the retro-orbital vein with 2 × 10⁹ plaque-forming units of a second-generation recombinant adenovirus (12, 13) containing the murine G6Pase cDNA under the control of the Rous sarcoma virus promoter (Ad-mG6Pase). Simultaneously, the glucose therapy was terminated. All of the 14-day-old G6Pase⁻/⁻ mice (n = 41) receiving Ad-mG6Pase infusion survived to 56 days of age. Therefore, the mice had a 100% survival rate up to 42 days post-infusion. Of the 10 animals that were followed up for 56–70 days post-infusion, only one animal died prematurely, generating a 90% 84-day survival rate of G6Pase⁻/⁻ mice under adenovirus-mediated gene therapy.

The survival of G6Pase⁻/⁻ mice under glucose therapy also markedly improved after weaning because mice tend to eat constantly and the mouse chow contains a high percentage of starch, the major component of a very successful dietary therapy in human GSD-1a patients (7). As a result, 56% of the weaned G6Pase⁻/⁻ mice lived to 42 days of age. However, only two control weaned G6Pase⁻/⁻ mice lived to 70 days of age, and only one to 84 days of age.

![Figure 1](image)
Hepatic G6Pase Expression—G6Pase is an ER membrane-associated enzyme with its active site facing the lumen (2, 3). Therefore, appropriate membrane insertion is required for activity. Moreover, the Glu-6-P substrate has to be translocated from the cytoplasm to the ER lumen before hydrolysis can occur (4). In earlier studies, we have demonstrated that Glu-6-P transport and hydrolysis are tightly coupled events and that G6Pase activity is required for efficient Glu-6-P transport into the ER by the G6PT (5, 6). Therefore, hepatic G6Pase expression was evaluated both by an increase in microsomal phosphohydrolase activity and by the activation of microsomal Glu-6-P transport.

G6Pase−/− mice were analyzed for expression of recombinant G6Pase by Northern and enzyme histochemical analyses of liver tissues before and after virus infusion. As expected, the G6Pase transcript was absent from the livers of control G6Pase−/− mice, but became detectable in the livers of Ad-mG6Pase-infused animals at 7–14 days post-infusion (data not shown). When livers from animals infused with Ad-mG6Pase for 14 days were sectioned and stained for G6Pase activity, the entire liver stained positive (Fig. 1A), albeit at a lower level than that of the G6Pase+/+ mice (Fig. 1C). In contrast, there was no detectable G6Pase activity in liver sections of age-matched control G6Pase−/− mice (Fig. 1A).

Glu-6-P phosphohydrolase activity in the liver reached as high as 19% of the activity in G6Pase+/+ mice at day 7 post-infusion (Table I). Hepatic G6Pase activity persisted for an additional 7 days before declining to 10.6% of control matched control G6Pase−/−, and G6Pase−/− mice at day 28 post-Ad-mG6Pase infusion (A). The error bars for the values indicated by open arrows and triangles and closed circles are merged within the symbols. B, time course of microsomal Glu-6-P uptake following Ad-mG6Pase infusion. The 3-min values of [U-14C]glucose accumulated in the intact microsomes of G6Pase−/− mice after Ad-mG6Pase infusion (△) are plotted against the time of post-Ad-mG6Pase infusion. Hepatic microsomal G6Pase activity (△) shown in Table I was included for comparison. Data are presented as the mean ± S.E.

Activation of Microsomal Glu-6-P Transport—Liver microsomes from G6Pase−/− mice transported Glu-6-P efficiently, indicating the presence of an intact G6Pase system (Fig. 2A). We have previously shown that G6Pase activity is required for efficient microsomal Glu-6-P uptake (5), and indeed, we observed no significant Glu-6-P uptake by liver microsomes of G6Pase−/− littermates (Fig. 2A). However, Ad-mG6Pase infusion activated microsomal Glu-6-P transport activity in the livers of G6Pase−/− mice (Fig. 2). It should be noted that despite a 19% restoration of hepatic G6Pase activity in G6Pase−/− mice at days 7–14 post-infusion, hepatic microsomal Glu-6-P transport activity at day 14 (28-day-old mice) was no detectable G6Pase activity in liver sections of age-matched control G6Pase−/− mice. G6Pase by Northern and enzyme histochemical analyses of liver tissues before and after virus infusion. As expected, the G6Pase transcript was absent from the livers of control mice at day 28 post-Ad-mG6Pase infusion (Fig. 2A), albeit at a lower level than that of the G6Pase+/+ mice (Fig. 1C). In contrast, there was no detectable G6Pase activity in liver sections of age-matched control G6Pase−/− mice (Fig. 1A).

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Gene Therapy in GSD-1a Mice

Ad-mG6Pase Infusion Corrects Pathological Manifestations of GSD-1a—Although our glucose therapy significantly improved the survival of G6Pase<sup>−/−</sup> mice, they grew slower than their G6Pase<sup>+/−</sup> or G6Pase<sup>++/−</sup> littersmates. At 14 days of age, the average weights of G6Pase<sup>−/−</sup> and G6Pase<sup>+/−</sup>/G6Pase<sup>++/−</sup> mice were 5.9 and 10.3 g, respectively (Fig. 3A). The body weights of G6Pase<sup>−/−</sup> mice increased rapidly after Ad-mG6Pase infusion, and the growth rate of the infused animals was comparable to that of G6Pase<sup>+/−</sup>/G6Pase<sup>++/−</sup> mice (Fig. 3A). The growth rate (Fig. 3A) of untreated G6Pase<sup>−/−</sup> mice that have survived weaning also increased presumably due to the combination of glucose therapy, food supplements, and the constant eating habit of mice.

Infusion of Ad-mG6Pase partially corrected liver (Fig. 3B) and kidney (Fig. 3C) enlargement seen in G6Pase<sup>−/−</sup> mice. The decrease in liver and kidney weights was accompanied by a concomitant reduction in glycogen contents in both organs (data not shown). Hematoxylin and eosin staining revealed that the G6Pase<sup>−/−</sup> mice had marked glycogen deposits in the hepatocytes of the liver (Fig. 4A) and both kidneys (Fig. 4D). Glycogen storage in both organs was considerably decreased after Ad-mG6Pase infusion (Fig. 4, B and E).

In addition to hypoglycemic seizures that could be relieved by immediate glucose injection, G6Pase<sup>−/−</sup> mice under glucose therapy manifested hypoglycemia, hyperlipidemia, and hyperuricemia in the first 28 days of life (Table II). As evidenced by the renewed growth of weaned G6Pase<sup>−/−</sup> mice, plasma glucose, cholesterol, triglyceride, and uric acid profiles were also markedly normalized (Table II). G6Pase gene replacement in G6Pase<sup>−/−</sup> mice resulted in the normalization of plasma glucose levels toward the normal control values in G6Pase<sup>+/−</sup> mice (Table II). Moreover, the hypoglycemic seizures frequently suffered by the untreated G6Pase<sup>−/−</sup> mice were not observed in Ad-mG6Pase-infused animals. Ad-mG6Pase infusion also elicited 68, 70, and 84% reductions in plasma cholesterol, triglyceride, and uric acid, respectively, at day 7 post-infusion (Table II). Plasma cholesterol and triglyceride were completely normalized at 14–28 days post-infusion.

**TABLE II**

| Age | Glucose<sup>a</sup> | Cholesterol<sup>b</sup> | Triglyceride<sup>c</sup> | Uric acid<sup>d</sup> |
|-----|------------------|-----------------|-----------------|-----------------|
| 21–28 days | 66.6 ± 15.6 | 519.9 ± 59.9 (7.0) | 238.2 ± 64.7 (4.5) | 7.16 ± 1.36 (7.2) |
| >28 days | 108.3 ± 12.7 | 115.9 ± 16.4 (1.6) | 56.6 ± 11.5 (1.1) | 1.83 ± 0.01 (1.8) |
| 21–84 days | 213.7 ± 12.7 | 73.9 ± 4.3 | 55.3 ± 5.0 | 1.00 ± 0.11 |
| >84 days | 109.3 ± 13.0 | 168.5 ± 23.2 (2.3) | 51.2 ± 2.5 (0.96) | 1.14 ± 0.31 (1.1) |

<sup>a</sup> p = 0.038 and 0.0025 for control G6Pase<sup>−/−</sup> (21–28 days old) and G6Pase<sup>−/−</sup> mice at days 7 and 14 post-infusion, respectively; p = 0.011, 0.33, and 0.13 for control G6Pase<sup>−/−</sup> (>28 days old) and G6Pase<sup>−/−</sup> mice at days 28, 56, and 70 post-infusion, respectively; and p < 0.0001 for G6Pase<sup>+/−</sup> mice and all others.

<sup>b</sup> p < 0.0001 for control G6Pase<sup>−/−</sup> mice (21–28 days old) and all others; p = 0.0005 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice at day 7 post-infusion; p = 0.012 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice (>28 days old); and p = 0.15–0.85 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice at days 14, 28, 56, and 70 post-infusion.

<sup>c</sup> p < 0.0001 for control G6Pase<sup>−/−</sup> mice (21–28 days old) and all others; and p > 0.5 for all other comparisons.

<sup>d</sup> p < 0.0001 for control G6Pase<sup>−/−</sup> mice (21–28 days old) and all others; p = 0.003 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice at day 7 post-infusion; p = 0.02 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice at day 7 post-infusion; p = 0.0001 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice (>28 days old); and p = 0.35 and 0.13 for G6Pase<sup>+/−</sup> and G6Pase<sup>−/−</sup> mice at days 7 and 28 post-infusion, respectively.

* ND, not determined.
DISCUSSION

We have used G6Pase−/− mice that manifest pathological features virtually identical to those of human GSD-1a patients to evaluate gene replacement therapy for this inheritable disorder. We demonstrated that a single administration of Ad-mG6Pase, a second-generation recombinant adenovirus containing the murine G6Pase cDNA, essentially corrected the abnormal plasma glucose, cholesterol, and triglyceride profiles in G6Pase−/− mice. Moreover, Ad-mG6Pase infusion partially alleviated liver and kidney enlargement manifested by GSD-1a mice and reduced glycogen deposition in both organs. Whereas only 15% of the 14-day-old G6Pase−/− mice under glucose therapy survived weaning, 100% of the age-matched G6Pase−/− mice infused with Ad-mG6Pase lived to 56 days of age, and 90% of the transgene-infused animals lived to 84 days of age. A limitation of the currently used adenoviral vector is the short-term expression of the transgene (18–21). Despite this limitation, the transient correction of metabolic abnormalities in G6Pase−/− mice achieved using the present system permits us to assess the feasibility of gene replacement therapy for GSD-1a. Our data suggest that gene therapy may be a viable option for the treatment of human GSD-1a patients when the G6Pase transgene expression can be sustained.

G6Pase is an ER transmembrane protein (2, 3), and an appropriate membrane insertion of the protein is required for enzymatic activity. In this study, we showed that survival of the G6Pase−/− mice was sustained immediately after Ad-mG6Pase infusion as the pups survived without glucose therapy. Hepatic G6Pase activity was restored to 3.6% of that in the G6Pase+/+ mice at day 2 post-infusion, suggesting that hypoglycemic seizures that threaten the survival of the G6Pase−/− mice could be prevented by low levels of G6Pase activity in the liver.

The active site of G6Pase faces the lumen of the ER (2, 3), and for Glu-6-P hydrolysis in vivo, the Glu-6-P substrate must be translocated from the cytoplasm to the ER lumen by the G6PT (4). We have previously shown that Glu-6-P transport and hydrolysis are tightly coupled events and that G6Pase activity greatly facilitates Glu-6-P uptake by the G6PT protein (5, 6). In this study, we demonstrated that the adenovirus-directed G6Pase expression in the livers of G6Pase−/− mice activated hepatic microsomal Glu-6-P transport activity, establishing the fundamental dependence of the G6PT and G6Pase on the hydrolysis of Glu-6-P.

Expression of the G6Pase gene is activated immediately following birth (2, 21). However, a surge in G6Pase mRNA and enzymatic activity is not accompanied by a simultaneous increase in the G6PT-mediated Glu-6-P uptake (21), despite the fact that a high level of G6PT expression has occurred since gestation day 19 (22). Instead, postnatal microsomal Glu-6-P transport activity increases only gradually to peak around postnatal day 28 (21). In this study, we showed that an increase in hepatic G6Pase activity (19% of G6Pase+/+ mice) in Ad-mG6Pase-infused G6Pase−/− mice was accompanied by a similar increase (25% of G6Pase+/+ mice) in microsomal Glu-6-P uptake activity at day 14 post-infusion when the mice were 28 days old (Fig. 2B). A comparable increase in microsomal Glu-6-P uptake was not accompanied by the increase in hepatic G6Pase activity at day 7 post-infusion when the mice were 21 days old. The discordance between G6PT/G6Pase expression and microsomal Glu-6-P transport activity suggests the involvement of additional factor(s) in modulating the microsomal Glu-6-P transport function. It has been reported that an increase in G6Pase mRNA by glucose is mediated, in part, by glucokinase, which catalyzes the phosphorylation of glucose to Glu-6-P (23). It is possible that the cytoplasmic Glu-6-P level, which is controlled to some extent by glucokinase in the liver, can regulate the G6PT protein to transport Glu-6-P. Interestingly, hepatic glucokinase mRNA and activity first appear in the liver only at postnatal day 15, and its highest expression is reached at postnatal day 30 (24). The role of glucokinase in microsomal Glu-6-P transport is currently under investigation.

It has been shown that liver transplantation into GSD-1a mice has corrected hypoglycemia and several other biochemical abnormalities associated with this disorder (10, 11). This is consistent with our present findings indicating that expression of the G6Pase transgene in liver to 19% of that in G6Pase−/− mice corrected growth retardation, liver and kidney enlargement, hypoglycemia, hyperlipidemia, and hyperuricemia manifested by GSD-1a mice and greatly improved the survival of these mice. In summary, we have used G6Pase−/− mice to assess gene replacement therapy for GSD-1a, and our results strongly suggest that this disorder can be corrected by gene therapy when vector systems that can direct sustained expression of the transgene are developed.

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