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Glycogen storage disease type Ia mice with less than 2% of normal hepatic glucose-6-phosphatase-α activity restored are at risk of developing hepatic tumors

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ABSTRACT

Glycogen storage disease type Ia (GSD-Ia), characterized by impaired glucose homeostasis and chronic risk of hepatocellular adenoma (HCA) and carcinoma (HCC), is caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC). We have previously shown that *G6pc* −/− mice receiving gene transfer mediated by rAAV-G6PC, a recombinant adeno-associated virus (rAAV) vector expressing G6Pase-α, and expressing 3–63% of normal hepatic G6Pase-α activity maintain glucose homeostasis and do not develop HCA/HCC. However, the threshold of hepatic G6Pase-α activity required to prevent tumor formation remained unknown. In this study, we constructed rAAV-co-G6PC, a rAAV vector expressing a codon-optimized (co) G6Pase-α and showed that rAAV-co-G6PC was more efficacious than rAAV-G6PC in directing hepatic G6Pase-α expression. Over an 88-week study, we showed that both rAAV-G6PC- and rAAV-co-G6PC-treated *G6pc* −/− mice expressing 3–33% of normal hepatic G6Pase-α activity (AAV mice) maintained glucose homeostasis, lacked HCA/HCC, and were protected against age-related obesity and insulin resistance. Of the eleven rAAV-G6PC/rAAV-co-G6PC-treated *G6pc* −/− mice harboring 0.9–2.4% of normal hepatic G6Pase-α activity (AAV-low mice), 3 expressing 0.9–1.3% of normal hepatic G6Pase-α activity developed HCA/HCC, while 8 did not (AAV-low-NT). Finally, we showed that the AAV-low-NT mice exhibited a phenotype indistinguishable from that of AAV mice expressing ≥3% of normal hepatic G6Pase-α activity. The results establish the threshold of hepatic G6Pase-α activity required to prevent HCA/HCC and show that GSD-Ia mice harboring <2% of normal hepatic G6Pase-α activity are at risk of tumor development.

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1. Introduction

Glycogen storage disease type Ia (GSD-Ia or von Gierke disease, MIM232200) is an autosomal recessive disorder caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC) that catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis of the liver, kidney, and intestine [1,2]. G6Pase-α is a hydrophobic protein anchored in the endoplasmic reticulum by 9 transmembrane helices with its active sites situated inside the lumen [3]. For catalysis, the G6P substrate must be transported from the cytoplasm into the endoplasmic reticulum lumen by a G6P transporter (G6PT), and the G6Pase-α/G6PT complex maintains interprandial blood glucose [1,2]. Patients affected by GSD-Ia are unable to maintain glucose homeostasis and present with fasting hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, lactic acidemia, and growth retardation [1,2]. Untreated, GSD-Ia is juvenile lethal. Dietary therapies [4,5] have enabled GSD-Ia patients to attain near normal growth and pubertal development. However, no current therapy is able to address the long-term complication of hepatocellular adenoma (HCA) that develops in 75% of GSD-I patients over 25 years-old [1,2,6–8]. In 10% cases, HCA undergoes malignant transformation to hepatocellular carcinoma (HCC). In GSD patients classified to date, 52% of HCA are inflammatory HCA, 28% β-catenin mutated HCA and 20% unclassified HCA [9,10].

Abbreviations: AAV, adeno-associated virus; ChREBP, carbohydrate response element binding protein; G6Pase-α, glucose-6-phosphatase-α; G6P, glucose-6-phosphate; G6PT, glucose-6-phosphate transporter; GPE, *G6PC* promoter and enhancer; GSD-Ia, glycogen storage disease type Ia; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; GLUL, glutamate-ammonia ligase; LGR5, leucine-rich repeat containing G protein-coupledreceptor 5; STAT3, signal transducer and activatorof transcription 3.

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We have examined the efficacy of gene therapy mediated by rAAV-G6PC, a recombinant adeno-associated virus (rAAV) pseudotype 2/8 vector expressing human G6Pase-α directed by the human *G6PC* promoter/enhancer (GPE) [11–13]. Systemic administration of rAAV-G6PC delivers the G6Pase-α transgene to the liver of *G6pc* −/− mice and provides a sustained correction of metabolic abnormalities over 90 weeks [12,13]. The rAAV-G6PC-treated *G6pc* −/− mice expressing 3–63% of normal (or wild-type) hepatic G6Pase-α activity maintain blood glucose homeostasis for 70–90 weeks, show no evidence of HCA/HCC, tolerate a 24-h fast, and are protected against age-related obesity and insulin resistance [12,13]. However, the threshold of hepatic G6Pase-α activity required to prevent tumor formation remained unknown. Studies have shown that codon optimization strategies have proven useful to increase translation efficiency [14–17]. We therefore generated a rAAV vector expressing a codon-optimized (co) human G6Pase (co-G6Pase) to determine if this might increase translation efficiency and enzyme activity.

In this study, we examined the efficacy of gene transfer in *G6pc* −/− mice mediated by rAAV-G6PC and rAAV-co-G6PC, and the minimal hepatic G6Pase-α required to prevent tumor formation. We show that the rAAV-co-G6PC vector is more effective than the rAAV-G6PC vector in directing hepatic G6Pase-α expression. All rAAV-G6PC/rAAV-co-G6PC-treated *G6pc* −/− mice expressing 3–33% of normal hepatic G6Pase-α activity (AAV mice) maintain glucose homeostasis, lack HCA/HCC, and are protected against age-related obesity and insulin resistance. Of the eleven rAAV-treated *G6pc* −/− mice harboring 0.9–2.4% of normal hepatic G6Pase-α activity (AAV-low mice), 3 expressing 0.9–1.3% of normal hepatic G6Pase-α activity (AAV-low-T mice) develop HCA/HCC and 8 did not (AAV-low-NT), showing that mice restoring <2% of normal hepatic G6Pase-α activity are at risk of developing hepatic tumors. We also showed that the AAV-low-NT mice exhibited a phenotype indistinguishable from that of AAV mice.

2. Materials and methods

2.1. Construction of pSVL and rAAV vectors and infusion of G6pc −/− mice

The human co-G6PC was synthesized by Life Technologies and subcloned into the pSVL vector, yielding pSVL-co-G6PC. The pTR-GPE-co-G6PC plasmid was constructed by replacing human G6PC at 5′-*Sbf*I and 3′-*Not*I sites in pTR-GPE-G6PC with the human co-G6PC cDNA. The rAAV vectors were produced at the University of Florida Powell Gene Therapy Center Vector Core Laboratory. All animal studies were conducted under an animal protocol approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee. The rAAV vector was infused into 2-week-old *G6pc* −/− mice as described previously [11–13]. The Age-matched *G6pc* +/+ and *G6pc* +/− mice with indistinguishable phenotype were used as controls. All mice were fed with the standard mouse chow (NIH-31 Open formula mouse/rat sterilizable diet) from ENVIGO (Fredrick, MD, USA). After weaning, the control and rAAV-treated *G6pc* −/− mice were housed separately. The male and female mice were also housed separately with 3 females per cage and 1–3 males per cage. The 3 tumor-bearing mice were housed in 3 different cages.

Hepatic G6Pase-α activity in 66–88-week-old *G6pc* +/+/*G6pc* +/− mice averaged 171.4 ± 5.7 units, and was designated as the normal value of hepatic G6Pase-α activity. With the exception of the two HCC-bearing mice, mouse liver samples were collected at sacrifice following a 24-h fast to ensure that hepatic glucose is produced mainly via gluconeogenesis.

2.2. Phosphohydrolase assay

Liver microsome isolation and microsomal G6Pase-α assay were as described previously [11–13]. In phosphohydrolase assays, reaction mixtures (100 μl) containing 50 mM cacodylate buffer, pH 6.5, 10 mM G6P and appropriate amounts of microsomal preparations were incubated at 30 °C for 10 min. 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 pre-incubating disrupted microsomal preparations at pH 5 for 10 min at 37 °C to inactivate the acid-labile G6Pase-α. One unit of G6Pase-α activity represents 1 nmol G6P hydrolysis per minute per mg microsomal protein. The 60–90 week-old wild-type mice typically averaged 170–180 units G6Pase-α activity in the livers [12,13].

2.3. Phenotype analysis

Body composition was assessed using the Bruker minispec NMR analyzer (Karlsruhe, Germany). The presence of HCA nodules in mice was confirmed by histological analysis of liver biopsy samples, using 5 or more separate sections per liver. Blood levels of glucose along with hepatic levels of glucose, triglyceride, lactate, and G6P were determined as described previously [12,13]. Insulin tolerance testing of mice consisted of a 4-h fast, prior to blood sampling, followed by intraperitoneal injection of insulin at 0.25 IU/kg, and repeated blood sampling via the tail vein for 1 h [13].

2.4. Quantitative real-time RT-PCR and Western-blot analysis

The mRNA expression was quantified by real-time RT-PCR using the TaqMan probes in an Applied Biosystems QuantStudio 3 Real-Time PCR System (Foster City, CA, USA). Data were normalized to Rpl19 RNA.

2.5. Analysis of ChREBP nuclear localization

The nuclear location of carbohydrate response element binding protein (ChREBP) in mouse liver sections was performed as described previously [13]. Mouse liver paraffin sections (10 μm thickness) were treated with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidases, then blocked with the Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA). For ChREBP detection, liver sections were incubated serially with a rabbit antibody against ChREBP and a biotinylated anti-rabbit IgG (Vector Laboratories). The resulting complexes were detected with an ABC kit using the DAB Substrate (Vector Laboratories). Sections were counterstained with hematoxylin (Sigma-Aldrich, St Louis, MO, USA) and visualized using a Zeiss Axioskop2 plus microscope equipped with 40×/0.50NA objectives (Carl Zeiss MicroImaging, Jena, Germany). Images were acquired using a Nikon DS-Fil digital camera and NIS-Elements F3.0 imaging software (Nikon, Tokyo, Japan). The percentage of cells in 10 randomly selected fields containing ChREBP positive nuclei was recorded.

2.6. Statistical analysis

The unpaired *t*-test was performed using the GraphPad Prism Program, version 4 (San Diego, CA, USA). Values were considered statistically significant at $p < 0.05$.

3. Results

3.1. Codon-optimized G6Pase-α exhibits increased enzymatic activity

To increase the levels of transgene expression, we constructed a codon-optimized human G6Pase-α construct, co-G6PC. Transient expression in COS-1 cells showed that the pSVL-co-G6PC construct directed 1.8-fold higher G6P hydrolytic activity, compared to the pSVL-G6PC construct (Fig. 1A). We then infused 2-week-old *G6pc* −/− mice with 6×10^{12} viral particles (vp)/kg of rAAV-G6PC or rAAV-co-G6PC vector. At age 12-weeks, hepatic microsomal G6Pase-α activity in rAAV-G6PC- and rAAV-co-G6PC-treated mice were 15% and 32%, respectively