SHORT COMMUNICATION

Liver-directed adeno-associated virus serotype 8 gene transfer resuces a lethal murine model of citrullinemia type 1

RJ Chandler1,5, TN Tarasenko2,5, K Cusmano-Ozog3, Q Sun4, VR Sutton4, CP Venditti1 and PJ McGuire2

INTRODUCTION

Citrullinemia type 1 (CTLN1) is an autosomal recessive disorder of metabolism caused by a deficiency of argininosuccinate synthetase. Despite optimal management, CTLN1 patients still suffer from lethal metabolic instability and experience life-threatening episodes of acute hyperammonemia. A murine model of CTLN1 (fold/fold) that displays lethality within the first 21 days of life was used to determine the efficacy of adeno-associated viral (AAV) gene transfer as a potential therapy. An AAV serotype 8 (AAV8) vector was engineered to express the human ASS1 cDNA under the control of a liver-specific promoter (thyroxine-binding globulin, TBG), AAV8-TBG-hASS1, and delivered to 7–10 days old mice via intraperitoneal injection. Greater than 95% of the mice were rescued from lethality and survival was extended beyond 100 days after receiving a single dose of vector. AAV8-TBG-hASS1 treatment resulted in liver-specific expression of hASS1, increased ASS1 enzyme activity, reduction in plasma ammonia and citrulline concentrations and significant phenotypic improvement of the fold/fold growth and skin phenotypes. These experiments highlight a gene transfer approach using AAV8 vector for liver-targeted gene therapy that could serve as a treatment for CTLN1.

Keywords: urea cycle disorders; citrullinemia; AAV8; hyperammonemia

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1Organic Acid Research Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA; 2Physician Scientist Development Program, Genetic Diseases Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA; 3Department of Genetics and Metabolism, Children’s National Medical Center, Washington, DC, USA and 4Biochemical Genetics Laboratory, Baylor College of Medicine, Houston, TX, USA. Correspondence: Dr PJ McGuire, National Human Genome Research Institute, National Institutes of Health, 49 Convent Drive, Room 4A62, Bethesda, MD 20892, USA.
E-mail: peter.mcguire@nih.gov

These authors contributed equally to this work.
The efficacy of gene delivery has been demonstrated in the ASS1 KO mouse using an E1-deleted adeno-associated vector, which carried the ASS1 cDNA driven by a ubiquitous CMV promoter. 13 Although the adenoviral treated mice did exhibit a modest increase in survival and metabolic improvement, mice treated twice with the adenovirus and supplemented with sodium benzoate survived on average, 40 days. Recent successes in the spf-ash mouse, a model of ornithine transcarbamylase deficiency, using adeno-associated viral (AAV) vectors capable of long-term transgene expression suggests that gene therapy for CTLN1 could be advanced to the clinic. 14–16

Herein, we describe the therapeutic efficacy of a liver-targeted single-strand AAV8 vector as a new gene therapy treatment for CTLN1 with the potential for translation to the clinic. The AAV8-treated homozygous fold mice are rescued from lethality, display reduced circulating metabolites and increased hepatic ASS enzyme activity. However, liver-directed gene therapy did not fully correct the biochemical phenotype of systemic ASS1 deficiency; arginine levels plummeted in treated fold/mice due to persistent renal deficiency. Our results provide the first evidence of the utility of systemic gene delivery for citrullinemia using AAV8.

RESULTS AND DISCUSSION

Unlike the adenovirus that used a ubiquitous CMV promoter for correction of the ASS1 KO mouse, 13 our AAV vector utilized a thyroxine-binding globulin (TBG) promoter to direct ASS1 expression to the liver. A total of 20 ASS1fold/fold (fold/fold) mice received an intraperitoneal injection of 1 × 1010 genome copies (GCs) of AAV8 carrying the human ASS1 cDNA (AAV8-hASS1) under the control of the liver-specific TBG promoter at 7–10 days of life. This time point was chosen owing to the ease of phenotypic identification of animals (that is, fur abnormalities) and agreement with time to diagnosis in CTLN1. The AAV8 dose was based on previous descriptions of gene-therapy-mediated rescue of murine models of methylmalonic and propionic acidemia. 17,18 All wild-type (WT) mice survived for the duration of study for up to 106 days (N = 25). Untreated fold/fold mice (N = 6) could be recognized at 7–10 days of life but were not recovered at weaning at 3–4 weeks, consistent with the original description of this model. Nearly all (N = 19) of the treated fold/fold mice (95%) survived the early lethality period (<28 days) up to 106 days (P < 0.01, Figure 1a), with the exception of a single-treated fold/fold mouse that died at 28 days. AAV8 gene therapy significantly prolonged survival when compared with the ASS1 mouse, 13 likely due to the ability of AAV vectors to provide long-term transgene expression in comparison to E1-deleted adenoviral vectors. 19

Fold/fold mice were not distinguishable from fold/+ or WT littermates at birth. As untreated fold/fold mice develop over time, a growth disparity becomes apparent in the rate of weight gain when compared with WT littermates. In a cross-sectional analysis of weights, fold/fold mice treated with AAV8-hASS1 followed a distinct growth curve (Figure 1b) similar to treated ASS1 KO mice, 13 albeit with greater weight gain. At ~80–90 days, fold/fold mice (mean = 13.5 g, s.d. = 2.1) weighed ~40% less than littermate controls (mean = 21.6 g, s.d. = 0.7). In addition to weight, overall length was also decreased compared with WT littermates (Figure 1c). Fold/fold was initially described for its abnormal patchy hair pattern (fold–follicular dystrophy). 7 With hepatic ASS1 correction, coat texture and fullness was still patchy at 26 days but significantly improved by 52 days.

With improvement in survival of treated fold/fold mice, we next looked at the liver 30 days post treatment to examine whether hASS1 mRNA was expressed and if hepatic ASS1 enzyme activity was increased. Using quantitative PCR, AAV-hASS1 vector was detectable on average at 567 copies/haploid genome in the treated animals, but was undetectable in WT and untreated animals (Figure 2a). Similar to the vector copy number, treated fold/fold demonstrated ASS1 mRNA levels 35 × (s.d. = 25.6) above untreated fold/fold (Figure 2b).

Figure 1. Survival, weight, length and coat in fold/fold treated with liver-targeted gene therapy. Fold/fold mice received 1 × 1010 GC/mouse at 7–10 days of life. (a) Survival in untreated fold/fold (N = 6), treated fold/fold (N = 20) and WT littermates (N = 25). (b) Cross-sectional analysis of weights in fold/fold (N = 19) and WT littermates (N = 20). (c) Length and coat texture in untreated fold/fold, treated fold/fold and WT littermates.
This is known as the intestinal–renal axis.1,20 Patients arginine levels by converting citrulline to arginine through ASS1 transduction or expression of the human mRNA.

Increased variability may be due to stochastic effects due to injection, elevated above WT. Similar to the CTLN1.5 Surprisingly, pretreatment plasma arginine levels in supplementation is often required after liver transplantation for with CTLN1 may display arginine deficiency on plasma amino-acid levels remained elevated in plasma ammonia levels by 4 after treatment with gene therapy in ASS1 KO mouse,13 the average plasma ammonia and citrulline levels were grossly elevated in untreated fold/fold. Blood samples taken ~1 month after treatment with gene therapy in fold/fold showed a reduction in plasma ammonia levels by >50% in treated animals (mean = 195.4 μmol l⁻¹, s.d. = 173.2, P = 0.03, Figure 2d).

As with ammonia, plasma citrulline levels were also reduced (Figure 2e). At baseline, plasma citrulline was grossly elevated (mean = 2754 μmol l⁻¹, s.d. = 111.9) in untreated fold/fold when compared with WT mice (mean = 110.9 μmol l⁻¹, s.d. = 22.5). Post gene therapy, plasma citrulline levels were reduced by 73% (mean = 756.3 μmol l⁻¹, s.d. = 844.0, P = 0.01) when compared with untreated fold/fold, although these levels still remained elevated above WT. Similar to the ASS1 KO mouse,13 the average plasma citrulline levels remained elevated in fold/fold. This variability may be due to stochastic effects due to injection, transduction or expression of the human mRNA.

The kidney has an important role in the maintenance of plasma arginine levels by converting citrulline to arginine through ASS1 and ASL. This is known as the intestinal–renal axis.1,20 Patients with CTLN1 may display arginine deficiency on plasma amino-acid profiles due to renal ASS1 enzyme deficiency. In addition, arginine supplementation is often required after liver transplantation for CTLN1.5 Surprisingly, pretreatment plasma arginine levels in fold/ fold were two times greater than WT (mean = 230.0 μmol l⁻¹, s.d. = 9.6, P < 0.01, Figure 2f). Original descriptions of the fold/fold model reported mean plasma arginine levels of 171 μmol l⁻¹, s.d. = 68, which can overlap with WT mice.7 We hypothesized that plasma arginine in fold/fold may be related to the large citrulline pool being fed through the hypomorphic ASS enzyme in the kidney; however, these data reflect a specific point in time and may not reflect overall arginine status. More importantly, with liver-targeted gene therapy, fold/fold plasma arginine levels plummeted on average to less than half of WT (mean = 26.8 μmol l⁻¹, s.d. = 22.0, P < 0.01) consistent with persistent renal ASS enzyme deficiency seen post liver transplantation.5 Regarding the ASS1 KO post gene therapy, marginal improvement was seen in plasma arginine.15 This discrepancy in plasma arginine levels between the treated fold/fold and ASS1 KO levels is likely related to the vectors used. In the ASS1 KO, the adenoviral vector had a ubiquitous promoter, which may transduce the kidney and result in an improved intestinal–renal axis.

In conclusion, these studies are the first to establish the efficacy of liver-directed gene therapy in the rescue of argininosuccinate synthetase deficiency using AAV8. Improvements in biochemical parameters and survival are clearly demonstrated. In addition, the importance of extrahepatic ASS1 deficiency in the maintenance of plasma arginine levels is suggested by the metabolic parameters observed in the treated fold/fold mice.

**METHODS**

Murine model of CTLN1

The fold mutation was initially described at the Jackson Laboratory in a production colony of P/J mice. Congenic (N4) B6EiP-fold/1 (stock number 006449) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA); herein referred to as fold. Fold mice display elevated plasma ammonia and massively elevated plasma citrulline concentrations. WT littermate animals were used as controls throughout.
AAV8 construction, production and delivery

The expression vector, pENN-AAV-TBG-PI-RBG (PennVector P1015) was obtained from the University of Pennsylvania Vector Core. This vector contains transcriptional control elements from the thyroxine-binding globulin (TBG) promoter, cloning sites for the insertion of a complementary DNA and the rabbit β-globin polyA signal. Terminal repeats from AAV serotype 2 flank the expression cassette. The human ASS1 cDNA was isolated from a human liver cDNA library by real-time PCR using ASS1-specific primers (listed below) and was sequenced verified. The ASS1 cDNA was then cloned into pENN-AAV-TBG-PI-RBG. This newly created vector AAV-TBG-PI-ASS1-RBG was packaged into AAV8, purified by cesium chloride centrifugation and titered by qPCR as previously described. Animal studies were reviewed and approved by the National Human Genome Research Institute Animal Care and Use Committee. Viral particles were diluted to a total volume of 100 μl with phosphate-buffered saline immediately before injection and 1 x 1010 GCs were administered via intraperitoneal injection at 7–10 days of age.

ASS1-Koz-Xhol CTCAGAGgccaccATGTCCAGCAAAGGCTCCGTG
ASS1-Stop-M1ul acgcgtGGGTTCTATTGCGAGTGC

Quantification of hepatic ASS1 mRNA expression

Total RNA was extracted from the liver using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), followed by DNA digestion (Ambion, Austin, TX, USA). Reverse transcription was performed using Applied Biosystems High capacity cDNA Transcription Kit. Quantitative real-time PCR was subsequently performed on the cDNA with TaqMan gene expression assays (murine β-actin (Mm00607939_s1) and murine Ass1 (Hs01597989_g1) from Applied Biosystems, Foster City, CA, USA). Samples were analyzed in an Applied Biosystems 7500 fast real-time PCR system, in accordance with the manufacturer’s protocol. All samples were analyzed in triplicate.

Vector GC number

GC number was measured by quantitative real-time PCR analysis. A standard curve was prepared using serial dilutions of the AAV plasmid carrying ASS1 cDNA. Genomic DNA was extracted from murine liver samples and murine genomic DNA was used to determine the vector GC number per mouse haploid genome.

Metabolic studies

Plasma was isolated from blood collected by retro-orbital bleeding. The samples were immediately centrifuged, and the plasma was removed and stored at – 80 °C for later analysis. Plasma citrulline and arginine were analyzed by ion exchange chromatography (Biochrom 30, Holliston, MA, USA). ASS1 activity was determined using a clinically available enzyme assay which measures the conversion of [14C]-aspartate into argininosuccinic acid22 (Clinical Biochemical Genetics, Baylor College of Medicine, Houston, TX, USA).

Statistical analyses

In all instances, P-values were considered significant if the value was <0.05. Kaplan–Meier survival curves were used to compare groups on the basis of treatment with or without liver-targeted gene replacement therapy. The weights between treated and untreated mice, and differences in metabolite and enzyme levels were assessed using a two-sided, two-tailed unpaired Student’s t-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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