Adeno-Associated Virus Serotype 8 Gene Transfer Rescues a Neonatal Lethal Murine Model of Propionic Acidemia

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Abstract

Propionic acidemia (PA) is an autosomal recessive disorder of metabolism caused by a deficiency of propionyl-coenzyme A carboxylase (PCC). Despite optimal dietary and cofactor therapy, PA patients still suffer from lethal metabolic instability and experience multisystemic complications. A murine model of PA (*Pcca*^{-/-}) of animals that uniformly die within the first 48 hr of life was used to determine the efficacy of adeno-associated viral (AAV) gene transfer as a potential therapy for PA. An AAV serotype 8 (AAV8) vector was engineered to express the human *PCCA* cDNA and delivered to newborn mice via an intrahepatic injection. Greater than 64% of the *Pcca*^{-/-} mice were rescued after AAV8-mediated gene transfer and survived until day of life 16 or beyond. Western analysis of liver extracts showed that PCC was completely absent from *Pcca*^{-/-} mice but was restored to greater than wild-type levels after AAV gene therapy. The treated *Pcca*^{-/-} mice also exhibited markedly reduced plasma levels of 2-methylcitrate compared with the untreated *Pcca*^{-/-} mice, which indicates significant PCC enzymatic activity was provided by gene transfer. At the time of this report, the oldest treated *Pcca*^{-/-} mice are over 6 months of age. In summary, AAV gene delivery of *PCCA* effectively rescues *Pcca*^{-/-} mice from neonatal lethality and substantially ameliorates metabolic markers of the disease. These experiments demonstrate a gene transfer approach using AAV8 that might be used as a treatment for PA, a devastating and often lethal disorder desperately in need of new therapeutic options.

Introduction

PROPIONIC ACIDEMIA (PA) is an organic acidemia caused by mutations in either the *PCCA* or the *PCCB* genes, which encode for the mitochondrial enzyme propionyl-coenzyme A (CoA) carboxylase (PCC) (Fenton *et al.*, 2001). PCC is composed of *PCCA* and *PCCB* subunits and is involved in the catabolism of propiogenic amino acids, particularly isoleucine, valine, methionine, and threonine, as well as odd-chain fatty acids. This biotin-dependent enzyme catalyzes the first step in the conversion of propionyl-CoA to D-methylmalonyl-CoA in the pathway of propionyl-CoA oxidation (Fig. 1).

Most frequently, PA presents in the neonatal period with hyperammonemia, vomiting, poor feeding, and hypotonia and progresses into a life-threatening metabolic crisis.

Patients who survive suffer from recurrent metabolic instability and can develop multisystemic complications including neurological impairment (Haas *et al.*, 1995; Nyhan *et al.*, 1999), cardiomyopathy (Massoud and Leonard, 1993; Bhan and Brody, 2001; Mardach *et al.*, 2005), and pancreatitis (Kahler *et al.*, 1994). Typical laboratory abnormalities include severe metabolic acidosis, hyperglycinemia, ketosis, and marked hyperammonemia that may require dialysis. In addition to propionic acid (Hommes *et al.*, 1968), 3-hydroxypropionate (Ando *et al.*, 1972a), 2-methylcitrate (Ando *et al.*, 1972b), and propionylcarnitine (Roe *et al.*, 1984) are propionyl-CoA-derived metabolites that are present in an increased concentration in the blood and urine of affected patients. Although the precise roles that specific metabolite(s) have in the pathophysiology of PA remains uncertain, these compounds are clinical biomarkers of metabolism in PA patients.

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FIG. 1. The pathway of propionyl-CoA to succinyl-CoA metabolism is displayed. PA results from deficiency of the enzyme PCC and is caused by mutations in the *PCCA* or *PCCB* gene. Isolated MMA can be caused by defects in any of several downstream steps in the pathway, including D-methylmalonyl-CoA epimerase (MCEE), MUT, or enzymes that impair the synthesis of adenosylcobalamin (MMAA and MMAB). OAA, oxaloacetic acid; TCA, tricarboxylic acid.

The standard treatment for PA is dietary restriction of amino acid precursors, L-carnitine supplementation to replenish diminished carnitine levels, and administration of metronidazole to reduce the generation of propionic acid by intestinal bacteria. Liver transplantation has been used to relieve the metabolic decompensations of PA in circumstances where the patient cannot be managed by standard treatment (Saudubray *et al.*, 1999; Yorifuji *et al.*, 2000). However, despite the aggressive nutritional and cofactor therapy, the long-term prognosis for patients with PA remains poor (van Der Meer *et al.*, 1996; Saudubray *et al.*, 1999; Perez-Cerda *et al.*, 2000). The inclusion of this disorder in expanded newborn screening programs highlights the need for new therapies to provide better long-term prognosis for patients with PA.

A murine model of PA, created by deleting a portion of the *Pcca* gene, has been established. The targeted allele does not produce *Pcca* RNA and behaves as a null mutation (Miyazaki *et al.*, 2001). *Pcca*^{-/-} mice recapitulate the severe neonatal manifestations of PA and uniformly die within the first 48 hr of life. A recent effort to test adenoviral gene delivery of the human *PCCA* cDNA to *Pcca*^{-/-} mice resulted in a significant but modest increase in life expectancy of 38 hr or less (Hofherr *et al.*, 2009). The same article also reported that adeno-associated virus (AAV) serotype 8 (AAV8)-mediated gene transfer of *PCCA* to *Pcca*^{-/-} mice resulted in a life expectancy increase of only 10 hr or less. Although these experiments demonstrate theoretical potential for gene therapy in PA, a method of gene transfer with a sustained therapeutic effect needs to be established before considering gene transfer as a practical therapeutic option for patients with PA.

We have previously demonstrated rescue of a neonatal lethal murine model of methylmalonic acidemia (MMA) for over 18 months using AAV8 gene transfer (Carrillo-Carrasco *et al.*, 2010; Chandler and Venditti, 2010). MMA is an organic acidemia caused by deficient activity of methylmalonyl-CoA mutase (MUT), an enzyme also needed for the conversion of propionyl-CoA to succinyl-CoA, and has a neonatal phenotype very similar to PA, in both patients and knock-out mice. To investigate the potential of viral gene transfer as a treatment for PA, we used the same methodology and vector design previously used in our successful AAV8 MMA gene therapy studies (Chandler and Venditti, 2010). In this report, we establish that AAV8-mediated gene delivery to *Pcca*^{-/-} mice can provide long-term rescue of the neonatal lethal phenotype, result in hepatic *PCCA* expression, and significantly reduce the levels of propionyl-CoA-derived metabolites. These results unambiguously demonstrate AAV8-mediated gene transfer as a potential treatment for PA.

Materials and Methods

Animal studies

Murine experiments were approved and performed according to the regulations and standards of the National Human Genome Research Institute Animal Care and Use Committee (Bethesda, MD). *Pcca* knock-out mice harbor a targeted mutation in the *Pcca* gene that removes a 2-kbp region of exon 4 and its intron (Miyazaki *et al.*, 2001; Hofherr *et al.*, 2009). *Pcca*^{+/-} mice on a mixed C57/BL6/129 background (Hofherr *et al.*, 2009) were re-derived onto FVB/N at our facility. The triply-mixed *Pcca*^{+/-} progeny have no disease manifestations and were intercrossed to generate *Pcca*^{-/-} mice, which uniformly die in the neonatal period as previously described.

Virus production and delivery

The viral construct, AAV2/8.CB7.CI.RBG, was graciously provided by the University of Pennsylvania Vector Core (Philadelphia, PA). This construct, into which the full-length human *PCCA* cDNA was cloned, contains transcriptional control elements from the cytomegalovirus enhancer/chicken β -actin promoter, cloning sites for the insertion of a cDNA, and the rabbit β -globin polyA signal. Inverted terminal repeats from AAV serotype 2 flank the expression cassette. The genome was packaged into an AAV8 capsid, purified by cesium chloride centrifugation, and titrated by quantitative polymerase chain reaction. AAV2/8.CB7.CI.EGFP.RBG, also provided by the University of Pennsylvania Vector Core, substitutes the green fluorescent protein (GFP) gene for *PCCA* and was used as a negative control. Vector genome copies (GC) of AAV8-PCCA (1×10^{10}) in a total volume of 10 μ l were delivered into the liver parenchyma of neonatal *Pcca*^{-/-} mice and control littermates using a 32-gauge needle during the first 8 hr of life.

Western blotting

Liver tissue samples were homogenized with a 2-ml Tenbroeck tissue grinder (Wheaton, Millville, NJ) in T-PER (Pierce Biotechnology, Rockford, IL) tissue protein extraction buffer in the presence of Halt (Pierce) protease inhibitor cocktail. Thirty micrograms of clarified extract was used in

western blot analysis and were probed with PCCA MaxPab polyclonal antibody (catalog number H00005095-B01P, Abnova, Walnut, CA) at a dilution of 1:1,000 with 2% bovine serum albumin and mouse monoclonal anti-OxPhos Complex III core II antibody (catalog number A-11143, Invitrogen, Carlsbad, CA) at a dilution of 1:3,000. Horseradish peroxidase-conjugated sheep anti-mouse antibody (catalog number NA931, GE Healthcare Life Sciences, Piscataway, NJ) was used as the secondary antibody at a dilution of 1:20,000 for PCCA detection and 1:30,000 for Complex III detection. Signal was visualized with West Pico chemiluminescent substrate (catalog number 34080, Pierce).

Metabolite levels

Blood samples were taken from sacrificed animals on day of life 1 or by retro-orbital sinus plexus sampling on day of life 28. Samples were centrifuged immediately after collection, diluted in water, and stored at -80°C in a screw-top tube for later analysis. Plasma 2-methylcitrate levels were measured by gas chromatography-mass spectrometry with stable isotopic internal calibration as previously described (Marcell *et al.*, 1985; Allen *et al.*, 1993).

Results

AAV8-PCCA gene transfer rescues the neonatal lethal phenotype

A total of 14 $Pcca^{-/-}$ mice received an intrahepatic injection of 1×10^{10} GC of AAV8 carrying the human PCCA cDNA AAV8-PCCA under the control of the ubiquitous chicken β -actin promoter during the first 8 hr of life. We chose a dose of 1×10^{10} GC because this dose is capable of rescuing the murine model of MMA (authors' unpublished data). Nine of the treated $Pcca^{-/-}$ mice (64%) survived the neonatal period and lived until day 16 of life (Fig. 2). Untreated $Pcca^{-/-}$ mice ($n=11$) and $Pcca^{-/-}$ mice that received AAV8-GFP ($n=3$)

FIG. 2. Survival of $Pcca^{-/-}$ mice: $Pcca^{-/-}$ untreated mice ($n=11$), $Pcca^{-/-}$ mice injected with 1×10^{10} GC of AAV8 carrying the human PCCA cDNA ($n=14$), and $Pcca^{-/-}$ mice injected with 1×10^{10} GC of AAV8 carrying GFP cDNA ($n=3$).

FIG. 3. Immunoblots of 30 μg of murine liver protein extract stained for Pcca and ubiquinol-cytochrome *c* reductase core protein II (Uqcrc2), which was used as a loading control. Lane 1 ($Pcca^{+/+}$) and Lane 2 ($Pcca^{-/-}$) are liver extracts from the respective untreated mice at day 1 of life. Lanes 3–5 are liver extracts from mice treated with 1×10^{10} GC of AAV8-PCCA at birth.

died by the second day of life (Fig. 2). Unfortunately, on day of life 16 two treated $Pcca^{-/-}$ mice received severe wounds as a result of fighting and were sacrificed. These mice as well as an additional treated $Pcca^{-/-}$ mouse that was sacrificed on day of life 43 were evaluated for hepatic PCCA protein expression. At the time of this report six of the AAV8-treated $Pcca^{-/-}$ mice were over 2 months old, with the oldest surviving $Pcca^{-/-}$ mice now 9 months of age.

PCCA protein is expressed in $Pcca^{-/-}$ mice following gene transfer

To determine if PCCA protein was being expressed in $Pcca^{-/-}$ mice following AAV8-PCCA gene delivery, an immunoblot of 30 μg of total liver protein was probed with a

FIG. 4. Mean plasma methylcitrate levels. Normal plasma ($n=9$) is from *Pcca*^{+/+} and *Pcca*^{+/-} mice at day of life 1. Because of neonatal lethality, *Pcca*^{+/+} plasma ($n=4$) was collected at day of life 1 from untreated *Pcca*^{-/-} mice ($n=5$). *Pcca*^{-/-} AAV8-PCCA plasma ($n=7$) was collected from *Pcca*^{-/-} mice that received an injection of AAV8 carrying the human PCCA cDNA between day of life 22 and 28. Error bars represent ± 1 SD. * $p=0.01$ (t test).

murine PCC polyclonal antibody that cross-reacts against both the murine (*Pcca*) and human (PCCA) orthologs. Significant levels of PCCA protein were detected on an immunoblot of liver extracts from the treated *Pcca*^{-/-} mice at both 16 and 43 days after birth (Fig. 3). *Pcca* protein was detected in the liver extracts from *Pcca*^{+/+} mice but not in the liver extracts from *Pcca*^{-/-} mice.

PCC activity detected in *Pcca*^{-/-} mice following gene transfer

In order to generate functional PCC enzyme, the human PCCA subunit expressed from the transgene must be able to form a heterocopolymer with the endogenous murine *Pccb* subunits. To determine if there was functional PCC enzymatic activity following AAV8-PCCA treatment, we measured plasma 2-methylcitrate levels in both treated and untreated *Pcca*^{-/-} mice (Fig. 4). 2-Methylcitrate is increased in PA because deficient PCC enzymatic activity causes accretion of the PCC substrate, propionyl-CoA (Fig. 1). The accumulating propionyl-CoA then condenses with oxaloacetate via the action of citrate synthase to form 2-methylcitrate. Therefore, increasing the amount of PCC enzymatic activity in the *Pcca*^{-/-} mice by gene transfer should diminish the concentration of propionyl-CoA and cause a corresponding drop in 2-methylcitrate levels. We observed a significant ($p=0.01$) decrease of plasma 2-methylcitrate ($12.4\mu\text{M}$) levels in the treated *Pcca*^{-/-} mice ($n=9$) at 21–28 days post-treatment in comparison with plasma 2-methylcitrate ($39.8\mu\text{M}$) levels in the untreated *Pcca*^{-/-} mice ($n=5$) at 1 day of life (Fig. 4), verifying that an increase in PCC enzymatic activity occurred after AAV8-PCCA gene transfer. However, the plasma 2-methylcitrate levels of the treated *Pcca*^{-/-} mice did not normalize to the levels (709 nM) seen in control mice (*Pcca*^{+/+} and *Pcca*^{+/-}). In addition, these treated *Pcca*^{-/-} mice exhibit an increased ability to oxidize propionate in comparison to *Mut*^{-/-} mice that also have a block in the same pathway (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/hum). The ability to oxidize propionate depends upon the presence of PCC activity (Fig. 1).

Discussion

Our previous gene therapy studies on MMA provided a rational framework for the experimental approaches under-

taken here to establish a viable method of gene transfer for patients with PA. We were able to rescue greater than 64% of the *Pcca*^{-/-} mice from neonatal death with a single intrahepatic injection of 1×10^{10} GC of AAV8-PCCA at birth. More than 40% of the treated *Pcca*^{-/-} mice have survived for more than 2 months. The oldest AAV8-PCCA-treated *Pcca*^{-/-} mice were 6 months old at the time of this report. The treated *Pcca*^{-/-} mice demonstrate robust expression of the PCCA protein and show significant reduction in the circulating levels of a disease-related biomarker, 2-methylcitrate. Our experiments demonstrate an AAV gene delivery approach that could be used as a possible treatment for PA, a severe organic acidemia that is frequently recalcitrant to medical management.

The murine models of both MMA and PA display neonatal lethality, and it is likely that other animal models lacking proteins required for propionyl-CoA oxidation will have a similar phenotype. Although AAV gene transfer affords a dramatic rescue of the lethal phenotype in murine models of MMA and PA, elevated levels of metabolites characteristic of the disorders in humans persist in the corrected mutant mice after gene transfer. Prolonged exposure to these metabolites is believed to contribute to the multi-systemic ailments that afflict the patients. Thus, minimal AAV-mediated rescue could provide a means to study the pathophysiology of the chronic human conditions, which would not otherwise be possible using a neonatal lethal animal model.

Propionyl-CoA oxidation involves several proteins, including PCCA, PCCB, MUT, MMAA and MMAB, and D-methylmalonyl-CoA epimerase (Fig. 1). A deficiency in any one of these enzymes impedes the conversion of propionyl-CoA to succinyl-CoA and results in a severe metabolic disease in humans. The successful long-term rescue of both murine models of MMA (*Mut* knock-out) and PA (*Pcca* knock-out) with AAV indicates that gene transfer for all genetic defects in the conversion of propionyl-CoA to succinyl-CoA would also be amenable to gene therapy.

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Author Disclosure Statement

No competing financial interests exist.

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