Minimal ureagenesis is necessary for survival in the murine model of hyperargininemia treated by AAV-based gene therapy

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INTRODUCTION

Urea is the major end-product of nitrogen metabolism in humans and terrestrial animals. The biochemical pathway required for its synthesis consists of six enzymes and two membrane transporters.1 Deficiencies in each of these proteins, or the urea cycle defects, have been identified. Each genetic defect gives rise to a discrete clinical and biochemical phenotype.1,2

Arginase deficiency is an autosomal recessive distal urea cycle disorder, resulting from a loss of arginase 1 (ARG1) activity. ARG1 deficiency typically presents in late infancy to the second year with a syndrome of microcephaly, spasticity, seizures, clonus, loss of ambulation and failure to thrive associated with hyperargininemia.3 Profound hyperammonemia is uncommon. Neonatal presentations are rare and often are associated with severe hyperammonemia.4,5 Long-term therapy for arginase deficiency involves a low-protein diet and administration of sodium benzoate and sodium or glycero phenylbutyrate. These dietary and pharmaceutical interventions can partially alleviate hyperargininemia; however, no completely effective therapy is available.

We have developed a gene therapy approach for ARG1−−/− animals. Our approach uses an adeno-associated viral (AAV) vector expressing murine ARG1 administered intravenously on the second day of life.6−8 We utilized AAV driven by the ubiquitously expressing chicken β-actin promoter/cytomegalovirus enhancer or the liver-specific promoter thyroxine-binding globulin, resulting in hepatic expression of ARG1. However, we observed a substantial loss of AAV vector genomes and consequent low levels of hepatic arginase activity because of frequent hepatocyte cell division during early life.9,10 Arginase activity can be detected by a colorimetric enzymatic assay, resulting in the production of urea; however, when the level of arginase expression is low, exact activity is difficult to quantify by this method.

The measurement of urea production via isotopic methods using metabolic tracers has had an important role in studying urea cycle flux in patients with defects in the urea cycle.11 Such studies could also be used to evaluate the efficacy of therapeutic interventions, in this case, AAV-based hepatic gene transfer in the treatment of arginase deficiency in a murine model. This would allow us to assess residual ureagenesis and define the effects of hepatic arginase expression in arginase-deficient mice.
minimal activity necessary for control of plasma ammonia, survival, longevity and normal cognitive development in these animals. The kinetic approach also avoids limitations of the conventional enzyme assay, which result from extremely low arginase activity. Furthermore, knowing the amount of activity that is necessary for survival and urea cycle functioning in arginine-deficient animals may be important in planning future human interventions.

In these studies, we used mass spectrometry to monitor the in vivo synthesis of [13C] urea following administration of [1-13C] acetate. Hepatic mitochondria, the location of the first steps of ureagenesis, quickly convert labeled [1-13C] acetate to H13CO3 in the tricarboxylic acid cycle. Some of the metabolite then becomes substrate for carbamoyl phosphate synthetase, yielding [13C] carbamoyl phosphate, which the urea cycle converts to [13C] urea. The remainder of the H13CO3 appears as 13CO2 in blood and in exhaled air. By measuring [13C] urea in blood of the mice, determination of the urea pool turnover is possible. This method of investigation has provided us with an assessment of how well the urea cycle is functioning in stable adult ARG1−/− animals treated by AAV-based liver-specific gene therapy. The purpose of this report is to present the outcomes and potential implications of this study.

RESULTS

Animal survival
Survival of animals undergoing interventions versus no therapy was performed. We observed no difference in survival of AAV-treated ARG1−/− mice versus littermate controls at the time of study (P = 0.43). Animals were euthanized at the completion of these studies. As expected, all untreated knockout animals perished before weaning (Figure 1) by about 2 weeks of life (P < 0.0001 when compared with AAV-treated ARG1−/− mice or controls).

Liver examination
We previously noted that AAV-treated animals were lean compared with their littermate controls. We therefore compared liver weights between the groups and found that this parameter, as a proportion of body weight, differed between groups with treated ARG1−/− livers being about 27% heavier than that of littermate controls. AAV-treated ARG1−/− mice had livers that weighed 5.45 ± 0.53 grams (n = 10) compared with that of littermate controls with 4.30 ± 0.30 g (n = 10) (P = 0.00001). In addition, gross examination of liver showed a tan color in the AAV-treated ARG1−/− and a reddish brown color in controls (Figures 2a and b). The organs were soft without evidence of cirrhosis and were indistinguishable from controls when examined by palpation alone.

Plasma ammonia and biochemical analysis
To determine in part how well the urea cycle was functioning, plasma ammonia concentration was measured before the administration of [1-13C] acetate at time 0. Mice were studied at 10–12 weeks of age. Baseline plasma ammonia concentration did differ between the two groups with AAV-treated ARG1−/− mice having circulating ammonia levels ~85% higher than that of littermate controls. Littermate controls (n = 10) had plasma ammonia concentrations on average of 104.03 ± 46.27 μmol l−1, whereas plasma ammonia concentrations in AAV-treated knockout mice (n = 9) were 192.35 ± 82.12 μmol l−1 (P = 0.01) (Figure 3).

We also noted changes in amino-acid levels before and after acetate administration (Table 1). AAV-treated ARG1−/− mice showed higher levels of plasma glutamine at baseline, but both littermate controls (n = 11) and AAV-treated ARG1−/− animals (n = 11) responded to acetate injection with a comparable (>20%) decline in the plasma glutamine concentration. Similarly, littermate controls (n = 11) showed a decline (>20%) of blood arginine; in contrast, acetate treatment evoked a slight increase (~3%) of blood arginine in AAV-treated ARG1−/− mice (n = 11). Similarly, citrulline declined in the littermate controls (n = 11) and increased in AAV-treated mice (n = 11). The AAV-treated animals (n = 6) demonstrated hypoorinthinemia compared with the litters (n = 11); circulating plasma ornithine in the AAV-treated ARG1−/− mice at baseline was only 57% of littermate controls. Plasma ornithine in the littermate controls demonstrated a decline of ~17% 30 min after acetate administration; however, ornithine only declined ~4% in the AAV-treated mice after acetate administration.

Hepatocellular arginase activity and ureagenesis
Our prior investigations indicated that hepatic arginase expression declined with animal growth, as AAV episomes were lost consequent to hepatocellular division. We examined hepatic arginase activity in littermate controls and AAV-treated ARG1−/− mice in these studies. Immunohistochemistry demonstrated scattered arginase-positive cells throughout the liver parenchyma (Figures 2c and d) compared with littermate controls; no collagen deposition consistent with fibrosis was present. Arginase expression was widespread in the latter animals. Ureagenesis was examined in AAV-treated ARG1−/− mice (n = 11) and compared with littermate controls (n = 11) by [1-13C] acetate administration. The findings demonstrated that the rate of ureagenesis is about 3.3% of control animals (Figure 4).

DISCUSSION

Urea is the major end-product of mammalian nitrogen metabolism. The ARG1 single point mutation in our mouse model results in no residual enzyme activity in the liver. We showed previously that ARG1 knockout mice were able to survive and have normal learning and cognitive function as adults with reduced hepatic enzyme activity after neonatal administration of an AAV vector expressing ARG1. However, in the studies conducted herein, we have demonstrated that the livers of the AAV-treated ARG1−/− mice weigh more than the littermate controls. Our group has previously demonstrated that hepatocytes from the ARG1−/− mice are two- to threefold larger than that of controls, suggesting that hepatocytes are undergoing hypertrophy and this may be the
reason why the organ weights from these animals were greater. The hepatocytes also have been demonstrated to contain a variety of intracytoplasmic inclusions, including large, well-defined, round or oval eosinophilic inclusions. 12

A key question is how much hepatic arginase activity is needed to normalize ureagenesis and allow survival of knockout mice. We addressed this problem by utilizing the stable isotope method to measure in vivo flux through the urea cycle. Our extensive experience with this approach has demonstrated its utility in human investigations. 11,13–17 It is important to understand the relationship between flux and enzyme activity in order to apply gene transfer technology to human trials and has been used in these studies to answer this question.

Elevated plasma glutamine, a finding common in most urea cycle disorders, also is increased in the AAV-treated ARG1−/− mice. This abnormality reflects the tendency to hyperammonemia in animals with a limited number of arginase-expressing cells in the liver and elsewhere. 6,8 These animals also are more vulnerable to administration of ammonium chloride, as previously demonstrated. 7 The administration of acetate to these animals and littermate controls caused a decline (~20%) of blood glutamine concentration, perhaps denoting an increase of N-acetylglutamate (synthesized from acetyl CoA and L-glutamate), an obligatory effector of carbamoyl phosphate synthetase 1, the initial step of the urea cycle. 18 The lowered blood glutamine also could reflect a sudden release of insulin. 19

Hepatic mitochondria convert CH313COO− to H13CO3 in the tricarboxylic acid cycle and then condense the latter with NH3 to carbamoyl phosphate, which in mitochondria combine with ornithine to yield citrulline. In the littermate controls, we detect a decline of circulating ornithine of about 18% as it is consumed in the generation of citrulline. Progressing through the urea cycle, plasma citrulline and arginine concentrations also decline (here ~22% from baseline for both) as arginase acts on arginine to generate [13C] urea and also regenerate ornithine.

However, in the AAV-treated ARG1−/− mice, a different biochemical picture emerges. Plasma arginine and citrulline levels are high because limited hepatic arginase enzymatic activity restricts regeneration of ornithine from arginine. Indeed, baseline ornithine is only ~50% of the value in controls. Although plasma ornithine does decline with the administration of acetate as in the littermate controls, the magnitude is substantially less, only about 4% from baseline. Conversely and unlike the littermate controls, plasma arginine and citrulline concentrations increase slightly as, we speculate, that there is more flux into the proximal urea cycle due to acetate administration, likely due to the limited hepatic arginase activity. Thus, the limited amount of arginase activity allows animal survival, 6 control of circulating ammonia and development of a normal central nervous system. 7 However, the metabolic defect still is associated with an increase in hepatic cell mass as seen in the AAV-treated ARG1−/− mice.

Although the findings described may have important implications for arginase deficiency, they are likely not broadly applicable to other disorders of the urea cycle, particularly the proximal disorders. Studies conducted with the ornithine transcarbamoylase deficient (spf) 20,21 and arginosuccinate synthetase deficient 22 murine models demonstrate that the residual expression of enzyme in hepatocytes after AAV administration in the neonatal period are insufficient to prevent hyperammonemia and death. However, in the studies conducted herein the measurement of ureagenesis in these AAV-treated ARG1−/− mice has now allowed for an improved understanding of the relationship between the
in that adult-onset model. 23 The current studies suggest that numbers of episomal AAV vector DNA, 6,8,9 a small amount remains following a single dose of neonatal AAV-expressing arginase 1. Our previous studies have demonstrated that arginase activity serves to provide minimal ureagenesis compared with residual arginase activity and in vivo metabolic competency, following a single dose of neonatal AAV-expressing arginase 1. The findings from these studies have several important implications and raise further questions. First, with the loss of large numbers of episomal AAV vector DNA, a small amount remains that serves to provide minimal ureagenesis compared with controls. Our prior studies of a conditional knockout of arginase 1 supported this concept and demonstrated that arginase activity of as little as 9% of controls appeared to be necessary for survival in that adult-onset model.23 The current studies suggest that <5% of control activity leads to the normal development in neonatal onset arginase de ciency; however, the urea cycle still is not functioning normally and the animals do remain vulnerable to a nitrogen load,7 but this is sufficient to provide long-term survival.

Second, plasma ornithine concentration remains low in the AAV-treated ARG1−/− mice. The question arises as to whether the administration of ornithine to AAV-treated ARG1−/− mice will improve function by increasing nitrogen flux into the urea cycle. Studies will commence to address this question; however we postulate that this treatment may improve proximal urea cycle function, thus, leading to lower circulating plasma ammonia. However, this will not address the limited arginase capacity provided by the relatively small number of arginase-expressing hepatocytes. Furthermore, the urea cycle takes place in the perportal hepatocytes and expression of arginase in hepatocytes by AAV-based expression is heterogenous in its location.

A final question is whether an improvement in urea cycle function will occur with a second administration of AAV-expressing arginase 1. Our previous studies have demonstrated that operational tolerance is established to transgene-encoded proteins with neonatal administration of AAV-expressing vectors in mice.6,10 Immunologic ignorance appears to be an important mechanism for lack of an immune response to AAV when administered in the neonatal period to mice (unpublished data); these fi ndings and that of others22 suggest that these animals would benefi t from readministration of AAV after adult organ size has been reached, typically by about 5–6 weeks of life.6 As an associated decline in episomal copy number would be expected in the human liver (albeit over a longer period than the murine liver where the rate of doubling is much more rapid), such questions will be important to address as therapies for arginase defi ciency and other metabolic disorders with AAV-based therapy are considered for clinical application.

MATERIALS AND METHODS

Materials

Sodium [1-13C] acetate (98.0 atom% excess) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Reagents for derivatization of urea and amino acids were from Pierce Chemical Co. (Rockford, IL, USA).

Mouse procedures

The targeted ARG1 allele contains a deletion in exon 4 of the ARG1 gene and were generated12 and backcrossed to achieve a homogeneous NIH-Swiss strain background as described previously.6 All mice were housed...
under specific pathogen-free conditions with food and water provided ad libitum. Mice were kept according to the National Institutes of Health guidelines and all experimental procedures were conducted in accordance with guidelines for the care and use of research animals at our institution. Arginase-deficient newborn pups on the second day of life were injected with 1.0 x 10^11 gc kg^-1 of AAVrh10 thyroxine-binding globulin-mArg1 (codon-optimized) diluted in pharmaceutical grade saline by the superficial facial vein. The injections were performed in a total volume of 50 µL. Mice aged 10–12 weeks were studied. Mice were fed standard mouse chow (Labdiet/PMI Nutrition International, St. Louis, MO, USA, (Picolab Rodent diet 20, Catalogue Number 5053)). At 8 am of the day of study, chow was removed. At 11 am, baseline blood was collected followed by intraperitoneal administration of 1% [1-13C] acetate (5.4 µg g^-1 weight) prepared in sterile pharmaceutical grade saline in a total volume of 500 µL. Thirty minutes later, blood was collected and the animals were euthanized. Plasma and serum were frozen immediately and stored at −80°C until analysis. Whole livers were removed and weighed.

PCR genotyping
Genomic DNA was prepared from tail tip by standard methods, and genotyping by PCR was performed as previously described.6

Biochemical analysis of serum
The concentration of amino acids was determined by high-performance liquid chromatography, utilizing pre-column derivatization with o-phthalaldehyde as previously described.7,8

Ammonia analysis of serum
Ammonia was determined in serum samples by reductive amination of 2-oxoglutarate and oxidation of nicotinamide adenine dinucleotide phosphate, using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA) using 10 µL for each sample tested. Results are presented as mean ± s.d.

Immunohistochemistry for arginase expression
The livers were removed from euthanized animals and placed in 4% paraformaldehyde for 18–24 hours. Immunohistochemistry was performed as previously described.6

Sodium 1-13C acetate loading study
The studies were performed in the morning after food was removed. Baseline samples of blood were collected as well as 30 min samples. Specimens were centrifuged at 4°C to isolate plasma. The measurement of [13C] urea was performed as previously described,10 with appropriate adjustment of volumes based on quantity of mouse plasma.

Statistical analysis
Survival curves were computed in each group of mice using the Kaplan–Meier method and compared across groups using the log-rank test. Calculations were made using software JMP PRO version 11 (SAS, Cary, NC, USA). t-test was utilized for other statistical analyses for other comparisons. P-values of < 0.05 were considered significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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