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Lethal phenotype in conditional late-onset arginase 1 deficiency in the mouse

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ABSTRACT

Human arginase deficiency is characterized by hyperargininemia and infrequent episodes of hyperammonemia, which lead to neurological impairment with spasticity, loss of ambulation, seizures, and severe mental and growth retardation; uncommonly, patients suffer early death from this disorder. In a murine targeted knockout model, onset of the phenotypic abnormality is heralded by weight loss at around day 15, and death occurs typically by postnatal day 17 with hyperargininemia and markedly elevated ammonia. This discrepancy between the more attenuated juvenile-onset human disease and the lethal neonatal murine model has remained suboptimal for studying and developing therapy for the more common presentation of arginase deficiency. These investigations aimed to address this issue by creating an adult conditional knockout mouse to determine whether later onset of arginase deficiency also resulted in lethality. Animal survival and ammonia levels, body weight, circulating amino acids, and tissue arginase levels were examined as outcome parameters after widespread *Cre*-recombinase activation in a conditional knockout model of arginase 1 deficiency. One hundred percent of adult female and 70% of adult male mice died an average of 21.0 and 21.6 days, respectively, after the initiation of tamoxifen administration. Animals demonstrated elevated circulating ammonia and arginine at the onset of phenotypic abnormalities. In addition, brain and liver amino acids demonstrated abnormalities. These studies demonstrate that (a) the absence of arginase in adult animals results in a disease profile (leading to death) similar to that of the targeted knockout and (b) the phenotypic abnormalities seen in the juvenile-onset model are not exclusive to the age of the animal but instead to the biochemistry of the disorder. This adult model will be useful for developing gene- and cell-based therapies for this disorder that will not be limited by the small animal size of neonatal therapy and for developing a better understanding of the characteristics of hyperargininemia.

1. Introduction

Arginase deficiency is a rare single-enzyme liver defect in which arginase 1 (ARG1) is lost. This is the final enzyme in the urea cycle, the major pathway for the detoxification of ammonia in mammals. In the liver, *ARG1* hydrolyzes arginine into ornithine, which re-enters the urea cycle as urea and then is excreted as waste. Multiple heterogeneous missense and nonsense mutations and deletions, resulting in an inability to detoxify ammonia and an elevation in arginine, have been described in diverse human populations [1–8]. Variability in penetrance is seen: affected children can present neonatally with severe hyperammonemia [4,9], but ARG1 deficiency usually presents in late infancy. The clinical presentation of hyperargininemia is quite distinct from those of other urea cycle disorders, and the occurrence of hyperammonemic encephalopathic episodes is uncommon. It usually presents with an insidious

Abbreviations: AAV, adeno-associated virus; ANOVA, analysis of variance; ARG, arginase; HPF, high-power field; OAT, ornithine amino transferase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline.

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onset and manifests with a progressive neurological syndrome with microcephaly, spasticity, seizures, clonus, spastic diplegia, and failure to thrive [10] and rarely [4,9], if ever, is fatal in the perinatal or neonatal period.

The neurologic manifestations seen in arginase deficiency may arise from the accumulation of metabolites of arginine (that is, guanidino compounds) or may result from hyperargininemia itself; the exact cause and the proximate neurotoxin or mechanism of injury to the brain that causes the progressive neurological deterioration and mental retardation are not known. The neurologic impairment and developmental regression are associated with corticospinal [11] and pyramidal tract deterioration; despite having severe developmental delay, affected children typically avoid the catastrophic hyperammonemic crises characteristic of the other single-enzyme liver defects and therefore tend to survive into adulthood [12]. The lack of frequent episodes of hyperammonemia is likely but unproven due to an increase in the second form of arginase, arginase 2, which compensates for the lack of arginase 1 [12]; this enzyme is expressed in extrahepatic tissues, mainly in the kidney and brain [12,13]. Long-term therapy rests on the provision of a low-protein diet and administration of sodium benzoate and sodium phenyl butyrate. However, these therapies are incomplete and only palliative and not curative.

To model arginase deficiency, we previously generated ARG1-deficient mice in our laboratory by replacing the active site of the *ARG1* gene, located on exon 4, with the neomycin resistance gene [14]. No *ARG1* RNA was found on Northern blot nor was any cross-reacting material detected. These mice completely lack liver arginase 1 activity and therefore replicate the severe, neonatal human phenotype. In contrast to the juvenile-onset disease in which human patients can survive into adulthood, our arginase-deficient NIH-Swiss mouse model had a mean survival of 17 days, and the longest-surviving animal died at 21 days of life [15]. Biochemically and behaviorally severe hyperammonemia, as measured by both serum assay [15] and cognitive testing [16], was evident. Plasma ammonia levels of untreated ARG1-deficient mice were increased more than 10-fold, and serum arginine levels were markedly elevated [14]; their livers were abnormal and had histopathologic features similar to those seen in human arginase-deficient patients who died from hyperammonemia. We were able to palliate the disease by delivery of the arginase gene by using an adeno-associated virus (AAV) vector shortly after birth [15,16]; hepatic arginase levels rose to a sufficient level to metabolize arginine and prevent hyperammonemia and death. Treated animals survived to one year before euthanasia [16].

However, despite these encouraging results, no effective animal model replicating the later-onset juvenile arginase deficiency phenotype exists nor does an adult model in which alternative methods of therapy may be examined. To study the pathophysiology of arginase deficiency systematically in an adult animal model of the disease, we induced widespread arginase 1 deficiency in adult animals by using a conditional arginase 1 knockout mouse strain [17]. The goals of the present studies were to conditionally delete the arginase gene in adult mice *via* gavage administration of tamoxifen and investigate whether the human neonatal or juvenile phenotype would be replicated in adult animals and to define an adult animal model that could be used to develop therapeutic approaches for this disorder, which currently has no adequate treatment.

2. Materials and methods

2.1. Mouse procedures

We generated mice that were hemizygous for a widespread *Cre*-recombinase (UBC-cre/ERT2; The Jackson Laboratory, Bar Harbor, ME, USA) with homozygosity for the Lox-P Arg1 insertion (at exons 7 and 8 of the *ARG1* gene [17] [floxed]). By backcrossing and further mating, we produced a homozygous inducible gene deletion of arginase 1 with tamoxifen as the inducing agent. All mice were housed under specific pathogen-free conditions; chow (PicoLab Rodent Diet 20 [5053], calories 23.55% protein, 11.922% fat, and carbohydrate 64.528%; LabDiet, St. Louis, MO, USA) and water were provided *ad libitum*. All mice were kept in accordance with 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. Scheduled blood sampling was taken from the retro-orbital plexus. Serum was frozen immediately and stored at −80 °C until analysis. Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) (20 mg/mL) in corn oil was administered by gavage. Mice were euthanized by isoflurane, and blood and tissues were collected. Tissues were snap-frozen for later analysis and also placed in 4% paraformaldehyde for immunohistochemistry.

2.2. Polymerase chain reaction genotyping

Genomic DNA was prepared from the tail tip by standard methods. Anion-exchange column-purified genomic DNA was subjected to polymerase chain reaction (PCR) for genotyping. Primer sets for our conditional arginase knockout (JAX strain C57BL/6-Arg1tm1Pmu/J) were oIMR9556 forward TGC GAG TTC ATG ACT AAG GTT and oIMR9557 reverse AAA GCT CAG GTG AAT CGG. PCR had the following cycle conditions: denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min for 35 cycles by using DNA polymerase (Takara, Mountain View, CA, USA, catalog number RR006A). Genotype for conditional *Cre*-recombinase was also confirmed by using the following primers: (JAX strain Tg(UBC-cre/ERT2)1Ejb/J): transgene primer forward 1084: GCG GTC TGG CAG TAA AAA CTA TC; transgene primer reverse 1085: GTG AAA CAG CAT TGC TGT CAC TT; internal positive control forward 7338: CTA GGC CAC AGA ATT GAA AGA TCT; and internal positive control reverse 7339: GTA GGT GGA AAT TCT AGC ATC ATC C. Cycle parameters were denaturation at 94 °C for 30 s, annealing at 51.7 °C for 1 min, and elongation at 72 °C for 2 min for 35 cycles. To confirm that lox P sites were intact, conditional knockout primers and conditions were developed: forward primer 5′-ACC TGC TGG GAA GGT ACG ATG CT-3′ and reverse primer 5′-AGG CTA CCT CTC TGG ATA CCT TTG C-3′. Cycle parameters were denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min for 30 cycles (1100-base pair amplicon product).

2.3. Biochemical and ammonia analysis of serum

Serum amino acid analysis was performed on a Biochrom 30 HPLC amino acid analyzer (Biochrom Ltd., Cambridge, UK). In brief, 30 to 50 μL of serum was mixed with equal volumes of Biochrom Seraprep and Lithium dilution buffer. Protein was precipitated by centrifugation, and 10 μL of supernatant was injected into the analyzer. Physiological amino acid standard (Sigma-Aldrich) was used to calibrate and determine analyte concentration. Analysis was performed by using EZchrom Elite software (Agilent Technologies, Santa Clara, CA, USA). Ammonia was determined in serum samples, by reductive amination of 2-oxoglutarate and oxidation of NADPH, by employing a commercial kit (Sigma-Aldrich, catalog number AA0100) and using 20 μL of serum for each sample tested. Determination of guanidino compound levels and urea was accomplished as previously described [16].

2.4. Biochemical analysis of liver tissue

Tissue amino acid levels were measured in tamoxifen-treated conditional arginase knockout mice before (baseline) and after (experimental) administration. For each sample, the tissue was weighed and homogenized in water to a concentration of 0.2 g/mL. The supernatant was then removed and sonicated, 1 mL was removed, and 70 mg of sulfosalicylic acid was added. The samples were then centrifuged.