Molecular Genetics and Metabolism Reports 9 (2016) 54–60

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

Liver-specific knockout of arginase-1 leads to a profound phenotype similar to inducible whole body arginase-1 deficiency

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ARTICLE INFO

Article history:
Received 15 September 2016
Accepted 5 October 2016
Available online xxxx

Keywords:
Arginase
Liver
Hepatocyte
Urea cycle
Gene therapy
Inducible knockout mice

ABSTRACT

Arginase-1 (Arg1) converts arginine to urea and ornithine in the distal step of the urea cycle in liver. We previously generated a tamoxifen-inducible Arg1 deficient mouse model (Arg1-Cre) that disrupts Arg1 expression throughout the whole body and leads to lethality ≈2 weeks after gene disruption. Here, we evaluate if liver-selective Arg1 loss is sufficient to recapitulate the phenotype observed in global Arg1 knockout mice, as well as to gauge the effectiveness of gene delivery or hepatocyte transplantation to rescue the phenotype. Liver-selective Arg1 deletion was induced by using an adeno-associated viral (AAV)-thyroxine binding globulin (TBG) promoter-Cre recombinase vector administered to Arg1 "floxed" mice; *Arg1*$^{fl/fl}$). An AAV vector expressing an Arg1-enhanced green fluorescent protein (Arg1-eGFP) transgene was used for gene delivery, while intrasplenic injection of wild-type (WT) C57BL/6 hepatocytes after partial hepatectomy was used for cell delivery to "rescue" tamoxifen-treated Arg1-Cre mice. The results indicate that liver-selective loss of Arg1 (>90% deficient) leads to a phenotype resembling the whole body knockout of Arg1 with lethality ≈3 weeks after Cre-induced gene disruption. Delivery of Arg1-eGFP AAV rescues more than half of Arg1 global knockout male mice (survival >4 months) but a significant proportion still succumb to the enzyme deficiency even though liver expression and enzyme activity of the fusion protein reach levels observed in WT animals. Significant Arg1 enzyme activity from engrafted WT hepatocytes into knockout livers can be achieved but not sufficient for rescuing the lethal phenotype. This raises a conundrum relating to liver-specific expression of Arg1. On the one hand, loss of expression in this organ appears to be both necessary and sufficient to explain the lethal phenotype of the genetic disorder in mice. On the other hand, gene and cell-directed therapies suggest that rescue of extra-hepatic Arg1 expression may also be necessary for disease correction. Further studies are needed in order to illuminate the detailed mechanisms for pathogenesis of Arg1-deficiency.

1. Introduction

In the urea cycle, arginase-1 (Arg1) catalyzes the distal step in the conversion of ammonia to urea. Patients with the genetic disorder of Arg1 deficiency display a unique phenotype relative to other urea cycle disorders, consisting of hyperargininemia and neurological deficits including some or all of the features of spastic diplegia, seizures, intellectual disability and failure to thrive [1,2]. Mouse models to study the human disorder have been created and all recapitulate the main biochemical feature of hyperargininemia [3–6]. However, the global Arg1 knockout (KO) exhibits a much more profound phenotype compared to the human disorder with all mice dying approximately 2 weeks after birth, presumably due to hyperammonemia [3]. To circumvent the neonatal lethality, three groups have generated similar, but not identical, inducible Arg1 KO mouse models with several differences that have been described recently [1,4–6]. These mice also die invariably 2–3 weeks after gene disruption. There are some differences in interpretation of the mechanisms for lethality with contributing factors including a wasting phenotype and also hyperammonemia [1].

We [7] and others [8–11] have focused on strategies to rescue the lethal phenotype of inducible and global Arg1 KO mouse models. Gene therapeutic approaches work best but none have rescued every feature of the genetic deficiency [7,9–11]. Understanding the mechanism(s) for the pathological features of genetic Arg1 deficiency in order to develop effective therapeutic strategies is paramount. An important question that remains unresolved is if it is the loss of Arg1 expression in liver, where Arg1 expression is by far the most abundant, that drives the

Abbreviations: Arg1, arginase-1; Arg1-eGFP, arginase-1 enhanced green fluorescent protein; AAV, adeno-associated virus; TBG, thyroxine-binding globulin; gc, genome copies; WT, wild-type; ip, intraperitoneal.

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http://dx.doi.org/10.1016/j.ymgmr.2016.10.003

neurological phenotype in humans and lethal phenotype in mice, respectively, or if it is due to the loss of extra-hepatic Arg1 expression that is also contributing to the pathological sequelae.

Herein, we interrogate this question by generating liver-specific Arg1 KO mice, in combination with various gene therapeutic efforts and hepatocyte cell transplant rescue experiments. While we have not yet determined specific mechanisms, we demonstrate that tissue-specific Arg1 deficiency can be just as severe as global Arg1 absence and have raised novel questions to be addressed in the field of rare urea cycle disorder research.

2. Materials and methods

2.1. Liver-specific Arg1 knockout mouse model

Arg1 floxed mice (*Arg1*$^{fl/fl}$; >8 week old male) with loxP sites flanking exons 7 and 8 of the *Arg1* gene [12] were injected with high (1.5×10^{11} genome copies (gc)), medium (5×10^{10} gc) or low (1.5×10^{10} gc) doses of an adeno-associated viral (AAV)-thyroxine binding globulin (TBG) promoter-Cre recombinase vector (obtained from University of Pennsylvania Gene Therapy Core Services; AAV8.TBG.PI.Cre.rBG) via the intraperitoneal (ip) route to induce liver-specific Arg1 deficiency.

2.2. Global inducible Arg1 knockout mouse model

Arg1-Cre mice, originally obtained from the Jackson Laboratory and bred in-house, derived from parental strains *Arg1*^{flox} (JAX 008817, C57BL/6-*Arg1*^{tm1Pmu}/J) and CreER^{T2} (JAX 008463, B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J) (male, 12–16 weeks old) were injected ip on 5 sequential days with tamoxifen to induce global Arg1 deficiency as previously described [4,7]. All procedures were reviewed and approved by the Queen's University Animal Care Committee (Funk 2011-048) and conformed to the Guidelines of the Canadian Council on Animal Care. Unless otherwise specified, water and standard rodent chow (containing 21.8% protein, 9% fat, 2.2% fiber, 5% minerals by weight; PicoLab Mouse Diet 20 (5058)) were provided ad libitum. Specific signs of health deterioration due to arginase deficiency were present in mice, regardless of gender, approximately 24–48 h before exhibiting severe distress, which allowed for humane euthanization by CO_2 inhalation [4,7]. Humane endpoints were defined as body weight loss of >15% relative to the weight at the time of the final tamoxifen administration, accompanied by a slightly hunched posture. Some mice could lose substantial weight in a single day when approaching the endpoint resulting in some exceeding the 15% threshold.

2.3. Gene therapeutic delivery of Arg1-eGFP AAV vector

The AAV vector for transgene delivery has been previously described [7]. Briefly, the rh10 serotype vector expressing an Arg1-enhanced green fluorescent protein (Arg1-eGFP) transgene from a strong promoter (hybrid cytomegalovirus enhancer/chicken β-actin) was used in male 8 weeks old Arg1-Cre mice at 1.5×10^{11} gc injected two weeks prior to tamoxifen-induced Arg1 knockout to allow for adequate time for transgene expression.

2.4. Two-thirds hepatectomy and intrasplenic injection of C57BL/6 wild-type hepatocytes

Mice (female, >8 weeks old) were conditioned with retrorsine (Sigma-Aldrich; 70 mg/kg ip, twice at 2 week intervals), a cell cycle inhibitor, to block proliferation of native hepatocytes [13]. Two-thirds partial hepatectomy [14] was carried out 2 weeks after the last retrorsine dose to create a selective growth advantage for transplanted WT hepatocytes. Two million donor cells obtained from immunocompatible female C57BL/6 mice were suspended in 0.1 ml of media (high glucose DMEM supplemented with 15 mM HEPES (pH 7.4), 10% FBS and 100 nM dexamethasone) and injected slowly over 20–30 s into the lower pole of the spleen as described [13,14]. 15 weeks later, allowing sufficient time for donor cell engraftment, the usual tamoxifen-induced Arg1 knockout procedure was carried out.

2.5. Biochemical measurements and Arg1 enzyme activity assay

Blood was collected from the submandibular vein. A 3.2 mm circle from a dried blood spot sample collected on Whatman 903™ filter paper card was punched into the designated well of a 96-well plate. Sample preparation was based on the method described by Turgeon et al. [15] with minor modification. Briefly, amino acids were extracted using a methanolic solution containing the following isotope-labeled amino acid internal standards: ^{15}N,2-^{13}C-glycine, d4-alanine, d8-valine, d3-leucine, d3-methionine, ^{13}C6-phenylalanine, ^{13}C6-tyrosine, d2-ornithine, d2-citrulline and 5-^{13}C-d4-arginine-HCl (Cambridge Isotope Laboratories). After evaporation under nitrogen, the residue was derivatized using 3.0 N HCl in n-butanol at 60 °C for 20 min. Excess reagent was evaporated to dryness using nitrogen followed by reconstitution with 80% acetonitrile. A 10 μl portion of sample was subjected to analysis by mass spectrometry.

The analytical system used in this study consisted of a Waters TQ Detector equipped with electrospray ionization (ESI) source, Waters 1525 μ Binary HPLC Pump and a Waters 2777C Sample Manager (Waters). Tandem mass spectrometric (MS/MS) analysis of amino acids was achieved using a combination of selected reaction monitoring and neutral loss of mass to charge (m/z) of 102 scans, with the ESI source being operated in the positive ion mode.

Arg1 enzyme activity in liver homogenates was assayed as described previously [4,7]. One unit of activity is defined as 10 nmol urea/μg protein.

2.6. Western blot analysis

Liver tissues were homogenized in a solution containing T-PER solution and 1× HALT protease inhibitor cocktail (40 μl/mg tissue). Homogenates were further diluted in T-PER to a concentration of 1 mg/ml protein with 2× Laemmli buffer. Protein samples (20–30 μg) were subjected to Western Blot analysis. Proteins separated by electrophoresis in 10% TGX FastCast acrylamide gels (Bio-Rad) were transferred to PVDF membrane (Bio-Rad, TurboBlot system) and probed with rabbit polyclonal *anti*-Arg1 (C-terminal peptide; 1:10,000; Abcam #ab91279) and mouse monoclonal *anti*-α-tubulin (1:5000; Sigma #T5168) antibodies. Immunoreactive proteins were detected using HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:7500; Sigma) enhanced chemiluminescence signal. Digitized images were recorded with a FluorChem 8900 instrument (Alpha Innotech, San Leandro, CA). Some images underwent semi-quantitation with publicly available Image J software (Version 1.47, NIH).

2.7. Immunofluorescence and pathological analysis of liver sections

Liver sections (6 μm) were deparaffinized and rehydrated with toluene and ethanol by routine procedures. Antigen retrieval was performed by boiling slides in 10 mM citrate buffer, pH 6.0, 0.02% Tween-20 (Arg1 staining) or Tris/EDTA buffer, pH 9.0, 0.05% Tween-20 (CD4 staining). After PBS rinsing, sections were permeabilized with 1× PBS + 0.2% Triton X-100 for 10 min at room temperature then blocked with 2% normal goat serum (Cedarlane) in PBS for 30–45 min. Sections were incubated with rabbit polyclonal *anti*-Arg1 (1:100; Abcam #ab91279), rabbit monoclonal (EPR19514) anti-CD4 (1:250; Abcam #ab183685) in 1–2% goat serum, and mouse monoclonal *anti*-glutamine synthetase (1:200; Abcam #ab64613) in serum, for 1 h at room temperature or overnight at 4 °C. Secondary antibodies, goat *anti*-rabbit IgG FITC (Arg1; Cedarlane), goat anti-rabbit IgG (H + L)