Abcb7, the gene responsible for X-linked sideroblastic anemia with ataxia, is essential for hematopoiesis

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Abstract

X-linked sideroblastic anemia with ataxia (XLSA/A) is a rare syndromic form of inherited sideroblastic anemia associated with spinocerebellar ataxia, and is due to mutations in the mitochondrial ATP-binding cassette transporter Abcb7. Here, we show that Abcb7 is essential for hematopoiesis and formally demonstrate that XLSA/A is due to partial loss of function mutations in Abcb7 that directly or indirectly inhibit heme biosynthesis.
Introduction

X-linked sideroblastic anemia with ataxia (XLSA/A) is a rare syndromic form of inherited sideroblastic anemia associated an early onset, non- or slowly-progressive, predominantly truncal, spinocerebellar ataxia, accompanied by severe, selective cerebellar hypoplasia.\textsuperscript{1-5} The anemia of XLSA/A is typically mild and may be overlooked until after the neurological presentation. The peripheral blood and bone marrow findings resemble mild typical X-linked sideroblastic anemia (XLSA) due to mutations in ALAS2.\textsuperscript{6} Affected males have a mildly decreased, or low normal hemoglobin with a mild microcytosis and an increased red blood cell distribution width (RDW), often with erythrocyte dimorphism and Pappenheimer bodies. Bone marrow examination shows ringed sideroblasts.

Mutation analysis in three XLSA/A families has demonstrated $ABCB7$ missense mutations in each.\textsuperscript{1,2,4} $ABCB7$ is the human orthologue of the yeast mitochondrial ATP binding cassette (ABC) transporter Atm1p, which has been implicated in the transport of a component required for the maturation of cytosolic iron-sulfur (Fe-S) cluster proteins out of mitochondria; yeast lacking $ATM1$ ($\Delta atm1$) are deficient in the holo-forms of cytosolic Fe-S proteins, are respiratory deficient, and accumulate pathologic iron deposits in mitochondria akin to mitochondrial iron seen in ringed sideroblasts.\textsuperscript{7-9} Complementation assays in yeast suggest that each of the human mutations is a mild partial loss of function allele.\textsuperscript{1,2}
By using conditional gene targeting in mice, we previously demonstrated the essential nature of Abcb7 in the development of many tissues, and confirmed its role in cytosolic Fe-S protein maturation in mammals. Here, we show that Abcb7 is essential for hematopoiesis and formally demonstrate that the XLSA/A anemia is due to partial loss of function alleles.

Materials and Methods

The generation of a conditionally targeted (‘‘floxed’’) allele of Abcb7 (Abcb7\(^{fl}\)) has been described previously. A targeted point mutant allele was made by site directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) of the original gene targeting construct to create a glutamic acid to lysine mutation at position 433 (E433K) mutation of the mouse protein corresponding to the hematologically most severe, E433K, human allele. All exons and intron/exon boundaries were re-sequenced in the mutagenized targeting construct. Newly targeted, homologously recombined embryonic stem cell colonies from the 129S4/SvJae background were analyzed for the mutation by sequencing genomic PCR products. Similar phenotypic results were obtained from C57BL/6 chimeric animals produced from multiple independent ES cell lines with or without the Neo\(^R\) cassette.

C57BL/6J-Mx1-Cre animals were obtained from Jackson Laboratories (Bar Harbor, ME) and bred to the previously described Abcb7\(^{fl}\) allele (REF) on a mixed 129S4/SvJae x 129S6/SvEvTac background. F3 animals were used for most experiments. Induction of Cre expression was achieved in 4 day or 4-week-old animals by subcutaneous injection of 200\(\mu\)g or intraperitoneal injection of 400\(\mu\)g, respectively, of polyinosine-polycytosine
(pI-pC) on alternate days for a total of three doses. Clinically ill animals were humanely euthanized in accordance with guidelines accepted by the Animal Care and Use Committee at Children’s Hospital Boston. As a case control, a sex-matched, Mx1-Cre-negative animal injected with pI-pC was phenotyped in parallel with each moribund experimental animal. Complete blood counts, zinc protoporphyrin quantification, and Gpi1 isozyme chimerism analyses were performed as previously described.\textsuperscript{13}

For electron microscopy studies, whole blood was processed as previously described.\textsuperscript{14} Electron microscopic thin sections were examined with and without lead and uranyl acetate staining on an FEI/Phillips EM 208S (FEI Electron Optics BV, Eindhoven, Netherlands) equipped with an AMT (Danvers, MA) digital camera. Light microscopic images were acquired on a Nikon Eclipse E600 microscope with a 100x/0.30 oil immersion lens and an RT Slider SPOT 2.3.1 camera (Diagnostic Instruments) using SPOT Advanced software (v. 3.5.9).

\textbf{Results}

In order to evaluate the effect of \textit{Abcb7} deletion on hematopoiesis, we bred the \textit{Abcb7}\textsuperscript{fl} allele to the interferon-\textalpha-inducible \textit{Mx1-Cre} line. As previously reported,\textsuperscript{12} following induction, we found highly variable systemic deletion, including near complete rearrangement in the bone marrow and liver, no effect in testes or brain, and intermediate levels of rearrangement in all other tissues examined (Supplemental Figure 1). Serial peripheral blood smears demonstrated the presence of a transient population of siderocytes that peaked at day 5 in \textit{Abcb7}\textsuperscript{fl} + Cre animals (Figure 1A and C, and Supplemental Figure 2); siderocytes were not present in animals without \textit{Cre}. 
Transmission electron micrographs of day 5 peripheral blood showed a subset of reticulocytes from experimental animals with clusters of swollen, pale damaged mitochondria; electron dense intramitochondrial deposits, typical of siderocytes, were not seen (Figure 1E). However, treatment of whole blood with acid ferrocyanide iron stain prior to processing the cells for electron microscopy highlighted the inner and outer membranes of abnormal mitochondria in mutant cells (Figure 1G). Consequently, there is iron deposited in mitochondria insufficient to produce electron-dense deposits, but adequate to produce a detectable histochemical reaction. Marrow ringed sideroblasts were not present in either group, as assessed by light microscopy iron staining, during the entire time course (data not shown).

One to two weeks following the disappearance of siderocytes, Abcb7fl+Cre animals became clinically ill, with signs including lethargy, rubor, epistaxis, and rectal bleeding, necessitating euthanasia. The median survival of +Cre newborns was 16 days (Figure 2A and Supplemental Figure 3). Post-mortem gross and microscopic examination typically showed histological evidence of gastrointestinal, urological, and/or intracranial hemorrhage with or without microscopic evidence of systemic bacterial infection. Consistent with these findings, complete blood counts (CBCs) revealed severe pancytopenia (Figure 2B), and the bone marrow was markedly hypocellular (Figure 2D). CBCs taken from animals at day 11 of the induction protocol, prior to the onset of clinical signs, showed that the platelet and white blood counts were less than 25% of control values (Figure 2B). While there was partial or near complete gene deletion in tissues outside the hematopoietic system, apart from characteristic hepatocellular iron...
deposits fully described elsewhere\textsuperscript{10,11} we did not observe gross or microscopic phenotype in other tissues. It is possible that with time we would see other abnormalities, but the uniformly rapidly fatal bone marrow failure phenotype precluded their ascertainment.

In order to circumvent the bone marrow-dependent lethality, we created a targeted point mutant allele corresponding to the hematologically most severe human E433K mutation.\textsuperscript{2} Male chimeras were, however, infertile due to testicular atrophy and incomplete spermatogenesis associated with interstitial iron deposition (not shown), which is a phenotype not reported in human subjects with XLSA/A. In other non-hematopoietic tissues, despite substantial chimerism, we observed no gross, or histological abnormality. In particular, animals did not appear ataxic or otherwise neurologically compromised. In the blood, however, a population of siderocytes was present in proportion to the degree of chimerism as assessed by Gpi1 isozyme analysis (Figure 3A). Similar to the $\textit{Abcb7}^\textit{fl}$ $\textit{+Mx1-Cre}$ animals, abnormal mitochondria were readily seen in reticulocytes, however iron deposition could only be demonstrated by acid ferrocyanide enhancement (Figure 3B and 3C), and marrow ringed sideroblasts were not visualized by either method (data not shown).

Our ability to characterize the biochemical phenotype of the $\textit{Abcb7}^{E433K}$ allele was severely restricted by the unexpected male infertility, which precluded germline propagation. Nonetheless, analysis of chimeric blood demonstrated that the proportion of
protoporphyrin IX chelated with zinc (zinc protoporphyrin IX [ZPP]) compared with iron (heme) was increased in animals with a greater proportion of mutant RBCs (Figure 3D).

In toto, our data demonstrate that Abcb7 is essential not only for erythropoiesis, but hematopoiesis in general, and that the XLSA/A anemia phenotype is due to partial loss of function alleles in ABCB7. As in several other murine models of sideroblastic anemia, including vitamin B6 (pyridoxine) nutritional deficiency, isoniazid (INH) toxicity (REF), flexed-tail (f), and Sod2 deficiency, we did not observe pathologic mitochondrial iron deposits in nucleated erythroid precursors characteristic of sideroblastic anemias in humans in either the Abcb7fl + Mx1-Cre or the Abcb7E433K model; true sideroblasts have only been seen in murine erythroid precursors lacking Alas2, and even then associated with peculiar, diffuse cytosolic iron staining. This would further suggest that there is something biologically distinctive between human and murine precursors with respect to mitochondrial iron handling and/or toxicity. For example, it is possible that in mice, mitochondrial iron accumulation, the cessation of heme biosynthesis, and mitochondrial senescence occur later in comparison to morphologic maturation than in humans. Furthermore, as ferrochelatase is required for the formation of zinc protoporphyrin IX, the observation that the RBC ZPP/Heme ratio increases in proportion to the contribution of Abcb7E433K mutant cells would suggest that Abcb7 has an effect on heme biosynthesis independent of a negative effect on ferrochelatase. Rather, it would appear that Abcb7 loss of function directly or indirectly alters the availability of reduced iron required to synthesize heme from protoporphyrin IX.


References


Figures:

Figure 1: **Transient siderocytosis following Mx1-Cre mediated Abcb7 gene deletion.**

(A) Serial peripheral blood siderocyte counts in 5 representative Abcb7fl + Mx1-Cre animals induced at 4 days of age. Iron-stained peripheral blood smears of (B) Abc7fl – Mx1-Cre and (C) Abc7fl +Mx1-Cre. Arrowheads indicate cells with siderotic granules. Transmission electron micrographs of reticulocytes in Abc7fl animals ± Mxl-Cre (D) – Cre, conventional EM stain; (E) +Cre, conventional EM stain; (F) –Cre, EM Fe stain; (G) +Cre EM Fe stain. Arrows indicate a representative mitochondrion in each field. Note the increased electron density of the mitochondrial membranes in G. Electron micrographs 24,000X original magnification.
Figure 2: Abcb7 is essential for hematopoiesis. (A) Survival curve of newborn Abc7fl animals ± MX1-Cre induced with pI-pC. (■) –Cre, (◆) +Cre. (B) Peripheral blood parameters of animals in (A) at day 11 (open bars) and immediately pre-mortem (closed bars). Values represent the mutant value expressed as a percentage of the control value. Hb, hemoglobin; Retic; absolute reticulocyte count; WBC, white blood cells; PLT, platelets. Femoral bone marrow in 4-week-old Abcb7fl ± Mxl-Cre case controlled animals induced with pI-pC. (C) –Cre, (D) +Cre. Note extreme hypocellularity in (D).
Figure 3: An Abcb7\textsuperscript{E433K} mutation results in siderocytosis with increased zinc protoporphyrin. (A) Siderocytes in the peripheral blood of an Abcb7\textsuperscript{E433K} chimera (Fe stain). Transmission electron micrographs of Abcb7\textsuperscript{E433K} chimera reticulocytes (B) unstained (C) Fe stain. Original magnification 44,000x (D) RBC zinc protoporphyrin to heme ratio as a function of Abcb7\textsuperscript{E433K} chimerism measured by Gpi1 isozyme. Normal is typically <100 ng/µg for mice.
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