Dietary L-leucine improves the anemia in a mouse model for Diamond-Blackfan anemia

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Running title: Leucine improves the anemia in DBA mice
ABSTRACT

Diamond-Blackfan anemia (DBA) is a congenital erythroid hypoplasia caused by a functional haploinsufficiency of genes encoding for ribosomal proteins. Recently, a case study reported a patient who became transfusion-independent in response to treatment with the amino acid L-leucine. Therefore, we have validated the therapeutic effect of L-leucine using our recently generated mouse model for RPS19-deficient DBA. Administration of L-leucine significantly improved the anemia in Rps19-deficient mice (19 % improvement in hemoglobin concentration; 18% increase in the number of erythrocytes), increased the bone marrow cellularity and alleviated stress hematopoiesis. Furthermore, the therapeutic response to L-leucine appeared specific for Rps19-deficient hematopoiesis and was associated with downregulation of p53 activity. Our study supports the rationale for clinical trials of L-leucine as a therapeutic agent for DBA.
INTRODUCTION

Diamond-Blackfan anemia (DBA) is a congenital erythroid hypoplasia characterized by macrocytic anemia with selective absence of erythroid precursors, physical abnormalities and cancer predisposition\(^1,^2,^3,^4\). Mutations in genes encoding for ribosomal proteins (RP) have been identified in approximately half of the DBA patients\(^5,^6,^7,^8,^9,^10,^11\). *RPS19* is the most common DBA gene (25 % of the patients), the majority of the mutations resulting in RPS19 haploinsufficiency\(^12,^13\).

Corticosteroids and transfusion form the main therapeutic regimen in DBA\(^4\). However, these treatments involve risks for serious side effects, and a high proportion of deaths are treatment-related. Recently, based on the theory of inefficient translation as the underlying cause for the severe anemia\(^14\), Pospisilova *et al.* reported one patient who became transfusion-independent in response to treatment with the amino acid L-leucine\(^15\). Leucine is an essential amino acid that plays an important role in the regulation of protein synthesis, and this response seems to involve the mammalian target of rapamycin (mTOR) pathway\(^16\). Furthermore, on a systemic level dietary leucine affects multiple metabolic and signaling pathways\(^17\). In the current study we have investigated the therapeutic potential of L-leucine in DBA using our recently generated mouse model for RPS19-deficient DBA\(^18\). L-leucine treatment significantly improves the number of erythrocytes and hemoglobin concentration in Rps19-deficient mice, and is associated with reduced p53 activity in hematopoietic progenitors.
METHODS

Mice
Rps19 deficiency was induced by feeding the transgenic mice with doxycycline-containing food (Bio-Serv; 200 mg/kg doxycycline). L-leucine (Sigma; 1.5 % w/v) was administered in the drinking water. Mice were maintained at Lund University animal facility and all animal experiments were performed with consent from the Lund University animal ethics committee.

Blood and bone marrow cellularity
Peripheral blood was collected from the tail vein in Microvette tubes (Sarstedt) and analyzed using Sysmex XE-5000. Bone marrow cells were isolated by crushing both femurs and tibiae in PBS containing 2 % fetal calf serum (GIBCO), stained with Türk’s solution (Merck) and counted in a Bürker chamber.

Flow cytometry
For a full list of antibodies see Supplemental table 1. For immunophenotypic analysis, approximately 3 x10^6 cells were stained with antibodies for 30 minutes on ice in dark. For Phospho-flow cytometry, 0.5 x10^6 cells were stained for surface markers as above. BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD; 554714) was used for intracellular staining according to manufacture’s instructions. Experiments were performed on FACS Aria™ and FACSCanto™ II cytometers (Becton Dickinson) and analyzed using FlowJo software (Tree Star, v9.3.3).

Quantitative real-time PCR
Total RNA was isolated from FACS-sorted cells using RNAeasy micro kit (Qiagen) and cDNA transcribed with SuperScript III reverse transcriptase (Invitrogen). Real-time PCR reactions were performed using pre-designed assays (Applied Biosystems; Supplemental table 2) with the exception of Rps19 that was quantified using the SYBR GreenER™ system (5'
Statistical analyses
Student’s \( t \) test was used to determine statistical significance, and two-tailed \( P \) values are shown.

RESULTS AND DISCUSSION

To study the therapeutic potential of L-leucine we took advantage of our recently generated mouse model for RPS19-deficient DBA\(^1\). This model contains an \( Rps19 \)-targeting shRNA (shRNA-D) expressed by a doxycycline-responsive promoter located downstream of the \textit{Collagen A1} gene (Supplemental figure 1). Experimental animals were bred homozygous for the shRNA-D (D/D), and littermates without shRNAs were used as controls.

Administration of doxycycline to transgenic Rps19 knockdown mice results in a rapid reduction in bone marrow cellularity, and following this initial shock Rps19-deficient mice are partly able to compensate for the erythroid defect\(^1\). Monitoring the cellularity in blood and bone marrow during the first weeks after induction of the phenotype provides an excellent approach to test novel therapeutic agents enhancing Rps19-deficient hematopoiesis.

We chose to administer 1.5 % (w/v) L-leucine in the drinking water, a regimen that doubles the concentration of leucine in serum\(^1\), and simultaneously fed the mice with doxycycline-containing food pellets in order to induce Rps19 deficiency (Figure 1A). L-leucine administration had no significant effect on weight gain, and we observed no reduction in \( Rps19 \) knockdown efficiency (Figure 1B, Supplemental figure 2). After two weeks of doxycycline administration Rps19-deficient mice showed a reduction in erythrocyte numbers and the hemoglobin concentration, and both parameters were significantly improved upon L-leucine treatment (Figure 1C). Reticulocytes were slightly increased but not statistically
significant. The effect on white blood cells appeared modest, while platelet count was reduced in both Rps19-deficient and control mice (Supplemental figure 3). Additionally, L-leucine had no effect on erythroid recovery in wild-type mice after sublethal irradiation or phenylhydrazine-induced hemolytic anemia (Supplemental Figure 4).

The hypocellular bone marrow in Rps19-deficient mice was significantly improved upon L-leucine treatment (Figure 1D). Simultaneously, L-leucine treatment decreased the frequencies of hematopoietic stem and progenitor compartments (Figure 1E), as assessed by flow cytometry (Supplemental figure 5). Strikingly, the frequencies of all erythroblasts in Rps19-deficient mice were significantly reduced upon L-leucine treatment (Figure 1F). Based on the similar reticulocyte count in untreated and L-leucine-treated Rps19-deficient mice, these findings indicate that L-leucine enhances the differentiation of Rps19-deficient erythroblasts into fully functional erythrocytes. The spleen size and the frequencies of erythroid precursors in Rps19-deficient mice were similar to controls, and not affected by L-leucine treatment (Supplemental Figure 6).

Studies in animal models have demonstrated a stimulatory role of L-leucine on protein synthesis in multiple tissues, which has been linked to the mTOR pathway. We used flow cytometry to quantify the phospho-Rps6 and phospho-4E-BP1, two downstream components of mTOR kinase, in myeloid progenitors and erythroid precursors (Figure 2A). No difference was observed between cells from Rps19-deficient and control mice when evaluating these parameters, and this was not modulated by L-leucine treatment. However, the absence of apparent changes in mTOR activity does not exclude the possibility of enhanced protein synthesis. As an example, chronic supplementation of leucine in the drinking water led to increased protein synthesis in various tissues of rats without adaptive changes in mTOR.

p53 has been implicated as the key sensor of abnormal ribosome biogenesis, and we have shown that the deletion of Trp53 rescues Rps19-deficient hematopoiesis. L-leucine administration led to dampened expression of previously identified p53 transcriptional targets in Rps19-deficient
hematopoietic progenitors (Figure 2B)\textsuperscript{18}, demonstrating the reduced activity of p53 upon L-leucine treatment.

In conclusion, we demonstrate that the administration of L-leucine improves the anemia in a mouse model for RPS19-deficient DBA, and the therapeutic effect is associated with reduced p53 activity in hematopoietic progenitors. Our study thus supports the role of L-leucine as a therapeutic agent in the treatment of DBA.

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AUTHORSHIP

Contribution: S.K. conceptualized the project and directed the research; P.J., S.D. and K.O. performed the experiments. P.J., J.F., D.B. and S.K. analyzed the data and wrote the manuscript.

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REFERENCES


FIGURE LEGENDS

Figure 1 L-leucine administration improves the anemia in Rps19-deficient mice. (A) Experimental strategy to test the effect of L-leucine in vivo: Rps19 deficiency was induced by feeding transgenic mice with doxycycline-containing food pellets (200mg/kg), while L-leucine was simultaneously supplied in the drinking water (1.5 % w/v). (B) Quantitative real-time PCR analysis of Rps19 in erythroid (preCFU-E/CFU-E) and myeloid (preGM/GMP) hematopoietic progenitors 14 days after doxycycline and L-leucine administration. (n = 5, 11 and 10 for Control, [D/D] and [D/D]+L-leucine, respectively). (C) Erythrocyte number, hemoglobin concentration, mean corpuscular volume (MCV) and reticulocyte number on day 14 (n = 12, 10, 38 and 34 for Control, Control+L-Leucine, [D/D] and [D/D]+L-leucine, respectively). (D) Bone marrow cellularity (n = 17, 25 and 28 for Control, [D/D] and [D/D]+L-leucine, respectively) and (E-F) hematopoietic stem and progenitor cell, and erythroblast frequencies on day 14 (n = 17, 16 and 18 for Control, [D/D] and [D/D]+L-leucine, respectively). Error bars represent standard deviation. HSC, hematopoietic stem cell; MkP,
megakaryocyte progenitor; GMP, granulocyte-macrophage progenitor; CFU-E, colony-forming unit-erythroid; EB, erythroblast.

Figure 2 L-leucine administration has no effect on mTOR but dampens the p53 activity in hematopoietic progenitors. (A) Flow cytometric quantification of phospho-Rps6 (Ser235/236) and phospho-4E-BP1 (Thr37/46) in myeloid progenitors (CD11b+c-Kit+), proerythroblasts (Ter119lowc-Kit+CD11b−) and mature erythroid precursors (Ter119+CD11b−c-Kit+) on day 14 (n = 6, 3, 5 and 7 for Control, Control+L-Leucine, [D/D] and [D/D]+L-leucine, respectively). Mean fluorescence intensity (MFI) relative to that of isotype control is presented. (B) p53 transcriptional targets were quantified using real-time PCR in preCFU-E/CFU-E and preGM/GMP populations sorted from individual mice on day 14 (n = 5, 13 and 13 for Control, [D/D] and [D/D]+L-leucine, respectively). Error bars represent standard deviation.
**Figure 2**

A.

- Panel A shows dot plots for SSC-A vs FSC-A, CD11b vs Ter119, and c-Kit vs Ter119 with FITC labeling. Different line colors represent unstained, isotype control, p4E-BP1, and pRps6.

- Panels B1 and B2 show bar graphs for p4E-BP1 (Istotype) and pRps6 (Istotype) with CD11b+ c-Kit+ ProEB and EEs.

- Graphs in Panel C display relative expression for Cdkn1a, Zmat3, Phlda3, PIP4A3, Bax, and Ccng1 with bars showing preCFU-E / CFU-E and preGM / GMP.

- Statistical significance is indicated with *P < 0.05 and **P < 0.01.
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