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Effect of 5-aminolevulinic acid on erythropoiesis: A preclinical *in vitro* characterization for the treatment of congenital sideroblastic anemia

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ABSTRACT

Congenital sideroblastic anemia (CSA) is a hereditary disorder characterized by microcytic anemia and bone marrow sideroblasts. The most common form of CSA is attributed to mutations in the X-linked gene *5-aminolevulinic acid synthase 2* (*ALAS2*). ALAS2 is a mitochondrial enzyme, which utilizes glycine and succinyl-CoA to form 5-aminolevulinic acid (ALA), a crucial precursor in heme synthesis. Therefore, ALA supplementation could be an effective therapeutic strategy to restore heme synthesis in CSA caused by *ALAS2* defects. In a preclinical study, we examined the effects of ALA in human erythroid cells, including K562 cells and human induced pluripotent stem cell-derived erythroid progenitor (HiDEP) cells. ALA treatment resulted in significant dose-dependent accumulation of heme in the K562 cell line. Concomitantly, the treatment substantially induced erythroid differentiation as assessed using benzidine staining. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis confirmed significant upregulation of heme-regulated genes, such as the globin genes [hemoglobin alpha (*HBA*) and hemoglobin gamma (*HBG*)] and the heme oxygenase 1 (*HMOX1*) gene, in K562 cells. Next, to investigate the mechanism by which ALA is transported into erythroid cells, quantitative RT-PCR analysis was performed on previously identified ALA transporters, including *solute carrier family 15 (oligopeptide transporter), member (SLC15A) 1*, *SLC15A2*, *solute carrier family 36 (proton/amino acid symporter), member (SLC36A1)*, and *solute carrier family 6 (neurotransmitter transporter), member 13 (SLC6A13)*. Our analysis revealed that *SLC36A1* was abundantly expressed in erythroid cells. Thus, gamma-aminobutyric acid (GABA) was added to K562 cells to competitively inhibit SLC36A1-mediated transport. GABA treatment significantly impeded the ALA-mediated increase in the number of hemoglobinized cells as well as the induction of *HBG*, *HBA*, and *HMOX1*. Finally, small-interfering RNA-mediated knockdown of ALAS2 in HiDEP cells considerably decreased the expression of *HBA*, *HBG*, and *HMOX1*, and these expression levels were rescued with ALA treatment. In summary, ALA appears to be transported into erythroid cells mainly by SLC36A1 and is utilized to generate heme. ALA may represent a novel therapeutic option for CSA treatment, particularly for cases harboring *ALAS2* mutations.

Abbreviations: CSA, congenital sideroblastic anemia; ALA, 5-aminolevulinic acid; ALAS2, 5-aminolevulinic acid synthase 2; HBA, hemoglobin alpha; HBG, hemoglobin gamma; HMOX1, heme oxygenase 1; GABA, gamma-aminobutyric acid; HiDEP, human induced pluripotent stem cell-derived erythroid progenitor; XLSA, X-linked sideroblastic anemia; FECH, ferrochelatase; PBGD, porphobilinogen deaminase; AlaAcBu, 1-(butyryloxy)ethyl-5-amino-4-oxopentanoate; RT-PCR, reverse transcription polymerase chain reaction.

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1. Introduction

5-Aminolevulinic acid (ALA), an important precursor of heme, is a natural amino acid biosynthesized in the mitochondria of both animals and plants [1]. The compound is synthesized from glycine and succinyl-CoA in mitochondria, which is catalyzed by two different ALA synthases (ALAS): one expressed ubiquitously (ALAS1) and the other expressed only by erythroid precursors (ALAS2) [2]. During synthesis, ALA is exported to the cytosol where it is converted to coproporphyrinogen III. All the remaining steps of heme biosynthesis take place inside the mitochondria. Coproporphyrinogen III is imported into the mitochondria, and is finally catalyzed into protoporphyrin IX. Subsequently, heme is generated by the insertion of ferrous iron into protoporphyrin IX, which is catalyzed by ferrochelatase (FECH). Heme synthesis mostly occurs in developing erythroblasts located in the bone marrow to produce hemoglobin, whereas approximately 15% of the daily synthesis occurs in the liver to form heme-containing enzymes, such as cytochrome P450 [3]. Consequently, mutations in the genes involved in the heme biosynthetic pathway can cause impairment of oxygen delivery, mitochondrial respiratory chain activity, and drug metabolism, leading to the onset of porphyria and congenital sideroblastic anemia (CSA) [3,4].

CSA is a hereditary microcytic anemia characterized by bone marrow sideroblasts with excess iron deposition in the mitochondria. The most common type of CSA is X-linked sideroblastic anemia (XLSA), which is caused by defects in the X-linked gene *ALAS2* [5–7]. Most of the XLSA-associated mutations in *ALAS2* are missense substitutions resulting in loss of functionality, whereas mutations in the *ALAS2* regulatory region, such as the promoter [8] and intron 1 [9], lead to decreased *ALAS2* expression. ALAS2 missense mutations commonly decrease the binding of pyridoxal 5′-phosphate (PLP; vitamin B6), which is a cofactor for ALAS2 enzymatic activity, thus accounting for the PLP responsiveness in XLSA patients carrying such mutations [7]. However, nearly half of XLSA cases are unresponsive to PLP [5,6,9]. Therefore, ALA supplementation could be a useful alternative therapeutic strategy for restoring heme synthesis in CSA disorders caused by defects in ALAS2.

As a preclinical study, we examined the effects of ALA in human erythroid cells. Furthermore, we investigated the molecular mechanism by which ALA is transported into erythroid cells.

2. Materials and methods

2.1. Cell culture and reagents

All cells were grown in a humidified incubator at 37 °C with 5% carbon dioxide. Human K562 erythroleukemia cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum (Biowest, Miami, FL) and 1% penicillin–streptomycin (Sigma, St. Louis, MO, USA). Human induced pluripotent stem (iPS) cell-derived erythroid progenitor (HiDEP) cell lines were cultured in StemSpan Serum-Free Expansion Medium (STEMCELL Technologies, Vancouver, BC, Canada) containing 3-U/mL erythropoietin, 1-μg/mL doxycycline (Sigma), and 1-μM dexamethasone (Sigma), as described previously [10]. ALA hydrochloride (SBI Pharmaceuticals Co., Ltd., Tokyo, Japan) and gamma-aminobutyric acid (GABA) (Sigma) were prepared with distilled water. Erythropoietin was a kind gift of Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan).

2.2. Extraction of mouse tissue RNA

Mouse heart, lung, liver, spleen, kidney, intestine, and muscle were frozen in liquid nitrogen immediately after isolation. Frozen tissues were homogenized and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). A magnetic activated cell sorter system (Miltenyi Biotec, Auburn, CA, USA) was used to separate mouse Ter119-positive erythroblasts from the bone marrow.

2.3. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was purified using TRIzol (Invitrogen), and 1 μg of purified total RNA was used to synthesize complementary DNA (cDNA) with ReverTra Ace qPCR RT Master Mix (TOYOBO). Reaction mixtures (20 μL) for real-time quantitative RT-PCR consisted of 2 μL of cDNA, 10 μL of Quantitect SYBR Green PCR Master Mix (QIAGEN), and appropriate primers. Product accumulation was monitored by measuring SYBR Green fluorescence and normalized relative to *GAPDH* messenger RNA (mRNA).

To evaluate expression levels of human and murine ALA transporters [i.e., solute carrier family 36 (proton/amino acid symporter), member (SLC36A1), solute carrier family 15 (oligopeptide transporter), member (SLC15A) 1, SLC15A2, and *solute carrier* family 6 (neurotransmitter transporter), *member 13* (SLC6A13)], an amplified cDNA fragment of each gene was cloned into the pGEM™-T Easy Vector (Promega, Madison, WI), and was used as an internal standard in quantitative RT-PCR. The plasmid copy number was calculated as follows: copy number (copy/μL) = $6.02 \times 10^{23} \times$ [plasmid DNA concentration (μg/μL)] $\times 10^{-6}$/[total plasmid size (base pair)] $\times$ 660. Primer sequences are available upon request.

2.4. Heme content

Heme content was determined fluorometrically as described previously [11]. In brief, cell pellets were suspended in 2-M oxalic acid and boiled (100 °C) for 30 min to dissociate protoporphyrin IX and iron from heme. Subsequently, fluorescence for protoporphyrin IX was measured at 400 nm (excitation) and 662 nm (emission). To exclude endogenous levels of protoporphyrin IX, the fluorescence based on unboiled samples were subtracted.

2.5. Assay of erythroid differentiation of K562 cells

Erythroid differentiation of K562 cells was scored by benzidine staining as described previously [12]. Benzidine (o-dianisidine) was obtained from Sigma. Benzidine-positive cells were quantified by light microscopy (n = 600). Viable cells were counted by trypan blue dye (Invitrogen) exclusion.

2.6. Microarray analysis

SurePrint G3 Human GE 8 × 60 K Microarrays (G4851B) (Agilent, Palo Alto, CA, USA) and Human Oligo chip 25 k (Toray, Tokyo, Japan) were used, respectively, for expression profiling of ALA-treated K562 cells and ALAS2-knockdowned HiDEP cells, as described previously [13]. Gene Ontology analysis was performed as previously described [13].

2.7. Silencing of ALAS2 gene expression by small interfering RNA (siRNA)

We used siGENOME SMARTpool (Thermo Scientific Dharmacon, Lafayette, CO) to perform siRNA-mediated transient knockdown in HiDEP cells. The antisense sequences of the siRNA for human *ALAS2* were CUAGCUGAAUUGAGCCUAA, GAUCCAAGGUAUCCGUAAC, CG