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Sustained enhancement of OCTN1 transporter expression in association with hydroxyurea induced gamma-globin expression in erythroid progenitors

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Abstract

The clinical benefits of hydroxyurea treatment in patients with sickle cell disease (SCD) are due largely to increased gamma-globin expression. However, mechanisms that control gamma-globin expression by hydroxyurea in erythroid progenitors are incompletely understood. Here, we investigated the role of two hydroxyurea transporters, urea transporter B (UTB) and organic cation/carnitine transporter 1 (OCTN1), in this process. Endogenous expression of both transporters peaked towards the end of erythroid differentiation. Unlike UTB, hydroxyurea-induced OCTN1 expression correlated positively with gamma-globin level and was sustained throughout the period of induction. These results highlight a potential major role for OCTN1 in the efficacy of hydroxyurea.

Keywords

Hydroxyurea; Urea Transporter B; Organic Cation/Carnitine Transporter; Gamma-globin; Erythroblasts

Introduction

Hydroxyurea (HU) is the only FDA-approved drug for the treatment of sickle cell disease (SCD). Clinical benefits of hydroxyurea are attributed largely to its ability to induce γ -

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Conflict of Interest:

The authors have declared no conflicts of interest exists

globin gene expression and increase fetal hemoglobin (HbF) production during erythropoiesis [1]. Transcriptional control of γ -globin expression by HU involving multiple transcription factors has been characterized [2–4]. However, factors that control the transport of HU into erythroid cells to enhance the production of HbF have never been defined.

Specific solute carrier membrane transporters modulate the transcellular movement and intracellular accumulation of HU. They include 3 organic anion transporting polypeptides (OATP), 2 organic cation/carnitine transporters (OCTN) and 2 urea transporters (UT) [5]. Transporters of the OATP family are found predominantly in the liver, intestine, and kidneys and modulate the pharmacokinetics of HU [6]. Among HU transporters, only two, UTB and OCTN1, are highly expressed in bone marrow and in erythrocytes [7, 8]. UTB is a bidirectional transporter that is important for maintaining balanced osmolality within erythrocytes by regulating urea transport [9, 10]. OCTN1 is an uptake transporter expressed in immature erythrocytes and in the K562 erythroid cell line [7, 11]. Hitherto, the expression pattern of UTB and OCTN1 during erythropoiesis, and the impact HU has on this process has never been described. In this, study, we reasoned that UTB and OCTN1 may be involved in HU-mediated γ -globin induction in erythroid progenitor cells.

Materials and Methods

Cell Culture

Mobilized CD34+ cells purified from peripheral blood of healthy human donors were purchased (Fred Hutchinson Cancer Research Center) and cultured in a 3-phase liquid erythroid differentiation assay. In Phase 1 cells were culture for 2 days in Serum-Free Expansion Media comprised of Iscove's Modified Dulbecco's medium (IMDM; Thermo Fisher) supplemented with 100 U/ml penicillin/streptomycin, 1 μ M B-mercaptoethanol, 25% BIT 9500 Serum Substitute, StemSpan CC110 (Stem Cell Technologies), 2mM L-glutamine, and 20ng/ml recombinant human Interlukin-3 (IL-3, Peprotech). In Phase 2, on differentiation day 0 cells were cultured in erythroid differentiation media comprised of IMDM supplemented with 10% Human AB Serum (Atlanta Biologicals), 1% Bovine Serum Albumin, 10 μ g/mL human recombinant insulin 0.5 μ g/mL Vitamin B12, 15 μ g/mL Folic Acid, 50nM B-mercaptoethanol (Sigma-Aldrich), 50U/mL penicillin/streptomycin (Thermo Fisher), 10ng/mL human recombinant IL-3, 10ng/mL Stem Cell Factor (SCF; Peprotech), and 2U/mL Erythropoietin (Janssen Biotech). On differentiation day 6 cells were plated in phase 3 media, which is similar to phase 2 media without IL-3 and SCF. A small aliquot of cells was collected at various time points for flow cytometric and Q-PCR analysis. Cytospin slide preparations were created and stained with Wrights-Giemsa hematologic stain (Sigma-Aldrich). For HU experiments, cells were cultured in media with 100 μ M HU or PBS starting on differentiation day 6 of culture.

Human Erythroleukemia cell lines (ATCC) were cultured in IMDM media supplement with 10% FBS and 100 U/mL penicillin/streptomycin. For HU experiments, 100 μ M HU or PBS vehicle was added to culture media and cultured for 72 hours.

Quantitative PCR

Cells were harvested, washed with 1xPBS and lysed in Qiazol Lysis reagent (Qiagen). Total RNA was isolated using the miRNeasy kit (Qiagen). RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher) and gene expression was measured using the following Taqman gene expression assays (Thermo Fisher): *KIT* assay Hs00174029_m1, *GYP A* assay Hs00266777_m1, *SLC41A1* assay Hs00998197_m1, *SLC22A4* assay Hs01548718_m1, *HBG2/HBG1* assay Hs00361131_g1, *18s* assay Hs99999901_s1. Fold change of expression was determined by comparative dCT methods using ribosomal *18s* gene as the endogenous control. Transcript copy number was determined by relative standard curve generated from oligonucleotides and normalized to *18s* transcript copy number.

Flow Cytometry

Cells were harvested and analyzed for surface expression using the following antibodies: APC conjugated anti-CD34, PE-conjugated anti-CD71, FITC-conjugated anti-glycophorin A (BD Biosciences). 200,000 cells were collected washed and stained with the respective antibodies. All samples were analyzed within 1 hour following antibody staining using the BD LSR Fortessa Cell Analyzer in combination with BD FACSDiva and FlowJo Software.

Results

While both UTB and OCTN1 are known to be present on erythroid cells, the expression pattern of these transporters has not been fully characterized during erythropoiesis. To characterize UTB and OCTN1 expression in erythroid progenitor cells, we established a robust assay to differentiate human CD34+ positive cells towards the erythroid lineage. Authenticity of our assay was reaffirmed by observation of distinct morphological features of erythroid cells at specific stages of maturation (Figure S1). In addition, expression of surface markers CD71 and glycophorin A (GYPA) were used to stage erythroid maturation by flow cytometry (Figure S2). With this model, we discovered that UTB (encoded by *SLC14A2* gene) mRNA level progressively increased with erythroid cell maturation. There were 100-fold more transcript copies in late-stage cultures comprised primarily of orthochromatic erythroblasts and early erythrocytes compared to earlier stages (Figure 1A). OCTN1 (encoded by *SLC22A4* gene) mRNA was maximally expressed in mid-stage cultures comprised predominantly of poly/orthochromatic erythroblasts with 4.5- to 12-fold more transcript copies than early-stage cultures comprised of basophilic erythroblasts (Figure 1B). In accordance with maturation, expression of the stem cell gene *KIT* decreased significantly by 13-fold with differentiation whereas expression of *GYP A*, significantly increased by greater than 1400-fold (Figure 1D and E).

Our next objective was to determine whether UTB and OCTN1 expression is altered by HU in erythroid precursors. Human erythroleukemic cells (HEL) exposed to HU for 72 hours had enhanced transporter expression compared to PBS vehicle-treated controls. UTB and OCTN1 gene expression increased significantly by 1.6-fold and 1.9-fold, respectively (Figure 2A and 2B). The increase in transporter expression was concomitant with a significant increase in γ -globin expression (Figure 2C). Analysis of the same cells revealed

that the enhanced OCTN1 expression, but not UTB, correlated positively with the induction of γ -globin gene expression ($R^2 = 0.56$, $P = 0.0048$, Figure 2D).

To validate the findings from HEL cells, we studied primary erythroid progenitors derived from CD34+ cells of 5 different human donors. Cells were exposed to HU during the mid-stages of erythroid development when greater than 83% of cells expressed both CD71 and GYPA erythroid surface antigens (Figure 3A). HU increased γ -globin gene expression by ~3 fold after 48 and 72 hours of treatment (Figure 3B). Expression of UTB increased by a mean 1.4-fold at the 48 hour time point, then declined at 72 hours (Figure 3C). On the contrary, the 1.8-fold enhanced OCTN1 gene expression by HU was sustained through 72 hours in parallel with the γ -globin induction (Figure 3D). These results in erythroid cell lines and primary cells suggest a linear relationship between the expression of the OCTN1 transporter and γ -globin in erythroid cells.

Discussion

Collectively, our data show, for the first time, an up-regulation of HU transporters in erythroid cells is concomitant with augmented γ -globin induction by HU. Along with the elucidation of endogenous transporter expression patterns, the present study suggests that UTB and OCTN1 transporters may be contributors to HU-mediated γ -globin induction. Both UTB and OCTN1 have been previously established as transporters of HU [5]. Presently, we show that their expression varies with erythroid cell development with a peak towards the end of differentiation. These findings are consistent with previous reports of high UTB expression in mature erythrocytes relative to other tissues and blood cells [9] and reports that OCTN1 expression precedes GYPA expression in hematopoiesis [7]. The stage-specific pattern of expression of UTB and OCTN1 suggests that entry and intracellular accumulation of HU varies during erythroid cell development. Given that the expression of γ -globin is also variable at different stages of erythroid maturation [12], variation in transporter expression likely has important ramification for efficacy of HU.

During erythroid differentiation, globin gene transcription occurs at high levels after the proerythroblast stage and plateau during polychromatic and orthochromatic stages [12]. We observe a similar precipitous rise in endogenous HU transporter expression when cell cultures are comprised of basophilic, polychromatic, and orthochromatic erythroblasts. Furthermore, HU-mediated γ -globin synthesis is prominent in basophilic erythroblasts [13]. It is induced by stem cell factor in late-stage erythroblasts [14], and by pomalidomide and lenalidomide during the mid-stages of erythropoiesis [15]. Our findings of HU transporter expression at the optimum stage of globin synthesis during erythropoiesis suggest that they play a central role in γ -globin induction by HU. OCTN1, in particular, may be critical in mediating the efficacious outcomes of HU given its sustained induction and significant correlation with γ -globin induction. In conclusion, this study raises for the first time a potential, hitherto unknown, role of transporters in the pharmacologic induction of fetal hemoglobin for the amelioration of SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hydroxyurea transporter expression is developmentally regulated during erythroid differentiation

(A–B) Quantitative real-time PCR of gene expression in erythroid cells at various days of culture shows that expression of urea transporter B (UTB) continually increases during differentiation whereas gene expression of organic cation/carnitine transporter 1 (OCTN1) is variable during different stages of erythropoiesis. (C–D) Differentiation of the cells is confirmed by the decrease of stem cell-specific gene *KIT* and the increase of erythroid-specific gene *GYPA* through 11 days in culture. (ANOVA Dunnett's multiple comparisons test; n=3–5 from 3 independent experiments *P=0.0002 or ** P< 0.0001 vs Day 0)

Figure 2. Hydroxyurea-mediated induction of transporter expression correlates to increased γ -globin expression in erythroid cell line

(A–C) Quantitative real-time PCR analysis shows that, UTB, OCTN1, and γ -globin gene expression are up regulated in HEL erythroid cell line following 72-hour exposure to 100 μ M hydroxyurea (HU) compared to PBS vehicle (V) treated cells. (D) Increase in γ -globin gene expression is significantly correlated with increase in OCTN1 gene expression (Student's t-test; n=12 from 4 independent experiments; *P=0.04, **P<0.01 or ***P<0.0001; Pearson's R^2 = 0.56; P= 0.0048).

Figure 3. Enhanced transporter expression by HU is concomitant to an increase γ -globin expression in primary erythroid cells

(A) Quantification of flow cytometry analysis shows the proportion of cells expressing erythroid markers CD71 and glycophorin A (GYPA) at 3, 6, and 11 days of culture. (B-D) Quantitative PCR analysis shows that mean gene expression of UTB, OCTN1, and γ -globin is significantly increased in primary human erythroid cells obtained from 5 different donors (represented by symbols) following 48- or 72- hour exposure to 100 μ M hydroxyurea. (Student's t-test; n=5 from 3 independent experiments; **P<0.01 or ***P<0.0005)