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The proinflammatory cytokine GM-CSF downregulates fetal hemoglobin expression by attenuating the cAMP-dependent pathway in sickle cell disease

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ABSTRACT

Although reduction in leukocyte counts following hydroxyurea therapy in sickle cell disease (SCD) predicts fetal hemoglobin (HbF) response, the underlying mechanism remains unknown. We previously reported that leukocyte counts are regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) in SCD patients. Here we examined the roles of GM-CSF in the regulation of HbF expression in SCD. Upon the analysis of retrospective data in 372 patients, HbF levels were inversely correlated with leukocyte counts and GM-CSF levels in SCD patients without hydroxyurea therapy, while HbF increments after hydroxyurea therapy correlated with a reduction in leukocyte counts, suggesting a negative effect of GM-CSF on HbF expression. Consistently, in vitro studies using primary erythroblasts showed that the addition of GM-CSF to erythroid cells decreased HbF expression. We next examined the intracellular signaling pathway through which GM-CSF reduced HbF expression. Treatment of erythroid cells with GM-CSF resulted in the reduction of intracellular cAMP levels and abrogated phosphorylation of cAMP response-element-binding-protein, suggesting attenuation of the cAMP-dependent pathway, while the phosphorylation levels of mitogen-activated protein kinases were not affected. This is compatible with our studies showing a role for the cAMP-dependent pathway in HbF expression. Together, these results demonstrate that GM-CSF plays a role in regulating both leukocyte count and HbF expression in SCD. Reduction in GM-CSF levels upon hydroxyurea therapy may be critical for efficient HbF induction. The results showing the involvement of GM-CSF in HbF expression may suggest possible mechanisms for hydroxyurea resistance in SCD.

Introduction

Clinical manifestations of patients with sickle cell disease (SCD) are primarily due to the polymerization of sickle hemoglobin, leading to vaso-occlusive crisis, chronic hemolysis, and ischemia-reperfusion injury associated with inflammation [1–3]. Although SCD patients share a common mutation in the β-globin gene, the clinical severity of SCD is extremely heterogeneous. The mechanisms underlying this clinical heterogeneity remain unknown.

Fetal hemoglobin (HbF) is expressed in patients with SCD at variable levels and known to alleviate the clinical severity of SCD [4]. HbF expression has been shown to be regulated by single nucleotide polymorphisms (SNPs) of multiple genetic loci [5–7], and it is likely that these genetic loci play a role in determining the levels of HbF production in SCD patients [8]. Leukocytosis is frequently observed in untreated patients even in the absence of bacterial infection [9], and an elevated base-line leukocyte count is associated with an increased risk of early death [10]. Severe complications are also predicted later in life for sickle cell children with high leukocyte counts [11]. These clinical observations suggest that both HbF levels and leukocyte counts have significant effects on the clinical severity of SCD.

Pharmacological stimulation of HbF expression, especially with hydroxyurea, is now a recognized therapeutic option for SCD patients with a severe clinical phenotype. However, the HbF response to hydroxyurea varies among patients. Discovering predictors of HbF response to hydroxyurea would permit clinicians to identify patients who would respond to hydroxyurea therapy and would increase the clinical effectiveness of hydroxyurea, which would, in turn, minimize adverse effects of the drug. A study by Charache and associates showed that significant predictors of high post-therapy HbF levels

Abbreviations: CREB, cAMP response element binding protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HbF, fetal hemoglobin; SCD, sickle cell disease; SNP, single nucleotide polymorphism; TTP, tristetrapolin.

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are post-therapy plasma hydroxyurea level, initial leukocyte counts, and initial HbF concentration [12]. Ware et al. also demonstrated that changes in blood counts in SCD children after hydroxyurea therapy indicate the HbF response to hydroxyurea [13]. However, the mechanisms by which these clinical variables serve as predictors of HbF response remain unknown.

Earlier studies by Croizat and Nagel reported elevated plasma GM-CSF levels in SCD patients who have low HbF levels [14,15]. They concluded that hematopoietic stress associated with low HbF levels contributes to increased levels of GM-CSF [15]. We recently reported a positive correlation between leukocyte counts and plasma GM-CSF levels in SCD patients [16]. Subsequently we found that leukocyte counts correlate inversely with HbF levels in steady-state SCD patients, suggesting the possibility that HbF expression in SCD patients is negatively affected by the mechanisms that sustain leukocyte counts.

Here we report that leukocyte counts and plasma GM-CSF levels inversely correlate with HbF levels in SCD patients, and that GM-CSF is a negative regulator for HbF expression, which is in sharp contrast to the conclusion by Croizat and Nagel [15]. Our in vitro studies using primary erythroid cells consistently showed that GM-CSF downregulates HbF expression. Moreover, we demonstrate that GM-CSF attenuates the cAMP-dependent pathway in both cytokine-dependent erythroid cells and primary erythroid cells; our previous studies showed the critical role of cyclic nucleotide-dependent pathways in sustaining HbF expression [17–19]. These results suggest that GM-CSF has regulatory roles in both leukocyte counts and HbF levels in SCD patients. GM-CSF is likely to downregulate HbF expression at least in part by attenuating the cAMP-dependent pathway. Potential mechanisms by which HbF response to hydroxyurea is regulated in the context of GM-CSF are discussed.

Materials and methods

SCD patients

Retrospective data on 372 SCD patients who were homozygous for the β^S mutation and were under clinical care at the Sickle Cell Center of Georgia Health Sciences University were analyzed in this study. Of these, 192 patients who had not received hydroxyurea or blood transfusions for at least the past 3 months were considered to be in steady-state, and 125 SCD patients who were receiving HU therapy (15 to 35 mg/kg/day) for at least 6 months were enrolled in this study. In addition, 63 pediatric SCD patients (Kuwait University) who had a mutation in the γ-globin gene promoter [20] and expressed high levels of HbF were included to examine the effects of leukocyte counts on HbF expression; none of these patients was receiving hydroxyurea. Clinical characteristics of these cohorts of SCD patients are shown in Supplementary Tables (Tables S1–S3). Hematologic data reported in this study were the average of individual data for at least 3 months. Informed consent was obtained from all subjects. The study was performed in accordance with the principles of the Declaration of Helsinki and approved by the institutional review boards of Georgia Health Sciences University and Kuwait University.

Measurement of plasma GM-CSF levels

Plasma levels of GM-CSF were measured as described previously [16]. Briefly, GM-CSF levels were determined using high sensitivity immunoenzymatic assay kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Complete blood counts and HbF measurements

Complete blood counts were performed using an Advia Hematology System 120 (Bayer Diagnostics, Tarrytown, NY, USA). HbF quantification was performed by cation-exchange high-pressure liquid chromatography (Synchropak CM 300) as described previously [21]. Increases in HbF levels in patients on hydroxyurea were determined by deducting pre-therapy HbF levels from post-therapy HbF levels.

Culture of TF-1 cells and CD34$^+$ cells and globin mRNA analysis

TF-1 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 mM HEPES. All cytokines were purchased from Peprotech (Rocky Hill, NJ) unless otherwise stated. TF-1 cells were cultured with 5 ng/ml GM-CSF or 2 U/ml erythropoietin (Amgen, Thousand Oaks, CA, USA) or both. CD34$^+$ cells were provided by NHLBI PEGT Hematopoietic Cell Processing Core (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). CD34$^+$ cells were cultured by the method described previously [19,22]. Briefly, CD34$^+$ cells were maintained for 14 days in Iscove's modified Dulbecco's medium containing 15% FBS, 15% human AB serum, 2 units/ml erythropoietin, 10 ng/ml stem cell factor, 10 mM β-mercaptoethanol, and 600 μg/ml holo-transferrin. To examine effects of GM-CSF, stem cell factor was depleted from cultures on days 5 and 10 to 100 ng/ml GM-CSF was added for the last 9 days before cell harvesting. Eighty to 90 percent of cells grown in the cultures expressed glycophorin A (CD235a) and CD71 as determined by flow cytometry. Total cytoplasmic RNA was extracted from nucleated erythroblasts using the method described by Chomoczynski and Sacchi [23]. Globin mRNAs were quantified by primer extension using a previously described method [24] with modifications [25,26].

Measurement of intracellular cAMP levels in erythroid cells

Intracellular levels of cAMP in TF-1 cells or primary erythroblasts were determined as described previously [17,27]. Briefly, 2×10^6 cells were incubated with 1 mM 3-isobutyl-1-methylxanthine for 30 min at room temperature. cAMP was extracted by suspending the cells with 0.5 M hydrocholoric acid. The supernatant was removed and the pH was neutralized by the addition of 8 M KOH. Intracellular cAMP levels were determined using a cAMP ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA).

Isolation of whole cell extracts from erythroblasts and Western blotting

Whole cellular extracts were prepared from TF-1 cells or primary erythroblasts, as described [18]. Briefly, 5 to 10×10^6 cells were suspended with 1×RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) supplemented by 1 mM PMSF, 0.1% SDS, and 10% (v/v) protein phosphatase inhibitor cocktail Set IV (EMD Chemicals, Gibbstown, NJ, USA). Western blotting was performed as described previously [28]. Briefly, 20 to 30 μg of the cellular extracts were separated on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). All antibodies used for Western blot analyses were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

For analysis of correlations with non-Gaussian distributed data, which included hematological data of SCD patients (Figs. 1 and 2), the Spearman correlation coefficient (r_s) was utilized. Assays for cAMP levels were performed in triplicate and the data were expressed as means ± SE. The data were analyzed by the Student *t* test. *P* values <0.05 were considered to be statistically significant.