ORIGINAL RESEARCH

Serum of sickle cell disease patients contains fetal hemoglobin silencing factors secreted from leukocytes

Tohru Ikuta[1]
Hassan Sellak[1]
Si-Yang Liu[2]
Nadine Odo[1]

[1]Department of Anesthesiology and Perioperative Medicine, Medical College of Georgia, Augusta University, Augusta, GA, USA; [2]Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta University, Augusta, GA, USA

Correspondence: Tohru Ikuta
Department of Anesthesiology and Perioperative Medicine, Medical College of Georgia, Augusta University, 1120 15th Street, BIW-2144, Augusta, GA 30912, USA
Tel +1 706 721 3098
Fax +1 706 721 8752
Email tikuta@augusta.edu

Background: The mechanisms that regulate fetal hemoglobin (HbF) expression in sickle cell disease (SCD) remain elusive. We previously showed that steady-state SCD patients with high HbF levels due to a *γ-globin* gene mutation demonstrate strong inverse correlations between HbF levels and leukocyte counts, suggesting that leukocytes play a role in regulating HbF in SCD.
Materials and methods: To further investigate the role of leukocytes in HbF expression in SCD, we examined the presence of HbF silencing factors in the serum of 82 SCD patients who received hydroxyurea (HU) therapy.
Results: HU-mediated HbF induction was associated with elevated total hemoglobin levels and improved red blood cell parameters, but there was no correlation with reticulocyte or platelet counts. Importantly, we again found that HU-induced HbF levels correlated with reductions in both neutrophils and lymphocytes/monocytes, indicating that these cell lineages may have a role in regulating HU-mediated HbF expression. Our in vitro studies using CD34+-derived primary erythroblasts found that patient serum preparations include HbF silencing factors that are distinct from granulocyte-macrophage colony-stimulating factor, and the activity of such factors decreases upon HU therapy.
Conclusion: Together, these results demonstrate the importance of leukocyte numbers in the regulation of HbF levels for SCD patients both in steady state and under HU therapy, and that leukocytes secrete HbF silencing factors that negatively affect HbF expression in erythroid-lineage cells in SCD.
Keywords: sickle cell disease, leukocytes, hydroxyurea, fetal hemoglobin, silencing factors

Introduction

The primary genetic defect in sickle cell disease (SCD) is a mutation of the *β-globin* gene.[1] It produces sickle hemoglobin, which polymerizes under low oxygen tension. This formation of sickle hemoglobin polymers is assumed to underlie vaso-occlusive crisis, chronic hemolysis, and ischemia–reperfusion injury associated with inflammation in this disorder.[2–4]

Despite sharing a common *β-globin* mutation, the clinical severity among patients with SCD is extremely heterogeneous and the underlying mechanisms remain unknown. Elevated levels of fetal hemoglobin (HbF) expression alleviate the clinical severity of SCD; however, expression levels are also variable.[5] HbF expression is regulated by single-nucleotide polymorphisms (SNPs) of multiple genetic loci,[6–8] which presumably help to determine the level of HbF production in SCD.[9] In addition to HbF, which is otherwise dormant in people without anemia, leukocytosis is frequently observed in untreated SCD patients even in the absence of bacterial infection.[10] An elevated baseline

leukocyte count is associated with a risk for early death.[11] Further, high leukocyte counts in children with SCD predict severe clinical complications later in life.[12] These clinical observations suggest that both HbF levels and leukocyte counts are consequential to the clinical severity of SCD.

Although the pharmacological stimulation of HbF expression by hydroxyurea (HU) is an established treatment for patients with clinically severe SCD,[13] HbF response to HU varies significantly and a number of SCD patients are resistant to HU therapy. Clarifying the predictors of HbF response to HU will allow clinicians to determine which patients are most likely to respond to HU therapy. This will in turn limit the toxicities associated with the treatment among those who would not benefit from the therapy. A study by Charache et al showed that initial leukocyte count and HbF concentration as well as post-therapy plasma HU levels are predictors of high post-therapy HbF levels.[14] Ware et al also demonstrated that greater changes in blood counts in SCD children on HU therapy result in better HbF response to HU.[15] However, the mechanisms by which these hematologic parameters predict HbF response remain elusive.

As to the mechanisms responsible for leukocytosis in SCD, we had reported a positive correlation between leukocyte count and plasma granulocyte-macrophage colony-stimulating factor (GM-CSF) levels in SCD patients,[16] indicating that plasma GM-CSF levels help to regulate leukocyte count. We subsequently analyzed retrospective data from SCD patients receiving HU therapy and found that the rate of HbF induction associated with HU therapy is proportional to the reduction of peripheral blood leukocyte count.[17] We also found that GM-CSF has a negative regulatory effect on HbF expression.[17] These results suggest that leukocytes are an important regulator for determining HbF response to HU.

To determine whether leukocytes are also involved in the regulation of HU-mediated HbF expression in SCD, in this study we analyzed retrospectively collected pre-HU and post-HU therapy hematological data. We found that HbF response was again associated with reduced leukocyte count, but not reticulocyte and platelet counts, suggesting that leukocytes likely play a critical role in HbF response to HU. Furthermore, our in vitro studies suggest that patient sera contain as-yet unidentified factors that appear to inhibit HbF expression in CD34$^+$-derived erythroid progenitors cultured with erythropoietin (Epo). Interestingly, the ability to inhibit HbF activity varied among steady-state patients and was significantly decreased upon HU therapy. This study has revealed novel aspects of the molecular mechanisms by which HU regulates HbF expression, which will help us to better understand HU resistance among SCD patients.[15,18]

Materials and methods
SCD patient blood serum
The study was performed in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board of Augusta University. We collected retrospective data on 337 adult SCD patients who were homozygous for the β^S mutation and under the care of the Sickle Cell Center of the Medical College of Georgia at Augusta University; the clinical characteristics of this cohort were reported previously.[17] Of these patients, 82 were receiving HU therapy (15–35 mg/kg/day) and had not received transfusions for at least 6 months. Hematologic values were reported as the average of at least 3 months of data. Serum preparations that had no visible hemolysis were obtained from multiple patients under HU therapy, before daily intake of HU to minimize carryover of HU. Written informed consent was obtained from all patients.

In vitro culture of human CD34$^+$-derived erythroid progenitor cells
Human CD34$^+$ cells obtained from the National Heart, Lung and Blood Institute Programs of Excellence in Gene Therapy Hematopoietic Cell Processing Core (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) were cultured by a method described previously[19] with minor modifications. Briefly, CD34$^+$ cells ($1–10 \times 10^4$ cells/mL) were cultured in Iscove's modified Dulbecco's medium containing 30% fetal bovine serum (FBS) or 30% human AB serum, 3 unit/mL Epo, 20 ng/mL stem cell factor, and 10 ng/mL interleukin 3. Culture media were replaced every 4 days. Anti-human GM-CSF antibody (Thermo Fisher Scientific, Waltham, MA, USA) was added to sera from SCD patients at a dilution ratio of 1:10 followed by a 30-minute incubation at room temperature before adding to culture media. Cells were harvested on day 14. Cell photographs were taken by EVOS FL Cell Imaging System (Advanced Microscopy Group, Bothell, WA, USA).

Flow cytometry
Cells were suspended in FACS buffer (phosphate-buffered saline containing 5% FBS and 0.1% sodium azide). Following incubation with human Fc blocker (Thermo Fisher Scientific) for 10 minutes at room temperature, cells were then washed twice in FACS buffer and stained at 4°C for 30 minutes with a phycoerythrin (PE)-labeled anti-human glycophorin A monoclonal antibody (CD235a PE) (BD Biosciences, San

Jose, CA, USA) and fluorescein isothiocyanate-labeled CD71 monoclonal antibody (BD Biosciences). Paraformaldehyde was added in an equal volume to a final concentration of 0.1% to fix cells for 30 minutes at room temperature. The expression of glycophorin A and CD71 on erythroblasts was analyzed in a Becton-Dickinson FACScan using Cell Quest software (Franklin Lakes, NJ, USA).[20] Data were represented as percent of positive cells.

Isolation of whole cell extracts from erythroblasts and immunoblotting

Whole cellular extracts were prepared from CD34+-derived erythroblasts as described.[21] Briefly, cells ($5–10 \times 10^6$ cells) were suspended with $1 \times$ RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) supplemented with 1 mM phenyl-methyl sulfonyl fluoride, 100 mM sodium orthovanadate, and protease inhibitor cocktail. Whole cellular extracts were obtained by centrifugation at $14,000 \times g$ for 15 minutes. Immunoblotting was performed as described previously.[22] Approximately 2–3 micrograms of cellular extracts were separated on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). All antibodies used for immunoblotting analyses were purchased from Santa Cruz Biotechnology unless otherwise stated. Protein bands were visualized by the Phototope HRP Western blot detection system (Cell Signaling Technology, Danvers, MA, USA) according to the protocol provided by the supplier. Immunoblotting for γ-globin expression was performed by using serum preparations with no visible hemolysis that were isolated from multiple SCD patients.

Real-time (RT)-PCR

Expression of γ-globin mRNA in human CD34+-derived erythroid progenitors treated with or without SCD patients' serum was examined by RT-PCR as described previously.[23] To determine expression levels of human γ-globin mRNA in primary erythroblasts, human CD34+ cells were cultured as described above. Total RNA was extracted from primary erythroblasts using RNeasy Mini Kit (Qiagen, Germantown, MD, USA), and cDNA was generated with the SuperScript II Reverse Transcriptase kit (Invitrogen). RT-PCR was carried out with the Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All amplifications were performed in triplicate, and 18S rRNA was used as internal control. Relative expression was quantitated using the standard $\Delta\Delta Ct$ method. The primers used were as follows: human γ-globin,

forward-5′-TGGATGATCTCAAGGGCAAC-3′; reverse-5′-TCAGTGGTATCTGGACA-3′; human 18S rRNA, forward-5′-TTGGAGGGCAAGTCTGGTG-3′; reverse-5′- CCGCTCCCAAGATCCAACTA-3′. All primers were designed and obtained from Integrated DNA Technologies (Coralville, IA, USA). RT-PCR for γ-globin mRNA expression was carried out by using several serum preparations with no visible hemolysis from multiple SCD patients.

Statistical analysis

The Spearman correlation coefficient (r_s) was used for correlations with non-Gaussian distributed data, which included hematologic values (Figures 1–4). Other data were analyzed by Student's t-test. P-values less than 0.05 were considered statistically significant.

Results
Response of HbF to HU is associated with hematologic improvements in SCD patients

Our previous study showed that reduced leukocyte counts in response to HU therapy is critical for efficient HU-mediated HbF induction.[17] To further investigate the mechanisms by which HbF response is regulated in SCD, we first examined whether HbF response was associated with hematologic improvements in a retrospective study of a cohort of SCD patients described previously.[17] Of these, 82 HU responders were selected. There were substantial correlations between the levels of HbF induction by HU and increases in the total hemoglobin levels, MCV, and MCHC (Figure 1A–C). This suggests that HU therapy significantly improves RBC parameters, suggesting its clinical effectiveness for SCD. Our cohort of SCD patients may be pathophysiologically comparable to those reviewed recently.[24]

HU-induced HbF levels are not correlated with reticulocyte or platelet counts

Next, we investigated whether HU-mediated HbF levels correlated with reticulocyte and platelet counts, both of which are assumed to play a role in modulating the pathophysiology of SCD.[25,26] However, we saw no significant correlations between HU-induced HbF levels and reticulocyte or platelet counts (Figure 2A and B), suggesting that the mechanisms controlling the numbers of these lineage cells in peripheral blood may not be relevant to the mechanisms by which HbF response is regulated by HU.
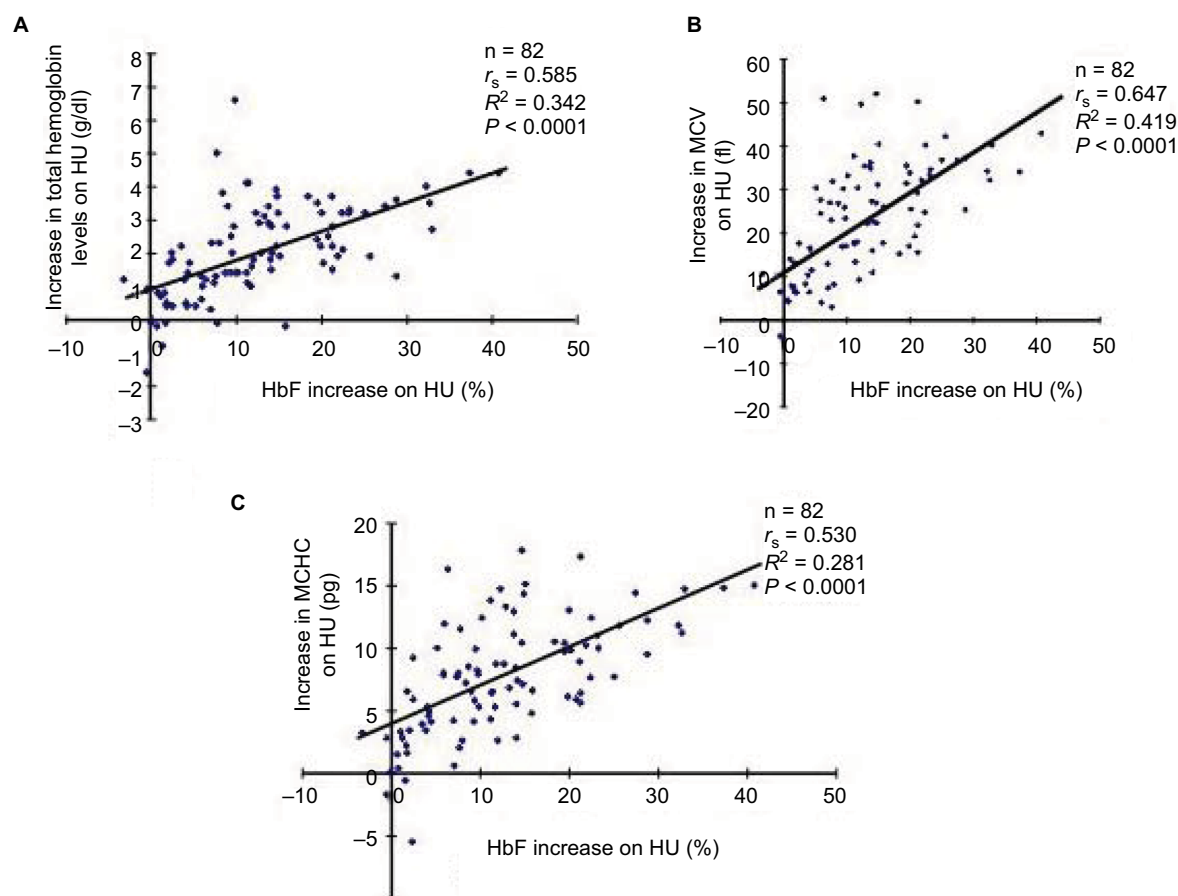
Figure 1 HbF response to HU is associated with hematological improvements in SCD patients.
Notes: Increases in HbF levels after HU therapy correlate with increases in total hemoglobin levels (**A**), MCV (**B**), and MCHC (**C**). Correlations were analyzed with 82 SCD patients who received HU therapy.
Abbreviations: HbF, fetal hemoglobin; HU, hydroxyurea; SCD, sickle cell disease.
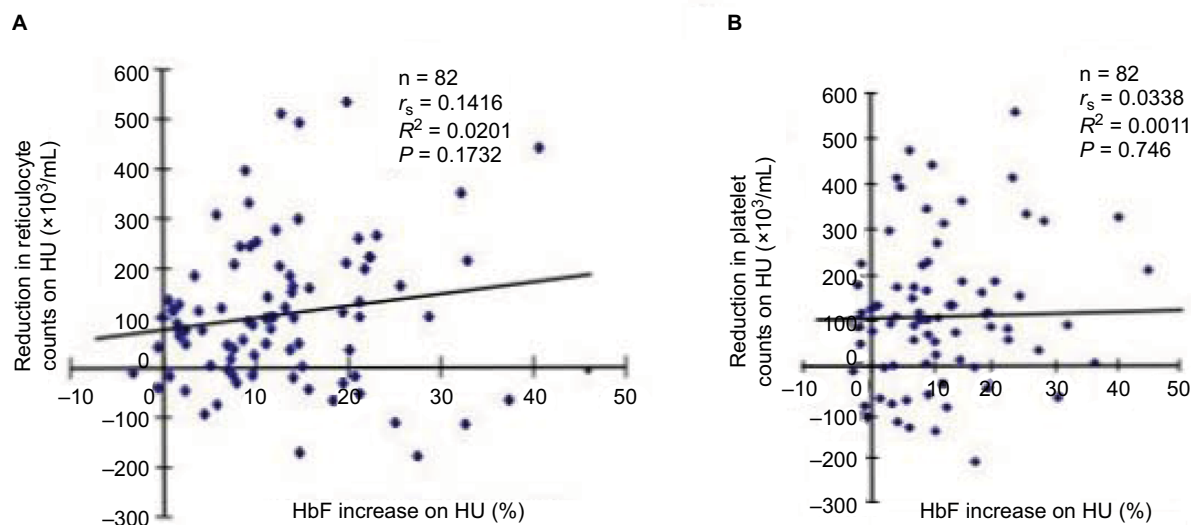

Figure 2 There were no correlations between HU-induced HbF increases and reductions in the number of reticulocytes (**A**) and platelets (**B**) among the SCD patients who received HU therapy.
Abbreviations: HU, hydroxyurea; HbF, fetal hemoglobin; SCD, sickle cell disease.

A

n = 82
$r_s = 0.197$
$R^2 = 0.039$
$P = 0.056$

B

n = 82
$r_s = 0.0294$
$R^2 = 0.0864$
$P < 0.004$

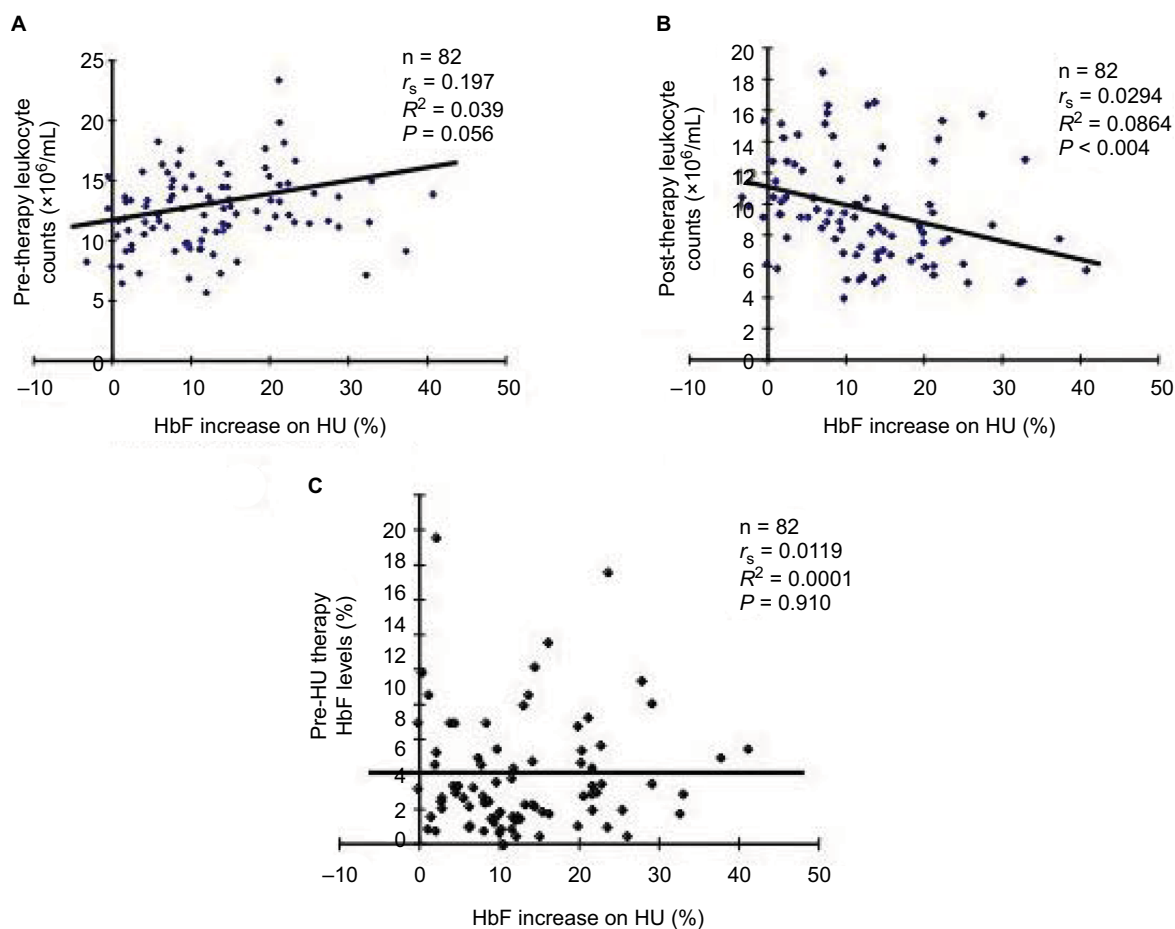**C**

n = 82
$r_s = 0.0119$
$R^2 = 0.0001$
$P = 0.910$

Figure 3 Correlations between the levels of HbF increases upon HU therapy and pre-HU therapy leukocyte counts (**A**), post-HU therapy leukocyte counts (**B**), or pre-HU therapy HbF levels (**C**). Eighty-two SCD patients who received HU therapy were analyzed.
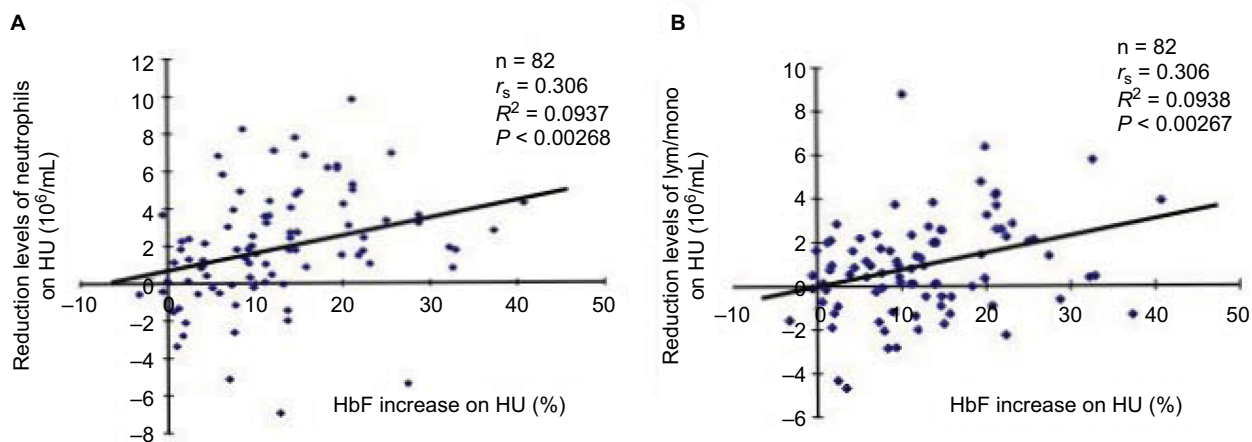**Abbreviations:** HU, hydroxyurea; HbF, fetal hemoglobin; SCD, sickle cell disease.

A

n = 82
$r_s = 0.306$
$R^2 = 0.0937$
$P < 0.00268$

B

n = 82
$r_s = 0.306$
$R^2 = 0.0938$
$P < 0.00267$

Figure 4 Correlations between neutrophil (**A**) or lymphocyte/monocyte counts (**B**) and HU-induced HbF levels.
Note: Eighty-two SCD patients who received HU therapy were analyzed.
Abbreviations: HU, hydroxyurea; HbF, fetal hemoglobin; SCD, sickle cell disease.

Associations between HU-induced HbF levels and leukocyte counts

We had previously found that HU-induced HbF correlates with reduced leukocyte counts,[17] suggesting that the mechanisms by which HU regulates HbF induction in SCD may be relevant to those controlling leukocyte numbers in peripheral blood. Charache et al demonstrated that predictors of HbF response include pre-therapy leukocyte counts and HbF levels.[14] However, we found that HU-mediated HbF induction levels did not correlate with pre-therapy leukocyte counts (Figure 3A, $P=0.056$) but did correlate with post-therapy leukocyte counts (Figure 3B, $P<0.004$). The HbF increase was not influenced by pre-therapy HbF levels (Figure 3C, NS), a result that was inconsistent with Charache et al's study.[14] This might reflect genetic and cellular differences in the cohorts studied.

To investigate the cell lineages of leukocytes involved in determining HbF response to HU therapy, we compared HU-mediated HbF increases and reductions of neutrophil or lymphocyte/monocyte counts (Figure 4). The correlation value between the HbF increases and the reduction levels of neutrophil counts ($P<0.00268$) was the almost the same as that between the HbF increases and lymphocyte/monocyte reductions ($P<0.00267$; Figure 4A and B). This suggests that both cell lineages are involved in determining HbF levels induced by HU.

HbF silencing factors that are present in blood serum of SCD patients

Importantly, the results shown in Figure 5, together with our previous finding that leukocyte counts of steady-state SCD patients with high HbF levels demonstrated a strong inversely correlation with HbF levels (N = 47, $R^2 = 0.229$, $P<0,0006$),[17] has led us to hypothesize that leukocytes may have a role in downregulating HbF expression in erythroid-lineage cells by secreting HbF silencing factors.

To detect the HbF silencing factors present in serum of SCD patients, we first determined whether CD34+ cells cultured with 30% human AB serum can be differentiated to erythroblasts as our previous studies had employed both FBS and human AB serum.[19,20,27] As shown in Figure 5A and B, 30% human AB serum permitted CD34+ cells to differentiate to erythroid-lineage cells to a degree similar to that by 30% FBS, as analyzed by flow cytometry on the basis of expression of glycophorin A and CD71. Next, we investigated the expression of both γ-globin and γ-globin mRNA in CD34+-derived erythroblasts incubated with FBS, normal human AB serum, or SCD patient serum (Figure 5C and D). The leukocyte

counts of SCD patients studied here were as follows: Pt.1, 7,800/µL; Pt. 2, 15,300/µL; Pt. 3, 11,800/µL; the counts represent average numbers for 3–6 months. Although CD34+-derived erythroblasts (lane 2) cultured with 30% human AB serum expressed γ-globin at a level similar to that of cells cultured with 30% FBS (lane 1), the cells that were cultured with the serum of SCD patients demonstrated significantly lower levels of γ-globin expression (lanes 3–6). It is of note that the levels of γ-globin expression was substantially varied among steady-state SCD patients without HU (lanes 3 and 4), and serum from a SCD patient who was receiving HU revealed a weaker HbF silencing effect (lane 5). Because we previously showed that the proinflammatory cytokine GM-CSF has an inhibitory effect on HbF expression in erythroid-lineage cells,[17] it is confirmed that such HbF silencing is not blocked by the addition of anti-GM-CSF antibody (lane 6), indicating that HbF silencing factors are distinct from GM-CSF. Next, we examined γ-globin mRNA expression levels in these erythroblast preparations by RT-PCR (Figure 5D). The results of γ-globin mRNA expression were in accord with those of immunoblotting shown in Figure 5C. Thus, it is likely that in SCD, leukocytes may secrete HbF silencing factors that negatively affect HbF expression in erythroid cells, and that HU may reduce the serum levels of such factors, which may be relevant to HU-mediated HbF induction in SCD patients.

Discussion

It is of paramount importance to elucidate the mechanisms by which HU regulates HbF expression in the context of SCD because the magnitude of HbF response to HU in SCD is remarkably heterogeneous and a large subset of patients are non-responders.[28] Furthermore, HU-induced HbF increases are eventually lost even in SCD patients who initially responded to HU and these underlying mechanisms also remain unknown.[15,18] A number of SNPs associated with HbF responders in SCD patients have been identified;[29] however, the significance and the implications of such SNPs are yet to be established.

We recently reported that HU-induced HbF levels correlate with reduced leukocyte counts.[17] This suggests that the mechanisms mediating HU-induced HbF expression may be relevant to those that control leukocyte counts. In this study, we performed a retrospective analysis of SCD patients for whom clinical data of pre-HU and post-HU therapy were available. We found that the levels of HbF induction following HU therapy are associated with the improvements of hematological parameters such as total hemoglobin, MCV,
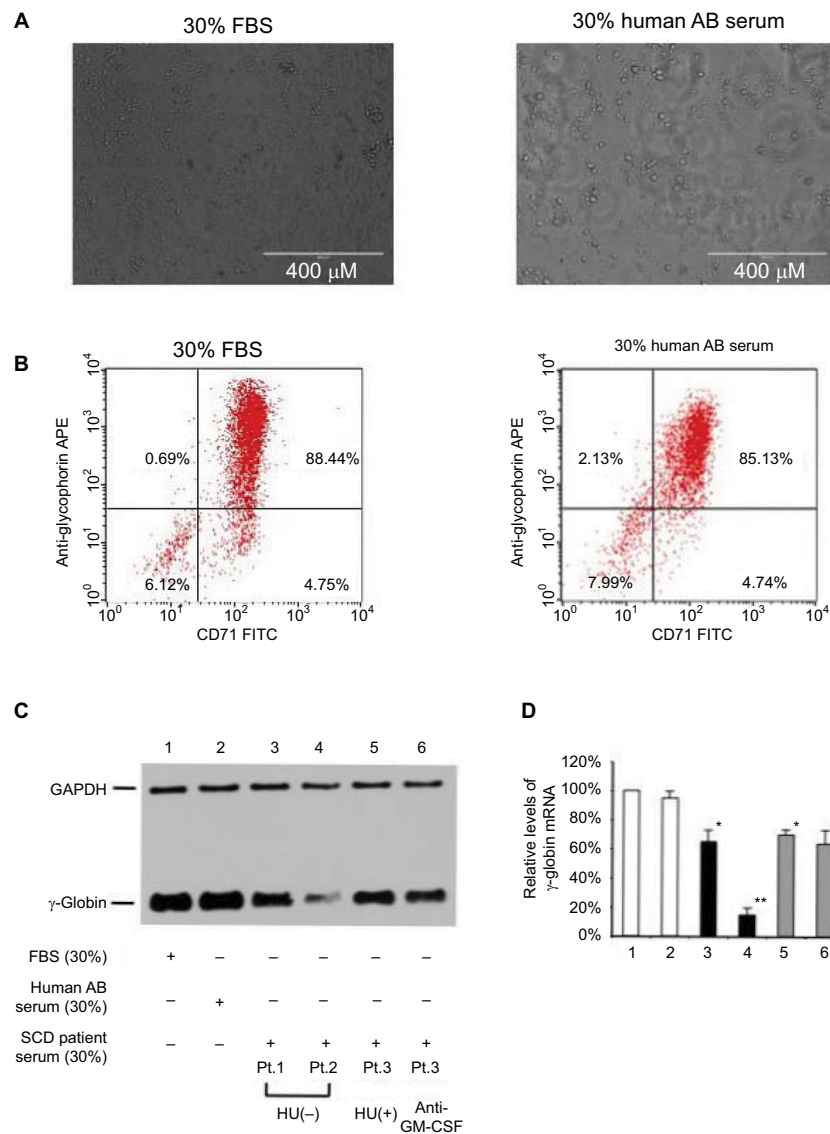
Figure 5 Presence of HbF silencing factors in serum of SCD patients.
Notes: (**A**) Culture of CD34$^+$-derived erythroblasts with 30% FBS (left panel) or 30% human AB serum (right panel). Cell photographs were taken on day 10 using EVOS FL Cell Imaging System. White bars indicate 400 μM. (**B**) Analysis of CD34$^+$-derived erythroblasts that were harvested on day 14 by flow cytometry using anti-glycophorin A and anti-CD71 antibodies. (**C**) Immunoblotting analysis of γ-globin and GAPDH in whole cell lysates prepared from CD34$^+$-derived erythroblasts. Lanes: 1, CD34$^+$-derived erythroblasts cultured with 30% FBS; 2, CD34$^+$-derived erythroblasts cultured with 30% human AB serum; 3 and 4, CD34$^+$-derived erythroblasts cultured with 30% serum from steady-state SCD patients (Pt.1 and 2); 5, CD34$^+$-derived erythroblasts cultured with 30% serum from SCD patients with HU therapy; 6, CD34$^+$-derived erythroblasts cultured with 30% serum from SCD patients with HU therapy that was incubated with anti-GM-CSF antibody as stated in Materials and methods. GAPDH was used for internal control. (**D**) Quantitation of γ-globin mRNA levels in CD34$^+$-derived erythroblasts cultured in (**C**) by RT-PCR. Lane designation is the same as that of (**C**). The γ-globin mRNA level of CD34$^+$-derived erythroblasts cultured with 30% FBS was set to 100%. *$P<0.05$ compared to lane 1. **$P<0.01$ compared to lane 1.
Abbreviations: HbF, fetal hemoglobin; SCD, sickle cell disease; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HU, hydroxyurea; GM-CSF, granulocyte-macrophage colony-stimulating factor; RT-PCR, real-time PCR.

and MCHC (Figure 1), suggesting that HbF response to HU is a legitimate marker for confirming the clinical effectiveness of HU therapy. By contrast, there were no significant correlations between HU-induced HbF levels and reticulocyte or platelet counts (Figure 2), suggesting that these lineage cells are unlikely to play a role in determining HbF response to HU; rather, their changes reflect secondary effects of HU therapy.

Previous studies have shown correlations between HU-induced HbF expression and leukocyte counts.[15,17,30] We also found that HbF expression levels of steady-state SCD patients with high HbF levels due to a mutation in the Gγ-globin gene promoter inversely correlate with leukocyte counts.[17] Also, a notion that HU-induced HbF expression may be relevant to the mechanisms controlling leukocyte counts is supported by an earlier report by Steinberg that SCD patients with high baseline leukocyte counts who exhibit a great reduction in leukocytes with HU therapy have more robust increases in HbF.[31] Thus, multiple lines of clinical evidence strongly
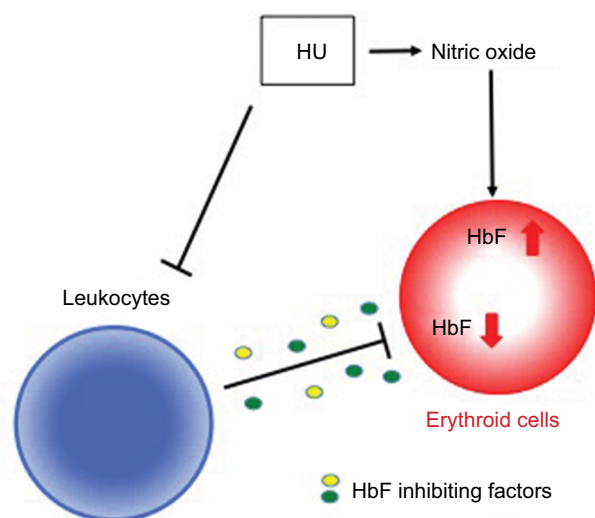
Figure 6 Possible model for the mechanisms that regulate HU-induced HbF expression.
Notes: HU has been shown to stimulate HbF expression in erythroid cells through the nitric oxide/sGC/cGMP pathway.[33–35] HU is expected to suppress the proliferation of leukocytes by inhibiting ribonucleotide reductase,[32] which in turn inhibits the production of HbF silencing factors.
Abbreviations: HU, hydroxyurea; HbF, fetal hemoglobin; sGC, soluble guanylate cyclase.

suggest that HbF expression is significantly influenced by the mechanisms controlling leukocyte count in the context of SCD.

The molecular and cellular mechanisms by which HU-induced HbF levels are associated with reduced leukocyte counts are not yet clear. Based on our study results, we hypothesize that leukocytes may secrete protein factors that bind to erythroid cells and inhibit HbF expression. Our in vitro studies (Figure 5) have demonstrated the presence of HbF silencing factors in the serum of SCD patients, whether or not they are receiving HU therapy. This is the first demonstration of HbF silencing factors in the serum of SCD patients. Interestingly, the HbF silencing activity in the serum varied significantly among SCD patients (Figure 5C, lanes 3 and 4). However, it can be argued that such factors may not in fact be secreted by leukocytes. Leukocyte numbers usually decrease in response to HU therapy in SCD because HU is a ribonucleotide reductase inhibitor.[32] Consistent with this clinical evidence, HbF silencing activities are also concomitantly reduced in someone with SCD who is receiving HU (Figure 5C lane 5). Further rigorous scrutiny is required to verify that HbF silencing factors derive from leukocytes. Importantly, such HbF silencing factors were not absorbed by anti-GM-CSF antibody (Figure 5C), suggesting that HbF silencing factors are distinct from GM-CSF, which we showed has a negative consequence on HbF expression.[17]

Our current model of the role of leukocytes in HU-mediated HbF expression in erythroid cells is summarized in Figure 6. We and others have shown that the soluble guanylate cyclase (sGC)-cGMP pathway plays a role in HU-induced HbF expression.[33–35] HU is assumed to augment HbF levels at least in part by generating nitric oxide and through the sGC-cGMP pathway. Although the sGC-cGMP pathway is also implicated in erythroid cells as a signaling mechanism for chemically induced HbF expression, it is still unknown whether extracellular signals from other lineage cells are transduced to erythroid cells and whether such signals are capable of modulating HbF expression by intracellular signaling pathways. In this study, we have clearly shown that HbF levels in SCD patients, whether they are receiving HU treatment, or not, are closely affected by leukocyte count in peripheral blood, presumably because of HbF silencing factors. As leukocyte count generally decreases in response to HU administration in SCD patients, it is reasonable to speculate that in addition to the nitric oxide/sGC-cGMP axis, HU might also increase HbF expression in SCD patients by inhibiting the secretion of HbF silencing factors from leukocytes. Collectively, there may be at least three groups of HbF regulatory proteins or pathways involved in HbF expression are possibly involved in inducing vaso-occlusive crisis therapy. One is GM-CSF, which is supposed to cause leukocytosis in SCD[16] and has a negative consequence on HbF expression.[17] Myeloid cytokines such as G-CSF or GM-CSF are possibly involved to induce vaso-occlusive crisis.[36,37] Second, HU is shown to stimulate HbF expression at least in part through the nitric oxide/sGC-cGMP axis.[33–35] This is consistent with prior vitro and in vivo findings showing that nitric oxide is generated from HU.[38,39] A third mechanism includes HbF silencing factors that are presumably released mainly from leukocytes, as reported in this study (Figure 5). HbF silencing factors may be cytokines or chemokines; further studies are necessary to precisely characterize HbF silencing factors.

Conclusion

This study has shown that HU-induced HbF expression is regulated at least in part by the mechanisms controlling leukocyte counts, and that HbF silencing factors that are secreted, possibly by leukocytes, may be involved in HU-regulated HbF expression in SCD. Thus, this study provides an important clue to the mechanisms by which HbF expression is regulated in the context of SCD patients receiving HU. It would be interesting to compare serum levels of cytokines or chemokines in SCD patients, both with and without HU

treatment. Further insight into HbF silencing factors might help us clarify the mechanisms underlying HU resistance as seen in a subset of SCD patients.[15,18]

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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