Hydroxyurea attenuates activated neutrophil-mediated sickle erythrocyte membrane phosphatidylserine exposure and adhesion to pulmonary vascular endothelium

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Submitted 14 September 2007; accepted in final form 9 November 2007

Haynes J Jr, Obiako B, Hester RB, Baliga BS, Stevens T. Hydroxyurea attenuates activated neutrophil-mediated sickle erythrocyte membrane phosphatidylserine exposure and adhesion to pulmonary vascular endothelium. Am J Physiol Heart Circ Physiol 294: H379–H385, 2008. First published November 9, 2007; doi:10.1152/ajpheart.01068.2007.—Activated neutrophils in vivo animal models (12–14, 16, 20 –22, 25, 26). The best characterized erythrocyte membrane receptors involved in erythrocyte-EC adhesion are very late-activating antigen-4 (VLA-4) and CD36 (15, 19, 25, 34, 35). An erythrocyte membrane nonreceptor mechanism implicated in erythrocyte-EC adhesion involves a subpopulation of erythrocytes that have phosphatidylserine (PS) on the external erythrocyte membrane (PS exposed) that binds to the subendothelial matrix protein, thrombospondin (27), and/or a PS receptor on vascular endothelium (8). We have previously reported that activated neutrophils increase SSRBC retention/adherence in the pulmonary circulation (12). We subsequently demonstrated that products released from activated neutrophils increase SSRBC PS exposure and retention/adherence compared with SSRBC controls (13). The increased retention/adherence seen with neutrophil activation was attenuated when lungs were perfused with SSRBCs cloaked with the calcium-dependent phospholipid-binding protein, annexin V. This mechanistically supports the role of erythrocyte PS exposure in SSRBC adherence to vascular endothelium.

Hydroxyurea (HU) is an antisickling agent that decreases vasoocclusive events such as sickle pain episodes and acute chest syndrome (4). The effects of HU are due in part to increased fetal hemoglobin (HbF) synthesis, which slows the rate at which sickle hemoglobin depolymerizes when deoxygenated. Decreased SSRBC adhesion to vascular endothelium (3), expression of VLA-4 and CD36 on stress reticulocytes (25), in vitro adhesion of SSRBCs to thrombospondin and laminin (15), and SSRBC PS exposure (7) have been reported when studying SSRBCs from individuals treated with HU. The role of HbF and HU in increased SSRBC-EC adhesion seen with neutrophil activation is not known. Setty et al. (31, 32) have reported under basal conditions that children with sickle cell anemia, who have higher proportions of F-cells, have a concomitant decrease in the number of erythrocytes positive for CD36 and VLA-4 (32), decreased erythrocyte PS exposure (31), and decreased erythrocyte-EC adhesion (32).

Based on the observations above, we postulate that the retention/adherence of SSRBCs (from individuals treated with HU for ≥ 1 yr) to pulmonary vascular endothelium is significantly less than that seen in both the SS and SS + F groups. No difference was observed between the SS and SS + F groups. At baseline, the proportion of HbF and F-cells did not differ between the SS + F and SS + HU groups. However, SSRBC treatment with activated neutrophil supernatant caused a twofold increase in PS-exposed SSRBCs in the SS control and no change in the SS + HU group. We conclude that 1) HU attenuates SSRBC retention/adherence in the pulmonary circulation seen in response neutrophil activation, 2) HU stabilizes SSRBC membrane PS, and 3) HU attenuation SSRBC retention/adherence in the pulmonary circulation occurs through a mechanism(s) independent of HbF.

Fetal hemoglobin; erythrocyte adhesion

IN SICKLE CELL ANEMIA, the adhesion of sickle red blood cells (SSRBCs) to vascular endothelium is thought to play a major role in the initiation of vasooclusion. This phenomenon has been described in cultured endothelial cells (ECs) and in ex vivo animal models (12–14, 16, 20–22, 25, 26). The best characterized erythrocyte membrane receptors involved in erythrocyte-EC adhesion are very late-activating antigen-4 (VLA-4) and CD36 (15, 19, 25, 34, 35). An erythrocyte membrane nonreceptor mechanism implicated in erythrocyte-EC adhesion involves a subpopulation of erythrocytes that were perfused with erythrocyte suspensions from SSRBC retention/adherence in isolated-perfused rat lungs. Lungs showed altered rheology and decreased numbers of vasoocclusive episodes. This improved hemoglobin (HbF) concentration has been associated with improved endothelium than non-PS-exposed cells. An increase in SSRBC fetal hemoglobin (HbF) (SS) from the SS control and no change in the SS/H11001 group. However, SSRBC treatment with activated neutrophil-mediated SSRBC retention/adherence in isolated-perfused rat lungs. Lungs were perfused with erythrocyte suspensions from 1) individuals homozygous for hemoglobin S with 0–7% HbF (SS), 2) with ≥ 8% HbF (SS + F), and 3) individuals homozygous for hemoglobin S treated with HU therapy for ≥ 1 yr (SS + HU). Retention of SSRBCs from the SS + HU group was significantly less than that seen in both the SS and SS + F groups. No difference was observed between the SS and SS + F groups. The percentage of HbF and F-cells did not differ between the SS + F and SS + HU groups. At baseline, the proportion of PS-exposed SSRBCs was not different between the SS and SS + HU groups. No difference was observed between the SS and SS + F groups. No difference was observed between the SS and SS + HU groups. At baseline, the proportion of HbF and F-cells did not differ between the SS + F and SS + HU groups. However, SSRBC treatment with activated neutrophil supernatant caused a twofold increase in PS-exposed SSRBCs in the SS control and no change in the SS + HU group. We conclude that 1) HU attenuates SSRBC retention/adherence in the pulmonary circulation seen in response neutrophil activation, 2) HU stabilizes SSRBC membrane PS, and 3) HU attenuation SSRBC retention/adherence in the pulmonary circulation occurs through a mechanism(s) independent of HbF.

IN SICKLE CELL ANEMIA, the adhesion of sickle red blood cells (SSRBCs) to vascular endothelium is thought to play a major role in the initiation of vasooclusion. This phenomenon has been described in cultured endothelial cells (ECs) and in ex vivo animal models (12–14, 16, 20–22, 25, 26). The best characterized erythrocyte membrane receptors involved in erythrocyte-EC adhesion are very late-activating antigen-4 (VLA-4) and CD36 (15, 19, 25, 34, 35). An erythrocyte membrane nonreceptor mechanism implicated in erythrocyte-EC adhesion involves a subpopulation of erythrocytes that...
(FITC) was purchased from R&D Systems (Minneapolis, MN). Glutaraldehyde and bovine serum albumin (BSA; 66,000 mol wt) were purchased from Sigma Immunochemicals (St. Louis, MO). Triton X-100, PE-Cy5 (Tri-color)-conjugated human HbF (anti-HbF), and mouse IgG-106 isotypic negative antibody control were purchased from Caltech (Burlingame, CA). Phycoerythrin (PE)-conjugated, anti-glycophorin-A (anti-GPA) antibody and the isotypic negative control antibody were purchased from Beckman-Coulter (Somerset, NJ).

Isolated perfused lung. Male Sprague-Dawley rats (275–350 g) were anesthetized with nembutal sodium (25 mg ip), and the lungs were removed for extracorporeal perfusion as previously described (11, 28). A tracheostomy was performed that permitted ventilation with a Harvard rodent ventilator (model 683) at 55 breaths/min with a tidal volume of 2.5 ml and 2.0 cmH₂O positive end-expiratory pressure. The inspired gas mixture was 21% O₂-5% CO₂-74% N₂ (room air gas). A median sternotomy was performed, heparin sodium (100 IU) was injected in the right ventricle, and cannulas were placed in the pulmonary artery and left ventricle. Heart, lungs, and mediastinal structures were removed en bloc and placed into a humidified chamber. Lungs were perfused by a Gilson Minipuls 2 peristaltic pump at a constant flow of 0.03 ml·g body wt⁻¹·min⁻¹. Lungs were perfused with a physiological salt solution (PSS) containing BSA. The PSS-BSA perfusate contained (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 22.5 NaHCO₃, 1.18 KH₂PO₄, 3.2 CaCl₂, and 5.5 glucose and 4 g/100 ml BSA. PSS-BSA (100 ml) was perfused through the lungs in a nonrecirculating fashion to remove residual blood cells and plasma. The perfusate was then changed to a PSS-BSA perfusate that contained red blood cells (RBCs) from individuals homozygous for hemoglobin S. The perfusate hematocrit was ~10%. Pulmonary arterial and venous pressures were continuously monitored with Cope pressure transducers (model 041-500-503) and recorded on a Grass polygraph recorder (model 7E). Zone 3 flow conditions (arterial > venous > alveolar pressures) were maintained throughout all experiments.

Blood collection. All participants gave informed consent, and the Human Use Committee approved the protocol. During steady state, blood samples (~40 ml) were obtained from individuals with homozygous sickle cell anemia in the sickle cell clinics at the University of South Alabama. Samples were collected in green top tubes containing heparin sodium and used immediately. Sickle cell anemia was documented by high-pressure liquid chromatography in each subject. The percent HbF was defined in 36 patients (24 patients not on HU and 12 patients on HU ≥1 yr) homozygous for hemoglobin S. From patients not on HU therapy, two groups were defined based on the percent HbF, HbF < 8% (SS, n = 12) and HbF ≥8% (SS + F, n = 12) and compared with the %HbF seen in patients treated with HU for ≥1 yr (SS + HU). These data were used to design subsequent studies. *P < 0.001 compared with SS + F and SS + HU. No significant difference was seen when comparing SS + F to SS + HU.

In isolated perfused lung, increased retention of SSRBCs can occur from increased adhesion to vascular endothelium and/or secondary to mechanical obstruction. Thus, throughout this article, the number of retained SSRBCs per gram of lung is referred to as retention/adherence.

Flow cytometric enumeration of F-cells and PS externalization. F-cells and the presence of PS-exposed erythrocytes were quantitated with a FACS Vantage flow cytometer (BD Biosciences, San Jose, CA) using 110 mW of the 488-nm line of an air-cooled argon laser (Spectra-Physics, Mountain View, CA). A 560-nm short-pass (SP) dichroic mirror (DM) was placed in front of FL1 and FL2; a 610 SP dichroic mirror (DM) was used to design subsequent studies. Routine hemostatological parameters are shown in Table 1.

RBC isolation and ⁵¹Cr labeling. Each whole blood sample was centrifuged (model 5682, Forma Scientific) at 4,100 rpm for 10 min following the removal of the plasma and buffy coat. Packed RBCs were then mixed at a 1:3 dilution with normal saline and washed three times. With each wash, the RBC suspension was centrifuged for 10 min at 3,000 rpm followed by the removal of the supernatant. Following the third wash, a complete removal of platelets and white blood cells was verified by microscopic examination of stained smears. Packed RBCs were resuspended in PBS (1 ml PBS/1 ml packed RBCs) and incubated with 100 μCi of ⁵¹Cr for 60 min in a shaker water bath at 37°C. Following incubation, the ⁵¹Cr-labeled RBCs were washed once with normal saline and resuspended in PSS-BSA to obtain a hematocrit of ~10%.

RBC retention. As previously described (12), cell counts of the ⁵¹Cr-labeled RBC suspension were obtained in a Cell-DYN 900 Coulter counter. The specific activity of the ⁵¹Cr-labeled RBC perfusate was calculated following gamma counts at 162–176 keV for 3 min and expressed as counts/min per RBC. The isolated lung was then perfused with the ⁵¹Cr-labeled RBC perfusate for 30 min. Following this, the lung was washed with 50 ml of RBC-free PSS-BSA under identical hemodynamic conditions. Perfusion was stopped, and the lung was dissected into lobes and weighed. Each lobe was counted for 3 min in a gamma counter. Retention/adherence (R) was calculated as R = specific activity of lung/specific activity of perfusate; R = (counts/min per gram of lung)/(counts/min per RBC); and R = retained RBC/gram of lung.

Fig. 1. Comparison of the percent fetal hemoglobin (HbF) in sickle anemia patients: presence and absence of hydroxyurea (HU) therapy. From routine hemoglobin evaluations using high-pressure liquid chromatography, the %HbF was defined in 36 patients (24 patients not on HU and 12 patients on HU ≥1 yr) from the sickle cell clinics in patients homozygous for hemoglobin S. From the patients not on HU therapy, two groups were defined based on the percent HbF, HbF < 8% (SS, n = 12) and HbF ≥8% (SS + F, n = 12) and compared with the %HbF seen in patients treated with HU for ≥1 yr (SS + HU). These data were used to design subsequent studies. *P < 0.001 compared with SS + F and SS + HU. No significant difference was seen when comparing SS + F to SS + HU.
Table 1. Comparison of hematological parameters

<table>
<thead>
<tr>
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<th>SS Control</th>
<th>SS + F</th>
<th>SS + HU</th>
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<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>12</td>
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<tr>
<td>Hemoglobin, g/dl</td>
<td>8.8 ± 0.3</td>
<td>10.0 ± 0.5</td>
<td>10.2 ± 0.4</td>
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<tr>
<td>Reticulocyte count, %</td>
<td>6.9 ± 1.1</td>
<td>8.1 ± 1.5</td>
<td>5.3 ± 0.8</td>
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<tr>
<td>Mean corpuscular volume</td>
<td>94.6 ± 2.5</td>
<td>89.6 ± 2.8</td>
<td>109 ± 4.0 †</td>
</tr>
<tr>
<td>Volume, FL</td>
<td></td>
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<tr>
<td>WBC count (×10⁶)</td>
<td>11.7 ± 0.9</td>
<td>12.8 ± 1.0</td>
<td>6.8 ± 0.6 ‡</td>
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Values are means ± SE; n, number of individual blood samples. SS homozygous for hemoglobin S; SS control, 0–7% hemoglobin F; SS + F, >8% hemoglobin F; SS + HU, on hydroxyurea therapy for 1 yr. †p < 0.01 compared with SS control. ‡p < 0.001 compared with SS + F controls.

the upper left and upper right quadrants (total number of GPA-positive cells) of this same dot plot.

Neutrophil isolation and activation. Heparinized whole blood (20 ml) from individuals with homozygous sickle cell anemia was diluted 1:1 with normal saline. Neutrophils were then isolated after dextran sedimentation and centrifugation on histopaque 1077 cushions as described previously (12). Briefly, dextran 500 was added to the 1:1 mix at one-fourth the volume of the 1:1 mix. This was allowed to incubate for 45 min at 37°C. The supernatant (which contains neutrophils) was aspirated into a 50-ml centrifuge tube and was underlayered with 10-ml of histopaque 1077. This was centrifuged at 1,250 rpm for 30 min. The supernatant was aspirated and discarded, leaving the neutrophil pellet. Sterile H2O (3 ml) was added to the neutrophil pellet and agitated for 30 s to lyse any residual RBCs. PBS (45 ml) was added immediately thereafter to stop the reaction. This was centrifuged at 1,250 rpm for 10 min. Finally, the supernatant was aspirated and discarded. This process of RBC lysis, followed by a PBS wash, was repeated until the neutrophil pellet was without visible evidence of RBC contamination. A neutrophil stock suspension was prepared with the neutrophil pellet suspended in 1 ml of sterile Hank’s balanced salt solution. A total neutrophil count was obtained using the Cell-DYN 900 Coulter counter. Final preparations contained 95% neutrophils, and viability was >95% as assessed by trypan blue dye exclusion. Phorbol myristate acetate (20 ng/ml) was added to the test tube containing the 1 ml stock suspension of neutrophils and allowed to incubate for 30 min before being added to the perfusate reservoir of lungs perfused with SSRCB suspensions. The final concentration of activated neutrophils was 200,000 cells/ml of perfusate. In studies evaluating PS-exposed cells, a volume of supernatant from activated neutrophil stock (equivalent to the volume calculated to contain ~200,000 cells/ml) was added to 1 μl of whole blood in 200 μl of PBS-BSA and allowed to incubate for 20 min before subsequent FACS analysis.

Statistics. All results are presented as means ± SE. Statistical analyses were performed using the unpaired Student’s t-test and one-way analysis of variance (ANOVA). Tukey’s test (37) was used for multiple comparisons when ANOVA indicated statistically significant differences between or within groups. Differences were considered to be significant when P < 0.05.

RESULTS

Hematological parameters of whole blood from homozygous sickle cell anemia patients with <8% fetal hemoglobin, ≥8% fetal hemoglobin, and HU therapy for ≥1 yr. The hematological characteristics of the whole blood from patients with homozygous sickle cell anemia treated with HU for ≥1 yr (SS + HU) were compared with the homozygous sickle cell anemia controls with 0–7% HbF (SS control) and ≥8% HbF (SS + F) (Table 1). Although the total hemoglobin concentrations tended to be higher in the SS + F and SS + HU groups than in the SS control, these differences were not significant. Similarly, no significant difference was observed in reticulocyte counts. Although no difference in the mean corpuscular volume was observed between the SS and SS + F controls, the mean corpuscular volume was significantly larger in the SS + HU group. Comparison of the total white blood cell counts revealed the counts in the SS + HU group were significantly lower than those seen in the SS and SS + F controls (P < 0.001).

The percent F-cells and hemoglobin F in individuals on HU are comparable with individuals with homozygous sickle cell anemia with elevated HbF. The percentage of F-cells was significantly lower in the SS-control group compared with SS + F (P < 0.01) and SS + HU (P < 0.01) groups (Fig. 2). The percentage of F-cells was highest in the SS + F (43.6 ± 9.0%) followed by the SS + F (41.5 ± 6.0%) and SS (9.7 ± 2.0%) groups. The percent F-cells did not differ significantly between the SS + F and SS + HU groups. Similarly to the percent F-cells, the percent HbF in the SS control (2.0 ± 0.8%) was much lower than that observed in the SS + F (19.0 ± 2.0%) and SS + HU (15.0 ± 2.0%) groups (Fig. 1). No difference in the percentage of HbF was observed between the SS + F and SS + HU groups. Results of studies performed simultaneously with the F-cell analysis that addressed retention/adherence in the isolated perfused lung are described below.

The retention/adherence of SSRBCs from individuals with homozygous sickle cell anemia on chronic HU is decreased compared with homozygous sickle cell anemia controls in isolated perfused lung. Studies were performed that compared the retention/adherence (expressed in RBC/g of lung) characteristics in lungs perfused with suspensions containing RBCs from individuals with homozygous sickle cell anemia with 0–7% HbF (SS), sickle cell anemia with ≥8% HbF (SS + F), and individuals with sickle cell anemia receiving HU for ≥1 yr (SS + HU) (Fig. 3). The difference in retention/adherence observed when comparing SS and SS + F-perfused lungs was not significant. Retention/adherence in the SS and SS + F groups was 5.0 ± 0.4 × 10³ and 4.0 ± 0.4 × 10³, respectively.
incubation of SS
increase in the percentage of PS-exposed cells. In contrast, supernatant from activated neutrophils resulted in a 123% neutrophil groups (Fig. 4). Incubation of control SSRBCs with P/H9021 in a recirculating fashion under constant flow conditions for 30 min, and the retention/adherence of erythrocytes was determined as previously described in MATERIALS AND METHODS, and comparisons between study groups were made. \( \Phi P < 0.001 \) compared with the SS and SS + F groups.

In contrast, retention/adherence in the SS and SS + F groups was significantly increased compared with that in SS + HU (\( P < 0.001 \)). These results suggest that the percent HbF is not a primary determinant of basal SSRBC retention/adherence in the pulmonary circulation and that HU decreases SSRBC retention/adherence through a mechanism independent of increased HbF synthesis.

**HU decreases activated neutrophil-mediated sickle erythrocyte PS exposure.** We have previously demonstrated that products of activated neutrophils increase SSRBC retention/adherence in the isolated perfused lung (12). We subsequently demonstrated that activated neutrophils increase SSRBC PS exposure and that annexin V (a calcium-dependent phospholipid-binding protein for PS) attenuates activated neutrophil-mediated retention/adherence (13). With the use of flow cytometry, double-labeling studies were performed with annexin V-FITC fluorescence and GPA to assess and compare the percent PS-exposed SSRBCs from patients on chronic HU with SS controls at baseline and following coinubation with supernatant from activated neutrophils. No difference in the percentage of PS-exposed cells was seen when comparing the SS control with the SS + HU ± supernatant from activated neutrophils groups (Fig. 4). Incubation of control SSRBCs with supernatant from activated neutrophils resulted in a 123% increase in the percentage of PS-exposed cells. In contrast, incubation of SS + HU erythrocytes with supernatant from activated neutrophils did not increase the percentage of PS-exposed erythrocytes significantly above control values (2.8 ± 0.5% and 4.2 ± 0.7%, respectively). Similar findings to those observed in assessing PS-exposed erythrocytes were found in parallel retention/adherence studies in isolated perfused lung (Fig. 5). A comparison of retention/adherence in the isolated lung perfused with activated neutrophils + SS control erythrocytes resulted in a 21% increase in retention/adherence above that observed in lungs perfused with SS control erythrocytes alone. In contrast, activated neutrophils did not increase erythrocyte retention/adherence in lungs perfused with erythrocytes from patients in the SS + HU group. These observations lend support to chronic HU therapy, having a stabilizing effect on SS erythrocyte membrane PS exposure in response to products released by activated neutrophils and/or HU therapy, having an inhibitory effect on actual neutrophils products released during activation.

![Fig. 3. Sickle red blood cells (SSRBCs) from patients treated with HU are less adhesive in the isolated perfused rat lung than SSRBCs from patients not treated with HU. Isolated rat lungs ventilated with a 21% O₂-5% CO₂-74% N₂ gas mixture were perfused with 10% hematocrit suspensions containing \( ^{31} \)Cr-labeled erythrocytes from control patients (no HU) homozygous for hemoglobin S with <8% HbF (SS, \( n = 12 \)) or ≥8% HbF (SS + F, \( n = 12 \)) or patients homozygous for hemoglobin S treated with HU for ≥1 yr (SS + HU, \( n = 12 \)) (see MATERIALS AND METHODS). Lungs were perfused in a recirculating fashion under constant flow conditions for 30 min, and the retention/adherence of erythrocytes was determined as previously described in MATERIALS AND METHODS, and comparisons between study groups were made. \( \Phi P < 0.001 \) compared with the SS and SS + F groups.](https://www.ajpheart.org/doi/10.1161/01.HYP.0000304278.60092.C0)

![Fig. 4. HU treatment attenuates sickle erythrocyte phosphatidylserine (PS) exposure. With the use of flow cytometry as described in MATERIALS AND METHODS, double-labeling studies were performed with the calcium-dependent phospholipid-binding protein, annexin V-FITC (25 \( \mu \)l/ml) and erythroid-specific marker glycophorin-A (12 \( \mu \)g/ml) to assess the number of PS-exposed SSRBCs relative to the total SSRBC population (expressed as %PS-exposed SSRBCs) in the absence and presence of supernatant from activated neutrophils (AN-S). Four groups of SSRBCs were assessed for %PS exposure and compared: SS control (\( n = 11 \)), SS + AN-S (\( n = 11 \)), SS + HU (\( n = 5 \)), and SS + HU + AN-S (\( n = 5 \)). \( \Phi P < 0.001 \) compared with SS control, SS + HU, and SS + HU + AN-S groups. No significant difference was seen between the SS, SS + HU, and SS + HU + AN-S groups.](https://www.ajpheart.org/doi/10.1161/01.HYP.0000304278.60092.C0)

![Fig. 5. Activated neutrophils (ANs) do not increase retention/adherence of SSRBC in isolated lungs perfused with cells from patients treated with HU. Isolated rat lungs ventilated with a 21% O₂-5% CO₂-74% N₂ gas mixture were perfused with paired 10% hematocrit suspensions containing \( ^{31} \)Cr-labeled erythrocytes from patients homozygous for hemoglobin S not treated with HU (SS) ± AN (\( n = 7 \), each) and treated with HU (SS + HU) ± AN (\( n = 4 \), each). In studies using neutrophils, the activation of neutrophils was achieved in the test tube by the addition of phorbol myristate acetate (20 ng/ml) to neutrophils suspended in 1 ml of HBSS. ANs were then added to the perfusate reservoir (200,000 cells/ml) at the initiation of lung perfusion. Lungs were perfused in a recirculating fashion under constant flow conditions for 30 min, and the retention/adherence of erythrocytes was determined as previously described in MATERIALS AND METHODS, and comparisons between study groups were made. \( \Phi P < 0.001 \) compared with SS. \( \Phi P < 0.001 \) compared with SS + HU and SS + HU + AN. \( \Phi P < 0.001 \) compared with SS + HU and SS + HU + AN. There was no significant difference between the SS + HU and SS + HU + AN groups.](https://www.ajpheart.org/doi/10.1161/01.HYP.0000304278.60092.C0)
Chronic HU therapy decreases sickle erythrocyte retention/adherence in lung circulation through effects exerted on the erythrocyte membrane. In Fig. 4, supernatant from activated neutrophils significantly increased the percentage of PS-exposed sickle erythrocytes compared with the sickle erythrocyte control. In contrast, the percent PS-exposed sickle erythrocytes was significantly lower following chronic HU treatment compared with sickle erythrocyte controls. When these cells were exposed to supernatant from autologous-activated neutrophils, the percentage of PS-exposed erythrocytes remained low and did not differ from the HU control. In Fig. 5, sickle erythrocyte retention/adherence was found to be inversely related to PS exposure depicted in Fig. 4. To address a potential effect of HU on neutrophils in augmenting sickle erythrocyte retention/adherence, studies were designed to assess the effect of activated heterologous neutrophils from normals (HbA) on retention/adherence (Fig. 6). Retention/adherence did not differ significantly between sickle erythrocytes obtained from two cohorts of patients on chronic HU, thus the data were pooled. A small insignificant increase in retention/adherence was seen comparing the SS + HU control to the SS + HU + supernatant from activated neutrophils (autologous, HU treated) at 1.17 × 10^8 and 1.82 × 10^8 SSRBC/g lung. When comparing the SS + HU control to the SS + HU + AN-A (from normals, HbA), the retention/adherence was not different at 1.17 × 10^8 and 0.99 × 10^8 SSRBC/g lung, respectively. This observation supports that the primary effects of HU observed in this study is on the erythrocyte membrane and not on the neutrophil.

**DISCUSSION**

HU is the only Food and Drug Administration-approved drug used in clinical practice that clearly decreases the incidence of painful vasoocclusive crisis and acute chest syndrome in sickle cell disease (4). Although the exact mechanism(s) by which HU produces its effects in vivo is not known, an increase in HbF synthesis and the proportion of F-cells, increased SSRBC deformability, improved SSRBC hydration (1), and decreased adherence of SSRBCs to vascular endothelium (3) are all potential contributors to the improvement in clinical symptoms.

This study assessed the relationship of HbF, PS exposure, and chronic HU treatment in modulating SSRBC-EC retention/adherence at steady state and in response to activated neutrophils in the pulmonary circulation. In isolated perfused rat lungs, the retention/adherence characteristics of SSRBCs from individuals with homozygous sickle cell anemia not on HU (HbF < 8%, and HbF ≥ 8%) were compared with retention/adherence of SSRBCs from individuals on HU for ≥1 yr. In this study we postulated that SSRBCs from patients treated with HU would demonstrate decreased SSRBC-EC adhesion in response products released from activated neutrophils compared with that in SSRBC controls. We further postulated that increased HbF and/or F-cells with decreased SSRBC PS exposure would correlate with the attenuation of neutrophil-mediated SSRBC retention/adherence. The data reported here demonstrate that erythrocyte retention/adherence in lungs perfused with SSRBCs from individuals on HU is significantly lower than observed in lungs perfused with SSRBCs from individuals not on HU independent of the percent HbF or F-cells. In contrast to SSRBCs from the control group (not on HU treatment), where autologous-activated neutrophils and/or their products increase SSRBC PS exposure and retention/adherence, supernatant from autologous-activated neutrophils and/or their products did not increase SSRBC PS exposure or retention/adherence in the HU treatment group. Similarly to autologous-activated neutrophils in the HU treatment group, increased retention/adherence of SSRBCs in the HU treatment group was not observed when perfused with activated neutrophil supernatant from normal (HbAA, no HU) donors.

Increased expression of CD36 and VLA-4 increases sickle reticulocyte adhesion to vascular endothelium (19, 35). PS-exposed SSRBCs have been demonstrated to be more adhesive to vascular endothelium than non-PS-exposed SSRBCs (13, 27, 33). In addition, we have recently demonstrated how activated neutrophil-membrane PS exposure and retention/adherence in lungs compared with that in SSRBC controls. This study also demonstrates that HU decreases the number of PS-exposed SSRBCs and SSRBC retention/adherence in perfused lung compared with that in SSRBC controls (not on HU). Based on these observations, the most plausible explanation for our finding of decreased retention/adherence of SSRBCs in the HU treatment group is decreased SSRBC membrane CD36, VLA-4, and PS exposure.

Unlike the SSRBC control, whose percentage of PS-exposed SSRBCs and retention/adherence significantly increased when circulated with activated neutrophils and/or their supernatant, SSRBC PS exposure and retention/adherence did not increase significantly above control values in the presence of activated neutrophils in the HU treatment group. This suggests that HU not only decreases SSRBC PS exposure under steady-state conditions but has a stabilizing effect on SSRBC membrane PS externalization in response to inflammatory stimuli. A second possibility is that HU exerts an inhibitory effect on activated neutrophil-mediated erythrocyte PS exposure. In regard to the latter, HU has been found to decrease neutrophil myeloperoxidase activity (30) and to normalize neutrophil L-selectin shedding and basal and poststimulation H_2O_2 production (2).
In this study our findings do not support a direct effect of HU on neutrophil-mediated increases in SSRBC retention/adherence. We observed that retention/adherence of SSRBCs in lungs perfused with activated neutrophil supernatant from normals (HbAA, no HU) did not differ from that seen in lungs perfused with activated neutrophil supernatant obtained from autologous donors (HbSS, on HU) or the HU control. If the reported effect of HU were due to altering neutrophil function, there would likely have been an increase in SSRBC retention/adherence seen with activated neutrophil supernatant obtained from normal donor neutrophils.

A strong linear correlation exists between the amount of HbF present and the proportion of F-cells. The proportion of F-cells in normal adults is reported to be 0.5% to 7% (36). At birth, ~80% of hemoglobin in erythrocytes is HbF and is replaced by adult forms of hemoglobin, i.e., HbA and HbS, around 6 mo of age (6). Whether or not elevated HbF in SSRBCs decreases erythrocyte adhesive properties in pulmonary circulation is not known, nor is it clear whether the attenuation of SSRBC retention/adherence seen with erythrocytes from individuals treated with HU is a function of elevated HbF. Setty et al. (31) have demonstrated an inverse correlation between the proportion of F-cells and the number of PS-exposed SSRBCs in children with sickle cell anemia. Setty et al. (33) also reported similar findings that demonstrated an inverse relationship between the proportion of F-cells and CD36 and VLA-4-positive erythrocytes. These studies suggest that the presence of increased HbF would inversely correlate with SSRBC retention/adherence in pulmonary circulation. This, however, was not seen. What was observed was that SSRBC retention/adherence in the SSRBC control (no HU) with <8% HbF did not differ from the SS control with ≥8% HbF. Although the level of HbF in the SS control with ≥8% HbF equaled that seen in the HU control, retention/adherence was 54% less than that seen in the SS control with elevated HbF. These findings, coupled with comparable increases in SSRBC retention/adherence in lungs perfused with SSRBCs with low Hbf or F-cells and increased Hbf or F-cells, strongly suggest that SSRBC adhesion to vascular endothelium occurs independently of the Hbf concentration and/or number of F-cells. It further suggests that the attenuation of activated neutrophil-mediated SSRBC-EC retention/adherence seen with erythrocytes from individuals treated with HU is not an affect related to Hbf or F-cells. This observation is further supported by two studies demonstrating that HU decreases erythrocyte adhesion receptors before significant increases in Hbf (3, 34).

Another explanation that could possibly explain our findings of decreased SSRBC retention/adherence in lung circulation and decreased erythrocyte PS exposure at baseline and in response to inflammatory stimuli in the chronic HU treatment group is HU modulation of inflammation through the regulation of nitric oxide synthesis (5, 18). Indeed, several investigators have reported data demonstrating in vivo nitric oxide formation (9) and increased plasma nitrite/nitrate and cGMP levels in sickle cell patients treated with HU (29). Increased nitric oxide production in patients treated with HU has been suggested to improve SSRBC rheology through vasodilatation, decreased expression of endothelial cell vascular cell adhesion molecule-1 (VCAM-1) (9), and increased Hbf synthesis (5, 18). The reaction of HU with hemoglobin to form nitric oxide was not investigated in this study. Thus we can only speculate that increased nitric oxide bioavailability may have played a role in the protective effect seen with chronic HU-treated SSRBCs. More studies will be required to answer the protective role of nitric oxide particularly when others have reported that, in physiological concentration, only small amounts of hemoglobin react with HU to form nitric oxide (10, 17, 23, 24).

In these studies, it is suggested that mechanisms beyond the direct reaction of HU and hemoglobin account for the increase in nitric oxide metabolites observed in patients treated with HU. In our study isolated rat lungs not previously exposed to HU, perfused with SSRBC suspensions not containing plasma or HU, and ventilated with a room-air gas mixture allowed the investigators to study only the effect of chronic HU treatment on the SSRBCs. This study design excludes HU effects on endothelial cell VCAM-1, plasma nitric oxide effects, and HbS polymerization. This coupled with the studies that compared SSRBC retention/adherence of cells with high Hbf (not on HU) to cells from patients on HU with high Hbf does not support a role for HU-nitric oxide-Hbf synthesis interactions in decreased retention/adherence of SSRBCs from patients treated with HU at baseline or in response to inflammatory stimuli.

In summary, HU decreases SSRBC retention/adherence in the pulmonary circulation under steady-state conditions and in response to inflammatory products released by activated neutrophils. Chronic HU treatment not only decreases the number of PS-exposed SSRBCs but also has a stabilizing effect on PS externalization in response to inflammatory stimuli. These effects on the erythrocyte membrane appear to occur independent of elevated Hbf and proportion of F-cells. Other inflammatory cells such as the macrophage are also likely involved but were not addressed in this study. This study provides additional data that lend to understanding further how HU exerts clinical efficacy beyond increasing Hbf synthesis.

ACKNOWLEDGMENTS

We thank Marilyn Chancellor for the preparation of this manuscript.

GRANTS

This work is supported by the Florence Foundation Research Development Grant Award.

REFERENCES


