Hydroxyurea therapy of a murine model of sickle cell anemia inhibits the progression of pneumococcal disease by down-modulating E-selectin

*Jeffrey D. Lebensburger,1 Thad Howard,1 Yunming Hu,2 Tamara I. Pestina,1 Geli Gao,2 Melissa Johnson,3 Stanislav S. Zakharenko,4 Russell E. Ware,7 Elaine I. Tuomanen,2 Derek A. Persons,1 and *Jason W. Rosch2

Departments of 1Hematology, 2Infectious Diseases, and 3Animal Imaging Center, and *Department of Developmental Neurobiology, St Jude Children's Research Hospital, Memphis, TN

Sickle cell anemia is characterized by chronic hemolytic anemia and vascular inflammation. Hydroxyurea therapy decreases vaso-occlusive complications of SCA and reduces mortality,1,2 but the mechanism of this benefit has been debated. Elevated white blood cell counts have been found to correlate with greater mortality in sickle cell disease (SCD),3 and early studies suggested hydroxyurea reduced leukocytosis.4,5 Subsequent studies found no statistical association between reduction of leukocytosis and mortality in SCA patients receiving hydroxyurea, implicating increased levels of hemoglobin F (HbF) as the main predictor of mortality.6 A recent placebo-controlled clinical trial of hydroxyurea in pediatric SCA patients confirmed a highly significant decrease in total white blood cell and absolute neutrophil counts after hydroxyurea treatment as well as increases in hemoglobin and HbF levels.7 A significant decrease in acute chest syndrome, which is oftentimes initiated by lung infection, also was observed in this study.7 Bacteremia was recorded 6 times in the placebo group but only 3 times in the hydroxyurea-treated patients;7 a trend that was not significant because of the low incidence. Although induction of HbF is generally accepted to be a major benefit of hydroxyurea therapy in SCA, the relative contribution of other factors, including decreased white blood cell counts, to positive outcomes remains a possibility that has yet to be explored.

Modulation of leukocytosis by hydroxyurea raises the question of infection risk in SCA. Children with SCA have a 400-fold greater risk of fulminant, lethal pneumococcal sepsis than their healthy peers or patients with other hemolytic anemias,8-10 a finding recapitulated in the sickle cell mouse model.11 Despite administration of penicillin prophylaxis, pneumococcal polysaccharide vaccine, and pneumococcal protein-conjugate vaccine, invasive pneumococcal disease continues to be a serious risk for patients with SCA.12 Progression of pneumococcal infection in SCD is accelerated by the widespread vascular inflammation that induces expression of endothelial receptors used for bacterial invasion.11,13,14 Recent studies have demonstrated pharmacologic reduction of vascular inflammation attenuates invasive infection.14 We sought to determine the impact of administration of hydroxyurea at a dose that reduces leukocytosis15 on the course of pneumococcal infection.

Because murine sickle cell models lack γ globin, the model also isolates effects of hydroxyurea that are independent of HbF induction.15 We demonstrate that hydroxyurea reduces lung inflammation and confers protection against pneumococcal challenge. Hydroxyurea therapy significantly reduces expression of E-selectin and decreases adhesion and extravasation of neutrophils. The proposed mechanism of protection was confirmed in E-selectin-deficient sickle cell mice that showed no additional protective benefit from hydroxyurea therapy. Patients with SCA receiving hydroxyurea therapy also were shown to have significantly reduced serum E-selectin. These data indicate that hydroxyurea affects leukocyte–endothelial interactions in SCA, resulting in protection against lethal pneumococcal sepsis.

Methods

Generation of sickle cell mice

All experiments using animals were performed with the prior approval of and in accordance with guidelines of the St Jude Institutional Animal Care


*J.D.L. and J.W.R. contributed equally to this work.

There is an Inside Blood commentary on this article in this issue.

The online version of the article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2012 by The American Society of Hematology
intravitral fluorescence microscopy

Cranial windows were installed overlying the cerebral cortex as described previously.19,20 Mice with cranial windows were anesthetized with 1 mg of ketamine and 1 mg of xylazine in a volume of 200 μL/mouse, immobilized on a stereotactic frame, and placed under an industrial-scale microscope (model MM-11, Nikon). One hour before surgery, mice were injected intravenously with TNFα, as described previously.19 Green fluorescent protein (GFP) neutrophils were prepared as described previously from C57BL/6-Tg(CAG-EGFP)131Osb/LeySop mice (stock 006567; The Jackson Laboratory).21 Video images were taken for 5 minutes after injection of GFP neutrophils using NIS Elements (Nikon). The number of adherent neutrophils was measured by an observer unaware of mouse type or treatment group.

in vivo 2-photon imaging

Two-photon laser-scanning microscopy was performed using an Ultima IV imaging system (Prairie Technologies), a Ti:sapphire Chameleon Ultra femtosecond-pulsed laser (Coherent), and 20× 0.95 numerical aperture, water-immersion infrared objectives (Olympus), as described previously.22,23 Surgery was performed over the auditory cortex, and cranial windows were installed as described under “intravitral fluorescence microscopy.” Rhodamine-dextran (10 000 MW, 1 mg/mouse; Invitrogen) was injected to visualize the vasculature along with the GFP neutrophils. Samples were excited at 900 nm, and consecutive 500 images were captured at a rate of 15 frames/second using the AOD module of the Ultima IV imaging system.

Measurement of inflammatory cytokines

Inflammatory cytokines were measured using the Milliplex map kits (Millipore) for murine and human samples. For both sample types, a CVD kit containing E-selectin, ICAM-1, and VCAM-1 was used.

Mouse challenge

Streptococcus pneumoniae strain D39x was grown on tryptic soy agar (EMD Biosciences) supplemented with 3% sheep blood or defined semisynthetic casein liquid media24,25 supplemented with 0.5% yeast extract. Hydroxyurea did not effect bacterial growth at concentrations up to 500μg/mL. Bacterial challenge studies were performed as described previously, except that lethal dose (LD)50 (rather than LD100) was chosen to observe pneumonia as well as sepsis. Bacteria were introduced by intranasal administration in 25 μL of saline: 5 to 8 × 105 CFUs for transplanted mice and 2 × 106 CFUs for nontransplanted mice. The E-selectin mice were challenged with 1 × 106 CFUs in 25 μL of saline. Mice were monitored daily for signs of infection and imaged in the IVIS Imaging System 100 System (Xenogen).27 Differences in the survival curves were calculated by Mantel-Cox log rank test. Bacterial density in blood was quantified at 24 hours after infection and compared by Mann-Whitney U test.

hydroxyurea human protocol

Children with SCA were enrolled in the Hydroxyurea Study of Long-Term Effects (HUSTLE, NCT 00305175). Subjects in the new cohort had baseline serum samples obtained before initiating hydroxyurea treatment. The hydroxyurea dose was initially 20 mg/kg/day, but it was adjusted to reach the maximal tolerated dose (MTD) as described previously.28 Follow-up samples were obtained after reaching MTD. All blood collections and analyses using HUSTLE samples were approved by the St Jude Investigative Review Board.

results

Impact of hydroxyurea on the course of pneumococcal infection

Sickle mice were treated with hydroxyurea for 4 weeks. Bacteria were administered intranasally to initiate colonization, and progression to pneumonia and hydroxyurea was continued daily after

flow cytometry

Bronchoalveolar lavage fluid was collected 6 hours after intratracheal administration of 106 ethanol-fixed pneumococci, and red blood cells were lysed using ACK lysis buffer (Lonza Walkersville Inc). Ethanol-fixed pneumococci were used to provide a consistent immune-stimulus because differential outgrowth of bacteria in the lungs can result in greater variability in the time points being studied. Single-cell suspensions were enumerated, and cells were blocked with 1% FBS and stained for surface CD11b and GR1 (eBioscience). Flow cytometry data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).
Treatment with hydroxyurea resulted in a significant delay in time to death (Figure 1A; \( P = .0028 \)), and the bacterial burden in the blood was significantly reduced (sickle/mock median = 4.2 \( \times \) \( 10^9 \) CFUs/mL vs sickle/hydroxyurea median below limit of detection of 103). Wild-type mice showed no protective effect of hydroxyurea therapy (Figure 1B).

Histopathologic findings in the lungs were consistent with survival data. In the mock-treated sickle animals, 80% showed severe interstitial pneumonia characterized by neutrophils filling the alveolar space, thickened alveolar walls, and the presence of foamy macrophages (Figure 2C). The vessels contained marked perivascular lymphocytes, plasma cells, sickled erythrocytes, and neutrophils. Vasculitis was common in more than 80% of the sections examined. In contrast, 80% of hydroxyurea-treated animals displayed modest interstitial pneumonia with few neutrophils in the alveolar space and less alveolar wall thickening (Figure 2D). The vascular lesions were more moderate in comparison with the mock-treated animals, with less perivascular infiltration by lymphocytes. Quantitation of the histopathologic signs of interstitial pneumonia and vasculitis showed significant reduction in the SCA, mock-transplanted mice undergoing hydroxyurea therapy (Figure 2E-F). Wild-type mice showed no changes in lung inflammation in

![Figure 1. Effect of hydroxyurea therapy on pneumococcal pneumonia.](image)

![Figure 2. Histopathology of SCA lungs after pneumococcal challenge.](image)
response to hydroxyurea (Figure 2E-F). These data indicate that sickle mice receiving hydroxyurea therapy have significantly reduced lung inflammation and damage in response to bacterial infection and improved survival.

**Effect of hydroxyurea on leukocytes**

Consistent with previous studies of hydroxyurea in both patients and murine models, a significant decrease in the number of circulating leukocytes, particularly neutrophils, was observed after hydroxyurea therapy (mean = 6.1 vs. 4.7 × 10³ cells/µL; *P* = .003). To ascertain the degree to which neutrophils could be recruited to the lung in response to a defined, nonreplicating inflammatory stimulus, killed pneumococci were administered intratracheally to mock- or hydroxyurea-treated sickle mice, and lung lavages were collected 6 hours after challenge. A significant (*P* < .05, Mann-Whitney) reduction in the number CD11b⁺/GR1⁺-positive neutrophils was seen in the lungs of the hydroxyurea-treated animals compared with the mock group (Figure 3A; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). To examine neutrophil adherence to the endothelia as a critical step in recruitment, GFP neutrophils were injected into sickle mice fitted with cranial windows to allow visual monitoring of adherent neutrophils in real time. Although SCA mice showed numerous neutrophils adherent to vessels (Figure 3B-C; supplemental Video 1), the hydroxyurea-treated animals had significantly (*P* < .05, Mann-Whitney) fewer adherent neutrophils (Figure 3B,D; supplemental Video 2). Similar observations were made when imaged using 2-photon microscopy wherein adherence and extravasation of the neutrophils were more apparent in the SCA mock animals (Figure 3E; supplemental Video 3) compared with the SCA animals treated with hydroxyurea (Figure 3F; supplemental Video 4). This indicates that the effect of hydroxyurea in the SCA mice was apparent at the level of neutrophil adherence and margination to endothelia and resulted in decreased pulmonary leukocytosis.

**Hydroxyurea decreases E-selectin**

Several host receptors are required for the binding and recruitment of neutrophils to the site of infection, including E-selectin and ICAM-1. We sought to determine whether any markers of vascular inflammation were altered in sickle mice with or without hydroxyurea therapy. We observed a significant (*P* < .001) decrease in the amount of soluble E-selectin in the serum of hydroxyurea-treated sickle mice (Figure 4A) but no significant differences in
soluble ICAM-1 or VCAM-1 levels between these 2 groups (supplemental Figure 2). Furthermore, we did not observe any significant decreases in the amount of soluble inflammatory cytokines, including TNFα, IL-6, IL-1β, or IL-12 (data not shown). The decreased amount of E-selectin also was observed in the lungs of sickle mice treated with hydroxyurea. Untreated SCA mice displayed high levels of E-selectin on pulmonary epithelia, underscoring the heightened pulmonary inflammation (Figure 4C). Hydroxyurea treatment reduced the amount of E-selectin found throughout the lung in 80% of the animals examined (Figure 4D), indicating that soluble E-selectin levels correlated well with decreased expression on the cellular surface. No discernible differences were observed for platelet activating factor receptor (PAFr) or P-selectin expression in the lung sections (data not shown).

To ascertain whether hydroxyurea attenuation of E-selectin expression was operative in human SCA patients receiving hydroxyurea therapy, serum samples collected before therapy and at MTD were analyzed for levels of soluble E-selectin, ICAM-1, and VCAM-1. In excellent agreement with the murine data, we observed a significant \((P < .001)\) decrease in E-selectin levels after hydroxyurea treatment in patients (Figure 4B) and no significant differences in either ICAM-1 or VCAM-1 (data not shown).

To confirm that the protective benefit of hydroxyurea in the murine SCA model was indeed dependent on E-selectin, we next undertook experiments in which E-selectin–deficient mice were engrafted with SCA bone marrow, generating an E-selectin–deficient SCA mouse. We recognize that E-selectin–deficient mice have poor leukocyte recruitment and fail to clear bacteria, resulting in increased sensitivity to systemic pneumococcal infection.\(^29,30\) However, even though the baseline survival is lower in E-selectin–deficient mice than in wild-type mice (with and without transplant to SCA), improved survival of the SCA E-selectin–deficient mice by hydroxyurea would indicate independence from E-selectin. The mice underwent hydroxyurea or saline therapy as described for the mice in Figure 1A, and they were subsequently challenged with pneumococci. Hydroxyurea was found to confer no significant protective benefit in SCA mice in the absence of E-selectin at either the level of mean time to death, overall survival (Figure 5A), or bacterial invasion into the blood (Figure 5B). These data indicate that hydroxyurea protects against pneumonia in SCD.

Figure 4. Effect of HU on E-selectin levels in lung and serum. (A) Measurements of soluble E-selectin in the serum of mice (SCA saline, \(n = 30\), mean \(= 145 \pm 45 \text{ ng/mL}\); SCA + HU, \(n = 35\), mean \(= 98.5 \pm 35.5 \text{ ng/mL}\)). (B) Measurements of soluble E-selectin in the serum of humans (SCA/pre-HU, \(n = 92\), mean \(= 77 \pm 60\); SCA/MTD, \(n = 57\), mean \(= 31 \pm 26\); healthy controls, \(n = 18\), mean \(= 22 \pm 14\)) undergoing HU therapy. All groups significantly different \((P < .001)\) from all other groups by 2-tailed \(t\) test (*compared with SCD mock; †compared with SCD + HU). (C-E) Representative sections of lung stained for E-selectin (purple) from SCA animals receiving saline (C), HU (D), or a negative control with no primary antibody (E). Magnification: left column, \(\times 4\); right column, \(\times 10\).

Figure 5. Lack of effect of hydroxyurea on pneumococcal pneumonia in E-selectin deficient SCA mice. (A) Mean overall survival of E-selectin–deficient (triangles; \(n = 9\)) vs wild-type, E-selectin–deficient/SCA mice treated with saline (circles; \(n = 21\)) or E-selectin–deficient/SCA mice treated with hydroxyurea (squares; \(n = 18\)). Mice were challenged with \(3 \times 10^6\) CFUs in 25 \(\mu\)L saline. No significant difference (Mantel-Cox log rank test, \(P = .744\)) was observed between the E-selectin–deficient/SCA mice untreated versus treated with hydroxyurea. (B) Log CFUs/mL of bacteria in blood of mice in Figure 5A at 24 hours after challenge. Each symbol is a mouse, and the bar is the mean. — indicates limit of detection. The asterisk indicates a statistically significant difference compared with SCA saline group \((P = .03\) for survival, \(P = .003\) for blood titers).
attenuation of the heightened levels of E-selectin in SCA mice is associated with improved survival.

Discussion

Hydroxyurea is increasingly used to treat patients with SCA to decrease the complications of anemia and microcirculatory vasculitis by inducing fetal hemoglobin. This study focuses on the HbF-independent effects of hydroxyurea that seem to play a significant role in decreasing the heightened susceptibility to invasive pneumococcal infection in this population. We demonstrate that hydroxyurea improved survival of SCA mice in a model of pneumonia and sepsis. Treatment induced a systemic decrease in neutrophil adhesion to activated endothelia that resulted in decreased neutrophil recruitment to the infected lung and significantly attenuated lung damage. There are several possible mechanisms for this effect.

Both the pulmonary and systemic vascular response to a variety of inflammatory stimuli is exacerbated in SCA mice. The fulminant course of pneumococcal infection in SCA derives, at least in part, from this vascular inflammation. Inflammation up-regulates receptors on endothelial cells, including the PAFr that is used by pneumococci to translocate across epithelia and endothelia as invasive disease progresses. Thus, the deterioration of pneumonia to bacteremia is accelerated in SCD mice in a PAFr-dependent manner, and anti-inflammatory therapy, such as statins, decreases PAFr expression and slows disease progression. However, hydroxyurea was shown in this study not to affect PAFr expression, suggesting that the mechanism of protection must be different.

Recent evidence has clarified the mechanistic reason for the severity of systemic pneumococcal disease. Functional asplenia is operative both in murine and human SCA, but hydroxyurea only improves spleen function when HbF levels are increased. The lack of HbF in the SCA murine model exposes the HbF-independent effects of hydroxyurea and thus focuses on the benefit of hydroxyurea independently of the spleen. In human patients, the induction of HbF and improved spleen function would potentially add to the protective benefit that we have modeled in the murine system.

Another major player determining the progression of any infection is the leukocyte response. SCA is characterized by a circulating leukocytosis and anti-inflammatory strategies that reduce the adhesion of leukocytes improve the microcirculatory pathophysiology of SCA mice. Clinical studies have indicated that cytotoxic agents, such as hydroxyurea, not only increase HbF production but also decrease leukocytosis because of myelosuppression of the bone marrow. Although hydroxyurea reduces the incidence of vaso-occlusive events in SCA, the impact on mortality or rate of infection has not been well characterized. There is a suggestion that patients receiving hydroxyurea therapy may have reduced pulmonary disease. We demonstrate that hydroxyurea not only decreased circulating leukocytosis but also strongly reduced leukocyte recruitment to the infected lung that was associated with significant protection from lung damage, reduced invasion of bacteria into the bloodstream, and improved survival from pneumococcal pneumonia in the mouse SCA model. This is consistent with the concept that although leukocytes are required to clear infection, high numbers of leukocytes can damage host tissues and lead to poor outcome despite bacteriologic cure.

The possible underlying mechanism for the effect of hydroxyurea on leukocyte recruitment was revealed to involve endothelial leukocyte adhesion molecules. Various adhesion molecules, including ICAM-1, VCAM, P-selectin, and E-selectin, are elevated in SCA patients. Hydroxyurea specifically decreased the expression of E-selectin on sickle mouse pulmonary epithelial and vascular endothelial cells. This effect was not observed for ICAM-1 or VCAM, similarly to previous findings in human SCA patients. We show that this mechanism was also evident in children with SCA, where hydroxyurea decreased basal leukocytosis and sE-selectin levels in serum. Sickle monocytes induce significantly higher levels of E-selectin in endothelial cells compared with monocytes from healthy controls. After hydroxyurea therapy in SCA patients as well as in mice, the number of circulating monocytes is reduced consistent with decreased sE-selectin, suggesting a potential mechanism for the observations of this study.

The recently completed pediatric phase 3 clinical trial of hydroxyurea (BABY HUG, clinicaltrials.gov, NCT 00006400) tested the clinical safety and efficacy of hydroxyurea therapy for infants with SCA. The incidence of sepsis and bacteremia was lower for infants receiving hydroxyurea (5 participants in placebo arm vs 2 participants in hydroxyurea arm; P = .26) but not statistically different in the BABY HUG trial. With a low incidence of pneumococcal infection, a larger sample size is required to power future studies evaluating the clinical efficacy of hydroxyurea in preventing pneumococcal sepsis. Recognizing the clinical safety of hydroxyurea in this setting and the potential to reduce the risk of pneumococcal sepsis suggested by the mouse SCA model should further add to the accumulating evidence that suggests hydroxyurea is a treatment of choice for patients with SCA. Pharmacologic targeting of inflammation to alleviate vaso-occlusive complications in SCA has attracted considerable attention and numerous therapies have shown great promise. Our data suggest that the decrease in leukocyte adhesion and migration in response to hydroxyurea therapy of SCA attenuates the host inflammatory status independent of HbF expression and has profound beneficial effects on the outcome of pneumococcal pneumonia. The observation that a similar decrease in sE-selectin was observed both in murine and human SCA subjects is encouraging. Because multiple approaches for reducing infection risk seem to have distinct mechanisms of action in the SCA host, there exists the exciting possibility that such therapies may have an additive effect and further reduce susceptibility to infection.

Acknowledgments

The authors thank the Animal Imaging Center for technical assistance with the cranial windows and Justin Burton for technical assistance with the injections. The Cell Sorting Core performed the FACS.

This work was supported by grants R01 HL-090941 (R.E.W.), U54 HL-070590 (R.E.W., D.A.P., E.I.T.), RO1 AI27913 (E.I.T.), R01 MH-079079 (S.S.Z.), and F32 AI082888 (J.W.R.); U54 HL-070590 Sickle Scholar Award (J.W.R.) from the National Institutes of Health; and the American Lebanese Syrian Associated Charities.

Authorship

Contribution: J.D.L., S.S.Z., E.I.T., D.A.P., and J.W.R. designed the experiments; T.H. and R.E.W. collected and analyzed the
human samples; J.D.L., T.I.P., G.G., M.J., S.S.Z., and J.W.R. performed the animal experiments; Y.H. performed the histology; and all authors assisted in writing the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for J.D.L. is Division of Pediatric Hematology and Oncology, University of Alabama at Birmingham, Birmingham, AL.

Correspondence: Jason W. Rosch, 262 Danny Thomas Pl, MS 320, Memphis, TN 38103; e-mail: jason.rosch@stjude.org.

References

17. Ware RE. How I use hydroxyurea to treat young patients with sickle cell anemia. Blood. 2010;116(10):1779-1786.

Hydroxyurea therapy of a murine model of sickle cell anemia inhibits the progression of pneumococcal disease by down-modulating E-selectin

Jeffrey D. Lebensburger, Thad Howard, Yunming Hu, Tamara I. Pestina, Geli Gao, Melissa Johnson, Stanislav S. Zakharenko, Russell E. Ware, Elaine I. Tuomanen, Derek A. Persons and Jason W. Rosch