Hydroxyurea corrects the dysregulated L-selectin expression and increased H_2O_2 production of polymorphonuclear neutrophils from patients with sickle cell anemia

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Impaired polymorphonuclear neutrophil (PMN) functions during sickle cell anemia (SCA) may have a pathogenic role in the onset of vasoocclusive events. We used flow cytometry to study, in whole blood, the adhesion molecule expression and respiratory burst of PMNs from children with SCA. Three different clinical groups were studied: (1) patients with no history of vasoocclusive events (n = 15); (2) patients with a history of vasoocclusive events (n = 17); and (3) patients receiving hydroxyurea therapy for severe vasoocclusive events (n = 9). Unstimulated PMNs showed decreased L selectin expression and increased H_2O_2 production whatever the severity of the disease, re-flecting PMN activation. This could contribute to endothelial activation reflected by abnormal plasma levels of soluble adhesion molecules (soluble intercellular adhesion molecule-1, sE selectin, and sL selectin). After stimulation with bacterial N-formyl peptides (N-formyl-methionyl-leucyl-phenylalanine [fMLP]), PMNs from untreated patients with a history of vasoocclusive events showed dysregulated L selectin shedding and increased H_2O_2 production. Furthermore, in these patients, tumor necrosis factor priming followed by fMLP stimulation induced an H_2O_2 production significantly higher than in the other patient groups and controls. These impairments could immobilize PMNs on the endothelium, thereby inducing reduced blood flow and fostering microvascular occlusion and vascular damage. In contrast, children treated with hydroxyurea showed near-normal basal and poststimulation H_2O_2 production as well as normal L selectin shedding after stimulation but no change in plasma levels of soluble adhesion molecules. To our knowledge, this is the first report showing major qualitative changes of PMN abnormalities upon hydroxyurea treatment in SCA patients. This strongly suggests that PMNs are a primary target of this drug. (Blood. 2002;99:2297-2303)

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Introduction

Sickle cell anemia (SCA) results from a single point mutation that substitutes valine for glutamic acid at the sixth position in the β-globin chain and produces hemoglobin S (Hb S).[1,2] The basic pathophysiologic mechanism, based upon the polymerization of deoxy-Hb S and the ensuing deformation, rigidification, and fragilization of the red cells, explains the 3 hallmarks of the disease: hemolytic anemia, vasoocclusive events, and progressive functional asplenia leading to increased susceptibility to infection. Even though all SCA patients have the same biochemical defect, they show wide variability in the frequency and clinical severity of vasoocclusive crises and remain asymptomatic for long periods, indicating that additional factors must contribute to the pathophysiology of vasoocclusion.[3] Abnormal red blood cell (RBC) adhesion to the endothelium has recently been forwarded as the probable initiating event of vasoocclusion, while variations in the endothelium activation state may modulate the unpredictable occurrence of the major complications.[4] Inflammation plays a major role in this process.[5] To date, hydroxyurea (HU) is the only drug known to reduce the frequency of vasoocclusive crises, acute chest syndromes (ACSs), and transfusion requirements.[6,7] However, the mode of action of HU remains largely unknown.

RBCs are not the only circulating blood cells to be involved in SCA. Thrombospondin produced by activated platelets is involved in RBC adhesion to the endothelium.[8] Monocytes from SCA patients activate endothelial cells in vitro, increasing endothelial expression of adhesion molecules and tissue factor.[9] Furthermore, abnormal adhesion between endothelial cells and polymorphonuclear neutrophils (PMNs) might participate in the early steps of the vasoocclusive process by decreasing the flow rate in the microvasculature. Two major lines of evidence point to the involvement of PMNs in the pathophysiology of SCA. First, a high PMN count is a characteristic feature of SCA patients at steady state, and polymorphonuclear leukocytosis is related to an increased risk of early death, ACSs, and stroke.[10,11] Second, HU decreases the PMN count,[12] and this is the biologic parameter most strongly linked to the beneficial effect of HU,[13] suggesting that PMNs may be a preferential drug target.

PMN extravasation into tissues requires the expression of adhesion molecules at the PMN surface and their counterparts on endothelial cells.[14,15] In addition, PMNs release large quantities of reactive oxygen species (ROS) in response to various stimuli, in the so-called respiratory burst.[16] This process is critical for bacterial killing and also potentiates inflammatory reactions, sometimes inducing severe host tissue injury when activation is excessive or inappropriate.[17,18] Inaccurate regulation of adhesion molecule

expression could therefore increase PMN binding to the endothelial surface, leading to inappropriate endothelial activation. Incubation of sickle erythrocytes (SS-RBCs) with endothelial cells in various in vitro models increases the expression of vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E selectin,[19,20] which are the endothelial counterreceptors of the blood cell adhesion molecules very late antigen-4, β2-integrin, and L selectin, respectively. SS-RBCs have been shown to adhere to PMNs and activate the respiratory burst in vitro.[21] However, contradictory results on the expression of the adhesion molecules CD11b/CD18 and L selectin at the PMN surface and on the PMN respiratory burst have been reported in patients with SCA.[22-26] These discrepancies could be due to the fact that most studies have involved PMNs isolated from their blood environment by means of various procedures that may alter cell responses.[27]

In this study PMNs from untreated SCA children with and without a history of vasoocclusive events were analyzed in fresh whole blood to minimize procedure-related changes in surface receptor expression. We also studied children treated with HU for major vasoocclusive events to examine the effects of HU on PMN functions. Flow cytometry, which can be used to study events at the single-cell level, was chosen to analyze adhesion molecule expression at the PMN surface (especially L selectin and CD11b/CD18) and the PMN respiratory burst (H_2O_2 production). These parameters were measured at baseline and after ex vivo stimulation. In parallel, we determined circulating levels of soluble adhesion molecules (sICAM-1, sE selectin, and sL selectin), which have been reported to reflect blood cell–endothelium interactions and endothelial damage.[28-30]

Patients, materials, and methods

Patients

We studied 41 children from sub-Saharan Africa with SCA followed at the SCA Center at Robert-Debré Hospital (Paris, France). Their clinical and hematologic characteristics are summarized in Table 1. To enter the study the patients had to be homozygous for the Hb S mutation, with complete medical records since at least age 6 months. The patients were divided into 3 groups. Children in the nonvasoocclusive category (non-VOC) had no history of painful crisis or ACSs (n = 15; median age, 7.4 years; range, 2.3-13.8 years) and no hospitalization in the previous 2 years and the year following the study. Children in the vasoocclusive category (VOC) (n = 17, median age 6.3 years; range, 1.9-9 years) had been hospitalized a median of 3 times per year (range, 2-4 times per year) for painful crisis or ACSs; the median number of hospitalization days per year was 18 days in the previous 2 years (range,

11-29 days). Only 8 of them had experienced an ACS since birth. Children in the HU treatment category (HU) (n = 9, median age 8.7 years; range, 5.7-13.7 years) were receiving the drug for past major vasoocclusive events (more than one ACS or recurrent severe painful crisis requiring morphinomimetic analgesics). They spent a median of 42 days per year in the hospital (range, 33-48 days prior HU therapy) and had been symptom-free for at least 10 months before the study. The median duration of HU therapy was 14 months (range, 12-43 months), and the median dose was 15 mg/kg/d (range, 11-17 mg/kg/d).

All patients were at steady state, ie, were free of any acute event 4 weeks prior to and after blood sampling, and transfusion-free for 3 months prior to and 1 month after blood sampling. They were all treated with preventive penicillin V and folate supplementation. No patients have had stroke or priapism in each category. There was no crossing over between non-VOC and VOC categories in the 2 years following the study. The complete medical history was recorded, and no systemic disease potentially altering PMN functions was identified. None of the patients had taken curative antibiotics or corticosteroids during the 6 months prior to blood sampling. Blood samples were obtained during a regular clinical consultation. Thirty heterozygous AS (sickle cell trait) subjects (median age, 39 years; range, 26-53 years) and 11 healthy African AA (normal hemoglobin) subjects (median age, 18 years; range, 5 to 39 years) served as controls. Complete blood counts including RBC indices and reticulocytes were performed, and fetal hemoglobin (HbF) was assayed.

After informed consent had been obtained from the patients, the parents, and the controls, whole blood was sampled, kept on ice, and transported immediately to the laboratory.

Reagents

The following reagents were used: human recombinant tumor necrosis factor-α (rhTNF-α; 2×10^5 U/mL; Genzyme, Cambridge, MA); 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Eastman Kodak, Rochester, NY); N-formyl-methionyl-leucyl-phenylalanine (fMLP) and endotoxin (lipopolysaccharide [LPS]) from *Escherichia coli* (055 = B5) (Sigma Chemical, St Louis, MO); R-phycoerythrin (PE)–conjugated monoclonal mouse antihuman CD11b antibody (Dakopatts, Glostrup, Denmark); fluorescein isothiocyanate (FITC)–conjugated monoclonal mouse antihuman CD18 antibody and peridinin chlorophyll protein (PerCP)–conjugated monoclonal mouse antihuman CD45 (Becton Dickinson Immunocytometry Systems, San Jose, CA); monoclonal mouse antibody to human L selectin (CD62L, Coulter Immunology, Hialeah, FL); and FITC-conjugated goat antimouse antibody (Nordic Immunology, Tilburg, The Netherlands).

Stock solutions of DCFH-DA (50 mM) and fMLP (10^{-2} M) were prepared in dimethylsulfoxide and stored at −20°C. The different solutions were diluted in phosphate-buffered saline (PBS) just before use.

Table 1. Characteristics of the study population

Blood cell parameters	Subjects*			
	African controls (n = 11)	Non-VOC group (n = 15)	VOC group (n = 17)	HU group (n = 9)
Leukocytes†	4.40 ± 0.29	12.50 ± 0.55‖	13.47 ± 1.10‖	10.12 ± 0.67‖¶
PMNs†	2.15 ± 0.24	6.13 ± 0.39‖	6.08 ± 0.51‖	5.05 ± 0.54‖
Monocytes†	0.30 ± 0.01	1.07 ± 0.02‖	0.92 ± 0.01‖	0.88 ± 0.01‖
Platelets†	266 ± 18	440 ± 29‖	444 ± 31‖	386 ± 29‖
Hb‡	124 ± 3	80 ± 2‖	75 ± 2‖	82 ± 4‖
HbF§	—	11.2 ± 1.4	8.3 ± 1.1#	12.4 ± 1.9

*Subjects described in "Patients, materials, and methods."
†Means ± SEM blood cell counts, × 10^9/L.
‡Means ± SEM of Hb values, g/L.
§Means ± SEM of HbF, %.
‖Significantly different from controls ($P < .05$).
¶Significantly different from the other patient groups ($P < .05$).
#Significantly different from HU-treated patients ($P < .05$).

Determination of adhesion molecule expression at the PMN surface

Whole-blood samples were either kept on ice or incubated with fMLP (10^{-6} M) or PBS at 37°C for 5 minutes. To study CD11b and CD18 expression on CD45^{high} cells, samples (100 μL) from each patient were incubated at 4°C for 30 minutes with the following reagent combination: PE–anti-CD11b/PerCP–anti-CD45 and FITC–anti-CD18/PerCP–anti-CD45. To study L selectin expression on CD45^{high} cells, samples were incubated with nonconjugated anti-CD62L antibody at 4°C for 30 minutes, washed with ice-cold PBS, and then incubated at 4°C for 30 minutes with FITC–goat antimouse antibody; after one wash in ice-cold PBS, samples were incubated with PerCP–anti-CD45 at 4°C for 30 minutes. Red cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson), and white cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Nonspecific antibody binding was determined on cells incubated with the same concentration of an irrelevant antibody of the same isotype.

H₂O₂ production

H₂O₂ production was measured by using a flow cytometric assay derived from the technique described by Bass et al[31] and others.[32] We checked that this assay could be used to compare PMN oxidative burst in subjects with different PMN counts (data not shown). One milliliter of fresh blood collected onto preservative-free lithium heparinate (10 U/mL) was preincubated for 15 minutes with DCFH-DA (100 μM) in a water bath at 37°C with gentle horizontal agitation. DCFH-DA diffuses into the cells and is hydrolyzed into DCFH; during the PMN respiratory burst, nonfluorescent intracellular DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) by H₂O₂ in the presence of peroxidase. The samples were then incubated with TNF-α (100 U/mL) or LPS (5 μg/mL) diluted in PBS, or with PBS alone, at 37°C for 30 minutes. fMLP diluted in PBS (10^{-6} M final concentration) or PBS was added for 5 minutes at 37°C. The reaction was stopped, and samples were incubated with PerCP–anti-CD45 antibody at 4°C for 30 minutes. Red cells were lysed with FACS lysing solution (Becton Dickinson). After one wash (400g, 5 minutes), white cells were suspended in 1% paraformaldehyde-PBS. The fixed samples were kept on ice until flow cytometric analysis on the same day. As previously reported, the FACS lysing solution modified neither the amount of DCF generated nor the expression of activation markers such as CR3, as shown by flow cytometry.[32] Moreover, PMN viability was not altered in our conditions, as assessed by means of flow cytometry in terms of propidium iodide exclusion. Finally, we checked that DCF did not diffuse out of the cells (data not shown).

Flow cytometry

We used a Becton Dickinson FACSCalibur with a 15-mW, 488-nm argon laser. PMN functions were analyzed using CellQuest software. A first gate was drawn on a forward- and side-scatter dot plot around the granulocyte population. The fluorescence of anti-CD45 antibody was used to identify CD45$^+$ cells and to gate out other cells and debris, such as SS reticulocytes (which are resistant to red cell lysis). A second gate was then drawn on CD45$^+$ cells. The purity of the gated cells was assessed by using FITC- or PE-conjugated CD3, CD14, and CD15 antibodies (Becton Dickinson). Acquisition was performed on the intersection of these 2 gates. A total of 5000 events were counted per sample, and the fluorescence pulses were amplified by 4-decade logarithmic amplifiers. The green fluorescence of DCF and FITC antibodies was recorded from 515 to 545 nm; the red fluorescence of PE–anti-CD11b was recorded from 563 to 607 nm. In all cases, unstained cells were used and the photomultiplier settings were adjusted so that the unstained cell population appeared in the lower left-hand corner of the fluorescence display. For dual-color analysis, single-cell controls were used to optimize signal compensation. All the results were obtained with a constant photomultiplier gain. The mean fluorescence intensity (MFI) was used to quantify cell responses.

Cytokine and soluble adhesion molecule assays

Blood was collected into sterile ethylenediaminetetraacetic acid–treated vacuum tubes, transported on ice to the laboratory, and immediately centrifuged at 1500g for 15 minutes at 4°C to avoid cytokine synthesis or breakdown in vitro. Plasma samples were stored at −70°C for no longer than 15 days before assay. Cytokines in plasma were assayed in duplicate by using immunoenzymatic assays for interleukin (IL)-8 and TNF-α, with a detection limit of 10 pg/mL (R&D, Abingdon, United Kingdom, for IL-8; and Immunotech, Marseille, France, for TNF-α). The assays were standardized by comparing the results to those of standards from the National Institute for Biological Standards and Control (England). Cytokine recovery was 102% ± 4% from normal plasma spiked with recombinant human cytokines. Soluble L selectin, sE selectin, and sICAM-1 were assayed in duplicate by using immunoenzymatic assays (R&D for sE selectin and sICAM-1; Immunotech for sL selectin) with respective detection limits of 15 ng/mL, 0.1 ng/mL, and 0.35 ng/mL for sL selectin, sE selectin, and sICAM-1.

Statistical analysis

Results are expressed as means ± SEM. The group means were compared using analysis of variance followed by a multiple comparison of means with the Mann-Whitney least-significant-difference procedure, and P values below .05 were considered significant. Correlations were identified by means of the Spearman rank correlation coefficient (ρ).

Results

Characteristics of the study population

As shown in Table 1, total leukocyte, PMN, and monocyte and platelet counts were significantly higher in the 3 groups of SCA patients than in healthy African controls. Patients treated with HU had significantly lower leukocyte counts than untreated patients, while the difference in the PMN count was borderline. The Hb level was significantly lower in the patients than in the healthy controls. The percentage of HbF was significantly higher in HU-treated patients than in untreated VOC children (12.4% vs 8.3%), and so was the median of the mean corpuscular volume values (92 vs 82 μm³). The reticulocyte count was significantly lower in HU-treated patients (285×10^9/L) than in untreated patients (389×10^9/L). The data for heterozygous AS parents did not differ from those in the African control group.

Expression of adhesion molecules at the PMN surface

L selectin expression at the PMN surface is a critical factor in the earlier rolling phase of interaction between PMN surface and endothelial cells leading to transendothelial migration.[15] As shown

Table 2. L Selectin expression at the surface of unstimulated and fMLP-stimulated PMNs from controls and patients

Subjects	MFI	
	Unstimulated PMNs	fMLP-stimulated PMNs
African controls (n = 11)	167 ± 18	68 ± 7
Non-VOC group (n = 15)	108 ± 12*	78 ± 10
VOC group (n = 17)	113 ± 16*	107 ± 16*†
HU group (n = 9)	99 ± 11*	50 ± 4

Whole blood was incubated with anti-CD62L antibody at 4°C for 30 minutes either after maintaining the samples at 4°C (unstimulated PMNs) or after incubation with PBS or fMLP (10^{-6} M) at 37°C for 5 minutes. The MFI was recorded as described in "Patients, materials, and methods." Values obtained after 5 minutes of incubation with PBS at 37°C did not differ from that obtained after maintaining the samples at 4°C. Values are given as means ± SEM.

*Significantly different from controls ($P <$ .05).

†Significantly different from HU-treated patients ($P <$ .05).

in Table 2, the MFI value for anti–L selectin antibody binding to unstimulated PMNs was significantly lower in all groups of SCA patients than in the healthy AA controls. In addition, L selectin expression correlated negatively with the PMN count ($\rho = 0.5$, $P = .0004$). Incubation with PBS alone at 37°C did not significantly modify L selectin expression relative to unstimulated PMNs maintained at 4°C (data not shown). As expected,[33] after stimulation with fMLP (10^{-6} M, 5 minutes, 37°C), L selectin was no longer detectable on PMNs from control subjects (Table 2). The MFI obtained with anti-L selectin antibody was lower than the MFI obtained with an irrelevant antibody of the same isotype (data not shown). L selectin shedding by PMNs from the non-VOC and HU patient groups was normal. In contrast, L selectin was still detectable after fMLP stimulation of PMNs from VOC patients, with an MFI significantly higher than that in control subjects and HU-treated patients.

The β2 integrins (eg, CD11b/CD18) play a major role in the second, high-affinity phase of the interaction between PMNs and endothelial cells.[14] CD11b/CD18 expression by unstimulated PMNs did not differ significantly between the control subjects and any of the SCA patient groups (Table 3). After fMLP stimulation, PMN CD11b/CD18 expression was slightly decreased in the SCA patients (Table 3). This may in part have been due to the decreased total β2-integrin content of immature cells, owing to hyperleukocytosis.[34]

No significant modification of adhesion molecule expression was observed in heterozygous AS subjects compared with control subjects (data not shown).

H₂O₂ production by PMNs in whole blood

As shown in Table 4, basal H_2O_2 production was significantly higher in unstimulated PMNs (samples incubated with DCFH-DA and treated with PBS) from untreated SCA patients than in PMNs from healthy controls, the latter expressing low background fluorescence. This increased basal H_2O_2 production was not observed in patients receiving HU.

TNF (100 U/mL, 30 minutes) added separately to whole blood induced barely detectable H_2O_2 production by PMNs from control subjects. A small but significant increase was observed in untreated SCA patients compared with the healthy controls. fMLP (10^{-6} M for 5 minutes) added to whole blood induced a slightly enhanced respiratory burst in the VOC group. Preincubation of whole blood with TNF, followed by stimulation with 10^{-6} M fMLP for 5 minutes at 37°C, induced strong H_2O_2 production by PMNs from control subjects, as expected.[32] No significant difference in the priming effect of TNF on the response to fMLP was observed between the non-VOC group and controls, while PMNs from VOC

Table 3. CD11b and CD18 expression at the surface of resting and fMLP-stimulated PMNs from controls and patients

Subjects	MFI			
	Unstimulated PMNs		fMLP-stimulated PMNs	
	CD11b	CD18	CD11b	CD18
African controls (n = 11)	242 ± 30	47 ± 4	2957 ± 130	333 ± 23
Non-VOC group (n = 15)	226 ± 22	49 ± 5	2356 ± 148*	240 ± 25*
VOC group (n = 17)	227 ± 15	44 ± 3	2545 ± 160	266 ± 17*
HU group (n = 9)	247 ± 40	49 ± 3	2425 ± 160*	259 ± 19*

Whole blood was incubated with anti-CD11b and anti-CD18 antibodies at 4°C for 30 minutes, either after maintaining the samples at 4°C (unstimulated PMNs) or after incubation with PBS or fMLP (10^{-6} M) at 37°C for 5 minutes. The MFI was recorded as described in "Patients, materials, and methods." Values are given as means ± SEM.

*Significantly different from controls ($P < .05$).

Table 4. H₂O₂ production by whole blood PMNs from controls and patients

Subjects	MFI of DCF content			
	PBS	TNF	fMLP	TNF + fMLP
African controls (n = 11)	14.4 ± 0.9	18.2 ± 2	24.9 ± 1.6	62.6 ± 6.5
Non-VOC group (n = 15)	23.1 ± 2.9*	28.6 ± 3.9*	30.6 ± 3.4	62.5 ± 8.7
VOC group (n = 17)	27.5 ± 3.5*	33.6 ± 4.1*	40.5 ± 5.5*	92.0 ± 7.1*†‡
HU group (n = 9)	19.7 ± 2.5	23.4 ± 3.6	31.5 ± 7.7	51.1 ± 7.2

After preincubation with DCFH-DA (100 μM) for 15 minutes at 37°C, whole blood was pretreated with PBS or TNF (100 U/mL) for 30 minutes and then treated with PBS or fMLP at 10^{-6} M for 5 minutes. The MFI of DCF was recorded as described in "Patients, materials, and methods." Values are given as means ± SEM.

*Significantly different from controls ($P < .05$).

†Significantly different from the non-VOC group ($P < .05$).

‡Significantly different from the HU-treated group ($P < .05$).

patients produced significantly larger amounts of H_2O_2 than PMNs from the other patient groups and the controls. Interestingly, in these priming conditions H_2O_2 production by PMNs from HU patients was not different from that of control PMNs and was significantly lower than in VOC patients. Similar results were obtained after LPS priming (5 μg/mL, 30 minutes) followed by fMLP stimulation (data not shown).

As noted above for adhesion molecule expression, no significant modification of H_2O_2 production by resting or stimulated PMNs was observed in heterozygous AS subjects.

Plasma levels of cytokines and soluble adhesion molecules

Because TNF and IL-8 are potent PMN activators,[35,36] we measured plasma levels of these 2 cytokines. No significant modification of TNF levels (Table 5) or IL-8 levels (data not shown) was found in SCA patients compared with controls. To investigate endothelial cell activation, we measured circulating levels of soluble adhesion molecules. Soluble ICAM-1 levels and sE selectin levels were significantly increased in both groups of untreated patients as compared with control subjects. We also observed a significant decrease in sL selectin levels in non-VOC patients compared with control subjects. No significant difference in soluble adhesion molecule levels was found between the 2 groups of untreated patients or between VOC patients and HU-treated patients (Table 5).

Discussion

These results show that circulating PMNs from SCA children are activated whatever the clinical expression of the disease. Indeed, unstimulated PMNs from these patients showed decreased L selectin expression and increased H_2O_2 production. Concomitantly, we found increased plasma levels of sICAM-1 and sE selectin, reflecting endothelial cell activation. These abnormalities did not increase with disease severity. However, PMNs from untreated patients with a history of vasoocclusive events (VOC patients) showed enhanced H_2O_2 production after stimulation with bacterial N-formyl peptides or TNF alone and after TNF priming followed by fMLP stimulation. In addition, PMNs from these VOC patients showed reduced L selectin shedding in response to fMLP. Note that values in the heterozygous AS parents did not differ from those in African AA controls (data not shown). HU treatment had a strong beneficial effect on the PMN abnormalities observed in VOC patients. Indeed, HU therapy was associated with near-normal

Table 5. Plasma levels of TNF and soluble adhesion molecules

Subjects	TNF, pg/mL	sICAM, ng/mL	sE selectin, ng/mL	sL selectin, ng/mL
African controls (n = 11)	18.5 ± 5.6	208 ± 53	92.0 ± 18.5	2296 ± 128
Non-VOC group (n = 15)	27.8 ± 5.8	514 ± 53*	198.0 ± 66.3*	1673 ± 166*
VOC group (n = 17)	29.5 ± 5.3	590 ± 69*	161.3 ± 10*	1813 ± 144
HU group (n = 9)	33.1 ± 10.3	578 ± 154*	120.4 ± 24.1	1619 ± 220*

Levels of sICAM-1, sE selectin, and sL selectin were determined with immunoassays with respective detection limits of 0.35 ng/mL, 0.1 ng/mL, and 15 ng/mL. Values are given as means ± SEM.

*Significantly different from control values ($P < .05$).

H_2O_2 production, both at basal state and after priming, and normal L selectin shedding after stimulation. In contrast, HU treatment did not affect sICAM plasma levels. To our knowledge, this is the first report showing qualitative PMN changes upon HU treatment, strongly suggesting a targeted effect of the drug on PMNs.

A major step in PMN migration from the circulation to inflammatory sites is the modulation of adhesion molecule expression on both PMNs and endothelial cells. In particular, proinflammatory mediator–induced shedding of L selectin (CD62L) and increased expression of β2 integrin (CD11b/CD18) at the PMN surface are major events in transendothelial migration.[14,15] Altered modulation of adhesion molecule expression could thus influence PMN migration and lead to inappropriate release of ROS, thereby initiating tissue injury. In transgenic SCA mice, Kaul and Hebbel[5] showed inappropriate activation of leukocytes upon hypoxia/reoxygenation but not in normal mice.

Our results confirm the decrease in L selectin expression at the surface of resting PMNs reported by Lard et al[26] in asymptomatic SCA patients. Diminished L selectin expression related to a shedding process has been reported to occur during PMN activation.[15] A similar decrease in L selectin expression, associated with normal CD11b/CD18 surface expression, has previously been reported in patients with inflammatory diseases other than SCA.[37,38] L selectin shedding depends at least in part on the action at the PMN surface of a protein disulfide isomerase, which regulates the susceptibility of L selectin cleavage by a metalloprotease.[39] This protein disulfide isomerase–mediated mechanism did not alter the expression of several other cell surface molecules. Because L selectin interacts with carbohydrates of the endothelial membrane, the decreased expression of this molecule at the PMN surface may lead to a decrease in the marginal pool of PMNs and thereby contribute to the hyperleukocytosis observed in SCA patients. Indeed, we found a negative correlation between L selectin expression and the PMN count. The impairment of L selectin expression by unstimulated cells did not increase with disease severity. In contrast, after stimulation with N-formyl peptides, significant L selectin dysregulation was observed on PMNs from VOC patients: L selectin was still detectable at the surface of stimulated PMNs from VOC patients, while it disappeared from the surface of stimulated PMNs from controls and non-VOC patients. Blockade of L selectin shedding has been reported to increase leukocyte exposure to the inflamed endothelium.[40] The dysregulated L selectin shedding observed in VOC patients could therefore immobilize PMNs on the endothelium and play a key role in the initiation and propagation of vasoocclusive events by slowing blood through the microvasculature, delaying the SS-RBC transit time, and increasing the likelihood of HbS polymerization within the microvasculature.

We also observed increased basal H_2O_2 production by whole-blood PMNs from SCA patients relative to PMNs from African healthy control subjects. In addition, H_2O_2 production by PMNs from SCA patients was barely increased after stimulation with TNF or fMLP alone. The contrast between this latter result and reports of an unaltered respiratory burst in PMNs from SCA patients[23,25] could at least in part be due to methodologic factors. Indeed, we studied PMNs in whole blood, while other studies used PMNs isolated from their blood environment by various procedures that may affect surface receptor expression and thereby alter cell responses. Activation due to isolation procedures might thus mask differences between patients and healthy controls. After TNF priming followed by fMLP stimulation, H_2O_2 production by PMNs from VOC patients was increased by the same degree as in controls and reached a level significantly higher than in the other patient groups, in which the effects of the 2 stimuli were only additive. This difference may be related to a relative response defect in the non-VOC group, although acquired PMN hyperresponsiveness in the VOC group cannot be ruled out at this stage. The increased ROS production observed after priming of PMNs from VOC patients may be related at least in part to potentiation of the oxidative burst by L selectin, which did not disappear after fMLP stimulation and has been reported to act as a signaling molecule.[41]

Decreased L selectin expression associated with increased H_2O_2 production in SCA patients could result from local production of proinflammatory cytokines such as TNF-α and IL-8, which are potent mediators of PMN activation.[35,36] However, in accordance with previous data[42-44] we observed no significant increase in TNF or IL-8 plasma levels in SCA patients. These results did not preclude a locally restricted production of these proinflammatory mediators. Several studies have described, in SCA patients, increased plasma level of platelet-activiating factor[45] and production of C3a and C5a,[46,47] which are potent activators of PMNs. Furthermore, Hofstra et al[21] showed that sickle RBCs specifically bind to neutrophils through an IgG-mediated process that activates PMNs and induces ROS release. Finally, SS reticulocytes show abnormal adhesion to the endothelium.[4] Thus, multiple mechanisms are ongoing in the vascular bed that may contribute to both PMN and endothelial activations.

Dysregulation of adhesion molecule expression and increased ROS production by resting and stimulated PMNs from SCA patients could increase endothelial activation and vascular damage. As previously reported by Blei et al,[48] we found increased levels of sICAM-1 and sE selectin, which have been reported to reflect endothelial damage.[28,29] The observed decreased sL selectin levels may reflect sL selectin sequestration by widespread binding to activated endothelium in microvascular beds.[49] This is supported by immunohistochemical findings showing that sL selectin specifically binds to the luminal surface of high endothelial venules at sites of inflammation.[49,50] Thus, increased levels of sICAM-1 and sE selectin, associated with low levels of sL selectin, probably reflect diffuse activation of the endothelium. These results are in keeping with previous data demonstrating the presence of increased numbers of activated circulating endothelial cells[51] and increased plasma levels of sVCAM-1.[52,53] Moreover, ROS generated by activated PMNs have been reported to stimulate VCAM-1

up-regulation on the vascular endothelium, a known attachment site for sickle reticulocytes.[54] Increased ROS levels produced by activated neutrophils could lead to endothelial damage, increased production of proinflammatory mediators, and increased adherence between endothelial cells and SS-RBCs through VCAM-1, leading to amplification of the process.

HU is the only drug known to decrease the frequency of painful crisis in patients with SCA.[6] It was given initially with the aim of increasing HbF production.[55] However, there is no correlation between the increase in HbF levels, which is late and highly variable from patient to patient, and the clinical benefit, which is early and at least almost constant.[56] Moreover, Bridges et al[57] demonstrated that HU decreased SS reticulocyte adhesion to endothelial cells and that this change preceded the increase in HbF. Following on from reports that HU reduces the PMN count,[12,13] we show for the first time that this drug also qualitatively affects PMN functions in SCA. Indeed, PMNs from children treated with HU for past major vasoocclusive events showed a correction of the decreased L selectin shedding found in untreated VOC children. This could limit PMN adhesion to the endothelium and explain the reduced severity and frequency of vasoocclusive events on HU. In addition, basal and stimulated H_2O_2 production by PMNs from patients treated with HU showed a return to control values, and this could also reduce endothelial injury. This result is in accordance with previous data demonstrating that, in vitro, HU decreases the oxidative burst of activated PMNs from healthy subjects.[58] The persistently high sICAM level in HU-treated patients could be due to interactions other than PMN endothelium.

In conclusion, we present evidence that whole-blood PMNs from homozygous SCA patients are activated, even in the absence of clinical complications. Reduced L selectin shedding and increased ROS production could immobilize PMNs on the endothelium, thereby inducing reduced blood flow, microvascular occlusion, and vascular damage. HU has a strong beneficial effect on these PMN abnormalities. Our data strongly suggest that PMNs are a primary target of this drug and that this beneficial effect on PMNs may be a key component of its clinical efficiency. Further studies of the mechanism of action of HU on PMNs may lead to the identification of new therapeutic targets.

Acknowledgments

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