Coagulation activation and inflammation in sickle cell disease-associated pulmonary hypertension

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ABSTRACT

Background
Pulmonary hypertension (PHT) is common in sickle cell disease (SCD). The purpose of this study was to determine whether markers of coagulation activation and inflammation are associated with PHT in SCD.

Design and Methods
This cross-sectional study was performed using a cohort of patients followed at an adult Sickle Cell Clinic. Pulmonary artery systolic pressure was determined by Doppler echocardiography, and the diagnosis of PHT was defined using age, sex and body mass index-adjusted reference ranges. Clinical laboratory examinations, including hematologic studies and biochemical tests, as well as various measures of coagulation activation, endothelial activation and inflammation, were conducted on SCD subjects and on healthy, race-matched control subjects without SCD.

Results
Patients with SCD (n=76) had higher plasma levels of markers of coagulation (thrombin-antithrombin complex, prothrombin fragment F1+2, D-dimer) and endothelial (soluble vascular endothelial cell adhesion molecule, sVCAM) activation compared with control subjects (n=6). SCD patients with PHT (n=26) had significantly higher levels of sVCAM compared with those patients without PHT (n=50). Although PHT patients showed increased plasma measures of coagulation activation, the differences were not statistically significant when compared to those of patients without PHT. HbSS patients with PHT also had a trend towards higher levels of other inflammatory cytokines (interleukins 6, 8 and 10) than HbSS patients without PHT. There was a modest negative correlation between hemoglobin and plasma measures of coagulation and endothelial activation, and modest positive correlations between markers of hemolysis and plasma measures of coagulation and endothelial activation.

Conclusions
SCD patients with PHT have higher levels of markers of endothelial activation and other inflammatory markers than patients without PHT. A trend towards an increased level of markers of coagulation activation was observed in SCD patients with PHT compared with that in patients without PHT. Markers of hemolysis are associated with coagulation activation and endothelial dysfunction in SCD patients. Clinical trials of anticoagulants and anti-inflammatory agents are warranted in SCD patients with PHT.

Key words: sickle cell disease, coagulation activation, endothelial activation, inflammation, pulmonary hypertension.

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Introduction

Pulmonary hypertension (PHT) is a common complication in patients with sickle cell disease (SCD), with a reported prevalence of approximately 30%.[1-4] Multiple studies have shown that PHT is associated with increased mortality in SCD patients.[2,4-7] The pathogenesis of PHT in SCD is probably due to a variety of factors. Recent evidence suggests a central role for chronic intravascular hemolysis, with associated scavenging of nitric oxide by cell-free plasma hemoglobin.[4,8,9] Arginase, which converts L-arginine (the substrate for nitric oxide synthesis) to ornithine, is also released following hemolysis.[10] Elevated arginase activity, and the resultant decrease in the arginine/ornithine ratio, is associated with PHT in SCD.[4,11] Although various studies have found no association between PHT and a history of acute chest syndrome,[3,4] chronic lung injury resulting from repeated episodes of acute chest syndrome may lead to the development of PHT due to chronic fibrotic pulmonary parenchymal damage, altered vascular tone, vascular proliferation, hypoxia and consequent pulmonary vasculopathy. Finally, pulmonary thromboembolism[12,13] and progressive endothelial damage with concentric pulmonary vascular intimal hyperplasia and *in situ* thrombosis[13,14] may also contribute to the pathogenesis of PHT in SCD. The aim of the present study was to determine whether coagulation activation and inflammation are associated with PHT in SCD. Furthermore, we aimed to assess correlations between measures of coagulation activation and inflammation with markers of hemolysis. To address these questions, we evaluated a cohort of patients followed at an adult Sickle Cell Clinic.

Design and Methods

Patients and study design

The patients studied represent a cohort followed at the Sickle Cell Clinic at The University of North Carolina, Chapel Hill, USA. Patient selection methods have been previously described.[7] Seventy-six patients (median age, 39 (interquartile range 31-47) years; 46 women) with SCD, and an additional six healthy, race-matched control subjects (median age 45 (interquartile range: 37-49) years; 4 women) without SCD were included in the analyses. Each enrolled patient with SCD was studied while in a non-crisis, steady state, had not experienced an episode of acute chest syndrome in the 4 weeks preceding enrollment and had no clinical evidence of congestive heart failure. The study was approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina, Chapel Hill, USA. Written informed consent was obtained from all subjects.

Echocardiography

Transthoracic Doppler echocardiography was performed in all SCD patients using a Hewlett-Packard 2500 Ultrasound System, with a 2.5/2.0 MHz ultrasound probe (Model 21215A) for recording continuous wave signals. Echocardiography was not performed in the control subjects. Echocardiograms were interpreted by a single cardiologist blinded to all the patients' data, except for the diagnosis of SCD. Multiple views (apical four-chamber, short axis and tricuspid inflow) were obtained to record optimal tricuspid flow signals. The tricuspid regurgitant jet velocity was measured using continuous wave Doppler echocardiography on at least three waveforms with well-defined velocity *envelopes* and an average value used for data analysis. The pulmonary artery systolic pressure (PASP) was calculated using the modified Bernoulli equation ($PASP=4V^2$ + right atrial pressure), with the right atrial pressure assumed to be 10 mmHg. The diagnosis of PHT in our study was based on PASP values adjusted for age, sex, and body mass index.[15] A subject was classified as having PHT if his/her PASP exceeded the upper limits of normal in the reference ranges.

Measurements of coagulation activation, endothelial activation and inflammation

Blood samples were drawn into citrate-containing tubes using a 21-gauge needle. Plasma aliquots immediately isolated from patients' samples were stored at -80°C. Commercially available enzyme-linked immunosorbent assay kits were used according to the manufacturers' recommendations to measure the following plasma markers of coagulation activation, endothelial activation and inflammation: (i) prothrombin fragment F1+2 (F1+2; Dade Behring, Marburg, Germany), (ii) thrombin-antithrombin complex (TAT; Dade Behring, Marburg, Germany), (iii) D-dimer (Asserachrom® D-Di; Diagnostica Stago, Asnières, France), (iv) human soluble vascular cell adhesion molecule-1 (sVCAM-1; R&D Systems, Minneapolis, Minnesota, USA), and (v) soluble CD40 ligand (sCD40L; Alexis Biochemicals, San Diego, CA, USA). Plasma levels of seven other inflammatory cytokines were measured in homozygous SCD patients (HbSS) according to the manufacturer's instructions using the Bio-Plex system from Bio-Rad Laboratories, which combines the principle of a sandwich immunoassay with Luminex fluorescent bead-based technology.[16]

Statistical analysis

Most of the data related to coagulation and inflammatory markers were not normally distributed; therefore, medians and interquartile ranges (25th and 75th percentiles) are used to describe the distributions of these variables stratified by SCD and PHT status. Wilcoxon's rank sum tests were used to compare continuous measures between SCD patients and controls, and to compare PHT patients with those SCD patients without

PHT. Kruskal-Wallis tests were used to compare continuous markers of coagulation activation among the three groups (PHT, no PHT, and healthy controls). Fisher's exact tests were used to compare categorical variables between controls and SCD patients. A large proportion of SCD patients had no measurable interleukin-2 or interleukin-4 (80-94%), therefore these variables were analyzed on the basis of detectable levels. Patients with or without PHT were compared with respect to those with detectable levels using Fisher's exact tests. Spearman's correlations were used to identify associations among measures of coagulation activation, endothelial activation, and inflammatory cytokines. All tests were two-sided (α=0.05). Statistical analyses were performed using SAS software (version 9.1; SAS Institute, Cary, NC, USA).

Results

Clinical and laboratory characteristics

Patients with SCD had higher white blood cell counts (9.2×10^9/L *vs* 7.1×10^9/L; p=0.02), platelet counts (399×10^9/L *vs* 251×10^9/L; p=0.002), reticulocyte counts (6.1% *vs* 1.7%; p=0.0002) and fetal hemoglobin levels (5.4% *vs* 0.5%; p=0.0009) than healthy control subjects (Table 1). Furthermore, SCD patients showed higher absolute neutrophil counts (4.8×10^9/L *vs.* 3.6×10^9/L; p=0.03) and absolute monocyte counts (0.45×10^9/L *vs.* 0.25×10^9/L; p=0.002) than healthy control subjects. When SCD patients with PHT were compared with those SCD patients without PHT, the former had higher white blood cell count counts (10.4×10^9/L *vs.* 8.7×10^9/L; p=0.05), absolute neutrophil counts (5.5×10^9/L *vs* 4.4×10^9/L; p=0.03), and absolute monocyte counts (0.55×10^9/L *vs* 0.4×10^9/L; p=0.02).

Markers of coagulation activation and inflammation

The plasma markers of coagulation activation assessed in this study were higher in SCD patients than in control subjects. SCD patients with and without PHT had significantly higher D-dimer levels than control subjects (1251.5 ng/mL [FEU] *vs.* 318 ng/mL [FEU]; p=0.02). The median values of TAT and F1+2 levels were also higher in SCD patients, although the differences were not statistically significant. When SCD patients with PHT were compared with those SCD patients without PHT, the median values of all three markers of coagulation activation were higher, but the differences were not statistically significant (Table 2). Levels of sCD40L (a marker of platelet activation and inflammation) and sVCAM-1 (a marker of endothelial activation) were higher in SCD patients than in control

Table 1. Patients' clinical and laboratory characteristics.

Characteristic	*Sickle cell disease (n=76) Median (25th,75th) or frequency (%)*	*Control (n=6) Median (25th,75th) or frequency (%)*	*p value**
Age (years)†	39 (31, 47)	45 (37, 49)	0.35
Gender (female)	46 (61)	4 (67)	1.00
Genotype			
SS	56 (74)	–	
Sβ⁰	4 (5)	–	
Sβ⁺	7 (9)	–	
SC	9 (12)	–	
White blood cell count ($\times10^9$/L)	9.2 (7.3, 11.5)	7.1 (4.4, 7.9)	0.024
Hemoglobin (g/dL)	8.8 (7.6, 10.5)	13.2 (12.2, 13.4)	<0.001
Platelet count ($\times10^9$/L)	399 (312, 487)	251 (219, 291)	0.002
Reticulocyte count (%)	6.1 (4.4, 9.2)	1.7 (1.3, 1.8)	<0.001
Absolute neutrophil count ($\times10^9$/L)	4.8 (3.8, 6.6)	3.6 (2.1, 4.5)	0.03
Absolute monocyte count ($\times10^9$/L)	0.45 (0.3, 0.65)	0.25 (0.2, 0.3)	0.01
Fetal hemoglobin (%)	5.4 (2.5, 9.6)	0.5 (0.3, 2.0)	<0.001
NT-proBNP (pg/mL)	98 (52, 186)	50 (50, 53)	0.006
Blood urea nitrogen (mg/dL)	9 (7, 14)	11 (8, 13)	0.52
Creatinine (mg/dL)	0.8 (0.65, 1.0)	1 (0.7, 1.1)	0.30
Total bilirubin (mg/dL)	1.8 (1.0, 3.1)	0.35 (0.3, 0.5)	<0.001
Direct bilirubin (mg/dL)	0.1 (0.1, 0.2)	0.1 (0.1, 0.1)	0.13
Indirect bilirubin (mg/dL)	1.7 (0.9, 2.9)	0.2 (0.2, 0.3)	0.001
Lactate dehydrogenase (U/L)	872 (652, 1424)	494 (376, 537)	0.001
Chronic NSAID use (Yes)	18 (24%)	0 (0%)	0.58
Chronic anticoagulant use (Yes)	12 (16%)	0 (0%)	0.58

**p values comparing SCD patients to control were obtained by Wilcoxon's rank sum tests; †pulmonary hypertension was defined using age, sex and body mass-index adjusted reference ranges.*

Table 2. Markers of coagulation activation, endothelial activation, and sCD40 ligand.

	Sickle cell disease (n=76)		*Control (n=6)*		
	PHT (n=26) Median (25th,75th)	*No PHT (n=50) Median (25th,75th)*	*Median (25th,75th)*	*p value* for 3 groups*	*pvalue° PHT vs. not*
TAT (ng/L)	7.1 (4.0, 15.2)	5.7 (3.5, 13.5)	3.1 (2.2, 5.4)	0.32	0.80
F1+2 (nmol/L)	1.2 (0.7, 1.9)	0.9 (0.7, 1.3)	0.55 (0.4, 1.0	0.16	0.26
D-dimer (ng/mL)	1603 (758, 2547)	1234 (679, 1744)	318 (295, 433)	0.043	0.32
Soluble CD40L (ng/mL)	6.7 (6.4, 8.6)	7.6 (6.2, 9.0)	2.8 (2.7, 3.1)	0.001	0.62
Soluble VCAM (ng/mL)	954 (714, 1553)	682 (507, 885)	374 (324, 446)	0.001	0.004

**p values comparing the three groups (PHT, no PHT, and healthy controls) were obtained by Kruskal-Wallis tests; °p values comparing PHT to no PHT were obtained by Wilcoxon's rank sum tests.*

Table 3. Cytokine levels in HbSS patients with and without pulmonary hypertension.

	PHT (n=19)	*No PHT (n=34)*	*p value**
Interleukin-2 (pg/mL) (% 0 or not detectable)	84%	82%	1.00 (Fisher's exact test)
Interleukin-4 (pg/mL) (% 0 or not detectable)	79%	94%	0.17 (Fisher's exact test)
	Median (25^{th},75^{th})	*Median* (25^{th},75^{th})	
Interleukin-6 (pg/mL)	4.7 (2.7, 7.8)	2.9 (1.5, 6.5)	0.06
Interleukin-8 (pg/mL)	5.4 (2.4, 15.5)	2.7 (0, 7.9)	0.07
Interleukin-10 (pg/mL)	0.57 (0.25, 1.2)	0.35 (0.03, 0.82)	0.10
IFN-γ (pg/mL)	31.3 (0, 85.7)	13.3 (0, 71.4)	0.76
TNFα (pg/mL)	1.9 (0, 7.8)	3.3 (0.98, 21.9)	0.25

**p values were obtained by Wilcoxon's rank sum tests. IQR: interquartile range (25^{th} percentile, 75^{th} percentile). IFN: interferon; TNF: tumor necrosis factor.*

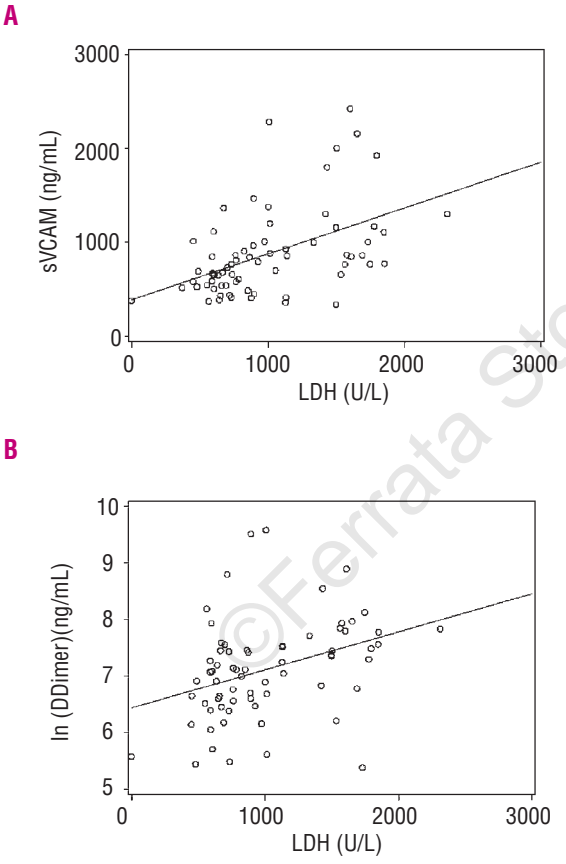

Figure 1A. The level of sVCAM is higher in SCD patients with PHT than in patients without PHT. sVCAM levels are positively correlated with lactate dehydrogenase in study patients with SCD, r_s=0.51 (p<0.001). 1B. Plasma measures of coagulation activation are correlated with markers of hemolysis. D-dimer levels are positively correlated with lactate dehydrogenase (LDH) concentration in the study patients with SCD, r_s=0.42 (p<0.001).

subjects. When SCD patients with PHT were compared with those patients without PHT, the former had higher median sVCAM-1 levels (954 ng/mL *vs.* 682 ng/mL; p=0.004), but there were no differences in the levels of sCD40L. Levels of other inflammatory cytokines were only measured in HbSS patients. Of the cytokines measured, HbSS patients with PHT had consistently higher median levels of interleukin-6 (4.7 ng/mL *vs.* 2.9 ng/mL; p=0.06), interleukin-8 (5.4 ng/mL *vs.* 2.7 ng/mL; p=0.07) and interleukin-10 (0.57 ng/mL *vs.* 0.35 ng/mL; p=0.10) compared with HbSS patients without PHT, although the differences were only of borderline statistical significance (Table 3).

Relationship of markers of coagulation and endothelial activation with markers of hemolysis

We looked for correlations between plasma measures of both coagulation and endothelial activation, and markers of hemolysis in SCD patients with and without PHT. Hemoglobin concentration showed modest negative correlations with TAT, F1+2, D-dimers and sVCAM (Table 4). Lactate dehydrogenase showed statistically significant, but modest positive correlations with TAT, F1+2, D-dimers and sVCAM (Figures 1A and 1B). Indirect bilirubin, another marker of hemolysis, showed a small positive correlation with D-dimers and sVCAM. sVCAM was positively correlated with D-dimers (r=0.24; p=0.045) but not with other measures of coagulation activation. Surprisingly, fetal hemoglobin was not correlated with TAT, F1+2, or D-dimers, although there appeared to be a small negative correlation with sVCAM (r=-0.22; p= 0.07).

Relationship of markers of coagulation and endothelial activation, and inflammatory cytokines with absolute monocyte count

Spearman's correlation coefficients were not significant for relationships between absolute monocyte count and any of the measures of coagulation or endothelial activation (Table 4). However, there were weak positive correlations between absolute monocyte count and interleukin-10 (r=0.28; p=0.05) and between sVCAM and interleukin-10 (r=0.28; p=0.04).

Discussion

Sickle cell disease is characterized by a chronic inflammatory state.[17,18] Patients exhibit elevated leukocyte counts, abnormal activation of granulocytes, monocytes, and endothelial cells,[19-21] and increased levels of multiple inflammatory mediators.[18,22-24] In addition, SCD is often referred to as a hypercoagulable state[25] because patients manifest increased thrombin and fibrin generation,[26,27] increased tissue factor procoagulant activity,[28] and increased platelet activation[19,22,27] even when they are in a non-crisis, steady state. Furthermore, thrombosis may contribute to the pathogenesis of several SCD-related complications. For example, stroke, caused by large vessel obstruction with superimposed thrombosis, often occurs in SCD patients.[29] Both pulmonary embolism and pregnancy-

related venous thromboembolism appear to occur more commonly in SCD patients than in appropriate control patients.[30,31] Finally, autopsies of SCD patients suggest that *in situ* thrombotic arteriopathy of small pulmonary arteries is present in most patients with PHT.[13]

In our cohort, we found increased levels of measured plasma markers of coagulation activation (D-dimers, F1+2, and TAT) in patients with SCD compared to the levels in healthy age- and race-matched controls. Although not statistically significant, the median values of these markers were higher in SCD patients with PHT than in those SCD patients without PHT. The median absolute monocyte count in SCD patients with PHT was also higher than that observed in SCD patients without PHT. Tissue factor is an important physiologic initiator of hemostasis and essentially all tissue factor procoagulant activity that can be assayed in the whole blood in normal individuals is present in the mononuclear cell fraction.[28] Monocytes are activated in SCD patients, increasing the expression of E-selectin, intercellular adhesion molecule 1, VCAM-1 and tissue factor on endothelial cells,[20] and are a source of tissue factor-positive microparticles in these patients.[32] The lack of correlation between plasma markers of coagulation activation and absolute monocyte count suggests that the monocyte count is not an appropriate surrogate for tissue factor procoagulant activity. This agrees with a previous report by Key *et al.*[28]

However, the correlations observed between markers of hemolysis and plasma measures of coagulation activation suggest that the activated blood coagulation present in SCD is due, at least in part, to hemolysis with resultant scavenging of nitric oxide by cell-free plasma hemoglobin. These results are consistent with a recent report that hemolysis and decreased nitric oxide bioavailability appear to contribute to platelet activation in SCD-associated PHT.[33] Several factors, including abnormal red blood cell phospholipid membrane asymmetry, with increased expression of phosphatidylserine,[34] and ischemia-reperfusion injury[35] appear to contribute to the hypercoagulability observed in SCD patients. Recent data suggest that type II phosphatidylserine red blood cells (highly phosphatidylserine-positive and including dense sickle cells) cause a 2-fold increase in endothelial tissue factor expression. This appears to be due to the increased hemolysis of these cells rather than to the physical interaction of type II phosphatidylserine red blood cells with the endothelium in patients with SCD.[36]

The higher levels of sVCAM observed in SCD patients with PHT compared to those in patients without PHT confirm the presence of increased endothelial dysfunction in these patients. In addition, the strong correlation between sVCAM and markers of hemolysis suggests that endothelial dysfunction may, in part, be a result of decreased nitric oxide availability, a finding in agreement with previous studies.[9,37] Nitric oxide decreases cytokine-induced endothelial activation by repression of VCAM-1 gene transcription.[38] The increased levels of interleukin-6, interleukin-8 and interleukin-10 observed in PHT patients in our study suggest a role for cytokines in the development of PHT in SCD. Although a variety of biological stimuli, including inflammatory cytokines, are known to up-regulate VCAM in vascular endothelial cells,[39-43] only interleukin-10 was weakly correlated with sVCAM in our study. While the reason for this correlation is not clear, the trend towards higher interleukin-10 levels in patients with PHT may represent a protective anti-inflammatory response to the increased inflammation observed in SCD patients with PHT. Interleukin-10 is an important physiological negative regulator of macrophage activation[44] and suppresses the release of various inflammatory mediators including tumor necrosis factor-α and NO by activated macrophages.[45,46] The administration of interleukin-10 ameliorates disease in models of endotoxemia,[47] transplantation[48] and autoimmunity.[49] Furthermore, elevated endogenous levels of interleukin-10 in humans appear to be a positive prognostic variable in autoimmune disease[50] and allogeneic transplant patients.[51]

The increased coagulation activation, endothelial dysfunction and inflammation observed in SCD patients with PHT have important therapeutic implications. In the absence of controlled studies, the majority of patients with SCD-associated PHT are treated with pharmacological agents known to be effective in idiopathic pulmonary arterial hypertension. Autopsy findings in SCD patients with PHT are similar to those in

Table 4. Correlations with markers of coagulation and endothelial activation.*

Variable	*TAT*	*F1+2*	*D-Dimers*	*sVCAM*
Hemoglobin (g/dL)	-0.24 (p=0.045)	-0.30 (p=0.012)	-0.26 (p=0.03)	-0.43 (p<0.001)
Total bilirubin (mg/dL)	0.21 (p=0.08)	0.14 (p=0.24)	0.32 (p=0.008)	0.36 (p=0.002)
Indirect bilirubin (mg/dL)	0.18 (p=0.15)	0.12 (p=0.35)	0.29 (p=0.02)	0.32 (p= 0.007)
Lactate dehydrogenase (U/L)	0.25 (p=0.04)	0.27 (p= 0.03)	0.42 (p<0.001)	0.51 (p<0.001)
Absolute monocyte count (x10^9/L)	0.09 (p=0.43)	0.03 (p=0.79)	0.05 (p=0.65)	0.03 (p=0.82)
Fetal hemoglobin (%)	-0.025 (p=0.84)	-0.13 (p=0.29)	-0.10 (p= 0.40)	-0.22 (p=0.07)

**Data shown as Spearman's correlation coefficients (r_s values) and p values are presented in parentheses.*

non-SCD patients who have idiopathic pulmonary arterial hypertension.[13,52] Several observational studies suggest that anticoagulation may be effective in the treatment of patients with idiopathic pulmonary arterial hypertension.[53-55] Given the coagulation activation and *in situ* thrombosis observed in SCD-associated PHT, anticoagulation may be an effective treatment in these patients. In addition, as these patients manifest endothelial dysfunction and increased inflammatory markers, statins, which possess anti-inflammatory and other favorable effects, may also be beneficial by improving endothelial function and decreasing tissue factor expression (and as a consequence, coagulation activation).[35] Treatment with simvastatin appears to improve 6-minute walk performance, cardiac output, and lowers right ventricular systolic pressures in non-SCD patients with pulmonary arterial hypertension.[56] Hydroxyurea may also be beneficial in SCD patients with PHT. In addition to improving red blood cell survival[57] and endothelial function,[58] treatment with hydroxyurea decreases the monocyte counts (and levels of inflammatory cytokines). Indeed, the prevalence of PHT in SCD patients on hydroxyurea has been reported to be lower than in those not receiving this agent.[7]

Although echocardiograms were not performed in the control subjects, it is our belief that these subjects did not have any clinically meaningful cardiac disease. This is because they enjoyed good overall health, with no abnormal symptoms which might suggest cardiac disease, and they all had normal N-terminal pro-brain natriuretic peptide (NT-proBNP) values. Normal values of this peptide are particularly important given that some reports show a correlation between natriuretic peptide values and right ventricular pressure overload.[59,60]

To summarize, SCD patients with PHT appear to have increased endothelial dysfunction, coagulation activation and inflammation compared with patients without PHT. The endothelial dysfunction, as well as coagulation activation, is due, at least in part, to hemolysis, with resultant scavenging of nitric oxide. Further studies evaluating the contribution of coagulation activation and inflammation to the pathogenesis of PHT in SCD are needed. In the absence of defined therapies for SCD-related PHT, our data suggest that clinical trials evaluating anticoagulants and anti-inflammatory agents are warranted.

Authorship and Disclosures

KIA: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, study supervision; CGM: statistical expertise, analysis and interpretation of data, critical revision of the manuscript for important intellectual content; CAH: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content; SJ: acquisition of data, critical revision of the manuscript for important intellectual content; HCW: acquisition of data; DS: acquisition of data, critical revision of the manuscript for important intellectual content; CS: technical support; AH: technical support, acquisition of data, critical revision of the manuscript for important intellectual content; LVP: critical revision of the manuscript for important intellectual content; EPO: critical revision of the manuscript for important intellectual content, study supervision.

The authors have previously published other unrelated findings from this patient cohort: Ataga *et al.* Pulmonary hypertension in patients with sickle cell disease: a longitudinal study. Br J Haematol 2006;134:109-15; this is referenced in the manuscript.[7]

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