Coagulation activation in sickle cell trait: an exploratory study

Chirag Amin,1 Soheir Adam,1 Micah J. Moolberry,1 Abdullah Kutlar,2 Ferdane Kutlar,2 Denise Esserman,3 Julia E. Brittain,4 Kenneth I. Ataga,1 Jen-Yea Chang,1 Alisa S. Wolberg1 and Nigel S. Key1,5

1Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, 2Department of Medicine, Georgia Regents University, Augusta, GA, 3Department of Biostatistics, School of Public Health, Yale University, New Haven, CT, 4Department of Cell Biology and Anatomy, Georgia Regents University, Augusta, GA, and 5Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, USA

Received 27 February 2015; accepted for publication 14 July 2015
Correspondence: Dr Nigel S. Key, Department of Medicine, Division of Haematology/Oncology, 1079 Genetic Medicine Building, CB #7035, Chapel Hill, NC 27599, USA.
E-mail: nigel_key@med.unc.edu

As many as 200 million people worldwide are heterozygous carriers of a mutation in the HBB gene (HBB E6V, also termed HbS mutation or ‘sickle gene’), resulting in a condition known as sickle cell trait (HbAS, hereafter termed AS) (Piel et al, 2013) (Dr. S. Grosse, personal communication, US Centers for Disease Control and Prevention, Atlanta, GA). Although traditionally viewed as a benign carrier state, AS may be a risk factor for certain adverse outcomes, including venous thromboembolism (VTE) (Tsaras et al, 2009; Key & Derebail, 2010; Key et al, 2015). In a large case–control study of VTE in African-American subjects, we reported that AS was associated with an increased risk of VTE, with an odds ratio (OR) of about 2; more strikingly, AS appeared to confer a particularly high risk for pulmonary embolism (PE) (OR ≈ 4) (Austin et al, 2007). In addition, a possible interaction between AS and hormonal contraceptive use may further heighten the risk of VTE in women of reproductive age (Austin et al, 2009). Other studies have also reported an association between AS and risk of VTE, particularly PE (Heller et al, 1979; Bucknor et al, 2014; Folsom et al, 2015). This association was deemed a high priority area for further research in a recent National Institutes of Health conference on sickle cell trait (Goldsmith et al, 2012).

It has previously been reported that plasma levels of the ‘pre-thrombotic’ biomarkers thrombin-antithrombin complexes (TAT), prothrombin fragments 1 + 2 and D-dimers are elevated in subjects with AS compared to race-matched controls (Westerman et al, 2002). One additional study demonstrated elevated TAT complex levels in AS compared to non race-matched controls (Helley et al, 1997). However, comparison with race-matched controls is essential as both pre-thrombotic markers, such as D-dimer (Lutsey et al, 2006), and plasma thrombin generation (Roberts et al, 2013) are higher in healthy individuals of African descent.

In the present report, we sought to confirm the presence of a pre-thrombotic state in healthy subjects with AS using plasma markers of coagulation activation. Simultaneously, we investigated possible mechanisms that might account for the

Summary

Recent epidemiologic data suggest that sickle cell trait (HbAS; AS) is a risk factor for venous thromboembolism. We conducted an exploratory study of healthy subjects with AS under baseline conditions to determine whether a chronic basal hyperactivation of coagulation exists, and if so, what mechanism(s) contribute to this state. Eighteen healthy AS individuals were compared to 22 African-American controls with a normal haemoglobin profile (HbAA; AA) and 17 patients with sickle cell disease (HbSS; SS). Plasma thrombin-antithrombin complexes and D-dimer levels were elevated in AS relative to AA patients (P = 0.0385 and P = 0.017, respectively), and as expected, were much higher in SS versus AA (P < 0.0001 for both). Thrombin generation in platelet poor plasma was indistinguishable between AA and AS subjects, whereas a paradoxical decrease in endogenous thrombin potential was observed in SS (P ≤ 0.0001). Whole blood tissue factor was elevated in SS compared to AA (P = 0.005), but did not differ between AA and AS. Plasma microparticle tissue factor activity was non-significantly elevated in AS (P = 0.051), but was clearly elevated in SS patients (P = 0.004) when compared to AA controls. Further studies in larger cohorts of subjects with sickle cell trait are needed to confirm the results of this preliminary investigation.

Keywords: sickle, venous thrombosis, tissue factor, coagulation, thrombin.

First published online 7 September 2015
doi: 10.1111/bjh.13641
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British Journal of Haematology, 2015, 171, 638–646
hypercoagulable state in AS subjects compared to healthy HbAA (AA) controls, as well as subjects with sickle cell disease (SCD; HbSS; SS), in whom a complex and multi-causal state of activation of coagulation has been well described (Ataga & Key, 2007; Lim et al., 2013). The choice of analytes was influenced by these prior studies, which have focused on both the trigger for coagulation activation in SS, such as circulating tissue factor (TF, also termed coagulation Factor III) (Key et al., 1998; Colella et al., 2012; Setty et al., 2012), as well as ‘downstream’ enhancement of thrombin generation capacity (Hemker et al., 2006; Gerotziafas et al., 2012; Lim et al., 2013; Noubouossie et al., 2013).

Methods

Study subjects

This exploratory cohort study was conducted at the University of North Carolina at Chapel Hill. Institutional Review Board approval was obtained and written informed consent was obtained from all study participants. The study included only African-Americans between the ages of 18 and 65 years. Ethnicity/race was self-declared.

Subjects were recruited into three study categories: Group 1 or controls (AA) included subjects without any haemoglobinoopathy; Group 2 included subjects with sickle cell trait (AS); and Group 3 included subjects with SCD (SS) with either HbSS or HbS-βthal genotype. Exclusion criteria included the presence of a prior history of VTE, current pregnancy or use of hormonal contraceptive therapy and current use of anticoagulants or anti-platelet agents (the latter within the past 7 days). Subjects with SCD were also excluded if they had experienced a recent (<4 weeks) pain crisis or and/or hospitalization, had recently (<3 months) undergone surgery, or if they had received blood transfusion within the last 3 months. No subject in any group had diabetes mellitus or hypertension, cancer or an acute or chronic inflammatory disorder.

Phlebotomy and plasma preparation

After discarding the first 3 ml of blood, blood was drawn into appropriate commercial vacutainer or pre-prepared tubes and rapidly mixed. Samples obtained from a less than fully satisfactory venipuncture were discarded. For D-dimer and TAT analysis, blood was drawn into 3-2% sodium citrate. For thrombin generation and clot formation analyses, blood was drawn into 3-2% sodium citrate/18.5 µg/ml corn trypsin inhibitor to prevent contact activation (Luddington & Baglin, 2004). In each case, platelet-free plasma was prepared by centrifugation, followed by a second centrifugation step at 13 000 g for 2 min. Plasma aliquots were stored at −80°C until batched analysis could be performed. Frozen plasma samples were thawed rapidly at 37°C immediately prior to assay and discarded after use.

Materials

Factor VIIa and factor X were from Enzyme Research Laboratories, Inc. (South Bend, IN, USA). Chromogenic substrate Pefachrome FXa 8595 was from Centerchem, Inc. (Norwalk, CT, USA). Re-lipidated recombinant human TF (Innovin®) was from Siemens Healthcare Diagnostics Products GmbH (Marburg, Germany). Immunbind TF enzyme-linked immunosorbent assay (ELISA) kits were from American Diagnostica, Inc (Stamford, CT, USA). An inhibitory monoclonal antibody (HTF1) to TF was a kind gift from Ronald Bach, PhD (Minneapolis VA Medical Center, Minneapolis, MN, USA). Corn trypsin inhibitor was from Hematologic Technologies, Inc. (Essex Junction, VT, USA). Unilamellar phospholipid vesicles [15% phosphatidylserine (PS), 41% phosphatidylycholine (PC) 41% and phosphatidylethanolamine (PE) 44%] were prepared as described (Hope et al., 1983).

Haematology profile

Routine haematological assays were performed using standard methods at the McClendon Clinical Laboratories at the University of North Carolina Memorial Hospital. Because the laboratory reports markedly low or undetectable serum hemoglobin values as ‘<220 mg/l’, this parameter was treated as a categorical variable, and observed values were divided into those that were below (<220 mg/l), within (220–1390 mg/l) and above (>1390 mg/l) the laboratory’s quoted normal reference range.

Plasma D-Dimer and thrombin-antithrombin (TAT) complexes

D-dimer on plasma samples was measured in the clinical laboratory using the HemosIL® D-Dimer HS immuno-turbid assay on an ACL TOP® coagulation analyser (Instrumentation Laboratory, Bedford, MA, USA) (Salvagno et al., 2008). Because the laboratory reports markedly low D-dimer values as ‘<150 µg/ml’, plasma D-dimer values were treated as a categorical variable, dividing observed values into those that were below (<150 µg/ml), within (150–229 µg/ml) and above (>229 µg/ml) the laboratory’s quoted normal reference range. Plasma TAT levels were determined by using an ELISA assay kit from Affinity Biologicals (Lancaster, ON, Canada).

Thrombin generation and fibrin formation in plasma

Thrombin generation was measured fluorometrically using Calibrated Automated Thrombography (CAT), as previously described (Machlus et al., 2009), using the TF and phospholipid reagents (final concentration, 1 pmol/l and 4 µmol/l, respectively) supplied by the manufacturer (Thrombinoscope BV, Maastricht, Netherlands). Parameters of interest included: (i) lag time; (ii) time to peak thrombin generation;
(iii) peak thrombin level; and (iv) area under the curve ['endo-
genous thrombin potential' (ETP)].

Fibrin formation was measured turbidimetrically by con-
tinuous measurement of absorbance at 405 nm in a Spectra-
max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Here, platelet-free plasma samples were re-calci-
fied, and clotting was initiated by addition of Innovin® (1:60 000
dilution, final) and phospholipid vesicles (125 μmol/l, final),
as previously described (Gray et al, 2011). Endpoints of
interest included: (i) onset of fibrin formation; (ii) rate of
fibrin formation; and (iii) absolute change in turbidity from
baseline.

Microparticle tissue factor activity

As previously described (Key & Mackman, 2010), microparti-
cles (MPs) were isolated from citrate anticoagulated platelet-
free plasma by centrifugation at 20 000 g for 30 min. The
pellet was re-suspended in HBSA (20 mmol/l HEPES, pH
7.4, 150 mmol/l NaCl, and 1 mg/ml bovine serum albumin)
and re-centrifuged at 20 000 g for 30 min, before final re-
suspension in 250 μl HBSA. MPs were incubated with factor
VIIa (100 pmol/l, final) and factor X (135 nmol/l, final), in
the presence of calcium (5 mmol/l, final) and anti-TF anti-
body (HTF1, 10 μg/ml final) or IgG control. Factor Xa gen-
eration was measured in aliquots by chromogenic substrate
cleavage as described. Specific TF activity (fmol/l) was determined
by subtracting the rate of factor Xa generated in the
presence of HTF1 from the rate of factor Xa generated in the
presence of IgG controls with reference to a standard curve
generated using lipidated recombinant human TF (In-
novin®). Effective TF concentration in each lot of Innovin®
was calculated as previously described (Johnson et al, 2009).

Whole blood tissue factor activity

As previously described (Key et al, 1998), frozen whole blood
samples were thawed at 37°C for 3 min, after which 250 μl
was added to 900 μl TBS-EDTA (20 mmol/l Tris-HCl, pH
7.5, 150 mmol/l NaCl, 100 mmol/l EDTA) and mixed by
vortexing. Following three freeze-thaw cycles, sample pellets
were prepared by high-speed centrifugation (10 000 g at
4°C for 60 min) and washed three times in TBS-EDTA
before final suspension of the pellet in 250 μl HBSA. One
hundred microlitre was incubated with HTF1 (4 μg/ml) or
control antibody for 60 min. Forty microlitre of each sample
was then added to a 96-well plate in duplicate. Forty microlit-
tre of factor mix (2 nmol/l factor VIIa and 300 nmol/l factor
X in HBSA with 5 mmol/l calcium) was then added to each
well. Plates were incubated at 37°C for 2 h before adding
10 μl of EDTA. Forty microlitre of the chromogenic sub-
strate, Pefachrome FXa 8595 (final concentration 0.3 nmol/l)
was added to each well. The rate of factor Xa cleavage of
the chromogenic substrate at room temperature in each well
was monitored in a ThermoMax® plate reader (Molecular
Devices, Sunnyvale, CA, USA). Innovin® at various concen-
trations was used as the TF standard. TF-dependent procoag-
ulant activity (pmol/l) was defined as the difference between
the rates of factor Xa cleavage of substrate in the absence/presence of HTF1.

Inflammatory markers and cytokines

Cytokine and chemokine levels in citrated plasma were deter-
mined by colourimetric bead assay using the Luminex system
and human-specific bead sets (R&D Systems, Minneapolis,
MN, USA). Results were interpolated from 5-parameter-fit
standard curves generated using the relevant recombinant
human proteins (R&D Systems).

Statistical analysis

The primary goal of the study was to explore differences in
coagulation and inflammatory parameters between subjects
with AS and matched controls (AA). Patients with SS were
included as a positive control and to examine whether any
gene dosing effects were evident. The study had planned to
enrol 20 subjects from each subtype (AA, AS and SS). As this
was an exploratory study, sample size was estimated from
prior studies that used similar assays in individuals with SS
disease (Westerman et al, 2002; Ataga et al, 2008).

Descriptive analyses were performed to explore the distri-
bution of each variable or analyte. Data for continuous vari-
ables were expressed as median values with interquartile
ranges (IQR), and compared between groups (AA vs. AS and
AA vs. SS) using the Mann–Whitney U test. For categorical
data (D-dimers and haptoglobin), groups were compared
using Fisher’s exact test. Spearman rank correlation was used
to determine correlation between variables; in the case of a
categorical variable (such as D-dimers), a rank of 1 was given
to values falling below the reference range, a rank of 2 to val-
ues within the range, and a rank of 3 to values above the
quoted reference range. Significance was established at the
0.05 level; no adjustments were made for multiple compar-
isons, because this was an exploratory study.

Results

Study subject characteristics

Fifty-seven African-American subjects were recruited: 22 AA,
18 AS and 17 SS. Age was comparable amongst the groups
with median ages of 37.5 (IQR: 32–45), 38.5 (IQR: 33–45)
and 41 (IQR: 24.5–47.5) years for AA, AS, and SS, respec-
tively. Ninety-one per cent of the AA controls were females,
while females accounted for 56% of the AS subjects and 53%
of the SS subjects.

As expected, haemoglobin levels were similar for AA and
AS subjects, but were substantially lower among those with
SS, in whom anaemia was accompanied by evidence of
Coagulation activation in sickle trait

Table I. Haematological data in study subjects.

<table>
<thead>
<tr>
<th>Assay</th>
<th>AA (n = 22)</th>
<th>AS (n = 17)</th>
<th>SS (n = 16)</th>
<th>P-value*</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x 10^9/l)</td>
<td>6-1 (5-0-7-4)</td>
<td>6-8 (5-8-7-1)</td>
<td>8-8 (6-7-12-4)</td>
<td>0.5422</td>
<td>0.0105</td>
</tr>
<tr>
<td>AMC (x 10^9/l)</td>
<td>0-3 (0-3-0-4)</td>
<td>0-4 (0-3-0-5)</td>
<td>0-6 (0-4-0-8)</td>
<td>0.0600</td>
<td>0.0023</td>
</tr>
<tr>
<td>ANC (x 10^3/l)</td>
<td>3-3 (2-2-4-0)</td>
<td>4-1 (3-2-4-4)</td>
<td>4-1 (3-2-6-0)</td>
<td>0.1563</td>
<td>0.0397</td>
</tr>
<tr>
<td>Platelet count (x 10^9/l)</td>
<td>285 (255-327)</td>
<td>325 (290-364)</td>
<td>399 (308-512)</td>
<td>0.0474</td>
<td>0.0052</td>
</tr>
<tr>
<td>Hb (g/l)</td>
<td>125 (121, 131)</td>
<td>124 (120-137)</td>
<td>86 (82-100)</td>
<td>0.7984</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDH (iu/l)</td>
<td>463 (436-525)</td>
<td>518 (454-558)</td>
<td>1022 (905-1144)</td>
<td>0.2549</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) or n (%).

AA, HbAA individuals (controls); AS, HbAS individuals (sickle cell trait); SS, HbSS individuals (sickle cell disease); WBC, white blood cell count; Hb, haemoglobin concentration; LDH, lactate dehydrogenase; ANC, absolute neutrophil count; AMC, absolute monocyte count.

*Comparison of AA to AS.
†Comparison of AA to SS.

Table II. Coagulation data in study subjects.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>AA (n = 22)</th>
<th>AS (n = 17)</th>
<th>SS (n = 16)</th>
<th>P-value*</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Dimer (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td>0.0171</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;150</td>
<td>14 (64%)</td>
<td>3 (18%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150–229</td>
<td>3 (14%)</td>
<td>4 (24%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;229</td>
<td>5 (23%)</td>
<td>10 (29%)</td>
<td>16 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT (nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood tissue factor activity (pmol/l)</td>
<td>0.02 (0.02-0.02)</td>
<td>0.03 (0.02-0.97)</td>
<td>3.04 (0.03-8.78)</td>
<td>0.0385</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Microparticle tissue factor activity (fmol/l)</td>
<td>0.08 (0.04-0.14)</td>
<td>0.09 (0.04-0.11)</td>
<td>0.23 (0.09-0.33)</td>
<td>0.7337</td>
<td>0.0048</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR) or n (%).

AA, HbAA individuals (controls); AS, HbAS individuals (sickle cell trait); SS, HbSS individuals (sickle cell disease); TAT, thrombin-antithrombin complex.

*Comparison of AA to AS.
†Comparison of AA to SS.

accelerated haemolysis – specifically, higher serum lactate dehydrogenase (LDH) and lower haptoglobin values (Table I). Reticulocyte counts were not obtained. There was a trend towards lower serum haptoglobin and, to a lesser extent, higher serum LDH values in AS compared to control subjects. Total white blood cell counts were also higher in the SS group; this was accounted for by an increase in both absolute neutrophil and absolute monocyte counts. There was a trend towards elevated absolute monocyte counts in AS subjects. Platelet counts were higher in the SS group, but it is noteworthy that they were also marginally higher (P = 0.0474) in AS subjects (median: 325 x 10^9/l; IQR: 290–364 x 10^9/l) compared to AA controls (median: 285 x 10^9/l, IQR: 255–327 x 10^9/l).

Coagulation is activated in vivo in both SS and AS

D-dimer levels were elevated in every patient with SS, with a highly significant increase compared to controls (P < 0.0001) (Table II). However, D-dimer levels were also elevated in AS relative to AA (P = 0.017). It should be noted that D-dimers were above the laboratory’s reference range in 5 of 22 (23%) subjects in the AA control group. This probably reflects the fact that D-dimer levels are elevated in African-Americans compared to white Americans (Lutsey et al, 2006), who comprise the large majority of healthy subjects from whom the reference ranges are derived in the clinical laboratory.

Plasma TAT complexes showed similar results, with a marked increase in SS (P < 0.0001) and a more modest but significant increase in AS (P = 0.0385) (Table II). Notably, in the case of both the AS and SS groups, the distribution of values included a substantial number of subjects whose TAT levels overlapped with AA controls, and the overall differences between the groups and controls was driven by the population of outliers (data not shown). For the entire group of study subjects, TAT complexes and D-dimers were moderately well correlated, with a Spearman correlation coefficient of 0.55 (P < 0.0001).

Thrombin generation capacity and fibrin formation in AS or SS plasma are not increased

We next examined whether the capacity for thrombin generation is increased in the plasma of AS and SS subjects
**Microvascular cell adhesion molecule 1 (VCAM1;** P<0.0001) was clearly increased in SS subjects (P=0.0734) (Fig 3A). MP-TF procoagulant activity showed a trend towards being elevated in AS subjects (P=0.051), and was clearly increased in SS subjects (P=0.004) compared to controls (Fig 3B).

**MP-TF activity levels and TAT complexes were only weakly correlated for the entire group (AA, AS and SS), with a coefficient of 0.30 (P=0.0295).** In addition, the correlation between MP-TF and D-dimers was only 0.28 (P=0.0381), but this may have been influenced by the fact that D-dimer was treated as a categorical variable with only three levels. WB-TF did not correlate with TAT or D-dimer as evidenced by coefficients of 0.01 (P=0.9215) and 0.16 (P=0.2442), respectively.

**Inflammatory markers and cytokine levels are not elevated in AS**

Several markers, including soluble CD40 ligand (P=0.0276), tumour necrosis factor (TNF-α; P=0.0003) and soluble vascular cell adhesion molecule 1 (VCAM1; P<0.0001)

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**Table III. Thrombin generation and plasma clot formation parameters.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AA (n = 21)</th>
<th>AS (n = 16)</th>
<th>SS (n = 17)</th>
<th>P-value*</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thrombin generation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>5.3 (4.8–6.3)</td>
<td>5.2 (4.7–5.7)</td>
<td>5.1 (4.2–5.5)</td>
<td>0.4433</td>
<td>1.052</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>9.9 (9.2–10.7)</td>
<td>10.4 (9.2–11.4)</td>
<td>8.1 (7.7–9.1)</td>
<td>0.6693</td>
<td>0.0001</td>
</tr>
<tr>
<td>Thrombin peak (nmol/l)</td>
<td>1636 (1288–2066)</td>
<td>1683 (1306–2331)</td>
<td>2032 (1731–2328)</td>
<td>0.6826</td>
<td>0.0887</td>
</tr>
<tr>
<td>ETP (nmol/l × min)</td>
<td>14960 (14313–17709)</td>
<td>16234 (13951–18515)</td>
<td>12175 (11700–13295)</td>
<td>0.4573</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Clotting</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset (min)</td>
<td>15.0 (12.0–16.5)</td>
<td>12.3 (10.4–15.4)</td>
<td>11.6 (8.9–14.8)</td>
<td>0.2802</td>
<td>0.0418</td>
</tr>
<tr>
<td>Rate (mOD/min)</td>
<td>78.6 (52.4–99.3)</td>
<td>60.8 (40.7–83.7)</td>
<td>85.8 (55.4–120.9)</td>
<td>0.2060</td>
<td>0.2943</td>
</tr>
<tr>
<td>Change in turbidity</td>
<td>0.70 (0.57–0.78)</td>
<td>0.68 (0.61–0.76)</td>
<td>0.67 (0.56–0.77)</td>
<td>0.9638</td>
<td>0.7277</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR).

AA, HbAA individuals (controls); AS, HbAS individuals (sickle cell trait); SS, HbSS individuals (sickle cell disease); ETP, endogenous thrombin potential; mOD, mean optical density.

*Comparison of AA to AS.

†Comparison of AA to SS.
were elevated in SS compared to AA controls, whereas epithelial-derived neutrophil-activating peptide 78 (ENA78 (CXCL5); \( P = 0.0276 \)) and granulocyte colony-stimulating factor (\( P = 0.0304 \)) were significantly reduced (Table S1). C-reactive protein, an acute phase response marker that has been reported to be elevated in SS patients (Singhal et al., 1993), was not increased in this cohort. The inflammatory markers C-reactive protein, TNF \( \alpha \), interleukin (IL) 1–\( \alpha \), IL1\( \beta \), IL6, and IL8 also did not differ between AA and AS subjects. Soluble P-selectin levels trended higher in both AS (\( P = 0.0551 \)) and SS (\( P = 0.0577 \)) subjects, but without an apparent ‘gene dosing effect’ observed in these groups.

**Discussion**

Elevated levels of plasma D-dimers, and to a lesser extent, TAT complexes in this study are consistent with a previous report that coagulation is more activated in healthy subjects with AS compared to race-matched controls (Westerman et al., 2002). However, further larger scale studies will be required to definitely establish this association. We also included a cohort of patients with SS, and observed the expected pronounced elevation in the pre-thrombotic markers in plasma (TAT complexes and D-dimers) compared to controls. Whether or not activation of coagulation in SS patients contributes to vaso-occlusive complications, such as pain crisis, stroke, or pulmonary hypertension remains an open question (Hillery & Panepinto, 2004; Ataga & Key, 2007; Pakhaz & Wun, 2014), but several pieces of evidence now support the conclusion that the risk of VTE is increased not only in SCD (Stein et al., 2006; Novelli et al., 2012; Naik et al., 2014), but also in sickle trait (Austin et al., 2007; Bucknor et al., 2014; Folsom et al., 2015). We examined whether thrombin-generating capacity in AS and SS plasma is increased in parallel with the increased levels of circulating TAT and D-dimers. Paradoxically, ETP was reduced in individuals with SS (Fig 1). Several groups have addressed the question whether thrombin generation in plasma is elevated in SCD, with mixed and sometimes opposite conclusions (Lim et al., 2013). Notably, an analogous discrepancy between elevated TAT and/or D-dimer in vivo and normal or decreased ETP ex vivo has been described elsewhere, for example in normal pregnancy (Eichinger et al., 1999). When performed in cell-free plasma, ETP is dependent on the net balance between levels of procoagulant and anticoagulant factors (Machlus et al., 2009), and therefore the combination of elevated procoagulants, such as FVIII and reduced anticoagulants (such as protein C and protein S) in SCD might be expected to result in a net increase in thrombin generation potential. Beta thalassaemia syndromes, particularly after splenectomy, represent another haemoglobinopathy that is associated with in vivo evidence of coagulation hyperactivation and a clinical risk of thrombotic events (Ataga et al., 2007). Tripodi et al. (2009) demonstrated that while whole blood thrombelastometry end points were suggestive of a hypercoagulable state in \( \beta \)-thalassaemia, plasma thrombin generation profiles were normal. The authors concluded that abnormalities in blood cell number or function – as opposed to plasma proteins – contribute to the hypercoagulability in \( \beta \)-thalassaemia. The apparent discrepancy between increased plasma TAT levels and reduced ETP in this study may be indicative of a similar situation in SCD.

Some of the well-recognized complications of SCT include hyposthenuria, renal papillary necrosis, renal medullary carcinoma, splenic infarction at high altitude, glaucoma following hyphema of the anterior chamber of the eye, exertional rhabdomyolysis and exercise-related sudden death (Tsaras et al., 2009; Hooper et al., 2010; Key & Derebail, 2010). Common to all these scenarios is the likelihood that sickling of
erythrocytes occurs in a vascular bed that may be either continuously or intermittently exposed to conditions that favour sickle haemoglobin polymerization, including hypoxia (e.g. in the anterior chamber of the eye or in the splenic microvasculature at high altitude), acidosis or hyperosmolarity (e.g. in the renal medulla).

We evaluated whether some of the mechanisms believed to contribute to activation of coagulation in SS (Ataga & Key, 2007) might also be at play in AS. By analogy to SCD, we evaluated whether circulating TF levels are elevated in individuals with SCT. We, and others have previously reported that cell-associated TF levels are elevated in patients with SCD (Key et al, 1998; Colella et al, 2012; Setty et al, 2012). The present study confirmed that finding, but failed to demonstrate any increase in WB-TF in AS subjects (Fig 3A). The finding of increased MP-TF activity in SS subjects (Fig 3B) is consistent with our previous observation (Key et al, 1998), but may be at odds with the study by van Beers et al (2009). These authors also found that erythrocyte and platelet MP numbers were elevated in SCD, but reported that thrombin generation was dependent on factor XI, but not factor VII, thereby implicating the ‘intrinsic’ coagulation pathway. This discrepant observation probably reflects technical differences, most notably the presence of plasma in the assay reported by van Beers et al (2009). In this study, we were surprised to find marginal, albeit insignificantly elevated levels of MP-TF procoagulant activity in subjects with AS (Fig 3B). However, the absent or modest correlations between WB-TF and MP-TF activities (respectively) question whether circulating TF is the dominant mechanism contributing to activation of coagulation in vivo. Indeed, the role of circulating MP-TF as a mediator of coagulation activation and thrombosis in human disease states has been extensively studied, although with mixed conclusions to date (Owens & Mackman, 2011; Geddings & Mackman, 2013; Lacroix et al, 2013).

In addition, we evaluated whether a number of pro-inflammatory cytokines are elevated in subjects with AS, as is the case in SS (Hoppe, 2014). It is well established that one mechanism contributing to the ‘cross talk’ between inflammation and coagulation is mediated by certain pro-inflammatory cytokines, such as TNF-α, and IL1 and 6 (Levi & van der Poll, 2010; Engelmann & Massberg, 2013; Sparkenbaugh & Pavlinski, 2013). However, in this study, we were unable to demonstrate any significant increase in these inflammatory markers in AS subjects. Consistent with our results, Tripette et al (2010) found no difference in baseline levels of multiple cytokines and soluble adhesion molecules [TNFα, IL6, soluble (s) Intercellular Adhesion Molecule 1 (ICAM1), sVCAM1, sP-selectin and sL-selectin] in patients with SCT, although differences were observed following a sub-maximal exercise protocol.

Several limitations of this study warrant discussion. We did not perform formal age- and sex-matching among the three cohorts, which resulted in a greater preponderance of females among the control group. Secondly, because this was designed as an exploratory study, the relatively small sample size may have limited our ability to draw definitive conclusions about the role of any single mechanism in the hyper-activation of coagulation in AS. Indeed, by analogy to the complexity of the hypercoagulability in SS, other candidate mechanisms may also contribute to the excessive activation of coagulation in subjects with AS. For example, we have recently evaluated erythrocyte PS expression in subjects with AS. While we have not observed any increase in AS subjects compared to controls at baseline in preliminary studies, we have detected a significant increase in red cell PS expression in AS subjects on haemodialysis compared to matched controls. Furthermore, red cell PS expression was highly correlated with plasma TAT levels (Brittain et al, 2010). These data suggest that a ‘second hit’, which might include extreme hypoxia in the renal medulla or the venous valvular pockets in the lower extremity (Sevitt, 1974; Hamer et al, 1981), may be required to perturb AS red cells, and thereby promote their ability to contribute to activation of coagulation. We are continuing to evaluate this and other possibilities with the ultimate goal of identifying biomarkers of thrombotic risk that may be present in sickle haemoglobinopathies.

Acknowledgements

The authors would like to thank Dr. David Barrow, Dell Strayhorn RN FNP, Susan Jones RN, Ben Hulkower, Patrick Moody and Laura Gray for their excellent technical assistance. The authors also acknowledge funding from the North Carolina TRACS Institute (UL1TR000083), U01HL117659 (NSK and KIA), T32HL007149 (CA, SA and MJM), K12HL087097 (MJJM), R01 HL094740 (ASW) and the Doris Duke Charitable Foundation (NSK), and Yale CTSA (UL1TR000142).

Authorship and disclosures

NSK was the principal investigator and takes primary responsibility for the paper. CA, SA and KIA recruited the study subjects; CA, MJJM, AK, FK, JEB, J-YC, and ASW performed the laboratory assays; DE was primarily responsible for statistical analysis; NSK coordinated the research; NSK and ASW wrote the paper. The authors report no potential conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Plasma inflammatory marker and cytokine levels.
References


