Platelet aggregation and physiological anticoagulants in sickle-cell disease

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تكدُّس الصفيحات ومضادات التخثر الفيزيولوجية في داء الخلايا المنجلية

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الخلاصة: قام الباحثون بتقييم 30 مريضاً بفقر الدم المنجلي في ما بين كانون الثاني/يناير 2002 وكانون الأول/ديسمبر 2004. وقد دخل هؤلاء المرضى إلى مستشفى الخبر، حيث كانوا يعانون من نوبة انغلاق وعائي بسبب مستويات مضادات الثرومبين III والبروتين C، والبروتين S. كما درسوا تكدُّس الصفيحات، وقمنا بتقييم مستويات الحالات المستقرة خلال فترة المتابعة، بالمقارنة مع 36 حالة شاهدة من البالغين. فثبت أن مستويات البروتين C، والبروتين S، ومضادات الثرومبين III في المجموعات الشاهدة أعلى بكثير منها في نوبات الانغلاق الوعائي، والحالات المستقرة ($P \geq 0.001$). وقد وجدت فروق يُعتدُّ بها إحصائياً بين المجموعات الشاهدة وبين المرضى بالنسبة لجميع عوامل تكدُّس الصفيحات باستثناء الإبينفرين. ولم تظهر أي فروق يُعتدُّ بها بين مستويات البروتين C والبروتين S ومضادات الثرومبين III، ومتغيِّرات تكدُّس الصفيحات لدى المرضى ذوي الحالات المستقرة وأولئك الذين يعانون من نوبة انغلاق وعائي.

ABSTRACT During the period January 2002–December 2004, we assessed 30 sickle-cell anaemia patients admitted to hospital in Al Khobar with vaso-occlusive crisis for levels of antithrombin (AT) III, protein C (PC) and protein S (PS). We also did platelet aggregation studies. Steady state levels were assessed during follow-up and compared with 36 adult controls. Levels of PC, PS and AT III in the control group were significantly higher than in those in vaso-occlusive crisis and those in steady state ($P < 0.001$). There was a statistically significant difference between controls and patients for all platelet aggregation factors except adrenaline. There was no significant difference between the levels of PC, PS, AT III and platelet aggregation variables in patients in the steady state and in vaso-occlusive crisis.

Agrégation plaquettaire et anticoagulants endogènes dans la drépanocytose

RÉSUMÉ Entre janvier 2002 et décembre 2004, nous avons évalué les taux d'antithrombine (AT) III, de protéine C (PC) et de protéine S (PS) chez 30 patients atteints de drépanocytose (ou anémie falciforme) en crise vaso-occlusive admis à l'hôpital d'Al Khobar. Nous avons également étudié chez ces patients l'agrégation plaquettaire. Les concentrations en phase stationnaire ont été évaluées au cours du suivi et comparées à celles de 36 témoins adultes. Dans ce groupe témoin, les taux de PC, de PS et d'AT III se sont avérés significativement supérieurs à ceux observés chez les drépanocytaires tant en crise vaso-occlusive qu'en phase stationnaire ($p < 0,001$). Il est apparu une différence statistiquement significative entre témoins et patients en ce qui concerne tous les facteurs d'agrégation plaquettaire, à l'exception de l'adrénaline. Les taux de PC, de PS et d'AT III et les variables de l'agrégation plaquettaire n'ont mis en évidence aucune différence significative entre les drépanocytaires en phase stationnaire et les patients en crise vaso-occlusive.

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Introduction

Sickle-cell anaemia (SCA) was first recognized as a haematological disorder more than 90 years ago [*1*]. The sickle-cell gene, haemoglobin S (HbS), is widespread; the highest incidences are found in equatorial Africa, parts of Sicily and Southern Italy, Northern Greece, Southern Turkey, the Middle East, Saudi Arabia (especially the Eastern province) and much of central India [*2,3*]. Progress in understanding of the disease was initially slow, but molecular research has led to advances in understanding the genetics, evolution and history of the disease. Unfortunately, despite these advances, the precise pathogenesis of the most common complication, vaso-occlusive crisis, has not yet been elucidated [*1*].

In Saudi Arabia, SCA is present throughout the country and is common in the Eastern and Southern regions, the frequency of the HbS gene being 25% in the Qatif oasis (Eastern province) [*2*]. Data from several studies indicate that in the Eastern province 20%–30% of newborns are heterozygous for the gene and 1.6%–2.3% may be homozygous, so the frequency of homozygosity for the sickle-cell gene should be around 1% [*4*]. One study showed overall prevalence of HbS homozygotes was 1.058% and HbS heterozygotes was 7.356%, with the Eastern province having the highest frequency [*5*].

The underlying pathophysiology of many of the clinical complications of SCA is poorly understood. It seems to be a multifactorial process involving increased blood viscosity, reduced red cell deformability, abnormal red cell adhesive properties, endothelial intimal proliferation, bone marrow or fat embolism and a chronic hypercoagulable state [*6*]. Numerous investigations have been conducted to elucidate the mechanisms responsible for the prothrombotic state.

Some reports on SCA patients during the steady state have shown increased platelet aggregability, elevated beta thromboglobulin, reduced levels of protein C (PC) and protein S (PS). Antithrombin (AT) III has been reported to be elevated, normal and reduced [*7,8*]. The changes reported during sickle-cell crisis included reduced antithrombin III levels [*7,8*]. Many conflicting reports concerning the physiological anticoagulants have been presented. Reduced PC and PS levels have been noted in the steady state [*8–11*], although some studies have reported no significant difference between steady state and vaso-occlusive crisis [*10,12*]. In contrast, significantly reduced levels in crisis compared to steady state have been also reported [*8*]. Antithrombin III has been reported to be significantly elevated in the steady state [*8*]. Other studies found normal levels [*11,12*]. There have been conflicting reports concerning differences between steady state and vaso-occlusive crisis levels [*7,8,11*].

Normal as well as decreased platelet aggregation has been reported in the steady state, while during vaso-occlusive crisis aggregation was reported to be diminished [*7,8,13–15*].

There is considerable evidence now that SCA is a hypercoagulable condition, especially in the steady state. This has been attributed to procoagulant properties of sickle red blood cells and their abnormal adherence to vascular endothelium as well as increased thrombin activity with defects in fibrinolysis [*6,8*].

The main objectives of this study were to assess platelet aggregation patterns and levels of PC, PS and AT III in SCA patients in the steady state and in vaso-occlusive crisis.

Methods

This study was carried out during the period January 2002–December 2004 at King Fahd Hospital of the University, Al-Khobar, one of the main cities in the Eastern province.

The participants in this study were adults (over 18 years) with SCA attending the haematology outpatient department and those who were admitted via the emergency room with vaso-occlusive crisis. Diagnosis was made on clinical grounds and by standard laboratory methods. Patients with a history of taking hydroxyurea were excluded from the study.

We randomly selected SCA patients in the steady state during their follow-up visits to the outpatient department (Group 1) and explained the study to them. About 50–60 patients were approached but a few changed their minds or did not show up. Any haemolysed or lipaemic blood samples were rejected. Data were complete for 30 participants in this group.

Group 2 comprised patients with vaso-occlusive crisis, selected during admission or while attending the emergency room. Many of these patients were admitted and discharged from the emergency room before we could complete the investigations. A number of patients had collapsed veins, making blood extraction difficult. In all, this group comprised 30 patients for whom all data were complete.

We also randomly selected 36 healthy volunteers (hospital staff, medical students and 10 male blood donors) as controls (Group 3). They comprised 20 males and 16 females aged 21–42 years without any history of thromboembolic disease and not on any medication. There were no refusals to participate. They were examined and the blood samples processed in the same time period as the specimens from patients.

A specially designed form was used to record patients' data, i.e. name, medical record number, age, sex, presence of splenomegaly, haemoglobin level, haemoglobin electrophoresis pattern, history of number of crises per year, frequency of blood transfusion, history of major thrombotic problems in patients or family members and history of hydroxyurea treatment (negative in all the patients tested). Data was taken from the hospital files and laboratory computer records.

Blood samples were collected from all participants with a minimum of stasis. Nine volumes of blood were added to 1 volume of sodium citrate (3.8%) for measurement of AT III, PC and PS. Samples were taken without delay to the haematology laboratory where plasma was separated and samples were either processed immediately or frozen to be tested in batches at a later stage. At testing, samples were thawed at 37 °C and tested within 1 hour. Any haemolysed or lipaemic samples were rejected. AT III, PC and PS functional assays were performed on a Dade Behring coagulation timer (Dade Behring, Marburg, Germany), and determined according to the manufacturer's instructions.

For platelet aggregation studies, a second sample of 9 mL venous blood was drawn and added to 1 mL 0.11% sodium citrate in a plastic tube. Platelet-rich plasma was obtained by centrifuging at room temperature for 10 minutes at 150 g. The platelet-rich plasma was carefully removed using a plastic pipette to avoid any contamination with red cells or buffy coat and placed in a stoppered plastic tube at room temperature. Platelet-poor plasma was prepared by centrifuging the remaining blood specimen for 15–20 minutes at 1500–2000 g. Platelet aggregation was done using a Bio/Data PAP-4 aggregation instrument (Bio/Data Corporation, Pennsylvania, United States of America), according to standard methods [*16*].

Statistical analysis

Results were entered into the computer using *SPSS*, version 10. Descriptive statistics, mean and standard deviation, were determined. The statistical significance of the difference in the mean of the studied groups was obtained using the *t*-test or Mann–Whitney test, depending on the normality of the data. Analysis of variance using Dunnett's test or the Kruskal–Wallis test was used to compare the 3 groups simultaneously.

P-value < 0.05 was considered significant throughout the study.

Results

There were a total of 30 SCA patients studied in the steady state (Group 1) (21 males, 9 females). Mean age was 24.53 [standard deviation (SD) 6.85] years. Thirty patients, 11 females and 19 males, mean age 25.97 (SD 7.72) years, were also studied during admission and/or emergency room visit for vaso-occlusive crisis (Group 2). The control group (Group 3) comprised 36 normal healthy adults, 20 males and 16 females, mean age 30.00 (SD 6.62) years.

The haemoglobin electrophoresis pattern in the majority of cases was HbS in association with haemoglobin F (HbF) and haemoglobin A_2. Six patients were double heterozygotes for SCA and β-thalassaemia (HbS/β^0). The steady state haemoglobin levels in these patients ranged from 6.4 g/dL to 10.7 g/dL.

The control group had a statistically significantly higher level of AT III, PC and PS levels and all platelet aggregation variables except for adrenaline compared to the SCA patients (Groups 1 and 2) (*P*-value < 0.05) (Table 1).

There was no statistically significant difference in the levels of any of the variables (AT III, PC, PS and platelet aggregation) between the SCA patients in the steady state (Group 1) and those in vaso-occlusive crisis (Group 2) (Table 1).

In Group I, there was no significant difference between males and females regarding physiological anticoagulants and platelet aggregation variables except for collagen and ristocetin: the values for males [mean 72.29% (SD 11.84) for collagen; mean 78.43% (SD 10.70) for ristocetin] were significantly higher than those for females [mean 61.00% (SD 10.44), $P = 0.02$ for collagen; mean 70.00% (SD 6.10), $P = 0.036$ for ristocetin]. In Group 2, there was no significant difference between these values for males and females.

The normal (reference) ranges for our laboratory for the methods and reagents used are:

- AT III (functional activity) 80%–120%
- PC (functional activity) 70%–130%
- PS (functional activity) 65%–140%
- platelet aggregation 40%–100%.

Discussion

Coagulation changes in SCA and their involvement in the pathogenesis of vaso-occlusive crisis have been the subject of conflicting reports. The possible role of platelet activation, platelet aggregation or platelet abnormalities, as well as the role of clotting factors, in the pathogenesis of vaso-occlusive phenomena has generated much interest and research. In one study, aggregation in response to adrenaline, collagen and adenosine diphosphate (ADP) in children with SCA was similar to, or not significantly different from, normal controls. Additionally, there was no increase in malondialdehyde generation, which is not

Table 1 **Comparison of physiological anticoagulants and platelet aggregation factors for 2 groups of sickle-cell anaemia patients and a control group**

Variable	Sickle-cell anaemia patients		*P*-value[a]	Controls	*P*-value[b]
	Group 1 Mean (SD)	Group 2 Mean (SD)		Group 3 Mean (SD)	
Physiological anticoagulant (%)					
Antithrombin	92.54 (12.27)	96.62 (15.07)	0.254	106.81 (10.88)	< 0.001
Protein C	91.75 (12.39)	86.18 (19.78)	0.197	105.87 (12.56)	< 0.001
Protein S	77.79 (14.91)	77.83 (23.35)	0.993	104.84 (14.32)	< 0.001
Platelet aggregation factor (%)					
ADP	67.87 (12.00)	66.57 (13.17)	0.691	75.33 (7.06)	0.002
Collagen	68.90 (12.42)	64.77 (10.77)	0.174	72.33 (7.41)	0.014
Adrenaline	70.67 (13.48)	66.23 (12.92)	0.199	72.78 (8.96)	0.81
Arachidonic acid	70.07 (12.39)	66.77 (10.42)	0.269	74.94 (6.94)	< 0.005
Ristocetin	75.90 (10.23)	72.67 (10.19)	0.225	79.61 (6.34)	0.009

Group 1 = patients in steady state; Group 2 = patients in vaso-occlusive crisis.
[a]*Comparing groups 1 and 2.*
[b]*Comparing all 3 groups.*
SD = standard deviation.
ADP = adenosine diphosphate.

consistent with the occurrence of a marked degree of platelet activation in SCA [*14*].

In our study, there was a statistically significant difference between normal controls and SCA patients for all platelet aggregation variables except adrenaline. Why only adrenaline does not differ cannot be explained; further studies would be needed. It has been suggested that reduced responsiveness to platelet aggregating agents reflects ongoing *in vivo* platelet activation and secretion, leading to depletion of platelet granule stores [*13*]. Stuart et al. reported no difference in platelet response to ADP in children with steady state SCA and normal controls; aggregation with ADP was, however, significantly lower during vaso-occlusive crisis than in the steady state [*17*]. In contrast to adults, it appears that platelet aggregability in children with steady state SCA has generally been reported to be normal or reduced [*13*]. On the other hand, Francis reported increased platelet aggregability in adults in the steady state [*8*]. Haut et al., however, reported normal aggregation in response to ADP, adrenaline, collagen and thrombin in adults with SCA [*18*].

Platelet aggregability during vaso-occlusive crisis has not been as extensively investigated as in the steady state. Our results concur with other published reports in that there was no significant difference between the steady state and vaso-occlusive crisis, and to some extent resembled the study by Buchanan and Holtkamp, who reported no significant differences in aggregation to adrenaline, ADP and collagen [*13*]. A study on SCA children, however, showed abnormal aggregation response to ristocetin but no significant differences with other agonists, compared to the control group,

as well as no difference between steady state and vaso-occlusive crisis [*7*]. Again, the significance of abnormal response to ristocetin alone is difficult to explain.

Reports concerning PC, PS and AT III have been even more debatable. Significantly reduced levels of PC and PS in SCA were shown in a 1991 study; the greatest level of deficiency was seen in patients with severe disease [*9*]. As in our study, however, there was no significant difference in the patients during the steady state and during episodes of vaso-occlusive crisis. In a study on children with SCA, PC and PS levels were reduced compared to the controls, while AT III levels did not differ [*6*]. Westerman et al. showed that PC and PS levels were decreased, but again they found no significant difference between steady state and vaso-occlusive crisis [*19*]. In another study on children with SCA, there was no significant difference in AT III levels when comparing steady state, final day of crisis and normal controls [*7*]. Karayalcin and Lanzkowsky, however, reported that mean PC levels were lower in HbSS (homozygous) children and adults and fell even lower during episodes of vaso-occlusive crisis [*20*].

These reports are rather inconclusive and conflicting and the studies were done in different settings. The significance and sources of coagulation abnormalities and their relationship to clinical severity and thromboembolic events in sickle-cell disease are not clear. Our study showed that the values of AT III, PC and PS in SCA patients were lower than in the controls, but there was no significant difference between SCA patients in the steady state and those in vaso-occlusive crisis, in concurrence with many previous studies.

The role of platelet activation or hyperactivity in patients with sickle-cell disease, during vaso-occlusive crisis using monoclonal antibodies to activation dependent antigens as well as plasma levels of platelet alpha granule constituents, β-thromboglobulin and platelet factor 4, which have been studied and shown to be increased in these patients as well as during crisis [*21,22*].

There was, however, no indication in this study or in previous studies whether the haemostatic abnormalities are primary or secondary, and it has not yet been demonstrated that they play a major role in vaso-occlusive crisis [*8,12,15,17,21*]. It does appear that changes in platelet aggregation and/or coagulation changes may play some role. What, and to what extent, still needs further research.

Although the number of patients studied was small, further studies would be interesting especially if they compared the same patients during steady state and during vaso-occlusive events, as well as comparing daily levels while patients were recovering from any vaso-occlusive event.

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