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Thrombotic events in patients with sickle cell anemia: relationship to Protein C, S and total homocysteine levels

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A B S T R A C T

Sickle cell disease (SCD) is a genetic disease characterized by hypercoagulable state and increased risk of thromboembolic events, a rare but significant complication of SCD. Protein C and S are natural anticoagulant proteins. Total homocysteine (tHcy) is an independent risk factor for venous thromboembolism and cardiovascular disease, and its level is therefore of interest in sickle cell disease. The aim of this study was to investigate the plasma levels of protein C and S levels and their relationship to homocysteine level in patients with sickle cell anemia compared to control subjects. In this study twenty patients (m=12, f=8) with sickle cell anemia, classified into sickle cell trait (Hb AS, n=15) and sickle cell disease (Hb SS, n=5), and twenty normal age-sex controls (m=12, f=8) were included. Protein C, S and homocysteine levels were measured using ELISA diagnostic kits technique. The data was statistically analysed by SPSS-17 and p values less than 0.05 were considered significant. Our results showed that mean tHcy levels were found to be significantly higher in patients (SS and/or AS) than in control group. No significant correlation was observed between tHcy with protein C or S. The mean value of protein C and S within normal range and statistically not significant in patients compared to controls, but significantly decreased in Hb SS patients. In conclusion, sickle cell anemia is associated with mild elevated tHcy level which may contribute to increased risk of hypercoagulability and thromboembolic complications.

Introduction

Sickle cell disease (SCD) is a genetic disease characterized by hypercoagulable state in which various hemostatic systems both in steady state and during vaso-occlusion are perturbed with increased activation of the coagulation system and

platelets, thrombin generation, and occurrence of thrombosis (Zohreh and Abbas, 2011; Rahimi et alo., 2008; Ataga et al., 2007; Moreira et al., 2006).

The pathogenesis of hypercoagulability is considered to be multifactorial. Altered components of hemostasis system in SCD have been suggested. Low plasma levels of protein C, protein S, and antithrombin III, elevated plasma levels of thrombin-antithrombin (TAT) complexes, prothrombin fragment 1+2 (F1+2), D-dimer complexes, and circulating antiphospholipid antibodies, platelet activation during vaso-occlusive crisis, abnormal external exposure of phosphatidylserine (PS) and adherence of sickle erythrocytes to the vascular endothelium, reducing NO level in the presence of hemolytic anemia, and increased tissue factor expression have been detected in SCD patients (Ataga, 2009; . Ataga and Orringer, 2003). These abnormalities of hemostatic system in SCD are leading to increase risk of thrombosis.
Total homocysteine (tHcy) is an independent risk factor for venous thromboembolism and cardiovascular disease (Lubinska et al., 2006 Cattaneo, 2006; Saeed et al., 2006; Virdis et al., 2002). Homocysteine has often been shown to be related to occlusive vascular disease independently of other known risk factors (den Heijer et al., 2005). Platelet aggregation, anticoagulant functions of plasma and vascular vasomotor function are altered in the presence of high plasma levels of Hcy (Medina et al., 2001). Homocysteine may inhibit thrombomodulin (Cattaneo, 1999) and protein C, S may be reduced in SS disease (Wright et al., 1997). Therefore, it is possible that raised homocysteine levels in SS disease predispose to the development of thrombosis (Houston et al., 1997) through inhibition of the protein C anticoagulant pathway (Van der Dijs et al., 1998). 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 (Prengler et al., 2002). Both pulmonary embolism and pregnancy-related venous thromboembolism appear to occur more commonly in SCD patients than in appropriate control patients (Stein et al., 2006; James et al., 2006).

Protein C and S are vitamin K-dependent protein with an essential natural anticoagulant functions. Protein C exists in an inactive form and is activated by thrombin-thrombomodulin complex. It's activated form (activated protein C, APC) controls the coagulation process by cleaving and inactivating factor VIIIa (FVIIIa) and FVa in the presence of protein S, which act as a cofactor for activated protein C, down-regulating clot formation and promoting fibrinolysis (Attvall et al., 2006).

In the present study we investigated the plasma protein C and S levels and their relationship to homocysteine level in patients with sickle cell anemia compared to control subjects.

Materials and Methods

Subjects

This study was conducted in Sana'a city, Yemen, from August to September, 2013. It included 60 subjects aged 1 to 18 years. The patient's group consisted of 20 patients with sickle cell anemia (12 male and 8 female) (mean age ±SD, 7.7±4.0; median, 7.9; ranged from 1 to 16; 95% CI, 5.8-9.6 years old). Diagnosis of patients was done by Hb electrophoresis, using SAS-1 Alkaline Hb Gel kit (Helena Bioscience

Europe, Gateshead, UK), and classified into sickle cell trait (Hb AS, n=15, m=8, f=7) and sickle cell disease (Hb SS, n=5, m=4, f=1). These patients were selected randomly from those referred to the out-patient's clinics of medical, pediatric and general surgery departments of Kuwait, Al-Gomhori, Al-Sabeen and Al-Thawra Hospitals. Also from patients attended National Centre of Public Health Laboratories (NCPHL) as well as to specialized medical laboratories, Al-Aulaqi, Med-Lab. and Al-Dubhani, most of these patients were referred by private clinics. The age–sex matched control group included 20 subjects (12 male and 8 female) (mean age ±SD, 8.6±4.6; median, 8.0; ranged from 2 – 18; 95% CI, 6.4-10.8 years old) as normal volunteers. All participants gave their informed consent to participate in this study.

Sample collection

Non-fasting venous blood samples (5 ml) were collected from each patient and control. From this 5 ml, 3 ml were put in plain tube and 2 ml in sodium citrated tube. Citrated samples were mixed well and separated by centrifugation within 20 minutes of collection at 3500 x g for 5 minutes. The separated plasma was stored frozen at -20°C for later analysis and estimation of protein C and S concentrations. Sample of plain tube was left to clot for 30 minutes and serum was separated by centrifugation at 3500 x g for 5 minutes. Determination of serum tHcy oncentrations were carried out immediately and the remaining serum samples were stored at – 20°C.

Biochemical Methods

Determination of Protein C

Plasma concentrations of protein C were determined by ELISA method (double antibody capture assay) using REAADS protein C antigen kit supplied by Corginex Inc. (Colorado, USA). The Intra-assay precision of assay was 7.0% with a mean accuracy 99.4%. The reference range for healthy subjects between 72-160 %.

Determination of Protein S

Plasma concentrations of free protein S were determined by ELISA method using REAADS Monoclonal free protein S kit supplied by Corginex Inc. (Colorado, USA). The Intra-assay precision of assay was 5.2% with a mean recovery 101.2%. The reference range for healthy subjects between 65-144 %.

Determination of Total Homocysteine (tHcy)

Serum tHcy concentrations were determined by Axis® Homocysteine enzyme immunoassay (EIA) reagent kit supplied by (Axis Biochemicals ASA, IBL-Hamburg, Germany). The Intra-assay precision coefficient of variation (CV) of this assay was 6.8% for average value 10.3 μmol/L. The reference ranges for adult male and female between 5 and 15μmol/L.

Statistical Analyses

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS software version 17.0 for Windows, Inc., Chicago, Illions, USA) to indicate the degree of significant between the mean values of the patient groups and the mean values of the corresponding controls. Descriptive data were given as mean ± standard deviation (SD). All tests were two-tailed and p values less than 0.05 were considered statistically significant. Pearson correlation coefficients (r) were calculated to quantify the relationship between tHcy and protein C and S.

Result and Discussion

Total homocysteine (tHcy)

Total Homocystein level was increased in 40% of patients with sickle cell anemia (n=8, Hb AS=5, Hb SS=3). There was a significant increased mean tHcy level in sickle cell patients by 95% compared to the control group (Mean ± SD, 17.4 ± 6.8 μmol/l; 95% confidence interval (CI), 14.2-20.6; standard error of mean, 1.5 vs 8.9 ± 1.8 μmol/l; 95% CI, 8.0-9.7; standard error of mean, 0.4, respectively; p=0.001) (Table1). In sickle cell patients, tHcy was ranged from 6.5 to 28.3 compared to control 4.0 to 11.75 μmol/l. tHcy was non-significantly correlated negatively with protein C (r= -0.175; p=0.460), S (r= -0.220; p=0.352) and positively with age (r= 0.004; p=0.987). Only three of the hyperhomocysteinemic patients had low protein C and S levels.

Protein C

Protein C was non-significantly decreased in 25% of patients with sickle cell anemia (n=5, Hb AS=2, Hb SS=3). Mean value of protein C within normal range and decreased in patients by 4.4 % compared to the control group (Mean±SD, 88.6±16.9 %; median, 91.0; ranged from 60.0 to 117.0 vs 92.7±7.9%, median, 92.0; ranged from 79.0 to 109.0, respectively; p=0.338) (Table 1). Protein C level was significantly correlated positively with protein S (r= 0.749; p=0.001) and non-significantly with tHcy and age. Protein C was significantly decreased in patients with Hb SS compared to control group (p=0.006).

Protein S

Free protein S was non-significantly decreased in 13.3% of patients with sickle cell anemia (n=4, Hb AS=2, Hb SS=2). Also, the mean value of protein S within normal range and decreased in patients by 2.9 % compared to the control group (Mean±SD, 82.0±16.6 %; median, 85.5; ranged from 51.0 to 107.0 vs 84.9±10.5 %, median, 83.0; ranged from 68.0 to 103.0, respectively; p=0.522). Protein S level was significantly correlated positively with protein C and non-significantly with tHcy and age. Protein S was significantly decreased in patients with Hb SS compared to control group (p=0.003).

In the present study, we observed that patients with sickle cell anemia have a surprisingly elevated homocysteine level. The mean value of protein C and S within normal range and statistically not significant. Patients with sickle cell anemia have significantly higher mean homocysteine level compared to control group and was non-significantly correlated with protein C, S and age. This observation was consistent with the results of other previous studies (Pandey et al., 2012; Meekoo et al., 2004; . van der Dijs et al., 2002). Two pediatric studies found no homocysteine differences compared with control subjects (Balasa et al., 1999; Rodriguez-Cortes et al., 1999). Interestingly, the authors of one of those studies later reported higher tHcy levels in SCD only among older children (Balasa et al., 2006). The results thus suggest that pediatric findings may vary with the age of the children and with geographic influences. The only study of adults with SCD reported higher tHcy levels in 49 patients compared with 16 control subjects (Lowenthal et al., 2000), raising the possibility that only adults and older children may be at risk for hyperhomocysteinemia.

Table.1 Comparison between levels of tHcy, protein C and protein S in sickle cell patients and control subjects

Parameters	Hb AS + SS patients (n=20) (Mean ± SD)	Hb AS (n=15) (Mean ± SD)	Hb SS (n=5) (Mean ± SD)	Control Subjects (n=20) (Mean ± SD)	P value
Age (Year)	7.7 ± 4.0	7.9 ± 4.4	6.9 ± 3.0	8.6 ± 4.6	0.523
Protein C (%)	88.6 ± 16.9	92.6 ± 14.8	76.6 ± 18.7	92.7 ± 7.9	0.338
Protein S (%)	82.0 ± 16.6	87.4 ± 13.8	65.8 ± 14.4	84.9 ± 10.5	0.522
Total homocysteine (tHcy) (μmol/L)	17.4 ± 6.8	16.2 ± 6.6	20.8 ± 6.9	8.9 ± 1.8	0.001

Ischemic complications are a major cause of morbidity and mortality in patients with sickle cell disease (Platt et al., 1994). Biochemical evidence supports the existence of a hypercoagulable, prothrombotic state in SCD patients, as evidenced by elevated levels of activated coagulation factors, increased factor VII turnover and thrombin-antithrombin complexes, and impaired anticoagulation mechanisms such as those in the protein C pathway (Ataga and Orringer, 2003; . Solovey et al., 2004; Westerman et al., 1999).

On the other hand, the mean value of protein C and S were non-significantly decreased and remain within normal range compared to control group. This observation was consistent with the result of other previous study (. Pandey et al., 2012). Marked significantly decreased protein C and S were found among patients with Hb SS and this observation was consistent with the results of other previous study (El-Hazmi et al., 1993). The protein C anticoagulant pathway is activated by complexes and prothrombin fragment 1+2 in the steady-state which is only partially reversed by transfusion. Onyemlukwe et al thrombin binding to thrombomodulin, with subsequent activation of protein C by the thrombin-thrombomodulin complex and EPCR. Evidence from patients with SCD suggests an impaired protein C pathway, accompanied by decreased blood levels of protein C and protein S (Westerman et al., 1999; Wright et al., 1997). Reduced activity of naturally occurring anticoagulants protein C and protein S may contribute to vaso-occlusion in sickle cell disease (SCD) (Schnog et al., 2004). El-Hazmi et al (1993) reported significantly reduced levels of proteins C and S in SCD patients with the highest prevalence of deficiency in patients with a severe form of disease and frequent episodes of crisis. Lower levels of the naturally occurring anticoagulants protein S and protein C which are found in SCD patients could be attributed to either hemostatic abnormalities or hepatic dysfunction. Liesner et al (1998) reported that children with SCD have a reduction in levels of the majority of the coagulation inhibitors (protein Cand S) and increased thrombin generation (thrombin - antithrombin (1992) described significantly lower level of serum AT-III in patients with SCD compared to controls. Bayazit et al (2001)

in a survey of SCA anemia patients in a steady state from Turkey found a significant lower level of protein C and AT levels in patients with SCA compared to controls. Also, they reported non significant lower levels of protein S in the patients than in the controls. They suggested that both hemostatic abnormalities and hepatic dysfunction contribute to low levels of natural coagulation inhibitors in SCA patients.

Conclusion

In conclusion, sickle cell anemia is associated with mild elevated tHcy level which may contribute to increased risk of hypercoagulability and thromboembolic complications.

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Competing interests

The authors declare no competing interests to disclose.

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