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Regular Article

Evaluation of the procoagulant activity of endogenous phospholipids in the platelet-free plasma of children with sickle cell disease using functional assays ☆

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ABSTRACT

Background: The mechanisms of hypercoagulability in sickle cell disease (SCD) are poorly understood.
Objective: We aimed to explore the procoagulant activity of endogenous phospholipids (ePL) in the platelet-free plasma of SCD children.
Methods: A factor Xa clotting time (XACT), thrombin generation (TG) and a capture-based assay for the detection of procoagulant microparticles (PMP) were used. Forty three SCD children (35 SS, 6 SC and 2 Sβ+) were evaluated at steady-state and compared to 20 controls. Fourteen patients were also evaluated during vaso-occlusive crisis. TG was performed using 10pM tissue factor without addition of exogenous phospholipids. A control condition was also performed using 10pM tissue factor and 4 μM phospholipids. Percentages of the test/control conditions were calculated for the peak height (% peak), endogenous thrombin potential (% ETP) and velocity index (% VI).
Results: XACT times were shorter, PMP levels, peak height and velocity index of thrombin generation were higher in SCD patients than controls. Lag time and ETP were not different between the two groups. % peak, % ETP and % VI were higher in patients than controls. Significant correlations were found between PMP levels and XACT, also between PMP levels and peak height, velocity index, ETP and their respective percentages to the control condition, but not with lag time. Double heterozygous patients showed intermediate values for XACT and TG parameters. No significant difference was observed when comparing patients at steady-state versus vaso-occlusive crisis.
Conclusion: High procoagulant activity of ePL was observed in the platelet-free plasma of SCD children, probably borne by procoagulant microparticles. This may contribute to a high hemostatic potential and predisposition to thrombotic complications in these patients.

Introduction

Sickle cell disease (SCD) is considered as a hypercoagulable state but the exact mechanism of this hypercoagulability is not yet fully understood. High levels of markers of *in vivo* thrombin and fibrin generation were previously reported, indicating a continuous activation of coagulation in these patients [1–6]. We postulated that an increased amount of procoagulant phospholipids could at least partially explain these observations. As shown by others, sickle red blood cells express high amount of phosphatidylserine (PS) on their surface [7,8], and increased levels of circulating microparticles deriving from various cells were detected in SCD patients [9–12]. Flow cytometry has been the most commonly used technique to evaluate the content of phospholipids in SCD, through the binding of annexin V on sickle red blood cells or microparticles surface [9,11,12]. Using this technique, the size, the amount and the cellular origin of phospholipids-bearing structures have been investigated. However, their contribution in thrombin generation in patients' plasma has not been documented. In the present study we have explored the procoagulant activity of endogenous phospholipids (ePL) in the platelet-free plasma of SCD and control children using a clotting time-based assay and a thrombin generation test. Procoagulant microparticles (PMP) levels were also measured. SCD children were evaluated at steady-state and during painful crisis.

Abbreviations: SCD, sickle cell disease; ePL, endogenous phospholipids; ETP, endogenous thrombin potential; PMP, procoagulant microparticles; XACT, factor Xa based clotting time.

☆ Preliminary results of this study were presented as a poster at the annual meeting of the American Society of Hematology, December 2010, Orlando, USA *(ASH annual meeting abstracts 2010; 116:1630).*

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Materials and Methods

Patients and controls

The study was approved by the local Ethics Committee and informed consent was obtained from each patient (when applicable) or from the two parents before enrolment. Samples were collected from SCD children who attended our outpatient clinic from February 2010 to February 2011. They were treated either by exchange transfusion therapy (ExT), hydroxyurea or no disease modifying treatment. They were evaluated in different clinical conditions: steady-state, within two days of admission for a vaso-occlusive crisis. Steady-state was defined as the absence of any clinical acute event within the four weeks preceding their routine visit. For controls, we used the remaining citrated plasma after completion of routine haemostatic tests before a minor elective surgery. They were free of any hemoglobin disease, hemolytic anemia or clinical manifestation of haemostatic disorder. Their routine coagulation tests, liver and kidney function tests, CRP and plasma LDH levels were checked normal. Controls were matched for sex and age. Demographics of patients and controls, and the distribution of SCD children according to hemoglobin genotype and to treatment regimen are presented in Table 1.

Sample collection and handling

Peripheral venous blood of patients and normal controls was collected into Vacutainer® tubes (BD, Plymouth, UK) containing buffered sodium citrate (0.109 M). Venipuncture was performed using a butterfly 21 G needle. Platelet-free plasma was prepared by a first centrifugation at 3200 *g* for 15 min followed by a second centrifugation of the supernatant at 16000 *g* for 2 min. All samples were stored at −80 °C. Prior to analysis, samples were rapidly thawed for 5 min in a water bath at 37 °C. Platelet-free plasma was prepared and handled within the hour after blood collection.

Clotting time-based assay

The clotting time-based assay was performed on the STA-R® automate (Roche Diagnostics, Belgium) using the kit STA®-Procoag-PPL (Stago, Paris, France). The principle of the test relies on a Factor Xa based clotting time assay (XACT) as previously described by Exner et al. [13]. This assay is designed so that the phospholipids present in the reaction mixture are provided by the patient's sample only. Briefly, 25 µl of patient's plasma is mixed with 25 µl of phospholipid depleted plasma and prewarmed at 37 °C. The phospholipid depleted plasma is provided in the test kit. Coagulation is triggered by the addition of an activating reagent containing factor Xa and calcium. Clotting time, expressed in seconds, is inversely proportional to the amount of procoagulant phospholipids present in the patient's sample.

Table 1
Demographics of normal controls and sickle cell disease patients.

Population	Number	Gender (Female/Male)	Age (years)
Normal controls	20	9/11	11 (4 – 17)
All SCD at StS	43	18/25	8 (4 – 11)
Hemoglobin genotype			
- SS	35	13/22	9 (5–11)
- SC + Sβ	8 (6SC + 2Sβ)	5/3	7 (3 – 9)
Treatment groups			
- HU	17	7/10	10 (6 – 11)
- ExT	13	1/12	10 (5 – 14)
- NoT	13	10/3	7 (3 – 9)
During VOC	14	7/7	10 (7 – 11)

The age is expressed as median (P25-P75), NC: normal controls, SCD: sickle cell disease, StS: steady-state, HU: hydroxyurea, ExT: exchange transfusion therapy, NoT: no disease modifying treatment, VOC: vaso-occlusive crisis.

Thrombin generation assay

TG was performed using calibrated automated thrombogram CAT® method as described by Hemker et al. [14]. Experiments were carried out within a propylene round-bottomed microtiter plate (Immulon 2HB®, Thermo Labsystems, Helsinki, Finland). TG was triggered by using first, 10 pM tissue factor alone (Innovin®, Dade Behring, Marburg, Germany) and secondly, as control condition, by using 10 pM tissue factor and 4 µM of synthetic phospholipids (Synapse BV, Maastricht, The Netherlands). These phospholipids consist of a mixture of 20% phosphatidylserine (PS), 20% phosphatidylethanolamine and 60% phosphatidylcholine. Tissue factor and synthetic phospholipids were reconstituted in Hepes-buffered saline which comprised 20 mM Hepes, 140 mM NaCl and pH 7.35. In practice, 20 µL of triggering solution or Thrombin Calibrator® (Thrombinoscope BV, Maastricht, The Netherlands) were added in 80 µL of platelets-free plasma in each sample or calibration well respectively. After 15 min of incubation at 37 °C, 20 µL of solubilized Z-Gly-Gly-Arg-AMC reagent Fluo-substrate® (Thrombinoscope BV, Maastricht, The Netherlands) diluted in calcium chloride containing reagent Fluo-buffer® (Thrombinoscope BV, Maastricht, The Netherlands) were automatically dispensed in each well. The reaction was monitored using a fluorometer (Fluoroskan® Ascent FL, Thermo Labsystems, Helsinki, Finland) with an excitation filter at 390 nm and an emission filter at 460 nm, at 37 °C. The thrombogram curve and the different TG parameters were automatically displayed by the dedicated software Thrombinoscope™ (Synapse BV, Maastricht, the Netherlands). All experiments were carried out by the same operator. Four TG parameters were analyzed: Lag Time, Peak height (Peak), Endogenous Thrombin Potential (ETP), and Velocity Index. We also expressed the results as percentages of Peak (% Peak), Velocity index (% VI) and ETP (% ETP). These percentages were calculated by dividing the value of the TG parameter obtained after triggering with 10 pM tissue factor alone by that of the control condition (10 pM tissue factor and 4 µM phospholipids).

Detection of procoagulant microparticles

PMP were detected and their activity was quantified using a capture-based assay (Zymuphen MP-Activity®, Hyphen Biomed, Neuville-sur-oise, France). Briefly, PMPs present in the sample bind to annexin V coated in microplate wells. Unbound materials are washed away. Then the factor Xa-Va mixture containing calcium and purified prothrombin are added. The amount of phospholipids on the surface of bound microparticles is then the limiting factor for conversion of prothrombin to thrombin. The amount of thrombin formed is measured via its specific activity on the thrombin substrate. Results are expressed as nanomoles/L (nM).

Statistical analyses

Results were expressed as median with interquartile range (IQR). Comparisons between SCD patients and normal controls were performed using Mann Whitney test. Kruskal-Wallis test was used when more than two groups had to be compared. Dunn's post test was performed to compare all pairs of groups when the Kruskal-Wallis test was significant. Wilcoxon matched pairs test was used to compare the same SCD patients in two different conditions (steady-state versus vaso-occlusive crisis or before versus after ExT). Spearman's coefficient was used to assess correlations between tests results. Statistical calculations were realized using the software Graphpad Prism® version 5 (GraphPad Software Inc, USA). A $p<0.05$ was considered significant.

Results

Overall 63 children aged from 2 to 20 years were enrolled in this study including 20 normal controls and 43 SCD patients at steady-state.