Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease

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ABSTRACT

Background
Sickle cell disease is characterized by a hypercoagulable state as a result of multiple factors, including chronic hemolysis and circulating cell-derived microparticles. There is still no consensus on the cellular origin of such microparticles and the exact mechanism by which they may enhance coagulation activation in sickle cell disease.

Design and Methods
In the present study, we analyzed the origin of circulating microparticles and their procoagulant phenotype during painful crises and steady state in 25 consecutive patients with sickle cell disease.

Results
The majority of microparticles originated from platelets (GPIIIa,CD61) and erythrocytes (glycophorin A,CD235), and their numbers did not differ significantly between crisis and steady state. Erythrocyte-derived microparticles strongly correlated with plasma levels of markers of hemolysis, i.e. hemoglobin (r=-0.58, $p<0.001$) and lactate dehydrogenase (r=0.59, $p<0.001$), von Willebrand factor as a marker of platelet/endothelial activation (r=0.44, $p<0.001$), and D-dimer and prothrombin fragment F1+2 (r=0.52, $p<0.001$ and r=0.59, $p<0.001$, respectively) as markers of fibrinolysis and coagulation activation. Thrombin generation depended on the total number of microparticles (r=0.63, $p<0.001$). Anti-human factor XI inhibited thrombin generation by about 50% ($p<0.001$), whereas anti-human factor VII was ineffective ($p>0.05$). The extent of factor XI inhibition was associated with erythrocyte-derived microparticles (r=0.50, $p=0.023$).

Conclusions
We conclude that the procoagulant state in sickle cell disease is partially explained by the factor XI-dependent procoagulant properties of circulating erythrocyte-derived microparticles.

Key words: microparticles, sickle cell disease, coagulation activation, hemolysis.

Introduction

Sickle cell disease (SCD) is characterized by chronic hemolysis and recurrent ischemia due to micro-vascular occlusion following the adhesion of erythrocytes and leukocytes to the vascular endothelium.[1] In addition, SCD is complicated by chronic coagulation and endothelial activation, resulting in a hypercoagulable state.[2] Although this hypercoagulability is considered to be multi-factorial, it has become increasingly clear that chronic hemolysis plays a pivotal role in this process and many other sickle cell-related complications. Also in other diseases characterized by chronic hemolysis, such as paroxysmal nocturnal hemoglobinuria and β-thalassemia, hemolysis has been related to coagulation activation and thrombotic complications.[2,3] Phospholipids have been demonstrated to trigger intrinsic coagulation.[4] This was confirmed in a recent study demonstrating that the hypercoagulable state in SCD is specifically linked to the rate of phosphatidylserine exposure on erythrocytes.[5]

Previous observations also suggested a possible contribution of circulating cell-derived microparticles to the hypercoagulable state in SCD.[6] Microparticles are small membrane vesicles released from cells by *budding* upon activation or during apoptosis; microparticles in the blood can originate from platelets, erythrocytes, leukocytes and endothelial cells.[7] Elevated numbers of circulating microparticles have been reported in patients suffering from a variety of diseases with vascular involvement and hypercoagulability, including SCD.[8-14] The exact mechanism by which circulating microparticles trigger coagulation in SCD does, however, remain unclear. The majority of circulating microparticles in SCD originate from erythrocytes and platelets and may support coagulation activation by exposure of phosphatidylserine to facilitate complex formation between coagulation factors in the coagulation activation cascade; an increased exposure of tissue factor has been demonstrated on monocyte-derived microparticles.[8,15] A more thorough understanding of the mechanism by which circulating microparticles affect coagulation and endothelial activation might be helpful in the development of new therapies in SCD.

In the present study, we established the cellular origin of circulating microparticles in patients with SCD during painful crises and during steady-state disease, and explored the relation of these microparticles with coagulation, fibrinolysis and endothelial activation.

Design and Methods

Patients

Consecutive adult sickle cell patients (HbSS, HbSβ[0/+]-thalassemia or HbSC, confirmed with high performance liquid chromatography), admitted with a painful crisis to the Academic Medical Center in Amsterdam were eligible for inclusion. A painful crisis was defined as hospital admission for the treatment of not otherwise explained pain in the extremities, back, abdomen, chest, or head.[16] Patients were asked to provide a blood sample every second day during admission to explore patterns in the number and origin of microparticles during painful crises. Patients included during a painful crisis were asked to provide a baseline blood sample during a subsequent visit to the outpatient clinic. Baseline (steady state) was defined as a period without pain or painful crisis for at least 4 weeks. Healthy controls were recruited as a reference group. All patients and controls gave written informed consent and this study was approved by the internal review board of the Academic Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Collection of blood samples

Blood samples were taken from the antecubital vein without a tourniquet through a 19-gauge needle with a Vacutainer system. Blood was collected into a 4.5 mL tube containing 0.105 M buffered sodium citrate (Becton Dickinson, San Jose, CA, USA). Within 15 min after collection, cells were removed by centrifugation (20 min at 1550 x g at 20°C) to prevent platelet disappearance and concurrent formation of platelet-derived microparticles. Platelet-poor plasma prepared this way is practically free of leukocytes and erythrocytes, and contains about 1% of the original number of platelets. Whether these remaining platelets are indeed small platelets, large platelet-derived microparticles or a mixture thereof does, however, remain a matter of debate. Their number increases about two-fold after freeze-thawing, which we checked in samples from several patients in the present study. Plasma aliquots of 0.25 mL were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Reagents and assays

Fluorescein isothiocyanate (FITC)-labeled IgG$_1$, phycoerythrin (PE)-labeled IgG$_1$, CD20-PE, CD14-PE and CD71-PE were obtained from Becton Dickinson (San Jose, CA, USA), IgG$_{2b}$-PE from Immuno Quality Products (Groningen, The Netherlands), CD61-FITC from Pharmingen (San Jose, CA, USA), CD54-PE and CD62P-PE from Beckman Coulter Inc. (Fullerton, CA, USA), CD62E-PE from Ancell Corporation (Bayport, MN, USA), CD106-FITC from Calbiochem (Gibbstown, NJ, USA), CD142 (tissue factor)-FITC from American Diagnostica Inc. (Stamford, CT, USA), CD144-FITC from Alexis Biochemicals (San Diego, CA, USA) and (anti-)glycophorin A (CD235) from DAKO (Glostrup, Denmark). Finally, allophycocyanin (APC)-conjugated annexin V was purchased from Caltag (Burlingame, CA, USA). Anti-factor VII, anti-factor XI and anti-tissue factor pathway inhibitor (TFPI) were obtained from Sanquin (Amsterdam, The Netherlands). Assays were performed as described by the manufacturer (Parameter human sP-Selectin Immunoassay by R&D Systems; Minneapolis, MN, USA). Platelet counts were determined with a Cell-Dyn 4000 (Abbott Diagnostics Division; Abbott Laboratories; Hoofddorp, The Netherlands). Markers of coagulation activation, fibrinolysis and endothelial activation [prothrombin fragment F$_{1+2}$ (F$_{1+2}$) Enzygnost, Dade Behring, Marburg,

Germany; von Willebrand factor (VWF-Ag) antibodies from DAKO, Glostrup, Denmark; D-dimer; Asserachrom D-Di, Roche, Almere, The Netherlands] were measured by enzyme-linked immunosorbent assay (ELISA).

Isolation of microparticles

A sample of 250 µL of frozen plasma was thawed on melting ice for 1 h and centrifuged for 30 min at 18,890 x g and 20°C to pellet the microparticles. After centrifugation, 225 µL of the supernatant were removed. The pellet and remaining supernatant were resuspended in 225 µL phosphate-buffered saline (PBS) containing citrate (154 mmol/L NaCl, 1.4 mmol/L phosphate, 10.9 mmol/L trisodium citrate, pH 7.4). After centrifugation for 30 min at 18,890 x g and 20°C, 225 µL of the supernatant were removed again. The microparticle pellet was then resuspended with 75 µL PBS-citrate.

Flowcytometry

Five microliters of the microparticle suspension was diluted in 35 µL $CaCl_2$ (2.5 mmol/L)-containing PBS. Then 5 µL APC-labeled annexin V were added to all tubes plus 5 µL of the cell-specific monoclonal antibody or isotype-matched control antibodies (total volume: 55 µL). The samples were incubated in the dark for 15 min at room temperature. After incubation, 900 µL of calcium-containing PBS were added to all tubes (except to the annexin V control, to which 900 µL of citrate-containing PBS were added). Samples were analyzed for 1 min in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA, USA). Both forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. The numbers of microparticles per milliliter were estimated as follows:

$$N_{MP}/mL = [955 \, \mu L/\mu L \text{ flow rate in 1 minute}] \times [100 \, \mu L/5 \, \mu L] \times [1{,}000 \, \mu L / 250 \, \mu L].$$

Microparticles were identified on the basis of their size and density and on their ability to bind cell-type specific CD antibodies and annexin V.[7] The gate settings were confirmed using beads of up to 1.0 µm. Background signal accounted for 3-5% of the total signal in a typical experiment. Annexin V measurements were corrected for auto-fluorescence. Labeling with cell-specific monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies by subtracting the amount of isotype-matched positive events from the total positive events.[13] The within-run and day-to-day coefficients of variation of the microparticle assay are 8% and 13%, respectively.

Thrombin generation

The thrombin generation test was used as described previously.[17] Briefly, microparticles were reconstituted in defibrinated (reptilase-treated) normal pool (microparticle-free) plasma. For the inhibition experiments, the defibrinated plasma and the microparticles were incubated separately for 30 min at room temper-

ature with 20 and 5 µL of antibodies against coagulation factors VII or XI, or TFPI, respectively. Anti-factor VII was used to inhibit the extrinsic pathway and anti-factor XI to inhibit the intrinsic pathway and the factor XI-dependent amplification loop. Plasma and microparticles were pooled after preincubation and incubated for an additional 10 min at 37°C. Thrombin generation was started (t=0) by addition of 30 µL $CaCl_2$ (16.7 mmol/L final concentration). At fixed intervals, 3 µL-aliquots were removed and added to 147 µL prewarmed chromogenic substrate Pefachrome TH-5114 (Pentapharm, Basel, Switzerland, final concentration 0.215 mmol/L) to measure the concentration of free thrombin. After 3 min, 90 µL of 1 mol/L citric acid were added to stop the conversion of Pefachrome TH-5114. The amount of p-nitroaniline generated was determined at $\lambda = 405$ nm with a Spectramax microplate reader (Molecular Devices, Union City, CA, USA). For quantitative analysis, the results are expressed as the area under the thrombin generation curve (AUC), calculated for the time interval between 0 and 15 min after addition of $CaCl_2$.

Statistics

Continuous data are expressed as medians with corresponding inter-quartile ranges. Between group differences were tested with the Mann-Whitney U test or Wilcoxon's rank test in the case of paired analyses. Categorical data are presented as percentages or numbers. Differences between groups of categorical data were tested with the χ^2 test. For correlation studies, Spearman's rank correlation coefficient was determined. To analyze data for possible confounding by multiple testing errors, correlations were also analyzed in mixed models with the patient as subjects. Furthermore, to explore any effect of genotype on the correlation studies, multivariate analyses were performed using both linear and mixed models including specific genotype groups (HbSS, HbSβ$^{0/+}$-thalassemia or HbSC) as factors.

Healthy controls were not included in the correlation studies and the mixed models. p values of 0.05 or less were considered statistically significant. Statistical analyses were performed using SPSS 12.0.2 (SPSS Inc, Chicago, IL, USA).

Results

Patients

A total of 25 consecutive patients with a painful crisis were included, with 13 also providing baseline samples. The patients' characteristics are presented in Table 1. The median duration of hospital admission was 8 days and none of the patients developed complications such as an acute chest syndrome, sepsis or renal failure. The median age of the controls (n=10) was 41 (range, 30-47) years and 60% were female. None of the patients was treated with chronic transfusion therapy.

Numbers and origin of microparticles

Median (inter-quartile range) numbers of microparti-

cles during painful crisis, steady state and in healthy controls are shown in Table 2. In steady state, during painful crisis and in healthy controls, the majority of microparticles originated from platelets (CD61$^+$) and erythrocytes [glycophorin A$^+$ (CD235$^+$)]. In contrast to platelet-derived microparticles, the numbers of erythrocyte-derived microparticles differed significantly between patients and controls. This difference was most marked between healthy controls and patients during a painful crisis (p<0.001). The erythrocyte-derived microparticles were strongly correlated with levels of lactate dehydrogenase (r=0.59, p<0.001) and hemoglobin (r=-0.58, p<0.001; Table 3). In the patients with SCD a distinct subset of transferrin receptor (CD71$^+$)-exposing microparticles was present, which were absent in healthy controls. Furthermore, neither microparticles originating from monocytes (CD14$^+$) nor endothelial cells (CD144$^+$, CD146$^+$, CD62E$^+$) were detectable, and also no microparticles exposing tissue factor could be identified. The different subpopulations of microparticles were comparable in size, as reflected by the forward scatter, and in phosphatidylserine distribution, as reflected by comparable fluorescence of annexin V in all groups.

Correlation of microparticles with markers of coagulation activation

The results of the vWF-Ag, D-dimer and F1+2 assays are shown in Table 4 (as a reference, the levels of D-dimer and F1+2 in the healthy controls were, respectively, 180 (170-532) µg/L and 159 (143-186) pmol/L). The total numbers of circulating microparticles were not correlated to any of the parameters reflecting platelet/endothelial activation (vWF-Ag), fibrinolysis (D-dimer) or coagulation activation (F$_{1+2}$). The platelet-derived microparticles and CD71$^+$ microparticles also did not show any correlation with these markers (Table 3). However, the number of erythrocyte-derived microparticles was strongly associated with markers of *in vivo* coagulation and fibrinolysis activation status as well as endothelial activation (Table 3; Figure 1). Both linear multivariate models as mixed models including genotype as a factor did not show any relation to genotype or any interaction with the described results.

Thrombin generation

The results of the thrombin generation assays are depicted in Figure 2. The AUC of the thrombin generation curve, representing the total amount of thrombin generated, correlated with the total number of circulating microparticles (r=0.63, p<0.001). Thrombin generation was unaffected by pre-incubation with anti-human factor VII but increased slightly in the presence of anti-TFPI (16%; p=0.01). In contrast, in the presence of anti-factor XI, thrombin generation decreased about 2-fold (p<0.001). The extent of this inhibition was significantly related to the number of erythrocyte-derived microparticles (r=0.50, p=0.023), but not to the numbers of platelet-derived or reticulocyte-derived microparticles (r=-0.03 and r=-0.10, respectively; p=NS). The absolute difference in thrombin generation (ΔAUC) between the experiments with and without factor XI

Table 1. Patients' characteristics.

	Painful crisis	Baseline
Number	25	13
Female (%)	56	54
Age (years)	31(26-41)	28(21-38)
Genotype*		
HbSS	10	5
Sβ^0-thal	2	2
Sβ$^+$-thal	5	3
HbSC	8	3
Blood parameters		
Hemoglobin (mmol/L)	9.7 (8.2-10.5)	9.4 (9.0-10.5)
Reticulocytes (%)	5.3 (4.3-6.0)	7.6 (3.3-9.8)
Platelets (×10^9/L)	273 (97-407)	239 (154-332)
Leukocytes (×10^9/L)	9.8 (9.0-13.8)	8.2 (5.3-12.0)
Lactate dehydrogenase (U/L)	374 (288-457)	287 (231-454)

Number of patients with specified genotype.

Table 2. Microparticle numbers during painful crises, in baseline conditions and in healthy controls.

	Painful crisis	Baseline	Controls
Number	25	13	10
Total microparticles (×10^6/mL)	5.5 (2.9-9.6)	6.1 (4.0-7.7)^a	3.6 (2.3-4.4)
Microparticles (×10^6/mL) positive for:			
CD71	0.25 (0.14-0.30)^b	0.24 (0.15-0.30)^a	0.00 (0.00-0.01)
Glycophorin A	0.41 (0.22-0.64)^b	0.33 (0.25-0.43)^a	0.13 (0.09-0.24)
CD61	5.0 (2.5-7.7)	5.5 (3.1-7.2)^a	3.2 (2.5-4.1)

Corrected for the number of events with isotype controls. ^{a}p≤0.05 versus controls.
^{b}p≤0.001 versus controls.

Table 3. Correlations between blood parameters, markers of blood activation and numbers of microparticles.

	Total microparticle	Microparticles stained for		
		Glycophorin A	CD61	CD71
Hematologic parameters				
Hemoglobin	0.01	-0.58^b	0.08	-0.15
Lactate dehydrogenase	-0.10	0.59^b	-0.17	0.05
Reticulocytes	0.49^a	0.32	0.40^a	0.76^b
Platelets	0.70^b	0.47^a	0.65^b	0.62^b
Markers of coagulation activation, fibrinolysis and endothelial activation				
vWF-Ag	-0.03	0.44^b	-0.07	0.00
F$_{1+2}$	-0.03	0.53^b	-0.07	-0.02
D-dimer	0.04	0.52b	-0.01	0.14

All values are Spearman's correlation coefficients. ^{a}p<0.05 ^{b}p<0.005.

Table 4. Markers of coagulation activation during painful crises and in baseline conditions.

|	Painful crisis	Baseline	p
F$_{1+2}$ (pmol/L)	307 (214-565)	213 (151-418)	0.06
D-dimer (µg/L)	2053 (911-3834)	1093 (599-2013)	0.04
vWF-ag (%)	193 (168-247)	141 (117-155)	0.03

Corrected for the number of events with isotype controls.

antibody correlated with the absolute number of glycophorin A$^+$ microparticles (r=0.55, p=0.002; Spearman's analyses, Figure 3).

Discussion

We analyzed the origin of circulating microparticles in patients with SCD during painful crises and steady state and studied the relationship of these microparticles with *in vivo* coagulation activation, hemolysis, fibrinolysis and endothelial activation. First, we demonstrated that almost all circulating microparticles were derived from erythrocytes and platelets, and that the total number of microparticles did not differ significantly between baseline conditions and during a painful crisis, although a shift towards more erythrocyte-derived microparticles was observed during the painful crises. The numbers of all types of microparticles were lower in healthy controls than in patients during baseline conditions. This difference, was, however, clearer for erythrocyte-derived microparticles than for platelet-derived microparticles. Furthermore, we identified a distinctive population of microparticles exposing CD71, the transferrin receptor, but lacking glycophorin A, which was correlated to the percentage
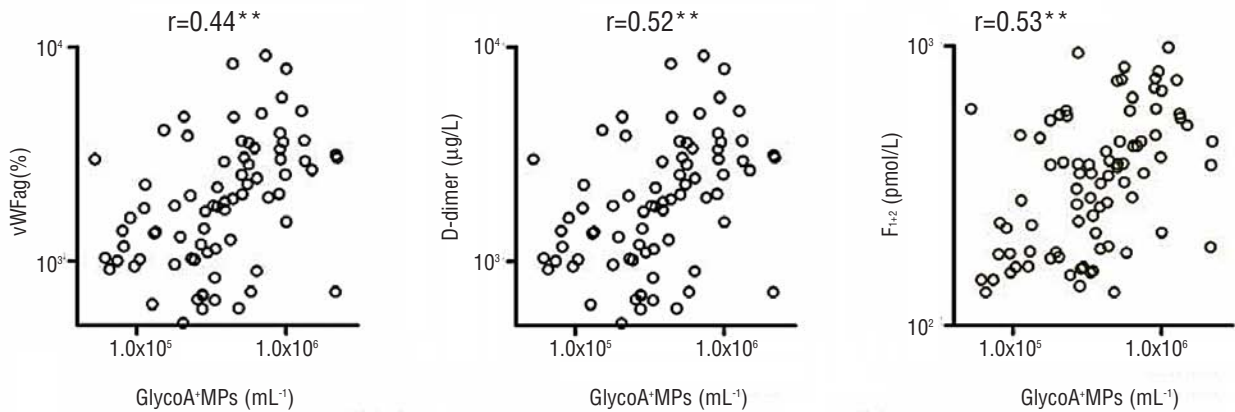
Figure 1. Correlations between glycophorin A$^+$ microparticles (GlycoA$^+$ MPs) and blood parameters. Correlations between glycophorin A$^+$ microparticles and markers of *in vivo* endothelial activation (vWF-Ag), fibrinolysis (D-dimer), and coagulation activation (F$_{1+2}$). Y- and X-axes are logarithmic. **p<0.005.
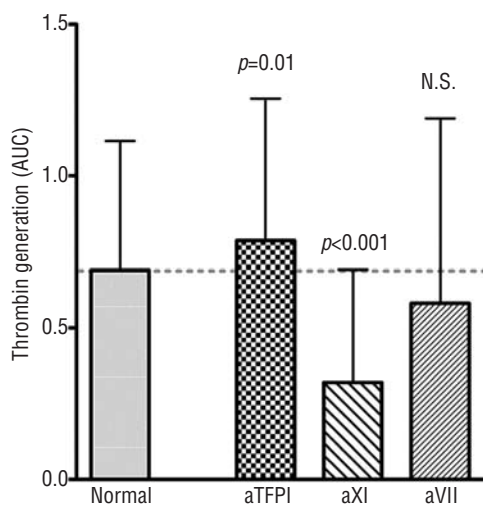
Figure 2. Thrombin generation. The gray (normal) bar shows the median (error bar: 75th quartile) of thrombin generation expressed as AUC after reconstitution of microparticles isolated from patient's blood to defibrinated and microparticle-free normal pool plasma. The "aTFPI", "aXI" and the "aVII" bars show the effects of the indicated antibodies on microparticle-induced thrombin generation.
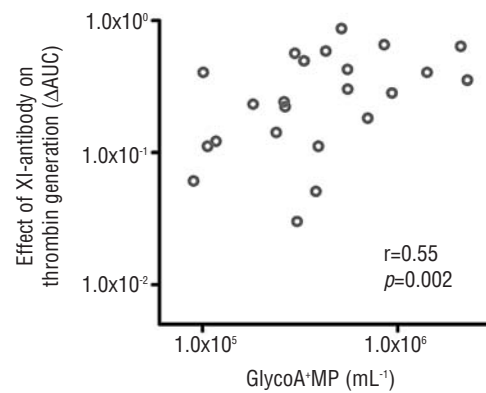
Figure 3. The effect of glycophorin A$^+$ microparticles (GlycoA$^+$ MP) and anti-human factor XI on thrombin generation. Correlation between the total number of glycophorin A$^+$ microparticles and the extent of inhibition of thrombin generation by anti-human factor XI (r=0.55 p=0.002). Y- and X-axes are logarithmic.

of reticulocytes. These CD71^{+} microparticles are probably selectively shed from reticulocytes during erythrocyte maturation.[18] The large difference in the number of circulating CD71^{+} microparticles between patients and controls most likely reflects the enormous difference in hematopoetic rate between these two groups of subjects. Apart from these microparticles, no other populations of circulating microparticles could be identified in the patients' plasma samples. In particular, no monocyte-derived (CD14^{+}) or endothelial cell-derived (CD144^{+}, CD146^{+}, CD62E^{+}) microparticles were identified. Also, no microparticles exposing tissue factor were detectable in our fractions. These data are in line with previous observations that high numbers of both erythrocyte-derived and platelet-derived microparticles are present in patients with SCD.[19,20] Our data contrast with previous findings by Shet et al.[8] who found endothelial- and monocyte-derived microparticles exposing tissue factor to be responsible for coagulation activation in SCD. In our study we were not able to detect this small subset of microparticles; this might be due to differences in the centrifugation forces used to isolate the microparticles.

While no correlation was observed between the total number of circulating microparticles and coagulation activation, erythrocyte-derived microparticles proved to be specifically related to *in vivo* coagulation, fibrinolysis and endothelial activation. These observations confirm those of previous studies of patients with thalassemia and paroxysmal nocturnal hemoglobinuria, pointing towards a direct relation between hemolytic anemia and hypercoagulability.[3,21] Using *ex vivo* experiments with hemolysates others showed that erythrocyte-derived microparticles enhance coagulation activation.[22] Furthermore, in splenectomized patients with idiopathic thrombocytopenic purpura, erythrocyte-derived microparticles are correlated with shortening of activated partial thromboplastin time and increased factor XI activity.[23] In SCD, Setty et al. had already demonstrated that only the number of phosphatidylserine-exposing erythrocytes correlated with *in vivo* markers of endothelial activation, fibrinolysis and coagulation activation, whereas this relation was absent with phosphatidylser-

ine-exposing platelets.[24] This discrepancy is probably explained by a qualitative difference in phosphatidylserine or other phospholipids between erythrocyte-derived microparticles and platelet-derived microparticles. Recently, it was shown that oxidized and unoxidized phospholipids have different effects on inhibition of coagulation.[25] In our thrombin generation experiments, we observed an almost 50% reduction in thrombin generation by anti-human factor XI. Factor XI plays an important role in enhancing thrombin generation, since trace amounts of thrombin can activate factor XI to factor XIa, which then augments thrombin generation via the tenase complex.[26] We, therefore, presume that the factor XI-mediated amplification occurs specifically by phosphatidylserine exposed on erythrocyte-derived microparticles.

Our present results do not exclude that small numbers of tissue factor-exposing microparticles are present in the plasma samples of SCD patients, since thrombin generation by isolated fractions of microparticles from these patients was enhanced when TFPI was blocked. Nevertheless, the amount of tissue factor present in such microparticles was insufficient to trigger tissue factor/VII-dependent coagulation activation in normal plasma, i.e. plasma containing physiological levels of TFPI. From our present study, we conclude that the procoagulant state in SCD is, at least in part, due to the procoagulant effects of circulating erythrocyte-derived microparticles. Their relation with activation of factor XI and the ability of anti-factor XI to block thrombin by microparticles isolated from plasma samples of SCD patients suggests an important role of factor XI-dependent thrombin generation in these patients.

Authorship and Disclosures

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