Clinical significance of circulating blood and endothelial cell microparticles in sickle-cell disease

Mutlu Kasar · Can Boğa · Mahmut Yeral · Suheyl Asma · Ilknur Kozanoglu · Hakan Ozdogu

Abstract Increased thrombocyte activation leads to a higher likelihood of coagulation in sickle-cell disease. On the other hand, chronic inflammation and endothelial cell activation promote vaso-occlusion. The effect of circulating microparticles derived from erythrocytes, monocytes, thrombocytes, and endothelial cells on the vaso-occlusive process is unclear. This study aims to analyze the relationship between sickle-cell disease and miscellaneous organ complications by defining the circulating microparticles during the steady-state and painful crisis periods in 45 patients with sickle-cell disease. Microparticle analysis was conducted using an eight-parameter flow cytometric method, using CD61 PERCP, CD142PE, CD106 FITC, CD14 APC-H7, CD235a FITC, and Annexin-V APC monoclonal antibodies. Microparticle levels of sickle-cell patients were found to be significantly higher during both painful crisis and steady-state situations compared with the control group (for all, $p < 0.001$). Among these microparticles, levels of erythrocyte microparticles (eMPs) were significantly higher during crisis than in the steady-state period (eMP steady state vs. painful crisis: 7.59 ± 12.24 vs. 7.59 ± 12.24, respectively; $p < 0.01$). Microparticles, including eMPs, were not affected by hydroxyurea treatment. Their level did not reflect the high frequency of crisis (>3 times/year). Thrombocyte microparticle levels were found to be higher in patients with nephropathia than in those without (48.05 ± 40.23 vs. 7.67 ± 6.75, respectively; $p < 0.049$). Circulating microparticles seem to be involved in the pathogenesis of sickle-cell disease. eMPs may help with the management of crisis. Thrombocyte microparticles might predict renal damage induced by vaso-occlusion.

Keywords Sickle-cell disease · Microparticle · Hydroxyurea · Vaso-occlusion

Introduction

Hemoglobin S is a mutant hemoglobin that arises when glutamic acid—the sixth normal amino acid of the beta (β) globulin chain—is replaced by valine. This hemoglobin cannot dissolve sufficiently and becomes polymerized when it undergoes a decrease in oxygen pressure. Hemoglobin consists of disturbed rigid erythrocytes [1, 2]. Sickle-cell disease is a chronic, inflammatory disease resulting from hemoglobin-S-related chronic hemolysis, microvascular circulatory disorder, and endothelial activation [3]. Membrane particles (0.1–1 μm in size) may be introduced into the blood circulation from erythrocytes, monocytes, thrombocytes, and endothelial cells, leading to rheologic problems and chronic inflammation. These structures, known as microparticles, carry cell membrane antigens and reflect miscellaneous cellular origins. In various benign and malignant diseases—specifically those regarding bleeding, transmitting infection, tissue damage,

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and immunological incidents—an emphasis is placed on the role of microparticles in coagulation activation [4, 5]. In sickle-cell disease, levels of microparticles derived from erythrocytes, thrombocytes, monocytes, and endothelial cells both during vaso-occlusive crisis and the stable period were increased in the circulation, and microparticles expressing tissue factor can reflect prothrombotic situation [6, 7]. In sickle-cell disease, the relationship between the microparticles that increase in blood with the clinical process of the disease and sickle-cell complications is not clear. This study aimed to investigate the effect of circulating microparticles derived from blood and endothelial cells on the clinical progress of homozygote sickle-cell patients.

Patients and methods

Study plan

This was a single-center, prospective and cross-sectional study. A total of 45 patients, 23 of whom are females, diagnosed with sickle-cell disease were involved in the study between 2009 and 2010. Thirty-two patients had homozygote Hb S disease, and 13 patients had sickle-cell $\beta^{+/0}$ thalassemia cases. Sickle-cell β^0 thalassemia is defined by the absence of normal β chains and, therefore, no hemoglobin A on hemoglobin electrophoresis because almost all the hemoglobin consists of Hb S. The diagnosis of sickle-cell β^+ thalassemia was straightforward. If hemoglobin A is detected as 5–30 % of the total hemoglobin in hemoglobin electrophoresis in patients who have homozygous hemoglobin S disease, whereas blood transfusion from normal individuals is absent [1]. Twenty-five of the cases were in 'painful crisis', whereas 30 of them were stabilized. Ten patients were in both painful crisis and under the stable condition. Hydroxyurea use was available in 17 patients (38 %). Frequency of crises were less 3/year in 30 (67 %) patients and above three in 15 (33 %) patients.

Features of the patient group are presented in Table 1.

In blood samples taken from patients during painful crisis and stable periods, the total microparticle level, as well as the levels of erythrocyte microparticles (eMPs), monocyte microparticles (MMPs), thrombocyte microparticles (tMPs), endothelial cell microparticles (EMPs), microparticles expressing total tissue factor, microparticles expressing the endothelium-derived tissue factor, and microparticles expressing the monocyte-derived tissue factor (MTF) were examined by flow cytometry. Thrombotic complications (deep venous thrombosis or pulmonary embolism), proteinuria, pulmonary hypertension, avascular necrosis, the frequency of painful crisis, and the hydroxyurea use of

patients were recorded, and analyses among subgroups were performed. Microparticle values among patients were compared with controls, including healthy individuals.

Features of the control group are presented in Table 2.

Painful crisis is defined as the admission of a patient to the hospital due to pain with no known cause—except for the patient with sickle-cell disease and medical

Table 1 Features of the patient group

Patient group	n
Total	45
Age (years)	28 (18–52)
Gender	
Female	23 (51 %)
Male	22 (49 %)
Disease	
Hb SS	32 (71 %)
Sickle-cell $\beta^{+/0}$ thalassemia	13 (29 %)
Examination period	
Painful crisis	25 (45 %)
Stable period	30 (55 %)
Hydroxyurea	
Available	17 (38 %)
Not avaliable	28 (62 %)
Thrombotic complication	
Available	2 (4 %)
Not avaliable	43 (96 %)
Nephropathia (microalbuminuria/proteinuira)	
Available	29 (61 %)
Not avaliable	12 (29 %)
Pulmonary hypertension	
Available	14 (33 %)
Not avaliable	28 (67 %)
Avascular necrosis	
Available	15 (33 %)
Not avaliable	30 (77 %)
Frequency of painful cris	
≤3 times/year	30 (67 %)
≤3 times/year	15 (33 %)

Table 2 Features of the control group

Control group	n
Total	20
Age	29 (18–41)
Gender	
Female	9 (45 %)
Male	11 (55 %

Fig. 1 Standard 1 and 2 μm marking with beads *FSC-A* forward scatter and *SSC-A* side scatter
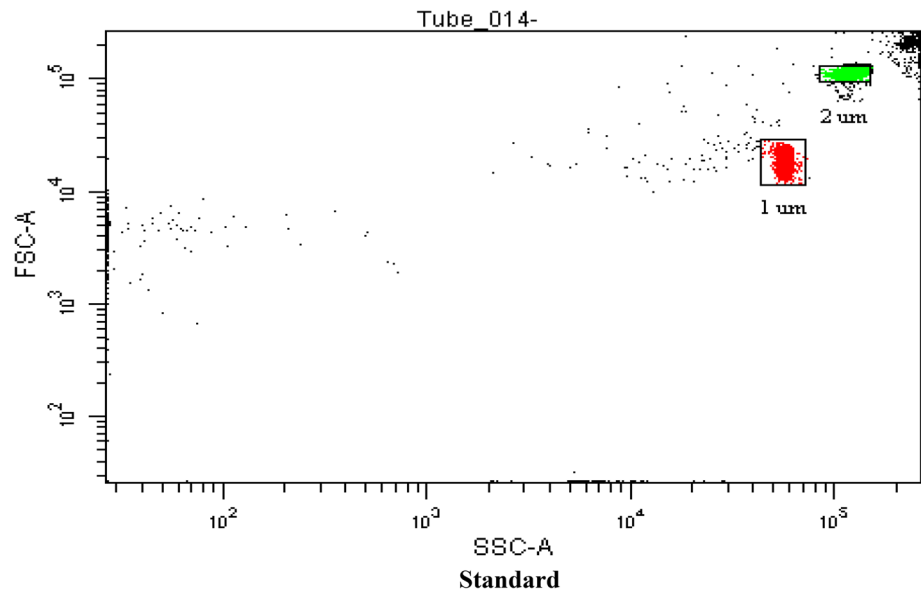
intervention with parenteral nonsteroid anti-inflammatory drugs, metamizole, and narcotics. Blood samples were taken while the patient was hospitalized for painful crisis. Blood samples were also taken from patients who did not have pain for the previous 4 weeks and did not undergo any erythrocyte change within the last 2 months because they were regarded to be in the steady-state period [6, 8]. Microalbuminuria was measured using a photometric method. Values at or greater than 30 mg/day were assessed as microalbuminuria. Kidney dysfunction as microalbuminuria and proteinuria were defined as nephropathia. Avascular necrosis was detected through magnetic resonance, found to be hypointense in T1 and hyperintense in T2, and defined as geographic, nonexpanding lesions with no detected contrast involvement. The calculation of pulmonary artery systolic pressure was made through tricuspid regurgitation with echocardiography in patients with no pulmonary obstruction or right ventricle outflow tract obstruction. A pulmonary artery pressure level greater than 25 mm Hg was regarded as pulmonary hypertension.

Patients with concomitant diabetes, cancer, acute coronary syndrome, sepsis, pregnancy, hypertension, and chronic kidney failure were excluded from the study.

All participants signed consent forms. Başkent University Faculty of Medicine, Research Ethics Committee approved the study.

Microparticle determination

Flow cytometry

All flow cytometry activities were conducted using a blue (wave length: 488 mm) and red (wave length: 633 mm) laser, eight-parameter Becton–Dickinson FACS CANTO II (BD Biosciences, San Jose, California, USA) device. For the analysis of microparticles, forward-scatter (FS) and side-scatter (SS) detectors were adjusted logarithmically. The threshold value of the cytometry was adjusted to detect 2-μm-, 1-μm-, and smaller cells. To this end, standard beads (1 vs. 2 μm; Polysciences Inc., PA, USA) were used to adjust the threshold value (Fig. 1).

Antibodies

Flow cytometry was conducted using anti-CD61 -PERCP, -CD142PE, -CD106 -FITC, -CD14 -APC-H7, -CD235a FITC, and -Annexin-V APC monoclonal antibodies. All antibodies were supplied by BD Biosciences. The following monoclonal antibodies were used: anti-CD61 for tMPs, -CD 235a for eMPs, -CD 106 for EMPs, -CD14 for MMPs, and -CD142 PE for MTF [5, 9–11].

In the presence of Annexin V calcium, phosphatidyl serine on the surface of microparticles is bound with high affinity. Thus, 2 nM/ml of binding solution (Binding Buffer, BD Biosciences) was utilized.

Isolation of microparticles

Three to 4 ml of blood were drawn from patients and those in the control group into 3.2 % sodium citrate tubes using a 21-gauge blood lancet without using a tourniquet. To obtain thrombocyte-rich plasma in 1 h, centrifugation was performed at 160 g at room temperature for 20 min. Thereafter, 2/3 of the plasma (thrombocyte-rich plasma) was centrifuged again at $1,500 \times g$ at room temperature for 20 min. Next, 2/3 of the plasma (thrombocyte-poor plasma) was taken and stored at −80 °C until the day of the study. When the day of the study arrived, thrombocyte-

Fig. 2 Analysis of negative control samples for endothelium (K1), thrombocyte (K2), monocyte (K3), tissue factor (K4) microparticle analyses
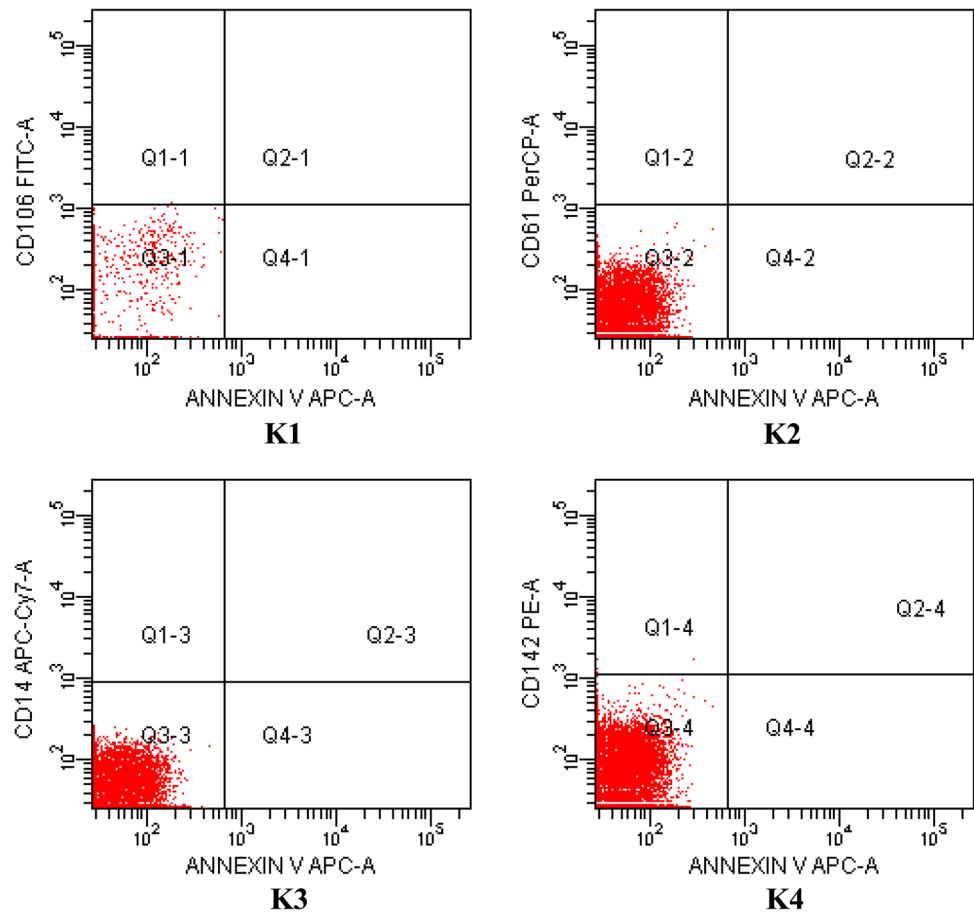
poor plasma was removed from the freezer, melted rapidly at 37 °C and centrifuged at $1,500 \times g$ at room temperature for 20 min. Next, 2/3 of the plasma (noncellular plasma) was removed and subjected to the next centrifugation stage ($13,000 \times g$ at room temperature for 2 min). The supernatant was then removed, and the remaining microparticle pellet was resuspended in 1 ml of Tris buffer (pH 7.4), followed by immediate analysis by flow cytometry [3].

Two tubes were prepared for flow cytometry. Monoclonal antibodies, suitable titrations of which had been determined beforehand, were then added to the tubes and incubated at room temperature in the dark for 20 min. Thereafter, flow cytometric analyses of all samples were conducted at low speed over a total duration of 1 min.

Analysis

Two microparticle standards were used for flow cytometry. For the first standard, cells less than 1 μm in diameter were chosen. For the second, Annexin-V-positive cells were regarded as microparticles and were included in the analysis. All analyses were conducted using the Becton–Dickinson FACS CANTO II FACSDiva software version 6.0 (BD Biosciences). For calculation of the number of

microparticles per ml, the following formula was used: NMP/ml = [1,000 μl/flow rate per 1 min] × [100 μl/5 μl] × [1,000 μl/250 μl] [8] (Fig. 2).

Statistical analysis

The data were analyzed using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA). After the compatibility of the data with a normal distribution was tested, the Mann–Whitney U test was used for the comparison of groups without a normal distribution for microparticle levels. The relationships among microparticle levels, hydroxyurea use, frequency of painful crisis, and complications (thrombotic complications, avascular necrosis, nephropathia, and pulmonary hypertension) were analyzed using Spearman's correlation test. A p value less than 0.05 was deemed to indicate statistical significance.

Results

Microparticle enumeration was completed in all patients. Circulating microparticles were at a higher level during the stable period than during the crisis period in some patients

Table 3 Genotypic and phenotypic characteristics of 45 cases with sickle cell disease

Case[a] (n)	Genotype	Clinical status	Microparticles (event per µl)							
			TMP	tMP	EMP	MMP	eMP	TTF	ETF	MTF
1	SS	Steady state	19.3	7.6	5.5	0.4	0.4	5.0	4.2	0.4
1	SS	Crisis	53.8	6.4	4.5	6.0	6.0	4.0	1.8	3.0
2	SS	Steady state	103.9	55.8	4.7	0.6	0.8	2.4	1.7	0.2
2	SS	Crisis	26.9	7.0	5.5	7.2	7.5	6.0	4.3	1.2
3	SS	Steady state	27.7	14.6	2.5	1.4	0.3	2.7	1.7	0.9
3	SS	Crisis	28.6	9.0	6.3	4.6	9.7	7.5	5.0	2.0
4	SS	Steady state	29.5	12.9	2.3	2.0	9.2	3.6	1.9	1.0
4	SS	Crisis	43.0	4.5	2.5	4.1	13	5.7	1.3	2.4
5	SS	Steady state	178.2	23.2	5.3	2.3	35.0	6.8	2.7	1.2
5	SS	Crisis	82.9	21.0	6.0	8.6	40.0	5.6	1.0	1.2
6	SS	Steady state	35	2.0	8.0	3.5	13.5	4.3	2.0	2.1
6	SS	Crisis	26.3	4.6	4.5	3.6	7.8	5.7	2.5	2.1
7	SS	Steady state	18.6	3.7	5.2	3.6	2.0	5.8	2.4	2.6
7	SS	Crisis	16.1	9.2	0.3	0.4	11.5	0.3	0.1	0.0
8	$S\beta^{0}$	Steady state	8.8	1.3	3.8	0.8	45.0	3.9	3.0	0.7
8	$S\beta^{0}$	Crisis	30.0	3.2	12.0	9.0	14.0	8.2	5.0	2.0
9	$S\beta^{0}$	Steady state	26.7	15.7	3.2	0.6	4.7	3.2	2.3	0.6
9	$S\beta^{0}$	Crisis	31.6	11.1	4.5	4.1	13.0	3.3	2.0	1.1
10	$S\beta^{0}$	Steady state	24.6	2.0	3.7	2.0	2.0	5.4	2.4	0.9
10	$S\beta^{0}$	Crisis	25.0	4.0	8.3	5.7	8.4	12.0	7.7	3.6
11	SS	Steady state	25.0	2.0	5.5	3.5	46.5	6.0	4.8	1.1
12	SS	Steady state	40.0	9.0	4.5	8.5	2.0	8.5	4.1	3.0
13	SS	Steady state	66.9	15.2	4.1	2.9	5.3	5.3	2.4	2.3
14	SS	Steady state	19.0	2.4	5.3	4.8	1.0	8.0	4.3	3.2
15	SS	Steady state	61.0	25.6	4.7	2.9	1.8	5.5	3.7	1.0
16	SS	Steady state	35.0	4.0	5.5	4.0	3.9	3.0	0.8	3.1
17	SS	Steady state	22.6	4.4	6.2	4.9	2.2	6.9	5.1	1.3
18	SS	Steady state	33.3	13.1	2.4	3.7	1.0	4.1	2.4	1.5
19	SS	Steady state	25.5	11.6	3.2	3.9	0.3	5.1	3.2	1.4
20	SS	Steady state	29.1	8.4	3.2	2.1	10.1	3.9	1.8	1.0
21	SS	Steady state	34.3	20.9	2.4	3.8	2.7	2.7	1.3	1.0
22	SS	Steady state	48.2	9.2	6.1	4.1	3.0	8.0	4.3	2.0
23	SS	Steady state	17.1	5.8	2.0	0.3	1.3	2.0	1.2	0.3
24	SS	Steady state	103.9	25.0	5.9	1.0	11.7	5.0	3.7	0.3
25	SS	Steady state	36.2	16.0	8.0	0.6	7.7	6.8	5.5	0.4
26	$S\beta^{0}$	Steady state	39.5	9.0	8.4	6.9	3.0	9.5	5.0	4.0
27	$S\beta^{0}$	Steady state	24.9	10.5	3.6	4.4	2.5	5.9	2.9	2.1
28	$S\beta^{0}$	Steady state	16.1	0.9	1.3	3.1	1.4	3.1	2.3	1.2
29	$S\beta^{0}$	Steady state	32.3	19.5	2.8	4.7	5.1	3.8	2.0	1.8
30	$S\beta^{0}$	Steady state	51.5	23.5	3.2	3.5	8.8	2.4	1.4	0.4
31	SS	Crisis	51.7	6.9	9.0	19.2	10.2	10.0	5.6	2.9
32	SS	Crisis	75.0	24.0	6.5	3.0	5.5	6.1	4.4	1.2
33	SS	Crisis	84.3	23.0	3.0	5.6	65.0	2.8	1.5	0.4
34	SS	Crisis	35.3	10.0	4.9	3.0	10.2	1.6	1.1	0.5
35	SS	Crisis	78.1	25.0	17.9	7.0	11.0	4.0	2.2	1.5
36	SS	Crisis	20.7	8.6	4.0	0.5	10.5	4.0	2.7	0.5
37	SS	Crisis	32.3	10.0	4.6	3.4	5.4	4.6	2.4	1.5

Table 3 continued

Case[a] (n)	Genotype	Clinical status	Microparticles (event per µl)							
			TMP	tMP	EMP	MMP	eMP	TTF	ETF	MTF
38	SS	Crisis	26.3	8.3	1.9	0.7	12.7	1.8	1.6	0.2
39	SS	Crisis	29.9	21.0	1.5	1.0	2.0	1.2	0.8	0.2
40	SS	Crisis	70.0	21.0	3.4	1.0	4.0	3.6	2.5	0.4
41	$S\beta^0$	Crisis	36.1	5.8	8.0	9.2	7.0	4.7	3.6	0.7
42	$S\beta^0$	Crisis	19.6	6.2	6.5	2.3	9.0	5.6	3.2	1.3
43	$S\beta^0$	Crisis	15.7	4.5	3.4	0.6	10.7	3.0	2.4	0.3
44	$S\beta^+$	Crisis	33.8	7.0	2.4	4.0	20.0	3.6	1.6	1.5
45	$S\beta^+$	Crisis	35.6	10.2	2.0	9.3	8.6	6.4	1.0	3.5

TMP total microparticle, *tMP* thrombocyte microparticle, *EMP* endothelial cell microparticle, *MMP* monocyte microparticle, *eMP* erythrocyte microparticle, *TTF* total tissue factor, *ETF* endothelial tissue factor, *MTF* monocyte tissue factor

[a] Cases 1–10 mentioned in the same case number because they included to the study both in stable and crisis condition

Table 4 Results related to microparticles in crisis period and control group

Microparticles	Crisis period (event per µl)	Control group (event per µl)	*p* value
n	25	20	
TMP	40.29 ± 21.42	6.14 ± 1.97	<0.001*
tMP	10.75 ± 7.14	1.59 ± 1.42	<0.001*
EMP	5.47 ± 3.75	2.46 ± 0.97	<0.001*
MMP	4.92 ± 4.14	0.40 ± 0.26	<0.001*
eMP	13.13 ± 12.93	0.10 ± 0.13	<0.001*
TTF	4.79 ± 2.68	2.50 ± 0.99	0.001*
ETF	2.67 ± 1.79	2.14 ± 0.87	0.599
MTF	1.40 ± 1.05	0.11 ± 0.13	<0.001*

TMP total microparticle, *tMP* thrombocyte microparticle, *EMP* endothelial cell microparticle, *MMP* monocyte microparticle, *eMP* erythrocyte microparticle, *TTF* total tissue factor, *ETF* endothelial tissue factor, *MTF* monocyte tissue factor

* Statistically significant

Table 5 Results related to microparticles in stable period and control group

Microparticles	Stable period (event per µl)	Control group (event per µl)	*p* value
n	30	20	
TMP	40.81 ± 34.30	6.14 ± 1.97	<0.001*
tMP	12.58 ± 11.10	1.59 ± 1.42	<0.001*
EMP	4.30 ± 1.70	2.46 ± 0.97	<0.001*
MMP	3.03 ± 1.93	0.40 ± 0.26	<0.001*
eMP	7.59 ± 12.24	0.10 ± 0.13	<0.001*
TTF	4.80 ± 1.93	2.50 ± 0.99	<0.001*
ETF	2.92 ± 1.27	2.14 ± 0.87	<0.023*
MTF	3.03 ± 1.93	0.11 ± 0.13	<0.001*

TMP total microparticle, *tMP* thrombocyte microparticle, *EMP* endothelial cell microparticle, *MMP* monocyte microparticle, *eMP* erythrocyte microparticle, *TTF* total tissue factor, *ETF* endothelial tissue factor, *MTF* monocyte tissue factor

* Statistically significant

under both stable and crisis conditions. Similar observations were made in other patients, including those with the genotype $S\beta^0$.

Features of the genotypic and phenotypic characteristics of 45 cases with sickle-cell disease are presented in Table 3.

The total microparticle levels, as well as the tMP, EMP, MMP, eMP, TTF, and MTF levels, were significantly higher in the crisis patient group than in the control group ($p < 0.001$). There was no significant difference between the crisis and control patient groups according to the endothelial cell tissue factor (ETF) levels.

Comparisons between the painful crisis and control patient groups according to the TMP, tMP, EMP, MMP, eMP, TTF, ETF, and MTF levels are presented in Table 4.

The TMP, tMP, EMP, MMP, eMP, TTF, ETF, and MTF levels were also significantly higher in the stable patient group than in the control group ($p < 0.001$).

Comparisons between the stable and control patient groups according to the TMP, tMP, EMP, MMP, eMP, TTF, ETF, and MTF levels are presented in Table 5.

When microparticle levels were compared according to painful crisis and steady state periods, the eMP level was found to be significantly higher in the crisis patient group than in the steady-state patient group ($p < 0.001$). However, there was no significant difference between the crisis and steady-state patient groups according to the TMP, tMP, EMP, MMP, TTF, ETF, and MTF levels (Table 6).

Comparisons between the stable and painful crisis patient groups according to the TMP, tMP, EMP, MMP, eMP, TTF, ETF, and MTF levels are presented in Table 5.

Table 6 Results related to microparticles in crisis and steady state period

Microparticles	Crisis period (event per µl)	Stable period (event per µl)	p value
n	25	30	
TMP	40.29 ± 21.42	40.81 ± 34.30	0.403
tMP	10.75 ± 7.14	12.58 ± 11.10	0.748
EMP	5.47 ± 3.75	4.30 ± 1.70	0.322
MMP	4.92 ± 4.14	3.03 ± 1.93	0.070
eMP	13.13 ± 12.93	7.59 ± 12.24	<0.001*
TTF	4.79 ± 2.68	4.80 ± 1.93	0.906
ETF	2.67 ± 1.79	2.92 ± 1.27	0.268
MTF	4.92 ± 4.14	3.03 ± 1.93	0.859

TMP total microparticle, *tMP* thrombocyte microparticle, *EMP* endothelial cell microparticle, *MMP* monocyte microparticle, *eMP* erythrocyte microparticle, *TTF* total tissue factor, *ETF* endothelial tissue factor, *MTF* monocyte tissue factor

* Statistically significant

The TMP levels of the steady-state patient group was observed to be higher in patients with nephropathia than in those without nephropathia, albeit not significantly so. The tMP levels were significantly higher in those with nephropathia than in those without (48.05 ± 40.23 vs. 7.67 ± 6.75, respectively; $p < 0.049$). There was no significant difference between those with nephropathia and those without nephropathia regarding the EMP, MMP, eMP, TTF, ETF, and MTF levels ($p > 0.05$).

In the steady-state patient group, the TMP, tMP, EMP, eMP, MMP, TTF, ETF, and MTF levels were not significantly different between those with thrombotic complications, pulmonary hypertension, and avascular necrosis and those without these conditions (for all, $p > 0.05$).

When the relationship between the microparticle levels and frequency of painful crisis was assessed, in the steady-state patient group, the TMP, tMP, EMP, eMP, TTF, ETF, and MTF levels were found to be not significantly different between those with a frequency of painful crisis ≤3 times/year and those with a frequency of painful crisis >3 times/year (For all, $p > 0.05$). The MMP level was significantly higher in those with a painful crisis frequency ≤3 times/year compared with those with a painful crisis frequency >3 times/year ($p = 0.006$).

When the relationship between the microparticle levels and hydroxyurea use was assessed, the eMP levels that had been high during the crisis period were not affected by hydroxyurea use ($p > 0.05$). In the steady-state patient group, the TMP, tMP, EMP, MMP, eMP, TTF, ETF, and MTF levels did not differ in the group using hydroxyurea compared with the group that did not (For all, $p > 0.05$).

Discussion

Sickle-cell disease is characterized by vaso-occlusion, leading to hemolytic anemia, an increased tendency towards infections, and ischemic tissue damage in almost all vascular areas. Furthermore, sickle-cell disease is also characterized by organ function disorders and premature death. Proinflammatory states, hypercoagulability, and endothelium function disorders contribute to vaso-occlusion [12]. Microparticles, levels of which are increased in many systemic diseases, and activation and apoptosis cases, have been examined in sickle-cell disease [6, 7]. Thrombocytes, erythrocytes, and endothelial cell-derived microparticles and microparticles consisting of tissue factor, result in an increase in hypercoagulability in sickle-cell disease.

Shet et al. [6] conducted a comprehensive study of sickle-cell disease and evaluated the total microparticle levels of 23 sickle-cell patients, 21 of whom were in painful crisis, and 16 patients with stable disease. They found that the TMP level was significantly higher in the painful crisis and stable condition groups compared with the healthy controls. In terms of the cellular sources of the microparticles, the levels of eMPs and MMPs were significantly higher both during the stable and crisis stages compared with in healthy individuals. The tMP, eMP, MMP, and EMP levels were higher during the crisis period compared with the stable period; however, no significant difference was found, which may be attributed to differences between individual patients. In our study, the TMP, EMP, MMP, eMP, tMP levels in the control group and patients during crisis or stable periods and were found to be significantly different. Thus, in sickle-cell disease, endothelial cells, erythrocytes, monocytes, and thrombocytes are in a constant state of activation. When the crisis and stable periods were compared, the eMP level was found to be increased during the crisis period, possibly due to mechanical decomposition resulting from sickling, complement-related cellular fractionations, and leukocyte-to-erythrocyte adhesion.

Expression of tissue factor by microparticles plays an important role in disease pathobiology. Tissue factor is the primary trigger of coagulation, and an increase in in vivo tissue factor expression promotes thrombosis in many diseases. In sickle-cell disease, monocytes, and, possibly, endothelium cells, express tissue factor abnormally, which may be related to symptoms such as stroke and pulmonary thrombosis [6].

Shet et al. found that the total tissue factor level was significantly higher than control subjects during both the crisis and stable periods in sickle-cell disease. They reported that microparticles expressing tissue factor are derived from endothelium cells and monocytes [5]. Van Beers et al. conducted a study of 25 painful crisis and 13

steady-state sickle-cell patients and found that most of the TMPs consisted of eMPs and tMPs. Additionally, unlike Shet et al. [13], they did not detect an increase in EMP and MMP levels, or tissue factor expression. In our study, most of the microparticles consisted of eMPs and tMPs. According to Shet et al., the TTF, ETF and MTF levels were higher during the crisis and steady-state periods. The high expression of tissue factor during both the crisis and stable periods indicates the existence of procoagulants in sickle-cell disease.

Because of the endothelial activation, the number of endothelial cells in the circulation increases [11, 14]. Vascular endothelium activation contributes to the pathogenesis of sickle-cell disease. Endothelium cell number and endothelium cell-derived tissue factor expression was demonstrated to be increased in the circulation [15, 16]. A study that assessed the antigens expressed by EMPs during activation and apoptosis revealed that the main markers of endothelial cells and EMPs during apoptosis are CD31, CD51/61, and CD105; however, levels of CD54, CD62E, and CD106 are also increased during activation [17]. Similar to our data, other studies evaluating sickle-cell disease reported no significant difference between the crisis and steady-state groups regarding the increase in EMP levels [2, 13].

Regarding the relationship between microparticles and organ damage, we could not identify any report in the literature that assessed proteinuria and microparticle levels. We did not include patients with end-stage kidney failure in our study, and the proteinuria level was at 67 % (29 patients). The difference in the tMP level was significant between those with nephropathia compared with those without nephropathia. The cause may be related to the increased thrombocyte activation in proteinuria. We did not identify a relationship between microparticles and complications such as pulmonary hypertension and avascular necrosis. Assessments in pediatric age groups before organ damage occurs may provide more accurate data regarding this issue.

Painful crisis refers to the period when endothelial activation, chronic inflammation, and the contact of sickle cells with the endothelium, thrombocytes, and inflammation cells occur at the greatest intensity, simultaneously with mechanical trauma to cells. During this period, blood microparticle levels are expected to be increased due to mechanical trauma. In our study, during the crisis period, the microparticle levels were not different from those during the steady-state period. This finding alone suggests the possibility that microparticle clearance is enhanced during the crisis period. We hypothesize that an inflammatory state during the crisis period may initiate removal of microparticles from the blood by accelerated macrophage activation. If such activity does indeed take place, it would not be selective with regards to the microparticle type and would not be related to the increase in tissue factor expression.

Hydroxyurea use may also be a contributing factor for microparticles level [18]. It has been reported that increased disease activity under inflammatory conditions can reduce the microparticle level by a secretory phospholipase mechanism, resulting in lysis of the microparticle membrane [19]. This mechanism might also be responsible for the decreased circulating microparticle levels during the crisis period.

In the present study, we evaluated various microparticles using conventional flow cytometry. Cells less than 1 μm in diameter that were positive for Annexin V were considered to be microparticles. Some groups defined microparticles as 2-μm or smaller particulates in current cytometry [9, 20]. However, because this size can also involve thrombocytes, we defined MPs as 1-μm-diameter or smaller particulates that are bound to Annexin V, similar to in previous reports. The lack of standardization of the methods of detection has led to variation in the reported size of microparticles [21]. This may complicate the differentiation of microparticles from other cell-derived vesicles, such as exosomes and apoptotic bodies or debris. New-generation technology that enables identification of smaller microparticles may overcome this limitation by characterization using flow cytometry, electron microscopy, Western blotting, or proteomics [21]. Microparticle staining by flow cytometry with a lipid marker such as calcein AM, PKH67, or bio-maleimide may provide optimal detection [22]. Recently, investigators have begun to use flow image cytometry for microparticle characterization, a technique that enables work interactively between images and analysis results from single or multiple images. All methods, including new technologies, have some disadvantages, such as artifacts due to the abundance of cell debris. In the present study, in addition to using conventional flow cytometry, the lack of assessment of hemoglobin S concentrations simultaneously with microparticle levels is a limitation.

In conclusion, this study supports the view that microparticles are important players in the pathogenesis of sickle-cell disease. We observed that eMPs may assist the management of crisis. We also identified that elevated tMP levels in peripheral blood during the stable phase may predict renal damage induced by vaso-occlusion in patients with sickle-cell disease. However, further investigation is necessary to develop and validate the results to gauge the use of MP in the patient management. Microparticles unaffected by the use of hydroxyurea or the development of crisis may be a preferable material for use in future sickle-cell disease research.

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