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RESEARCH

Microparticle profile during painful crisis and steady state in children with sickle cell anemia

Orak hücreli anemili çocuklarda ağrılı kriz ve kriz dışı dönemde mikropartikül profili

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Abstract

Purpose: Sickle cell anemia is a disease characterized by hemolytic anemia, hypercoagulopathy, and painful crises. Microparticles are 0.1-1 μm sized membrane particles derived during cellular activation or apoptotic phases of the cell cycle. In this study, we investigated the role of microparticles on clinical state and prognosis during painful crisis and steady state in children with sickle cell anemia.

Materials and Methods: Patients with sickle cell anemia who were followed up in Çukurova University Pediatric Hematology Department and presented with a painful crisis were included in the study. Children without any systemic disease were included in the control group. Total microparticle levels, erythrocyte (CD235a), endothelial (CD106), and monocyte (CD14) microparticle levels, and tissue factor expressing (CD142) microparticle levels were analyzed.

Results: A total of 29 patients with sickle cell anemia who presented with a painful crisis were included in the study. In addition, blood samples were collected from 26 of these patients in a steady state. Blood samples were obtained from 18 healthy children as the control group. Total microparticle levels were significantly higher in sickle cell anemia patients in painful crises than in control group. Erythrocyte and monocyte microparticle levels were significantly higher in patients with a painful crisis than in a steady state. Endothelial and tissue factor expressing microparticle levels were higher during a painful crisis than steady state, although not at statistically significant levels. Microparticle levels were lower in patients with hydroxyurea treatment than those without, although it was not a statistically significant difference.

Conclusion: Total microparticles as well as erythrocyte and monocyte microparticles were high in sickle cell

Öz

Amaç: Orak hücreli anemi, hemolitik anemi, hiperkoagülopati ve ağrılı krizlerle karakterize bir hastalıktır. Mikropartiküller, hücre döngüsünün hücresel aktivasyonu veya apoptotik fazları sırasında hücrelerden dökülen 0.1-1 μm boyutlarında membran parçacıklarıdır. Bu çalışmada, orak hücreli anemisi olan çocuklarda kriz ve kriz dışı durumda mikropartiküllerin klinik durum ve prognoz üzerindeki rolünü araştırdık.

Gereç ve Yöntem: Çukurova Üniversitesi Pediatrik Hematoloji Bölümünde takipli ve ağrılı kriz ile başvuran orak hücreli anemi tanılı hastalar çalışmaya dahil edildi. Herhangi bir sistemik hastalığı olmayan çocuklar kontrol grubuna dahil edildi. Hastaların ve kontrol grubunun total mikropartikül düzeyleri, eritrosit (CD235a), endotel (CD106), monosit (CD14) mikropartikül düzeyleri ve doku faktörü eksprese eden (CD142) mikropartikül düzeyleri analiz edildi.

Bulgular: Orak hücreli anemi tanısı olan ve ağrılı kriz ile başvuran toplam 29 hasta çalışmaya dahil edildi. Ayrıca bu hastaların 26'sının ağrılı kriz dışı durumda kan örnekleri alındı. Kontrol grubu olan 18 sağlıklı çocuktan kan örnekleri alındı. Total mikropartikül düzeyleri ağrılı krizdeki orak hücreli anemi hastalarında kontrol grubuna göre anlamlı olarak yüksek bulundu. Eritrosit ve monosit mikropartikül düzeyleri ağrılı krizde kriz dışı döneme göre anlamlı olarak yüksekti. Endotelyal ve doku faktörü eksprese eden mikropartikül seviyeleri, istatistiksel olarak anlamlı olmasa da ağrılı krizde kriz dışı döneme göre daha yüksekti. Mikropartikül seviyeleri hidroksiüre tedavisi alan hastalarda almayanlara göre daha düşüktü, ancak istatistiksel olarak anlamlı bulunmadı.

Sonuç: Orak hücreli anemi hastalarında ağrılı kriz sırasında total mikropartiküller, eritrosit ve monosit mikropartikülleri yüksek bulunmuştur. Mikropartiküllerin

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anemia patients during a painful crisis. Studies involving larger numbers of patients are needed to better understand the role of microparticles in the pathophysiology of sickle cell anemia and their association with painful crises.
Keywords: Child, microparticle, sickle cell anemia

orak hücreli aneminin patofizyolojisindeki rolünü ve ağrılı krizlerle ilişkisini daha iyi anlamak için daha fazla sayıda hastayı içeren çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Çocuk, mikropartikül, orak hücreli anemi çocuk, mikropartikül, orak hücreli anemi

INTRODUCTION

Sickle cell anemia (SCA) is a hereditary disease characterized by chronic hemolytic anemia and recurrent painful crises[1]. Hemoglobin S (HbS) is mutant hemoglobin with glutamic acid replacing valine as the 6th amino acid in ß globulin. HbS cannot adequately dissolve in low oxygen pressure and causes polymerization and rigid erythrocyte formation[2,3].

Microparticles are membrane-derived small budding vesicles generated during cellular activation and apoptosis. Microparticles do not contain a nucleus but carry specific surface antigens. Recognition of these particles occurs by size and antigens presentation[4,5]. Microparticles are present in low concentrations in normal plasma and participate in homeostasis as well as pathological states[6].

Circulating microparticles may be derived from different kinds of cells. Microparticles derived from erythrocytes express CD235a, leucocytes express CD45, granulocytes express lactoferrin or CD66b, lymphocytes express CD4, CD6, and CD20, and monocytes express CD14 on their surfaces[5,7]. CD31, CD51, CD105, CD144, and CD146 antibodies are used for detecting levels of endothelial microparticles[8]. High endothelial microparticle levels are associated with various pathological conditions such as atherosclerosis, diabetes mellitus and sepsis. Increased platelet microparticle formation contributes to the inflammatory role of platelets in various clinical conditions[6].

Many factors related to the activation of the anionic phospholipid surface, particularly apoptosis and endothelial damage in SCA, lead to an increase in the number of circulating microparticles. The study of microparticles in SCA is an ongoing area of research because the pathophysiological roles of microparticles in the manifestations of SCA are still unknown particularly in children. The erythrocytes, monocytes, thrombocytes, and endothelial microparticle levels both during a vaso-occlusive crisis and stable periods are increased in patients with SCA, like in many cardiovascular and metabolic diseases[4,9]. As recently reported, microparticles are associated with several complications in patients with SCA[10-12]. The measurement of microparticle levels could be an early marker of complications and prognosis in children with SCA.

The study hypothesized that the profile of microparticles would be higher in SCA patients compared to healthy children and that increased microparticles levels may be a biomarker of SCA complications. Therefore, the study evaluated the relationship of microparticles with complications and their effect on prognosis by measuring the levels of blood and endothelium-derived microparticles in children with SCA during crisis and steady states. This study contributes to the literature by revealing the microparticle profile of children with SCA during painful crises and at a steady state, as well as the microparticle profile in the presence of disease-modifying therapy and complications.

MATERIALS AND METHODS

Sample and Procedure

This study was a prospective study from a single center. Blood samples were collected from 29 pediatric patients with SCA during a painful crisis and from 26 of them in a steady state who were followed up at Çukurova University Pediatric Hematology Department. Eighteen age- and sex-matched healthy children without any systemic disease or drug use were enrolled as a control group.

A painful crisis is described as the admission of SCA patients for pain without any other significant causes. Patients were called for steady-state blood samples at least four weeks after the end of the painful crisis period. Blood samples of patients in a painful crisis were collected at admission, those of patients in stable condition were collected on recall, and all samples were analyzed at the same time.

Blood samples of both patient (painful crisis and steady-state period) and control groups were analyzed for total microparticles, endothelial

microparticles, monocyte microparticles, erythrocyte microparticles, and tissue factor expressing microparticles with flow cytometry. Hydroxyurea usage (10mg/kg/dose), frequency of painful crises, and presence of avascular necrosis of the femur or humerus were recorded.

The inclusion criteria for the patient group were admission with a painful crisis and the same patient was in a stable condition for at least 4 weeks after the end of the painful crisis. The inclusion criterion for the children in the control group was the absence of any signs of disease. Exclusion criteria were admissions for reasons other than a painful crisis, the presence of concomitant chronic kidney disease and hypertension in the patient group, and clinical or laboratory evidence of infection in the control group. A total of three patients had acute chest syndrome and were excluded from the study.

All children and their parents were informed about the study and their informed consent was obtained before the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Çukurova University Faculty of Medicine, Ethics Committee approved this study on 21/01/2011, Number 14.

Flow cytometry

All flow cytometry activities were conducted using a blue (wavelength: 488 mm) and red (wavelength: 633 mm) laser with an 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 cytometry was adjusted to detect 2 μm-, 1 μm-, and smaller cells[13].

Monoclonal antibodies used in flow cytometry were CD106 FITCH, CD235a FITCH, CD142PE, CD14 APC-H7, and ANNEXIN V APC. Total microparticle (TMP) was the total number of blood microparticles in the subjects. CD106 was used for endothelial microparticle analysis (EMP), CD235a for erythrocyte microparticle (eMP) analysis, CD142 for tissue factor-related microparticle (TTF) analysis, and CD14 for monocyte microparticle (MMP) analysis. All antibodies were obtained from BD Biosciences. Threshold levels for flow cytometry were arranged for the detection of 2 μm, 1 μm, or smaller cells.

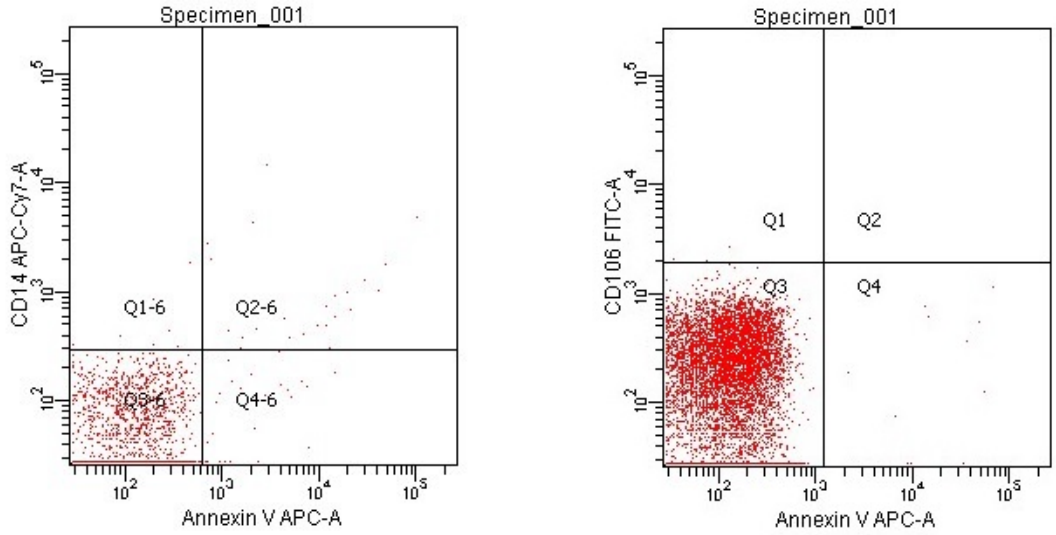

Figure 1. Measurement of monocytes and endothelial microparticles by flow cytometry.

For the selection of microparticles in flow cytometry, two standard protocols were performed. In the first protocol, cells smaller than 1μm were chosen; in the second protocol, Annexin V positive cells were accepted as microparticles and analyzed. All analyses were conducted using the Becton–Dickinson FACS CANTO II FACSDiva software version 6.0 (BD Biosciences). Microparticle number was calculated using the following formula: NMP (number of microparticles)/ml = [1,000 μl/flow rate per 1 min] x [100 μl/5 μl] x [1,000 μl/250 μl][13] (Figure 1).

The flow cytometry was conducted at the Apheresis Unit of Başkent University Dr. Turgut Noyan Training and Research Centre. Microparticle analyses of the patient and control groups were performed by a faculty member (IK) of the Department of Physiology at the Apheresis Unit of Başkent University Dr. Turgut Noyan Training and Research Centre.

Statistical analysis

The G* Power 3.1 statistical analysis program (Erdfelder, Foul & Buchner, Düsseldorf, Germany) was used to calculate the power of the study. The study population was determined as 28 by setting the effect size to 0.5, significance level to 0.05, power (1-β)=0.80 at the 95% confidence level.

SPSS 18.0 software program was used for statistical analysis of data. Normally distributed continuous variables were shown as the mean and standard deviation and as the median, minimum, and maximum when non-normally distributed. The Shapiro-Wilk test was used to test whether the distribution was normal for continuous variables. Categorical variables were reported as numbers and percentages. Categorical measures of groups were compared using the chi-square test. The Student's t-test was used to compare age, blood parameters, and EMP levels in the patient and control groups. In addition, it was used to compare EMP levels in groups determined according to the frequency of painful crises, hydroxyurea use, and the presence of avascular necrosis. The Mann-Whitney U test was used to compare TMP, eMP, TTF and MMP levels in patient and control groups. In addition, it was used to compare TMP, eMP, TTF and MMP levels in groups determined according to the frequency of painful crises, hydroxyurea use, and the presence of avascular necrosis. The paired t-test was used to compare EMP levels in painful crises and steady-state groups. The Wilcoxon signed-rank test was used to compare TMP, eMP, TTF and MMP levels in painful crises and steady-state groups. The level of statistical significance for all tests was 0.05.

RESULTS

Twenty-nine SCA patients and 18 control individuals were included in this study. Thirteen of the 29 SCA patients (44.8%) were male and 16 (55.2%) were female. In the control group, nine (50%) were male and 9 (50%) were female (Table 1). Six patients (21%) were using hydroxyurea. The frequency of crises was less than 1/year in five patients (17%), 1-3 times/year in 14 patients (48%), and more than three times/year in 10 patients (35%). The presence of avascular necrosis was observed in seven (24%) patients.

Table 1. Characteristics of sickle cell anemia patients and healthy controls

Variable		Patient group (n=29)	Control group (n=18)	p value
Sex	Female	16 (55.2)	9 (50)	0.482
	Male	13 (44.8)	9 (50)	
Age	Mean ± SD	11.62±3.56	11.7±4.3	0.855
Blood parameters Mean ± SD	Hemoglobin (g/dl)	8.9±1.6	12.2±1.1	<0.001*
	Hematocrit (%)	26.6±5.1	36.3±3.7	<0.001*
	Thrombocyte (/mm^3)	393793.1±183478	317555.6±90967.5	0.065
	Leucocyte (/mm^3)	11152.8±3269.4	7287.2±1877.8	<0.001*

SD: standard deviation, *Statistically significant

eMP levels of SCA patients were significantly higher in the crisis period than in the steady state (p=0.043). In addition, MMP levels of SCA patients were significantly higher in the crisis period than in the steady state (p=0.011). Total microparticle (TMP) levels and EMP levels of SCA patients were higher in the crisis period than in the steady state, although it was not statistically significant (p=0.770 and

p=0.061, respectively). Similarly, TTF levels of SCA patients were higher in the crisis period but this difference was not statistically significant (p=0.367).

Mean TMP, EMP, TTF, and MMP levels were higher in SCA patients during the steady state than in the control group, although the differences were not statistically significant (p=0.063, p=0.232, p=0.218, and p=0.249, respectively). Only, mean eMP levels were significantly higher in SCA patients than in the control group (p=0.001).

Mean TMP, eMP, TTF, and MMP levels were significantly higher in SCA patients during the crisis period than in the control group (p=0.03, p<0.001, p=0.009, and p=0006, respectively). Similarly, although not statistically significant, EMP levels were higher in SCA patients during the crisis period (p=0.249). Comparison of mean TMP levels and microparticle subtypes of SCA patients during the crisis period, steady state, and control group are shown in Table 2.

Table 2. Comparison of microparticle levels of the patient and control groups

Measures	Groups			p values		
	Sickle cell anemia patients		Control			
	Painful crisis	Steady state		a	b	c
	Mean±SD Median (Min-Max)	Mean±SD Median (Min-Max)	Mean±SD Median (Min-Max)			
TMP	29798.5±40567.6 17158 (2005-211615)	24425.8±23120.5 16715 (65-78802)	10219.6±5856.5 10441 (2015-19327)	0.770	0.030*	0.063
EMP	14.1±13.3 8.0 (1.0-48.0)	8.2±4.2 8.0 (1.0-20.0)	6.5±3.0 6.0 (2.0-13.0)	0.061	0.249	0.232
eMP	89.2±148.8 23.0 (2.0-616.0)	23.7±18.6 17.0 (3.0-90.0)	3.8±2.2 4.0 (1.0-8.0)	0.043*	<0.001*	<0.001*
TTF	38.8±82.7 18.0 (1.0-460.0)	22.3±21.1 13.0 (1.0-85.0)	12.2±10.9 8.0 (2.0-40.0)	0.367	0.009*	0.218
MMP	40.1±80.8 17.0 (1.0-430.0)	9.9±10.7 5.0 (1.0-32.0)	8.3±4.6 8.0 (4.0-25.0)	0.011*	0.006*	0.249

TMP: Total microparticle, EMP: Endothelial microparticle, eMP: erythrocyte microparticle, TTF: total tissue factor, MMP: monocyte microparticle; [a] Crisis period versus steady state, [b] crisis period versus control, [c] steady state versus control; *Statistically significant

TMP, EMP, eMP, TTF, and MMP levels tended to be lower in patients using hydroxyurea, however, there was no statistically significant difference (p=0.429, p=0.614, p=0.324, p=0.324, and p=0.196, respectively). Similarly, there was no statistically significant difference between the incidence of painful crisis (comparison of 0-3 times/year and >4 times/year) and TMP, EMP, eMP, MMP, and TTF levels (p=0.491, p=0.560, p=0.874, p=0.751, and p=0.164, respectively). Furthermore, there was no statistically significant difference in TMP, EMP, eMP, TTF, and MMP levels depending on whether there was humerus or femur avascular necrosis (p=0.409, p=0.900, p=0.486, p=0.486, and p=0.753, respectively).

DISCUSSION

SCA is characterized by vaso-occlusion, ischemic tissue damage, and organ function disorders[1,2]. Proinflammatory states and hypercoagulability are important contributing factors for vaso-occlusion in SCA[2]. Elevated microparticle levels in SCA patients are reported in the literature[4,9,10,13]. Microparticles cause increased inflammation and hypercoagulability in patient with SCA[8,9].

Shet et al. reported higher total microparticle levels in 21 adult SCA patients in a painful crisis and 16 SCA patients in a non-crisis period. They found a significant increase in TMP levels in SCA patients during the crisis period and non-crisis period compared to the healthy control group. They also reported that eMP and MMP levels were significantly higher in crisis and non-crisis periods than in the healthy control group[4].

Kasar et al. performed the most comprehensive study in Türkiye. In their study, 45 adult SCA patients were

included[13]. TMP, eMP, MMP, EMP and thrombocyte microparticle levels were higher both in the crisis and non-crisis periods in SCA patients than in the healthy group. According to the comparison of the painful crisis period and non-crisis periods, only eMP levels were higher in the crisis period. To eliminate personal differences, ten patients who gave samples both in a crisis and non-crisis period were analyzed and eMP, MMP, and TMP levels were higher in the crisis period[13]. Consistent with these studies, in the present study, TMP levels were higher in SCA patients in the crisis period compared to the steady state, but this difference was not significant. In addition, TMP levels were significantly higher in SCA patients during a crisis period than in healthy controls in this study.

Van Beers et al. reported that eMP levels in SCA patients during a crisis period were higher than in healthy controls[9]. Similarly, Tantawy et al. reported higher eMP levels in 50 SCA children during a crisis period compared to 40 healthy controls[10]. Recently, Boulassel et al. reported that the levels of erythrocyte and platelet microparticles were substantially higher in steady state SCA patients compared to healthy controls and the clinical phenotype did not significantly affect erythrocyte and platelet microparticle levels[14]. eMP levels in SCA patients during a painful crisis period were higher than in steady state or healthy controls in the present study. This finding was consistent with previously mentioned studies[4,9,10,13,15]. As previously reported[4,13], MMP levels were higher in SCA patients during a crisis period than in steady state and healthy controls in this study. This significant increase in eMP and MMP levels during the crisis period is thought to be related to the sickling of erythrocytes and the cellular degradation of erythrocytes by the complement system.

Endothelial cells have an important role in vascular pathologies including vaso-occlusion in SCA patients during a painful crisis[15]. Solovey et al. reported that endothelial-derived tissue factor in SCA patients was significantly higher than in healthy controls[16]. Contrary to this but similar to other reported studies, the present study found no significant difference between crisis and stable periods regarding the increase in EMP levels[13]. The reason for these different results may be the number and characteristics of the patients included in the study.

TTF may contribute to thrombotic symptoms by activating the coagulation system. Shet et al. and Kasar et al. reported higher TTF levels in SCA patients during a crisis period than in healthy controls[4,13]. Similarly, the present study found that TTFs were higher in SCA patients in a crisis period than in healthy controls, and although not statistically significant, they were also higher in steady-state SCA patients than in the healthy group. This suggests that TTFs are high in SCA due to the pro-coagulant state.

Hydroxyurea is a frequently used antimetabolite in severe SCA patients and increases fetal hemoglobin (HbF) levels. The effect of hydroxyurea treatment on circulating microparticles remains unclear. Some reported studies demonstrate increased levels of TMP, eMP, MMP, and EMP in hydroxycarbamide-treated patients[18,19], whereas others have reported reduced levels of TMP, eMP and platelet microparticles in hydroxycarbamide-treated patients[10,11,19-21]. Falanga et al. reported an inverse relationship between HbF concentration and microparticle formation, particularly those of platelet and erythrocyte origin[22]. In addition, one study reported that plasma microparticles released during a crisis increased endothelial ICAM-1 (Intercellular Adhesion Molecule 1) levels and neutrophil adhesion in SCA patients, whereas hydroxyurea treatment abolished their proinflammatory properties through its effects on microparticles[23]. Although the results in the literature are not similar, TMP, eMP, MMP, and EMP levels in SCA patients receiving hydroxyurea tended to be lower than those who did not, but this difference was not statistically significant. An inadequate number of studies and different techniques for the isolation and analysis of microparticles can explain this difference in the literature. Long-term studies in pediatric patients with SCA will clarify these different results.

Children with SCA have an increased risk of avascular necrosis of large joints, which may be due to thrombotic vascular occlusion. Marsh et al. reported microparticle levels 2.3-fold higher in SCA patients with femoral head avascular necrosis than those without. They suggested that microparticles might be a clinically useful biomarker of femoral head avascular necrosis in patients with SCA[12]. However, we did not find any significant difference in the microparticle levels according to the presence or absence of avascular necrosis.

Garnier et al. reported they did not detect an association between microparticle levels and occurrences of painful vaso-occlusive crises[21]. Similarly, the present study did not find any significant relationship between the incidence of

crisis and microparticle levels. In contrast, Kasar et al. reported a significant increase in MMP levels with an increase in crisis incidence, however, TMP, eMP, and EMP did not correlate with the incidence of crisis[13].

This study has some limitations. These are the inclusion of a relatively small number of patients from a single center in the study and the lack of long-term follow-up results. However, this study is one of the few studies in the literature addressing circulating microparticle levels in children with SCA. The results in the literature are inconsistent because of an inadequate number of studies.

In conclusion, this study showed that TMP, eMP and MMP levels were high in pediatric SCA patients during a painful crisis. Multicenter studies involving larger numbers of patients should be performed to better understand the role of microparticles in the pathophysiology of SCA and their association with painful crises. Elucidation of the role of microparticles in the pathophysiology of SCA and the development of complications may make microparticles an important biomarker in SCA in the future. A complete understanding of the role of microparticles in SCA pathogenesis may lead to the development of novel therapeutic methods targeting microparticles.

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