High levels of endothelial progenitor cells can be associated with thrombosis in patients with Behçet's disease

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ABSTRACT

Objective. *Behçet's disease (BD) is a systemic disorder characterised by vasculitis. Endothelial progenitor cells are derived from the bone marrow and contribute to new vessel formation. The aim of this study was to investigate the level of endothelial progenitor cells in BD and BD-associated conditions.*

Methods. *A total of 74 subjects were included in this study, of whom 44 and 30 subjects were patients with BD or healthy subjects, respectively. Endothelial progenitor cells were defined and measured by flow cytometry according to the expression of CD146, CD31 and CD34. We separated BD patients according to the active disease, pathergy test results, thrombosis and gender. MedCalc 12.5 software programme was used for statistical analyses.*

Results. *The level of endothelial progenitor cells was comparable in patients with BD and healthy subjects (p=0.849). It was also comparable in patients with active or inactive BD (p=0.320). The level of endothelial progenitor cells was higher in patients with thrombosis (p=0.04). There was no statistical significant difference between pathergy positive and negative patients (p=0.969). The level of endothelial progenitor cells was not correlated with age, C-reactive protein, erythrocyte sedimentation rate, white blood cells and disease duration (p>0.05).*

Conclusion. *The level of endothelial progenitor cells was significantly higher in BD patients with thrombosis. On the other hand, they were not associated with disease activity, pathergy test and other conditions. EPCs may be a useful marker for thrombosis in patients with BD. In our opinion, this is the most expected result in this study.*

Introduction

Behçet's disease (BD) is a systemic vasculitis disorder which is characterised by recurrent oral aphthous ulcers, genital ulcers, ocular lesions, and skin lesions. In addition to the specific signs of disease it can affect other organ systems such as vascular, gastrointestinal, and neurological systems. The etiology of the disease is unknown but genetic, immunologic and environmental factors are thought to be associated with disease. Gender distribution and clinical expression of disorder varies according to the ethnic origin. Clinical manifestations are important for diagnosis of BD (1, 2).

BD is an inflammatory-associated disorder and has a specific histopathological vasculitis pattern characterised by prominent neutrophil and monocyte infiltration in perivascular regions. Vascular system is primarily affected by this disorder and both venous and arterial systems are affected (3).

Endothelial progenitor cells (EPCs) are bone marrow derived immature cells which take part in neovascularisation and vascular homeostasis. They are composed of several cell types including mainly hematopoietic and mesenchymal stem cells. The level of circulating EPCs is low in normal medical conditions but rapidly increases in case of vascular injuries and tissue ischemia. In response to pathological stimulation of vascular injury or tissue ischemia, they migrate to the peripheral circulation and develop into mature endothelial cells (4, 5).

In this study we aimed to investigate the level of EPCs in BD and BD-associated conditions.

Materials and methods

This case-control study was carried out in the Rheumatology outpatient clinics of Başkent University School of Medicine in Adana from 1st January 2011 to 1st May 2011. The institutional review board of the hospital approved this experiment, and informed consent was obtained from all subjects. All proce-

dures were followed in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

A total of 74 subjects from both genders, with a minimum age of 18 years old, were included. The study group was comprised of 44 patients with BD and the control group was comprised of 30 healthy subjects. Patients with a diagnosis of chronic disease other than BD, malignancy, pregnancy, infection, recent surgery or trauma were excluded. Diagnosis of BD was based on the new international criteria (2). Pathergy test was performed with a disposable 26 gauge needle injecting 0.1 ml normal saline at the flexor aspect of left forearm, approximately 2 inches below the elbow crease and read at 48 hours. Activity of BD was determined according to the activity index of the Behçet's Disease Research Committee of Japan (6).

Vascular lesions such as thrombosis in present study were not acute thrombosis. They were detected with GE Logic 9 high resolution doppler ultrasonography (GE medical systems, Milwaukee, USA) and/or MR (magnetic resonance) venography. MR venograms were performed on 1.5-T scanners GEMedical Systems, Milwaukee, ABD) using a contiguous 2D time-of-flight (TOF) MR angiographic technique.

A peripheral blood sample was collected from each subject and placed into an Ethylenediaminetetraacetic acid (EDTA) tube via a 21-gauge needle. Anticoagulated blood samples were kept at 4°C and analysed by flow cytometry within 4 hours of venesection. Peripheral whole blood cells were prepared by a lyse/wash procedure and were then evaluated by flow cytometry (FACSCantoII, Becton Dickinson, California, USA). The use of this procedure minimises the cell loss, making it possible to perform the measurement of the all EPCs in the blood samples. A multistep manual technique was used to detect and quantify EPCs. Assessment of a minimum of 400.000 cells/peripheral blood sample was considered informative. Anti-CD146 PE, anti-CD31 FITC, anti-CD45 APC- Cy7 and anti-CD34 PE- Cy7 monoclonal antibodies were used for the detection of EPCs in this study. All monoclonal antibodies were taken from Becton Dickinson BioScience (California, USA). We used Cell-Dyn 3700 Hematology Analyzer (Abbott Laboratories, Chicago, IL, USA) for white blood cell counts. Cells were identified as total endothelial cells (ECs) if they were $CD146^{+}/CD31^{+}/CD45^{-}$ cells. Cells were identified as endothelial progenitor cells if they were CD146+/CD31+/$CD45^{-}/CD34^{+}$ cells. Finally, cells were

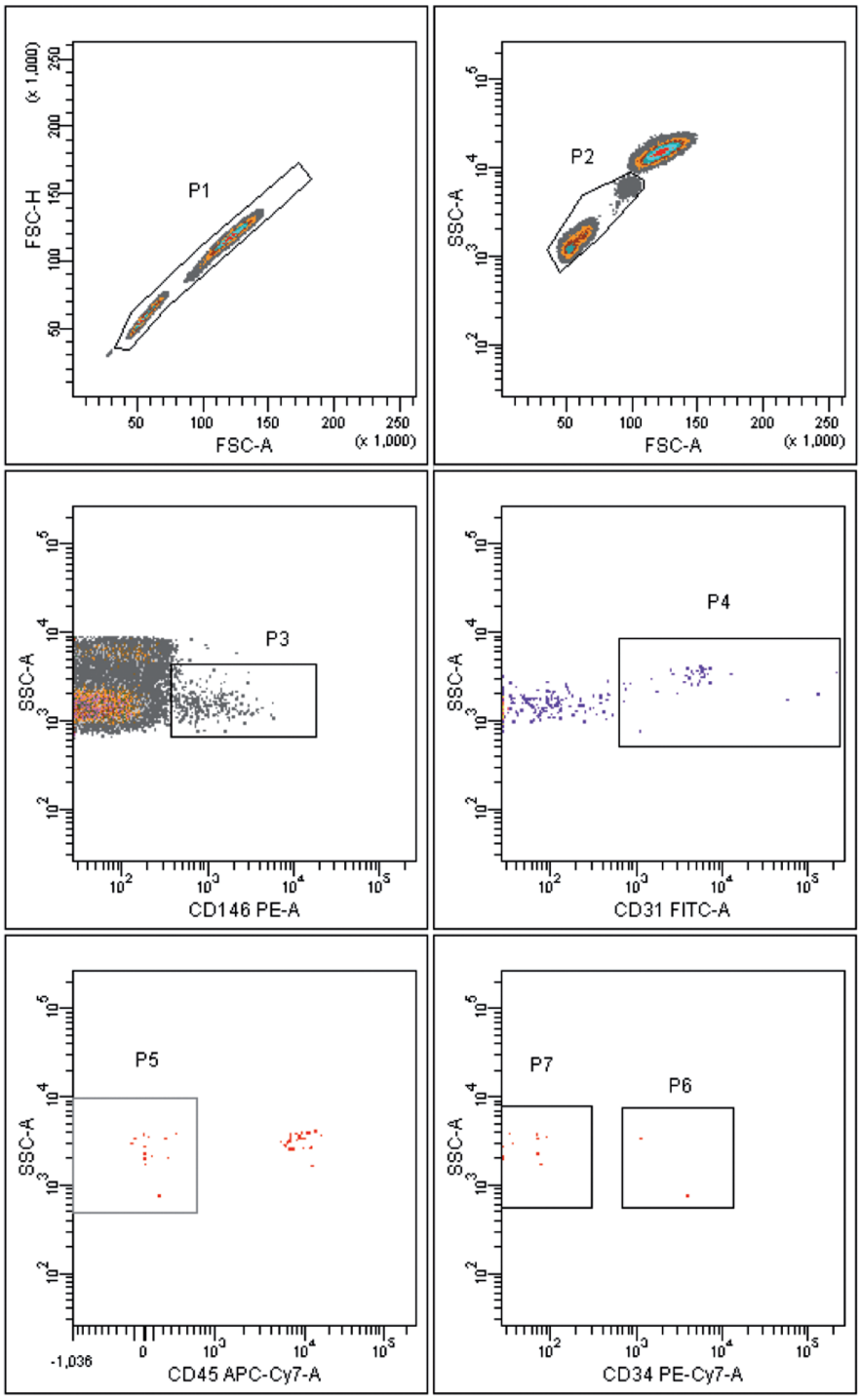

Fig. 1. Gating and quantification of endothelial cells (Non-viable cells, platelets, debris, and non-specific binding were excluded by FSC-A and FSC-H graphic (P1), P1 was met with FS-SS graphic and mononuclear cells were chosen (P2). CD146+/CD31+/CD45- cells were determined as endothelial cells (P3, P4, P5). CD34+ cells were determined as endothelial progenitor cells while CD34- cells were determined as mature endothelial cells (P6, P7).)

identified as mature endothelial cells if they were $CD146^+/CD31^+/CD45^-/CD34^-$ cells (Fig. 1). Flow cytometry data were analysed with DIVA software (Becton Dickinson, San Jose, California, USA). Non-viable cells, platelets, debris, and non-specific binding were excluded from the analyses by isotopic control and consecutive gating.

The MedCalc 12.5 software (MedCalc, Turkey) was used for all statistical analyses, and the data were reported as the mean ± SD. The Kolmogorov-Smirnov test was used to show the normal distribution of quantitative measurements; the Chi-square was used to test the statistical significance of the differences in frequencies; and the *t*-test or Mann Whitney U-test was used for the comparison of the quantitative measurements between the two groups. The correlation coefficient was used to analyse the degree of association between the two variables (Pearson correlation coefficient (r) with *p*-value and 95% CI for r). A log transformation was used for variables that were not normally distributed, and a *p*-value of less than 0.05 was considered to be statistically significant.

Table I. Characteristics of patients and control (The clinical features were positive anytime during the disease period).

	Behçet's disease (n=44)	Healthy subjects (n=30)	*p*-value
Age (years)	37.0 ± 11.6	37.2 ± 10.3	0.943
Female, n (%)	18 (40.9%)	15 (50%)	0.592
EPCs	2.84 ± 2.6	2.73 ± 1.9	0.849
Smoking, n (%)	12 (27.3%)	7 (23.3%)	0.907
Disease duration (months)	65.6 ± 83.9		
ESR (mm/h)	15.0 ± 11.4		
CRP (mg/L)	6.7 ± 8.5		
WBC /μL	7860 ± 2285		
Oral aphthae, n (%)	16 (36.4%)		
Genital ulcers, n (%)	13 (29.5%)		
Skin lesions, n (%)	12 (27.3%)		
Uveitis, n (%)	16 (36.4%)		
CNS involvement, n (%)	5 (11.4%)		
GIS involvement, n (%)	6 (13.6%)		
Joint involvement, n (%)	10 (22.7%)		
CVS involvement, n (%)	3 (6.8%)		
Thrombosis, n (%)	13 (29.5%)		
Erythema nodosum n (%)	1 (2.2%)		

EPCs: Endothelial progenitor cells.

Table II. Comparison of patients according to the sex.

	Women (n=18)	Men (n=26)	*p*-value
Age (years)	39.3 ± 14.0	35.4 ± 9.6	0.271
EPCs	2.38 ± 1.42	3.15 ± 3.17	0.344

Table III. Comparison of Behçet's disease patients according to the active disease.

	Active disease (n=13)	Inactive disease (n=31)	*p*-value
Age (years)	32.7 ± 9.0	38.8 ± 12.2	0.115
Female, n (%)	4 (30.8%)	14 (45.2%)	0.582
EPCs	3.09 ± 2.66	2.23 ± 2.45	0.320

Results

There were 33 (44.6%) women and 41 (55.4%) men in this case-control study. The mean age was 37.1±11.0 years old. Groups were matched in terms of age and sex (p=0.943, 0.592, respectively). There was no statistical significant difference between groups according to the level of endothelial progenitor (p=0.849) (Table I). Table I shows the properties of patients and healthy controls. The clinical features of patients were positive anytime during the disease period.

The number of women with BD was 18 (40.9%) while the number of men with BD was 26 (59.1%). Women and men were comparable in terms of age, level of endothelial progenitor cells (p=0.271, 0.344, respectively) (Table II).

According to the activity index of the Behçet's Disease Research Committee of Japan, there were 13 (29.5%) and 31 (70.5%) patients with active or inactive BD, respectively. The mean age and sex distribution of patients with active or inactive BD were comparable (p=0.115, 0.582, respectively). The level of endothelial progenitor cells were also comparable in patients with active or inactive BD (p=0.320) (Table III).

Pathergy test was positive in 21 (47.7%) of 44 patients. The mean age and sex distribution were comparable in patients with positive or negative pathergy test (p=0.399, 0.194, respectively). There was no statistical significant difference between groups according to the level of endothelial progenitor cells (p=0969). Frequency of active diseases in patients with positive or negative pathergy test were comparable (p=0.393) (Table IV).

Thrombosis was detected in 13 (29.5%) of 44 BD patients. There were no statistical significant difference according to the mean age and sex distribution of BD patients with or without thrombosis (p=0.725, 0.582, respectively). The level of endothelial progenitor cells was higher in patients with thrombosis. The difference was statistically significant (p=0.04) (Table V). Table VI shows the classification of vascular involvement. There were 3 (23%) patients with superficial thrombophlebitis, 4 (30.7%) patients with popliteal vein thrombosis, 1 (7.6%) patients with hepatic vein thrombosis, 1 (7.6%) patients with vena cava superior thrombosis, 1 (7.6%) femoral vein thrombosis.

The level of EPCs was not correlated with age, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood cells (WBC) and disease duration in patients with BD (p>0.05, for each) (Table VII).

Discussion

Behçet's disease is a systemic vascu-

Table IV. Comparison of Behçet's disease patients according to the thrombosis.

	Thrombosis (+) (n=13)	Thrombosis (-) (n=31)	*p*-value
Age (years)	36.0 ± 10.1	37.4 ± 12.3	0.725
Female, n (%)	2 (15.4%)	16 (51.6%)	0.582
EPCs	4.07 ± 3.14	2.32 ± 2.19	0.04

Table V. Comparison of Behçet's disease patients according to the pathergy test.

	Pathergy test (+) (n=21)	Pathergy test (-) (n=23)	*p*-value
Age (years)	35.4 ± 11.0	38.4 ± 12.2	0.399
Female, n (%)	6 (%28.6)	12 (%52.2)	0.194
EPCs	2.85 ± 3.13	2.82 ± 2.08	0.969
Active disease, n (%)	8 (38%)	5 (21.7%)	0.393

Table VI. Clasification of vascular involvement.

Vascular involvement	The number of patients n=13	Female/Male
Superficial thrombophlebitis	3 (23%)	1/2
Popliteal vein thrombosis	4 (30.7%)	0/4
Hepatic vein thrombosis	1 (7.6%)	1/0
Vena cava superior thrombosis	1 (7.6%)	0/1
Femoral vein thrombosis	1 (7.6%)	0/1
Sinus thrombosis	3 (23%)	1/2

Table VII. Correlation of endothelial progenitor cells with age, CRP, ESR, WBC and disease duration.

	Endothelial progenitor cells
Age	*p*=0.812 r=-0.035
CRP	*p*=0.435 r=-0.120
ESR	*p*=0.382 r=-0.134
WBC	*p*=0.113 r=-0.244
Disease duration	*p*=0.394 r=0.131

litides that affects both small and large arteries and veins. Inflammation of vessel walls is main mechanism that results in venous thrombosis, arterial obstruction or aneurisma (7). Although BD is a systemic disorder no specific serologic marker has been identified and additionally there are no laboratory markers that correlate well with this disorder. In this study we investigated the levels of EPCs in BD and BD-associated conditions. We have shown that the level of EPCs in BD was comparable with healthy controls. Endothelial dysfunction is one of the major causes that affect the level of endothelial cells negatively (8). Therefore, we excluded other diseases which are associated with endothelial dysfunction such as coronary artery disease, diabetes, renal failure etc. Due to aging is an effective factor that affects the level of EPCs (9) we compared BD patients with age and sex matched control subjects. Our observation on the link between BD and the level of EPCs is in agreement with the results of Fadini *et al*. study. Although they have shown a mild reduction in the level of EPCs in patients with BD the difference is not statistically significant. In addition, low level of EPCs in their study may due to the different putative progenitor cell populations which they quantified (10).

In present study we have also shown that the level of EPCs is not associated with disease activity, pathergy test and gender. To our knowledge, this is the first study that investigates the level of EPCs in BD according to the pathergy test and gender. Pathergy test is the non-specific hyperactivity of the skin. The perivascular and periadnexal lymphohistiocytic infiltration of varying intensity and their penetration in the deep dermis and moderate neutrophilic infiltration in the dermis are also important findings of pathergy test. In histopathology of the test, the presence of the vasculitis and neutrophilic vascular reaction are definitive but not a requirement (11). The absence of neutrophilic vascular reaction may be responsible for the result which we have shown in this study. Previous studies have reported that EPCs levels are higher in women than men (12, 13), but these studies were not about BD. There have been a few studies that investigated EPCs-disease activity association in BD. The results of these studies were inconsistent. For example in the Fadini *et al*. study, they reported that low levels of EPCs correlate with disease activity (10). On the other hand, in the Kutlay *et al*. study, circulating endothelial cells were reported to be high in active period of BD (14).

Endothelial activation in affected blood vessels is a mediator of vascular inflammation as well as thrombosis in BD (15). In this study, we have demonstrated that the level of EPCs was significantly higher in BD patients with thrombosis. In our opinion, this is the most expected result in this study and according to our knowledge this is also the first study that investigates the level of EPCs in BD patients with thrombosis. In accordance with this result circulating endothelial cells were reported to be high in a patient with BD complicated with cerebral thrombophlebitis (16). Further reinforcing this link, high levels of EPCs were reported in acute ischaemic events like myocardial infarction and in other vascular injuries (17, 18). We understood from these results that thrombosis or vascular injuries are the main determiners of EPCs levels irrespective of underlying disease.

Lastly, the levels of EPCs were not correlated with age, CRP, ESR, WBC and disease duration in this study. Similarly, in the Fadini *et al*. study, they did not find a correlation between age and EPCs levels. On the contrary, they found a negative correlation between disease duration and EPCs levels. C-reactive protein, ESR and WBC levels may be associated with the levels of EPCs dur-

ing acute inflammation. To further reinforce this link, thrombosis in this study was not of the acute type.

Circulating endothelial cells are found to be associated with ANCA-associated vasculitis (19) and previous studies have shown that acute inflammation increases the EPCs levels while the chronic inflammation or long lasting diseases decrease them (20, 21). According to these studies, it is difficult to say that vasculitis are associated with high or low levels of EPCs. Therefore, we can find high or low or normal levels of EPCs in vasculitis due to acute or chronic or other properties of inflammation. Consequently, we believe that acute inflammation on vessel walls is the most effective factor on EPCs levels.

This study did have some limitations too. First, it would have been beneficial if the sample size had been larger. Second, patients could have been compared according to the acute or chronic inflammation. Third, the majority of patients were on treatment and this condition affects the EPCs. Fourth, histologic examination of involved tissue in patients with BD frequently reveals a vasculitis, although this finding may not be demonstrated in all lesions, it would have been benefical if the patients had been separated according to the demonstrated vasculitis. Finally, further investigations are needed to support this study.

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