Circulated Activated Platelets and Increased Platelet Reactivity in Patients With Behçet's Disease

Servet Akar,* Mehmet Ali Özcan,† Halil Ateş,† Oğuz Gürler,* Inci Alacacioglu,† G. Hayri Özsan,† Nurullah Akkoç,* Sebnem Özkan,‡ Fatih Demirkan,† and Fatos Onen*

*Department of Internal Medicine, Division of Romatology, †Department of Internal Medicine, Division of Hematology, and ‡Department of Dermatology, School of Medicine, Dokuz Eylul University, Izmir, Turkey

Summary: Behçet's disease (BD) is a multisystem disorder. Venous as well as arterial thrombosis is a common complication of BD but exact pathogenetic mechanism of the thrombotic tendency is not well known. This study aimed to evaluate circulating activated platelets and platelet reactivity in Behçet's patients. Twenty-two Behçet's patients (4 female, 18 male; mean age 38.6 ± 10.9 years) and 20 control subjects (8 female, 12 male; mean age 38.8 ± 9.4 years) were included. Those patients who had hypertension, hyperlipidemia, peripheral or coronary artery disease, hepatic or renal function abnormality, and who were using aspirin and other platelet-active drugs were excluded. Platelet activity and reactivity to adenosine diphosphate (ADP) were measured by whole blood flow cytometry. We assessed markers of platelet degranulation (P-selectin; CD62P) and the activated glycoprotein IIb/IIIa receptor (PAC1 binding to fibrinogen binding site) before and after stimulation with ADP. Platelet P-selectin expression was not significantly different between patients and control subjects both at baseline (p=0.420) and after stimulation (p=0.56). Baseline (p=0.001) and ADP-stimulated (p=0.003) PAC1 binding was significantly higher in Behçet's patients than in the control group. Clinical activity has no effect on P-selectin expression and PAC1 binding. There is evidence of platelet activity and hyperreactivity in patients with BD and this may contribute to a prothrombotic state. In addition to aspirin, other antiplatelet drugs may be useful in the prevention and treatment of thrombosis in Behçet's patients.
Key Words: Behçet's disease—Platelet activity—Platelet reactivity—Platelet degranulation—Glycoprotein IIb/IIIa receptor.

Behçet's disease (BD) is an inflammatory disorder characterized by recurrent oral and genital ulcers, skin lesions, and uveitis. It is now recognized as a multisystem vasculitis that can affect all sizes and types of vessels (1,2). Vascular involvement is reported to occur in up to one third of patients (3,4). Venous thrombosis, which is uncommon in other vasculitis, is a frequent manifestation of BD (1,3,4). The exact pathogenic mechanism underlying the thrombotic tendency is still unclear. Vascular endothelial cell injury and resultant endothelial dysfunction seems to play a key role in the prothrombotic state in BD (4–6). Investigations of the hemostatic parameters have shown activation of both coagulation and fibrinolytic systems suggesting endothelial activation or injury (4–9).

Although the coagulation system, fibrinolytic activity, and thrombophilic factors were studied extensively in patients with BD, platelet function, which is a major component of hemostasis and thrombosis, was evaluated only in a few reports (10–13). Several studies have reported increased platelet adhesion and aggregation, particularly in response to adenosine diphosphate (ADP) in patients with BD (10,12). In these reports platelet functions were analyzed by using an aggregometer.

The evaluation of platelet function by whole blood flow cytometry, a powerful laboratory technique, is becoming more popular and allows determining the activation states of in vivo platelets by binding of an activation-dependent

Address correspondence to Fatih Demirkan, MD, Dokuz Eylül Üniversitesi, Tıp Fakültesi, Hematoloji-Onkoloji Bilim Dalı, 35340 İnciraltı, İzmir, Turkey; e-mail: fatih.demirkan@deu.edu.tr.

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monoclonal antibody. In addition to this assessment, inclusion of an exogenous agonist in the assay enables analysis of the reactivity of circulating platelets in vitro (14–16). The two most widely studied types of activation-dependent monoclonal antibodies are those directed against granule membrane proteins and those directed against conformational changes in the glycoprotein (GP) IIb/IIIa complex (CD41/CD61). P-selectin (CD62P) is constitutively expressed in the α-granule membrane of platelets and Weibel-Palade bodies of endothelial cells, and following activation it is expressed on the platelet surface and mediates mainly adhesion of activated platelets to other platelets in addition to endothelial cells and leucocytes. Therefore a P-selectin–specific monoclonal antibody binds only to degranulated platelets. Because surface P-selectin rapidly shed from platelets, it may not be an ideal marker for the detection of circulating degranulated platelets (14–17). Platelet activation leads to an increase of the number of GPIIb/IIIa complexes on the platelet surface membrane and also conformational changes generating neoepitopes that can bind both fibrinogen and von Willebrand factor. Thus monoclonal antibody PAC1 binds only stimulated GPIIb/IIIa complex, not to resting platelets (16).

The aim of this study was to evaluate the in vivo platelet activation status and in vitro reactivity of platelets to ADP by using whole blood flow cytometry in patients with BD and to search for a possible relation of platelet functions with disease activity.

PATIENTS AND METHODS

Twenty-two Behçet's patients (18 male, 4 female) and 20 control subjects (12 male, 8 female) were included in the study. All patients fulfilled three or more of the International Study Group Criteria for the diagnosis of Behçet's disease (18). Those patients who had uncontrolled hypertension, hyperlipidemia, peripheral or coronary artery disease, abnormal hepatic or renal function tests, and who were using aspirin and other platelet active drugs were excluded. Patients with at least two of the following findings in the preceding 1 month were accepted as having clinically active disease: oral ulcer, genital ulcer, eye lesions, skin lesions, thrombophlebitis, or arthritis.

Complete blood count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) levels were also studied in the patients with BD.

This study was approved by the Clinical and Laboratory Studies Ethical Committee of the University Hospital and all patients and control subjects gave their informed consent.

Sample Collection

At 8:00–9:00 AM following overnight fasting and 30 minutes of resting, venous blood samples were obtained without tourniquet application in vacutainer tubes that contained 3.2% sodium citrate. First, 2 mL of blood drawn was discarded in order to eliminate possible activated platelets. All studies were performed within 10 minutes after collection of blood.

Activation of Whole Blood

A total of 0.45 mL of whole blood was added to the tubes containing 50 uL of ADP solution. After swirling gently in order to mix we left the tubes for incubation at room temperature for 2 minutes.

Immunofluorescence Staining of Nonactivated Whole Blood

CD61 Per CP was added to all three tubes for platelet gating. Then platelet activation-dependent monoclonal antibodies of PAC1 FITC and CD62 PE were added. To one of the tubes to prevent nonspecific PAC1 binding 10 mL of RGDS solution was also added. A total of 5 uL of whole blood suspension was carefully added to the bottom of all tubes by using a fresh micropipette tip each time. Without vortex mixing, the tubes were gently mixed. Then the tubes were incubated in the dark for 15 to 20 minutes at room temperature. Following the incubation period 1 mL of cold 1% paraformaldehyde was added to each tube and they were finally stored at 2–8°C in the dark for at least 2 hours.

Immunofluorescence Staining of Activated Whole Blood

All immunfluorescence staining procedures defined above were repeated for ADP-activated whole blood samples as well.

Flowcytometric Analysis

Platelet activation from whole blood specimens, by using a three-color monoclonal antibody technique, was performed by using Becton Dickinson cytometry (BD FacsCalibur; San Jose, CA) (19). PAC1 and CD62 positivities were given as stained cell percentages.

Statistical Analysis

Continuous variables were presented as mean ± standard deviation (SD) values and the differences between the groups were analyzed with the Mann-Whitney *U* test. Comparison of the categorical data between two groups was performed with the χ^2 test. Correlations were evaluated by Pearson's correlation test. A probability value of <0.05 was considered statistically significant.

RESULTS

The patient and control groups did not differ significantly with respect to age and sex. Demographic characteristics and clinical findings of the patients and the control subjects are summarized in Tables 1 and 2. Eighteen patients were taking medication; 14 were receiving colchicine, 4 corticosteroid, 4 azathioprine, and 3 cyclosporine at the time of the study.

Baseline Platelet Activation Status

Although in vivo platelet P-selectin expression was not significantly different between BD patients and control groups (p=0.420) (Fig. 1), the activation status of the GPIIb-IIIa complex, which is reflected by the binding of PAC1 (monoclonal antibody), was significantly increased in BD patients (p=0.001) (Fig. 2). Both P-selectin expression and PAC1 binding were similar in clinically active and inactive BD patients (p values were 0.093 and 0.381, respectively) (Figs. 3 and 4).

In Vitro Platelet Reactivity

The platelet PAC1 binding was significantly higher in patients with BD after in vitro ADP stimulation (p= 0.003) (Fig. 2). The platelet P-selectin expression after stimulation was higher in control subjects, but this difference did not reach statistical significance (p= 0.056) (Fig. 1).

Clinical disease activity has no effect on reactivity regarding P-selectin expression and PAC1

TABLE 1. Demographic and Some of the Clinical Characteristics of Behçet's Disease Patients and Control Subjects

	Behçet's Patients	Controls
M/F	18/4	12/8
Age (yr)	38.6 ± 10.9	38.8 ± 9.4
Disease duration (yr)	9.6 ± 9.9	
ESR (mm/hr) (range)	19.1 (2–82)	
CRP (mg/L) (range)	9.0 (0.3–54.7)	

TABLE 2. Clinical Characteristics of Behçet's Disease Patients Including Clinical Activity at the Time of Study

	Ever	Preceding Month
Oral ulcer (%)	100	77.3
Genital ulcer (%)	95.5	22.7
Uveitis (%)	40.9	9.1
Skin lesions (%)	86.4	45.5
Thrombosis (%)	13.6	—
Arthritis (%)	36.4	4.5
Patergy positivity (%)	47.6	
HLA-B51	6/8	

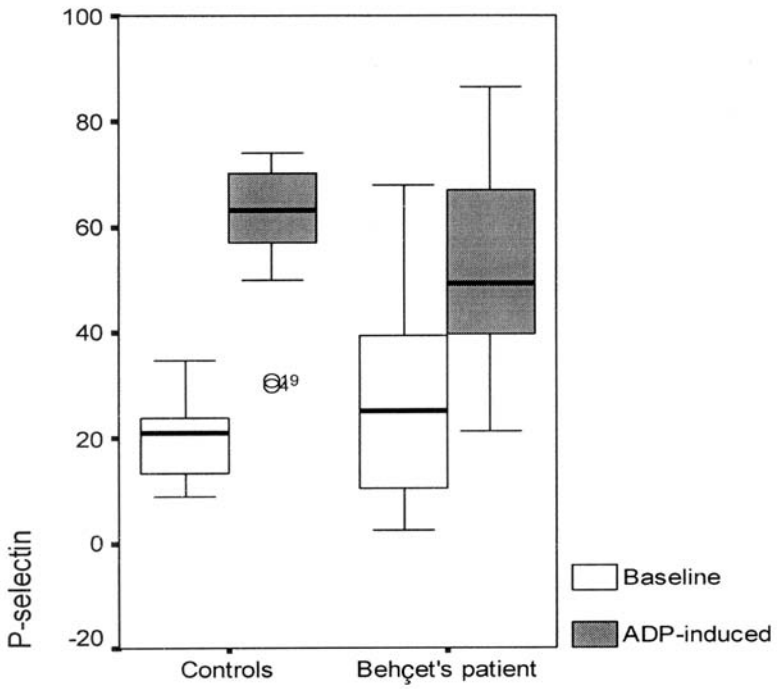


FIG. 1. Platelet surface P-selectin expression of Behçet's disease patients and control subjects in baseline and ADP-induced condition. The boxes represent interquartile ranges, lines across the boxes indicates median values, the whiskers are lines that extend from the boxes to the highest and lowest values excluding outliers.

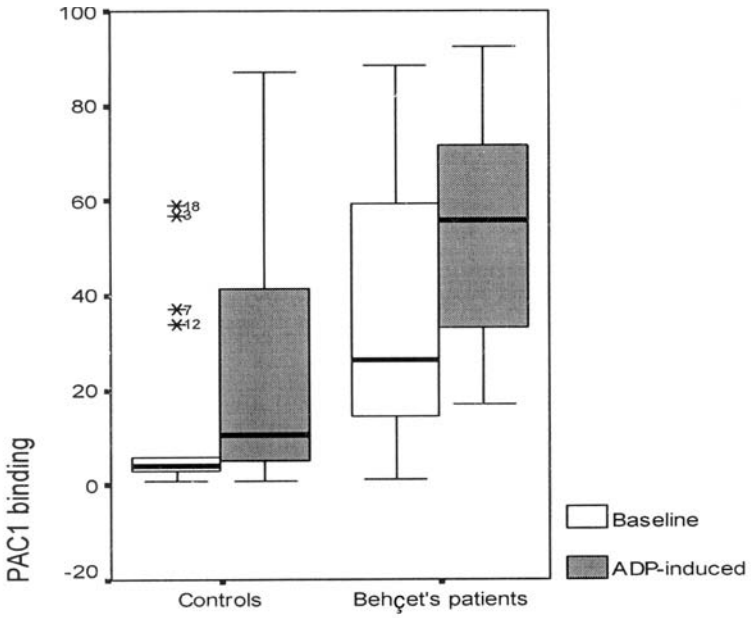


FIG. 2. PAC1 binding platelets from Behçet's disease patients and control subjects in baseline and ADP-induced condition. The boxes represent interquartile ranges, lines across the boxes indicates median values, the whiskers are lines that extend from the boxes to the highest and lowest values excluding outliers.

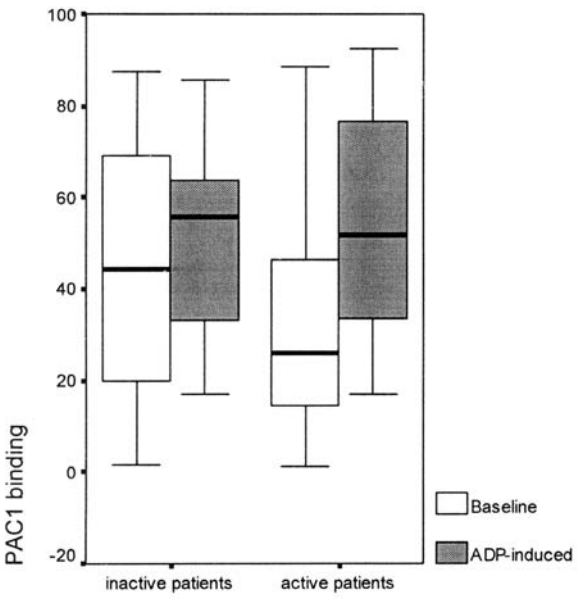


FIG. 3. Platelet surface P-selectin expression of clinically active and inactive Behçet's disease patients.

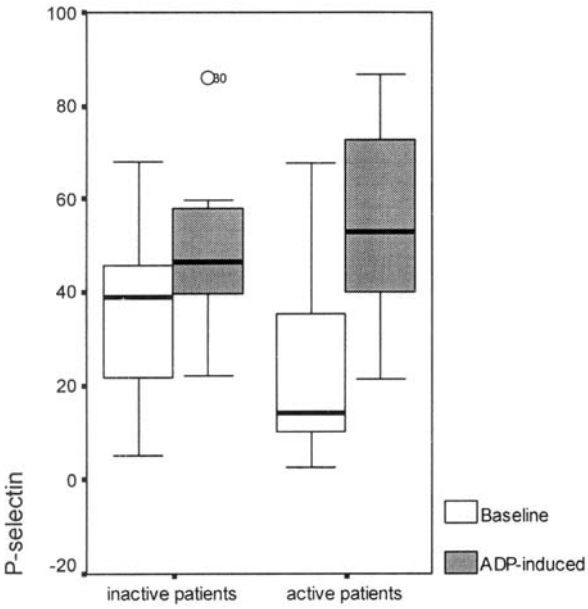


FIG. 4. Baseline and ADP-induced platelet PAC1 binding of Behçet's disease patients with and without clinical activity.

binding as seen in Figs. 3 and 4 (p values were 0.497 and 0.628, respectively). Medical therapy has no effect on baseline or ADP-stimulated platelet PAC1 binding and P-selectin expression.

DISCUSSION

The exact pathogenetic mechanism of thrombotic complications of BD is unknown. Although there is evidence of activation of coagulation and fibrinolytic processes (4,6,7), thrombophilic factors may not be the primary event in explaining the thrombosis in BD (1,7). Platelet functions that carry essential importance in hemostasis and thrombosis were evaluated by using whole blood flow cytometry.

This study not only demonstrates the presence of circulated activated platelets in patients with BD, but also provides evidence of enhanced platelet reactivity as demonstrated by increased PAC1 binding in response to ADP stimulation. No differences were observed between platelets from BD patients and normal controls regarding the expression of granule membrane protein P-selectin.

GPIIb/IIIa complex, a platelet-specific integrin, is essential for platelet aggregation. In resting platelets, GPIIb/IIIa receptors do not bind fibrinogen. Activation of GPIIb/IIIa is required for the binding of soluble ligands. Thrombin, ADP, collagen, thrombospondin, thromboxane A_2, or immune complexes are able to activate this integrin receptor (20,21). Following activation, GPIIb/IIIa undergoes a conformational change and clusters to increase affinity and avidity for fibrinogen (inside-out signaling). Postligand binding events regulated by integrin receptor GPIIb/IIIa (outside-in signaling) include platelet degranulation, spreading, stabilization of large platelet aggregates, and possibly platelet procoagulant activity (22).

There have been only a few reports investigating platelet functions (mainly platelet aggregation) in patients with BD (10–12,23). In these reports, platelet aggregation was analyzed by using aggregometry and yielded contradictory results. Nakano et al (12) found that most of the patients with BD had spontaneous small-size platelet aggregation when compared with normal individuals. In addition aggregation response to ADP was found to be increased (10), unchanged (11), or impaired for low dose ADP (23). Platelet aggregometry may show whether a particular clinical condition results in changes of platelet reactivity but cannot determine whether

the condition directly activates platelets. Besides, the manipulation of samples may result in artifactual in vitro platelet activation and aggregometry may be relatively insensitive for either detecting preexisting aggregates or monitoring the early phase of aggregation (24). To our knowledge, this is the first report comparing in vivo platelet activation status of BD patients with control subjects by flow cytometry. Flow cytometry is a direct method for the measurement of platelet function that avoids in vitro artifactual platelet activation and assesses the activation states of individual platelets (16).

In flow cytometric evaluation, PAC1 binds selectively and with high affinity to the fibrinogen binding site exposed as a result of inside-out signaling events in the GPIIb/IIIa receptor (25). Thus, increased PAC1 binding in BD patients indicates the presence of circulating active platelets (16).

P-selectin, a component of α granule membrane, is translocated to the surface membrane upon platelet activation. It mediates the binding of platelets to the other platelets and leukocytes as well as endothelial cells (17). It has been shown that circulated degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function as demonstrated by the participation in platelet aggregation, exposure of the fibrinogen binding site on the GPIIb/IIIa complex, and generation of procoagulant platelet-derived microparticules by these platelets that had shed surface P-selectin (26). Activation-dependent increase in surface P-selectin expression is not reversible over time in vitro (27). Therefore, platelet surface P-selectin was not considered to be an ideal marker of circulating degranulated platelets (15,16). Measurement of plasma-soluble P-selectin concentration may reflect the circulated degranulated platelets. Previously, plasma concentrations of P-selectin as well as E-selectin were found to be increased in patients with BD (28). A limitation of this study is that we did not evaluate the plasma-soluble P-selectin concentration concurrently and thus we could not reach a firm conclusion about P-selectin expression of the platelets from BD patients.

In a recent study, platelet P-selectin expression was investigated in thrombotic and nonthrombotic patients with BD by using flow cytometry (13). In this study, P-selectin expression was found to be higher in thrombotic BD patients regardless of the clinical disease activity, but the authors could not reach a conclusion on whether P-selectin expression is higher in BD because of the lack of a control group. In the present study, most of the thrombotic BD patients were excluded because of their aspirin and/or other platelet active drug usage. Therefore, we could not compare the platelet activation status and reactivity between thrombotic and nonthrombotic BD patients.

The cellular mechanisms responsible for these "primed" platelets in BD patients remain unknown. The results of the investigations about prostacylin and thromboxane B_2 (TxB_2) that are a measure of the free thromboxane A_2 levels in BD patients were conflicting. In a previous study, plasma levels of prostaglandin (PG) $F_{1\alpha}$, which is a more stable inactive metabolite of prostacyclin, were investigated in BD patients with or without vascular involvement during their active phase and found to be decreased compared to controls (29). But later both PG $F_{1\alpha}$ and TxB_2 were reported to be high (30), and the authors interpreted this finding as activation of both proaggregant and protective antiaggregant pathways. In another study, platelet sensitivity to prostacyclin was reported to be decreased despite no change in stimulated TxB_2 generation and platelet aggregation in patients with BD (11). Thus, in patients with BD, changes in arachidonic acid metabolism remain unproven and platelet activation and hyperreactivity appear to be independent of the arachidonate pathways. Increased thrombin generation in BD patients has been shown (7). It is known that thrombin can cause up to a 50% increase in the number of GPIIb/IIIa molecules exposed on the platelets (31). The association and pathophysiological relation of the increased thrombin and GPIIb/IIIa receptor activation remain to be elucidated. Currently, activation of endothelial cells by perivascular infiltrates and resultant disturbance in the vascular bed seems to be a more realistic explanation for in vivo platelet activation seen in BD patients (5).

In conclusion, our data demonstrate that platelets in BD patients are active and more reactive than normal controls and this may contribute to the thrombotic tendency. Aspirin as an antiplatelet agent blocks only one of the several signal transduction pathways leading to platelet activation, and further work is required to determine other antiplatelet regimens, particularly those blocking the GPIIb/IIIa receptors that influence platelet activation status in BD patients.

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