Platelet Activation and Red Blood Cell Phosphatidylserine Exposure Evaluated by Flow Cytometry in Patients with Behçet’s Disease: Are They Related to Thrombotic Events?

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Introduction

Behçet’s disease (BD) is a chronic multi-system inflammatory disorder characterised by recurrent oral and genital ulcers and uveitis. It involves a variety of organs including joints, the gastrointestinal tract, the skin and the central nervous and vascular systems [1]. Thrombotic complications have been reported in 10–45% of patients with BD [2] involving both arterial and venous thromboses, although venous thromboses are more frequent [2–4]. Several factors have been studied to establish the aetiology of atherothrombotic events in these patients, as the prothrombotic mechanisms are not well defined. These include thrombophilia [5–7], hypofibrinolysis [8, 9], endothelial injury [10, 11] and alterations in blood rheology [12, 13].

In reality, the distinction between arterial and venous thrombosis tends to be blurred. Heparin, which is used in the treatment of venous thrombosis, has also shown to be effective in acute coronary syndromes [14], probably due to its inhibitor effect on platelet activation [15]. On the other hand, aspirin, the antiplatelet drug of choice in the treatment of arterial thrombosis, also reduces the incidence of venous thromboembolisms [16]. These facts suggest that platelets, apart from their role in arterial thrombosis, play a part in venous thrombosis [17]. Therefore, the detection of circulating activated
platelets could be a sensitive method to help detect those patients with BD who, due to having a higher risk of thrombosis, could be potential candidates for antiplatelet therapy.

The study of platelet activation markers is of interest from the point of view of the formation of the clot. For example, P-selectin, or CD62, which is expressed in active platelets, regulates their adhesion to neutrophils and monocytes [18, 19] and to the endothelium [20, 21], which enables the formation of large irreversible platelet aggregates [22]. When the cell is activated, the movement of phospholipids across the membranes begins and leads to phosphatidylinerine (PS) exposure in the external layer of its membrane [23]. This loss of membrane phospholipid asymmetry induces the formation of the prothrombinase complex, which helps in the formation of the clot by generating thrombin [24, 25]. The PS exposure on the erythrocyte membrane can be associated with the exposure on the platelet membrane [26] and is generally accompanied by the activation of enzymes from the caspase family, which help in the formation of platelet-derived microparticles (PDMPs) [27]. The presence of PDMPs is associated with a prothrombotic hypercoagulability state, as they also have prothrombinase activity [28]. Circulating platelets are susceptible to various stimuli in vessels, and a certain portion of such cells may form circulating platelet microaggregates (PDMAs) [29]. The involvement of platelet and red blood cell (RBC) activation in the development of thrombotic events in BD patients has been scarcely studied; therefore, we consider that the present study will be of interest. To establish whether BD patients have a higher percentage of circulating activated platelets than the control group, several activation markers have been studied. These include CD62 exposure on the platelet surface, morphological changes of the platelet, formation of PDMPs and PDMAs. In addition, PS exposure on the erythrocyte surface was also studied, given that this cell is the most abundant in circulating blood and exercises an influence on platelet function through cell-cell interactions [26]. In addition, the presence of activated RBCs could be related to the presence of activated platelets. In fact, it is well known that blood cells interact between them, making the RBC-platelet interaction specially relevant regarding the big relative number of RBCs. Changes in the RBC membrane contribute to a prothrombotic state, thus enhancing thrombus formation [24, 25, 30].

The aim of the present study is to establish whether the cells evaluated (platelets and RBC) are involved in the pathogenic mechanism of the greater thrombotic tendency characteristic of BD.

**Subjects and Methods**

**Patients and Controls**

The patient group consisted of 72 patients with BD (39 males, 33 females, aged 46.5 ± 12.5 years when sampling) recruited from the records of La Fe and General Hospital between 1990 and 2005. All patients fulfilled 3 or more of the International Study Group criteria for the diagnosis of BD (International 90). The mean duration of disease was 8.6 ± 5.8 years (range 1–16). The distribution of clinical manifestations from the onset of the disease had been as follows: mouth ulcers 100%, genital ulcers 70%, arthralgia 64%, fever 50%, ocular involvement 46% (33% uveitis and 13% others), cutaneous involvement 42%, thrombotic events 28%, neurological events 28% and gastrointestinal involvement 12%. Patients were inactive or showed minimum activity (mild aphthosis or arthralgia). Mild aphthosis was defined as the presence of some isolated aphthae in the 4 weeks before sampling, without other concomitant signs or symptoms of disease activity. Twenty of the 72 patients with BD had a documented history of deep vein thrombosis. The age at which they suffered the event was 38.5 ± 15.3 years and the time elapsed from when the events took place until their recruitment was 6.3 ± 4.2 years (interval 1–11). Thromboses were located in the lower limbs (n = 12), the iliac and caval vein plus pulmonary embolisms (n = 3), the cerebral sinus (n = 2), the intracardiac area (n = 2) and in the upper limbs (n = 1). Seven patients had suffered repeated episodes of superficial phlebitis; 4 had suffered more than one deep vein thrombotic episode and were therefore on long-term oral anticoagulant therapy with acenocoumarol. In these 2 patients, acenocoumarol was replaced with low molecular weight heparin 20 days before sampling; this was not administered on the day before sampling but immediately after. None of the patients had malignancies, or renal or hepatic dysfunction. Sampling took place at least 6 months after any thrombotic event in order to avoid the acute phase. All thrombotic events had been assessed clinically and were confirmed using objective methods (clinical findings, Doppler ultrasound, venography, computed tomography or magnetic resonance imaging). At the time of recruitment, none of the patients were under immunosuppressive medication.

The control group consisted of 72 healthy volunteers (34 males and 38 females, aged 43.8 ± 11.0 years) matched for age and gender with the patients, and from the same geographical area. They were either members of the staff at our hospital or patients visiting our hospital for a medical check-up. The presence of previous thrombotic events was ruled out by means of a validated questionnaire [31].

Given the influence of cardiovascular risk factors on atherosclerotic events, these were recorded and matched for both groups. Subjects were considered having a risk factor if they were obese (body mass index >30), smokers (>1 cigarette/day), if they had hypertension (diastolic blood pressure >90 mm Hg), hyperlipidaemia (total cholesterol >5.7 mmol or triglycerides >2.0 mmol) or diabetes (fasting glucose concentration >7.0 mmol), or were re-

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Cell Activation and Behçet’s Disease

Pathophysiol Haemost Thromb
2007–08;36:18–22
Table 1. Mean value, standard deviation and statistical significance of the parameters studied in patients with BD and the control group

<table>
<thead>
<tr>
<th></th>
<th>BD patients (n = 72)</th>
<th>Control group (n = 72)</th>
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<tbody>
<tr>
<td>PS, % RBCs PS+</td>
<td>0.81 ± 0.75*</td>
<td>0.70 ± 0.73</td>
</tr>
<tr>
<td>P-selectin, % plt CD62+</td>
<td>1.98 ± 1.57*</td>
<td>1.91 ± 1.41</td>
</tr>
<tr>
<td>PDMPs/5,000 plt</td>
<td>179 ± 83*</td>
<td>168 ± 91</td>
</tr>
<tr>
<td>PDMAs/5,000 plt</td>
<td>228 ± 108*</td>
<td>197 ± 109</td>
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</tbody>
</table>

*Not significant versus the control group. plt = Platelets.

Table 2. Mean value and standard deviation of the forward and side scatter light (logarithmic scale) of the platelets in the patient and control groups

<table>
<thead>
<tr>
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<th>BD patients (n = 72)</th>
<th>Control group (n = 72)</th>
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<tbody>
<tr>
<td>FS (log)</td>
<td>22.6 ± 2.45*</td>
<td>23.26 ± 2.45</td>
</tr>
<tr>
<td>SS (log)</td>
<td>2.83 ± 0.38*</td>
<td>2.73 ± 0.37</td>
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*Not significant versus the control group. FS = Forward scatter light; SS = Side scatter light; log = Logarithmic.

Results

Results obtained in the present investigation comparing the whole patient group with the controls are summarised in table 1, in which no statistically significant differences were observed in the expression of PS on the erythrocyte surface of the patients compared with the controls (0.81 ± 0.75 vs. 0.70 ± 0.73%). As regards the platelet population, the patients did not show any significant differences in the percentage of cells that expressed CD62 on their surface when compared with healthy controls (1.98 ± 1.57 vs. 1.91 ± 1.41%). Similarly, there were no significant differences in the number of PDMPs and PDMAs detected per 5,000 platelets between the two groups (179 ± 83 vs. 168 ± 91 and 228 ± 108 vs. 197 ± 109, respectively.
ly), although there appeared to be a trend towards patients having a larger number than controls. No statistically significant correlations were observed between the cytometric parameters studied (results not shown).

Table 2 shows the values of forward scatter and side scatter light (logarithmic scale) corresponding to the patient and control population. As can be seen in this table, there are no statistically significant differences associated with the size, complexity or roughness of the platelets between patients and controls. In fact, the value of the forward scatter light was directly related to the size of the cell, i.e. 22.6 ± 2.45 versus 23.26 ± 2.45. Something similar is observed while comparing the values obtained from the side scatter light, associated with the complexity of the cell surface, which were 2.83 ± 0.38 and 2.73 ± 0.37, respectively. However, comparing the 20 patients who had suffered thrombotic events with the rest of the patients studied (n = 52) (table 3), it can be seen that the patients with any previous thrombotic event had a higher CD62 expression and a higher number of circulating PDMAs than the patients without thrombosis (2.68 ± 1.99% and 252 ± 143 vs. 1.75 ± 1.24% and 177 ± 91, respectively; p < 0.05). Looking at the platelet morphology (forward and side scatter light of the corresponding gate), we did not find differences between patients with and without thrombosis related to these cytometric parameters (results not shown).

Discussion

It is currently postulated that in the absence of exogenous agonists, whole blood flow cytometry allows the state of in vivo circulating activated platelets to be detected. Accepting this assumption, the results of the present study show that no significant differences were observed in the spontaneous activation of circulating platelets, while comparing BD patients with controls. Neither were there any significant differences between the two groups as regards the formation of PDMPs or PDMAs. The values of the forward and the side scatter light, which allow morphological features such as the size and roughness of the cells to be measured, showed that there were no significant differences in the platelet population between patients and controls. Given that it is a well-established fact that platelet activation is accompanied by a change in shape and size, the result of the present study, having not detected any morphological differences in the platelets between patients and controls, seems to confirm that there need not be any differences associated with their activation either. These results are in agreement with other authors, who, by using aggregation techniques, indicate that platelets from patients with BD show normal activity [35]; thus, platelet activation does not seem to play a major role in the tendency for thrombosis in BD, and the pathogenesis of such thrombotic events is still not well understood. Our results are in agreement with other authors, who also detected a higher expression of CD62 in the platelets of BD patients with thrombosis [36]. This increase in expression could have significant pathogenic effects, since CD62-positive platelets are attached to neutrophils and monocytes [18, 19] resulting in endothelial adhesion [20, 21] which help to form and stabilise large platelet aggregates [22]. Therefore, CD62 may contribute to endothelial injury and thrombosis development.

The presence of a higher number of spontaneous PDMPs in BD patients with previous thrombosis can, at least partly, be attributed to the fact that these patients show shear stress changes in their circulation. This haemorheological change may be sufficient to stimulate platelets and thus lead to the formation of microaggregates, as has been described in other diseases [37]. These microaggregates are considered potentially dangerous because they could aggravate thrombus formation, leading to vascular occlusions by the formation of a plateletrich thrombus [38]. These two parameters (CD62 and PDMPs) seem to be related to the presence of previous thrombosis, given that both parameters play a major role in the tendency for thrombosis. Therefore, BD patients with a previous thrombosis should constitute a subsidiary group to receive prophylactic treatment in order to avoid new thrombotic events, which represents an added difficulty as these patients are often resistant to standard antithrombotic and antiplatelet treatment.

**Table 3.** Mean value, standard deviation and statistical significance of the parameters studied in BD patients with or without thrombosis

<table>
<thead>
<tr>
<th></th>
<th>BD with thrombosis (n = 20)</th>
<th>BD without thrombosis (n = 52)</th>
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</thead>
<tbody>
<tr>
<td>PS, % RBC PS+</td>
<td>0.83 ± 0.76*</td>
<td>0.80 ± 0.72</td>
</tr>
<tr>
<td>P-selectin, % plt CD62+</td>
<td>2.68 ± 1.99**</td>
<td>1.75 ± 1.24</td>
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<td>PDMPs/5,000 plt</td>
<td>191 ± 107*</td>
<td>175 ± 73</td>
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<tr>
<td>PDMAs/5,000 plt</td>
<td>252 ± 143**</td>
<td>177 ± 91</td>
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</table>

*Not significant versus BD patients without thrombosis; **p < 0.05 versus BD patients without thrombosis. plt = Platelets.
References


