Crosstalk between inflammation, iron metabolism and endothelial function in Behçet’s disease

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Abstract. Behçet’s disease (BD) is a rare chronic vasculitis of unclear etiology. It has been suggested that inflammatory response has an important role in BD pathophysiology. Herein, we aimed to study the interplay between inflammation, iron metabolism and endothelial function in BD and search for its putative association with disease activity. Twenty five patients clinically diagnosed with BD were selected and twenty four healthy age-sex matched individuals participated as controls. Results showed an increase of total number of circulating white blood cells and neutrophils, serum transferrin, total iron binding capacity, myeloperoxidase (MPO), ceruloplasmin (Cp), C reactive protein, β2 microglobulin and Cp surface expression in peripheral blood monocytes in BD patients comparatively to healthy individuals (p < 0.05). Of notice, the alterations observed were associated to disease activity status. No significant differences between the two groups were found in serum nitric oxide concentration. The results obtained suggest an important contribution from innate immunity in the pathogenesis of this disease. In particular, surface expression of leukocyte-derived Cp may constitute a new and relevant biomarker to understand BD etiology.

Keywords: Behçet’s disease, inflammation, iron metabolism, endothelial function, disease activity

1. Introduction

Behçet’s disease (BD) is a rare chronic, inflammatory multisystemic disorder characterized mainly by recurrent oral and genital ulcers, cutaneous lesions, vasculitis and uveitis [43,45]. The diagnosis of this disease is still based on clinical criteria as established by the International Study Group for BD (ISGBD)

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The search for a specific laboratorial test for the diagnosis of BD has been recurrently attempted but it remains to be found. The etiology of BD has not been fully clarified. The most widely accepted hypothesis for BD pathogenesis is the triggering of a profound inflammatory response by an infectious agent in a genetically susceptible host. In fact, predisposition to BD has been associated to polymorphisms in HLA-B gene, specifically HLAB*51, but its underlying biological mechanisms are still unknown. An "auto-inflammatory" model to explain BD immunopathogenesis was also suggested. In previous studies, various functions of polymorphonuclear cells (PMN) in peripheral blood were shown to be increased in this disease. Activated neutrophils were also reported to release substantial amounts of myeloperoxidase (MPO) which can contribute to tissue damage observed in BD. On the other hand, nitric oxide (NO) has been recognized as a potent vasodilator that modulates the early vascular response of the acute inflammatory reaction. Previous studies aimed to investigate the role of NO in BD clinical presentation, but the results obtained were inconclusive.

A major contributor to the inflammatory response is ceruloplasmin (Cp), which is an abundant multicopper plasma glycoprotein that participates in the acute phase reaction and contributes to the systemic oxidant/anti-oxidant balance. Although the liver is the predominant source of circulating Cp, extra-hepatic expression has been demonstrated in many tissues. Particularly, a new glycosylphosphatidylinositol (GPI)-anchored form of Cp (GPI-Cp) was previously shown to be expressed mainly in the mammalian central nervous system. Recent results from our group showed that human peripheral blood mononuclear cells constitutively express both secreted Cp and GPI-Cp isoform, suggesting a close link between inflammation and oxidative biology. Interestingly, Cp was found to be increased in the serum of BD patients. Nevertheless, apart from our own previous study of peripheral blood lymphocytes (PBL)-derived Cp expression in BD patients, to our knowledge there was no further investigation into the possible role of leukocyte-associated Cp in this disease.

Herein, we aimed to study inflammatory response in BD and search for its putative association with the disease activity. In particular, we intend to investigate new features of leukocyte biology that could have a role in BD pathogenesis. Thus, Cp expression at the cell membrane of the three major circulating leukocyte subpopulations (PMN, PBL, and monocytes) was measured. Given the close functional relationship between Cp, inflammation and iron (Fe) homeostasis, measurement of Fe metabolism biomarkers was also performed. Furthermore, endothelial function was evaluated through the measurement of serum NO concentration in order to clarify its physiological relevance in BD.

2. Material and methods

2.1. Patients

A total of 25 BD patients, fulfilling the International Clinical Criteria proposed by the ISGBD were selected from the Instituto Português de Reumatologia (IPR) outpatient clinic. All BD patients had complete ophthalmologic and clinical examination to define ophthalmic and systemic involvement, as well as disease activity status at the time of recruitment. The disease inactivity was defined as the absence of any clinical symptoms of the disease in the previous month (not considering irreversible damage, but including mucocutaneous, eye, joint, vascular, intestinal and/or neurological manifestations). Based on the clinical examination patients were classified according to the presence of symptoms such as uveitis, arthritis and thrombophlebitis and to clinical severity of disease as: severe (patients with posterior uveitis or
retinal vasculitis); moderate (patients with deep phlebothrombosis or anterior uveitis); and mild (patients with mucocutaneous lesions or superficial thrombophlebitis or arthritis). Furthermore, BD patients were classified according to the onset age of the disease diagnosis (early onset if the disease was diagnosed earlier than 25 years-old, and late onset when the diagnostic was made later than 25 years-old).

Twenty-four age and sex-matched healthy volunteers were also enrolled and were selected from the Hospital Reynaldo dos Santos (HRS).

For genetic characterization, 23 total blood samples from BD patients and 1021 controls recruited from the central and south region of Portugal were studied.

This study protocol was approved by the local Ethics Committee and all patients signed an informed consent.

2.2. HLA Genotyping

Patients’ genomic DNA was extracted from total blood leukocytes through Salting-Out method. HLA-B*51 allele was identified by polymerase chain reaction with sequence specific primers (PCR-SSP) based on methods previously described [3].

2.3. Hematological, biochemical and immunological characterization

Cell blood count was performed in EDTA collected peripheral blood using an automated hematology counter (Coulter MAXM) and included white blood cell count (WBC) and differential leukocyte cell count.

Serum Fe and transferrin (Tf) concentrations were measured in a Cobas Integra® 400 (Roche) by colorimetric and immunoturbidimetric assays, respectively. Serum ferritin (Ft) concentration was measured by immunoluminescence using an IMMULITE® automated analyser (Diagnostic Products Corporation). Cp, C reactive protein (CRP) and β2 microglobulin (β2 m) were measured by nephelometry using a Beckman Immage analyzer.

Quantitative determination of MPO in serum was performed using an ELISA commercial kit (BIOXYTECH®) according to the manufactur’s protocol.

NO serum concentration was measured based in Griess reaction using a colorimetric assay (R&D®).

2.4. Immunophenotype and flow cytometry analysis of surface ceruloplasmin expression in peripheral blood leukocytes

WBCs were obtained from fresh peripheral blood collected by venous puncture in EDTA-coated tubes. Immunofluorescence staining, with fluorochrome labeled antibodies (BD, USA, except otherwise indicated) was applied in whole blood samples using a lyse/wash procedure. Cells were stained for Cp using the rabbit anti-human Cp (DakoCytomation, Denmark) as primary antibody followed by incubation with a swine F(ab’)2 anti-rabbit FITC-conjugated secondary antibody (DakoCytomation, Denmark). PMN were gated based on their physical characteristics in forward-scatter (FSC) versus side-scatter (SSC). Peripheral blood monocytes (MN) and PBL were gated based on their expression of FITC-CD45 and PE-CD14 (BD Leucogate™ USA). Flow cytometry analysis was performed using a FACSCalibur (BD, USA), and data processed using the CellQuest™ Pro Software. All samples were analyzed using the same voltage settings. Following correction for non-specific binding, data was expressed in fluorescence.
arbitrary units (FAU) resulting from the ratio between the mean fluorescence intensity (MFI) of stained cells and the MFI of non-stained cells in the same population.

2.5. Statistical analysis

All results were given as median and quartiles (lower 25% quartile - upper 75% quartile) for continuous variables and as proportions of categorical variables. Evaluation of the statistical differences between groups was performed using a general linear model ANOVA. Multiple comparisons between groups were performed using the Turkey Post-hoc Test.

For genetic data analysis, comparisons of HLA-B*51 frequencies between patients and controls and between disease severity/symptomatology groups were performed using the Pearson chi-square test or the exact Fisher’s exact test when appropriate.

Values of $p \leq 0.05$ were accepted as statistically significant. SPSS 20.0 software was used to perform all statistical analysis (SPSS inc, 2005).

3. Results

3.1. Characterization of study groups

The clinical features and genetic characterization of the groups studied are summarized in Table 1. Comparison between active (BDA) and inactive (BDI) BD patients showed there was no significant difference in the prevalence of disease symptomatology neither in the onset of the disease diagnosis. However, the prevalence of disease severity was significantly different between the two sub-groups of patients (Table 1). In fact, the group of BDI patients showed a higher percentage of severe patients (56%) and a lower percentage (13%) of mild patients compared to BDA (22% of severe and 56% of mild patients) individuals. Therefore, in spite the absence of any clinical symptoms of the disease by the time of the enrollment in the study, BDI patients presented the more severe manifestations of the disease.

Analysis of genetic data of all subjects enrolled, showed that a higher frequency of HLA-B*51 was observed in BD patients compared to controls (Table 1). Moreover, analysis of HLA-B*51 frequency according to clinical severity showed a significant increase in mild BD sub-group compared to controls (75% and 19%, respectively; $p = 0.001$) but not with severe or moderate sub-groups (33% and 43%, respectively). The analysis of HLA-B*51 frequency according to disease symptomatology didn’t show any significant differences between controls and any of the BD sub-groups considered.

3.2. Inflammatory parameters

Results obtained from evaluation of inflammatory status in peripheral blood of all participants in the study are shown in Table 2. Analysis of hematological data showed that the total number of circulating WBCs and neutrophils was significantly higher in all BD patients compared to controls. A significant increase of serum MPO concentration was also found in BD patients compared to healthy individuals. Furthermore, the comparison of serum inflammatory proteins between these two groups showed a significant increase of Cp, CRP and β2m in BD patients versus controls.
Table 1
Clinical features and genetic characterization of the enrolled subjects in the study

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Controls (n = 24)</th>
<th>BD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All BD (n = 25)</td>
<td>BDI (n = 9)</td>
</tr>
<tr>
<td>Sex (f/m)</td>
<td>11/13</td>
<td>16/9</td>
</tr>
<tr>
<td>Age (y)</td>
<td>38 ± 9</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>Clinical Severity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild, (n)</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>Moderate, (n)</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>Severe, (n)</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>Disease Onset</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early onset (≤25 years), (n)</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>Late onset (&gt;25 years), (n)</td>
<td>–</td>
<td>16</td>
</tr>
<tr>
<td>Disease Symptomatology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uveitis, (n)</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>Arthritis, (n)</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>Thrombophlebitis, (n)</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azathioprine, (n)</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>Colchicine, (n)</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>NSAIDs, (n)</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Corticosteroids, (n)</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>Genetic characterization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-B*51 (%)</td>
<td>19.8</td>
<td>47b</td>
</tr>
</tbody>
</table>

Data are expressed as count and frequency, except otherwise indicated. *p < 0.05 vs BDI patients; **p < 0.05 vs Controls; n- number of individuals, BDA- active BD patients; BDI- inactive BD patients

NSAIDs – Non-steroidal anti-inflammatory drug.

The results of Cp surface expression measured in the major subpopulations of peripheral blood leukocytes are presented in Figure 1. The data obtained showed PMN cells from BD patients exhibit the higher Cp surface expression compared to all other leukocyte subpopulations analyzed. This pattern of expression was also observed in healthy individuals while no significant differences were found between these two groups. However and of notice, a higher Cp expression at the surface of peripheral blood monocytes was found in patients with BD compared to healthy individuals. This increase was mainly due to the significant raise observed in Cp expressed at surface of monocytes from BDA patients (Figure 1).

3.3. Biomarkers of iron metabolism

Measurement of several Fe metabolism biomarkers in serum from all participants in the study showed a significant increase in serum Tf and Total Iron Binding Capacity (TIBC) of patients comparatively to controls (Table 2). No significant differences were found in Fe and Ft serum concentrations when both groups were compared.
### Table 2

Inflammatory, endothelial and iron metabolism markers measured in serum from enrolled subjects. In patients with Behçet’s Disease (BD) biochemical parameters were also studied according to disease activity

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 24)</th>
<th>All BD (n=25)</th>
<th>BDI (n = 9)</th>
<th>BDA (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>0.20 (0.10–0.30)</td>
<td>0.45 a (0.19–0.74)</td>
<td>0.52 (0.12–1.0)</td>
<td>0.43 a (0.21–0.75)</td>
</tr>
<tr>
<td>Cp (mg/dl)</td>
<td>30 (25–36)</td>
<td>39 (32–50)</td>
<td>34 (29–47)</td>
<td>41 (33–50)</td>
</tr>
<tr>
<td>β2 m (μg/l)</td>
<td>938 (866–1038)</td>
<td>1145 a (1040–1447)</td>
<td>1419 a (1057–1587)</td>
<td>1120 a (1024–1241)</td>
</tr>
<tr>
<td>Total WBCs (×10^6/ml)</td>
<td>6.2 (5.4–7.1)</td>
<td>7.5 (6.1–9.1)</td>
<td>7.4 (6.1–8.4)</td>
<td>8.1 (6.1–9.6)</td>
</tr>
<tr>
<td>Neutrophils (×10^6/ml)</td>
<td>2 m (938)</td>
<td>1145 a (1040–1447)</td>
<td>1419 a (1057–1587)</td>
<td>1120 a (1024–1241)</td>
</tr>
<tr>
<td>Lymphocytes (×10^6/ml)</td>
<td>0.88 (0.55–1.5)</td>
<td>1.5 (0.95–2.1)</td>
<td>1.8 (1.1–2.6)</td>
<td>1.4 (0.82–1.5)</td>
</tr>
<tr>
<td>Monocytes (×10^6/ml)</td>
<td>0.40 (0.35–0.50)</td>
<td>0.50 (0.40–0.65)</td>
<td>0.60 (0.45–0.70)</td>
<td>0.45 (0.40–0.60)</td>
</tr>
<tr>
<td>MPO (ng/ml)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Iron Metabolism</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fe (μg/dL)</td>
<td>105 (85–119)</td>
<td>103 (73–129)</td>
<td>96 (81–108)</td>
<td>113 (67–141)</td>
</tr>
<tr>
<td>Ft (mg/dl)</td>
<td>67 (30–114)</td>
<td>81 (24–149)</td>
<td>81 (19–185)</td>
<td>74 (26–142)</td>
</tr>
<tr>
<td>Tf (mg/dl)</td>
<td>231 (226–273)</td>
<td>305 (270–330)</td>
<td>294 (256–331)</td>
<td>307 (274–333)</td>
</tr>
<tr>
<td>TIBC (mg/dl)</td>
<td>316 (283–341)</td>
<td>381 (356–413)</td>
<td>368 (320–414)</td>
<td>386 (341–415)</td>
</tr>
<tr>
<td><strong>Endothelial Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO (μmol/L)</td>
<td>90 (72–113)</td>
<td>73 (44–99)</td>
<td>73 (44–88)</td>
<td>74 (45–115)</td>
</tr>
</tbody>
</table>

Cp – Ceruloplasmin; CRP – C Reactive Protein; β2 m – β2microglobulin; Ft – ferritin; MPO – myeloperoxidase; NO – nitric oxide; Tf – transferrin; TIBC – total iron binding capacity; WBC – White Blood Cell Count; BDA- active BD patients; BDI- inactive BD patients. Data are expressed as median and quartiles (lower 25% quartile - upper 75% quartile). *p < 0.05 vs Controls.

#### 3.4. Evaluation of endothelial function

No significant difference was found in serum NO concentrations measured in BD patients compared to healthy individuals. The results obtained showed however a tendency for a decrease in NO concentrations measured in serum of patients with BD (Table 2).

#### 3.5. Associations between clinical status and laboratory characterization

In order to search for a putative biomarker of disease activity in BD all the parameters studied were further analyzed in BDA and BDI sub-groups of patients (Table 2). Analysis of the data obtained showed that comparatively to controls, BDA patients exhibit a significant increase in several inflammatory biomarkers including the number of circulating WBCs and neutrophils, and serum CRP, β2 m and Cp concentrations. The expression of Cp inflammatory protein was also significantly increased at the surface of peripheral blood monocytes in BDA patients compared to controls. Moreover, both BDA and BDI patients showed a significant higher serum Tf concentration and TIBC compared to controls.

No significant differences were observed between BDA and BDI groups concerning the measurement of all parameters.
4. Discussion

Previous reports suggested that BD is caused by unknown environmental factor(s), against a background of genetic susceptibility [19], which has motivated the search for genetic associations with this disease [43]. No convincing evidence supports this hypothesis although the association between BD and HLAB51 is the most commonly reported [32], as observed in HLA genotyped patients in this study. An important and still controversial question is whether HLA-B51 is a marker of susceptibility and/or severity in BD [1, 15, 28]. Herein, analysis of HLA-B*51 frequency according to disease severity showed a significant increase of this allele in patients with mild disease compared to healthy individuals. However, no association was found according to disease symptomatology. These results support previous observations in mild BD Portuguese patients [3].

Besides the evidence that BD could have a strong genetic background [14], the inflammatory process is known to be a major contributor for the physiopathology of this rare disease. In this study, we found increased concentrations of the inflammatory proteins Cp, CRP and $\beta_2$ m in BD patients, namely in BDA patients, compared to healthy individuals. The concentration of $\beta_2$ m was increased also in BDI patients compared to healthy individuals, supporting further the widely reported chronic inflammatory characteristics of this pathology.

Abnormalities of leukocyte biology were proposed as important factors in BD pathogenesis [5], which is supported by the data obtained in the present study. A significant higher number of circulating WBCs, mainly due to a significant increase of neutrophils, was found in BD patients. Of notice,
the significant increase observed in these inflammatory circulating cells was observed in active patients. Previous studies [9, 34] had shown that neutrophils are hyperactive in BD, being implicated as mediators in tissue and vascular damage. Herein, increased serum levels of MPO were also found in BD patients.

Measurement of NO concentration in serum did not show any significant difference between these two groups, although a tendency for decreased NO levels in BD patients was observed. These results are possibly due to the metabolic interaction between endothelial function and inflammatory response. Previously, both decreased [4, 35] and increased [20, 42] NO levels were reported in BD patients. The decrease of NO concentrations could reflect a diminished NO availability as a consequence of endothelial dysfunction that would compromise regular endothelial NO production [25] and further aggravate the vascular severity of the disease [40]. However, under particular conditions inflammatory cytokines could stimulate inducible form of nitric oxide synthetase, which is expressed in circulating leukocytes and platelets [12, 36] to produce NO, contributing to maintain its levels in blood [40]. Due to the vascular and inflammatory characteristics of BD, one could assume that both situations may occur in the studied patients, explaining the inexistence of significant alterations compared to healthy individuals.

Systemic pro-oxidant/anti-oxidant balance is tightly linked to prevention of free radical reactions mediated by transition metals. In this context, Fe-binding and Fe oxidizing proteins have been suggested as major antioxidants in plasma [16]. Herein, in addition to a higher concentration of circulating Cp a significant increase of serum Tf and TIBC was also observed in BD patients (both BDA and BDI), suggesting its role as a protective mechanism against potential oxidative damage. Previously, it has been repeatedly reported that activity of Cp is increased in BD [22, 41] which give further support to our present findings. In fact, Cp is an acute phase protein and the raise in inflammatory status observed in this disease could continuously stimulate Cp hepatic synthesis.

Although Cp is frequently addressed as a serum protein secreted by the liver we previously showed that immune cells constitutively express both Cp isoforms [2, 24]. In the present report we found that PMN are the circulating leukocytes with the higher Cp surface expression. In this context, although we presently cannot measure the Cp isoform secreted by peripheral blood leukocytes one can suggest that the significant increase of WBCs, particularly neutrophils, observed in BD patients could contribute to the raise of Cp measured in the periphery. Moreover and of notice, a significant increase of surface Cp expression was found in circulating monocytes from BD patients, particularly in active ones. Cp has been shown to exhibit also potent pro-oxidative activity [29] and may contribute to the macrophage-mediated host defense against invasive organisms or foreign materials. A possible alternative function of monocyte-derived Cp may arise from its generally proposed role in Fe transport through its ferroxidase activity driving Fe homeostasis in a direction unfavorable to the infectious organism. The observation that Cp has bactericidal activity is consistent with this mechanism [21] and could support previous suggestions of the involvement of infection in the etiology of BD.

To our knowledge, this is the first report showing significant alterations in the expression of both isoforms of Cp in patients with BD, namely the expression of the membrane-anchored isoform at the surface of peripheral blood monocytes. The results obtained highlight the important contribution from the functional relationship between innate immunity, inflammation and Fe metabolism in this disease, suggesting new biological pathways implicated in its pathogenesis. The low sample size herein analyzed should be however regarded as a limitation of the present study. Thus, the physiological consequences of these observations need to be further investigated in order to clarify its role in this rare pathology.
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Clin Exp Immunol
Blood Cells Mol Dis
Neutrophil-potentiating factors released from stimulated lymphocytes, special reference to the increase in
