Neutrophil Activation Promotes Fibrinogen Oxidation and Thrombus Formation in Behçet Disease

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Background—Behçet disease (BD) is a systemic vasculitis with a broad range of organ involvement, characterized by a multisystemic, immune-inflammatory disorder involving vessels of all sizes and often complicated by thrombosis. Systemic redox imbalance and circulating neutrophil hyperactivation have been observed in BD patients and are thought to be responsible for impaired coagulation. We here focused on the pathogenetic mechanisms potentially linking immune cell activation and thrombosis, and specifically examined whether neutrophil activation can affect fibrinogen modifications and consequently elicit thrombosis.

Methods and Results—Blood samples were collected from 98 consecutive BD patients attending our dedicated Center and from 70 age- and sex-matched healthy controls; in all patients fibrinogen function and structure, fibrin susceptibility to plasmin-lysis, plasma redox status, leukocyte oxidative stress markers, and possible reactive oxygen species sources were examined. Thrombin-catalyzed fibrin formation and fibrin susceptibility to plasmin-induced lysis were significantly impaired in BD patients ($P<0.001$). These findings were associated with increased plasma oxidative stress markers ($P<0.001$) and with a marked carbonylation of fibrinogen ($P<0.001$), whose secondary structure appeared deeply modified. Neutrophils displayed an enhanced NADPH oxidase activity and increased reactive oxygen species production ($P<0.001$), which significantly correlated with fibrinogen carbonylation level ($r^2=0.33, P<0.0001$), residual $\beta$-band intensity ($r^2=0.07, P<0.01$), and fibrinogen clotting ability ($r^2=0.073, P<0.01$)

Conclusions—In BD patients, altered fibrinogen structure and impaired fibrinogen function are associated with neutrophil activation and enhanced reactive oxygen species production whose primary source is represented by neutrophil NADPH oxidase. (Circulation. 2016;133:302-311. DOI: 10.1161/CIRCULATIONAHA.115.017738.)

Key Words: fibrinogen ■ neutrophils ■ oxidative stress ■ thrombosis

Chronic inflammation is a well-known risk factor for the development of thrombosis. However, the details of the complex crosstalk between inflammation and hemostasis are far to be elucidated. Some interesting animal models of inflammation-induced thrombosis exist, but human models have not been yet proposed.

Clinical Perspective on p 311

Among the systemic inflammatory diseases characterized by thrombotic tendency, Behçet disease (BD) is a peculiar systemic vasculitis with frequent mucocutaneous, ocular, gastrointestinal, and cerebral lesions; recurrent thrombosis occurs more frequently in male patients with active disease, and represents an important cause of morbidity and mortality. Cardiovascular events affect up to 45% of BD patients involving both arterial and venous vessels of all sizes, but deep and superficial vein thrombosis of the lower extremities are the most common vascular manifestations of the disease.

An overall imbalance in blood redox status (assessed by ischemia-modified albumin, advanced oxidation protein products, and pro-oxidant/antioxidant balance) has been reported in BD. Moreover, systemic inflammation more than usual thrombophilic factors is thought to be the main trigger of thrombosis in this condition and seems to be mainly mediated by T lymphocytes, monocytes, neutrophils, and proinflammatory cytokines along with endothelial cell dysfunction. In particular, neutrophils can mediate tissue injury in different ways: actually, neutrophils in BD display an intrinsic hyperactivation, probably HLAB*51-related, and usually participate in perivascular infiltration in BD lesions. Based on these pathogenetic concepts and clinical experience, the European League Against Rheumatism (EULAR) recommendations...
for the management of BD suggest that thrombosis should be treated with immunosuppression rather than anticoagulation,17 as an inflammation-induced thrombosis.

Recent studies have provided evidence that fibrinogen plays a multifaceted role in inflammatory responses and autoimmunity.18,19 The ability of fibrinogen to participate in the inflammatory response depends on its specific interaction with integrins, the leukocyte cell surface adhesion receptors. M2 (CD11b/CD18, Mac-1) and X2 (CD11c/CD18, p150,95) are the main fibrinogen receptors and are expressed on neutrophils, monocytes, macrophages and several subsets of lymphocytes.20 In BD, endothelial cell dysfunction, increased reactive oxygen species (ROS) production,21 and neutrophil hyperfunction have been reported,15 together with an impaired fibrinolysis.22

In this study, to highlight the mechanisms of inflammation-induced thrombosis, we investigated fibrinogen modifications, fibrin susceptibility to plasmin-lysis, plasma redox status, leukocyte oxidative stress, and possible ROS sources in a population of BD patients. Moreover, we explored fibrinogen structure and its possible relationship with neutrophil-dependent ROS production.

Methods

Patients Enrollment

From November 2009 until June 2014, 98 patients with BD who attended the Florence Behçet Center (47 males and 51 females) and 70 age-matched healthy control subjects, were included in the study (Table 1). All the patients were diagnosed as having Behçet disease according to International Study Group criteria.23 Patients with other autoimmune diseases, active infections, or neoplastic conditions were excluded. Blood samples were collected from patients without immunosuppressive therapy and only prednisone assumption was allowed. In colchicine-treated patients (21 males and 27 females), therapy was suspended at least 7 days before blood sample collection. The study protocol was approved by local Ethical Committee and informed consent was obtained from all subject enrolled. Demographic and clinical characteristics of the population studied are summarized in Table 1.

Sample Collection

Blood samples were collected in Vacutainer tubes containing 0.109 mol/L buffered trisodium citrate (1:10) or EDTA (0.17 mol/L). After centrifugation (1500g for 15 minutes at 4°C), aliquots of plasma were used for experiments or stored at 80°C for further analysis. Another aliquot of sodium citrate plasma was used for fibrinogen purification.

Fibrinogen Purification

Fibrinogen was purified using the previously described ethanol precipitation method.24 After the purification procedure, fibrinogen concentration was determined by ultraviolet spectroscopy at a wave length of 280 nm, assuming an extinction coefficient of 1.51 mg/mL. The yield of purified fibrinogen was not statistically different between patients and controls (9.7±1.4 versus 8.9±1.9, mg/10 mL of plasma, respectively). The purity of the fibrinogen preparations (from 10 mL of citrated plasma) was assessed by densitometry of Coomassie-stained polyacrylamide gels after electrophoresis under reducing conditions. In our purification procedures, the amount of fibrinogen, expressed as a percentage of total protein content, yielded 97.1±1.4% of total protein content in controls and 94.0%±1.1% in Behçet patients. No significant statistical difference was observed in the purification yield between controls and patients.

Protein Concentration Assay

Protein concentration in the samples was determined using the Bradford assay.25 A standard curve of bovine serum albumin (0–15 μg protein/200 μL volume) was used.

Protein Carboxyl Determination

Oxidative modification on plasma proteins and on purified fibrinogen fractions was assessed based on carbonyl content using 2,4-dinitrophenylhydrazine (DNPH), as previously reported.26 DNPH reacts with protein carbonyl (PC), forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically. Briefly, plasma (100 μL), after incubation with DNPH (400 μL), was precipitated with trichloroacetic acid and the pellet washed several times with a 1:1 mixture of ethanol/ethyl-acetate. Finally, the pellet was resuspended in 500 μL of guanidine hydrochloride and measured at 370 nm. PC content was calculated by using a molar extinction coefficient of 22000 mol/L cm−1. The results, expressed in terms of nmol/mL of PC, were then normalized for protein concentration.

Thiobarbituric Acid Reactive Substances Estimation

Plasma thiobarbituric acid reactive substances (TBARS) levels were measured using a TBARS assay kit (OXI-TEK, ENZO, USA) in accordance with the manufacturer’s instructions. Briefly, the adduct generated by reacting malondialdehyde with thiobarbituric acid after 1h at 95°C was measured spectrophotometrically, with excitation at 530 nm and emission at 550 nm. TBARS were expressed in terms of malondialdehyde equivalent (nmol/mL) and then normalized for protein concentration.

Circular Dichroism Spectra of Purified Fibrinogen Extracts

Circular dichroism spectra were recorded at 25°C in 0.2 cm quartz cells from 250 to 195 nm (farUV), using a protein concentration of 1 mg/mL. Samples were filtered through 0.22 μm filters and 5 spectra recorded for each sample. Molar ellipticity values [θ] were calculated according to the following equation: [θ] (deg·cm²dmol⁻¹) = [θ] (MRW) /[10(l/c)], where 0 is the displacement from the baseline value X to the full range in degrees; MRW is the mean residue weight of the amino acids; (l) is the path length of the cell (cm); and (c) is protein concentration (g/mL).27

Thrombin-Catalyzed Fibrinogen and Polymerization Assays

For functional analysis, purified fibrinogen fractions stored at −80°C and not previously thawed were used. Fibrin polymerization was monitored at 595 nm in a 96-well micro titer plate reader (model 550, Bio-Rad Milan, Italy) at 25°C, as previously described.28 Before the polymerization assay, control and patient fibrinogen samples were extensively dialyzed against 100 mmol/L Tris/HCl buffer, pH 7.4, and diluted to a final concentration of 1 mg/mL. To each reaction (in triplicate), 240 μL of fibrinogen (1 mg/mL) in 100 mmol/L Tris/HCl, 5 mmol/L CaCl₂, pH7.4 was added. The polymerization reaction was started by adding 60 μL thrombin (at a final concentration of 0.25 U/mL). Absorbance was monitored for 90 minutes at 25°C.

Absorbance curves were characterized using the following parameters: (1) the maximum slope (Vmax), calculated as the slope of the steepest part of the polymerization curve (using 15 time points), which represents the rate of lateral protofibril association; (2) the lag phase, measured as the time elapsed until an increase in absorbance was seen, which reflects the time to the start of lateral fibril aggregation; (3) maximum absorbance (MaxAbs) of the growing clot, recorded 60 minutes after polymerization was initiated, which reflects an average fibrin fiber size and the number of protofibrils per fiber.29
Table 1. International Study Group Criteria and Noncriteria Characteristics of the Patients With Behçet Disease: Demographic and Clinical Features

<table>
<thead>
<tr>
<th>International Study Group criteria for Behçet disease</th>
<th>Major criteria</th>
<th>n (%) patients = 98/98 (100%)</th>
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<tbody>
<tr>
<td>Recurrent oral ulcerations: minor aphthous, major aphthous or herpetiform ulceration observed by physician or patient, which recurred at least 3 times in one 12-mo period</td>
<td>n (%) patients = 98/98 (100%)</td>
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<tr>
<td>+ Minus criteria</td>
<td></td>
<td></td>
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<tr>
<td>Recurrent genital ulceration: aphthous ulceration or scarring observed by physician or patient</td>
<td>2 minor criteria</td>
<td>n (%) patients = 73/98 (74%)</td>
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<tr>
<td>Genital ulcers + Eye lesions 14 (19%)</td>
<td>Genital ulcers + Eye lesions + Skin lesions 16 (64%)</td>
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<tr>
<td>Eye lesion: anterior uveitis, posterior uveitis, or cells in vitreous on slit lamp examination or retinal vasculitis observed by ophthalmologist</td>
<td>Genital ulcers + Eye lesions 14 (19%)</td>
<td>Genital ulcers + Eye lesions + Skin lesions 16 (64%)</td>
</tr>
<tr>
<td>Skin lesions: erythema nodosum observed by physician or patient, pseudofolliculitis or papulopustular lesions, or acneform nodules observed by physician in postadolescent patients not on corticosteroid treatment</td>
<td>Skin lesions + Eye lesions 25 (34%)</td>
<td>Skin lesions + Eye lesions + Pathergy test 7 (28%)</td>
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<tr>
<td>Positive pathergy test read by physician 24–48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin lesions + Pathergy test 8 (11%)</td>
<td>Eye lesions + Pathergy test 1 (1%)</td>
<td></td>
</tr>
<tr>
<td>Other noncriteria demographic and clinical features of the patients with Behçet disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>51/98 (52%)</td>
<td></td>
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<tr>
<td>Females</td>
<td>47/98 (48%)</td>
<td></td>
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<tr>
<td>HLAB*51</td>
<td>53/98 (54%)</td>
<td></td>
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<tr>
<td>Vascular involvement</td>
<td>31/98 (32%)</td>
<td></td>
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<tr>
<td>Articular involvement</td>
<td>55/98 (56%)</td>
<td></td>
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<tr>
<td>Gastrointestinal involvement</td>
<td>36/98 (37%)</td>
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<tr>
<td>Central nervous system involvement</td>
<td>18/98 (18%)</td>
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Determination of Fibrin Structure Based on Label-Free Differential Interference Contrast Microscopy

Fibrin clots prepared as described above were analyzed by differential interference contrast (DIC) microscopy. This is a label-free microscopy technique with a high sensitivity to thin cellular material, even when it is located within thick tissue. DIC microscopy is superb for observing transparent objects and very thin filaments or sharp interfaces, which produce good contrast even when their diameter falls below the resolution limit of the optical system. DIC microscopy causes one side of an object to appear bright while the other side appears darker. This shadow effect gives a pseudothree-dimensional appearance to the specimen, at excellent resolution.

Purified fibrinogen fractions stored at −80°C and not previously thawed were used. Before polymerization, control and patient fibrinogen samples were extensively dialyzed against 100 mmol/L Tris/HCl buffer, pH 7.4, and diluted to a final concentration of 1 mg/mL. To each reaction (in duplicate) 240 μL of fibrinogen (1 mg/mL) in 100 mmol/L Tris/HCl, 5 mmol/L CaCl₂, pH 7.4, was seeded on glass coverslips. The polymerization reaction was started by adding 60 μL of thrombin (at a final concentration of 0.25 U/mL) at 25°C. After 90 minutes the sample was analyzed using a Leica TCS SP5 microscope (Mannheim, Germany), using DIC. The observations for each examined sample were obtained using a Leica Plan Apo 63x oil immersion objective.

Fibrin Digestion With Plasmin and Electrophoretic Analysis of Plasmin Digests

Fibrin clots were prepared in microcentrifuge tubes by incubating bovine thrombin (12 U/mL; final concentration) with fibrinogen (2 mg/mL; final concentration) in 20 μL of 100 mmol/L Tris/HCl, 5 mmol/L CaCl₂, pH 7.4, for 1 h at 25°C. Plasmin was then added (5 μL of 100 μg/mL), and the fibrin clots were digested over a period of 6 h at 37°C, as previously described. The digestion reaction was terminated by adding 10 μL of lithium dodecylsulfate gel electrophoresis sample buffer. The same lot of thrombin and of plasmin were used for all experiments. Samples were heated at 70°C for 10 minutes under reducing conditions (50 mmol/L dithiothreitol). Then, aliquots from each digest (equivalent to10 μg of fibrin) were loaded onto 4%–12% Bis-Tris gels. After electrophoresis, gels were stained with Coomassie blue. Band intensities of stained gels were quantified by densitometry using the Chemi-Doc system and Quantity-One software (Bio-Rad, Milan, Italy). Data were expressed as the ratio between the densitometric reading of the purified protein at a given digestion time and that of the undigested protein (time 0 for incubation with plasmin).

Assessment of ROS Generation by Flow Cytometry

After collection, 100 μL EDTA-anticoagulated blood samples was resuspended in 2 mL of BD FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA), gently mixed, and incubated at RT in the dark for 10 minutes, following manufacturer’s protocol. Next, the cells were centrifuged, the supernatant discarded, and cells washed twice in PBS. To determine the level of intracellular ROS generation, cells were incubated with H₂DCF-DA (2.5 μmol/L; Invitrogen, Carlsbad, CA) in RPMI without serum and phenol red for 15 minutes at 37°C. After labeling, cells were washed and resuspended in PBS and analyzed immediately using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA). The sample flow rate was adjusted to about 1000 cells/s. For a single analysis, the fluorescence properties of 20,000 leukocytes were collected. The respective gates were defined using the distinctive forward-scatter and side-scatter properties of 20,000 leukocytes.
properties of the individual cell populations. Moreover, the viability of the cells was controlled by flow cytometry with propidium iodide staining and was found to exceed 95%. Data were analyzed using BD FACSDiva software (Becton-Dickinson, San Jose, CA).

Leukocyte Subpopulation Cell Sorting
After collection, 5 mL EDTA-anticoagulated blood samples was resuspended in 100 mL of BD FACS Lysing Solution (Becton Dickinson Biosciences), gently mixed, and incubated at RT in the dark for 10 minutes, following manufacturer’s protocol. Next, cells were centrifuged, supernatant discarded, and cells washed twice in PBS. For cell sorting, cells were resuspended in 2.5 mL 1640 RPMI (containing glutamine, supplemented with 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, Cellegro, Washington, DC) and flow-sorted with a FACS Aria (Becton Dickinson Biosciences). The sample flow rate was adjusted to about 10,000 cells/s. Forward and side scatter were used for identification of leukocyte subpopulations (lymphocytes, monocytes and neutrophils). In our experimental design, the use of label-free leukocyte subpopulations is mandatory for all planned experiments (ie, incubation of leukocyte subpopulations with fibrinogen and leukocyte subpopulations NADPH oxidase activity).

Forward and side scatter were also used to exclude debris and dead cells. Purity of each leukocyte subpopulation was >95% as confirmed by postsorting FACS analysis with specific antibodies. Moreover, the viability of the cells was controlled by flow cytometry with propidium iodide staining and was found to exceed 95%. Data were analyzed using BD FACSDiva software (Becton-Dickinson).

Leukocyte Subsets
FACS-sorted leukocyte subset (lymphocytes, monocytes and neutrophils). In our experimental design, the use of label-free leukocyte subpopulations is mandatory for all planned experiments (ie, incubation of leukocyte subpopulations with fibrinogen and leukocyte subpopulations NADPH oxidase activity).

For in vitro leukocyte-induced fibrinogen carbonylation assay, each FACS-sorted leukocyte subset (lymphocytes, monocytes and neutrophils; 7.5×10⁶ cells) was incubated with 0.4 mg of fibrinogen in 200 μL 1640 RPMI (in presence or absence of the NADPH oxidase inhibitor apocynin 1.5 mmol/L) for 3h at 37°C. After centrifugation (1500g for 10 minutes), the supernatant was collected and used for the estimation of carbonyl content.

Statistical Analysis
To assess the quality of analyses, all the experiments were performed 3 times on the same sample, each 1 with 3 replicates. After assessing the low intraexperiment and interexperiment variability and the reproducibility of measures (Repeated measures ANOVA, data not shown), each value per subject was calculated as the overall mean of the means of the 3 experiments. Therefore, group means and SD were calculated by using the overall mean for each subject as single value in the calculations.

Table 2. Oxidative Stress Markers

<table>
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<tr>
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<th>Controls n=70</th>
<th>BD Patients n=98</th>
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<tbody>
<tr>
<td>Plasma PC (nmol/mg)</td>
<td>11.57±3.42</td>
<td>18.91±5.73</td>
</tr>
<tr>
<td>Plasma TBARS (nmol/mL)</td>
<td>0.610±0.070</td>
<td>1.905±0.961</td>
</tr>
<tr>
<td>Fibrinogen PC (nmol/mg)</td>
<td>0.450±0.050</td>
<td>1.941±0.817</td>
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</table>

Group mean±SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. PC indicates protein carbonyl; and TBARS, thiobarbituric acid reactive substances.

*Significant difference vs control at the P<0.01 level (Student t test).

NADPH Oxidase Activity Assay
NADPH oxidase activity assay was performed on each leukocyte subset using a Lumat LB 9507 single-tube luminometer (Berthold Technologies, GmbH & Co, Bad Wildbad, Germany). After washing with phosphate-buffered saline, 7.5x10⁴ cells were resuspended in 150 μl Krebs-HEPES buffer (99 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1 mmol/L KH₂PO₄, 1.9 mmol/L CaCl₂, 25 mmol/L NaHCO₃, 20 mmol/L HEPES, 11.1 mmol/L glucose, pH 7.44) and incubated for 10 minutes at 37°C. Then, lucigenin was added to the sample to a final concentration of 25 μmol/L. Luminescence was recorded continuously for 30 minutes. Each sample was also assayed in the presence of the NADPH inhibitor diphenyleneiodonium chloride, DPI (20 μmol/L) to obtain basal luminescence values. NADPH oxidase activity was expressed as relative units of luminescence (RLU)/s.

In Vitro Fibrinogen Carbonylation Induced by Leukocyte Subsets
For in vitro leukocyte-induced fibrinogen carbonylation assay, each FACS-sorted leukocyte subset (lymphocytes, monocytes and neutrophils; 7.5x10⁶ cells) was incubated with 0.4 mg of fibrinogen in 200 μL 1640 RPMI (in presence or absence of the NADPH oxidase inhibitor apocynin 1.5 mmol/L) for 3h at 37°C. After centrifugation (1500g for 10 minutes), the supernatant was collected and used for the estimation of carbonyl content.

In Vitro Fibrinogen Carbonylation Induced by Leukocyte Subsets

The obtained leukocyte subpopulation were used for NADPH oxidase activity assay and for in vitro leukocyte subpopulation-induced fibrinogen carbonylation assay.

Figure 1. A. Lymphocyte/monocyte/neutrophil ROS production in controls and BD patients. Group mean±SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. B. Pearson rank correlation analysis in BD patients, comparing fibrinogen carbonyl content and lymphocytes/monocytes/neutrophils ROS. ROS production and fibrinogen carbonyl content was measured as described in the Materials and Methods section. Each point represents the overall mean of different experiments/replicates for each subject. *Significant difference vs control at the P<0.01 level (Student t test). BD indicates Behçet disease; PC, protein carbonyl; and ROS, reactive oxygen species.
All data presented a normal distribution. Statistical significance was evaluated with a standard unpaired Student t test (2-tailed; \(P<0.05\)) when appropriate. For multiple-comparison analysis, 1-way ANOVA followed by the Bonferroni post hoc test was applied when appropriate. Correlation analysis was performed using the Pearson’s test. All statistical operations data were processed using the Graph Pad Prism 5 software. A value of \(P<0.05\) was considered to be as statistically significant.

**Results**

**Oxidative Stress Markers in Plasma, Lymphocyte, Monocyte, and Neutrophil Fractions and in Purified Fibrinogen**

The main plasma oxidative stress markers—namely PC, accounting for protein oxidative damage, and TBARS, an index of lipid peroxidation—were significantly increased in BD patients compared with healthy controls (\(P<0.001\)). Purified fibrinogen obtained from patients showed a significantly increased extent of carbonylation as compared to that obtained from healthy controls (\(P<0.01\); Table 2). ROS production in BD patients in lymphocyte and monocyte fractions was almost 2-fold and in neutrophils was 3-fold compared with healthy controls (Figure 1A).

Interestingly, only neutrophil ROS production positively and significantly correlated with fibrinogen PC content (\(P<0.0001, r^2=0.3327\)), whereas monocyte/lymphocyte ROS production did not (Figure 1B). ROS production in granulocyte/monocyte/lymphocyte populations did not show any significant correlation with total plasma PC content (data not shown).

**Circular Dichroism Spectra: Analysis of Secondary Structure**

The secondary protein structure, mainly represented by \(\alpha\)-helices and \(\beta\)-pleated sheets, deeply influences protein function. In our study, secondary protein structure was analyzed by far-UV circular dichroism spectroscopy (Figure 2). In control subjects the observed spectrum for fibrinogen suggested a typical \(\alpha\)-helical structure with minima at 208 nm and at 222 nm. Fibrinogen from BD patients displayed an altered circular dichroism spectrum mainly consisting in a decrease in the negative peak in the 215 to 225 nm region, therefore suggesting a decrease in \(\alpha\)-helical content (Figure 2), which can account for functional protein modifications.

**Fibrinogen Polymerization and Fibrin Formation**

Fibrinogen functioning was assessed by evaluating the kinetics of fibrin polymerization. Representative curves of thrombin-catalyzed fibrinogen polymerization are shown in Figure 3A. In BD patients, the ability of fibrinogen to undergo clotting was diminished: significant differences in BD patients versus controls were found in the main parameters which characterize the polymerization curves (as described in the Methods section; ie, Lag time, Vmax, and Max Abs \(P<0.01\)), suggesting a different clot structure. In Figure 3B the significant correlation between fibrinogen PC and Max Abs is reported \(P<0.0001, r^2=0.2894\). When polymerization kinetics was assayed in patients’ plasma, the obtained Max Abs was significantly reduced as compared with that of controls \(P<0.01\), data not shown).

ROS production in the leukocyte subpopulations correlated with Max Abs, which resulted from fibrinogen polymerization curves. No significant correlation was found between Lymphocyte/Monocyte ROS production and Max Abs (Figure 3C and 3D). Conversely, Max Abs significantly correlated \(P<0.01\) with neutrophil ROS production (Figure 3E).

**Differential Interference Contrast Microscopy**

To evaluate the structure of the fibrin network in BD patients and controls, fibrin samples obtained from BD patients and controls were analyzed by differential interference
contrast (DIC) microscopy. Representative images are shown in Figure 4. After 90 minutes a tight fibrin network is still evident after plasmin-induced lysis in fibrin obtained from BD patients compared with controls.

Fibrin Susceptibility to Plasmin-Induced Lysis
To evaluate fibrin susceptibility to plasmin-induced lysis, we focused on the degradation rate of the fibrin $\beta$ chain; the degradation before and 3h and 6h of plasmin digestion in BD patients and controls is reported in Figure 5A. In BD patients the relative band intensity at each plasmin incubation time was significantly increased with respect to controls ($P<0.01$; Figure 5B). Moreover, in BD patients, the correlation between fibrinogen carbonyl content and the relative fibrin $\beta$ chain intensity after 6h of plasmin digestion was shown to be significant ($P<0.001$, $r^2=0.1254$, Figure 5C). The correlation between ROS production in leukocyte fractions and residual fibrin $\beta$ chain after 6h of plasmin digestion is shown in Figure 5D, 5E, and 5F. No significant correlation was found between lymphocyte or monocyte ROS production and residual fibrin $\beta$ chain (Figure 5D and 5E). Conversely, a significant correlation ($P<0.01$, $r^2=0.073$) was found when neutrophils were analyzed (Figure 5F).

NADPH Oxidase Activity in Lymphocyte, Monocyte, and Neutrophil Fractions
The activity of NADPH oxidase, one of the major physiological ROS source, was measured in leukocytes obtained from BD patients and controls. NADPH oxidase activity measured in lymphocyte, monocyte, and neutrophil fractions, in the presence or absence of a specific NADPH oxidase inhibitor, revealed only in BD neutrophils a significantly increased (about five-fold) enzyme activity as compared with that of neutrophils obtained from controls (Figure 6).

Neutrophils Are Responsible for Fibrinogen Carbonylation
In the in vitro experiments equal amounts of lymphocytes, monocytes, and neutrophils from BD patients were incubated with human fibrinogen for 3h incubation at 37°C. Interestingly, a significantly increased PC content ($P<0.01$) was observed only in fibrinogen samples incubated with patients’ neutrophils (Figure 7A). Simultaneously, fibrinogen samples incubated with the different leukocyte fractions were tested for susceptibility to plasmin-induced lysis. Only fibrinogen incubated with patients’ neutrophils showed a marked resistance to plasmin-induced lysis as evidenced by increased residual $\beta$ chain intensity compared to controls after 6h of incubation with plasmin (Figure 7B).
Discussion

Although inflammation-induced thrombosis is a well-known process, its pathogenic mechanisms still remain a matter of debate. BD may be considered a prototype of systemic inflammatory disease causing thrombosis. In fact, thrombosis, which is the most common vascular manifestation in this condition, responds to immunosuppressive treatment rather than anticoagulation.

The association among oxidative stress, inflammation, and endothelial dysfunction has been repeatedly suggested and blood oxidative stress markers (lipid peroxidation markers, protein carbonyls) have been indicated as prognostic tools in vasculitis and particularly in BD. Only few studies until now have explored the possible blood ROS sources in BD, whereas it is unknown whether oxidative stress may affect the structure and function of fibrinogen, a plasma protein involved both in inflammation and coagulation and particularly susceptible to oxidation.

Inflammation-induced endothelial injury emerges as a key factor connecting chronic inflammation and thrombosis. However, the mechanisms underlying this pathogenetic correlation remain to be elucidated. Our results indicate that leukocyte ROS production, particularly by neutrophils, is significantly enhanced in BD patients. This finding is associated with increased plasma levels of oxidative stress markers and in particular with a greater extent of fibrinogen carbonylation. In the blood of BD patients only neutrophil-derived ROS (but not lymphocyte- or monocyte-derived ROS) showed a significant correlation with fibrinogen carbonyl content, so suggesting that neutrophil activation drives fibrinogen oxidation.

Neutrophils play a critical role in host defense by the engagement of specific surface receptors, with a multitude of ligands including fibrinogen. It has been reported that neutrophils and monocytes promote vascular injury through the generation of inflammatory mediators, tissue infiltration, and oxidation of lipoproteins and other proteins. Neutrophils release serine proteases promoting efficient thrombus formation in mice by stabilizing fibrin deposition, likely by proteolytic inactivation of fibrinogen.

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Inflammation-induced endothelial injury emerges as a key factor connecting chronic inflammation and thrombosis. However, the mechanisms underlying this pathogenetic correlation remain to be elucidated. Our results indicate that leukocyte ROS production, particularly by neutrophils, is significantly enhanced in BD patients. This finding is associated with increased plasma levels of oxidative stress markers and in particular with a greater extent of fibrinogen carbonylation. In the blood of BD patients only neutrophil-derived ROS (but not lymphocyte- or monocyte-derived ROS) showed a significant correlation with fibrinogen carbonyl content, so suggesting that neutrophil activation drives fibrinogen oxidation.

Neutrophils play a critical role in host defense by the engagement of specific surface receptors, with a multitude of ligands including fibrinogen. It has been reported that neutrophils and monocytes promote vascular injury through the generation of inflammatory mediators, tissue infiltration, and oxidation of lipoproteins and other proteins. Neutrophils release serine proteases promoting efficient thrombus formation in mice by stabilizing fibrin deposition, likely by proteolytic inactivation of fibrinogen.

Figure 4. Representative differential interference contrast (DIC) Microscopy images of fibrin from a BD patient and a control subject. The same fibrinogen concentration was used in these subjects, who were matched for age and sex. DIC microscopy analyses were performed as described in the Materials and Methods section. BD indicates Behçet disease.

Figure 5. A, Representative gel of fibrin degradation after 0 h, 3 h, and 6 h of plasmin digestion, using fibrinogen purified from BD patients and controls. B, Residual fibrin β chain intensity after 6 h of plasmin digestion in fibrinogen purified from controls and BD patients. Group mean±SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. *Significant difference vs control (P<0.01, Student t test). Plasmin-induced fibrin digestion and electrophoretic analyses of plasmin-digests were performed as described in the Materials and Methods section. C, In BD patients, Pearson rank correlation analysis comparing BD fibrinogen carbonyl content and residual β chain intensity after 6 h of plasmin digestion showed a statistical significance (C, P<0.001 r²=0.1254). Each point represents the overall mean of different experiments/replicates for each BD patient. D–F, Pearson rank correlation analysis comparing lymphocytes/monocytes/neutrophils ROS and Max Abs in BD patients. Each point represents the overall mean of different experiments/replicates for each BD patient. BD indicates Behçet disease.
endogenous anticoagulants. Moreover, in neutrophils, soluble fibrinogen can trigger, through an integrin-dependent mechanism, an activating signal that promotes degranulation, phagocytosis enhancement, and apoptosis delay. These events may deeply affect the fate of the inflammatory response. In the reported in vitro experiments, purified fibrinogen resulted markedly carbonylated when incubated with neutrophils derived from BD patients, but not with monocytes or lymphocytes from the same patients.

At the same time, a marked increase in NADPH oxidase activity was observed only in the neutrophil fraction. NADPH oxidase-derived ROS are key players in oxidative stress and inflammation, and the NOX complex (the major isoforms of NADPH oxidase) is considered to be a major source of ROS in phagocytic polymorphonuclear neutrophils and monocytes.

In the present study, both plasma oxidative markers and fibrinogen PC were markedly and significantly increased in BD patients when compared with control subjects. In BD, fibrinogen PC significantly correlated with neutrophil-derived ROS, but not with lymphocyte- or monocyte-derived ROS. In this context, it has been reported that fibrinogen is more prone to oxidation than albumin, and on oxidation, clot formation rate has been observed to be decreased. Other investigators reported that exposure of fibrinogen to oxidizing conditions (ie, Fe³⁺-ascorbate) promoted fibrin formation, enhanced platelet aggregation, and supported less efficient plasminogen activation by tissue plasminogen activator.

Figure 6. NADPH oxidase activity was assessed by luminometric assay (as described in the Materials and Methods section) in lymphocytes/monocytes/neutrophils from BD patients and controls. All assays were performed in the absence and in the presence of a specific NADPH oxidase inhibitor (apocynin). Group mean±SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. *Significant difference vs control (P<0.01, ANOVA-Bonferroni test). BD indicates Behçet disease.

Figure 7. A, Leukocyte-dependent fibrinogen (FG) carbonylation. Lymphocytes/monocytes/neutrophils from BD patients were incubated with purified control fibrinogen and its carbonyl content was then estimated. All assays were performed in the absence or presence of a specific NADPH oxidase inhibitor (as reported in the Materials and Methods section). Group mean±SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. **Significant difference vs FG + lymphocytes and # indicates significance vs Fibrinogen + monocytes (P<0.01, ANOVA-Bonferroni test). BD indicates Behçet disease.

B, Fibrin susceptibility to plasmin-induced lysis. Representative gel of fibrin degradation after 0 h, 3 h, and 6 h of plasmin digestion, using control fibrinogen incubated with lymphocytes/monocytes/neutrophils from BD patients. Residual fibrin β chain intensity after 6 h of plasmin digestion in control fibrinogen incubated with lymphocytes/monocytes/neutrophils from BD patients. Group mean±SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. *Significant difference vs control (P<0.01, ANOVA-Bonferroni test). BD indicates Behçet disease.
In our study, the relationship between oxidized fibrinogen and fibrinogen function has been explored by measuring the clotting ability of purified fibrinogen, using an in vitro thrombin-catalyzed polymerization assay. In this system, the magnitude of the turbidity increase relates to the architecture of the formed clot and the altered maximum absorbance of fibrin polymerization reflects the formation of thinner and more compact fibers. When we assessed, in BD patients, thrombin-catalyzed fibrin polymerization, a slower rate and turbidity was observed compared with healthy controls, as indicated by decreased Vmax and Max abs values. In particular, this latter parameter was significantly and inversely correlated with fibrinogen carbonyl content suggesting a direct influence of carbonylation on fibrin polymerization. Moreover, in BD patients, only neutrophil (but not lymphocyte or monocyte) ROS production inversely and significantly correlated with turbidity/Max Abs parameters in clotting assays.

When fibrinogen secondary structure was investigated by far-UV circular dichroism spectroscopy, a reduction in α-helical content was detected in BD patients. Considering that protein functionality largely depends on its secondary structure, this finding suggests that carbonylation promotes the formation of a species less rich in α-helix, which may specifically affect the biological activity of fibrinogen. Our observation concerning the modified fibrinogen function is in keeping with our and previous reports, showing that fibrinogen oxidation impairs the capacity of isolated fibrinogen to form a fibrin clot under the effect of thrombin.\textsuperscript{36,38,39} To study another important feature of fibrinogen function in relation to carbonylation, we determined, both in BD patients and controls, fibrin resistance to plasmin-induced lysis. In BD patients, fibrin showed a marked resistance to lysis and its degradation was significantly decreased with respect to healthy controls. Moreover, fibrin resistance to lysis significantly correlated with fibrinogen PC and with neutrophil ROS production (but not with lymphocyte-or monocyte-derived ROS).

Interestingly, 1 of our major findings is a strong and positive correlation between fibrinogen PC and residual β-chain intensity after plasmin-induced lysis. Moreover, a significant correlation between residual β-chain intensity after plasmin-induced lysis and neutrophil ROS production was found.

The reported findings are consistent with the observation, in patients with acute coronary syndromes, that clots composed of dense networks are more resistant to lysis and these features correlate with inflammation and oxidative stress.\textsuperscript{41} Hence, clot structure, analyzed by electron and differential interference contrast microscopy, revealed, in BD patients, an altered clot architecture mainly characterized by a tight fibrin network composed of filaments with slightly decreased average fiber size that are resistant to plasmin-induced lysis when compared with control subjects. Clots composed of thin fibers and small pores have been suggested to be more thrombogenic;\textsuperscript{44,45} but the mechanisms underlying the formation of these abnormal fibrin clots have not yet been established. The posttranslational oxidative modifications of fibrinogen could play an important role in this context.\textsuperscript{46}

In conclusion, our data highlight that neutrophil activation promotes fibrinogen oxidation and thrombus formation in BD. In particular, our results suggest that an altered fibrinogen structure and an impaired fibrinogen function are associated with neutrophil activation and enhanced ROS production whose primary source is represented by neutrophil NADPH oxidase. BD is a systemic vasculitis\textsuperscript{12} frequently complicated by recurrent thrombosis, especially in male patients with active disease.\textsuperscript{16} Our results were observed in BD patients regardless of the presence of vascular involvement. Furthermore, all patients had inactive disease, thus suggesting that BD is per se a model of inflammation-induced thrombosis. Altogether, these data may improve our understanding of the pathogenesis of inflammation-induced thrombosis and may suggest potential targets for innovative therapeutic approaches.

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Disclosures

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References

Behçet disease (BD) is a systemic inflammatory disease which frequently affects medium-sized and large vessels, histologically characterized by neutrophil-rich inflammatory infiltrates. BD is often complicated by both arterial and venous thrombosis, which is often resistant to common anticoagulation therapy and rarely causes embolism. The pathogenesis of BD-related thrombosis is still unclear. In this study, we demonstrate that in BD patients neutrophils have a significantly increased reactive oxygen species production as a result of abnormal NADPH-oxidase activity; reactive oxygen species promote fibrinogen carboxylation and secondary structure modification, which ultimately cause altered fibrinogen clotting ability and reduced fibrin susceptibility to plasmin-induced lysis. These data offer a new model of inflammation-induced thrombosis and support current therapeutic concepts regarding the use of immunosuppressive (rather than anticoagulation) therapy in BD-related thrombosis. The model of inflammation-induced thrombosis warrants further investigation in BD and in other autoimmune diseases.
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