CLINICAL SCIENCE

Serum homocysteine level is increased and correlated with endothelin-1 and nitric oxide in Behçet’s disease

H Er, C Evereklioglu, T Cumurcu, Y Türköz, E Özerol, K Şahin, S Doganay

Background/aims: Behçet’s disease (BD) is a systemic inflammatory vasculitis of young adults with unknown aetiology, characterised by endothelial dysfunction and occlusion in both deep venous and retinal circulation. Ocular involvement occurs in 70% of cases and is characterised by periphlebitis, periarthritis, vascular occlusion, and thrombosis leading to blindness despite vigorous treatment. Endothelin-1 (ET-1) is a vasoconstricting peptide while nitric oxide (NO) is a relaxing molecule and both are released by endothelium for blood flow regulation. Homocysteinaemia is a newly defined term connected to the increased risk of atherothrombotic and atherosclerotic systemic and retinal vascular occlusive diseases, and its role in the course of BD has not been previously described. The authors aimed to detect serum total homocysteine (tHcy), ET-1, and NO in BD and to assess if tHcy, ET-1, and NO are associated with ocular BD or disease activity.

Methods: 43 consecutive patients with ocular (n = 27) or non-ocular (n = 16) BD (36.95 (SD 9.80) years, 22 male, 21 female) satisfying international criteria and 25 age and sex matched healthy control subjects (37.88 (8.73) years, 13 male, 12 female) without a history of systemic or retinal venous thrombosis were included in this study. Patients were examined by two ophthalmologists with an interest in BD. Serum tHcy, ET-1, and NO concentrations were measured in both groups. Hyperhomocysteinaemia was defined as a tHcy level above the 95th percentile in the control group. Patients were divided into active and inactive period by acute phase reactants including α, antitrypsin, α1 antitrypsin, serum γ globulin, erythrocyte sedimentation rate, and neutrophil count.

Results: The overall mean serum tHcy, ET-1, and NO levels were significantly higher in patients with BD than in control subjects (tHcy = 15.83 (4.44) v 7.96 (2.66) ng/ml, p <0.001; ET-1 = 17.47 (4.33) v 5.74 (2.34) µmol/l, p <0.001; NO = 37.60 (10.31) v 27.08 (7.76) µmol/l, p <0.001). Serum tHcy, ET-1, and NO levels were significantly higher in active patients than in inactive patients and control subjects. In addition, among patients with ocular BD, the mean tHcy levels were significantly increased and correlated with ET-1 and NO levels when compared with non-ocular disease and control subjects. All acute phase reactant levels were significantly higher in active period than in inactive stage and controls.

Conclusions: Elevated tHcy may be responsible for the endothelial damage in BD and may be an additional risk factor for the development of retinal vascular occlusive disease, contributing to the poor visual outcome in these patients. Assessment of tHcy may be important in the investigation and management of patients with BD, especially with ocular disease.

Homocysteinaemia (Hcy) is an intermediary amino acid formed during the conversion of methionine to cysteine. Hyperhomocysteinaemia refers to mild to moderate elevation of Hcy in blood or serum. High levels of Hcy causes lipid peroxidation, vascular endothelial injury, impaired vasomotor regulation, prothrombotic surface, and therefore atherothrombogenesis. Furthermore, raised Hcy levels have recently been suggested as a risk factor for non-arteritic anterior ischaemic optic neuropathy and retinal vascular occlusive disease with thromboembolism. Two recently identified cellular mediators endothelin-1 (ET-1) and nitric oxide (NO) are released from endothelium. ET-1 induces vasoconstriction and contributes to ocular pathological manifestations, promoting retinal capillary non-perfusion and ischaemia. On the other hand, NO induces vasorelaxation and inhibits platelet adhesion. The combination of the paracrine effects of ET-1 along with release of NO are likely to have a relevant physiological role in the regulation of systemic and retinal blood flow.

We have recently reported for the first time that some vasoregulatory molecules such adenomedullin and NO are involved in ocular BD.

Abbreviations: BD, Behçet’s disease; CV, coefficients of variance; EUSA, enzyme linked immunosorbent assay; ESR, erythrocyte sedimentation rate; ET-1, endothelin-1; Hcy, homocysteine; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; tHcy, total homocysteine
might participate in the reparatory vessel endothelium mechanisms during the course of BD. Furthermore, our results demonstrated that adenomedullin was correlated with disease chronicity whereas NO was correlated with disease activity, suggesting NO as a possible new activity marker. Since homocysteinaemia participates in thrombogenic events and retinal vascular occlusive disease by direct toxic effect on endothelial cells, we considered it as an interesting target of investigation, and its role in the aetiopathogenesis of BD has not been previously investigated. In addition, we assessed whether the Hcy level is associated with ocular disease and disease activity. We also evaluated if the Hcy level is correlated with the two endothelium derived ET-1 and NO levels during the course of the disease. To our knowledge, this is the first report on Hcy levels during the course of BD.

MATERIALS AND METHODS

Patients

A total of 43 consecutive patients with ocular (n = 27) or non-ocular (n = 16) BD (36.95 (SD 9.80) years (range 17–57), 22 male and 21 female) attended our Behçet’s disease clinic and 25 age and sex matched healthy control subjects (37.88 (8.73) years (19–57), 13 male and 12 female) from a similar ethnic background were included in the present study. All BD patients fulfilled the criteria of the International Study Group for Behçet’s Disease.

Since there was no clinically acceptable scoring system and laboratory screening profile to define the severity of BD, clinical and laboratory findings were used to classify the patients as active or inactive. Erythrocyte sedimentation rate (ESR), neutrophil count, and acute phase reactants (αα, α2 antitrypsin and αα, macroglobulin) were determined and constituted the laboratory findings as described before. In clinical evaluation, worsening of clinical symptoms at the time of study with having at least three of the major findings (retinal vasculitis, oral or genital ulcer, pseudofollicular cutaneous lesions; pathergy test positivity) were considered to be in the active stage of the disease. The diagnosis of uveitis was made according to the International Uveitis Study Group.

Patients’ details were obtained from case notes and ocular examinations were performed by two experienced ophthalmologists using a standard procedure. In particular, a history of systemic thrombosis and evidence for retinal vascular occlusion was sought. Where the posterior segment could not be visualised, patients with an end stage ocular disease (phthisical or completely blind) were assumed to have suffered vaso-occlusive disease of the retina.

Control subjects

Twenty five hospital based healthy control subjects from a similar ethnic background were age and sex matched and had no history or clinical evidence of any systemic or ocular disease. Exclusion criteria were hepatic and renal diseases (chronic renal failure), thyroid disease, diabetes, essential hypertension, congestive heart disease, psychiatric illness, and pregnancies. Since drugs (fibrates, carbamazepine, phenytoin, methotrexate, corticosteroids, cytokotexis, non-steroidals, etc) could affect the levels of measured parameters, detailed histories of medication were obtained in both groups and such patients were not included in the study. None of the subjects had nutritional deficiency or took vitamin supplementation.

All participants in both groups gave their written informed consent and approval of the hospital’s institutional ethics review board was obtained. The laboratory personnel were maintained masked to the clinical diagnosis and group of the subjects, matching each blood sample by letter coding, and so were the clinicians to subsequent Hcy, ET-1, and NO level determinations until the end of the study.

Blood samples

Whole blood samples (totally 10 ml) were drawn using a 25 gauge needle from a peripheral vein, avoiding haemolysis, into plain tubes in the morning hours (08:00–10:00) after an overnight fast and 30 minutes of supine rest. None of the patients and controls had received any topical or systemic medication at least 2 weeks before blood collection. Following an immediate centrifugation of the first three quarters of the blood samples (7.5 ml) at 800 g for 10 minutes at 4°C, serum was collected and kept at −70°C until use.

Serum Hcy analysis

Since the concentration of Hcy in serum increases artificially over time if blood is stored uncooled and uncentrifuged immediately after sampling, owing to the time and temperature dependent release of Hcy from erythrocytes, the blood samples were cooled and centrifuged immediately after collection and the erythrocytes removed. Serum total homocysteine (tHcy) levels were determined by enzyme linked immunosorbent assay (ELISA) kit (Homocysteine Enzyme Immunoassay Kit, Bio-Rad Lab, Oslo, Norway). It is an easy to use immunoassay for the assessment of tHcy levels in serum. The tHcy enzyme immunoassay has highly specific enzymatic sample pretreatment, convenient microplate format, standardised immunoassay procedure, parallel sample processing, and wide dynamic range. Test results correlate well with reference high performance liquid chromatography (HPLC) at a level of at least 0.98.

In addition to the total mean serum levels for Hcy, hyperhomocysteinaemia was defined as a serum tHcy level above the 95th percentile in the control group as described before.

Serum ET-1 analysis

Duplicate serum samples from each subject were assayed for ET-1 by a commercially available ELISA kit (Endothelin-1 Enzyme Immunoassay Kit, Cayman Chem, Ann Arbor, MI, USA). It is a sandwich enzyme immunoassay that permits ET-1 measurements with a detection limit of ∼1.5 µmol/ml. The intra-assay and interassay coefficients of variance (CV) were both less than 10%. The assay allows sensitive and specific (100%) analysis of ET-1 in serum.

Serum total nitrite (NO₂− + NO₃−) analysis

NO is a labile compound with a brief half life and is rapidly converted to the stable end products nitrate (NO₃−) and nitrite (NO₂−) in typical oxygenated aqueous solutions, and is subsequently excreted into urine. Because an excellent and sensitive colorimetric reagent (the Griess reagent) exists for the determination of NO₃−, it is a common practice to use enzymatic or chemical reduction to convert all NO₃− to NO₂−. This is made by NADP (NADPH) dependent nitrate reductase enzyme in the presence of nitrate reductase and NADP, thereby circumventing some of the potential problems reported for NO₃− measurement using nicotinamide adenine dinucleotide phosphate (NADP) dependent nitrate reductases.

In short, serum (250 µl) was incubated at room temperature with 250 µl of substrate buffer (imidazole 0.1 mol/l, reduced NADP (NADPH) 210 µmol/l, flavine adenine dinucleotide, 3.8 µmol/l, pH 7.6) in the presence of nitrate reductase (Aspergillus niger, Sigma) for 45 minutes to convert NO₃− to NO₂−. Excess reduced NADPH, which interferes with the chemical detection of NO₂−, was oxidised by continuation of the incubation of 5 µg
(1 ml) of lactate dehydrogenase (LDH, Sigma), 0.2 mmol/l (120 µl) pyruvate (Sigma), and 79 ml of water. Total nitrite was then analysed by reacting the samples with Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H₃PO₄ spectroquant; Merck, Darmstadt, Germany). Reacted samples were treated with 500 µl of trichloroacetic acid (20%), centrifuged for 15 minutes at 8000 g, and the absorbance at 548 nm was compared with that of NaNO₂ standard (0–100 µmol/l). This method can be used to accurately measure as little as 1 µmol of NO₂⁻ (final concentration in the assay). Very little of the sample is required (5–85 µl for most samples).

Neutrophil count, ESR, and acute phase reactants analysis

The other part of the blood samples (2.5 ml) with EDTA (1 mg/ml) anticoagulant was used for the neutrophil counting by an automated blood counter (Coulter-STKS, Luton, UK). The ESR was determined by the classic Westergren method. The other acute phase reactant (α₁ antitrypsin and α₂ macroglobulin) concentrations were measured in the serum by a Behring nephelometer 100 analyser (Messer Grasheim, Frankfurt, Germany).

Statistics

Results were expressed as means (SD) and were analysed statistically by using Wilcoxon and the Mann-Whitney U test as indicated. p Values below 0.05 were considered to be significant. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows (SPSS Inc, version 8.0, Chicago, IL, USA).

RESULTS

All BD patients included in the present study fulfilled the stated diagnostic criteria. Twenty seven of the 43 (62.7%) had the ocular type of BD with chronic intraocular manifestations of the disease. All patients with BD had oral aphthous stomatitis. Cutaneous lesions were present in 40 patients (93.0%). Eighty eight per cent of cases (n = 38) had genital ulceration and articular symptoms were found in 35 (81.3%). Pathergy test was positive in 23 (53.4%). Nine patients (20.9%) complained of neurological symptoms and two (4.6%) had gastrointestinal complications. Twenty of 43 patients were in the active stage whereas the remaining 23 were in the inactive period.

The overall serum tHcy levels in patients with BD (15.83 (4.44) ng/ml) were higher than healthy control subjects (7.96 (2.66) ng/ml) and the difference was significant (p <0.001). Serum tHcy levels in active subjects (17.79 (3.90) ng/ml) were also significantly (p = 0.006) higher than in inactive patients (14.13 (4.23) ng/ml) and control subjects (p <0.001). In addition, patients with the ocular type of BD have significantly (p <0.001) higher tHcy levels compared with non-ocular BD subjects (18.25 (4.20) versus 13.53 (3.34) ng/ml; Table 1).

The 95th percentile of the tHcy levels in the control group was 12.40 ng/ml. Of the 43 subjects with BD 34 (79.0%) had serum tHcy levels above the 95th percentile in the control group. Hyperhomocysteinaemia was present in 25 (95.5%) of the 27 subjects with ocular BD and nine (56.2%) of the 16 patients with non-ocular BD. Of the 27 ocular patients, 22 were defined as having retinal vascular occlusive disease. Of these, vascular narrowing, obliteration, or vein occlusion was directly observed in 20 patients. The remaining two patients had phthisis bulbi. Twenty of the 22 (90.9%) subjects with retinal vascular occlusive disease had serum tHcy levels above the 95th percentile in the controls.

The overall serum ET-1 levels in patients with BD (17.47 (4.33) µmol/ml) were significantly (p <0.001) higher when compared with control subjects (5.74 (2.34) µmol/ml). In addition, active and ocular BD patients also had significantly higher serum ET-1 concentrations when compared with those in inactive stage and non-ocular disease (19.17 (3.71) versus 15.99 (4.35) µmol/ml; p = 0.014; and 19.40 (5.02) versus 15.62 (2.48) µmol/ml, p = 0.003, respectively). Overall serum total

### Table 1 Serum homocysteine, endothelin-1, and nitric oxide levels in patients with Behçet’s disease and controls with statistical comparisons

<table>
<thead>
<tr>
<th></th>
<th>All BD (n=43)</th>
<th>Active BD (n=20)</th>
<th>Inactive BD (n=23)</th>
<th>Ocular BD (n=27)</th>
<th>Non-ocular BD (n=16)</th>
<th>Control (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36.95 (9.80)</td>
<td>37.35 (9.39)</td>
<td>36.60 (10.34)</td>
<td>38.80 (9.84)</td>
<td>35.18 (9.65)</td>
<td>37.88 (8.73)</td>
</tr>
<tr>
<td>tHcy (ng/ml)</td>
<td>15.83 (4.44)</td>
<td>17.79 (3.90)</td>
<td>14.13 (4.23)</td>
<td>18.25 (4.20)§</td>
<td>13.53 (3.34)</td>
<td>7.96 (2.66)</td>
</tr>
<tr>
<td>ET-1 (µmol/ml)</td>
<td>17.47 (4.33)</td>
<td>19.17 (3.71)†‡</td>
<td>15.99 (4.35)</td>
<td>19.40 (5.02)§</td>
<td>16.62 (2.48)</td>
<td>5.74 (2.34)</td>
</tr>
<tr>
<td>NO (µmol/l)</td>
<td>37.60 (10.31)‖ 41.15 (7.96)†</td>
<td>34.52 (10.76)</td>
<td>42.00 (10.62)‖</td>
<td>33.40 (7.48)</td>
<td>27.08 (7.76)</td>
<td></td>
</tr>
</tbody>
</table>

§Significantly different from the inactive stage by Wilcoxon test (for each, p <0.01) and controls (Mann-Whitney U test; for each, p <0.001).

†Significantly different from the inactives (Wilcoxon test; for each, p <0.05) and controls (Mann-Whitney U test; for each, p <0.001).

‖Significantly different from the non-ocular patients (Wilcoxon test; for each, p <0.01) and controls (Mann-Whitney U test; for each, p <0.001).

### Table 2 Neutrophil count, erythrocyte sedimentation rate, and acute phase reactants (α₁ antitrypsin, α₂ macroglobulin) in patients with active or inactive Behçet’s disease and controls

<table>
<thead>
<tr>
<th></th>
<th>Active BD (n=20)</th>
<th>Inactive BD (n=23)</th>
<th>Healthy controls (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils (10³/ml)</td>
<td>5.64 (1.41)††‡</td>
<td>3.63 (0.78)‡</td>
<td>2.81 (0.41)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>32.88 (8.36)‡‡</td>
<td>18.26 (6.15)†</td>
<td>7.48 (1.85)</td>
</tr>
<tr>
<td>α₁ antitrypsin (mg/dl)</td>
<td>219.34 (39.42)‡</td>
<td>163.19 (20.42)‖‡</td>
<td>121.64 (14.15)</td>
</tr>
<tr>
<td>α₂ macroglobulin (mg/dl)</td>
<td>269.52 (31.58)‖</td>
<td>201.89 (28.54)</td>
<td>165.11 (23.91)</td>
</tr>
</tbody>
</table>

†Significantly different from the inactive stage by Wilcoxon test (for each, p <0.01).

‡Significantly different from the controls by Mann-Whitney U test (for each, p <0.01).
Nitric oxide (NO) levels are increased in BD and correlated with disease activity.9 The results of this study for NO confirm our previous study. Our present findings imply further that NO levels may be another indicator for ocular disease. The levels of ET-1 were also significantly higher among patients with ocular disease in the active stage, and among patients with vaso-occlusive disease compared with non-ocular patients. Whether retinal vascular occlusion in patients with BD is mainly caused by an obliterator peripheralis, thrombosis, or both, remains to be specified.

The main factor responsible for the increased frequency of thrombosis is thought to be endothelial dysfunction in BD.4 Venular endothelium regulates vascular tone by the roles of both vasoconstrictor and vasodilator molecules. In subjects with endothelial dysfunction, vasodilator responses are lost, exacerbating the vasoconstrictor response. ET-1 is a potent vasoconstrictor peptide produced by vascular endothelium and participates in inflammation, cellular injury, and vascular events.9 It has been demonstrated that many cytokines and chemokines induce ET-1 release by endothelium.23 In addition, elevated ET-1 levels are a well known marker of vascular endothelial dysfunction and contribute to ocular pathological manifestations, promoting retinal capillary closure and ischemia.15 16 In this respect, Iannaccone et al have recently demonstrated that increased circulating ET-1 levels in patients with retinal vein occlusion may be a marker of the occlusive event. They suggested that ET-1 homeostasis might be relevant to retinal vein occlusion pathogenesis and retinal ischemic manifestations. Our findings clearly demonstrated that serum ET-1 levels were higher in BD, especially in the active period. In addition, ocular BD patients had significantly higher ET-1 levels than non-ocular subjects. Furthermore, serum tHcy levels were correlated with ET-1 levels at all comparisons. Therefore, these findings suggest that ET-1 and tHcy may be interrelated in endothelial cell activities in BD. On the other hand, NO, synthesised mainly by the endothelium, is a very important molecule for the vascular system. Two important functions of NO are platelet adhesion inhibition and endothelial vasorelaxation.13 Since large amounts of NO are released as a response to systemic immunoinflammatory diseases, we think that the increased NO levels found in our BD patients are a result of inflammatory stimuli, which are the characteristic feature of the disease. Some cytokines known to be involved in the upregulation of endothelial cells may be responsible for the increased NO production. It is possible that increased ET-1 levels could directly favour vascular retinal occlusions. In this process, synthesised ET-1 may outweigh the amount of NO. This observation was possibly caused not only by endothelial cells, but also by polymorphonuclear leucocytes and macrophages found in inflammation since these cells are also known to be involved in the pathogenesis of BD.24 In addition, since increased tHcy and ET-1 have vasoconstricting properties with atherothrombogenic activity, NO may be increased to compensate for these effects with its vasodilatory and adhesion inhibitory properties.

The mechanism of Hcy associated vascular thrombosis and endothelial toxicity is not fully understood. Possible mechanisms include promotion of platelet activation and atherogenesis by oxidative injury, increased adhesiveness, enhanced coagulability, and vascular matrix damage.25 Important in this respect is the prominent features of BD including oblitative retinal vasculitis and thrombosis, the major cause of blindness. Likewise, systemic venous occlusion occurs in 30–40% of all patients.7 It has been demonstrated that coagulation indices including fibrinogen, von Willebrand factor, and plasminogen activator activity are increased in BD,26 but the underlying cause of this thrombotic state and the extent to which it may promote endothelial damage and perivasculitis remain unknown. Furthermore, the association of factor V Leiden, an important mediator in this pathogenesis, has more recently been demonstrated in BD, especially in ocular disease.27 A prevailing hypothesis is that Hcy promotes the
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Clotting cascade via several actions—inactivation of protein C, activation of coagulation factor V, increased vascular smooth muscle cell proliferation, and inhibition of thrombomodulin. Therefore, it is plausible that there may be an association between serum tHcy and BD and our results are consistent with these hypotheses. The high percentage of patients with bilateral vaso-occlusive disease who manifested hyperhomocysteinaemia will have to be confirmed by other studies. As a result, tHcy may be another risk factor for the development of vascular disease in BD patients, especially in retinal vascular occlusive disease.

In conclusion, we demonstrated for the first time that serum tHcy levels are increased in BD and correlated with disease activity. Likewise, tHcy levels were higher among patients with ocular disease compared with non-ocular disease and healthy control subjects. In addition, serum tHcy levels are positively correlated with serum ET-1 and NO levels at all comparisons. If thrombogenetic events do indeed contribute to retinal or systemic vaso-occlusion in BD, and if higher tHcy levels are responsible for this, anticoagulant therapy and the role of some vitamins, known to affect tHcy levels, may warrant investigation. The clinical inference is that measurement, treatment, and monitoring of tHcy levels may be valuable in the management of patients with BD, not only in active stage but also in those with ocular disease.

References

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