Asymmetric Dimethylarginine and Nitric Oxide Levels as Signs of Endothelial Dysfunction in Behcet’s Disease

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Abstract. Behcet’s disease (BD) has been known for many years, yet the etiology of the systemic vasculitis remains unknown. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthase. ADMA is involved in endothelial dysfunction in various vascular diseases and its level in BD is unclear. This study was performed to evaluate the relationship between ADMA and NO levels in plasma of patients with BD. There were 3 groups of 30 subjects: (a) controls, (b) BD patients with mucocutaneous involvement, and (c) BD patients with vascular involvement. Plasma NO levels were assayed by spectrophotometry and plasma ADMA levels were assayed by an ELISA test. Plasma ADMA levels were higher in both groups of BD patients than in the controls; the ADMA levels were higher in the BD patients with vascular involvement than in the mucocutaneous group. Plasma NO levels were lower in both groups of BD patients than in controls; plasma NO levels were lower in the BD patients with vascular involvement than in the mucocutaneous group. In the combined groups of 60 BD patients, there was significant inverse correlation between the plasma concentrations of ADMA and NO (r = -0.570, p <0.001). Plasma lipid profiles did not differ significantly between the BD patients and the controls. These results are evidence for increased plasma ADMA levels and decreased plasma NO levels as risk factors for cardiovascular events in BD patients. Inhibition of NO synthesis by ADMA may contribute to vascular involvement in BD.

Keywords: nitric oxide, Behcet’s disease, asymmetric dimethylarginine (ADMA), vasculitis, endothelial inflammation

Introduction

Behcet’s disease (BD) was described in 1937 by a Turkish dermatologist, Hulusi Behcet [1]. The estimated prevalence of BD is 8-10/10,000 people in Japan, the Middle East, and some Mediterranean countries, while it is less common in the populations of North Europe and America [2]. BD is an inflammatory disorder with unknown etiology [2,3]. Perivasculitis is a characteristic feature of BD, and up to 25% of BD patients have systemic venous thrombosis [3-7]. Endothelial dysfunction is considered to play a major role in the development of these lesions.

Nitric oxide (NO), a molecule of key importance for the vascular system, is synthesized by endothelial cells. NO is a mediator of immunity and inflammation, and its functions include inhibition of platelet adhesion and endothelial vasodilatation [8]. NO is produced by 3 isoforms of nitric oxide synthase (NOS): type I neuronal (nNOS), type II inducible (iNOS), and type III endothelial (eNOS)

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Abbreviations: ADMA, asymmetric dimethylarginine; BD, Behcet’s disease; CRP, C reactive protein; DDAH, dimethylaminohydrolase; ESR, erythrocyte sedimentation rate; NO, nitric oxide; NOS, nitric oxide synthase.

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[9]. Only iNOS is a Ca^{2+}-independent isoform; it is induced by cytokines during inflammatory and infectious processes and produces large amounts of NO [10].

Risk factors for endothelial dysfunction in vascular diseases impact upon endothelial function by decreasing NO bioavailability [11,12]. NO has an important effect on regulation of systemic blood pressure and local blood flow [12]. Reduced bioavailability of NO is believed to play a role in atherosclerotic vascular damage [8,9,12]. Dimethylarginines, which exist as symmetric and asymmetric molecules, are analogues of L-arginine [13]. Asymmetric dimethylarginine (ADMA) is an endogenous analogue of L-arginine that can modulate NO production, whereas symmetric dimethylarginine is biologically inactive [14] and is an endogenous competitive inhibitor of NO synthase [13]. Increased plasma ADMA level has been observed in vascular diseases and is considered to be a vascular risk factor [12,14]. ADMA inhibits the production of NO in cultured endothelial cells and isolated blood vessels [15,16]. Although studies have indicated an association between high ADMA levels and endothelial dysfunction [13,14], its role in the pathogenesis of BD is unclear. There have been many studies on oxidants and endothelial dysfunction in patients with BD [9,11-13], but few data are available on plasma NO and ADMA levels in BD patients.

Delineation of the pathogenic mechanism is important to the treatment of BD. The present study was aimed at comparing plasma NO and ADMA levels in BD patients vs healthy controls matched by age and sex, as well as measuring other biochemical parameters in patients with BD.

Materials and Methods

**Chemicals.** Except for the NO, ADMA, and CRP assay kits, reagents were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA) and organic solvents from Merck KGaA (Darmstadt, Germany). The total NO assay kit was obtained from Active Motif, Inc. (Carlsbad, CA, USA). The ADMA ELISA assay kit was obtained from Cardio Vasics Medical Science Labs (Palo Alto, CA, USA). The CRP assay kit was obtained from Alfa Wasserman BV (Woerden, The Netherlands). Reagent solutions were prepared each day and kept in a refrigerator at 4°C. The reagents were equilibrated at room temperature for 0.5 hr before use.

**Subjects.** The protocol was approved by the Ethics Committee of the Medical Faculty and all subjects volunteered for the trial. There were 60 BD patients (39 men, 21 women; age 22 to 57 yr; mean age 31 yr) including 30 with mucocutaneous BD and 30 with vascular involvement. None of the patients had alcohol abuse problems. Prior to the blood collection, the patients had not received any systemic therapy that might affect cellular immunity. The control group consisted of 30 healthy volunteers precisely matched for age and sex. The women who were included in the study had not been taking oral contraceptives for >6 mo prior to the blood collection.

The BD patients were assigned to 2 groups according to the predominance of vascular or mucocutaneous involvement based upon their hospital records. According to the recommendations of the International Study Group (ISG), the criteria for diagnosis of BD were the presence of 2 of 4 features, including (a) recurrent genital ulceration, (b) eye lesions, (c) positive inflammation pathergy test, and (d) skin lesions in addition to recurrent ulceration [17]. At the time of the study the patients who met at least three ISG criteria and who had C reactive protein (CRP) titers >8.0 mg/L and erythrocyte sedimentation rates (ESR) >20 mm/hr were considered to be in the active stage of the disease.

**Blood collection.** After informed consent was obtained from all patients and control subjects, whole blood samples (4 ml) were drawn with a 25-gauge needle from a peripheral vein, avoiding hemolysis, into EDTA-containing tubes. Blood collection occurred in the morning hours (8-10 am) after overnight fast and 30 min of supine rest.

One-half of the blood sample was used immediately for hematological analyses. The other half was centrifuged at 800 x G for 10 min; the supernatant plasma was removed for assays of biochemical and inflammatory markers, which were performed within 6 hr after collection. Plasmas were stored for <3 mo at -20°C pending the NO and ADMA assays.

**NO and ADMA assays.** Total NO assay was performed by spectrophotometry at 540 nm using a NO assay kit according to the manufacturer’s instructions. The assay was based on nitrate and nitrite determinations. Plasma ADMA level was determined by an ADMA ELISA kit according to the manufacturer’s instructions. The results were expressed in µmol/L.

**Other assays.** Blood neutrophils were counted with an automated blood counter (Coulter General S system 2, Beckman-Coulter Co, Miami, FL, USA). Blood ESR was determined by the Westergreen method. Plasma CRP and fibrinogen assays were performed with a Dade-Behring BN Prospective analyzer (Louisville, KY, USA). Plasma triglycerides, glucose, urea, creatinine, HDL-, LDL- and total cholesterol assays were determined by routine procedures using an automated analyzer (Hitachi Co., Tokyo, Japan).

**Statistics.** Results are expressed as means ± SD. Statistical analyses were performed using SPSS version 9.0 software (SPSS Inc. Chicago, IL, USA). The Chi-square test and the independent-sample t test were used to calculate p values.
ANOVA was used for multiple comparisons. Correlation coefficients were obtained by linear regression analysis; p values <0.05 were regarded as significant.

Results

The overall means in male vs female patients were not significantly different for any of the investigated parameters. ANOVA showed that the relationship of assay results in patients vs healthy controls was similar for men and women. Therefore, the data for men and women were combined.

Oral lesions were present in all BD cases (100%). Skin lesions were present in 96%, articular symptoms in 92.3%, genital ulcerations in 90.3%, and ocular involvement in 50% of BD patients. The pathery test was positive in 86.7% of BD patients. Neurologic symptoms were recorded in 13.4% of BD patients, and 4 patients (6.7%) had neuro-BD. Systolic and diastolic blood pressures did not differ significantly in the control group vs the groups of BD patients with vascular or mucocutaneous involvement.

As shown in Table 1, mean plasma ADMA levels were significantly higher in the entire group of 60 BD patients vs the controls (p <0.001) and mean plasma NO levels were significantly lower in the entire group of 60 BD patients vs the controls (p <0.001). In the 60 BD patients, there was significant inverse correlation between the plasma levels of ADMA and NO (r = -0.570, p <0.001). In all cases, the residual analyses indicated that the standard normal and equal variance assumptions were adequately satisfied.

Mean plasma ADMA levels were significantly higher in the 30 BD patients with vascular involvement vs the 30 BD patients with mucocutaneous involvement (p <0.01). Mean plasma NO levels were significantly lower in the 30 BD patients with vascular involvement vs the 30 BD patients with mucocutaneous involvement (p <0.05) (Table 1).

As shown in Table 2, the mean concentrations of plasma lipid constituents and the plasma levels of glucose, creatinine, and urea did not differ significantly between the BD patients and the controls. Measurements of plasma CRP and blood ESR were significantly higher in the BD patients vs the controls (p <0.001), although the blood total leukocyte counts (WBC) did not differ in the BD patients vs controls (Table 3).

| Table 1. Nitric oxide (NO) and asymmetric dimethylarginine (ADMA) levels in patients with Behcet’s disease (BD). |
| Subjects | plasma NO | plasma ADMA |
| Healthy controls | 27.50 ± 2.53 | 0.86 ± 0.56 |
| n = 30 | | |
| Mucocutaneous BD patients (n = 30) | 21.23 ± 2.15 | 2.59 ± 0.83 |
| Vascular BD patients | 15.04 ± 1.31 | 3.77 ± 1.26 |
| n = 30 | | |
| All BD patients | 18.13 ± 2.05 | 3.18 ± 1.21 |
| n = 60 | | |

| Table 2. Biochemical constituents in plasma of controls and patients with mucocutaneous BD and vascular BD. |
| Plasma constituents | Healthy controls (n = 30) | Mucocutaneous BD (n = 30) | Vascular BD (n = 30) |
| Glucose (mg/dl) | 79.0 ± 8.9 | 79.6 ± 7.2 | 83.5 ± 7.3 |
| Cholesterol (total) (mg/dl) | 180.4 ± 17.6 | 179.7 ± 17.5 | 171.9 ± 24.9 |
| Triglycerides (mg/dl) | 113.4 ± 57.7 | 120.3 ± 36.4 | 104.5 ± 49.1 |
| HDL-cholesterol (mg/dl) | 44.4 ± 10.1 | 39.5 ± 12.6 | 41.8 ± 11.1 |
| LDL-cholesterol (mg/dl) | 114.7 ± 17.9 | 112.7 ± 27.4 | 102.2 ± 21.1 |
| Creatinine (mg/dl) | 0.90 ± 0.11 | 0.93 ± 0.14 | 0.84 ± 0.18 |
| Urea (BUN) (mg/dl) | 13.8 ± 3.7 | 18.8 ± 4.8 | 12.9 ± 4.4 |

Data are means ± SD; results are expressed in µmol/L.

\( ^a \) p <0.001 vs healthy controls.

\( ^b \) p <0.05 vs mucocutaneous BD patients.

\( ^c \) p <0.01 vs mucocutaneous BD patients.
Table 3. Levels of plasma C-reactive protein (CRP), blood erythrocyte sedimentation rate (ESR), and blood leukocyte count (WBC) in healthy control subjects and patients with Behcet’s disease (BD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>BD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 30)</td>
<td>(n = 60)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>5.38 ± 2.25</td>
<td>32.10 ± 18.70*</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>6.63 ± 3.33</td>
<td>35.45 ± 11.15*</td>
</tr>
<tr>
<td>WBC (10^3/mm^3)</td>
<td>7.50 ± 2.11</td>
<td>8.91 ± 3.18</td>
</tr>
</tbody>
</table>

Data are means ± SD.
* p <0.001 vs controls.

Discussion

In patients with BD, risk of endothelial dysfunction is substantially increased [5-7]. In addition to the well known cardiovascular risk parameters (such as elevated levels of CRP and fibrinogen), there are several newly defined cardiovascular risk factors [3,5,6]. Evidence increasingly suggests that ADMA is also a potential candidate, which enhances vascular complications in patients with inflammatory diseases that resemble BD [13,21]. The results of the present study indicate that decreased levels of plasma NO and increased levels of plasma ADMA are associated with vascular involvement in BD patients. The inflammatory character of BD is attended by inhibited activity of endothelial dimethylaminohydrolase (DDAH), causing accumulation of ADMA and inhibition of NO synthesis [20].

NO, which is also known as endothelial-derived relaxing factor, is a gas that transmits signals in the organism and is responsible for the cytotoxicity of activated macrophages [19,21]. NO has a complex role in immune functions. Immunologic and inflammatory stimuli induce the peroxidation of NO over long periods, and NO exerts cytotoxic and cytostatic effects not only against invading cells, but also against healthy cells [21]. Important in this respect has been the recent focus on the role of the endothelium in inflammation. Primary actions of NO include vasodilatation and inhibition of platelet aggregation [20,21].

Two isoforms of NOS (eNOS and nNOS) are constitutive and Ca^{2+}-calmodulin dependent. They are rapidly activated by intracellular Ca^{2+} fluxes and produce small quantities of NO. On the other hand, the Ca^{2+}-independent isofom, iNOS, which is located in endothelial cells and macrophages, is involved in cellular immunity. When iNOS is activated by inflammatory cytokines, interferon gamma, bacterial lipopolysaccharides, and endotoxins during inflammatory and infectious processes, large amounts of NO are generated [4,8,10].

Endothelial dysfunction appears to be responsible for the increased frequency of thrombosis in BD. Chambers et al [5] reported that decreased activity of endothelial-derived NO leads to vasoconstriction, platelet aggregation, and monocyte adhesion and that, separately or in combination, these may lead to the vascular dysfunction seen in BD. Kiraz et al [22] reported that NO levels are higher in BD disease than controls, and that NO-associated injury of tissues, particularly the endothelium, may be important in the etiopathogenesis of vasculitis in BD. Evereklioglu et al [4] observed a significant difference in serum NO levels between patients and controls. They suggested that increased NO production might be responsible for the overall inflammatory process of BD and concluded that NO was related to the presence of BD. However, we found that plasma NO levels are lower in patients with BD than in controls, and are lower in BD patients with vascular involvement vs mucocutaneous involvement. Our findings are consistent with the reports of Buldanlioglu et al [19] and Orem et al [23], who also noted decreased NO production in BD patients compared to control groups.

Reduction of NO production is associated with increased platelet aggregation and leukocyte adhesion that contribute to vascular dysfunction and thrombosis [24]. The inhibition of NO synthesis accelerates vascular involvement [11], while long-term supplementation with L-arginine inhibits atherosclerosis and vascular involvement [25]. Increased plasma concentration of ADMA occurs in vascular involvement [11], skin lesions of systemic sclerosis [26], and inflammation [18,27].
These disorders are all risk factors for the development of BD [2]. To our knowledge, there is no previous report on the relationships between ADMA and BD and vascular involvement. In the present study, we found an inverse correlation between plasma ADMA and NO levels. Previously, it has been demonstrated that under certain conditions, nitrosylation, caused by NO itself, may diminish DDAH enzyme activity and lead to accumulation of ADMA [20,28,29].

CRP is an active component of the phagocytic system of polymorphonuclear (PMN) leukocytes. When neutrophils and macrophages are stimulated by pathogens, cytokines and other mediators of inflammation, such as CRP, are liberated from granules into the cytoplasm and play an important role in destroying phagocytosed materials. CRP and ESR levels are sensitive markers of inflammation and they reflect the activity of BD. Kokcam and Naziroglu [3] and Proni et al [31] found that the superoxide scavenging activity of PMN is lower in BD patients with elevated ESR and CRP levels than in control subjects.

In conclusion, the finding that ADMA accumulation may be a factor in the pathogenesis of BD may have important implications for therapy. ADMA competes with L-arginine for binding to NO synthase [30]. The administration of L-arginine reverses the endothelial dysfunction associated with vascular risk factor [25]. Therefore, we suggest that L-arginine might be considered as a potential therapy for Behcet’s disease.

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References

different patterns in heart transplant recipients and individuals with essential hypertension. Transplantation 2002;74:1395-1400.


