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Serum nitric oxide, asymmetric dimethylarginine, and plasma homocysteine levels in active Behçet's disease

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Aim: Behçet's disease is a relapsing vasculitis characterized by endothelial dysfunction. This study was conducted to determine the levels of nitric oxide (NO) and 2 related parameters, asymmetric dimethylarginine (ADMA) and homocysteine levels, in relation to the pathogenesis of Behçet's disease.

Materials and methods: A total of 49 Behçet's patients, comprising 26 patients with the active disease and 23 patients with the inactive disease, and a healthy control group of 24 individuals participated in this study. International Study Group Diagnostic Criteria were followed in the diagnosis of Behçet's disease; patients who had at least 2 signs were considered as having the active disease.

Results: Serum NO and plasma homocysteine levels were found to be significantly higher in patients with active Behçet's disease compared to patients with inactive Behçet's disease and healthy controls. In active and inactive Behçet's patients, serum ADMA levels were significantly higher than those of healthy controls. No statistically significant difference was found between patients with inactive Behçet's and healthy controls with respect to serum homocysteine levels.

Conclusion: The increased serum levels of ADMA presumably cause endothelial dysfunction because of a deficiency in NO production, which also appears to be involved in the vasculitis of Behçet's disease.

Key words: Nitric oxide, asymmetric dimethylarginine, homocysteine, Behçet's disease

Introduction

Behçet's disease (BD) is a chronic systemic disorder characterized by oral ulcerative lesions, ocular and cutaneous manifestations, neurologic features, arthritis, and cardiovascular involvement. The main histopathology of BD is vasculitis and neutrophilic or monocytic vascular inflammation; it can involve large, medium, or small vessels (1). Nitric oxide (NO), a molecule synthesized by endothelial cells, plays a pivotal role as a regulator during the onset of immunological and inflammatory reactions (2,3). Besides its vasodilator effects on vascular smooth muscle cells, it acts as a signaling molecule in endothelial and nerve cells and as a killer molecule in activated immune cells. NO is the most important molecule as it regulates the functions of the endothelium and maintains the health of endothelial functions. Endothelial dysfunction due to the reduced bioavailability of NO is involved in the course of atherosclerotic cardiovascular disease. NO is synthesized from L-arginine via the action of NO synthase. Dimethylarginines, which exist as symmetric and asymmetric molecules, are analogs

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of L-arginine (4). Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthases (NOS). ADMA levels are elevated in conditions associated with an increased risk of atherosclerosis, such as hyperhomocysteinemia, impaired renal function, and obesity (5–7). In vitro as well as clinical data suggest that by inhibition of endothelial NO synthesis, ADMA may contribute directly to endothelial dysfunction (2,4). An increased plasma ADMA level has been observed in vascular diseases and is considered to be a vascular risk factor (8). Homocysteine (Hcy), a sulfur-containing amino acid, is primarily derived from demethylation of dietary methionine. Increased plasma Hcy levels have become widely accepted as an independent risk factor for cardiovascular disease (9). Previous in vivo and in vitro studies suggest that Hcy limits the bioavailability of NO. The increased plasma levels of ADMA presumably cause endothelial dysfunction because of a deficiency in NO production, which also appears to be involved in the vasculitis of BD (5,10). The purpose of this study was to determine the serum levels of NO, ADMA, and Hcy in patients with BD and to evaluate its correlation with disease activity.

Materials and methods

Patients with BD were recruited from the Departments of Ophthalmology and Dermatology. Included in the study were 49 patients, 14 males and 35 females, who fulfilled the International Study Group Criteria for the Diagnosis of BD (1). The approval of the ethics committee was obtained, along with informed consent forms from all patients and controls. Twenty-four age- and sex-matched healthy subjects of similar ethnic origin, 7 males and 17 females, were also included in the study as a control group. The mean age was 34.1 ± 8.9 years in the patient group and 34.5 ± 6.2 years in the control group. BD patients were divided into active and inactive periods by clinical findings. Patients with at least 2 of the major symptoms (oral ulcers, genital ulcers, skin lesions, and eye involvement) were regarded as being in the disease's active period (1,11), and subjects who had been lesion-free for at least 30 days were regarded as being in the disease's inactive period. There were 26 patients (20 females and 6 males, mean age 34.2 ± 9.0 years) in the active and 23 patients (16 females and 7 males, mean age 34.0 ± 8.8 years) in the inactive period of BD. Patients with folic acid or vitamin B_{12} deficiency, diabetes mellitus, hyperlipidemia, chronic hepatitis, renal failure, and chronic alcoholism were excluded from the study. Moreover, patients using some medications and vitamins such as vitamin B_{12} and folic acid, methotrexate, and L-dopa were not included in this study. Whole blood samples (approximately 8 mL) were drawn from a peripheral vein in the morning hours (0800 to 1000 hours) after an overnight fast for use in NO and ADMA assays and for Hcy assay with K-EDTA. After centrifuging the blood samples at 2000 × *g* for 10 min, serum and plasma samples were collected and kept at −80 °C until use.

Serum concentrations of ADMA were measured by immunochemical quantification with a commercial enzyme-linked immunosorbent assay (ELISA) kit (ADMA® ELISA, DLD Diagnostika GmbH, Germany). Serum NO levels were also determined by the colorimetric method (colorimetric assay, Oxford Biomedical Research, USA). Plasma Hcy concentrations were measured by a commercially available microparticle enzyme immunoassay (AxSYM analyzer, Abbott Laboratories, USA).

Statistical analysis was performed with SPSS 13 for Windows (SPSS Inc., USA). The Mann–Whitney U test was used to compare the data obtained from patient and control groups. In addition, the Kruskal–Wallis test was used for triple comparison of the data obtained from active, inactive, and control patients. Paired comparison of the groups with statistically significant results was performed using the Mann–Whitney U test and adjustment for multiple testing was made by applying a Bonferroni correction. $P < 0.05$ was regarded as statistically significant.

Results

All results are summarized in Tables 1 and 2. Serum ADMA levels in patients with BD were significantly ($P < 0.001$) higher when compared with control subjects. In addition, patients with inactive BD also had significantly higher serum ADMA concentrations when compared with those in the active stage. Despite the high levels of NO compared with the control group, this was not statistically

Table 1. Serum NO, ADMA, and plasma Hcy levels in BD patients and healthy controls with statistical comparisons (Mann–Whitney U Test, mean ± standard deviation).

	NO (µmol/L)	ADMA (µmol/L)	Homocysteine (µmol/L)
Behçet's disease (n = 49)	23.1 ± 7.5	1.01 ± 0.32	11.8 ± 6.6
Healthy controls (n = 24)	25.4 ± 8.1	0.55 ± 0.09	7.8 ± 1.6
	P = 0.250	P < 0.001	P = 0.011

significant (P = 0.250). When the serum NO levels for Behçet's patients in both active and inactive periods were compared to those in the control group, there were significant differences (P < 0.001). Plasma Hcy levels in patients with BD were higher than in the healthy control subjects and the difference was statistically significant (P = 0.011). Plasma Hcy levels in patients with active BD were also significantly higher than in patients with inactive BD (P = 0.001) and insignificantly higher than in control subjects.

Discussion

BD is a recurring inflammatory disorder characterized by 4 major findings: oral aphthous ulcers, ocular lesions, skin lesions, and genital ulcerations, with inflammation also occasionally occurring in other tissues and organs such as the cardiovascular system, central nervous system, gastrointestinal tract, lungs, kidneys, and joints (1,2). We have learned much about the diagnosis, follow-up, and treatment of BD in the past 20–30 years, although we are far from a cure for the disease. Current studies are mainly performed to invent novel markers for the follow-up of the disease activity and for the response to the treatment of BD.

NO is a vital molecule in the vascular system that is synthesized by the endothelium. Many vascular diseases were reported to be associated with dysregulation of NO. It was reported that vascular inflammation is the main pathophysiologic mechanism in BD (3,12). Vascular inflammation leads to dysfunction and destruction of the endothelium. Several markers of endothelial dysfunction, like decreased NO, were reported to be associated with the presence of BD (11,13). 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 (12). Several studies investigating NO levels in BD were reported. Some of these studies reported increased NO levels in BD, whereas others reported decreased levels. Yapışlar et al. (14) reported increased platelet aggregation and decreased NO levels in 33 patients with BD, whereas Evereklioglu et al. (15) reported increased NO levels in patients with BD compared to controls, especially during acute exacerbations. In this study, we found an insignificant decrease in NO levels in patients with BD compared to controls. However, NO levels were increased in the active BD group compared to the inactive disease group and the control group. NO levels were significantly decreased in the inactive disease group compared to the controls. The findings of our study are comparable with the findings of

Table 2. Serum NO, ADMA, and plasma Hcy levels in active or inactive BD patients and healthy controls (Kruskal–Wallis Test, mean ± standard deviation).

	NO (µmol/L)	ADMA (µmol/L)	Homocysteine (µmol/L)
Active BD (n = 26)	31.3 ± 9.9	0.91 ± 0.28	13.7 ± 6.6
Inactive BD (n = 23)	15 ± 5.1	1.11 ± 0.37*	9.9 ± 6.7
Healthy controls (n = 24)	25.4 ± 8.1	0.55 ± 0.09	7.8 ± 1.6**
	P < 0.001	P < 0.001	P = 0.001

Bilateral comparisons statistically significant except for *inactive vs. active BD not significant, and **healthy controls vs. inactive BD not significant.

Evereklioglu et al (15). The activity level of BD is critical in studies evaluating NO levels in BD. As the disease activity is the main determinant of NO levels in BD, previous studies that assessed NO levels in BD by comparing BD and control groups without checking the disease activity would not be reliable.

It is well known that ADMA leads to endothelial dysfunction by decreasing NO levels in BD. Therefore, it may be postulated that increased ADMA might lead to endothelial dysfunction by decreasing NO. In their previous report, Sahin et al. (16) revealed increased ADMA levels in BD patients with vascular and mucocutaneous involvement compared to control subjects. Their study showed significantly increased ADMA levels in BD patients with vascular involvement compared to BD patients with mucocutaneous involvement and to control subjects. We found significantly increased ADMA levels in patients with BD compared to control subjects in the present study, which was comparable to the findings of Sahin et al. Our inactive BD group had higher ADMA levels than our active BD group. Decreased NO and increased ADMA levels in the inactive BD group in the present study might be explained by the fact that ADMA is the competitive inhibitor of NOS, although lower ADMA levels in the active BD group compared to the inactive BD group seem to be controversial. Although studies have indicated an association between high ADMA levels and endothelial dysfunction (8), ADMA's role in the pathogenesis of BD is unclear. The increased level of ADMA in BD patients is thought to be associated with increased synthesis and/or decreased breakdown of ADMA molecules. It was suggested that oxidative stress can cause the level of ADMA to rise, increasing methylation of L-arginine and inhibiting enzymes from breaking down ADMA (16,17). It was shown that the parameters indicative of oxidative stress increase in BD (13). Therefore, oxidative stress can be blamed for the increased ADMA levels in BD. Because reactive oxygen species are produced at sites of endothelial inflammation, parameters of oxidative stress are increased in BD (18). Increased oxidative stress leads to increased levels of ADMA by increasing L-arginine methylation and by decreasing the activity of dimethylarginine dimethylaminohydrolase (DDAH). Increased levels of ADMA might be associated with increased oxidative stress in BD. Furthermore, increased ADMA levels lead to increased oxidative stress in the endothelium by decreasing NO synthesis in a vicious cycle (19). 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). Given that BD is a type of vasculitis, ADMA levels are increased during the inflammatory process and our results are consistent with those of other studies. Kökçam and Bakar Dertlioğlu (2) also found that NO and ADMA levels of the patients with active BD and the ADMA levels of the patients with inactive BD were significantly higher than those of the healthy controls. A recent study reported that treatment with acetylsalicylic acid (ASA, 150 mg/day), a drug with antiinflammatory, antithrombotic, and analgesic therapeutic properties, significantly reduced ADMA levels related to the antioxidant protection of ASA in volunteer participants. Moreover, their study showed that NO levels increased slightly but not significantly and that Hcy levels reduced slightly after ASA treatment compared to the baseline values (21).

Hcy, found in several forms in plasma, is the product of methionine metabolism and is an independent risk factor for cardiovascular diseases. Hcy leads to accelerated atherosclerosis by destruction of endothelium cells as well as induction of proliferation of vascular smooth muscle cells (10,22). Endothelial cells neutralize the toxic effects of Hcy by secreting Hcy-binding NO. This protective effect of NO is lost in the case of long-lasting exposure of the endothelium to hyperhomocysteinemia, since Hcy decreases secretion of endothelial NOS by increasing lipid peroxidation. Consequently, impaired NO production causes the endothelium to be exposed to Hcy-mediated oxidative injury, which results in endothelial dysfunction (23,24). It was postulated that Hcy increases ADMA levels by acting in 3 steps. The first step is the increasing of the activity of protein arginine methyltransferase, the enzyme that transfers methyl residue to the arginine in proteins. The second step is the decreasing of the activity of DDAH, the enzyme that degrades ADMA. The third step is the increasing of protein turnover. By way of these mechanisms, Hcy increases ADMA levels and decreases NO levels. Previous studies reported increased Hcy levels in endothelial dysfunction (9,25).

Significantly increased Hcy levels in BD patients with vascular involvement, compared to control subjects and BD patients with mucocutaneous involvement, were reported in 2 studies (26,27). Sarican et al. (28) reported significantly increased Hcy levels in patients with active BD compared to controls and patients with inactive BD, and they could not find any significant difference between the last 2 groups with respect to Hcy levels. Comparable to those findings, Er et al. reported increased Hcy levels in BD patients with ocular involvement compared to BD patients without ocular involvement and in active BD compared to inactive BD (29).

Vasculitis-associated endothelial dysfunction is recognized as the main cause of increased thrombosis in BD, although its pathogenesis remains to be elucidated. The main evidence for this proposal is increased thrombomodulin, which is induced by endothelial dysfunction, in patients with BD. It is well-known that hyperhomocysteinemia is an independent risk factor for thrombosis. Hyperhomocysteinemia increases thrombosis by its toxic effects on endothelial cells, by increasing free radical formation, by inhibiting vasodilation, and by activating the coagulation system (16,30).

In conclusion, identifying the etiopathogenic role of NO would aid in the development of novel therapeutic approaches in BD. For this reason, further studies on NO metabolism, oxidative stress, and inflammation in BD subgroups would improve the diagnosis and treatment of the disease.

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