Serum prolidase enzyme activity and oxidative status in patients with Behçet's disease

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Objectives: To assess serum prolidase enzyme activity and oxidative stress in patients with Behçet's disease (BD).

Methods: The study population consisted of BD patients ($n = 42$) and healthy participants ($n = 29$). BD patients were classified as active ($n = 18$) or inactive ($n = 24$) according to disease activity. Serum prolidase enzyme activity, total antioxidant status (TAS), total oxidative status (TOS), oxidative stress index (OSI), and malondialdehyde (MDA) levels were measured.

Results: In BD patients with active disease, serum prolidase activity was significantly higher compared with the inactive and control participants. Serum prolidase activity was also significantly higher in all BD patients in comparison with controls. Serum prolidase activity was also positively correlated with OSI, C-reactive protein, and active BD. MDA, TOS, and OSI levels were all significantly higher in the BD group when compared with the healthy control participants. Serum TAS levels were significantly lower in BD patients in comparison with healthy controls.

Conclusion: High prolidase activity may indicate critical biological activities relevant to pathological events in BD, and this activity may be a biological indicator of disease. Further studies are needed to verify these findings.

Keywords: Prolidase, Oxidative status, Behçet disease

Introduction

Behçet's disease (BD) is a chronic progressive disorder that affects many bodily systems. BD is characterized by endothelial dysfunction and the production of excessive reactive oxygen species (ROS).¹ Common manifestations include oral and genital ulcers, skin lesions, uveitis, and arthritis.^{2,3} Immunoregulatory abnormalities have been proposed as possible pathogenic mechanisms, but the etiopathogenesis of BD remains unknown.⁴

Mucocutaneous lesions in BD are characterized by vasculitis with intravascular conglomerates of polymorphonuclear leukocytes, endothelial cell swelling, and fibrinoid necrosis.⁵ ROS released from active neutrophils and high concentrations of lipid peroxide products are responsible for endothelial dysfunction in BD.^{1,6} Increased ROS production and decreased

levels of antioxidants have also been reported in BD patients.^{7,8} In addition, the measurement of breakdown products, such as malondialdehyde (MDA), is commonly used to determine the degree of ROS-induced lipid peroxidation,^{9,10} and MDA levels are higher in BD patients.⁴ There is also a significant correlation between total antioxidant status (TAS) and total oxidative status (TOS) in BD patients.²

Prolidase, an enzyme found in human and animal tissues, plays an important role in collagen catabolism and synthesis. Prolidase activity is altered in fibrotic disease and chronic wounds and affected by oxidative stress.^{11,12} Several studies also reported that prolidase, via the expression of growth factors and transcription factors (e.g. vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 α (HIF-1 α)), may be an important factor in many physiological and pathophysiological processes, including wound healing, inflammation, and angiogenesis.¹³ It has also been reported that inhibitors of prolidase activity

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also inhibit transforming growth factor-beta 1 and the expression of its receptors.¹³ Prolidase overexpression also increases nuclear HIF-1 α and VEGF levels.¹⁴ VEGF is an important factor in angiogenesis and inflammation, which are codependent mechanisms that contribute to BD pathophysiology.¹⁵

Prolidase activity may play a role in BD pathogenesis because of its effects on VEGF. However, the relationship between BD and prolidase activity is unclear and has not been extensively studied. The aim of this study is to investigate serum prolidase enzyme activity in BD patients and determine its relationship with serum oxidative status.

Materials and methods

This study was conducted between May 2012 and May 2013 at the Department of Physical Therapy and Rehabilitation, Faculty of Medicine, Dicle University, Diyarbakir, Turkey. BD patients ($n = 42$) and healthy volunteers ($n = 29$) were included in this study. Diagnoses were made according to the criteria of the International Study Group for Behçet's disease.¹⁶ These criteria require recurrent oral ulceration and ≥ 2 other findings, such as recurrent genital ulceration, ocular inflammation, skin lesions, or positive pathergy test. All patients underwent complete ophthalmologic and systemic examinations to define ophthalmic and systemic involvement and disease activity. All patients underwent routine blood tests and determination of erythrocyte sedimentation rate (ESR), TAS, MDA, and serum prolidase activity. Although some studies report using the clinical activity index,¹⁷ other studies report disease activity.^{18,19} In this study, clinical activity was assessed according to the methods used in previous studies. Patients were classified with active ($n = 18$) or inactive BD ($n = 24$) according to the clinical and laboratory findings. Active BD was defined by worsening clinical symptoms at clinical evaluation and ≥ 3 other major criteria (e.g. oral or genital ulcerations, anterior/posterior panuveitis, papulopustular or pseudofollicular skin lesions, positive pathergy test). The control group included sex- and age-matched healthy volunteers without systemic and ocular pathologies ($n = 29$).

Patients with the following conditions were excluded from this study: inflammatory rheumatic disease; infectious or endocrine-related arthropathy; pregnancy; lactation; chronic disorders, including coronary artery disease, diabetes mellitus, hypertension, dyslipidemia, and renal disease; malignant, systemic, or local infection; and history of chronic alcohol consumption or smoking within the past year. All BD patients were receiving colchicine treatment (1.5 mg/kg/day); in addition, 18 patients also received azathioprine (100–150 mg/kg/day) and cyclosporine (100–150 mg/kg/day) during the study period.

The local ethics committee approved this study. All recruited participants provided informed consent before participation.

Biochemical analyses

Fasting blood samples were immediately centrifuged at $4000 \times g$ for 10 minutes, and serum was transferred to Eppendorf tubes and stored at -50°C until analysis. Serum prolidase activity (U/l) was measured using the spectrophotometric method,²⁰ which determines prolidase activity according to the prolidase-produced proline level. The supernatant was diluted 2-fold with serum physiological saline (0.9% NaCl). Twenty-five microliters of the mixture was preincubated with 75 μl of the preincubated solution (50 mmol/l Tris HCl buffer (pH 7.0) containing 1 mmol/l glutathione and 50 mmol/l MnCl_2) at 37°C for 30 minutes. The reaction mixture, which contained 144 mmol/l glycine-proline (100 μl (pH 7.8)), was incubated with 100 μl of the preincubated sample for 5 minutes at 37°C . To stop the incubation reaction, 1 ml glacial acetic acid was added. Acetic acid undergoes the typical chemical reactions of carboxylic acid. Upon treatment with a standard base, acetic acid converts to metal acetate and water and all enzymatic activity stops. After adding 300 μl Tris HCl buffer (pH 7.8) and 1 ml ninhydrin solution (3.0 g/dl ninhydrin dissolved in 0.5 mol/l orthophosphoric acid), the mixture was incubated at 90°C for 20 minutes and cooled on ice. Absorbance was measured at a 515 nm to determine the concentration of proline according to the method proposed by Myara *et al.*,²¹ which is a modified version of Chinard's method.²² The intra- and interassay coefficients of variation of this assay were both $<7\%$.

The TAS and TOS levels of the serum samples were evaluated using the automated methods developed by Erel.^{23,24} TAS results are expressed as mmol Trolox equivalent/l, and the TOS results are expressed as $\mu\text{mol H}_2\text{O}_2$ equivalent/l. The percent ratio of TOS:TAS was used as the oxidative stress index (OSI).²⁵ The OSI value was calculated using the following formula: OSI (arbitrary units) = TOS (nmol H_2O_2 equivalent/l)/TAS (nmol Trolox equivalent/l).²⁶ MDA was measured according to the procedure described by Ohkawa *et al.*²⁷

Statistical analysis

All data are expressed as the mean $\pm$ 1 standard deviation. The 1-sample Kolmogorov–Smirnov test was used to determine whether the data demonstrated a normal distribution. Intergroup comparisons were performed using the Mann–Whitney *U*-test and Student's *t*-test. Relationships between variables were analyzed using either Pearson or Spearman correlation analyses, depending on the distributions of the

variables. In this study, $P < 0.05$ is considered significant. All data were analyzed using SPSS statistical software (version 18.0 for Windows; IBM Corporation, Armonk, NY, USA).

Results

In this study, 25 males and 17 females comprised the BD patients. Seven patients demonstrated vascular involvement and all 42 patients were diagnosed with thrombophlebitis; however, 0 patients presented with arterial aneurysm. The central nervous system was compromised in one patient with cerebral venous thrombosis. Ocular involvement was observed in 29 patients. The mean duration of BD involvement was 5.6 ± 6.5 years, and 29 (69%) patients demonstrated positive pathergy tests. The clinical features of the BD patients are presented in Table 1.

The mean ages of the BD patients and the control group participants were 34.1 ± 9.7 and 33.2 ± 9.5 years, respectively. There were no statistically significant differences between the groups in terms of age or sex. Higher serum white blood cell ($P = 0.02$), C-reactive protein (CRP) ($P = 0.01$), and neutrophil activity levels ($P = 0.02$) were observed in BD patients in comparison with the healthy participants. However, there were no statistically significant differences between groups in terms of mean hematocrit or ESR. The demographic and laboratory data of the BD and control groups are presented in Table 2.

Serum prolidase enzyme activity ($P = 0.013$), MDA ($P = 0.029$), TOS ($P = 0.014$), and OSI ($P = 0.001$) were all significantly higher in BD patients. Serum prolidase enzyme activity was significantly higher in active BD patients than patients with inactive disease ($P = 0.021$). Fig. 1 shows the mean prolidase enzyme activity levels of the active and inactive patients. Serum TAS levels were significantly lower in BD patients than healthy controls ($P = 0.001$). The serum prolidase activity, TAS, TOS, OSI, and MDA levels of the BD patients and healthy participants are shown in Table 3.

Serum prolidase activity was positively correlated with OSI ($P = 0.05$; $r = 0.243$), CRP ($P = 0.037$; $r = 0.237$), and active BD ($P = 0.01$; $r = 0.39$).

Table 1 Clinical features of BD patients

Clinical features	n	%
Oral ulcer	42	100
Skin involvement	36	85.7
Genital ulcer	33	78.5
Arthritis	18	42.8
Ocular involvement	29	69
Positive pathergy test	29	69
Thrombophlebitis	7	16.6
CNS involvement	1	2.3

BD, Behçet's disease; CNS, central nervous system.

Table 2 Demographic and laboratory data of patients with Behçet's disease and control participants

	BD (n = 42)	Control (n = 29)	P
Age (y)	34.11 ± 9.78	33.24 ± 9.51	0.29
Sex (female/male)	17/25	10/19	0.63
Active/inactive disease	18/24	—	—
Neutrophil activity	7.15 ± 9.43	4.40 ± 1.65	0.02
Mean hematocrit (%)	39.75 ± 3.94	39.26 ± 3.66	0.66
Mean CRP (mg/dl)	1.39 ± 1.10	0.71 ± 0.46	0.01
Mean ESR (mm/hour)	11.60 ± 7.41	9.12 ± 5.91	0.14
Mean WBC (K/UI)	8.73 ± 2.92	7.15 ± 2.16	0.02

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cell count; BD, Behçet's disease.

Figure 1 Serum prolidase enzyme activity in patients with active and inactive BD. BD, Behçet's disease.

Table 3 Serum TAS, TOS, OSI, MDA, and prolidase activity in BD patients and control participants

	BD (n = 42)	Control (n = 29)	P
Prolidase (U/l)	4.68 ± 0.59	3.96 ± 1.19	0.013
TAS (nmol Trolox Equiv./l)	1.54 ± 0.18	1.72 ± 0.23	0.001
TOS (nmol H ₂ O ₂ Equiv./l)	15.37 ± 6.44	11.58 ± 5.87	0.014
OSI	10.01 ± 4.03	6.79 ± 3.35	0.001
MDA (nmol/g)	5.81 ± 2.40	4.61 ± 2.33	0.029

TAS, total antioxidant capacity; TOS, total oxidative status; OSI, oxidative stress index; MDA, malondialdehyde; BD, Behçet's disease.

However, serum prolidase activity did not demonstrate any significant correlation with TAS, MDA, or white blood cell count.

Discussion

BD is an inflammatory disease that is characterized by recurrent oral aphthous ulcers and numerous systemic manifestations. The underlying cause of BD remains unknown, but it is associated with the pathogenesis of diseases related to endothelial cell dysfunction, humoral immune defects, and immune system dysregulation.²⁸ Many of the clinical manifestations of

BD are associated with vasculitis, and endothelial dysfunction is a characteristic of BD.²⁹ Endothelial activation mediates vascular inflammation and thrombosis in BD,^{30,31} and increased oxidative stress has been observed in BD patients.¹⁷ Abnormal neutrophil function and ROS have been proposed as factors involved in the etiology and pathophysiology of BD. Vascular and endothelial tissue damage in BD might reflect the enhanced production of ROS by activated neutrophils.³² A recent study reported that papulopustular BD lesions might be associated with leukocytoclastic vasculitis and IgM, IgG, C3, and fibrin deposits, which is consistent with immune complex vasculitis.³³

Currently, oxidant/antioxidant status,^{2,34} plasma and erythrocyte MDA levels,^{6,35} and advanced oxidation protein products¹⁹ are commonly measured as indexes of oxidative stress in BD patients. In this study, we measured serum prolidase enzyme activity in BD patients. To the best of our knowledge, this is the first study to report significantly higher serum prolidase activity in BD patients in comparison with healthy controls.

Prolidase participates in collagen metabolism and is expressed in both human and animal tissues. Prolidase plays a vital role in end-stage collagen catabolism, as well as the degradation of dietary proteins,³⁶ and participates in the final rate-limiting step of collagen catabolism.³⁷ Prolidase activity increases during extracellular matrix remodeling, which demonstrates a high rate of collagen turnover.³⁸ In fibroblast cultures, increased prolidase activity is triggered by increased cell density.³⁹ Altered prolidase activity is also associated with oxidative stress and fibrosis-characterized diseases.^{40,41} Several studies suggest that prolidase, via the regulation of growth factors and transcription factors, may be an important factor in many physiological and pathophysiological processes, including wound healing, inflammation, and angiogenesis.¹³ In this study, we observed significantly higher serum prolidase activity levels in BD patients compared with healthy controls. Additionally, serum prolidase enzyme activity was significantly higher in active BD patients compared with inactive patients. Serum prolidase activity was also positively correlated with active BD status, CRP, and OSI.

ROS could also cause reversible or irreversible damage in all types of biochemical materials. Biological membranes and intracellular components are easily affected by free radicals. The oxidation of membrane lipids is one of the primary mechanisms of oxidative cellular damage. The measurement of breakdown products, such as MDA, is the most common approach for determining the degree of lipid oxidation. Recently, studies have reported that

MDA is significantly higher in BD patients compared with healthy controls.^{6,34,42} Buldanlioglu *et al.* suggest that this increase in MDA confirms increased oxidative stress in BD patients.⁴ The results of this study show that serum MDA is significantly higher in BD patients, and may confirm increased oxidative stress in BD patients. High MDA also shows that BD patients are under oxidative stress.

TAS is an indicator of the overall function of the enzymatic and nonenzymatic antioxidant molecules in plasma. TAS is also a marker of superoxide radical-retaining activity in plasma. In this study, serum TAS levels were significantly lower in BD patients compared with healthy controls. Similarly, Onur *et al.* reported decreased TAS levels in BD patients with respect to healthy controls.³⁵ Buldanlioglu *et al.* also reported that serum TAS levels are significantly lower in BD patients than controls.⁴ These results show that the antioxidant defense system is deficient or inadequate in BD patients. However, here we report significantly higher serum TOS and OSI levels in BD patients. In some studies, lower plasma TAS levels were found in patients with active and inactive BD in comparison with control groups;^{4,43} however, no differences between active and inactive groups were reported. Likewise, we found lower plasma TAS levels in patients with active BD in comparison with inactive BD, but the difference was insignificant.

All of our patients were using drugs to treat BD. These drugs included colchicine, azathioprine, and cyclosporine, and these drugs may impact oxidative status. However, we did not ask our patients to stop taking their medications for extended periods of time before blood sample collection. All patients were drug-free on the day that blood samples were drawn; however, this is an inadequate amount of time to clear all drug effects. Although data is lacking on this topic, the potential effects of medications on serum prolidase activity, TAS, TOS, and MDA should be kept in mind when evaluating our results.

In another study, psoriasis patients received topical and systemic immunosuppressant treatments, and no significant differences in serum prolidase activity were reported between groups.⁴⁴ Another study suggests that nonsteroid anti-inflammatory drugs affect collagen metabolism by inhibiting prolidase activity in collagen-synthesizing cells.⁴⁵ Colchicine is an anti-inflammatory drug, and its effects on prolidase activity are variable.⁴⁶ All of our patients were using colchicine, and future studies may demonstrate more powerful results if only patients without significant medical histories are included.

Acute phase reactants, such as CRP, ESR, and white blood cells, are important markers that may aid in the diagnosis and determination of medical

treatment in patients with inflammatory diseases. In this study, we found that the mean neutrophil count, CRP, and white blood cell count were statically higher in the BD group compared with the control group, but ESR did not differ between groups.

This study is a preliminary investigation of prolidase activity in BD patients, and some limitations must be taken into account. This study includes a limited number of patients and was not randomized. Importantly, BD patients were accustomed to receiving certain therapies to manage their conditions. To further clarify these issues, prospective and randomized studies with larger samples are needed.

Conclusion

Serum prolidase enzyme activity and oxidative stress were both significantly higher in BD patients in comparison to healthy controls. Serum prolidase activity was significantly higher in patients with active disease compared with the inactive and control groups. These data demonstrate a previously unreported association between serum prolidase enzyme activity and BD. Increased prolidase activity may be associated with critical biological activities relevant to the pathology of BD, especially during the active period. However, randomized, controlled clinical studies are needed that include a larger number of patients.

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