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Anticoagulant and Fibrinolytic Disorders in Patients with Behçet's Disease and Recurrent Aphthous Ulcer

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Abstract

Behçet's disease (BD) is a chronic multisystemic inflammatory disorder characterized by recurrent oral and genital aphthous ulcers, uveitis and skin lesions. Recurrent aphthous ulcer (RAU) is the most prevalent oral mucosal disease in humans. The pathogenesis and thrombopoiesis of BD and RAU have not been fully clarified. To reveal the haemostatic dysfunctions in the patients with BD and RAU, we evaluated the levels of coagulant, anticoagulant and fibrinolytic parameters in these patients.

Factor VIII clotting activity (FVIII:c), protein C antigen (PC:Ag), total protein S antigen (TPS:Ag), tissue-type plasminogen activator antigen (t-PA:Ag), plasminogen activator inhibitor-1 antigen (PAI-1:Ag) and D-dimer were detected in 24 BD, 58 RAU patients and 50 controls. Results showed that levels of PC:Ag, TPS:Ag, PAI-1:Ag and D-dimer were significantly elevated in both BD and RAU patients compared with controls ($P < 0.01$). PAI-1:Ag was even higher in BD patients than in RAU patients (74.99 ± 12.28 *vs*. 69.57 ± 13.11, $P < 0.05$), whereas the level of t-PA:Ag was significantly reduced in patients with BD and RAU ($P < 0.01$). In patients with RAU, PC:Ag was lower in major aphthous ulcer (MjAU) group than in minor aphthous ulcer (MiAU) group ($P < 0.05$). The expression of FVIII:c was significantly elevated in MiAU patients compared with controls ($P < 0.01$), while no difference was observed between MjAU patients and controls ($P > 0.05$). Our studies showed that there were anticoagulant and fibrinolytic disorders in BD patients, which may be responsible for diminished fibrinolysis in BD. Some haemostatic parameters may be correlated with the severity of RAU.

Key Words: Behçet's disease, recurrent aphthous ulcer, coagulation, anticoagulation, fibrinolysis

Introduction

Behçet's disease (BD) is a chronic multisystemic inflammatory disorder characterized by recurrent oral and genital aphthous ulcers, uveitis and skin lesions (18). BD can affect nearly all systems and organs, including the vascular system, central nervous system, gastrointestinal tract, lungs, kidneys and joints (19). This disease is distributed worldwide, but a higher prevalence was found among the Asian and Eurasian populations along the Silk Route stretching to countries of the Mediterranean region (23). The etiology and pathogenesis of BD have not been fully clarified. However, several factors, such as immune dysregulation, genetic susceptibility, inflammatory mediators, infectious agents and oxidative stress, are assumed to be involved in the onset of this disease.

BD is a vasculitis affecting vessels of different types, sizes and localizations. Vasculitis and perivascular inflammatory infiltrates are the predominant

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histopathologic lesions of BD. Thrombophilia or thrombophlebilis involving small and large veins is very common, whereas arteritis is rare. Venous thrombosis appeared to be the major vascular involvement in 7-33% of patients with BD, and represents 85-93% of vasculo-BD (10). Vasculitic endothelial injury may trigger or enhance the pathological hemostatic process and thrombophilic factors such as protein C, factor VIII, t-PA and PAI-1, could contribute to thrombosis in BD (12). Although haemostatic studies in BD support an imbalance towards a prothrombotic state at different levels, a defect specific to BD that can explain this thrombotic tendency has not been defined so far (2, 31).

Recurrent aphthous ulcer (RAU), also known as recurrent aphthous stomatits, recurrent oral ulcer, or simple or complex aphthosis, is the most prevalent oral mucosal disease in humans. RAU occurs in men and women of all ages, races and geographic regions and it is estimated that approximately 20% individuals has at least once been afflicted with aphthous ulcers (6). Lehner classified the lesions of RAU into three groups—minor aphthous ulcers (MiAU), major aphthous ulcers (MjAU) and herpetiform ulcers (HU) (14). Attacks may be precipitated by local trauma, stress, food intake, drugs, hormonal changes and vitamin and trace element deficiencies. Local and systemic conditions and genetic, immunological and microbial factors all may play a role in the pathogenesis of RAU. However, to date, no principal cause has been discovered (21). These ulcers are both clinically and histologically identical to those observed in BD (11), and Bang *et al*. have purposed that highly recurrent RAS can be a warning signal for BD (3) although the exact relationship between these diseases is still unknown.

This study aimed to compare the levels of coagulant, anticoagulant and fibrinolytic parameters of BD patients to RAU patients and healthy controls, which could help to reveal the haemostatic dysfunctions in the patients. The study would provide the theoretical basis for further research on the mechanism of the pathogenesis and thrombopoiesis of BD and RAU.

Materials and Methods

Subjects

Patients and controls were recruited from Stomatology Hospital of Shandong University (Jinan, Shandong, PRC) from October 2006 to June 2008. The patient group comprised 24 patients with BD (14 male and 10 female, aged 15 to 59 years, median age 30 years) and 58 patients with RAU (25 male and 33 female, aged 12 to 65 years, median age 35 years). All patients with BD fulfilled the criteria for diagnosis of Behçet's disease according to the International Study Group (ISG)*. Six of these patients with BD had histories of thrombotic complication. Thirteen patients were in the active phase of the disease, and eleven patients were inactive. Patients with RAU had only an aphthous oral ulcer, recurring at least three times a year. RAU patients consisted of 35 patients with minor aphthous ulcer (MiAU), 19 with major aphthous ulcer (MjAU) and 4 with herpetiform ulcer (HU) according to Lehner's classification. None of the patients with RAU had vascular complication. All of the BD and RAU patients were taking no drugs that could affect coagulation within two weeks before blood collection, though some patients had used corticosteroids and/or immunosuppressives two months before. The control group consisted of 50 healthy individuals (29 male and 21 female, aged 19 to 52 years, median age 32 years) from the same geographical area as the patients. All patients and controls gave their written informed consent for this study, and the study protocol was approved by the local institutional ethics committee.

Blood Samples

Blood samples were drawn from subjects after an overnight fast and were collected in tubes containing 3.8% trisodium citrate, which were centrifuged within one hour. The samples were centrifuged at 3,000 rpm at 4°C for 15 min and the separated plasma was stored immediately at –80°C.

Analysis of Factor VIII Clotting Activity

Factor VIII clotting activity (FVIII:c) assays were performed on an ACL-200 full automatic coagulation analyzer (Coulter Coagulation System, Miami, FL, USA) with reagents and protocols from the manufacturer (Beckman Coulter, Fullerton, CA, USA), and were expressed as a percentage of reference activity.

Analysis of Protein C, Protein S, t-PA, PAI-1 and D-Dimer

Measurements of protein C antigen (PC:Ag), total protein S antigen (TPS:Ag), tissue-type plasminogen activator antigen (t-PA:Ag), plasminogen activator inhibitor-1 antigen (PAI-1:Ag) and D-dimer were performed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's in-

*Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. Lancet 335: 1078-1080, 1990.

Table 1. Expression of FVIII:c, PC:Ag, TPS:Ag and D-dimer in patients with BD or RAU and healthy controls

	BD (n = 24)	RAU (n = 58)	Controls (n = 50)
FVIII:c (%)	91.54 ± 33.46	92.88 ± 36.87	80.82 ± 14.76
PC:Ag (mg/L)	4.70 ± 0.84*	4.68 ± 0.94*	2.41 ± 0.76
TPS:Ag (mg/L)	40.03 ± 4.47*	39.68 ± 5.05*	22.71 ± 6.07
D-dimer (mg/L)	0.57 ± 0.30*	0.59 ± 0.24*	0.32 ± 0.11

FVIII:c, factor VIII clotting activity; PC:Ag, protein C antigen; TPS:Ag, total protein S antigen. BD, subjects with Behcet's disease; RAU, subjects with recurrent aphthous ulcer; Controls, healthy subjects. Data were expressed as means ± SD; *$P < 0.01$, compared with controls.

structions (Sunbio, Shanghai, PRC).

Statistical Analysis

Data were expressed as means ± SD. Statistical analysis was performed using one-way ANOVA, Dunnett *t*-test and LSD test. $P < 0.05$ was considered statistically significant.

Results

Increased Expression of PC:Ag, TPS:Ag and D-dimer in Patients with BD or RAU

The levels of FVIII:c, PC:Ag, TPS:Ag and D-dimer were presented in Table 1. Significantly increased expression of PC:Ag, TPS:Ag and D-dimer was observed in patients with BD as well as in patients with RAU compared with that in healthy controls ($P < 0.01$). But no difference was found between BD patients and RAU patients. Though the levels of FVIII:c were higher in BD or RAU patients than in healthy controls, there was no significant difference between patients and controls ($P > 0.05$).

Reduced Expression of t-PA and Elevated Expression of PAI-1 in Patients with BD or RAU

As shown in Fig. 1a, the levels of t-PA:Ag were significantly lower in patients with BD and RAU (12.63 ± 4.53 and 13.40 ± 8.57) than in healthy controls (19.58 ± 7.15; $P < 0.01$). On the contrary, the expression of PAI-1:Ag was significantly elevated in both BD patients (74.99 ± 12.28) and RAU patients (69.57 ± 13.11) compared with that in healthy controls (33.81 ± 4.86; $P < 0.01$; Fig. 1b). Furthermore, such elevation was significantly higher in BD patients than in RAU patients ($P < 0.05$; Fig. 1b).

Anticoagulant and Fibrinolytic Factors in Patients with MiAU and MjAU

To further investigate the expression of anticoagulant and fibrinolytic factors in patients with RAU, we classified RAU patients into MiAU and MjAU groups. The results showed that PC:Ag, TPS:Ag, D-dimer and PAI-1:Ag were significantly increased in both MiAU and MjAU patients compared with controls ($P < 0.01$; Table 2) whereas the expression of t-PA:Ag was significantly decreased in patients with MiAU

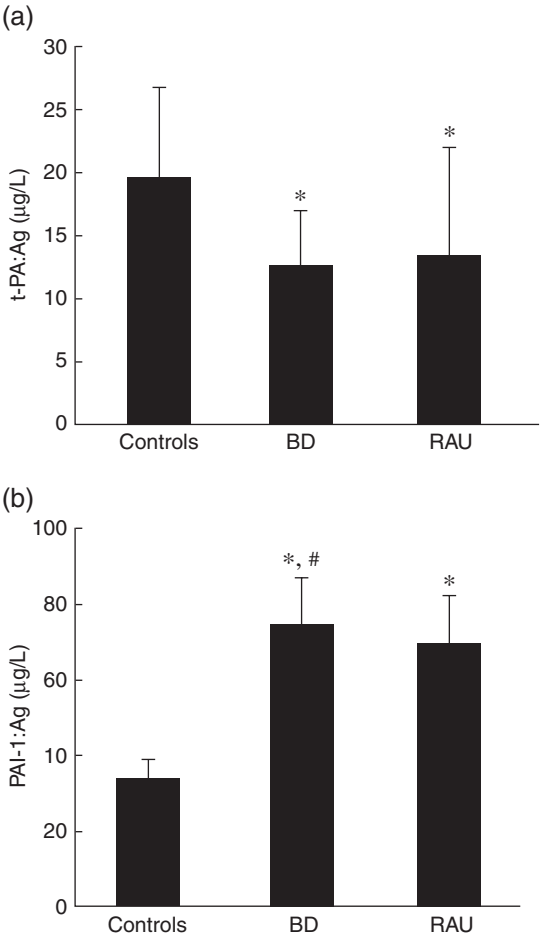

Fig. 1. The expression of t-PA:Ag and PAI-1:Ag in BD and RAU. (a) The expression of t-PA:Ag was significantly lower in patients with BD and RAU than in healthy controls. (b) The expression of PAI-1:Ag was significantly elevated in both BD and RAU patients. Results represent the means ± SD. *$P < 0.01$, compared with controls. #$P < 0.05$, compared with RAU patients.

Table 2. Expression of PC:Ag, TPS:Ag, D-dimer, PAI-1:Ag and t-PA:Ag in patients with MiAU, MjAU and in healthy controls

	MiAU (n = 35)	MjAU (n = 19)	Controls (n = 50)
PC:Ag (mg/L)	4.92 ± 0.95*	4.30 ± 0.80*, #	2.41 ± 0.76
TPS:Ag (mg/L)	40.80 ± 4.86*	38.73 ± 4.88*	22.71 ± 6.07
D-dimer (mg/L)	0.59 ± 0.24*	0.57 ± 0.28*	0.32 ± 0.11
PAI-1:Ag (μg/L)	66.83 ± 14.15*	72.47 ± 10.84*	33.81 ± 4.86
t-PA:Ag (μg/L)	14.31 ± 9.97*	12.24 ± 6.17*	19.58 ± 7.15

PC:Ag, protein C antigen; TPS:Ag, total protein S antigen; PAI-1:Ag, plasminogen activator inhibitor-1 antigen; t-PA:Ag, tissue-type plasminogen activator antigen. MiAU, subjects with minor aphthous ulcer; MjAU, subjects with major aphthous ulcer; Controls, healthy subjects. Data were expressed as means ± SD; *$P < 0.01$, compared with controls; #$P < 0.05$, compared with MiAU.

and MjAU ($P < 0.01$; Table 2). The level of PC:Ag was lower in the MjAU group than in the MiAU group ($P < 0.05$; Fig. 2a). The expression of FVIII:c was significantly elevated in MiAU patients (98.00 ± 37.08) compared with controls (80.82 ± 14.76; $P < 0.01$; Fig. 2b), while no difference was observed between MjAU patients (88.26 ± 37.82) and controls (80.82 ± 14.76; $P > 0.05$; Fig. 2b).

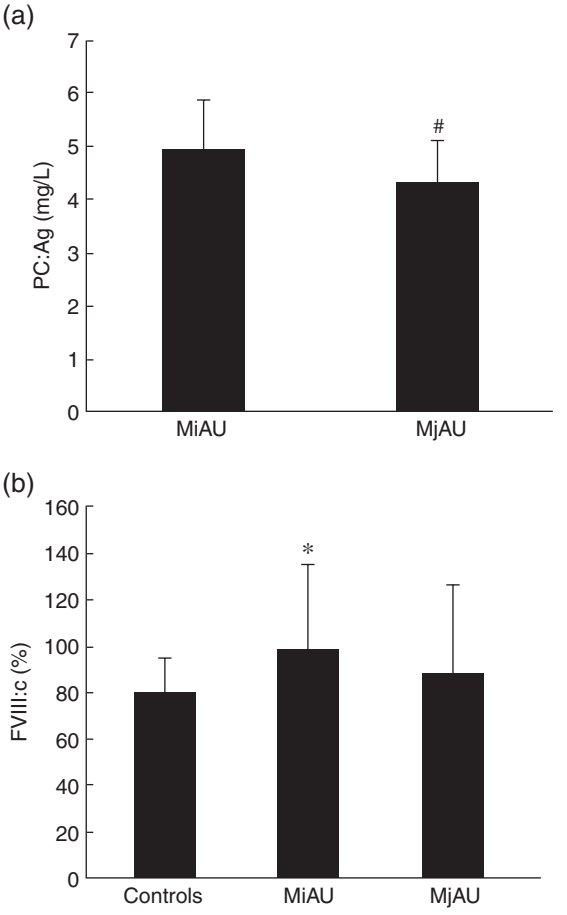

Fig. 2. The expression of FVIII:c and PC:Ag in MiAU and MjAU. (a) The level of PC:Ag was lower in the MjAU group than in the MiAU group. (b) The expression of FVIII:c was significantly elevated in MiAU patients. Data were expressed as means ± SD. #$P < 0.05$, compared with MiAU patients. *$P < 0.01$, compared with controls.

Discussion

Behçet's disease gives rise to a chronic relapsing systemic vasculitis involving arteries and veins of various sizes. The vascular involvement may consist of thrombophlebitis, deep vein thrombosis and arterial obstruction (1). Venous or arterial thrombosis occurs in 25% (10~37%) of patients. Venous thrombosis is more common than arterial thrombosis (88% *vs.* 12%). Deep and superficial venous thrombosis of the legs predominates (16). Why such thrombosis is so common in patients with BD is still unclear.

Endothelial cell injury or dysfunction seems to be a triggering factor in the prothrombotic state of BD (8, 9). Both Ozoran *et al*. (25) and Demirer *et al*. (4) found that the level of von Willebrand factor (vWF)—an endothelial product—was significantly higher in patients with BD, which supports endothelial destruction due to vasculitis related with BD. Demirer *et al*. (4) also reported significantly higher levels of tissue plasminogen activator (t-PA) in 127 patients with BD compared with 24 healthy age-matched controls. However, Ozoran *et al*. (25) found that t-PA levels were significantly lower in the active BD patient group than in the inactive and control groups with higher levels of plasminogen activator inhibitor (PAI). High levels of PAI from the accumulation of thrombocytes on the damaged surface of endothelium were thought to lead to a decrease in t-PA levels and inhibition of fibrinolytic activity. Hampton *et al*. (7) reported a higher plasma concentration of PAI-1 in BD patients, but no difference in t-PA. However, Yurdakul *et al*. (30) found the t-PA levels in BD with acute deep vein thrombosis (ADVT) were significantly

lower than those in patients with ADVT due to other causes, while PAI-1 levels did not show significant differences between the groups. In our study, the levels of t-PA were significantly lower in patients with BD than in healthy controls. On the contrary, PAI-1 expression was significantly elevated in BD patients. We also found increased expression of D-dimer in patients with BD. These results suggested that reduced t-PA and increased PAI-1 secretion from endothelial cells (as a result of vascular damage) might be responsible for diminished fibrinolysis in BD patients.

We also detected the factor VIII clotting activity in patients with BD. Though the levels of FVIII:c were higher in BD and RAU patients (91.54 ± 33.46% and 92.88 ± 36.87%) than in healthy controls (80.82 ± 14.76%), there was no significant difference between patients and controls. This was consistent with no difference of factor VIII coagulant activity reported by Hampton *et al*. (7). However, Leiba *et al*. (15) found that BD patients with thrombosis had significantly higher mean levels of factor VIII than those without thrombosis. Probst *et al*. (26) also reported a raised mean value of factor VIII in patients diagnosed with ocular BD. Most striking was 79% of patients had levels of factor VIII above 130% and 67% of patients with factor VIII activity levels above 150% correlated with a five-fold increase in risk of thrombosis.

Other coagulation parameters have also been studied. Most studies were unable to demonstrate abnormality of coagulation specific to BD (4, 7). In a study of 96 patients with BD, no differences were found with respect to fibrinogen level, prothrombin time (PT), and partial thromboplastin time (PTT) among patients with or without vascular attacks and controls. Furthermore, there was no correlation between the activity of disease and coagulation parameters (29). Espinosa *et al*. (5) studied 38 patients with BD (13 with venous thrombosis) and found the mean level of prothrombin fragments 1 + 2 was elevated in BD patients. But this level did not differ between patients with or without thrombosis. Similarly, Ozatli *et al*. (24) described significantly higher levels of activated FVII and fibrinogen in patients with BD, but no correlation with histories of thrombosis was found.

The role of protein S, protein C or antithrombin III levels in the pathogenesis of thromboembolic complications in BD is debatable. Most studies were unable to demonstrate lower levels or reduced activity of these proteins (4, 7, 17, 20, 28, 29). Mader *et al*. (17) reported that patients with BD did not have decreased protein C, protein S, antithrombin III activity or activated protein C resistance. Lee *et al*. (13) found that the level of antithrombin III was significantly lower in BD patients but no differences were observed in the levels of protein C or protein S activities. However, our results showed significantly increased expression of protein C and total protein S in patients with BD as well as in patients with RAU compared with healthy controls. These results were consistent with those reported by Navarro *et al*. (22). In the study of 39 BD patients, protein S level was higher in patients than in controls, whereas activated protein C (APC) level was significantly lower in patients than in controls. Reduced APC levels were associated with the high incidence of venous thromboembolism in BD (22). These differences could be due to a different disease activity status in different series.

Recurrent aphthous ulcer occurs in all patients diagnosed with BD. Oral ulceration is the initial clinical feature of up to 86.5% of adults and children with BD (1). However, it is difficult to predict with certainty those patients initially presenting with RAU who will subsequently proceed to develop multisystem involvement as part of BD (27). Bang *et al*. (3) examined the prognosis of the clinical relevance of recurrent oral ulceration in BD and found that approximately half the patients, who were initially diagnosed as RAU-only, developed other manifestations of BD in an average of 7.7 years after onset. They reported that highly recurrent RAU had appeared to be a warning signal for BD. Thus, the presence of a relatively specific laboratory marker may substantially facilitate the diagnosis of BD, and possibly support a diagnosis before all disease manifestations have occurred.

In our study, coagulant, anticoagulant and fibrinolytic parameters were detected in BD patients compared with RAU patients. The expression of protein C, protein S and D-dimer was increased, while t-PA was reduced in patients with RAU as well as in patients with BD. However, no difference was observed between RAU patients and BD patients. Remarkably, the expression of PAI-1 was significantly elevated in both BD (74.99 ± 12.28) and RAU patients (69.57 ± 13.11) compared with that in healthy controls (33.81 ± 4.86). Furthermore, such elevation was even higher in BD patients than in RAU patients ($P < 0.05$). The results suggest the abnormal fibrinolytic activity in BD is due to increased inhibition of tissue plasminogen activator.

All three types of RAU, minor, major and herpetiform, can be found in BD and there are no features which differentiate the oral ulcers in BD from those of RAU. We further observed the above parameters in different types of RAU. Interestingly, the levels of protein C and FVIII clotting activity were lower in the MjAU group than in the MiAU group. These data suggested that the levels of protein C and FVIII clotting activity might be correlated with

the severity of RAU.

In conclusion, there are abnormalities of anticoagulant and fibrinolytic factors in this group of patients with BD and RAU. Reduced t-PA and increased PAI-1 secretion from endothelial cells may be responsible for diminished fibrinolysis in BD. Some haemostatic parameters may be correlated with the severity of RAU. Further studies with larger patient populations, particularly with a large number of patients with vascular complications, are essential reaching a definitive conclusion about the role of anticoagulant and fibrinolytic disorders in BD and RAU.

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