Vascular endothelial growth factor and monocyte chemoattractant protein-1 in Behçet's patients with venous thrombosis

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ABSTRACT

Objective. Vascular lesions can in volve both arterial and venous systems which are often the major causes com plicating the disease course of Behçet's disease (BD). Vascular endothelial growth factor (VEGF) is a stimulant of angiogenesis secondary to ischemia while monocyte chemoattractant pro tein 1 (MCP-1) is induced by shear stresses leading to vascular collateral development. MCP-1 has been also shown to contribute to the recanaliza tion of venous thrombi. Tumor necrosis factor-alpha (TNF- α) is known to play a major role in the pathogenesis of BD. Furthermore, up-regulation of secreted MCP-1 and VEGF was observed fol lowing stimulation with TNF-a. In view of the above functions of VEGF, MCP-1 and TNF- α , we hypothesized that these factors may be important in the pathogenesis of thrombosis seen in BD.

Methods. A total of 36 patients with a diagnosis of BD were studied. BD pa tients were separated into 3 groups with respect to vascular involvement. Group BD-AT (n=9) with acute throm bosis, BD-CT (n = 12) with chronic thrombosis and BD-MC (n = 15) with mucocutaneous involvement only. The control group (group H) was com prised of 20 healthy persons. In addi tion, patients with acute, DC-AT (n=11) and patients with chronic DC-CT(n = 9) thrombosis without BD served as disease controls. Serum measurements of VEGF, MCP-1 and TNF-a were per formed by quantitative sandwich ELI -SA. The acute phase reactants, includ ing erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were also measured.

Results. The levels of VEGF were sig nificantly higher in the patients in group BD-AT than either in group BD- *CTor BD-MC* (p = 0.03 and p < 0.001, respectively). However, no significant difference was found for VEGF levels of thrombotic patients regarding the cause (BD-AT vs. DC-AT, p = 0.063; BD-CT vs. DC-CT, p = 0.084) or the stage of thrombosis (DC-AT vs. DC-CT, p > 0.05). Both BD patients and disease controls with acute thrombosis had significantly higher levels of MCP-1 as compared to corresponding chron ic thrombosis patients (BD-AT vs. DC-*CT*; p < 0.001; *DC*-*AT* vs. *DC*-*CT*, p <0.001). Patients with BD and disease controls had significantly higher serum TNF- α level when compared with healthy subjects. No significant differ ence with respect to serum TNF- α level was noted when patient subgroups with BD and disease controls were com pared with each other. Serum levels of VEGF, MCP-1, and TNF- α were not found to be correlated with either ESR or CRP (p > 0.05).

Conclusions. Increased levels of VEGF and MCP-1 detected in BD thrombosis suggest the possible role of those angiogenic cytokines in the path - ogenesis. Although not specific for BD, detection of VEGF or MCP-1 levels seems to serve as an assay for differen - tiation of BD patients with acute thrombosis from chronic.

Introduction

Behçet's disease (BD) is a systemic vasculitis of obscure origin, characterized mainly by recurrent oral and genital ulcers and uveitis (1). However, the disease can present with articular, neurological, pulmonary, intestinal, and vascular manifestations other than this classical triad (2). Vascular lesions can involve both arterial and venous systems which are often the major cause complicating the disease course of BD (3). Although all types and sizes of vessels can be involved, veins are affected more than the arteries. The most common patterns of vascular involvement are deep and superficial venous thrombosis of the extremities, which occur in approximately in 25% of patients (4). Definite mechanism of thrombotic tendency of BD is poorly understood. Endothelial cell injury and resultant endothelial dysfunction seem to play crucial role in the prothrombotic state of BD, but the mechanisms by which endothelial lesions activate the coagulation pathways are not known (5-7).

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a potent angiogenic cytokine that stimulates functional changes in endothelial cells including increased vascular permeability to fluids and plasma proteins, endothelial cell migration, von Willebrand factor release, and enhanced procoagulant activity. VEGF expression is regulated by a variety of growth factors and cytokines, including interleukin-1 (IL-1), interleukin- 6 (IL-6), tumor necrosis factor alpha (TNF-), transforming growth factor beta (TGF-), prostaglandin E₂ and nitric oxide (8-10). However, the most potent stimulant of VEGF expression is tissue hypoxia (11, 12). Defective oxygenation of cells, as can be observed during pathological events like cancer, stroke, and vasculitis stimulates the expression of VEGF which in turn leads to budding, sprouting and the formation of capillary networks, namely angiogenesis (13).

Monocyte chemoattractant protein 1 (MCP-1) is a strong chemoattractant protein for monocytes and binds to the monocyte via CCR-2 receptor. MCP-1 also increases transendothelial migration of monocytes (14). MCP-1 actively participates in the process of arteriogenesis in which preexisting collateral arterioles turn into functional collateral arteries. Increase in shear stresses in the pre-existing collateral arteries causes a marked activation of the endothelium with increases in the expression of MCP-1 (14-16). In addition to its functions on the arteries, MCP-1 is also known to participate actively to the organization and resolution of venous thrombi (13).

Elevated levels of TNF- have been previously demonstrated in the sera of patients with BD. Amelioration of clinical manifestations with the use of monoclonal antibodies targeting TNF-

, also suggests that TNF- could be one of the triggering factors in the cascade network of cytokines that is activated during the course of BD (1). On the other hand, no previous study was found to address specifically the contribution of TNF- in the pathogenesis of BD with vascular involvement. In addition, TNF- , as a proinflammatory cytokine has been reported to induce and modulate the expression of several angiogenic cytokines including VEGF and MCP-1 (8,13).

One salient feature of thrombosis seen in BD is its decreased ability for recanalization which leads to the sequel formation with the development of chronic organized thrombus within the vessel wall (17,18).

In view of the above cited functions of VEGF, MCP-1 and TNF- in the process of organization and resolution of venous thrombi, and due to the lack of clear-cut evidence regarding the etiology of thrombosis seen in BD, we hypothesized that these cytokines may be important in the pathogenesis of Behcet's thrombosis and difference in the levels of these cytokines as compared to thrombosis due to other causes might be responsible for the diverse course and outcome of thrombosis seen in BD.

To test this hypothesis, we have measured the VEGF, MCP-1, and TNFlevels in the serum of 21 BD patients with thrombosis and 15 BD patients with mucocutaneous symptoms only, and compared them with 20 patients having thrombosis without BD and 20 healthy subjects.

Patients and methods

Patients

Thirty-six patients diagnosed as BD were included in this study. All patients have fulfilled the International Study Group criteria for Behçet's disease (19). Patients with the involvement of systems other than mucocutaneous or vascular were not included in the study. Patients were divided into three subgroups according to the existence and stage (acute vs. chronic) of deep vein thrombosis (DVT) of the lower extremities. Patients with acute DVT (group BD-AT, 9 men; mean age, 25.8 ± 4.9) were defined as those who had developed symptoms of DVT in the last 15 days and in whom the diagnosis was confirmed by one of the radiological modalities (Doppler ultrasonography, computerized tomography, angiography). Patients with chronic DVT (group BD-CT, 12 men; mean age, 25.3 ±4.4) were defined as those who had been free of DVT related symptoms in the preceding 15 days but in whom the radiological modalities disclosed sequelae of prior thrombosis. Patients who had not encountered any other symptoms but mucocutaneous findings since the onset of the disease were labeled as mucocutaneous BD (group BD-MC, 15 men; mean age, 23.7 ± 4.2).

We also analyzed a group of 20 patients with a DVT in whom any acquired thrombophilic disorders (including antiphospholipid syndrome) could not be diagnosed by clinical and laboratory investigations. Acute versus chronic differentiation was made by applying the same criterion used for the patients with BD.

Exclusion criteria for all subjects were the existence of ischemic heart disease, ischemic cerebral disease, hepatic and renal diseases, thyroid disease, hyperlipidemia, and malignant disease. Eleven of these patients had acute (group DC-AT, 11 men; mean age, 24.6 ± 3.2) and 9 had chronic DVT(group DC-CT, 9 men; mean age, 33.3 ± 6.7). Healthy controls (group H, 20 men; mean age, 26 ± 5.3) were recruited from the hospital staff and deemed healthy by way of careful history and examination. The study was approved by the local ethics committee and all participants gave written informed consent. Laboratory findings included ESR and CRP.

Cytokine determination

Sera were obtained and frozen at -70 °C for batch analysis. Measurement of cytokine levels in sera from all patients and controls was carried out at the same time. Analysis of VEGF (KHG-0112, Biosource International), MCP-1

Table I. Patient characteristics. Values are means \pm SD or actual values. Values given in parentheses represent the percentage of the total number in each group. BD-AT, Behçet's disease with acute thrombosis; BD-CT, Behçet's disease with chronic thrombosis; DC-AT, diseased control with acute thrombosis; DC-CT, diseased control with chronic thrombosis; H, healthy controls. EN, erythema nodosum.

	BD-AT	BD-CT	BD-MC	DC-AT	DC-CT	Н
n	9	12	15	11	9	20
Male/female	9/0	12/0	15/0	11/0	9/0	20/0
Age (years)	25.8 ± 4.9	25.3 ± 4.4	23.7 ± 4.2	24.6 ± 3.2	33.3 ± 6.7	26.0 ± 5.3
Oral aphtae (n)	8 (89)	10 (83)	15 (100)	-	-	-
Genital ulcer (n)	4 (44)	5 (42)	8 (53)	-	-	-
Folliculitis (n)	7 (78)	9 (75)	13 (77)	-	-	-
EN (n)	5 (56)	6 (50)	7 (47)	-	-	-
Pathergy (n)	6 (67)	7 (58)	5 (33)	-	-	-

Table II. Serum VEGF, MCP-1 and TNF- concentrations in patients with Behçet's disease, diseased controls and healthy subjects. Results are presented as median and interquartile (in parenthesis) values. VEGF, vascular endothelial growth factor; MCP-1, monocyte chemoattractant protein 1; TNF-, tumor necrosis factor alpha; BD-AT, Behçet's disease with acute thrombosis; BD-CT, Behçet's disease with chronic thrombosis; DC-AT, diseased control with acute thrombosis; DC-CT, diseased control with chronic thrombosis; H, healthy controls.

V	/EGF	(pg/ml)	MCP-1	(pg/ml)	TNF-	(pg/ml)
BD-AT(n=9) 3	81.40	(489.33)	145.66	(55.51)	16.90	(7.93)
BD-CT(n = 12) 2	210.52	(159.62)	77.18	(35.15)	14.02	(12.93)
BD-MC (n = 15) 1	03.04	(95.74)	94.91	(25.94)	14.74	(6.48)
DC-AT(n = 11) 2	200.71	(299.46)	176.39	(39.47)	17.62	(8.69)
DC-CT(n= 9) 2	214.72	(172.96)	92.72	(116.53)	16.90	(15.49)
H (n = 20) 5	5.60	(19.71)	49.98	(9.39)	5.07	(4.43)

Results are presented as median and interquartile (in parenthesis) values. VEGF: vascular endothelial growth factor; MVCP-1: monocyte chemoattractant protein 1; TNF- : tumor necrosis factor alpha; BD-AT: Behçet's disease with acute thrombosis; BD-CT: Behçet's disease with chronic thrombosis; DC-AT: diseased control with acute thrombosis; DC-CT: diseased control with chronic thrombosis; H: healthy controls.



Fig. 1. The distribution of vascular endothelial growth factor (VEGF) levels in the serum samples of Behçet's patients with acute thrombosis (BD-AT), Behçet's patients with chronic thrombosis (BD-CT), diseased controls with acute thrombosis (DC-AT), diseased controls with chronic thrombosis (DC-CT), and healthy controls (H). Short horizontal lines represent the median values and longer lines mark significant differences and p values. Median VEGF levels of all patient subgroups with Behçet's disease and diseased controls were significantly higher than those of the healthy controls (* p < 0.001).

(KHC1012, Biosource International), and TNF- (KHC3012, Biosource International) were performed by ELISA according to the manufacturer's instructions. Intra-assay and inter-assay variances of all assays were 5% and 10%, respectively.

Statistical analysis

As none of the data showed the characteristics of normal distribution, results are presented as median and interquartile range values. Results were analyzed statistically using the Kruskal Wallis test, Kolmogorov-Simirnov Z test or the Mann-Whitney U test as indicated. The criterion for statistical significance was set at P < 0.05. Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (version 11.0, SPSS Inc., Chicago, IL).

Results

There were no significant differences in age or sex ratio among all study groups. Among the subgroups of BD patients no significant differences were noted with respect to the frequencies of mucocutaneous findings (Table I). CRP was found to be higher in group BD-AT than BD-CT (z: -2.111, p = 0.034). However, no such difference for ESR was noted when all study subgroups compared with each other (P > 0.05, for each). VEGF, MCP-1 and TNF- were detectable in all serum samples of the study subjects. The measured levels of VEGF, MCP-1 and TNF- were found to be significantly different among the study groups (2 = 54.75, p < 0.001; 2 = 53.23, p < 0.001; and $^{2} = 32.88$, p < 0.001, respectively). Median VEGF levels of patients with BD-AT, BD-CT and BD-MC were significantly higher than those of the healthy controls (p < p0.001, p < 0.001 and p < 0.001, respectively) (Table II, Fig 1). The levels were significantly higher in the patients in group BD-AT than either in group BD-CT or BD-MC (p = 0.03 and p <0.001, respectively). There was also significant difference between the samples from group BD-CTand group BD-MC (p = 0.004) (Fig. 1).

The VEGF levels of the patients either in group DC-AT or DC-CTwere signif-



Fig. 2. The distribution of monocyte chemoattractant protein-1 (MCP-1) levels in the serum samples of Behçet's patients with acute thrombosis (BD-AT), Behçet's patients with chronic thrombosis (BD-CT), diseased controls with acute thrombosis (DC-AT), diseased controls with chronic thrombosis (DC-CT), and healthy controls (H). Short horizontal lines represent the median values and longer lines mark significant differences and p values. Median MCP-1 levels of all patient subgroups with Behçet's disease and diseased controls were significantly higher than those of the healthy controls (* p < 0.001).

icantly higher than those of the healthy controls (p < 0.001 and p < 0.001, respectively) (Fig. 1). However, there was no significant difference between the samples from group DC-AT and DC-CT (p > 0.05). The median VEGF level of the patients in group BD-AT was higher than that of the patients with DC-AT, however it did not reach statistical significance (p = 0.063). No significant difference was observed between the group BD-CT and DC-CT (p > 0.05) (Fig 1). Furthermore, the serum VEGF levels did not correlate with either ESR or CRP (r_s =0.048, p = 0.679 and r_s = 0.095, p = 0.414, respectively). Each subgroup of BD patients and disease controls had higher concentration of serum MCP-1 compared to healthy controls (BD-AT vs. H; p < 0.001, BD-



Fig. 3. The distribution of tumor necrosis factor-alpha (TNF-) levels in the serum samples of Behçet's patients with acute thrombosis (BD-AT), Behçet's patients with chronic thrombosis (BD-CT), diseased controls with acute thrombosis (DC-AT), diseased controls with chronic thrombosis (DC-CT), and healthy controls (H). Short horizontal lines represent the median values. Median TNF- levels of all patient subgroups with Behçet's disease and diseased controls were significantly higher than those of the healthy controls (* BD-AT vs. H; p< 0.001, BD-CTvs. H; p = 0.007, BD-MC vs. H; p < 0.001, DC-ATvs. H; p < 0.001, DC-CTvs. H; p = 0.008).

CTvs. H; p < 0.001, BD-MC vs. H; p < 0.001, DC-AT vs. H; p < 0.001, DC-CT vs. H; p < 0.001) (Table II, Fig. 2), but the levels did not correlate with either ESR or CRP(rs = 0.168, p = 0.146 andrs = 0.214, p = 0.064, respectively). With respect to BD patients, concentration of MCP-1 was significantly increased in BD-AT serum samples compared to BD-CT and BD-MC (p < 0.001, p = 0.001, respectively) (Fig. 2). The median serum MCP-1 level in patients with DC-AT was significantly higher when compared with DC-CT (p < 0.001). The median level in the serum of DC-AT patients was significantly higher than that of those with BD-AT (p = 0.025), whereas no significant difference was observed between the levels of the patients in group DC-CT and group BD-CT(p > 0.05) (Fig. 2). Serum TNF- levels of all groups were higher than that of the healthy controls (BD-AT vs. H; p<0.001, BD-CTvs. H; p=0.007, BD-MC vs. H; p<0.001, DC-AT vs. H; p < 0.001, DC-CT vs. H; p = 0.008) (Table II, Fig. 3). However, no correlation was demonstrated between the serum TNF- levels and ESR or

CRP (rs =-0.096, p=0.408 and rs = 0.001, p = 0.994, respectively). Among the subgroups of patients with BD and disease controls no significant differences were detected with respect to serum TNF- levels (Fig 3). TNF- serum levels were not found to be correlated either with VEGF or MCP-1 levels (rs=-0.032, p=0.710 and rs= 0.074, p = 0.342, respectively).

Discussion

Venous thrombosis appeared to be the major vascular involvement reported in 25% of cases with BD. Although all veins of any location can be involved, deep veins of the lower extremities are affected more often than the others (2-4). We found that, compared with heal-thy control subjects, patients with BD and those with other causes of thrombosis have increased serum VEGF levels.

In the case of BD, patients with either acute or chronic thrombosis had significantly higher VEGF concentrations than the patients with mucocutaneous involvement. Furthermore, VEGF lev-

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els of BD patients with acute thrombosis were higher than those of BD patients in chronic stage. No such differences were noticed for VEGF levels in patients with thrombosis due to other causes with respect to the stage of thrombosis (acute vs. chronic). In addition, there were no differences in VEGF levels when subgroups of BD patients with thrombosis were compared with the corresponding subgroups of patients with other causes of thrombosis.

VEGF is an endothelial-cell specific potent mitogen that induces angiogenesis and vascular hyperpermeability (8). The source of the elevated VEGF in BD patients is likely to be multifaceted. VEGF is secreted from several types of cells, including vascular endothelial cells, in addition to macrophages, lymphocytes, and neutrophils (20-22). All these cell types have been shown to join vigorously in the course of BD (23-26). Among these cells, endothelial cells warrant a special consideration. Prothrombotic state is a fundamental constituent of BD and the endothelial dysfunction seems to be key event causing this situation (5-7). Several studies have also shown that various markers of endothelial dysfunction were found to be increased more prominently in Behçet's patients with widespread involvement than in patients with mucocutaneous involvement only (27-30). Increased VEGF levels in the circulation and its correlation with the disease activity have been demonstrated in a number of diseases characterized by endothelial dysfunction, such as Wegener's granulomatosis, Henoch-Schonlein purpura and antiphospholipid syndrome (31-33). In this respect, VEGF can also be considered as a marker of endothelial dysfunction. These findings may help to clarify, at least in part, the highest serum VEGF levels found in our BD patients with acute thrombosis, while the lowest levels in patients with merely mucocutaneous disease.

Our findings are also in line with a recent study demonstrating an increased VEGF levels and its correlation with the disease activity in a mixed group of patients with BD. Although none of the patients included in that study had had

vascular involvement, VEGF levels were found to be increased more dramatically in a subgroup of patients having widespread involvement and particularly ocular disease (34). Moreover, the major inducer of VEGF expression is hypoxia (12,35). Increased VEGF levels in the circulation have been demonstrated in several diseases, such as stroke, myocardial infarction, and peripheral artery disease which are all characterized by hypoxia (36-38). These data are in support with ours as showing the absence of difference in VEGF levels with respect to the cause of thrombosis. Hence, VEGF does not seem to be specific for a particular disease, but most likely is a non-specific marker for vascular disorders in which endothelial dysfunction and/ or hypoxia occurs. Platelets secrete proteins during aggregation that are important for coagulation, inflammation and vessel repair. Recently, it has been reported that megakaryocytes and platelets contain VEGF in their cytoplasm and it is secreted by aggregating platelets (39). Together with the fact that platelet aggregation occurs predominantly at sites of endothelial injury, these data may enlighten the elevated VEGF levels of patients with thrombosis. Finally, numerous cytokines, including IL-1, IL-6, and TNF- were implicated in the up-regulation of VEGF expression (9). A number of studies demonstrating increased levels of these cytokines in the serum of BD patients have been well known which may further explain the increased VEGF levels found in those patients (40, 41).

In serum of both patients with BD and disease controls, we found significantly raised serum levels MCP-1 compared with healthy controls. Although precise measurement of MCP-1 levels in the peripheral blood posses some short-comings due to its binding to erythrocytes, linear correlation between measured values of MCP-1 in the plasma and whole blood had been reported.

In the case of BD, patients with acute thrombosis had significantly higher concentrations of MCP-1 than the patients with chronic thrombosis. Similarly, in disease controls, patients at the acute phase of thrombosis had higher MCP-1 levels as compared to the patients at chronic phase. Thrombus organization after venous thrombosis leading to recanalization occurs at a variable rate. Although the mechanisms by which the venous lumen is restored are still being elucidated, monocytes are known to participate dynamically in the process of recanalization (42). In this respect, MCP-1 is known to contribute to the organization and resolution of venous thrombi by attracting monocytes (13). Endothelial cells are the major source of endogenous MCP-1 and these cells have been shown to express mRNAfor MCP-1 after stimulation by several pro-inflammatory cytokines including IL-1, TNF- (43). Furthermore VEGF has been shown to induce the expression of MCP-1, which may act in concert with MCP-1 in the process of recanalization and angiogenesis (44). Contrary to the previously published findings, we could not demonstrate any correlation between the serum levels of TNF- and MCP-1. Likewise, no correlation was found between the serum levels of MCP-1 and VEGF. Humphries et al. have found an increased endogenous MCP-1 levels during natural resolution of venous thrombi with the highest levels observed at the first week. They also revealed that injection of MCP-1 into the thrombus increased the thrombus organization scores and reduced the thrombus area (45). These observations are in conformity with our observation that higher MCP-1 levels found at the acute phase of venous thrombosis, indicating an active process of recanalization at an earlier stage.

When patients having acute thrombosis compared with regard to the cause of thrombosis, patients with thrombosis due to other causes were found to have higher levels of serum MCP-1 than patients with BD. Relatively lower levels of MCP-1 in BD patients might also explain why these patients are less prone to spontaneous recanalization than patients with thrombosis resulting from other causes.

We demonstrated increased serum TNF- levels both in BD patients and disease controls. Nevertheless, no significant difference was noted when pa-

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tient subgroups were compared with each other. TNF- is believed to play a pivotal role in BD and blockade of its activity has favorable effects in patients refractory to conventional immunosuppressive drugs (46). On the other hand, correlation of serum TNF- levels with the disease activity in BD is an area of debate; some studies suggest a correlation (47) while the others found no prevailing association between the two (48,49). In the present study, TNF- levels were not found to be correlated either with VEGF or MCP-1. Likewise, the lack of any correlation between the serum VEGF, MCP-1 levels and acute phase response (ESR,CRP) in this study may suggest that TNF- and inflammation do not seem to be major factors in the secretion of VEGF and MCP-1 in Behçet's thrombosis as well as thrombosis due to other causes.

In conclusion, the present study indicated that VEGF and MCP-1 may play a role during the course of venous thrombosis in BD. Although this study was not designed to differentiate acute thrombosis from the chronic one in patients with BD, determination of serum VEGF and MCP-1 levels seems to serve as a helpful tool in that purpose.

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