The role of heat shock protein 60, vascular endothelial growth factor and antiphospholipid antibodies in Behcet’s disease

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Background. Behcet’s disease is recognized as a systemic inflammatory disease of unknown etiology. T cells in this disease proliferated vigorously in response to a specific peptide of human heat shock protein (hsp 60) in an antigen-specific fashion producing Th1-like proinflammatory and/or inflammatory cytokines. Vascular endothelial growth factor (VEGF) is a cytokine participating in inflammation with endothelial cell effects. One of the prominent features of Behcet’s disease is vasculitis as a result of endothelial dysfunction. Antiphospholipid antibodies (aPL ab) may play a role in the development of thrombosis by inhibiting production of prostacyclin by endothelial cells. Our aim is to investigate the role of HSP 60, VEGF and antiphospholipid antibodies in Behcet’s disease and their relation to clinical manifestations and disease activity.

Material and Methods. Thirty patients with Behcet’s disease were included in the study; 17 of them were in active stage and 13 were in the inactive one. Fifteen age and sex matched healthy volunteers served as controls. Complete clinical examination; routine laboratory investigations and Doppler examination were done. Serum levels of HSP 60, VEGF and antiphospholipid antibodies were performed. Results. Serum levels of HSP 60, VEGF and antiphospholipid antibodies were significantly higher in patients than in controls, however their level did not correlate with disease activity. Serum level of VEGF correlated significantly with the presence of vascular manifestations and ocular affection. Serum level of antiphospholipid antibodies was greater in patients with thrombosis.

Conclusion. HSP60 has an important role in etiopathogenesis of Behcet’s disease which shed a new light on its autoimmune nature. VEGF and antiphospholipid antibodies may contribute to vascular damage and endothelial dysfunction seen in Behcet’s disease. Elevated serum level of VEGF may be an additional risk factor for development of ocular disease contributing to poor visual outcome.

Key words. HSP60 - VEGF - aPLab - Behcet’s disease
antigen that is derived from mammalian/bacterial 60/65 KDa HSP. Sequence homology and cross reactivity between microbial and human HSP led to the concept that HSP might be involved in the etiopathogenesis of BD. Vascular endothelial growth factor (VEGF) is a 34-45 KDa glycoprotein with similarity to platelet derived growth factor. It is a potent cytokine that modulates angiogenesis and vasculogenesis by acting as an essential mitogen for vascular endothelial cells. Because VEGF is a potent mitogen for dermal and ocular microvascular endothelial cells, its expression may be important in the vascular bed of Behcet’s patients.

Antiphospholipid antibodies (aPLab) may have a pathogenetic role in the development of vascular complications in BD. APLab represent a heterogenous group of immunoglobulins that include (lupus anticoagulant and anticardiolipin antibodies). They react with negatively charged rarely with neutral phospholipids. They play an important role in the development of thrombosis by inhibiting production of prostacyclin by endothelial cells.

MATERIALS AND METHODS

The study was conducted on thirty patients with Behcet’s disease (satisfying International study group criteria) recruited from the Dermatology, Rheumatology outpatient clinics and Vascular surgery department, Kasr El Aini Hospital during the period from March 2004 to April 2005. Fifteen healthy volunteers served as controls.

Clinical assessment

Clinical assessment of all patients was done. Dermatological examination for all skin lesions was performed in addition, ocular examination was done.

Both clinical and laboratory findings (ESR, neutrophilic count) were used to classify the patients as having active (n=17) or inactive disease (n=13).

Clinically, the presence of three of five major findings on admission (skin lesions, + pathergy test, uveitis, oral and genital ulcers) was considered to indicate the active stage of the disease.

Investigations

Routine laboratory investigations were done including; complete blood picture, ESR, liver and kidney functions, urine analysis. Vascular involvement was assessed by Doppler ultrasound.

Serum level of HSP60 was estimated using the Stressgen’s HSP60 Eliza kit; a quantitative sandwich immunoassay.

Serum level of VEGF was estimated by Accucyte Eliza kit, a competitive enzyme immunoassay.

Serum level of aPLab was determined by an indirect solid phase Eliza. The kit was provided by BL Diagnostika, Germany.

Statistical analysis

The statistical analysis was done using a Machintosh L.C.III computer and Stat-view statistical package. Statistical analysis were done according to Knapp and Miller (1992). Tests used included student (t) test for comparing means of two groups, Chi square ($\chi^2$) test for comparing qualitative data. Correlation and regression analysis (r) were also used.

RESULTS

Thirty Behcet’s patients with age ranging from 17-50 ys (mean=32.6±9.14) and a male to female ratio was (4:1) were included in this study. The disease duration ranged from 1 to 25 ys (mean=6.92±6.65). Fifteen healthy volunteers served as controls. Their ages ranged from 18 to 47 ys (mean=30.13±12.32) with a male to female ratio (4:1).

The clinical manifestations of Behcet’s disease patients are shown in Table (1).

Serum level of HSP60, VEGF and aPLab in all patients were significantly higher than that of controls (p<0.0001, <0.0001 and <0.01 respectively) (Table 2).

Seventeen patients were in the active stage of the disease whereas the remaining 13 patients were in the inactive stage. Neutrophil count and ESR concentration were significantly higher in patients with active disease than the inactive
one (p<0.01, <0.005 respectively) (Table 3).

Serum level of HSP 60, VEGF and antiphospholipid antibodies were higher in the active group than in the inactive one but the results were not statistically significant (p>0.05).

Comparing serum levels of HSP60, VEGF and aPLab according to presence or absence of clinical manifestations showed that serum level of VEGF was significantly higher in patients with vascular manifestations and in those with ocular affection (p<0.01, <0.005 respectively) (Table 4). In addition, serum level of aPLab was significantly higher in patients having vascular affection and thrombosis (p<0.005).

There was no correlation between serum level of HSP 60 and VEGF nor with aPLab, but a positive correlation was found between serum level of VEGF and aPLab (r=0.78, p<0.0001).

Table 1. Clinical manifestations of Behcet's disease patients.

<table>
<thead>
<tr>
<th>Item</th>
<th>No of patients (30)</th>
<th>Percent of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral ulcers</td>
<td>30</td>
<td>100%</td>
</tr>
<tr>
<td>Genital ulcers</td>
<td>28</td>
<td>93.3%</td>
</tr>
<tr>
<td>Skin rashes: Papulopustular, pseudofolliculitis</td>
<td>16</td>
<td>53.3%</td>
</tr>
<tr>
<td>erythema nodosum Palpable purpura</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin pathergy test</td>
<td>16</td>
<td>53.3%</td>
</tr>
<tr>
<td>Eye findings: uveitis retinal vasculitis</td>
<td>15</td>
<td>50%</td>
</tr>
<tr>
<td>Vascular findings:</td>
<td>15</td>
<td>50%</td>
</tr>
<tr>
<td>Venous: sup. Thrombosis, DVT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial: aneurysms, thrombosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac manifestations</td>
<td>6</td>
<td>20%</td>
</tr>
<tr>
<td>Chest manifestations</td>
<td>8</td>
<td>26.6%</td>
</tr>
<tr>
<td>Neurological manifestations:</td>
<td>14</td>
<td>46.6%</td>
</tr>
<tr>
<td>- ataxia, migraine, stroke</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Articular manifestation</td>
<td>12</td>
<td>40%</td>
</tr>
</tbody>
</table>

Table 2. Comparison between Behcet's disease patients and control.

<table>
<thead>
<tr>
<th></th>
<th>Patients(30)</th>
<th>Control(15)</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean level</td>
<td>Mean level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP 60 ng/ml</td>
<td>14.8±8.88</td>
<td>5.13±1.73</td>
<td>4.16</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>VEGF pg/ml</td>
<td>430.54±99.33</td>
<td>84.07±30.22</td>
<td>13.14</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>aPLab Gpl/ml</td>
<td>12.62±7.24</td>
<td>4.18±2.36</td>
<td>2.44</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

* : significant

Table 3. Comparison between patients with active and inactive stage of the disease as regards laboratory parameters.

<table>
<thead>
<tr>
<th></th>
<th>Inactive (n=13)</th>
<th>Active (n=17)</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>12.11±2.13</td>
<td>11.78±1.62</td>
<td>0.48</td>
<td>n.s</td>
</tr>
<tr>
<td>Neutrophil count</td>
<td>6.59±1.63</td>
<td>8.24±1.99</td>
<td>2.42</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>ESR</td>
<td>23.3±16.32</td>
<td>52.76±30.82</td>
<td>3.12</td>
<td>&lt;0.005*</td>
</tr>
<tr>
<td>Platelet</td>
<td>267.15±72.22</td>
<td>344.65±137.12</td>
<td>1.85</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>HSP 60</td>
<td>16.72±13.22</td>
<td>13.35±2.58</td>
<td>1.03</td>
<td>n.s</td>
</tr>
<tr>
<td>VEGF</td>
<td>424.52±88.32</td>
<td>435.14±109.45</td>
<td>0.29</td>
<td>n.s</td>
</tr>
<tr>
<td>aPLab</td>
<td>11.45±9.11</td>
<td>13.52±8.3</td>
<td>0.42</td>
<td>n.s</td>
</tr>
</tbody>
</table>

* : significant
n.s : non significant
Table 4. Comparison of mean VEGF level in Behcet’s disease patients according to presence or absence of some clinical manifestations.

<table>
<thead>
<tr>
<th></th>
<th>Presence</th>
<th>Absence</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genital ulcers</td>
<td>431.03±97.1</td>
<td>426.07±142.95</td>
<td>0.08</td>
<td>n.s</td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>448.32±81.97</td>
<td>426.09±104.28</td>
<td>0.48</td>
<td>n.s</td>
</tr>
<tr>
<td>Eye</td>
<td>485.45±98.17</td>
<td>388.55±79.56</td>
<td>2.99</td>
<td>&lt;0.005*</td>
</tr>
<tr>
<td>Arthritis</td>
<td>430.01±103.46</td>
<td>430.8±99.93</td>
<td>0.02</td>
<td>n.s</td>
</tr>
<tr>
<td>Vascular</td>
<td>476.49±92.27</td>
<td>390.32±89.44</td>
<td>2.59</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

*: significant
n.s.: non significant

**DISCUSSION**

HSPs are highly conserved molecules inducible by any form of cellular stress. They can bind cellular proteins to act as chaperones stabilizing protein and preventing denaturation (6). However, because of great homology between bacterial and human HSP, they have been claimed to cause autoimmunity (18).

Recently several mycobacterial HSP 65 peptides and their human analogues HSP60 have been shown to stimulate lymphoproliferative response in patients with BD in a specific manner (4).

In this study, we have found significantly elevated serum level of HSP 60 in Behcet’s patients than in control but the elevation did not correlate with disease activity. This is in agreement with Kibaroglu et al (16) and Direskeneli et al (6) who reported increased serum level of HSP60 in Behcet’s disease.

It has been shown that selected peptides derived from the sequences of HSP60 induce significant proliferation of T cells in patients with Behcet’s disease. Hu et al (17) have shown that both oral and nasal administration of HSP60 peptide induce uveitis which is a common feature of Behcet’s disease (18).

A molecular mimicry mechanism induces and/or exacerbate Behcet’s disease; self HSP60 and/or microbial HSP homologous to the self HSP60 activates self reactive T cells specific to the HSP peptides 19. Furthermore, HSP peptides stimulated oligoclonal T cells expansion producing Th1 cytokines. Ergun et al 4 have found increased TCR γδ cells that proliferate with overlapping mycobacterial-human HSP peptides in Behcet’s disease and regulate αβ T cells (4).

Cytokines are the major mediators of immunologic and inflammatory reactions. They mediate delayed type hypersensitivity, macrophage activation and activation and/or recruitment of neutrophils. Thus exacerbating acute inflammation in skin lesions (19).

VEGF is a potent endothelium-specific cytokine that potently stimulates inflammation, angiogenesis, microvascular hyperpermeability and endothelium-dependant vasodilatation as well as endothelium NO production (20,21).

Increased levels of VEGF have been reported in many autoimmune and infectious inflammatory diseases (22). The most prominent feature of Behcet’s syndrome is systemic dermal and ocular vasculitis with endothelial cell dysfunction (23). As VEGF has a direct effect on endothelial cells and it is produced by cells participating in the pathophysiology of Behcet’s syndrome, such as neutrophils, macrophages and endothelial cells, we expected elevated plasma VEGF levels in patients with Behcet’s syndrome. In this study, we demonstrated that plasma VEGF levels were higher in patients with Behcet’s syndrome than in age and sex matched healthy control subjects. Moreover its level correlated with the presence of vascular and ocular manifestations in Behcet’s patients. This is in agreement with Cekman et al (9) who found elevated level of VEGF in Behcet’s disease that correlated with ocular manifestations (9).

Many possible mechanisms or factors may be responsible for the increased plasma VEGF concentrations found in this study. Vascular thrombogenesis occurs in the course of Behcet’s syndrome, thus platelets may contribute to higher plasma VEGF levels, hydrogen peroxide released by activated...
neutrophils causes VEGF production by macrophages. Proinflammatory cytokines, which are known to participate in the course of Behcet’s syndrome (TNFα, soluble IL2 receptor, IL6, IL8 and nitric oxide), upregulates endothelial cells as well as VEGF production.

VEGF promotes inflammatory processes by mobilizing leukocyte (25) or, more recently, it has been shown that VEGF activates endothelial cell exocytosis of Weibel-Palade bodies, releasing vasoactive substances capable of causing vascular thrombosis and inflammation (26).

It is also known that VEGF production up-regulates NO synthase expression in endothelial cells and increases endothelial release of NO (16). NO could damage host cells and tissues either directly and/or following reaction with other free radicals. It plays a critical role in the development of thrombotic events or other pathophysiological changes in patients with Behcet’s disease (27).

APL ab exerts a procoagulant effect resulting in thrombosis mainly of the larger veins and arteries. Indeed, one of the key features in antiphospholipid syndrome (APS) is vascular thrombosis occurring in 23% to 58% of patients with aPL ab (28).

Previous reports have failed to find an association between APL ab and thrombosis (29).

However, Erkamova et al (30) have found an association between presence of APL ab and retinal vascular thrombosis in a group of Behcet’s disease. Moreover, Kang et al (31) have found an elevated aPLab level in patients with Behcet’s disease. Differences in results could be attributed to regional determinants whether environmental or genetic that may affect the presence of aPLab as they do for other antibodies (32). In this study, a significantly elevated level of aPLab was found in patients than in controls, and the elevation correlated with presence of vascular thrombosis. APLab play an important role in the development of thrombosis by inhibiting production of prostacyclin by endothelial cells. Others have found that aPL ab may activate endothelial cells, thus creating a hypercoagulable state that precedes and contributes to thrombosis 33. Thus aPLab may serve as an additional marker for a risk of development of vasculitis and thrombosis (33).

A positive correlation was found, in this study, between VEGF and aPLab both of which are related to the presence of vascular manifestations. This correlation was not previously reported. However, Williams et al 34 have found elevated level of VEGF in APS and suggested its role in the pathogenesis of thrombosis in antiphospholipid syndrome (34).

In conclusion, increased HSP60 has pathologic significance in BD. The specificity and primary function of HSP60 as a potential pathogenic factor needs to be clarified because this may lead to emergence of new treatment modalities such as its use in oral vaccination to induce tolerization and prevent relapses.

High plasma levels of VEGF in patients with Behcet’s syndrome, suggests a role for VEGF in dermal and ocular vascular events in the course of the disease.

As ocular Behcet’s syndrome is essentially diagnosed on the basis of clinical observation, VEGF could be used as a promising marker of ocular vaso-occlusive disease with neovascularization. Further investigations of the precise role of VEGF in the pathogenesis of Behcet’s syndrome may lead to novel therapies with antibodies or other inhibitors of VEGF.

Moreover, aPLab may serve as an additional marker for a risk of development of thrombosis.

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