SHORT COMMUNICATION

Angiogenic cytokines in induced sputum of patients with sarcoidosis

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Background and objective: Sarcoidosis is a systemic granulomatous disorder of unknown aetiology involving multiple organs and often associated with non-granulomatous microangiopathic lesions in various organs. Increased angiogenesis-inducing ability of activated alveolar macrophages was found in bronchoalveolar specimens from patients with pulmonary sarcoidosis and from patients with extrapulmonary involvement. In contrast, decreased levels of vascular endothelial growth factor (VEGF) were found in BAL fluid recovered from sarcoid-associated pulmonary fibrosis. This study evaluated whether sarcoidosis is associated with abnormalities of VEGF and IL-8 in induced sputum (IS) samples.

Methods: Twenty-three sarcoid patients and 13 controls performed IS. CD4/CD8 T-cell subsets were measured, as were pulmonary function tests and VEGF and IL-8.

Results: Sarcoid patients showed significantly higher mean %lymphocytes (P = 0.04), significantly higher mean CD4/CD8 ratio (P = 0.0001) and significantly lower VEGF levels (P = 0.03) than controls. Patients with stages III–IV sarcoidosis showed a lower level of VEGF compared with those with stages I–II sarcoidosis (P = 0.048). IL-8 was detected in 10/35 samples and positively correlated with % neutrophils (P = 0.054) and eosinophils (P = 0.045). VEGF immunohistochemical staining showed a mixed pattern of expression in the same tissue samples and was low in fibrotic tissue areas.

Conclusion: VEGF in IS samples may reflect impairment in angiogenesis associated with the extent of sarcoid fibrosis and functional disorders.

Key words: angiogenesis, IL-8, induced sputum, sarcoidosis, VEGF.

INTRODUCTION

Vascular endothelial growth factor (VEGF), a dimeric glycoprotein with a MW of 34 000 to 42 000, consists of two disulfide-linked peptide chains with identical N-termini.1,2 VEGF possesses potent vascular permeability-enhancing activity. On a molar basis, it is 50 000 times more potent than histamine.1 It is a specific mitogen and chemotactic factor for vascular endothelial cells3 and for the degradation of extracellular matrix and vascular permeability-enhancing activity.5,6 The existence of multiple activities embodied in the same protein suggests that VEGF may play multifunctional roles in angiogenesis, which is an integral part of physiological/beneficial and pathological/detrimental tissue repair responses.

The mechanisms that maintain lung alveolar capillary structure and function are not completely known. It has been reported that inhibition of VEGF receptors causes endothelial cell apoptosis and emphysema, suggesting that VEGF receptor signalling is required for the maintenance of the alveolar structure and that alveolar septal cell apoptosis contributes to the pathogenesis of emphysema.7 On the other hand, increased expression of VEGF has been demonstrated in the pulmonary arteries of smokers and patients with moderate COPD.8

Although IPF, sarcoidosis and other interstitial lung diseases are members of the same disease group, they have notable differences in their immunopathogenesis, clinical course, prognosis and response to...
steroid treatment. Many studies have supported the hypothesis that these differences may be related to distinct angiogenic and angiostatic profiles.15,16 The relative roles played by new vessel formation and vascular regression in IPF are unclear.11,12 A relationship between the plasma concentrations of the angiogenic cytokines (VEGF, IL-8 and endothelin-1 (ET-1)) and the clinical parameters of disease progression over a 6-month period has been demonstrated in patients with idiopathic interstitial pneumonias.13 Immunoreactivity of biopsies of IPF patients revealed that VEGF and IL-8 were minimal in highly fibrotic regions, thereby showing a correlation between interstitial capillary density and histologic severity.14 The absence of vessels in the fibroblastic foci of IPF described by Cosgrove et al.15 was explained as being due to pigment epithelium-derived factor inhibition of new vessel formation.

Sarcoidosis is a systemic granulomatous disorder of unknown aetiology involving multiple organs and often associated with non-granulomatous microangioptic lesions in various organs.16 Increased angiogenesis-inducing ability of activated alveolar macrophages was found in BAL specimens from patients with pulmonary sarcoidosis17 and from patients with extrapulmonary involvement.18 In contrast, decreased levels of VEGF were found in BAL fluid recovered from sarcoid-associated pulmonary fibrosis.19 Vascular endothelial growth factor has been studied as a genetic prognostic factor20 as well as a marker for determining treatment approaches in sarcoidosis.16,21 Serum levels of VEGF have been correlated with the baseline high-resolution CT fibrosis score.13 The present study used an non-invasive approach (induced sputum, IS) to retrieve angiogenic cytokines in the sputum of patients with sarcoidosis in order to study the involvement of these cytokines in the immunopathology of this disease.

METHODS

Study subjects

The sarcoid study group consisted of 22 non-smoker patients who had been diagnosed clinically and radiologically, and all had a positive transbronchial biopsy (TBB) which showed non-caseating granuloma. Patients were staged based on their CXR findings: 3 patients were classified as being in Stage I, 12 patients in Stage II, 6 in Stage III and 1 in Stage IV. No patient had received medications prior to recruitment into the study, or had an atopic background. The control group comprised 13 apparently healthy non-smokers without any respiratory disease who were recruited from the hospital staff. All participants signed informed consent forms and the study was approved by the Institutional Review Board.

Pulmonary function tests

Pulmonary function tests (spirometry, lung volumes and diffusion capacity) were performed by a Masterlab (Masterlab E. Jaeger, Wurzburg, Germany). The measurements were carried out by standard protocols according to American Thoracic Society guidelines.22

Sputum induction

Sputum induction was performed with an aerosol of hypertonic saline generated by a Ultrasonic Nebulizer—Model Omron U1 (Omron Health Care, Bannockburn, IL, USA) with an output of 0.5 mL/min and particle size having a <5 μ aerodynamic mass median diameter, using a slightly modified method of Pin et al.23 Briefly, subjects inhaled nebulized 3% saline for up to 20 min by ultrasonic nebulizer through a mouthpiece without a valve or noseclip. The patient was encouraged to cough and expectorate sputum into a sterile plastic container. The nebulization was stopped after 20 min or earlier if the sputum sample was of sufficiently good quality.

Sputum examination

The method of Popov et al.24 was used with some modifications. Briefly, sputum was processed as soon as possible within two hours of collection. Dithiothreitol (DTT-Sputalysin, Calbiochem Corp., San Diego, CA, USA) was added and mixed mechanically with the sputum in a shaking water bath at 37°C for complete homogenization. The cell suspension with phosphate buffered solution was filtered through a 52 μ nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) diluted with RPMI supplemented with foetal calf serum to achieve a concentration of 10^4/μL. One drop was placed in each cytocentrifuge cup already in place in a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA) and cytopsins were prepared at 1000 RPMI supplemented with 10% foetal calf serum (Biological Industries, Beit Haemek, Israel) for 5 min. Separate cytospin slides were stained by Giemsst. Three hundred non-squamous cells were counted and the results were expressed as a percentage of the total non-squamous cell count. Only samples containing ≤20% squamous cells were used.

Evaluation of the phenotype of sputum cells

Flow cytometric analysis was performed on a dual FACS 440 equipped with an Ar+ and Kr laser (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were collected and analysed using the Consort VAX and Disp4 and Disp2D programs (Becton-Dickinson). The information was collected on a logarithmic scale. The selection of lymphocyte population was based on a side-scatter and the expression of CD45. Lymphocytic subsets were identified by monoclonal antibodies as follows: CD3 = total T cells, CD4 = T helper cells and CD8 = T suppressor-cytotoxic cells. Monoclonal antibodies were directly conjugated to either phycoerythrin or fluorescein isothiocyanate. The
cells were incubated for 10 min with Epics Coulter Q-Prep and read either immediately or after 24 h.

**Measurement of VEGF and IL-8 in sputum**

Determinations of the absolute value of IL-8 and VEGF in the IS samples were performed by ELISA (R&D System Inc., Minneapolis, MN, USA). Spike experiments were done with pure metabolites in order to assess the efficacy of recovery. Only 10–12% of protein was found to be equally denaturated by DTT in all samples.

**Immunohistochemistry—VEGF**

Vascular endothelial growth factor was detected in five TBB using an anti-human VEGF antibody (at a dilution of I:160), and a mouse monoclonal antibody (MAB293; R&D Systems). Antigen retrieval was performed at 95°C in citrate buffer pH 6.0, 6.4 M sodium citrate dehydrate and 1.6 M citric acid monohydrate for 40 min. The slides were cooled at room temperature for 20 min and washed three times for 3 min each with Tris buffer pH 7.6, 0.15 M sodium chloride and 0.05 M Trizma HU. They were peroxidase-blocked for 5 min and washed as above. They were then incubated for 30 min with the primary antibody, followed by the secondary antibody (visualization reagent), then followed by the substrate-chromogen solution (3,3′-diaminobenzidine) and finally counter-stained with haematoxylin. Paraffin sections of normal lung sections known to express VEGF were used as a positive control. For the negative control, the primary antibodies were replaced with a non-specific negative control antibody. Ten different granulomatous regions were counted in each biopsy for immunopositive stained cells.

**Statistical analysis**

The levels of cytokines were compared using Student’s t-test or the Mann–Whitney U-test according to parametric or non-parametric distribution. Spearman’s rank or Pearson’s correlation was calculated to assess the correlation coefficient (r) and the P-value for statistical significance.

### Table 1 Demographic and pulmonary function test results of study participants

<table>
<thead>
<tr>
<th></th>
<th>TLC</th>
<th>DLCO%</th>
<th>FEV1/FVC</th>
<th>FEV1</th>
<th>ITGV</th>
<th>RV</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA Stage I</td>
<td>93 ± 16</td>
<td>0.014</td>
<td>0.010</td>
<td>84 ± 16</td>
<td>94 ± 21</td>
<td>103 ± 30</td>
<td>48 ± 11</td>
</tr>
<tr>
<td>SA stages II–III</td>
<td>87 ± 14</td>
<td>0.017</td>
<td>0.011</td>
<td>74 ± 14</td>
<td>84 ± 11</td>
<td>107 ± 25</td>
<td>51 ± 6</td>
</tr>
<tr>
<td>Controls</td>
<td>100 ± 16</td>
<td>0.001</td>
<td>ns</td>
<td>106 ± 16</td>
<td>104 ± 18</td>
<td>121 ± 27</td>
<td>42 ± 15</td>
</tr>
<tr>
<td>P-value</td>
<td>ns</td>
<td>0.006</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
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</tbody>
</table>

DLCO% and FEV1% were significantly different between sarcoid patients (SA) and controls. ns, not significant.

**RESULTS**

**Demographics and pulmonary function test results**

The sarcoid group comprised 14 women and eight men with a mean age of 41.1 ± 15.5 years (range 26–57). The control group comprised six women and seven men with a mean age of 48.2 ± 9.9 years (range 38–58) (P = not significant). The mean FEV1 of the sarcoid group was 80.8 ± 15.9% of predicted (range 51–104%) compared with 105.7 ± 13.3% (106–124%) for controls (P = 0.001). DLCO values ranged 54–97% of predicted for the sarcoid patients and 77–109% of predicted for the controls (79.9 ± 14.7% vs 93.2 ± 11.47%, P = 0.009; DLCO and FEV1 correlation r = 0.25, P = 0.006). TLC was decreased in 3 of the 22 sarcoid patients, while it was normal for all the controls (P = not significant) (Table 1).

**Differential cell counts in sputum cells**

Lymphocytes were significantly higher in the sarcoid patients (23.6 ± 12.4%) than in controls (14.4 ± 10.2%) (P = 0.01), but no differences were found for the other cells. The CD4/CD8 ratio was 5.3 ± 3.4 for sarcoid patients and 2.1 ± 0.48 for controls (P = 0.001). This difference was mainly caused by significant differences in CD4 T cells, which were 53.4 ± 18.6% in the sarcoid group and 30.9 ± 12.9% in the control group (P = 0.033). No significant difference was found in the number of CD8 T cells (15 ± 13% sarcoid and 14.5 ± 5.1% controls, P = 0.9) (Table 2).

**VEGF and IL-8 levels in sputum**

VEGF levels were significantly lower in the sarcoid patients (111.4 ± 91.9 pg/mL) than in controls (228.9 ± 167.8 pg/mL, P = 0.03). When the sarcoid patients were categorized by clinical stage, the mean VEGF level of the stages I–II patients (n = 15) was significantly higher than the mean VEGF level of the stages III–IV (n = 7) patients (132.2 ± 102 vs 66.9 ± 43.4 pg/mL, P = 0.048). IL-8 was detected in 10 of the 35 samples but there were no significant differences between the sarcoid and control groups (Fig. 1a,b).
respectively). A positive correlation was found between IL-8 levels and % eosinophils (r = 0.369; P = 0.045) while a borderline correlation was found between IL-8 and % neutrophils (r = 0.377 P = 0.054). There was a weak correlation between VEGF levels and FEV1/FVC (r = 0.43 P = 0.058) (Table 3). No other significant correlations were found.

**VEGF immunohistochemical staining**

In order to understand the differential secretion of VEGF in the sarcoid patients, five tissue samples recovered from the TBB were immunostained. The non-fibrotic areas showed 6.14 ± 2.3 vs 0.48 ± 0.8 immunopositive cells in more fibrotic lesions (P = 0.005). Representative sections with strong, mild and negative stained granulomatous regions associated with areas that showed different grades of fibrosis in the same sample are displayed in Figure 2a–c.

**DISCUSSION**

The main finding of this study was reduced levels of VEGF in the IS samples of patients with sarcoidosis compared with the IS samples of healthy controls. Similar data have been reported from bronchoalveolar studies, but the present study represents the first demonstration of the utility of a non-invasive approach (IS) for studying angiogenesis in sarcoidosis. IL-8 was detected in one-third of the IS samples; however, these were positively correlated with the secretion of VEGF.

The present study included patients with tissue-proven sarcoidosis in different radiological stages. The IS cellular parameters, including differential cell counts and T-cell subsets, confirm the presence of increased lymphocytosis with CD4 positive T-lymphocyte predominance.25-27 Moreover, several studies have confirmed that the level of several cytokines in IS samples reflect those present in the lining fluid recovered by BAL. Moodley et al.27 showed that the CD4/CD8 ratio and tumour necrosis factor-α levels in the IS of sarcoid patients correlated with those in BAL fluid and also paralleled changes with treatment. The information obtained by IS on neutrophils elastase in cystic fibrosis28 and on IL-8 in recovered from the TBB were immunostained. The non-fibrotic areas showed 6.14 ± 2.3 vs 0.48 ± 0.8 immunopositive cells in more fibrotic lesions (P = 0.005). Representative sections with strong, mild and negative stained granulomatous regions associated with areas that showed different grades of fibrosis in the same sample are displayed in Figure 2a–c.

**Table 2** Cell populations in the induced sputum samples of study participants

<table>
<thead>
<tr>
<th></th>
<th>Eos (%)</th>
<th>Ly (%)</th>
<th>Mac (%)</th>
<th>Neut (%)</th>
<th>CD4/CD8</th>
<th>CD8 (%)</th>
<th>CD4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA (n = 22)</td>
<td>3.6 ± 5.5</td>
<td>24 ± 12</td>
<td>27 ± 21</td>
<td>45 ± 21</td>
<td>5.3 ± 3.4*</td>
<td>15 ± 13</td>
<td>53 ± 18*</td>
</tr>
<tr>
<td>SA Stage I</td>
<td>4 ± 6</td>
<td>24 ± 13</td>
<td>27 ± 26</td>
<td>45 ± 23</td>
<td>5.9 ± 3.6</td>
<td>12 ± 8</td>
<td>54 ± 18</td>
</tr>
<tr>
<td>SA stages II–III</td>
<td>2.7 ± 4</td>
<td>24 ± 12</td>
<td>28 ± 22</td>
<td>45 ± 20</td>
<td>4 ± 2.7</td>
<td>21 ± 19</td>
<td>50 ± 20</td>
</tr>
<tr>
<td>Controls (n = 13)</td>
<td>3.4 ± 2.3</td>
<td>14 ± 10</td>
<td>32 ± 22</td>
<td>47 ± 25</td>
<td>2.1 ± 0.5</td>
<td>14 ± 5</td>
<td>31 ± 13</td>
</tr>
</tbody>
</table>

Lymphocytes (Ly) and the CD4/CD8 ratio were significantly different in sarcoid patients (SA) and controls (P < 0.01).

**Table 3** Correlations1 between cytokines levels and the number of specific cell types

<table>
<thead>
<tr>
<th></th>
<th>Vascular endothelial growth factor</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>r = 0.493</td>
<td>r = 0.369</td>
<td>r = 0.377</td>
</tr>
<tr>
<td>P = 0.003</td>
<td>P = 0.045</td>
<td>P = 0.054</td>
<td></td>
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</table>

1Non-parametric Mann–Whitney test. Significance was set at P < 0.05.

The IS samples of healthy controls (CO). A significant difference was found between levels in SA patients and levels in CO (*P = 0.03) and between levels in SA stages I–II and levels in SA stages III–IV (**P = 0.048). Levels of IL-8 in patients with SA and CO. There were no significant differences in the levels of IL-8 between the patient groups.

**Figure 1** (a) Levels of vascular endothelial growth factor (pg/mL) in patients with sarcoid (SA) and controls (CO). A significant difference was found between levels in SA patients and levels in CO (*P = 0.03) and between levels in SA stages I–II and levels in SA stages III–IV (**P = 0.048). (b) Levels of IL-8 in patients with SA and CO. There were no significant differences in the levels of IL-8 between the patient groups.

The levels of both cytokines (VEGF and IL-8) were well correlated (r = 0.493 P = 0.003). A significant positive correlation was found between IL-8 levels and % eosinophils (r = 0.369; P = 0.045) while a borderline correlation was found between IL-8 and % neutrophils (r = 0.377 P = 0.054). There was a weak correlation between VEGF levels and FEV1/FVC (r = 0.43 P = 0.058) (Table 3). No other significant correlations were found.
lung-transplanted patients\textsuperscript{29} is comparable or complementary to BAL findings. The present study measured VEGF levels in IS, whereas others had conducted these measurements using BAL\textsuperscript{19} Both methodologies have shown that the levels of secreted VEGF are lower in patients with pulmonary fibrosis and sarcoidosis than in controls. Moreover, the levels of VEGF in stages III–IV patients were significantly lower than the levels in stages I–II patients, indicating a less fibrotic parenchymal disorder for the latter.

Maitre et al. showed that the decrease in secretion of VEGF is parallel to the reduction in VEGF mRNA levels in tissue lesions in animal models of ARDS\textsuperscript{30} and Abadie et al. demonstrated that it is negatively correlated with endothelial cell apoptosis in lung specimens retrieved from ARDS patients.\textsuperscript{31} Another possible explanation for the decreased VEGF level is proteolytic degradation. Interstitial lung diseases are associated with chronic inflammation in the lungs, thus it is possible that these proteases may degrade VEGF and lead to the decreased level of VEGF in BALF. Supporting this, it has been shown that the concentrations of MMP-9 and the molar ratio MMP-9/TIMP-1 are significantly increased in the supernatants recovered from the IS of patients with SA.\textsuperscript{32}

Ageing also influences the levels of VEGF in BAL fluid, with levels declining significantly with advancing age.\textsuperscript{33} In the current study, there was no significant difference in age between the sarcoid and healthy control groups, so age alone can not explain the reduced levels of VEGF in IS samples of the sarcoid patients.

There was no correlation between VEGF secretion and differential cell counts in the IS samples, raising the question of what is the main cellular source of the secreted protein. The endothelial cell has been considered important in the secretion of angiogenic factors and in the response to them.\textsuperscript{34} Although VEGF was demonstrated by RT-PCR to exist in various sources, including human peripheral blood leucocytes,\textsuperscript{35} the expression of VEGF receptors has been reported to be limited to endothelial cells\textsuperscript{34} On the other hand, IL-8, an angiogenic member of the CXC (chemokine X cytokine) chemokine family, was correlated with the presence of neutrophils and eosinophils in IS samples.\textsuperscript{36} Kituchi et al.\textsuperscript{37} recently showed that the neutrophilic inflammation observed in severe asthma is often associated with IL-8 and that neutrophils can secrete a variety of mediators that may augment the migration of eosinophils.

No significant correlation was found between the VEGF levels and pulmonary function. This was explained by Winterbauer and Hutchinson who reported that the fibrotic changes that appeared in the different radiological stages in sarcoid patients were only slightly reflected in a decreased DL\textsubscript{CO} capacity while all other flow and lung volume parameters were normal.\textsuperscript{38} Interestingly, in the present study, the positively immunostained granulomatous regions were associated with areas that showed less fibrosis and the negatively stained area with fibrotic areas in the same sample. A similar pattern was observed in surgical lung biopsies of usual interstitial pneumonias in which a strikingly heterogeneous and non-uniform fibrosing process was one of the most characteristic features, together with alternating zones of fibrosis, honeycomb change and intervening patches of normal lung.\textsuperscript{12} The role of VEGF in the fibrotic process remains contradictory. VEGF seems to have different effects, depending on its level, location and the underlying pathologic process in lung tissue.\textsuperscript{18} Ebina et al.\textsuperscript{14} described the increased expression of VEGF in capillary endothelial cells and alveolar type II epithelial cells in highly vascularized alveolar septa, while fibroblasts and leucocytes in fibrotic lesions were faintly immunoreactive. This suggests a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Vascular epithelial growth factor (pg/mL) immunostaining of sarcoid biopsy. (a) Fibrotic area associated with negative staining; (b) mixed area associated with intermediate staining; (c) normal areas associated with positive staining.}
\end{figure}
possible role for VEGF in the vascular heterogeneity of IPF and raises the question of whether the increase in vascular density observed in the least fibrotic areas is a consequence of the development of the fibrogenic process or represents a compensatory mechanism.  
11 This compartmentalization is not reflected in peripheral samples, since plasma VEGF concentrations did not differ between patients with IPF and controls.  

In conclusion, these data support the concept that non-invasively recovered IS samples from airways reflect the interstitial microenvironment of parenchymal lung diseases and that IS can be considered a useful tool for investigating the immunopathological mechanisms in those diseases.

ACKNOWLEDGMENT

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