Circulating vascular endothelial growth factor and systemic inflammatory markers in patients with stable and exacerbated chronic obstructive pulmonary disease

Arschang VALIPOUR*, Martin SCHREDER†, Michael WOLZT‡, Sleman SALIBA‡, Sonja KAPIOTISS§, Philipp EICKHOFF* and Otto Chris BURGHUBER*

*Department of Respiratory and Critical Care Medicine, Ludwig Boltzmann Institute for Chronic Obstructive Pulmonary Disease, Otto-Wagner-Hospital, Vienna, Austria, †Department of Clinical Pharmacology, Medical University Vienna, Vienna, Austria, ‡Department of Mathematics, Technical University of Kaiserslautern, Kaiserslautern, Germany, and §Clinical Institute for Medical and Chemical Laboratory Diagnostics, Medical University Vienna, Vienna, Austria

ABSTRACT

The aim of the present study was to assess circulating levels of VEGF (vascular endothelial growth factor), a biomarker with prognostic significance in cardiovascular disease, and markers of systemic inflammation in patients with stable and exacerbated COPD (chronic obstructive pulmonary disease). Lung function parameters, arterial blood gas analysis and circulating levels of VEGF, IL-6 (interleukin-6), TNF-α (tumour necrosis factor-α), CRP (C-reactive protein), fibrinogen and the peripheral blood neutrophil cell count were assessed in 30 patients on admission to the hospital for acute exacerbation of COPD, in 30 age-, gender- and BMI (body mass index)-matched patients with stable COPD, and 30 matched controls with normal lung function. Patients with acute exacerbated COPD had higher circulating concentrations of VEGF (P < 0.001), IL-6 (P < 0.05) and CRP (P < 0.01) and an increased blood neutrophil cell count (P < 0.05) compared with patients with stable COPD and healthy controls. VEGF levels in exacerbated COPD correlated with systemic inflammatory markers, such as CRP (r = 0.61, P < 0.005), IL-6 (r = 0.46; P < 0.01) and fibrinogen (r = 0.39, P < 0.05). In patients with stable COPD, there was a significant relationship between circulating VEGF levels and the percentage of the predicted FEV1 (forced expiratory volume in 1 s) (r = 0.47, P < 0.01). Recovery from the exacerbation resulted in a significant decrease in both circulating VEGF levels and markers of systemic inflammation. In conclusion, circulating levels of VEGF and markers of systemic inflammation are up-regulated in patients with acute exacerbated COPD and decrease after recovery from the exacerbation.

INTRODUCTION

VEGF (vascular endothelial growth factor), a soluble heparin-binding glycoprotein, is a cytokine with potent angiogenic properties, enhancing vascular permeability and modulating thrombogenicity [1]. VEGF further serves as an endothelial cell survival factor, protecting endothelial cells against apoptosis and delaying endothelial

Key words: cardiovascular disease, chronic obstructive pulmonary disease (COPD), C-reactive protein (CRP), systemic inflammation, vascular endothelial growth factor VEGF.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; IL-6, interleukin 6; IQR, interquartile range; LTOT, long-term oxygen treatment; PaO2, arterial partial pressure of oxygen; PaCO2, arterial partial pressure of carbon dioxide; PBNC, peripheral blood neutrophil cell; PEF, peak expiratory flow; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor; VEGFppr, VEGF in platelet-rich plasma; VEGFppp, VEGF in platelet-poor plasma; VEGFser, VEGF in serum.

Correspondence: Dr Arschang Valipour (email arschang.valipour@wienkav.at).
cell senescence [2]. The biological properties of VEGF have led to interest in its role within the lung in both health [3] and disease [4]. In fact, studies have suggested that the expression of VEGF in lung tissue obtained from patients with COPD (chronic obstructive pulmonary disease) may play a role in the pathogenesis of the disease [5,6]. Kasahara et al. [5] have shown that VEGF protein expression is decreased in lung specimens obtained from patients with an emphysematous type of COPD. The authors have suggested that the absence of VEGF, which is a trophic factor for endothelial cells, contributes to the development of emphysema. Consistent with these findings, Kanazawa et al. [7] observed a positive relationship between VEGF concentrations in induced sputum samples and FEV₁ (forced expiratory volume in 1 s) in patients with stable COPD.

Over the last few years, VEGF levels have also been measured in the peripheral circulation as a biomarker of neovascularization and/or vascular remodelling. Several studies have reported increased circulating VEGF levels in conditions in which tissue hypoxia and/or inflammation are critical, such as coronary heart disease [8], sepsis [9], cancer [10] and rheumatoid diseases [11]. Furthermore, there is accumulating evidence that circulating VEGF levels have prognostic significance, particularly in CVD (cardiovascular disease) [12], which could provide a mechanism to link the association between inflammation and promotion of CVD. Both hypoxia and inflammation are also relevant to COPD. Thus the aims of the present study were 3-fold: (i) to determine circulating levels of VEGF and systemic inflammation in patients with COPD and appropriate controls, (ii) to assess the effects of acute deteriorations of respiratory health in COPD on circulating VEGF and systemic inflammatory markers during and after recovery from an acute exacerbation of COPD, and (iii) to assess the relationship between markers of systemic inflammation, hypoxaemia and lung function parameters with circulating VEGF levels in both stable and exacerbated COPD.

**MATERIALS AND METHODS**

The protocol was approved by the Ethics Committee of the Vienna City Council, and informed consent was obtained from each subject.

**Selection of patients**

The study population consisted of patients with acute exacerbated COPD admitted to the hospital, patients with stable COPD and control subjects without airflow obstruction.

Patients with acute exacerbated COPD

During an enrolment period of 12 months, a total of 90 patients admitted to the Otto-Wagner-Hospital because of acute exacerbated COPD were screened for eligibility [13]. Acute exacerbation was defined according to recommended international criteria [14]. We excluded patients who had conditions that could potentially affect circulating VEGF levels. Accordingly, 12 patients were excluded due to pneumonia, acute respiratory failure \((n = 8)\), heart failure \((n = 7)\), acute myocardial infarction \((n = 2)\) and interstitial lung disease \((n = 4)\). A total of seven patients were receiving oral anticoagulant therapy, five patients had recent revascularization, four patients had preceding lung surgery, five patients had renal failure, three patients had a history of cancer, and three patients were suffering from an autoimmune disease. Therefore 30 patients with acute exacerbated COPD were included in the final analysis. Suitable patients were recruited into the study within 24 h of admission. Pre-exacerbation pulmonary function tests were evaluated to corroborate the diagnosis of COPD.

Blood samples for cytokine measurements and arterial blood gas analysis with subjects breathing room air were taken before the initiation of exacerbation therapy. After blood sampling, all patients were treated with nebulized bronchodilator therapy, prednisolone and theophylline. Other treatments, such as antibiotics, were administered based on clinical need. A second blood sample was collected from the patients in a clinically stable state 6 weeks after hospital discharge. Spirometric measurements (V6200; Sensormedics) of FEV₁, FVC (forced vital capacity), FEV₁/FVC and PEF (peak expiratory flow) were performed at the follow-up visit to confirm the diagnosis of COPD. Predicted values for spirometric parameters were those of an Austrian population [15].

Patients with stable COPD

A total of 30 patients with stable COPD were recruited from the hospital outpatient clinic. These were matched with respect to age, gender, BMI (body mass index), smoking history (pack years) and FEV₁ to patients with exacerbated COPD. A stable condition was defined as a stable disease without reported exacerbations and at least three stable visits during the previous 6 months without any changes in respiratory medication and by the absence of an infection prior to inclusion in the study. On the basis of high-resolution computed tomography and lung function tests performed, most of these patients \((n = 23)\) were diagnosed with COPD of an emphysema type.

**Control subjects**

A total of 30 healthy subjects matched with respect to age, gender and BMI to patients with COPD comprised the control group. The controls had normal pulmonary function.

**Blood sampling preparations**

To assess the relative contribution of platelets to VEGF release into the circulation, concentrations of VEGFser
(VEGF in serum), VEGFprp (VEGF in thrombin-stimulated platelet-rich plasma) and VEGFppp (VEGF in non-stimulated platelet-poor plasma) were measured. Approx. 30 ml of venous blood was collected from the subjects’ cubital vein and divided into 3-ml plastic blood collection tubes containing either EDTA (for full blood count), trisodium citrate [for VEGFppp, VEGFprp, IL-6 (interleukin-6) and TNF-α (tumour necrosis factor-α)] or no anticoagulant (VEGFser). Serum and platelet-poor plasma samples were centrifuged at 10°C for 10 min at 3500 g. The supernatant was collected, aliquoted and frozen at −85°C until assay of VEGFppp, IL-6 and TNF-α.

VEGFprp was prepared after thrombin-activation. Venous blood was drawn into sodium-citrate-containing tubes (0.31 % w/v, final concentration) and centrifuged at 10°C for 15 min at 180 g. The supernatant platelet-rich plasma was separated and 4 units/ml thrombin was added to 1 ml of platelet-rich plasma. After clotting, samples were centrifuged at 10°C for 10 min at 3500 g, and the supernatant was removed, aliquoted and stored at −85°C.

All blood samples were processed within 30 min and frozen within 1 h after collection. Laboratory staff were blinded to the clinical data.

**Laboratory measurements**

All measurements were carried out in duplicate as a batch test. The human VEGF immunoassay (R&D Systems) was calibrated with a monoclonal antibody raised against recombinant VEGF165, the major isoform of soluble VEGF. The lower detection limit of the system was 9.0 pg/ml; intra- and inter-assay variations were 6.7 and 8.8 % respectively. IL-6 was measured using high-sensitivity quantitative ELISA kits (R&D Systems), and results are expressed as pg/ml. TNF-α was measured using high-sensitivity quantitative ELISA kits (R&D Systems). Concentrations below the LOQ (limit of quantification) were set to LOQ/2. Cell counts were performed with an automated haematology analyser (SE-9500; Sysmex). Analysis of the platelet-rich plasma preparations showed them to be 98–100 % pure. Plasma fibrinogen was determined according to the Clauss method. CRP (C-reactive protein) levels were assessed using an automated analyser (Hitachi 917; Boehringer Mannheim).

**Statistical analysis**

Clinical data with a normal distribution are presented as means (S.D.). Laboratory data are all reported as medians [IQR (interquartile range)]. Analysis between groups was performed using a χ² test or ANOVA, followed by post-hoc analysis as appropriate. For analysis of follow-up data in patients with exacerbated COPD, a Mann–Whitney U test was performed. Variables that correlated significantly with VEGFser levels in univariate analysis (Spearman’s rank correlation) were included in a backward stepwise multiple regression analysis. In these models, we forced in age, gender, BMI, PaO₂ (arterial partial pressure of oxygen), inhaled steroid use, pack years of smoking, hypertension and platelet count as covariates to adjust for their potential effects on VEGFser. A P value < 0.05 was considered statistically significant.

**RESULTS**

**Patient characteristics**

The characteristics of patients and controls are summarized in Table 1. The groups were well-matched with respect to age, gender and BMI. By definition, lung function parameters were normal in controls and significantly decreased in patients with COPD. There was no significant difference in the proportion of patients with oxygen therapy, hypertension, inhaled bronchodilator or corticosteroid use between patients with exacerbated COPD and those with stable COPD. Mean (S.D.) duration of hospitalization for patients with exacerbated COPD was 10.6 (3.7) days.

**VEGF measurements**

Coefficients of variation of the duplicate ELISAs were generally <5 %. The median (IQR) concentration of VEGFser [359 (201–639) pg/ml] was significantly higher (P < 0.001) than the corresponding concentration of
Table 2  Circulating cytokine concentrations and other laboratory markers in the study population

<table>
<thead>
<tr>
<th>Cytokine/marker</th>
<th>Exacerbated COPD (n = 30)</th>
<th>Stable COPD (n = 30)</th>
<th>Control subjects (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFs (pg/ml)</td>
<td>602 (457–883)**</td>
<td>229 (151–310)</td>
<td>238 (134–143)</td>
</tr>
<tr>
<td>VEGFpp (pg/ml)</td>
<td>275 (169–431)**</td>
<td>110 (42–188)</td>
<td>155 (61–210)</td>
</tr>
<tr>
<td>VEGFppp (pg/ml)</td>
<td>22 (13–28)*</td>
<td>16 (13–19)</td>
<td>11 (5–21)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>3.5 (0.8–6.2)*</td>
<td>2.2 (1.7–2.9)†</td>
<td>1.2 (0.7–1.4)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>6.0 (1–31)**</td>
<td>4.0 (2–6)</td>
<td>1.5 (0–5)</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>419 (329–470)†</td>
<td>424 (358–459)†</td>
<td>360 (326–393)</td>
</tr>
<tr>
<td>PBNC count (×10^3 cells/μl)</td>
<td>9.5 (6–12)*</td>
<td>7.0 (5–9)†</td>
<td>4.0 (4–6)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>14.0 (13–15)</td>
<td>15.0 (13–16)</td>
<td>14.3 (13–15)</td>
</tr>
<tr>
<td>Platelet count (×10^9 cells/μl)</td>
<td>279 (194–356)</td>
<td>273 (218–316)</td>
<td>268 (196–300)</td>
</tr>
</tbody>
</table>

VEGFppp [178 (90–302) pg/ml], despite a higher platelet count in the latter [432 (350–591) × 10^3 platelets/μl of platelet-rich plasma compared with 251 (202–309) × 10^3 platelets/μl of serum; P < 0.01]. There was a significant correlation between VEGFs and VEGFppp (r = 0.681; P < 0.001), and between VEGFs and VEGFppp (r = 0.474; P < 0.01). The correlations did not change significantly when the results from the control subjects were subtracted.

Circulating VEGF and systemic inflammatory markers in the study groups

The groups were well-matched with respect to haemoglobin concentrations and platelet count (Table 2). Overall, VEGF levels were significantly higher (P < 0.01) in patients with acute exacerbated COPD than in patients with stable COPD or controls with normal lung function. These findings were paralleled by an increase in CRP, PBNC (peripheral blood neutrophil cell) count and circulating IL-6 levels in patients with exacerbated COPD. Fibrinogen levels were significantly higher in patients with COPD compared with controls (P < 0.01 for both), with no significant difference between patients with stable and exacerbated COPD. There were no statistically significant differences in TNF-α concentrations between the groups.

Circulating VEGF and systemic inflammation at clinical recovery

There was a significant decrease in VEGFs levels from 602 (457–883) during acute exacerbation to 490 (378–699) pg/ml (P = 0.032) at 6 weeks after hospital discharge (Figure 1). There was a similar decrease in VEGFpp and VEGFppp levels (results not shown). The decrease in circulating VEGF was accompanied by a significant decrease in CRP [6 (1–31)] compared with 1 (1–4) mg/l; P = 0.013] and fibrinogen [419 (329–470)] compared with 311 (249–401) mg/l; P = 0.012] concentrations. Furthermore, there was a decrease in PBNC count and circulating IL-6 levels; however, these changes did not achieve statistical significance.

Correlation analysis for patients with stable and exacerbated COPD

For patients with exacerbated COPD, positive correlations were found between VEGFs and CRP, IL-6, PBNC count and fibrinogen (Table 3). Stepwise multiple regression analysis revealed that CRP was the strongest
Table 3  Correlation coefficients of VEGFser with inflammatory markers, lung function parameters and oxygenation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with exacerbated COPD</th>
<th>Patients with stable COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/l)</td>
<td>0.61***</td>
<td>0.15</td>
</tr>
<tr>
<td>PBNC count (&lt; 10³ cells/μl)</td>
<td>0.46**</td>
<td>0.46**</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.46**</td>
<td>0.07</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>0.39*</td>
<td>0.02</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.21</td>
<td>0.02</td>
</tr>
<tr>
<td>FEV₁ (litres)</td>
<td>-0.23</td>
<td>0.36*</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>-0.28</td>
<td>0.47**</td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>0.25</td>
<td>0.09</td>
</tr>
</tbody>
</table>

is cumulating evidence that neutrophils contribute to circulating VEGF [21,22]. According to Kusumanto et al. [22], approx. 60% of total circulating VEGF resides in neutrophils, whereas only 34% resides in platelets. Although we have not assessed the relative contribution of neutrophils to VEGF liberation into the circulation, we observed a strong relationship between levels of VEGFser and PBNC count, which may support a role for neutrophils in blood-borne VEGF. In disease conditions, the amount of neutrophil-derived VEGF may increase further. Neutrophils may secrete VEGF in response to acute inflammatory stimuli associated with viral [23] and bacterial [24] infections, which are frequently associated with exacerbations of COPD.

**DISCUSSION**

The present study has investigated circulating levels of VEGF and markers of systemic inflammation in carefully selected patients with stable COPD, patients with acute exacerbated COPD, and age-, gender- and BMI-matched controls with normal lung function.

The origin of circulating VEGF

Platelets have been proposed as the major source of circulating VEGF [16]. VEGF is released from platelets during blood clotting [17], haemostatic plug formation [18] and in response to inflammatory stimuli [19]. Concentrations of VEGFser in the present study, however, were significantly higher than the levels of VEGFprp, despite a higher platelet count in the latter. These findings suggest that, in addition to platelets, a further VEGF pool exists in serum that cannot be explained by platelet activation alone. In a previous study from our laboratory, Mittermayer et al. [20] observed a significant increase in circulating VEGF levels in healthy volunteers after experimentally induced systemic inflammation. VEGF levels in that study were related to markers of neutrophil activation rather than clot formation, suggesting a mechanism of VEGF release different from that of the coagulation cascade. There
sputum samples, our present findings may suggest a similar association between VEGF concentrations and airflow obstruction in patients with the emphysematous type of COPD, as reported previously [7].

Compared with stable COPD, patients with acute exacerbated COPD in the present study had higher circulating concentrations of VEGF and increased systemic inflammation. Furthermore, we observed a significant relationship between markers of systemic inflammation, such as CRP, IL-6 and fibrinogen, with levels of VEGFser in acute exacerbated COPD.

Low-grade systemic inflammation is common in patients with COPD [30]. The degree of systemic inflammation associated with COPD tends to increase over time [31] and is, furthermore, up-regulated during acute exacerbations [26,32]. Systemic inflammation in COPD has been associated with an increased risk of developing CVD [33]. One mechanism for this may be an increase in procoagulatory factors, such as IL-6 and fibrinogen, both of which have also been linked to VEGF [19,34,35]. Increased VEGF expression, in turn, may support prothrombotic effects via expression of the plasminogen activator inhibitor, von Willebrand factor and tissue factor [36,37]. In fact, there is evidence that circulating VEGF levels are increased in patients with acute coronary syndrome [8,12]. In a study by Heeschen et al. [12], patients with acute myocardial ischaemia and serum VEGF levels > 500 pg/ml had the lowest 6-month survival rates. Similarly, Slevin et al. [38] demonstrated increased concentrations of VEGFser (588 ± 121 pg/ml) in patients with acute ischaemic stroke. Expression of VEGF in that study correlated with both infarct volume and clinical disability. Levels of VEGF detected in acute exacerbated COPD are comparable with those associated with individuals at high risk of cardiovascular morbidity and mortality. The significant relationship in our present study between CRP, a marker strongly predictive for the development and prognosis of CVD, and VEGFser supports this relationship further.

A recent study [26] similarly assessed several circulating biomarkers, including VEGF, in patients with COPD during a stable state as well during an exacerbation. In contrast with our findings, however, Hurst et al. [26] did not observe a significant difference in median plasma VEGF levels between baseline (0.01 pg/ml) and exacerbation (0.01 pg/ml). Several methodological reasons may account for the discrepancy between our findings. (i) These authors have measured only free plasma levels of VEGF and may, therefore, have missed assessing the contribution of platelets and neutrophils to circulating VEGF levels. (ii) Hurst and co-workers used an antibody microarray to assess 36 different biomarkers in their patients. Compared with conventional ELISAs, the former is basically a qualitative exploratory technique with an overall lower sensitivity, particularly in the low picogram range, whereas the latter is a more accurate method to specifically quantify protein levels [39,40]. Moreover, the ELISA results in our present study were verified by duplicates in all cases. (iii) All patients in the present study were admitted to hospital because of acute exacerbated COPD, whereas only 3% of the patients studied by Hurst et al. [26] required hospitalization, indicating differences in the severity of exacerbation and, therefore, potential differences in the stimulus for VEGF release between the studies.

Finally, in the present study, we observed that recovery from an exacerbation resulted in a decrease in circulating VEGF levels and systemic inflammatory markers in patients with COPD. Similar results have been obtained by McColley et al. [41], who have shown that antibiotic therapy decreased VEGF levels and inflammatory markers in patients with cystic fibrosis. As all of our patients received systemic steroids and most received antibiotic therapy, we have to acknowledge that we cannot identify the effects of either on VEGF or systemic inflammation. Furthermore the impact of the observed changes in levels of VEGFser on patient outcomes has yet to be determined in longitudinal studies.

Potential limitations of the study

On a molecular level, hypoxia is an important stimulus for VEGF production [1]; however, we did not observe a relationship between circulating VEGF levels and arterial oxygenation in our present study. In this context, it has to be acknowledged that the hypoxic stimulus for VEGF production was decreased, as practically half of the patients with COPD were on LTOT. On the other hand, there is experimental evidence that both platelets and neutrophils, the predominant sources of circulating VEGF, do not release VEGF in response to hypoxia [42]. In addition, it has been shown previously that the transcription of VEGF may be inhibited in patients with chronic hypoxia [43]. Thus we suggest that hypoxaemia associated with COPD does not appear to be an independent stimulus for circulating VEGF.

Furthermore, we have to acknowledge that we did not perform echocardiography to rule out pulmonary hypertension, a potential confounder of circulating VEGF levels in patients with COPD. Tanaseanu et al. [44] have recently reported elevated VEGF plasma concentrations in patients with COPD with pulmonary hypertension compared with those without. Nevertheless, given the close matching of the patient groups and the strong association between changes in systemic inflammation and circulating VEGF levels, it is unlikely that the presence of pulmonary hypertension significantly biased our findings.

Conclusions

Our present findings suggest an up-regulation of systemic inflammation and circulating VEGF levels in patients with acute exacerbated COPD. Given the compelling
evidence implicating vascular factors in the pathogenesis of both chronic lung and CVD processes, VEGF may be an important factor linking COPD with cardiovascular pathology.

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