Immunohistochemical Evaluation of Growth Factors in the Peripheral Blood Vessels of Patients with Severe COPD and Osteoporosis

Ludmila G. Ugay¹, Evgeniya A. Kochetkova¹, Vera A. Nevzorova¹, Igor V. Manzulo²,³,⁵*, Yuliya V. Maistrovskaia¹, Eugeni V. Shepichev ⁴

¹State Budget Educational Institution of Higher Professional Education «Pacific State Medical University» of the Ministry of Public Health Russian Federation (PSMU), Vladivostok, Ostryakova Avenue 2, 690002, Russia.
²A.V. Zhirmunsky Institute of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 17 Palchevskii str., 690059, Russia
³School of Biomedicine, Far Eastern Federal University, Vladivostok, 8 Sukhanova str., 690950, Russia
⁴Medical center of Far Eastern Federal University Vladivostok, Russkiy ostrov, Village Ayax 10.
⁵Laboratory of Pharmacology, Institute of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 17 Palchevskii str., 690059, Russia.

*Corresponding Author: Igor V. Manzulo

Abstract

The expression of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGFβ) in the blood vessels of striated muscle specimens of 20 severe chronic obstructive pulmonary diseases (COPD) with osteoporosis and healthy subjects were assessed by immunohistochemistry method with using immunoperoxidase reaction with monoclonal and polyclonal specific antibodies. The serum levels of VEGF and TGFβ were studied in 74 severe COPD patients and 36 healthy individuals using immynoenzyme (ELISA) methods. Elevated level in TGF-β and reduced serum VEGF were associated with low bone mineral content and muscle mass in the COPD patients. Increased expressions of TGF-β2 and VEGF were observed in the vascular wall in the severe COPD group with osteoporosis. Intensities of staining in the arterial structures were higher in the COPD group, which were primarily presented in the endothelial and muscular layers. Thus, our study strengthens the hypothesis that change tissue and serum markers of the vascular remodeling are involved in pathophysiology of bone damage in severe COPD.

Keywords: Chronic Obstructive Pulmonary Disease, Osteoporosis, Vascular Endothelial Growth Factor, Transforming Growth Factor Beta, Vascular Dysfunction.

Introduction

According to modern concepts, chronic obstructive pulmonary disease is considered a multifactorial disease with clinical manifestations that are determined by diverse systemic effects, including osteoporosis [1]. Despite the bone loss in the patients with COPD is attributable to the systemic manifestations of COPD, the issues relate to the pathogenesis of osteoporosis in COPD patients are an area of an active discussion [2].

The COPD is a pressing problem, as this disease entails the physical performance limitations and disability. The disease starts in the bronchial mucosa: the secretary apparatus function changes in response to the external disease-producing factors (mucus hyper-secretion, changes in the bronchial secretion). In combination with an infection, body responses lead to the damage of bronchi, bronchioles and alveoli. The protease-antiprotease imbalance, as well as
the defects in the antioxidant defense of lungs, exacerbates the damage [3]. Interestingly, several studies have drawn attention to changes in serum markers during the vascular remodeling and the development of endothelial dysfunction that might be involved in the development of the osteoporosis in association with various diseases, such as carotid atherosclerosis, peripheral arterial disease, cardiovascular disease, and stroke [4-6]. Previous research has focused on the contributions of recruited inflammatory cells and their secreted mediators to the development of vascular remodeling [7]. Although many studies have demonstrated a link between coronary artery disease and osteoporosis, there are limited clinical data that suggest that endothelial dysfunction is associated with osteoporosis. Little is known about the contribution of vascular remodeling to bone damage and muscle wasting in COPD.

Because vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) have been implicated in the pathogenesis of COPD (Kierszniewska-Stepień et al., 2006; Santos et al., 2003; Beghe et al., 2006; Chi-Huei Chiang et al., 2014) the focus of the present study was on examining the expressions of these two molecules in intercostal muscles and their serum levels in patients with severe COPD with osteoporosis.

Materials and Methods

Subjects

The study population consisted of 74 subjects, including 36 healthy individuals (controls) and 38 patients with severe COPD and osteoporosis. The subjects were consecutively enrolled at the Department of Thoracic Surgery in the University Hospital. All participants underwent appropriate clinical and laboratory evaluations. All patients were clinically stable without evidence of lung infection at the time of the examinations. All participants provided written informed consent prior to their participation. The procedures were approved by the local Human Research Ethics Committee and were performed in accordance with the Declaration of Helsinki.

Bone Composition

The bone mineral density at the femoral neck (FN) and the lumbar spine (LS) expressed T-scores in standard deviation (SD) units, the bone mineral content (BMC) and skeletal muscle mass (expressed as lean mass) were determined by dual-energy X-ray absorptiometry (DEXA, GE Lunar “Prodigy”, USA) and according to standard protocols by a single experienced operator.

According to the recommendations of the WHO, T-score between -1 and -2.5 SD was considered osteopenia, T-score below -2.5 SD was diagnosed as osteoporosis, and T-score greater than -1.0 SD was confirmed as normal BMDs.

Biochemical Analyses

Blood samples were obtained from all individuals. The serum was separated and stored at -80°C prior to the analyses. Peripheral blood samples were collected into sterile glass tubes in the morning after an overnight fast. The samples were allowed to coagulate at room temperature for 30 min and were then centrifuged at 2500×g for 20 min.

The serum was separated and stored at −70°C before analysis. Prior to the analysis, the serum samples were slowly thawed and gently mixed. The simples were analyzed in duplicates. The serum VEGF and TGFβ2 levels were measured in duplicate with an ELISA test system (Quantikine, R&D Systems, Inc., Minneapolis, USA).

Immunohistochemistry

For the immunohistochemical analysis, we used biopsy materials (intercostal muscles) from 15 COPD (GOLD III) patients with bullous emphysema of lungs who had undergone thoracoscopic resection. The extractions of similar biopsied materials from the control group were intraoperatively performed in apparently healthy volunteers with corresponding ages and genders without respiratory pathologies who were undergoing surgeries related to traumatic rib fractures. To evaluate the level of growth factor secretion, we used immunoperoxidase reactions to reveal VEGF and TGF β2. The applied immunohistochemically method consisted of the following stages: tissue sample fixation (4% paraformaldehyde prepared in 0.1 M phosphate buffer, pH 7.2) for 12 hours and subsequent washing; the material was then placed in paraffin according to a standard procedure; a series of paraffin sections of 7 µm in thickness were obtained; pre-incubation with solutions that blocked endogenous peroxidase activities and
non-specific antibody binding were performed; the sections were treated in primary antibody solution (4°C, 24 hours); and treatment with primary antibodies for 1 hour at room temperature and the immunoperoxidase reaction were then performed. The materials used in the procedure included the following: primary mouse monoclonal antibodies to VEGF (Abcam ab1316, USA) and TGF beta 2 (Abcam ab36495, USA); these antibodies were diluted at 1:100 and 1:200, respectively. Secondary antibodies conjugated with horseradish peroxidase (Vector Laboratories, PI 2000 (anti-mouse), 1:100) were used according to the producers’ guidelines. For the immunoperoxidase reactions, chromogene (DAB Substrate Kit, Abcam ab64238, USA) was used. After staining, the sections were thoroughly washed in distilled water, dehydrated and placed into balsam according to the standard procedure. The obtained samples were studied with an AxioScope A1 light microscope (Carl Zeiss, Germany) and photographed with an AxioCam Icc3 digital camera (Carl Zeiss, Germany).

Statistical Analysis
The clinical and biochemical features of the population are presented as the means ± SDs. The COPD patients and healthy controls were compared using Student’s two-tailed unpaired t-tests. The Pearson coefficient was used to measure the linear correlations between variables. All analyses were performed using the Statistical Package for the of Social Sciences (SPSS) version 14.0 (SPSS Inc., USA) for Windows and the Microsoft Excel 2010 program. A value of p<0.05 was considered statistically significant.

Results
Clinical Findings
The clinical, biochemical, and body composition parameters of the patients with the severe COPD are illustrated in table 1. There was no significant difference in age between the COPD and healthy groups. The patients with COPD exhibited significantly higher TGF-β2 levels than did the healthy subjects, whereas the serum levels of VEGF were lower in the COPD group (Table 1).

Table 1: Clinical characteristics of the patients with COPD and the healthy subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy subjects</th>
<th>Severe COPD patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>36</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>54.93±5.92</td>
<td>54.54±6.03</td>
<td>0.896</td>
</tr>
<tr>
<td>Male/female</td>
<td>20/16</td>
<td>22/16</td>
<td></td>
</tr>
<tr>
<td>Smoking status (current/ex/never smokers)</td>
<td>10/11/15</td>
<td>28/6/4</td>
<td></td>
</tr>
<tr>
<td>Smoking index (pack-years)</td>
<td>27.71±7.52</td>
<td>57.13±12.23</td>
<td>na</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td>101.42±6.53</td>
<td>38.72±6.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>98.63±11.22</td>
<td>63.61±8.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>81.32±6.71</td>
<td>44.74±6.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DLCO (%)</td>
<td>96.73±12.62</td>
<td>46.31±11.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PaO2 (mmHg)</td>
<td>97.43±5.62</td>
<td>71.31±8.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PaCO2 (mmHg)</td>
<td>38.31±2.43</td>
<td>49.41±7.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.32±1.94</td>
<td>23.63±2.94</td>
<td>0.217</td>
</tr>
<tr>
<td>BMC (kg)</td>
<td>3.74±0.76</td>
<td>2.39±0.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>58.27±4.52</td>
<td>46.34±5.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TGF-β2 (ng/ml)</td>
<td>429.97±44.89</td>
<td>492.11±29.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>165.05±10.86</td>
<td>148.36±15.07</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations
COPD, chronic obstructive pulmonary disease; BMI, body mass index; FEV1, forced expiratory volume in one second; FVC, forced volume capacity; DLCO, single-breath diffusing capacity of carbon monoxide; PaO2, arterial partial pressure of oxygen; PaCO2, arterial partial pressure of carbon dioxide; BMC, bone mineral content; TGF-β2, transforming growth factors β1 and β2; VEGF, vascular endothelial growth factor. The univariate analysis revealed a negative correlation between the serum TGF-β2 concentration and the FEV1% (Fig. 1A). Additionally, the serum concentrations of TGF-β2 were significantly correlated with the emphysema marker carbon monoxide diffusion capacity (DLCO) (Fig. 1B).
Fig. 1: Relationships of serum TGF with pulmonary functional parameters in severe COPD patients. FEV1: forced expiratory volume in one second; DLCO: single-breath diffusing capacity for carbon monoxide; TGF-β2: transforming growth factors β2.

We observed a negative correlation between the serum TGF-β2 concentrations and the BMC (Fig. 2A). The serum TGF-β2 level was also negatively correlated with the lean mass (Fig. 2B). Significant positive correlations were observed between the serum VEGF levels and the BMC (Fig. 2C). Reduced serum VEGF levels were related to reduced skeletal lean mass (Fig. 2D).

Immunohistochemical Findings

We studied the TGF-β2 and VEGF expressions in the muscular arteries of the patients with severe COPD and the healthy subjects. The quantity of TGF in the immune positive arteries was 89.4±6.53% in the COPD patient samples and 36.12±6.82% in the control group, and this difference was significant difference. TGF-β2 expression in the vessel wall was observed in both groups. In the severe COPD group, evident increases in the TGF-β2 histochemical activities were observed in the endothelial (52.93±8.92%) and smooth muscular layers (87.34±8.51%), and these parameters were significantly greater than those in the healthy subjects (37.52±5.41% and 33.73±4.25%, respectively).

The percentage of VEGF-positive muscular arteries in the control group samples was significantly reduced (44.13±8.22%) compared with that in the severe COPD samples (93.21±7.34%). Moreover, the staining intensities of the intima (84.82±8.31%) and muscular layers (72.43±9.34%) of vessels in the COPD samples were much greater than those in the control group (32.31±3.42% and 18.64±9.13%, respectively). In our study, the elevated level of TGF-β2 and reduced serum concentration of VEGF were associated with low bone mineral content and muscle loss in the COPD patients.

The immunohistochemical results of the current study revealed that elevated expressions of TGF-82 and VEGF were observed in the vascular wall in the severe COPD group with osteoporosis compared with the control group.
Discussion

Presently, it is known that the vascular endothelium is an active metabolic system that supports vessel homeostasis via modulation of the vascular tone, regulation of the transportation of diluted substances to the vascular wall cells, the formation of the extracellular matrix and the control chemotactic, inflammatory and reparative processes in response to local damage. In the COPD, there is damage to the vascular endothelium that is directly related to structural changes of the vascular bed. Normally functioning endothelium is known to synthesize and secrete different biologically active substances, vasodilating agents, vasoconstrictors and growth factors.

However, with the growth of the endothelial dysfunction during COPD endothelin-1 and the growth factors, which are responsible for persistent vasoconstriction, proliferation and apoptosis of the endothelial cells, and in addition to the hypertrophy of the smooth muscular cells, are expressed numerously on endothelium surface with modified phenotype. The combination of these changes leads to the development of progressive vascular remodeling that in turn causes the progressive worsening of pulmonary function and sustained severe airway obstruction in the case of the severe COPD [8]. In support of the abovementioned process, several papers have reported increases in arterial stiffness in COPD patients [9].

Moreover, arterial stiffness is associated with disease severity. Also the bronchial wall is more vascularized in COPD patients compared to healthy individuals [10, 11]. An important role in COPD pathophysiology also belongs to angiogenic growth factors [12] that have a wide spectrum of biological influence on many cells; specifically, these, angiogenic growth factors stimulate or inhibit mitogenesis, chemotaxis and cell differentiation.

The best-studied endothelium-specific stimulator of angiogenesis is VEGF. The literature has described divergent effects of VEGF; in one set of conditions, VEGF acts as a protector, while in different conditions, it becomes a damaging factor. In the presence of an inadequate oxygen supply and a decrease in vascular blood flow, VEGF assumes the functions of a signal protein and plays an important role in improving vascular function and angiogenesis and thus helps restore the oxygen supply to the tissue [13]. Hence, healthy smokers exhibit a slight increase in the VEGF level that represents a compensatory defense mechanism. In contrast, in the case of pulmonary pathology, VEGF hyper production induces excessive cell proliferation [14]. In the case of experimental pulmonary emphysema, damage to the intracellular signaling controlled by VEGF has been noted [15]. Studies have demonstrated different results regarding VEGF, COPD patients with chronic bronchitis had the increased levels of VEGF and its soluble receptors in sputum [16] and peripheral blood [12, 17].

In contrast, COPD patients with the emphysematous phenotype exhibit a decrease in the concentration of this angiogenic growth marker [3, 18]. Moreover, according to Hosseini and Kierszniewska-Stepień, the elevation of the VEGF level in the blood serum is associated with disease severity [17, 18]. Other researchers have not found any deviations in the performance of this marker in COPD [19, 20]. In addition to the clinical results, the immunohistochemical research has demonstrated an increase in VEGF concentration in the supernatant from pulmonary fibroblasts and a reduced expression of VEGF receptors in the pulmonary arteries of COPD patients [7], which indicate that also VEGF may have different roles depending on disease progression and disease severity. In contrast, the research group of Lee revealed increases in VEGF expression in the bronchial epithelium, endothelium and vascular smooth muscles in the patients with chronic bronchitis [21].

Clinical studies with immunohistochemical analyses have demonstrated that changes in VEGF expression under hypoxic environment play an important role in vascular remodeling even in the early stages of the disease. For example, increased VEGF expression in the muscular pulmonary vessels is observed even among patients with mild COPD and smokers with regular pulmonary function compared with non-smokers. This expression is strictly related to the vascular wall thickness. Another study indicated the same results for patients with moderate COPD [15].

However, patients with severe pulmonary emphysema exhibit lower immunohistochemical VEGF expression in the pulmonary vessels and a decreased in its protein content
in the lung parenchyma despite intense vascular remodeling [14]. TGF-b is another multifunctional growth and differentiation factor that can control diverse biological processes [13]. The role of TGF-β as a vascular endothelium regulator is complex and controversial. The basic angiogenic effects of TGF-β have been demonstrated in vivo, and its cellular functions have also been clarified in vitro. These findings thus demonstrate the ability of TGF-β to suppress angiogenesis and induce apoptosis in endothelial cells [13].

Experiments proven that the introduction of TGF-β is accompanied by the disorganization and irregularity of the vascular pattern in whole mounted tissues of the mice (Sartori et al., 2014). An associated participates of the TGF-β and VEGF in vascular remodeling is not be excluded. Early in vivo studies demonstrated that the angiogenic effects might be mediated by the paracrine induction of VEGF [22]. In physiological conditions, the intensity of VEGF secretion by hematopoietic cells directly depends on the TGF-β level. Recently, the team of Fang demonstrated a novel mechanism by which TGF-b promotes angiogenesis in vivo via recruitment of paracrine VEGF expressing hematopoietic effector cells.

This VEGF mediated mechanism of action for TGF-b may affect the angiogenic balance during processes such as inflammatory conditions and tumor growth where TGF-b activity is upregulated. Interestingly, the vascular effects induced by TGF-β are significantly inhibited by VEGF neutralizing antibodies [13]. Therefore, the regulation of vascular remodeling is not limited only by the local production of different growth factors, including VEGF and TGF, and the intensities of their expressions on the surfaces of endothelial cells.

The intensity of vascular changes in COPD patients can also depend on the abilities of the growth factors to modulate the paracrine signaling systems that are necessary for cellular metabolism and remodeling. In the present study, the severe COPD patients exhibited a significantly higher level of TGF-β2 and a lower percentage of VEGF in the blood serum compared with the healthy group.

Additionally, divergent associations between the circulating TGF-β2, VEGF and the parameters of bronchial obstruction were observed. Interestingly, only the serum level of TGF-β2 was evidently correlated with the functional marker of pulmonary emphysema DLCO. The obtained results do not contradict those of other studies. Additionally, after a series of experimental and clinical studies, it was demonstrated that damage to endothelial function with the development of vascular remodeling can cause extravascular systemic effects, for example, changes in bone structure [4, 5]. There is increasing evidence for a common lineage and close interaction between vascular endothelial cells and bone cells. Moreover, it has been demonstrated that the osteogenetic differentiation of mesenchymal progenitor cells into osteoblast precursors is strictly controlled by endothelial cells.

Moreover, the influence of VEGF on signal transduction between the vascular network and bone tissue has been demonstrated as has the role of this growth factor in intramembranous osteogenesis. In contrast, the osteoblast and osteoclast surfaces have VEGF receptors (i.e., VEGFR-1 and VEGFR-2) and the VEGF-related co-receptor neuropilin, and the ability of osteogenic cells to produce VEGF has been detected [23, 24]. In vitro, VEGF and its receptors induce the chemotaxis, differentiation, proliferation and survival of osteoblasts and thus participate in an early stage of bone remodeling [1, 23, 25, 26].

These findings prove that VEGF can regulate bone formation by affecting osteoblasts in addition to its role in coupling angiogenesis and endochondral bone formation, but the evidence for the role of osteoblast-derived VEGF in bone development has only recently been obtained. Therefore, vascular factors regulate osteoclast and osteoblast activities [27]. Additionally, blood vessels can be used for the transport of circulating osteoblasts and osteoclast precursors to areas of active remodeling. Currently, much attention is being focused on the hypothesis of the close relation between angiogenesis and bone remodeling in different pathological conditions [1].

Nevertheless, the issue of vascular cell involvement in the initiation of bone resorption is currently unresolved. Generally, the role of vascular dysfunction in osteopenic syndrome development has been studied in a series of experimental [6] and clinical trials based on the connections of arterial stiffness and some markers of vascular remodeling with BMD [4, 5], and these studies have
demonstrated the importance of the endothelium in sustaining bone tissue homeostasis. Laroshe et al. was among the first to demonstrate that showed the number of thick-walled vessels, arterioles or arterial capillaries is strongly reduced in the femoral heads of patients with femoral neck fractures [28].

Similar results were demonstrated in later experimental studies [29]. Consequently, the loss of endothelial vasodilator function can be a contributing factor to both vascular and bone remodeling.

Nevertheless, the data regarding the influences of vascular remodeling on bone resorption and formation in different diseases remain controversial. In this context, we assumed that soluble and tissue growth factors that are engaged in vascular remodeling participate in the pathophysiology of the damaged bone metabolism in severe COPD.

Conclusion

Therefore, although of the influence of the vasoconstrictor reserve on BMD changes in COPD patients has not been sufficiently studied, endothelial dysfunction should not be considered only a pathogenetic mechanism of COPD development but also a cause of osteoporosis that might explain the correlation between these two pathogenic conditions. Osteopenic syndrome development in COPD is the result of the complex sequence of reactions in which the roles of endothelial and smooth muscular cells should be considered because these cells are involved in vascular remodeling. However, the level of endothelial dysfunction involvement in osteopenic syndrome has yet to be defined. The early treatment of vascular dysfunction might be a potential preventative strategy for avoiding bone loss in COPD patients.

Acknowledgements

This study was supported by a grant from Russian Science Foundation (No. 14-33-00009).

References


