Lymphangiogenesis in COPD: Another link in the pathogenesis of the disease

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Summary
Background: New lymphatic vessels are associated with tissue injury and repair. Recent studies have shown increased lymphatic follicles formation in the lungs of COPD patients. We hypothesized that lymphatic vascular remodeling could be part of COPD pathogenesis.

Aim: To investigate the lymphangiogenetic process in COPD we measured the lymphatic microvessel density (LMVD), the lymphatic invasion (LI), and their correlation with clinical and laboratory parameters.

Methods: Lung tissue from 20 COPD patients and 20 non-COPD smokers was immunohistochemically stained for D2-40 (lymphatic endothelial cell marker), and LYVE-1 (lymphatic endothelial hyaluronan receptor 1). Both groups had similar age and smoking history.

Results: D2-40 and LYVE-1 were expressed in all specimens. Lymphatic invasion was presented only in COPD specimens. Lymphatic microvessel density (LMVD) as revealed by D2-40 and LYVE-1 markers was statistically significantly higher in COPD patients when compared with non-COPD smokers. Both markers (D2-40, LYVE-1) were correlated with FEV1 (% pred) ($R^2 = 0.415$, $R^2 = 0.605$, respectively).

Conclusions: We report for the first time high lymphatic microvessel density and lymphatic invasion in COPD patients, related to the degree of airway obstruction. Our findings could provide novel insights in the pathogenesis of the disease.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major public health and economical problem worldwide. It affects about 10% of the general population but its prevalence among smokers may reach as much as 50%. Smoking, occupational exposures, environmental pollution as well as certain genetic variations are some of the risk factors for the disease. COPD is associated with epithelial mucous metaplasia, inflammation, parenchymal destruction and extensive lung remodeling in a way that is difficult to maintain normal lung function. In a recent study Hogg et al demonstrated that the disease progression correlates with the accumulation of mucous exudates within the lumen and infiltration of the walls by inflammatory cells that form lymphoid follicles. These changes participate in the process of "remodeling" that increases the thickness of the airways' walls. The lymphoid structures are generally the driver point of chronic inflammation. Inflammatory cells and various molecules (e.g. IL-1β, TNF-α, VEGF-C) have been recognized to play important roles in lymphangiogenesis (formation of lymphatic vessels) in experimental models and human diseases. In addition, soluble molecules, such as hyaluronic acid, involved in lung injury and repair can also induce lymphangiogenesis.

Recently, El-Chemaly et al in order to test the hypothesis whether lymphangiogenesis is part of the "remodeling" in idiopathic pulmonary fibrosis (IPF) demonstrated that the alveolar microenvironment in IPF contains soluble factors (e.g. hyaluronic acid) and cells (e.g. CD11b + macrophages) that enhance lymphangiogenesis. Although inflammatory cells, such as macrophages, and lung mediators such as hyaluronic acid are common features in COPD, no study thus far, investigated lymphangiogenesis in COPD.

At present, the identification of lymphatic vessels in normal tissue and in vascular malformations has been considerably improved by the newly discovered lymphatic endothelial markers, D2-40 and LYVE-1. We have chosen the LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) and the D2-40 (a marker of lymphatic endothelial cells) in order to evaluate the lymphatic microvessel density (LMVD) and the presence of lymphatic invasion (L.I) in COPD. LYVE-1 binds to hyaluronan on the luminal surface of lymphatic vessels, while D2-40 specifically reacts with lymphatic vessels, allowing identification of lymphatic vessel invasion. Our findings demonstrated an intense lymphangiogenic process in COPD, significantly correlated with the degree of airway obstruction.

Methods

We have studied 40 smokers who underwent open lung surgery for the excision of solitary pulmonary nodule. Twenty subjects were diagnosed with COPD and 20 were smokers without COPD. Table 1 presents the anthropometric and spirometric data of the patients studied. The surgical specimens were collected from the subpleural parenchyma at least 5 cm away from the solitary nodule, from an uninvolved segment. Formalin-fixed paraffin-embedded blocks of lung tissue were selected from each case on the basis of containing viable COPD tissue and normal controls. None of the specimens contained neoplastic tissue.

Medical records (sex, age, spirometry values, and disease stage) were thoroughly reviewed. Diagnosis and staging of COPD patients were based upon GOLD classification (Global Initiative for Chronic Obstructive Lung Disease). All the procedures followed were in accordance with international recommendations concerning human research and ethical standards and were approved by the Ethics Committee of the University Hospital of Heraklion, Crete, Greece. Informed consent was obtained from all patients participating in the study.

**LYVE-1 protocol**

Immunostaining of the formalin-fixed, paraffin embedded tissue sections was performed (EnVision System, DAKO). Consecutive 4 μm thick sections were sliced from each tissue block and used for the immunohistochemical study. Briefly, sections were deparaffinized and dehydrated to distilled water. Microwave treatment was performed twice in citrate buffer (pH = 6), at 300 W, for 15 min. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in absolute methanol for 7 min in room temperature. Then, the slides were washed with distilled water, and Tris Buffer Saline (TBS) (DAKO). The LYVE-1 (Lymphatic Vessel Endothelial Receptor 1, R&D Systems), primary antibody was used, with an incubation time of 60 min at room temperature. The optimal dilution (1/50) was defined on the basis of a titration experiment. Samples were washed for 5 min. The incubation of the secondary monoclonal antibody against LYVE-1 followed, for 15 min. Slides were washed with TBS for 5 min, incubated with peroxidase streptavidin for 15 min, and then washed again for 5 min.
Finally, the chromogenic substrate (DAB, LSAB +, DAKO) was applied, followed by counterstaining with hematoxylin 10% (2–5 min), dehydration, mounting and coverslipping.

**D2-40 protocol**

Formalin-fixed, paraffin embedded lung tissue sections of 4 μm were used. The automated slide processing system BenchMark XT IHC/ISH Staining Module (Ventana Medical Systems, Inc., Tucson, Arizona) was used for the optimization and performance evaluation of the D2-40 lymphatic marker (BioCare Medical). A protocol was established so that the entire assay procedure consisting of baking, deparaffinization, pretreatment, cell conditioning, titration, stringency wash, amplification, signal detection, counterstaining and post counterstaining was completed as a one-step fully automated assay (XT iVIEW DAB v3 procedure). Specifically, the slide processing system automatically performed the process starting with tissue deparaffinization followed by three cycles of cell conditioning (short-8 min conditioning, mild-30 min conditioning, and standard-60 min conditioning). Titration took place by placing the primary antibody D2-40 (an epitope of podoplanin; a marker of lymphatic endothelial cells) (BioCare Medical, 1/50 dilution) and then incubation for 32 min. Antibody amplification in several stages followed, and finally, counterstaining with hematoxylin 10% (4 min), application of bluing reagent, incubation for 4 min and coverslipping were performed.

**Assessment of D2-40 and LYVE-1 expression**

Two experienced pathologists examined the slides without any knowledge of the clinicopathological data. Examination was simultaneously performed by means of a double headed light microscope. Both pathologists had to reach a consensus on what constituted a single vessel prior to its inclusion in the count. The inter-observer coefficient of variation was less than 10%.

Briefly, D2-40 and LYVE-1 expression was scored as the percentage of stained cells in ten representative high power fields (at least 1000 cells). The criteria for positiveness were a cytoplasmatic staining irrespective of percentage of stained cells in ten representative high power fields. The inter-observer coefficient of variation was less than 10%.

Regarding D2-40 and LYVE-1 quantitative expression, the three most vascular areas (hot spots) within a section were selected for evaluation on a light microscope (Olympus BX-51) in low magnification (×100). The labeled vessels with the anti-D2-40 and anti-LYVE-1 monoclonal Abs were counted under a light microscope at ×400 magnification. The average counts exhibited the lymphatic microvessel density (LMVD). The median of all densities was measured for each antibody and was further used as a cut-off value for high and low expression. The score was defined as follows: low expression D2-40; LMVD < 81, low expression LYVE-1; LMVD < 594, high expression D2-40; LMVD ≥ 81, high expression LYVE-1; LMVD ≥ 594. Areas of necrosis were excluded. Statistical significance was additionally investigated by the presence of emboli within cells stained with D2-40 and LYVE-1.

**Statistical analysis**

The normality was evaluated using Shapiro–Wilk criterion. For the not normally distributed variables, non parametric methods analysis was used. Continuous variables were expressed as median, while categorical variables as absolute and relative frequencies. Mann–Whitney test and Kruskal–Wallis test were applied for the analysis of group differences within continuous variables. Correlation between continuous variables was assessed with Spearman’s correlation coefficient. A two sided p-value of less than 0.05 was considered statistically significant. The Statistical Package for Social Sciences (SPSS) program, version 16.0 (Chicago, Illinois, USA) was used for statistical analysis.

**Results**

We retrospectively evaluated 40 subjects; 20 COPD patients and 20 non-COPD smokers. All subjects were matched for age and had a similar smoking exposure (Table 1). The majority of COPD patients (14/20) were in stages 1 and 2 (GOLD).

**D2-40 expression**

All COPD patients showed increased LMVD_{D2-40} (≥81) expression as compared with non-COPD smokers [LMVD_{D2-40} (<81)]. Lymphatic invasion (L.I.) was exhibited only in COPD patients. Fig. 1ai shows a typical COPD smoker with emphysematous changes and abnormal enlargement of the air spaces presenting high LMVD_{D2-40} (≥81). Fig. 1aii shows a COPD smoker with typical findings of bronchitis with small bronchioles infiltrated by mononuclear inflammatory cells and scattered lumen occlusion by mucus plugging, presenting high LMVD_{D2-40} (≥81) expression. Fig. 1b shows a representative tissue specimens of a non-COPD smoker exhibiting low LMVD_{D2-40} (<81) expression.

LMVD_{D2-40} expression was statistically significantly increased in patients with COPD as compared with non-COPD smokers (Mann–Whitney test, \( U < 0.001, p < 0.001 \)) (Fig. 2). In detail, in the D2-40 marker, 35% of COPD patients exhibited increased expression (+++), 40% medium (+++), and 25% low (+). In non-COPD smokers the D2-40 expression was high (+++) only in 5% of the samples, medium (+++) in 70% and low (+) in 25% of the cases.

Furthermore, LMVD_{D2-40} was significantly correlated with FEV1 (% pred) (Correlation coefficient Spearman = r5 = −0.7, p < 0.001) as it is shown in Fig. 3. Interestingly, COPD patients exhibiting increased LMVD_{D2-40} ≥ 81 expression had lower FEV1 (% pred) values (median 53), while subjects with low LMVD_{D2-40} < 81 expression had higher FEV1 (% pred) values (median 93), (Fig. 3).
LYVE-1 expression

All COPD patients showed increased $\text{LMVD}_{\text{D}2-40} \geq 594$ expression as compared with non-COPD smokers. In detail, 60% of COPD patients exhibited high (+++), 35% medium (++), and 5% low (+) LYVE-1 expression, while 35% of non-COPD smokers exhibited high (+++), 40% medium (++), and 25% low (+) LYVE-1 expression.

Figure 1  ai A representative tissue sample of a COPD patient showing abnormal enlargement of the air spaces (emphysema) and high $\text{LMVD}_{\text{D}2-40} \geq 81$ expression. The D2-40 marker stained selectively the walls of the lymphatic vessels (magnification $\times 40$). aii A tissue sample of a COPD patient exhibiting typical findings of bronchitis with small bronchioles infiltrated by mononuclear inflammatory cells and scattered lumen occlusion by mucus plugging and high $\text{LMVD}_{\text{D}2-40} \geq 81$ expression (magnification $\times 100$). b A tissue sample of a typical smoker without COPD with low $\text{LMVD}_{\text{D}2-40} < 81$ expression (magnification $\times 100$).

Figure 2  Median and standard deviation of $\text{LMVD}_{\text{D}2-40}$ in smokers with and without COPD ($p < 0.001$).

Figure 3  Scatter-plot between Lymphatic Microvascular density D2-40 and FEV1 (% pred) in COPD and non-COPD smokers ($r = -0.7, p < 0.001$).
Fig. 4a shows a typical COPD smoker with emphysematous changes and abnormal enlargement of the air spaces presenting high \( \text{LMVD}_{\text{LYVE-1}} \) (≥594) expression, while Fig. 4b shows a representative tissue specimens of a non-COPD smoker with low \( \text{LMVD}_{\text{LYVE-1}} \) (<594) expression. \( \text{LMVD}_{\text{LYVE-1}} \) was statistically significantly increased in patients with COPD versus non-COPD smokers (Mann–Whitney test, \( U < 0.001, p < 0.001 \)) (Fig. 5).

Regarding spirometric data, \( \text{LMVD}_{\text{LYVE-1}} \) was significantly correlated with FEV1 (% pred) values (Correlation coefficient Spearman \( r_S = -0.8, p < 0.001 \)) (Fig. 6). Interestingly, COPD patients presenting increased \( \text{LMVD}_{\text{LYVE-1}} \) ≥594 expression had lower FEV1 (% pred) values (median 53) as compared to subjects with low \( \text{LMVD}_{\text{LYVE-1}} \) <594 expression and higher FEV1 (% pred) values (median 93), (Fig. 6).

Discussion

To the best of our knowledge, this is the first study to report high lymphatic microvessel density (LMVD) and lymphatic invasion (L.I.) in COPD patients. Additionally, both LMVD and LI were related to the degree of airway obstruction.

Lymphangiogenesis plays an important physiological role in homeostasis, metabolism and immunity.\(^{22}\) Lymphatic vessel formation has been implicated in a number of pathological conditions including neoplasm metastasis, edema, impaired wound healing, rheumatoid arthritis, psoriasis, lung fibrosis and asthma. In addition, lymphoid follicles (LF), known to participate in adaptive humoral and T-cell–mediated responses to antigens, are induced by chronic inflammation or infection.\(^{22,23}\) Increased number of LF was found in advanced COPD.\(^{2,3}\) However, the cellular and molecular mechanisms that contribute to the formation of LF and lymphatic vessels in the lungs of COPD subjects are not entirely understood.

Figure 5  Median values of \( \text{LMVD}_{\text{LYVE-1}} \) in smokers with and without COPD \( (p < 0.001) \).

Figure 6  Scatter-plot between Lymphatic Microvascular Density LYVE-1 and FEV1 (%pred) in COPD and non-COPD smokers \( (r = -0.8, p < 0.001) \).

Figure 4  a A typical tissue sample of a COPD smoker exhibiting emphysematous changes, abnormal enlargement of the air spaces, and high \( \text{LMVD}_{\text{LYVE-1}} \) (≥594) expression (magnification x100). b A typical tissue sample of a non-COPD smoker showing low \( \text{LMVD}_{\text{LYVE-1}} \) (<594) expression (x200).
Our results showed increased expression of LYVE-1 and D2-40 monoclonal antibodies (which selectively recognize the lymphatic endothelium), in COPD patients compared with non-COPD smokers, and this was correlated with the severity of the disease.

We believe that our data may possibly be interpreted by two ways; firstly, could point out a novel link in the autoimmunity component of COPD, and secondly, could reveal an impaired wound healing process leading to the abnormal lung tissue remodeling, or both.

The model that we propose could be the following: Initially, cigarette smoke injuries lung epithelial cells which release "danger signals" that act as ligands for toll-like receptors (TLRs) in the epithelium. This model has been initially introduced with the discovery of the endogenous, non-foreign "alarm signals", including altered host DNA, RNA, and proteins. Additionally, the by-products from host inflammatory cells such as reactive oxygen species may further promote tissue injury, and "auto-antigen" production. This chain of events may cause dendritic cells to mature and to migrate to local lymph organs, where, if the conditions are favorable, T-cell activation may result. Our findings exhibiting high microvessel density in the lungs of COPD patients may indicate another component of the regional adaptive immune response. Thus, the network of the new lymphatic vessels in the lungs of COPD patients might be simply the "short cut" which facilitates the "journey" of "danger signals" toward regional lymph nodes; in order to obtain a quicker and constant immune response. It has been proposed that COPD has an autoimmune component and that LF participate in this process. Up to now, there is scant information regarding the presence of lymphatics and lymphangiogenesis in the pathogenesis of chronic lung diseases, such as COPD.

Baluk et al investigated the role of lymphangiogenesis in a mouse model of chronic airway inflammation. They found extensive growth of lymphatic vessels in infected airways. Inhibition of lymphatic growth exacerbated mucosal edema and reduced the hypertrophy of draining lymph nodes, demonstrating that when lymphangiogenesis is impaired, airway inflammation may lead to bronchial lymphedema and airflow obstruction.

An alternative explanation for our data might be that lymphangiogenesis in COPD represent another component of the abnormal remodeling process. In this study, lymphatic microvessel density (LMVD) was increased as the disease progressed while lymphatic invasion (L.I) was present only in COPD specimens. Normal lung parenchyma is generally devoid of lymphatics, except for lymphatic vessels within bronchovascular tissue. Studies of lymphatics in healthy human lung are rare. Pusztaszeri et al found that D2-40 reactivity followed the bronchovascular distribution, and was nonexistent or rare in the alveoli. Yamashita et al showed that normal lymphatic vessels are in close proximity with the large bronchovascular trees and not present in the alveolar spaces. More recently, Kambochuer et al showed that about 3.6%–19% of alveolar spaces had an associated D2-40-reactive lymphatic vessel, in areas of healthy lung in the vicinity of excised tumors.

Another interesting point of this study was that LMVD of D2-40 and LMVD of LYVE-1 markers were negatively correlated with FEV1 (% pred) as shown in Figs. 3 and 5, respectively. COPD patients who presented increased lymphangiogenesis had lower FEV1 (% pred) values as compared to smokers without COPD and low lymphangiogenesis. Likewise, Hoshino et al found more vessels and a larger vascular area in patients with atopic, mild to moderate asthma when compared with control subjects. They also reported an inverse correlation between FEV1 (% pred) and/or airway responsiveness and the percentage of vessel area. In agreement with our results, Hashimoto et al reported increased vascularity in the medium and small airways of patients with asthma which was correlated with disease severity in mild and moderate asthma.

The above findings strongly suggest that airway vascularity (angiogenesis and lymphangiogenesis) is enhanced and correlates with disease severity providing evidence that the endothelium is proliferating contributing to the airway remodeling.

In conclusion, our results demonstrated significantly increased expression of the lymphatic endothelial markers D2-40 and LYVE-1 in COPD patients when compared with non-COPD smokers, and this was related to the severity of the disease. Although lymphatic vascular remodeling has not been studied up to now in COPD, it appears that lung lymphangiogenesis constitute another link in the adaptive immune response or/and airway remodeling as the disease progresses. Better understanding of the above pathways may put forward novel therapeutic targets in COPD.

**Author contributions**

Dr Hardavella: contributed to the analysis and interpretation of data, and preparation of manuscripts drafts.

Dr Tzortzaki: contributed to study design, acquisition and interpretation of data, and preparation of manuscripts drafts.

Dr Siozopoulou: contributed to data analysis and interpretation.

Dr Galanis: contributed to the interpretation of data and statistical analysis.

Dr Vlachaki: contributed to the acquisition of data.

Mrs Avgusti contributed to the acquisition of data.

Dr Stefanou: contributed to the analysis and interpretation of data.

Dr Siafakas: contributed to the conception and design of the study, interpretation of data and preparation of manuscripts drafts.

**Conflict of interest statement**

On behalf of all authors I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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