Abstract. Despite increasing knowledge on the cellular and molecular mechanisms involved in pulmonary fibrosis, its therapeutic options are still limited. The study of lymphangiogenesis has contributed to a better understanding of tumor growth and metastasis, with a major impact upon changes in therapeutic strategies and this was followed by the research of lymphatic vessels in other pathological conditions. Some data support the possible role of lymphangiogenesis in the pathogenesis of lung fibrosis. However, at the time of diagnosis for each patient with a fibrotic interstitial lung disease, it is necessary to predict the prognosis and to choose for individual targeted-therapy. Our aim was the characterization of lymphangiogenesis as a useful tool to stratify patients with lung fibrosis. We evaluated the presence, morphology and density of D2-40-positive lymphatic vessels and co-localization of D2-40/Ki67 in pulmonary fibrosis with different degrees of severity and without a specific etiology. Lymphatic vessel density did not correlate with severity grade and ranged between 4.66 to 38.33 vessels/×40 field, with the highest value in degree III of fibrosis. An intense proliferative activity of lymphatic endothelial cells was found in 24% (6 out of 25) of cases. The morphology of lymphatics and the presence of splitting combined with the proliferative activity of endothelial cell pillars suggested two different mechanisms in the formation new lymphatic vessels. Our results support the hypothesis that the activity and ongoing evolution of fibrosis can be predicted through the characterization of lymphangiogenesis but its presence or absence cannot predict the severity of fibrosis.

In recent years, much evidence has been accumulated to support the role of lymphangiogenesis in the progression and metastasis of human tumors. It has been shown that lymphangiogenesis is a key event in the spread of tumor cells via intratumoral and peritumoral lymphatic vessels. This process is governed by growth factors, and particularly by vascular endothelial growth factor (VEGF)-C and -D and their cognate receptor, VEGFR3. The characterization of lymphangiogenesis in a pathological condition implies not only determination of the number of lymphatic vessels, but also the proliferation of lymphatic endothelial cells.

In addition to the remarkable contribution of lymphangiogenesis to knowledge of the natural history of malignant tumor, some studies were initiated on lymphatic vessels and their formation in the other fatal disorders, such as pulmonary fibrosis (1). Moreover, the high mortality rate of pulmonary fibrosis has generated hypotheses regarding the pathogenic similarities between these diseases (2, 3). The current literature shows differences in long-term survival of patients with lung fibrosis, but the pathogenic mechanisms and individual prognosis are still controversial (4, 5).

There are several difficulties in the assessment of both fibrosis and lymphangiogenesis that could explain the reduced amount of data about the involvement of lymphatics in the lung tissue remodeling that characterizes the irreversible nature of fibrosis. Pulmonary fibrosis was frequently recognized as an end stage of several diffuse parenchymal lung diseases. In recent years, according to the American Thoracic Society/European Respiratory Society guidelines, pulmonary fibrosis has been associated with idiopathic pulmonary fibrosis (IPF) and characterized as the honeycomb lung by the usual interstitial pneumonia (UIP) pattern, an end stage of the disease (6, 7). This is why controversy appears concerning the difference between
‘early’ and ‘late’ UIP and also regarding the concept of mild, moderate and severe IPF, although it is recognized as a progressive disease.

On the other hand, the study of lymphatics was significantly improved only when new markers and some specific antibodies were introduced (8). It was established that a precise localization of the pre-existing lymphatics is necessary for the evaluation of lymphangiogenesis. Immunohistochemical studies using the D2-40 monoclonal antibody have showed the distribution of lymphatic vessels in the lungs with more accuracy, even though the existence of intralobular lymphatic vessels is still controversial. Recently, Kambouchner and Bernaudin (9) demonstrated the presence of the intralobular lymphatic network and that about 3.6% to 19% alveolar spaces have an associated D2-40 reactive lymphatic vessel. Nonetheless, the majority of the studies showed that normal alveolar spaces were devoid of lymphatic vessels, but in IPF, lung lymphatics were observed in close proximity to the alveolar space (10, 11).

Furthermore, D2-40 reactivity follows the bronchovascular, interlobular and sub-pleural distribution, the same as with the lung interstitium, and corresponds to the structure of the secondary pulmonary lobule (12). However, it is the underlying anatomy in the interpretation of abnormalities on high-resolution computed tomography that suggests fibrosis. The relationship between the remodeling of lymphatic vessels and the fibrotic aspect by this imaging modality has not yet been determined (13). The scarce information regarding lymphangiogenesis and lung tissue fibrosis has not completely shed light on its interrelationship with other pathogenic mechanisms, or whether it is a primary or secondary event in the development of the disease process, leading to remodeling and to the ‘honeycomb’ lung.

In our study, we evaluated the presence, distribution, morphology, and proliferative status of lymphatic vessels in relation to the degree of fibrosis in an attempt to identify a new and discriminating tool for predicting the prognosis in individual patients.

Materials and Methods

Patients and specimens. In this study, we included specimens obtained by video-assisted thoracoscopic surgery from 25 patients (14 men and 11 women) with pulmonary fibrosis, without a specific etiology, and five cases as the control group. Biopsies from the control cases were taken from patients operated on for other lesions of the lung. Informed consent was obtained from all participants according to the Declaration of Helsinki.

The morphological diagnosis and the degree of fibrosis were established by identification and, subsequently, by a quantitative assessment of four histological features: lung architecture, chronic inflammatory infiltrate (in terms of presence, density and distribution), macrophages (density and type), and the presence and extension of fibrosis. A scale from ‘0’ to ‘5’ was used to score each lesion and the total pathological fibrotic score was generated by adding these together. In this way, the cases were divided into degrees of severity from ‘I’ to ‘IV’, according to previous data published by the same team (14).

The specimens were fixed in 10% buffered formalin for 48 h and then embedded in paraffin, using the conventional histological procedure. The primary processing was completely standardized using the Shandon embedding center (Thermo-Shandon, Runcorn, Cheshire, United Kingdom). Serial sections were performed from each paraffin block, 3 μm in thickness. Sections from each case were stained with hematoxylin–eosin (HE), Masson’s trichrome stain and Gordon–Sweet silver stain. All reagents were from Sigma, (St. Louis, MO, USA).

Immunohistochemistry. Immunohistochemical detection of lymphatic vessels was performed using the monoclonal mouse antibody to human D2-40 (clone D2-40, dilution 1:200) (Dako, Glostrup, Denmark). Briefly, sections were de-waxed in benzene and rehydrated through decreasing, graded alcohol solutions. Antigen retrieval was performed by microwave, using citrate buffer (pH 6) for 30 min in a PT Link module bath (Dako). Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide solution for 10 min. Slides were washed with phosphate-buffered saline followed by incubation with anti D2-40 for 30 min, and application of the streptavidin–biotin complex. The final product of reaction was visualized by activated diaminobenzidine and nuclei were stained with Lillie’s hematoxilin.

Firstly, we assessed the distribution and density of D2-40-reactive vessels in normal lung samples. Secondly, the fibrotic samples were evaluated. Lymphatic microvessel density (LMVD) was determined by counting all immunostained vessels in three separate fields of fibrosis with the highest number (hot spots) at a high magnification (×200). The hot spot areas were identified after the stained sections were scanned at low magnification (×40). The arithmetical average was the final result in each case. Microscopic images were captured as JPEG format and processed using the Nikon Lucia G software (Nikon, Tokyo, Japan). The alveolar cells and epithelial basal cells of the bronchiolar mucosa were also stained with the D2-40 monoclonal antibody and we used them as internal positive control.

Co-localization of D2-40 and Ki67 (polyclonal, ready-to-use; Dako) for the proliferative characterization of lymphatic vessels was obtained by performing double immunostaining method using the Doublestain Envision kit (Dako). Nuclei were stained in brown for Ki67, and the cytoplasm of lymphatic endothelial cells was stained in red for D2-40. Only co-localization was taken into account to evaluate the proliferation of lymphatics. The full immunohistochemical procedure was performed with Dako Cytomation Autostainer (Dako Cytomation, Glostrup, Denmark).

Results

In the normal lung samples, we focused on assessing the distribution of lymphatic vessels after their identification by D2-40 immunostaining. In normal lung tissue, lymphatic vessels were constantly found in the subpleural spaces (Figure 1a), in the interlobular septa (Figure 1b), in the peribronchiolar interstitium (Figure 1c), and in the adventitia of large vessels (Figure 1d). Lymphatic vessels of various diameters were also associated with the small intralobular
arteries. Moreover, very small D2-40-positive lymphatic vessels were occasionally observed in the interalveolar septa. The sub-pleural lymphatic vessels were found in close proximity to the pulmonary parenchyma and away from the medium-sized blood vessels. They had a distinct lumen, without tissue pillars or septa, and different diameters. The perivascular lymphatic vessels were more numerous than the sub-pleural vessels. The peribronchial lymphatic vessels had a smaller size than perivascular vessels and some of these were collapsed. The lymphatics found in the interalveolar septa were frequently vessels of smaller size, but with an open lumen. From a large to a small size, all lymphatic vessels found in the normal lung did not exhibit endothelial proliferation, while the blood endothelial cells presented an occasional proliferation. In the normal lung, LMVD ranged between five and seven vessels/field.

According to the staging system which was described, the cases with fibrosis were sub-divided as follows: five cases with degree II, 15 cases with degree III and five cases with degree IV. We noticed D2-40-positive lymphatic structures in all of these cases, with variable distribution and no relationship between them and the extent of the disease. LMVD varied between 4.66 and 38.33 vessels/field, with the highest value in degree III fibrosis. Lymphatic density did not decline in the advanced stage of fibrosis and it did not correlate with the degree of pulmonary fibrosis (Table I).

Co-localization of D2-40 and Ki67 characterized proliferative activity of the lymphatic endothelial cells and was observed in only 24% cases with different degrees of severity: one case of degree II, four cases of degree III and one of degree IV. The D2-40/Ki67-positive lymphatics were peripherally distributed, they were absent from areas with severe fibrosis, and were not associated with the highest values of microvascular density.

The structure of lymphatic vessels in pulmonary fibrosis was significantly different from that of the normal lung. The following D2-40-positive lymphatic structures were observed: isolated lymphatic endothelial cells with cytoplasmic vacuoles (Figure 2a), buds of new lymphatics (Figure 2b), lymphatic cords (Figure 2c) and lymphatic vessels with lumen (Figure 2d). Additionally, the transition from non-lumeneinized lymphatic cord to lumenized vessel was observed along the same structure, through the presence of both the cordon and lumenization (Figure 2e). Altogether, these aspects suggested sprouting as being the potential mechanism of new vessel formation. We also noticed the division of the existing lymphatic lumen through the formation (Figure 2f) and insertion (Figure 2g) of transluminal pillars that reflects intussusceptive expansion and remodeling.

A particular aspect of the intussusceptive lymphatic vessel growth was in regard to the two different types of the intraluminal septa: D2-40-negative connective tissue pillars and the D2-40-positive pillared endothelial cells which, in isolated cases, have been associated with proliferative activity (Figure 2h). The morphological aspects that suggest these two mechanisms for new lymphatic vessel formation were observed simultaneously in samples from the same case. Massive accumulation of macrophages was observed only around the proliferating lymphatic vessels during the sprouting process in all cases (Figure 3).

**Discussion**

Currently, despite the fact that pulmonary fibrosis is defined as an irreversible disorganization of the lung architecture, the most important goal of therapy is to control disease progression (15). One major difficulty for this issue is that only a few prognostic indicators can predict its clinical behavior. The studies have shown that IPF, the most common pulmonary fibrotic condition, is characterized by expression of proliferation, of tissue re-modeling and of myofibroblast genes and it can be clearly distinguished from other interstitial pneumonias with an inflammatory pathway of fibrosis (16). Even so, targeted-therapy did not improve survival (17). These findings support the hypothesis that there are many mechanisms involved in the pathogenesis of fibrosis that are yet unknown (18). The involvement of lymphatics in the lung remodeling process and the possibility of lymphangiogenesis being involved and interrelated with other pathogenic fibrosis pathways are not well known.

Only scant data are available in the literature concerning the relationship between lymphangiogenesis and pulmonary fibrosis in its different stages (19, 20, 21). In our study, we established the presence and the variability of lymphangiogenesis in pulmonary fibrosis, whereas other studies have pointed-out only the absence of lymphatics in
lungs with severe fibrosis (22). The immunohistochemical assessment with the monoclonal D2-40 antibody showed an increased number of lymphatic vessels although the quantitative changes (LMVD) did not correlate with the degree of fibrosis. D2-40 is highly sensitive but not fully-specific for the lymphatic endothelium. In cases with pulmonary fibrosis, three other cell types are stained (alveolar cells and basal cells, both used as internal positive control, and myofibroblasts). These features may create problems in the counting of very small lymphatics, particularly the interalveolar ones. This is why we took into account only lymphatics with distinct lumen to calculate the LMVD. Based on our results, LMVD is a useful tool for characterizing pulmonary fibrosis, although the number of cases is quite low. On the other hand, no correlation was found between LMVD and the degree of fibrosis, and this could be considered a limit of the present finding and implies the need for the addition of new cases.

Endothelial lymphatic cells in the proliferative state were only rarely found, but this constitutes considerable evidence for the presence of active lymphangiogenesis in pulmonary fibrosis. We have shown definite co-localization of D2-40 and Ki67 in lymphatics from patients with pulmonary fibrosis, but not in the normal lung. To the best of our knowledge, this is the first demonstration of lymphatic endothelial cell proliferation in this condition.

The origin of new lymphatics is still controversial (23). Nonetheless, our results suggest two different mechanisms for their formation. The structure of lymphatics and their presence along the same lymphatic vessel of both cord and permeable lumen suggests that the sprouting process may be responsible for the expansion of the lymphatic network in lung fibrosis. The D2-40-/Ki67-positive multicellular endothelial cords could be an intermediate step in the formation of a mature vessel, while the isolated endothelial cell may be the first step. The high density of Ki67-positive lymphatic endothelial cells within the cords support proliferation and is not an artifact induced by the histological technique. On the other hand, we also observed the intussusceptive phenomenon: the insertion of lymphatic endothelial cells into the lumen of a mature vessel. This particular and original aspect, as a second distinct mechanism of lymphatic formation in pulmonary fibrosis, was observed in two different variants: with and without lymphatic endothelial cell proliferation. Intussusception with endothelial proliferation may be specific for

Figure 1. Distribution of D2-40-positive lymphatic vessels in the normal lung. a: Small-sized subpleural lymphatic vessels, close to normal lung parenchyma with regular lumen. b: Neighboring blood vessels were negative for D2-40 (arrow). c: Peribronchiolar lymphatic vessels with a collapsed and large lumen and a massive accumulation of macrophages surrounding these vessels. Note the presence of macrophages in the wall of some lymphatic vessels. d: Lymphatic vessels in the adventitia of large blood vessels.
pulmonary fibrosis since the proliferative aspect has been found in the pillars or on the vessel circumference. This aspect was not mentioned in other pathological conditions until now. We also found pre-existing lymphatic vessels in the interalveolar space where secondary lymphangiogenesis can start.

Controversial results concerning the possibility of a de novo formation of lymphatic vessels can be found in the literature. It has recently been shown that macrophages can differentiate into lymphatic endothelial cells (24). In our study, we found massive accumulation of macrophages in the vicinity of lymphatic vessels. We may speculate that the association of macrophages with lymphatics, thought to promote fibrosis, and also lymphangiogenesis could directly be activated by resident mesenchymal cells, through the production of lymphangiogenic growth factors.
At the time of the biopsy, we found early lymphangiogenesis in degree II fibrosis and, furthermore, the association of this with irreversible lesions in degree IV fibrosis. This suggests that lymphangiogenesis is involved in the critical steps of fibrosis progression. In addition, pulmonary fibrosis – a progressive and unpredictable process – could be characterized by lymphangiogenesis as a marker of ongoing fibrosis. The existence of lymphangiogenesis is a negative prognostic factor, suggesting the destruction of normal tissue architecture. It should also be regarded as an additional pathway of pathogenic fibrosis and might be a useful tool for early identification of this disease.

What the precise role of new lymphatic vessels is in pulmonary fibrosis is unclear. Moreover, whether growth factors are involved in this process and their source remains unknown. The progressive fibrotic remodeling of the alveolar epithelial–mesenchymal unit and replacement with bronchiolar cysts was explained by some authors through lymphatic persistence with collagen absorption, via fluid drainage (19, 20). In this way, lymphangiogenesis may function as a compensatory mechanism and only when lymphatics are compromised by tissue features do cases with dense fibrosis appear. However, further studies are necessary to confirm these findings.

Conclusion

In our study, we detected lymphangiogenesis by LMVD and endothelial proliferation status in all cases with pulmonary fibrosis. LMVD does not correlate with the degree of fibrosis, but seems to be an indicator of the active process of fibrosis. LMVD does not correlate with the degree of endothelial proliferation status in all cases with pulmonary fibrosis. Moreover, further studies are necessary to confirm these findings.

References


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