Myofibroblast induction and microvascular alteration in scleroderma lung fibrosis

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Abstract

Objective

Scleroderma (SSc) is an autoimmune connective tissue disorder characterized by progressive fibrosis of the skin and internal organs. The leading cause of death in SSc patients is pulmonary dysfunction as a result of interstitial fibrosis and pulmonary vasculopathy. Our objective was to evaluate histopathological abnormalities associated with the development of pulmonary fibrosis in SSc.

Methods

Postmortem SSc lung tissue from various stages of fibrosis and tissue from normal lung were analyzed by Masson’s trichrome staining and immunohistochemistry. Monoclonal antibodies against smooth muscle-α actin (myofibroblast marker), von Willebrand Factor, platelet endothelial cell adhesion molecule-1 (endothelial cell markers), or caldesmon (smooth muscle cell marker) were employed.

Results

We found that in the early active stages of SSc lung fibrosis two major types of cellular abnormalities occur. One is the induction of a large number of smooth muscle α-actin-positive myofibroblasts in interstitia. The other is the excessive formation of alveolar capillaries (hypervascularity) accompanied by an increase in the number of microvascular endothelial cells. The vascular abnormality also involves the development of microvessels that are irregular in size and shape. However, the population of myofibroblasts and capillary endothelial cells decline as the fibrosis progresses to its most marked, later stages.

Conclusion

We conclude that the induction of myofibroblasts and the overdevelopment of capillary microvessels characterize the progression of lung fibrosis in SSc. Using these histological alterations as criteria, therefore we have divided the fibrosis formed in the SSc lungs into four pathological stages. These results suggest that both fibroblast over-proliferation and vascular abnormality play an important role in the pathogenesis of lung fibrosis in SSc.

Key words

Myofibroblast, hypervascularity, pulmonary fibrosis, systemic sclerosis.

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Introduction

Scleroderma (systemic sclerosis, SSc) is an autoimmune disease characterized by vascular injury and progressive fibrosis of the skin and visceral organs (1-3). Pulmonary involvement occurs more frequently in SSc than in other connective tissue disease (4) and is the leading cause of death in SSc patients. The most common pulmonary manifestation of SSc is fibrosing alveolitis, occurring in approximately 80% of patients and having a histologic pattern of nonspecific interstitial pneumonia (NSIP) or usual interstitial pneumonia (UIP)(4). Pulmonary fibrosis in SSc is diffuse and characterized by variable degrees of inflammatory cell infiltration of the alveoli, interstitium and the peribronchiolar tissues (1,5). The pathology of pulmonary fibrosis in SSc demonstrates excessive fibroproliferation and deposition of various extracellular matrix (ECM) proteins by mesenchymal cells (1, 6, and 7). The composition of ECM in SSc lung consists largely of collagen I and III; however, synthesis of non-collagenous matrix molecules such as fibronectin and tenasin is also elevated in SSc (6-8).

Out in vitro tissue culture studies have demonstrated that the cell type responsible for producing elevated amounts of ECM is the SSc lung myofibroblast (7). Myofibroblasts are activated fibroblasts that are ultrastructurally and metabolically distinctive cells. These cells possess characteristics of both smooth muscle cells and fibroblasts, and express smooth-muscle contractile proteins such as smooth muscle α-actin (sm α-actin) (9,10). Myofibroblasts have been detected in bronchoalveolar lavage (BAL) fluid of SSc patients (7) and in cell cultures derived from post-mortem SSc lung explants (8,11-13). However, this cell type has never been shown in vivo in lung tissues undergoing SSc fibrosis. Although it has been suggested that myofibroblasts play crucial roles in tissue remodeling, wound healing, and various fibrotic disorders including pulmonary fibrosis (9-12, 14), when and how this particular cell type appears during the disease process has always been puzzling. One of the difficulties is the transitory appearance of myofibroblasts in the tissue, and a second is their morphological similarity to other fibroblast cell types. In this study, we have performed stage-by-stage observation of lung tissues during the progression of SSc fibrosis. This approach allows us to demonstrate the appearance of myofibroblasts as well as the proportion of myofibroblasts within the total fibroblast cell population during the development and progression of lung fibrosis in SSc.

Activation of endothelial cells and angiogenesis have been reported in a number of physiological and pathological processes such as inflammation, wound healing, malignant tumor growth, and various fibrosing disorders (15,16). Angiogenesis is critical to the growth of malignant cells in tumors, and the inhibition of angiogenesis results in the reversal of tumor growth (16). In the case of fibrosis, aberrant vascular growth has been observed during the development of idiopathic pulmonary fibrosis (IPF) (17), and microvascular injury has been noticed in various organs affected by SSc fibrosis (18). However, the timing, type, and scope of vascular change in fibrotic lung disorders are largely unknown. In this study, we show that the overdevelopment as well as structural abnormalities occur particularly and prevalently in interstitial pulmonary microvessels. We find that vascular alterations arise in early stages of fibrosis and correlate with accumulation of extracellular matrix proteins and abnormal proliferation of myofibroblasts. The results strongly suggest that both vascular and fibroblast abnormalities play important roles in the pathogenesis of SSc lung fibrosis.

Material and methods

Lung tissues

Tissues from 3 patients with SSc and 3 normal individuals were examined. Lung tissues were collected postmortem from SSc patients who fulfilled the American College of Rheumatology criteria for SSc and had evidence of lung involvement. The diagnosis of interstitial lung fibrosis was confirmed by histological examination of post-mortem lung tissue. Tissue from patient C.B. (female, age 48) was obtained at...
open lung biopsy, 11 years after the onset of limited cutaneous SSc complicated by interstitial lung disease and pulmonary arterial hypertension that, subsequently, required single lung transplantation. Lung tissue was obtained at autopsy from patient D.D. (male, age 61), approximately 20 years following the onset of limited cutaneous SSc complicated by severe interstitial lung disease and pulmonary arterial hypertension. Other lung tissue was obtained at autopsy from patient J.M. (female, age 71), approximately 1 year following the onset of limited cutaneous SSc complicated by severe pulmonary fibrosis. Tissues were collected from multiple parts of lung, in order to make comparisons between the normal control and the diseased lungs from SSc with various degrees of fibrosis within the same patient.

**Immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The 4 μm paraffin sections were collected on poly L-lysine coated slides, deparaffinized in xylene, rehydrated through a degrading series of ethanol before staining. Masson’s trichrome staining was used for histological evaluation, and immunohistochemistry was used to detect the presence of cells with myofibroblast, smooth muscle cell, and/or endothelial cell marker. For immunostaining, tissue sections were permeated to ensure antibody infiltration by treating in cold methanol for 10 minutes at −20º C. Prior to immunostaining, non-specific binding sites were blocked for 30 minutes in PBS containing 3% bovine serum albumin and 1% normal goat serum. Sections were incubated overnight with the following primary antibodies: anti-smooth muscle α-actin monoclonal antibody (clone 1A4, Sigma Immunochemicals, St. Louis, MO), For PECAM-1/CD31 staining, an antigen retrieval step was added prior to the blocking step by treating sections with a Target Unmasking Fluid (TUF, BD Pharmimogen, San Diego, CA) for 5 minutes at 95º C. After incubation with primary antibodies the endogenous peroxidase activity was suppressed for 30 minutes with peroxidase suppressor (ImmunoPure Peroxidase Suppressor Kit, Pierce, Rockford, IL). Tissue sections were then incubated with secondary antibodies, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson Immunoresearch Lab, Inc., West Grove, PA) for one hour at 8 μg/ml concentration. Color development was based on metal-enhanced 3,3’-diaminobenzidine (DAB) substrate (Metal Enhanced DAB Substrate Kit, Pearce, Rockford, IL). Tissue sections were counterstained for 30 sec using hematoxylin, a nucleus-sensitive dye (Harris Hematoxylin, Polyclinic, Bay Shore, NY), and mounted using cytoseal (Stephan Scientific, Kalamazoo, MI). All specimens were evaluated without knowledge of the patient’s clinical status.

**Image analysis**

Specimens were viewed under a Zeiss microscope (Axiovert 35) using a bright-field mode. The microscope images were obtained digitally (Spot RT Color Digital Camera, Diagnostics Instruments, Sterling Heights, MI), and they were processed to computer via software, Spot RT/version 3.2/advanced. If necessary, the collected image data were edited using Adobe PhotoShop 6.0, an image processing software.

**Numeric analyses**

For fibroblast cell counting, we performed Hematoxylin staining and immunohistochemistry using vWF or PECAM-1/CD31. The number of microvessels, expressing both endothelial markers but lacking smooth muscle layers, was scored similarly as above. To determine the severity of fibrosis in the lung, we performed Masson’s Trichrome staining on tissue section. Then, the thickness of alveoli septa was measured, if the thickening was due to the increase of acellular deposits accumulated in interstitia determined by Trichrome staining.

**Results**

In the present study we compared fibroblast, endothelial cell and ECM deposition in normal lungs and lungs in different stages of fibrosis from scleroderma patients. The pattern of disease in the three cases was that of non-specific interstitial pneumonia. Based on the histomorphological characteristics of SSc lung, we were able to divide SSc lung fibrosis into four stages.

**Normal lung tissue**

In normal lungs we observed thin layers of alveolar structures composed of septa, vascular components, and connective tissues. Alveolar septa were thin, allowing maximal air to occupy the lung (Fig. 1). The trichrome differential staining highlighted the fibrotic, extracellular matrix regions as blue. The quantity of ECM was very low and consistent throughout the normal lung (Fig. 1).

We observed that the normal alveolar septa contained very few interstitial cells, which were either fibroblasts or myofibroblasts. The two cell types shared a similar morphology, but myofibroblasts expressed smooth muscle-α-actin, unlike other interstitial fibroblasts (Fig.1). We were able to identify these two fibroblast cell types in the lung by performing immunostaining and nuclear staining on the same tissue section. In contrast, smooth muscle cells expressed the smooth muscle differentiation markers caldesmon (data not shown), in addition to smooth muscle α-actin. The location of smooth muscle cells was restricted almost exclusively to the blood vessels, where they form bundles of

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Fig. 1. Comparison of lung tissue from normal and systemic sclerosis (SSc, scleroderma) subjects. SSc lung fibrosis is divided into four stages according to extent of histopathological changes. Masson’s trichrome staining (left panel) shows the progression of fibrosis; ECM is stained blue, whereas cytoplasm and nuclei are red and black, respectively. Other panels illustrate immunohistochemical staining with antibody specific for smooth muscle α-actin (sm α-actin), caldesmon, von Willebrand factor (vWF) or platelet endothelial cell adhesion molecule (PECAM/CD31) post-stained with hematoxylin, shown as brown or blue staining, respectively. Myofibroblasts (sm α-positive cells) are abundant in the early fibrotic stage, SSc I. Both myofibroblasts and smooth muscle cells express sm α-actin, but myofibroblasts (arrows) are present in non-muscle locations. Vascular smooth muscle cells express an additional marker, caldesmon. In the second phase of SSc lung fibrosis (SSc II) endothelial cells stained positively for vWF and PECAM/CD31 are also abundant and there are numerous abnormal capillaries (arrows identify large, malformed capillaries). In the later phases, SSc III and SSc IV, myofibroblasts and endothelial cells are no longer apparent. Images were viewed by x 400 magnification.
muscle fibers in an orderly fashion (Fig. 1).

To study the vascular structures we used endothelial cell-specific markers, the von Willebrand Factor (vWF) and the platelet endothelial cell adhesion molecule-1 (also known as PECAM-1, CD31, or endoCAM). Both anti-vWF and anti-PECAM-1/CD31 antibodies recognized a layer of endothelial cells localized at the luminal surface of vascular tissues (Figs. 1 and 2). The number and distribution of the large and small blood vessels was constant throughout the normal lung, and the morphology of the pulmonary capillaries varied little from one to another.

**SSc lung fibrosis – Stage I**
The most noticeable change, the thickening of the alveolar septa, was observed in the early stage of SSc lung fibrosis. The affected lung accumulated an abnormally large amount of ECM proteins, mostly in the interstitium; blue trichrome stain identified excessive acellular deposits produced in the SSc lung (Fig. 1). At this stage most of the alveolar structures were still preserved, however, noticeable changes also occurred at the cellular level. In the early stage of fibrotic lung transformation, we observed that a large number of fibroblast cells populated the interstitium. Among the fibroblasts, the number of smooth muscle α-actin-positive myofibroblasts was particularly increased (Figs. 1, 2A, and 3A), whereas such cells were rarely observed in the normal adult lung. Within the patient tissues collected, we found that the myofibroblast population in the diseased lung significantly outnumbered the fibroblast population lacking a smooth muscle α-actin marker (Fig. 3A). Myofibroblasts were detected as individual cells scattered throughout the interstitium or in the vicinity of the vasculature, the regions of ECM accumulation. Smooth muscle cells expressing both caldesmon and smooth muscle α-actin served as an internal control. These muscle cells were restricted to the blood vessels, exhibiting no structural abnormality in the larger blood vessels (Fig. 2).

The total number of microvessels in the alveolar septa was clearly increased and the overdevelopment of blood vessels was accompanied by an increase in the number of endothelial cells expressing the vWF and PECAM-1/CD31 markers (Figs. 1, 2B, and 3B). The microvessels were often found in the alveolar septa as well as in the interstitium, where they formed numerous bubble-like structures (Figs. 1 and 2B). They consisted mostly of capillaries and small-sized blood vessels, each with definable endothelia and lumen. In addition, we found that among the overdeveloped microvessels a number of capillaries were abnormal in shape and size. Capillaries found in areas of SSc lung fibrosis were often larger and contained more endothelial cells than the capillaries found in normal lung (Figs. 1 and 2B). Some vessels even exhibited signs of inter-vascular fusion (Fig. 2B), and others formed “multi-bubbles”. Small capillaries were formed irregularly within bubbles of larger vessels (Fig. 2B). Surprisingly, we found many of the overdeveloped microvessels to contain blood cells in their lumina, despite their structural irregularity. This suggests the presence of a functional circulation through them.

Taken together, we have demonstrated that the overdevelopment of microvessels arises early and prominently in SSc lung fibrosis, and involves the development of numerous abnormal and irregular vascular structures.

**SSc lung tissue – Stage III**
This stage represents a transitional...
phase between stages II and IV but with distinct pathological characteristics. Most notably, the ECM accumulation has become so excessive that massive deposits occupy the largest portion of the lung. In addition, cellular portions of the tissues have undergone further changes, such as cellular de-differentiation and tissue degeneration.

In stage III we observed a significant reduction in the number of myofibroblasts, whereas a few fibroblasts lacking smooth muscle α-actin were still present in the interstitium (Fig. 1 and 3A). Similarly, the vascular markers (vWF or PECAM-1/CD31) demonstrated that the number of vascular endothelial cells was reduced and accompanied by degeneration of the vascular system (Fig. 1 and 3B). These changes collectively altered the morphology, density, and distribution of the vasculature. Overall, at this stage we observed that the tissue components no longer maintained their structural integrity and the progression of fibrosis had critically damaged the most vital structures of the lung, including the alveolar and vascular systems.

**SSc lung tissue – Stage IV**

In the final stage of SSc lung fibrosis, accumulation of fibrous extracellular deposits had progressed further (Figs. 1 and 3). Most of the cellular tissues were cleared from the lung and replaced by acellular, extracellular matrices. This loss included almost all of the fibroblasts and myofibroblasts, as well as non-muscle type fibroblasts that had disappeared from the lung (Fig. 1 and 3A). Likewise, epithelial cells and endothelial cells also disappeared, and alveolar septa or vessels were no longer recognizable in the tissue (Fig. 1 and 3B). We detected only partial and very weak vascular marker signals from the endothelial cells of residual tissues. The lung had been transformed irreversibly to a contracted fibrous organ lacking alveoli and vasculature. Therefore, based on histomorphological criteria we determine this to be the end stage of lung fibrosis in the SSc.

**Discussion**

Pulmonary fibrosis in scleroderma is often progressive and irreversible (3). It is characterized by microvascular and tissue injury, inflammation and excessive deposition of extracellular matrix components, eventually resulting in severe distortion of normal lung architecture (fibrosis and/or “honeycomb lung”) (1,5). Symmetric, bibasilar interstitial fibrosis affecting alveolar septa, bronchial walls, and interstitium is the most frequent postmortem histopathologic finding (5).

We demonstrate that in the early, active phases of SSc lung fibrosis the total population of fibroblasts is increased, and this supports the previous concept of a fibroblast abnormality in this disease (19). Furthermore, we show that the selective induction of a particular fibroblast type, the myofibroblast, occurs along with the fibrotic transformation of the lung. When the total number of fibroblasts increases in the earlier
phases of fibrosis, the ratio of myofibroblasts to other fibroblast types stays persistently higher. This degree of myofibroblast dominance has not yet been reported in other fibrotic disorders, although the presence of myofibroblasts has been observed in various pulmonary diseases including hypersensitivity pneumonitis, idiopathic bronchiolitis obliterans with organizing pneumonia (BOOP), idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP) (9,10,14,20) acute respiratory distress syndrome (ARDS) and sarcoidosis (10). The cases we present here all have the histologic pattern of non-specific interstitial pneumonia (NSIP) and therefore do not contain the “fibroblast foci” seen in the usual interstitial pneumonia (UIP).

Myofibroblasts are mesenchymal cells that contribute to tissue remodeling, collagen deposition or fibrosis, and contractility of the lung parenchyma (9, 10, 20-22). We have previously shown that fibroblasts cultured from bronchoalveolar lavage (BAL) fluid and from the lung tissue of scleroderma patients exhibit a myofibroblast phenotype (7, 11-13). In culture, SSc lung myofibroblasts express more ECM proteins, including collagen I and III, fibronectin, and tenascin C, than do normal lung fibroblasts (7, 8). These cells show greater proliferative capacity upon exposure to transforming growth factor-β1 (TGF-β1) and platelet derived growth factor-AA(PDGF-AA) than do normal lung fibroblasts (7, 23), and they are mainly responsible for excessive fibroproliferation in the lung (23-27). The present study demonstrates that SSc myofibroblasts aggressively populate pulmonary interstitia, where accumulation of ECM proteins is observed. The evidence leads us to hypothesize that myofibroblasts possess the capability to remodel the extracellular composition of the tissue, thereby leading to fibrosis. The fact that myofibroblasts disperse individually within the tissue may suggest the migratory character of these cells, and supports their potential role in tissue remodeling. Others have also observed the migratory character of myofibroblasts during tissue remodeling (9,28-30), and α-smooth muscle actin has been shown to be responsible for this migratory behavior (32).

In many fibrotic diseases, various origins of α-smooth muscle actin positive myofibroblasts have been postulated (pericytes, endothelial cells, epithelial cells, smooth muscle cells, fibrocytes and fibroblasts), but their actual origin(s) still remains unknown. Myofibroblasts exhibit different cytoskeletal proteins (α-smooth muscle actin, vimentin, desmin, myosin) during cell development and pathologic processes (11, 12, 24-27). Previously, we have reported the absence of desmin and smooth muscle myosin in myofibroblasts cultured from bronchoalveolar lavage fluid from SSc patient (7), suggesting that these cells do not originate from smooth muscle cells. Additionally, several lines of reasoning suggest that interstitial fibroblasts, under the influence of various stimuli, differentiate to a myofibroblast phenotype (11, 12, 27-37). In our recent studies in vitro, we reported that the serine protease, thrombin, which is present in elevated concentrations in BAL fluid from SSc patients (38), induces α-smooth muscle actin in normal lung fibroblasts and differentiates them to a contractile myofibroblast phenotype (11-13).

Along with myofibroblast induction, we also found that in the early stages of fibrosis the number of alveolar microvessels significantly increases. The increase is selective because it mostly occurs in microvessels but not in larger blood vessels. Numerous alveolar capillaries are overdeveloped and many of them present structural abnormalities. The induction and overdevelopment of microvessels appears with the increased number of vascular endothelial cells. This suggests that neovascularization or angiogenesis takes place during the course of fibrosis in SSc. These data are supported by the observation of high levels of circulating vascular endothelial growth factor (VEGF) and other angiogenic factors, chemokines and their receptors over-expressed in SSc patients (39, 40-44). Additionally, our immunohistochemical observations indicate that many of these overdeveloped microvessels might transport blood despite their structural deviation. However, we cannot completely rule out the other possibility that the vascular overdevelopment might result from activating a non-functional micro-capillary bed said to pre-exist in alveolar septa (15).

Endothelial injury and obliteratorive microvascular lesions are present in all of the involved organs in SSc (18). However, information on the vascular changes in SSc lung is controversial. Some reports suggest that angiogenesis is taking place in the SSc lung (41). Focal angiogenesis has been observed in the interstitia of SSc patients with fibrosing alveolitis (44). Similarly, enhanced endothelial proliferation and neovascularization have been observed in acute respiratory syndromes (ARDS), idiopathic pulmonary fibrosis (IPF) and bleomycin-induced animal models of pulmonary fibrosis (45-49). On the other hand, others have reported the reduction of vascular density and the redistribution of microvessels in the pulmonary interstitium in SSc (42, 43). Decreased angiogenesis has been linked to the high level of angiostatic endostatin found in the serum of SSc patient (43).

In the present study, we observed both neovascularization in the early stages of fibrosis and the structural disintegration of vasculature and loss of endothelial cell numbers in the later stages of lung fibrosis. The reduction of both mesenchymal and endothelial cells correlated with the degeneration of lung tissue and progression of fibrosis. The reversal of mesenchymal and endothelial cell excess has recently been reported with progression of lung fibrosis (40,50-52), which might result from selective cell death and/or degeneration of the tissue (53,54). Our stage-by-stage approach with analysis of lung tissue from different stages of fibrosis within the same patient demonstrates that the presence of myofibroblasts, endothelial cells and vascular distribution vary widely depending on the severity of fibrosis. In our opinion, such variations probably explain the disagreement regarding the role of angiogenesis in SSc in various studies.

To summarize, the current study demonstrates that in lung fibrosis associated with SSc a large population of myo-
fibroblasts arises transiently in the tissue during earlier stages of the disease, and their number decreases sharply as fibrosis progresses. Numerous alveolar capillaries also develop at the earlier stages of the disease, many of them with structural abnormalities. Most of the original and newly formed microvessels disappear as the lung transforms from a soft, cellular organ to an acellular, fibrotic one. These temporally progressive abnormalities warrant further investigation, since they may provide key information relevant to the etiology and/or pathogenesis of the development of pulmonary fibrosis in SSc.

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