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Usual interstitial pneumonia and smoking-related interstitial fibrosis display epithelial to mesenchymal transition in fibroblastic foci

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Double-staining immunohisto-chemistry

Summary
Background: Fibroblastic foci (FF) are a major histological feature of usual interstitial pneumonia (UIP) in idiopathic pulmonary fibrosis (IPF) and collagen vascular diseases (non-IPF). In addition, FF are occasionally associated with smoking-related interstitial fibrosis (SRIF). Recent studies have suggested a role for epithelial to mesenchymal transition (EMT) in pulmonary fibrogenesis.
Methods: Here, we investigated whether EMT was present in patients with IPF ($n = 19$), non-IPF ($n = 17$), and SRIF ($n = 16$) using morphometric immunohistochemistry, electron microscopy, and confocal microscopy. All patients had received lung biopsies or lobectomies for lung cancer.
Results: IPF and non-IPF patients displayed restrictive lung function patterns, whereas those with SRIF presented mixed patterns. Cells within FF presented high number of alpha-smooth muscle actin (αSMA)-staining cells; however, the foci of IPF patients showed comparatively lower number. Moreover, colocalization of thyroid transcription factor-1 (TTF1) and αSMA within FF showed low number of staining cells for IPF and SRIF in comparison to non-IPF ($p < 0.01$). Nevertheless, all groups displayed colocalization of high rate of $TTF1^{+}$-cells and

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low rate of αSMA$^+$-cells within hyperplastic epithelioid cells in FF. Also, we observed areas with low proportion of TTF1$^+$cells and αSMA$^+$cells, which were present in SRIF and non-IPF more often than IPF ($p < 0.001$). Electron microscopy revealed small breaks in the alveolar basal lamina, which allowed epithelioid cells to directly contact the collagenous matrix and fibroblasts. Three-dimensional reconstruction revealed intense αSMA staining within some epithelioid cells, suggesting that they had gained a mesenchymal phenotype.
Conclusions: These findings constitute the first report of EMT in SRIF and suggest that EMT occurs more prominently in SRIF and non-IPF than IPF.

Introduction

Fibroblastic foci (FF) of pulmonary fibrosis are small focal areas of young, myxoid-appearing matrix that contain aggregates of collagen-producing myofibroblasts undergoing active proliferation [1—3]. These foci are often identified at the transition zone between normal uninvolved lung tissue and abnormal fibrotic regions. FF have been hypothesized to represent local remodeling events during acute lung injury [2,4,5] and are believed to recapitulate processes occurring during the healing of skin wounds [6]. In particular, FF are clinically and biologically important in disease progression, with the amount of FF observed in surgical lung biopsies directly correlating with progressive physiologic deterioration and shortened survival in patients with IPF [7,8]. However, the role of FF in SRIF and non-IPF patients is not established.

FF are a major histological feature of usual interstitial pneumonia (UIP). Examples of causes of UIP include systemic sclerosis/scleroderma (non-IPF), idiopathic pulmonary fibrosis (IPF), rheumatoid arthritis, asbestosis and chronic nitrofurantoin toxicity [9,10]. In addition, FF are occasionally found in patients with smoking-related interstitial fibrosis (SRIF) [11] and may play a critical role in the development of this fibrosing lung disease. Although FF are clinically relevant, the mechanisms involved in their cellular origin and formation remain ill defined.

Recent *in vitro* and animal studies have suggested that epithelial to mesenchymal transition (EMT) of alveolar epithelial cells occurs during pulmonary fibrogenesis. EMT is the process by which epithelial cells lose their phenotypic characteristics and acquire features of mesenchymal cells, such as fibroblasts and myofibroblasts. Indeed, pulmonary fibrosis-associated EMT has been observed with experimental models [12,13], as well as *in vitro* [14,15] and human studies [16—18]. Moreover, EMT has been identified during embryonic differentiation [19], tumor progression [20], renal fibrosis [21], and liver fibrosis [22]. Therefore, we hypothesized that EMT might represent a general phenomenon occurring during pulmonary fibrogenesis, which is not only IPF specific. Here, we employed immunohistochemistry, electron microscopy, and confocal microscopy to investigate whether EMT was present in tissue sections from IPF, non-IPF, and SRIF patients.

Materials and methods

Characteristics of human subjects and inclusion criteria

Between 2006 and 2011, we consecutively enrolled patients with IPF ($n = 19$), non-IPF ($n = 17$), and SRIF ($n = 16$) who underwent open or thoracoscopic lung biopsy or lobectomy for lung cancer in our hospitals. Three lung pathologists (ATF, VLC, and HP), who were unaware of the clinical and physiologic findings, independently reviewed the lung biopsies. In cases where the classification by these pathologists differed, a consensus opinion on the overall histopathologic pattern was reached. Histologic features of UIP were based on a previously published report [23] and the criteria of the American Thoracic Society/European Respiratory Society (ATS/ERS) classification [9]. SRIF represented a secondary diagnosis in smoking patients with co-existing neoplastic disease, and its histologic features were based on a previously published report by Katzeinstein and colleagues [11]. Both non-IPF and IPF patients presented with the characteristic histopathologic pattern associated with UIP. The study protocol was approved by the Ethical Committee of Graz University, Austria (Number 24—135 ex 11/12). Notably, patients in an accelerated phase of interstitial pneumonia were excluded. Also, all non-IPF patients fulfilled the histological and clinical criteria for systemic sclerosis.

Immunohistochemistry

To simultaneously characterize epithelial and mesenchymal markers we employed a double-staining immunohistochemistry protocol, which utilized antibodies against thyroid transcription factor-1 (TTF1; mouse monoclonal, DAKO, Clone 8G7G3/1) and alpha-smooth muscle actin (αSMA; mouse monoclonal, DAKO Clone 1A4). Briefly, sections were deparaffinized, fixed in acetone (5 min, 22 C), and rehydrated in phosphate-buffered saline (PBS; pH 7.4) for 10 min. Immunohistochemistry was performed using a horseradish peroxidase labeled streptavidin biotin kit (HRP-LSAB, DAKO), as recommended by the manufacturer. After incubation with blocking solution (5 min), the sections were incubated for 30 min with one primary antibody (diluent from DAKO), followed by sequential 15 min incubations with biotinylated antibody (goat anti-mouse) and