Chronic hypersensitivity pneumonitis (HP) causes progressive and irreversible pulmonary fibrosis, a disease also observed in conjunction with idiopathic pulmonary fibrosis (IPF). Previous studies have demonstrated that the myofibroblast, a cell type whose origins involve the epithelial-mesenchymal transition (EMT), may play a role in the pathogenesis of IPF. The goal of this study was to determine whether EMT has a role in the pathogenesis of chronic HP. Lung specimens from a chronic HP model and from patients with chronic HP were analyzed. Cellular co-localization of epithelial and mesenchymal markers on the same alveolar epithelial cells (AECs) were examined using immunohistochemistry and cadherin switching by western blotting as indicators of EMT. EMT cells in the AECs were significantly more prevalent in lung specimens from Th2-prone A/J mice than in specimens from Th1-prone C57BL/6 mice. The percentage of EMT cells was correlated with the mRNA expressions of IL-13 and TGF-β1, the fibrosis score, and the collagen content in the A/J mice. In human, EMT cells in the AECs were significantly more prevalent in lungs specimens from patients with usual interstitial pneumonia (UIP) pattern than in specimens from patients with nonspecific interstitial pneumonia (NSIP) pattern at the moderate stage of fibrosis. In conclusion, EMT may play an important role in the fibrotic process of chronic HP under the Th2-biased environment.

Key words: hypersensitivity pneumonitis, myofibroblast, epithelial-mesenchymal transition, pulmonary fibrosis, murine model

Introduction

Hypersensitivity pneumonitis (HP) is an interstitial lung disease immunologically induced by the inhalation of organic dusts and certain inorganic chemicals. HP is clinically subclassified into acute and chronic forms. The chronic form of HP is thought to be induced by low antigen doses and to occur in individuals susceptible to the antigen. Our group has found that the usual interstitial pneumonia (UIP) pattern and partly nonspecific interstitial pneumonia (NSIP) pattern are correlated with the clinical course of the disease and prognosis.

Th2 immune polarization was recently identified as one of the causes of disease progression in patients with pulmonary fibrosis. Th2 cytokines such as interleukin (IL)-4 and IL-13 enhance the fibrotic processes by activating fibroblast proliferation and collagen production via transforming growth factor-β (TGF-β), whereas interferon-γ (IFN-γ), a Th1 cytokine, inhibits these processes. We and other investigators have recently observed Th2-biased immune responses in chemokines in the UIP pattern of chronic HP patients, as previously observed in IPF. We also found that a Th2-predominant immune response may be more relevant to the development of the UIP pattern than to the development of the NSIP pattern. Th2-biased genetic backgrounds may play an important role in the fibrosing processes in the murine model of chronic HP.

Fibroblasts are largely responsible for the augmentation of the collagen/matrix synthesis and deposition seen in pulmonary fibrosis. While the origin of the lung fibroblasts during pulmonary fibrosis has not been well
defined, three potential sources have been identified: the proliferation of resident lung interstitial fibroblasts, the differentiation of progenitor cells from bone marrow, or the transition of epithelial cells to a fibroblast phenotype, a process termed epithelial-mesenchymal transition (EMT)\textsuperscript{14}. The fundamental EMT phenomenon consists of a loss of normal epithelial integrity, for example, a loss of cell-to-cell adhesion molecules and their polarity, together with a gain of a mesenchymal phenotype, for example, migratory capacity\textsuperscript{15}. Evidence from the initial report on EMT identified the process as one of the causes of renal interstitial fibrosis\textsuperscript{16}. Specifically, EMT proved to be a major source of fibroblasts: more than one-third of all disease-related fibroblasts originated from the tubular epithelial cells at injury sites\textsuperscript{16}. TGF-β induced EMT in a primary culture of alveolar epithelial cells of rats, and the co-expression of epithelial and mesenchymal markers was observed in and around fibroblastic foci on the lung specimens of IPF patients\textsuperscript{17}. Little is known, however, about EMT in the development of chronic HP.

The present study was conducted to investigate the presence of EMT and to examine whether EMT is relevant to the pathogenesis of chronic HP.

Materials and Methods

Mice

C57BL/6 (B6) and A/J mice were purchased from Sankyo Medical Animal Supply (Sankyo Lab. Co., Tokyo Japan). The mice were bred in the animal facility of the Tokyo Medical and Dental University under specific pathogen-free conditions and included in the study after reaching 6 to 8 weeks of age. The University Committee on the Use and Care of Animals approved the protocol for this study.

Preparation of pigeon dropping extracts (PDE)

Pigeon-dropping extracts (PDE) were obtained according to the previously described method\textsuperscript{18}. Briefly, fresh pigeon droppings were stirred with a 20-fold volume of phosphate buffered saline solution (PBS) (pH 7.4) for 24 h, then dialyzed against distilled water. The extracts were then sterilized with filtration (Millex-GV; Millipore, Bedford, MA, USA) and lyophilized.

Murine model of chronic HP

The murine model of chronic HP was generated as previously described\textsuperscript{13}. Briefly, mice were immunized and boosted by a subcutaneous injection of 8 μg of PDE adsorbed onto alum (imjectAlum, Pierce, Rockford, IL, USA). Next, 8 μg of PDE dissolved in 40 μl of 0.9% saline was applied at the tip of the mouse’s nose and involuntarily inhaled. This instillation procedure was conducted 3 days per week for either 6 or 12 weeks (n=5 in each group). Control mice were subcutaneously injected with physiologic saline with alum and administered an equal volume of physiologic saline solution without PDE 3 days per week.

Immunoistochemical studies

Double immunohistochemistry was performed to assess the co-expression of epithelial and mesenchymal markers in lung specimens from the murine model of chronic HP and from patients with chronic HP, as previously described\textsuperscript{13}. Thyroid transcription factor-1 (TTF-1) and α-smooth muscle actin (α-SMA) were selected together as an epithelial marker and mesenchymal marker, respectively, because this combination proved to be more sensitive than other markers such as E-cadherin (epithelial marker), pancytokeratin (epithelial marker), vimentin (mesenchymal marker), and N-cadherin (mesenchymal marker). To confirm the co-localization of TTF-1 and α-SMA in an alveolar epithelial cell, cell images were collected by immunofluorescent staining with an OLYMPUS ® FV500 confocal microscope in the Tokyo Medical and Dental University Imaging Facility. TTF-1 in the nucleus was visualized with Alexa Fluor 546 goat anti-rabbit IgG. α-SMA in the cytoplasm was visualized with Streptavidin-Alexa Fluor 488-conjugate (Molecular Probes, Eugene, OR, USA). A Z-stack image was acquired for three-dimensional reconstruction. The number of immunoreactive cells of bronchiolar and alveolar epithelial cells in 20 fields was counted under a microscope at 200X magnification. Among all the epithelial cells observed, those judged to be positive for both TTF-1 in the nucleus and for α-SMA in the cytoplasm were defined as EMT\textsuperscript{17}. The percentage of double-positive cells was determined by the following formula: (TTF-1 + α-SMA) positive cells / TTF-1 positive cells x 100 (%). To prevent observer bias, the evaluation of immunoreactivity in each field was determined independently by two observers.

Alveolar epithelial type II cell study in the mouse model

Crude cell suspensions were prepared from A/J mice treated with PDE (n=4) and normal saline (n=4). Following anesthesia, the mice were exsanguinated and the pulmonary vasculature was perfused with saline via the right ventricle until the effluent was free of blood.
EMT in chronic HP.

The trachea was cannulated with 20-gauge tubing and the lungs were filled with 1-2 ml of dispase. After infusing 0.45 ml of low-melting-point agarose via the trachea, the lungs were immediately covered with crushed ice, incubated for 2 min, placed in 2 ml dispase, and incubated for another 45 min at 24°C. Next, the lung tissue was teased from the airways and minced in Dulbecco’s modified Eagle’s medium (DMEM) with 0.01% DNase. The minced lung was gently swirled for 10 min and passed successively through 100-µm and 40-µm Falcon cell strainers. The filtered suspension was centrifuged at 130g for 8 min and resuspended in 25 mM HEPES buffered DMEM. The cell pellet was resuspended in buffer containing PBS pH 7.2, 0.5% BSA, and 2 mM EDTA. ATII cells were purified by negative selection with the MACS system using CD45 MicroBeads (Miltenyi Biotec) and CD16/32 antibody (Miltenyi Biotec).

ATII cell proteins were extracted by RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitors. Equal protein concentrations were subjected to western blotting. After the samples were loaded on 10% polyacrylamide gels (Bio-Rad) and electrophoresed, the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). To examine the cadherin switching in ATII cells, the membranes were probed with primary antibodies (1:500 for E-cadherin (mouse IgG1, Invitrogen), 1:2000 for N-cadherin (mouse IgG 1, BD), 1:1000 for β-catenin (rabbit IgG, Cell Signaling Technology), 1:400 for β-actin (mouse IgG1, Abcam)). Goat anti-mouse IgG-H+L (Cy2) (Abcam) and goat anti-rabbit IgG-Cy3 (GE healthcare) were used as secondary antibodies. The multiplexed secondary antibody signals were detected by scanning the membranes with a Molecular Imager FX system (Bio-Rad). Results were expressed relative to β-actin band density used as a loading control.

Analysis of mRNA expression based on the real-time quantitative polymerase chain reaction in the murine chronic HP model

The expression levels of the IL-4, IL-13, and TGF-β1 genes in the lung of the murine model of chronic HP were determined by a quantitative real-time reverse transcription-polymerase chain reaction (PCR), as previously described. For IL-4, the forward primer was 5’-GAATGTACCAGGAGTGAAGAGG-3’ and the reverse primer was 5’-GCTCAATGTGGTTCAGGTT-3’. For TGF-β1, the forward primer was 5’-AGCCCGAGGCGGACTACTAT-3’ and the reverse primer was 5’-TCCACATGTTGCTCCACACT-3’. The PCR was performed in the MiniOpticon PCR detection system (Bio-Rad, Hercules, CA, USA) operated by MJ Opticon Monitor v3.1 analysis software (Bio-Rad) using ready-made fluorogenic probes (SYBR green; Bio-Rad). Gene expression was quantified relative to the expression of GAPDH, by the ∆∆CT method, according to the manufacturer’s protocols.

Semiquantitation of histological findings of fibrotic changes in the murine model of chronic HP

For the semiquantitative histological analysis of fibrotic changes induced by PDE, a numerical Ashcroft fibrosis scale was adapted. Twenty fields on each lung section were observed at a X10 objective in each successive field, and each field was assessed individually for the severity of fibrotic changes and allotted a score from 0 (normal) to 8 (total fibrosis) on a predetermined scale of severity. The mean of the scores from all fields was calculated as the fibrosis score. To prevent observer bias, all histological specimens were randomly numbered and interpreted in a blinded fashion. Each specimen was scored independently by two observers (K.M. and Y.M.). The means of their individual scores were considered the fibrosis scores.

Measurement of collagen content of the right lung in the murine model of chronic HP

The collagen content of the right lung was determined by the Sircol Collagen Assay kit (Biocolor, Belfast, Northern Ireland, UK) according to the manufacturer’s instructions.

Sample collection in patients with chronic HP

Lung specimens were obtained from 22 patients with chronic HP by thoracoscopic lung biopsy. All cases exhibited clinical, radiological, and physiological alterations consistent with chronic HP. Two pathologists (T.T. and T.A.) categorized the cases as either the UIP pattern (11 patients) or NSIP pattern (11 patients) based definitive pathological findings according to the 2002 ATS/ERS international consensus classification. From then, the patients with the NSIP pattern were subdivided into two groups: the
fibrotic NSIP pattern (6 patients) and the cellular NSIP pattern (5 patients). The diagnostic criteria for chronic HP were previously described. Normal lung specimens were obtained during a lobectomy for the removal of primary lung tumors. No histopathological evidence of disease was found in any of the resected specimen samples. This study was approved by the institutional review board. Appropriate written informed consent was obtained from all of the subjects.

Grading of fibrosis in patients with chronic HP

The grade of fibrosis in each field was also assessed using the previously described criteria, with slight modifications. Fibrosis was graded from 0 to 5 (0, normal lung; 1, minimal fibrous thickening of the alveolar or bronchiolar walls; 2, moderate thickening of the walls without obvious damage to the lung architecture; 3, increased fibrosis with definite damage to the lung structure, and the formation of fibrous bands or small fibrous masses; 4, severe distortion of the structure with large fibrous areas, (“honeycomb lung” is placed in this category); 5, total fibrous obliteration throughout the field).

Statistical analysis

The percentages of double-positive cells were expressed as mean ± SEM. Differences in the percentages of double-positive cells between PDE-exposed mice and control mice, and between B6 and A/J mice, were analyzed by the Mann-Whitney U test at each time point (6 weeks and 12 weeks). Similarly, differences in the percentages of double-positive cells between the two time points (6 weeks and 12 weeks) were analyzed by the Mann-Whitney U test in each strain (B6 and A/J mice). Spearman’s rank correlations were calculated to determine the correlation between the percentages of double-positive cells in the murine model and the mRNA expressions of the IL-4, IL-13, and TGF-β1 cytokines, the fibrosis score, and the measured collagen content. Differences in the percentages of double-positive cells between the histologic patterns or between the fibrosis grades were analyzed in the chronic HP patients by the Kruskal-Wallis test followed by the Dunn’s multiple comparison test. The statistics were analyzed using the statistical software package GraphPad Prism 5 (ver. 5; GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at P < 0.05.

Results

Assessment of histology in the murine model of chronic HP

Lung specimens revealed that PDE exposure induced a marked infiltration of inflammatory cells into the peribronchiolar and perivascular areas in A/J and B6. In particular, the lung specimens from the A/J mice showed a remarkable structural alteration of the alveoli and a thickening of the alveolar walls after 12 weeks. This histological change was similar to the change to the NSIP pattern observed in the human subjects (Figure 1A). The lung specimens from the B6 mice showed an accumulation of macrophages in the airspace, but no significant structural distortion was observed (Figure 1D). No significant infiltration of inflammatory cells was observed in the lung specimens from the control mice (Figure 1G). In the immunohistochemistry for TTF-1 and α-SMA, some of the alveolar epithelial cells in the PDE-exposed mice co-expressed TTF-1 in the nucleus and α-SMA in the cytoplasm (Figure 1C, F indicated by the black arrowhead). Very few cells were positive for α-SMA in the lung specimens from the control mice (Figure 1H,I).

Co-expression of epithelial and mesenchymal markers in the murine model of chronic HP

Paraffin-embedded, archived lung tissue samples from the murine model of chronic HP were examined. Spearman’s rank correlations test was used to assess interobserver variability before final values were determined by consensus. The interobserver variability for the evaluation of the extent of staining was 0.81, indicating good agreement between the observers. The percentages of cells double-positively stained for TTF-1 and α-SMA were significantly higher in the PDE mice (Figure 2A, black bars) than in the control mice (Figure 2A, open bars) at each time point (6 weeks and 12 weeks) and in each strain (B6 and A/J mice). The percentages of double-positive cells after 6 weeks of exposure to PDE did not significantly differ between the B6 and A/J mice; but the percentages after 12 weeks of exposure to PDE were significantly higher in the A/J mice (21.32±1.03%) than in the B6 mice (8.48±1.60%) (p=0.0079). In the A/J mice, the percentages of double-positive cells at 12 weeks (21.32±1.03%) were significantly higher than the percentages at 6 weeks (11.34±1.55%) (p=0.0079). In the B6 mice, however, the difference in the percentages between 6 weeks and 12 weeks was not significant.
EMT in chronic HP.

Alveolar epithelial type II cells after 12 weeks of exposure to PDE or normal saline were examined in the A/J mice (n=4). The AT2 cell yields in the final suspensions from each mouse were 8-10×10^5 cells, with a purity of 85-90%. The expressions of E-cadherin, N-cadherin, and β-catenin were analyzed by western blotting (Figure 3A). The expressions of E-cadherin (Figure 3B) and β-catenin (Figure 3C) did not differ between the PDE-exposed and control mice, while the expression of N-cadherin was significantly higher in the PDE-exposed mice than in the controls (0.125±0.028 vs. 0.057±0.024, p=0.0286). (Figure 3D).

EMT and Th2 cytokines in the murine model of chronic HP

The correlation between the percentages of EMT cells and mRNA expressions of the IL-4, IL-13, and TGF-β1 cytokines were evaluated in the murine model. In A/J mice, the percentages of EMT cells were significantly correlated with the expression levels of both IL-13 (r=0.7697, p=0.0126) (Figure 4B) and TGF-β1 (r=0.6565, p=0.0438) (Figure 4C). There was no correlation, however, between the percentages of EMT cells and the expression levels of IL-4 (r=0.18, p=0.6) (Figure 4A). No correlations were found between the percentages of EMT cells and the expression levels of the three cytokines in the B6 mice: IL-4 (r=0.1641).

Figure 1: Representative images of lung specimens from A/J mice treated with PDE (A: H&E staining; B and C: double immunohistochemistry), C57BL/6 mice treated with PDE (D: H&E staining; E and F: double immunohistochemistry), and A/J mice treated with normal saline (G: H&E staining; H and I: double immunohistochemistry). Cells co-expressing TTF-1 in the nucleus (pink) and α-SMA in the cytoplasm (brown) are indicated by the arrowhead. Scale bar = 100 μm (A, B, D, E, G, and H) or 50 μm (C, F, and I).
EMT and fibrosis in the murine model of chronic HP

Fibrosis in the lung was analyzed semiquantitatively based on the numerical fibrosis score and the measured collagen content, then the correlation between the percentages of EMT cells and the fibrosis score or measured collagen content was evaluated (Figure 5). In the A/J mice, the percentages of EMT cells were significantly correlated with the measured collagen content ($r=0.8424, p=0.0037$) (Figure 5A) and fibrosis score ($r=0.7477, p=0.0174$) (Figure 5B). In contrast, the percentages of EMT cells were not correlated with the measured collagen content ($r=0.2188, p=0.5367$) (Figure 5C) or fibrosis score ($r=0.5596, p=0.0963$) in the B6 mice (Figure 5D).

Assessment of histology in chronic HP patients

Figure 6 shows representative histological features of the patients with the UIP pattern. The lesion often showed scattered fibroblastic foci composed of aggregates of fibroblastic or myofibroblastic cells covered with type 2 pneumocytes (Figure 6A). In the immunohistochemistry for epithelial and mesenchymal markers, double-positive signals for TTF-1 and α-SMA were found in the alveolar and bronchiolar epithelial cells (Figure 6B), particularly in the alveolar epithelial cells covering the fibroblastic foci in specimens assessed as grade 3 fibrosis (Figure 6C, indicated by the black arrowhead). Confocal microscopy revealed co-localization of TTF-1 and α-SMA in the alveolar epithelial cells (Figure 6D).

Co-expression of epithelial and mesenchymal markers in chronic HP Patients

Double immunohistochemical staining for epithelial
marker (TTF-1) and mesenchymal marker (α-SMA) was examined by the same method in the murine model. Double-positive cells for TTF-1 and α-SMA were counted in a microscopic observation of epithelial cells in 20 fields at 200X magnification. Interobserver variability in the evaluation of the extent of staining was 0.9, indicating good agreement between the observers. The number of double-positive cells was higher in UIP pattern (Figure 7E,F) than in cellular NSIP pattern (Figure 7A,B) or fibrotic NSIP pattern (Figure 7C,D). Staining of the normal lung specimens revealed no co-expression of cells for either marker (data not shown). The percentages of double-positive cells (mean ± SEM) associated with a histologic pattern and grade of fibrosis were shown in Table 1. For the UIP pattern, the percentages of double-positive cells stained for TTF-1 and α-SMA were significantly higher in grade 3 (35.19 ±2.13%) than in the other grades (p<0.05) (Figure 8C). For the fibrotic NSIP pattern, the percentages of double-positive cells were significantly higher in grade 3 fibrosis (18.33±2.55%) than in the other grades (p<0.05) (Figure 8B). For the cellular NSIP pattern, the differences between grade 3 (13.36±1.66%) and the other grades were not significant (Figure 8A). In

Table 1. Percentages of EMT cells in fibrosing grades and histological patterns.*

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<th>grade 1</th>
<th>grade 2</th>
<th>grade 3</th>
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<tr>
<td>c-NSIP</td>
<td>8.62±1.27</td>
<td>9.08±1.05</td>
<td>13.36±1.66</td>
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<tr>
<td>f-NSIP</td>
<td>9.11±0.58</td>
<td>10.2±0.96</td>
<td>18.33±2.55</td>
</tr>
<tr>
<td>UIP</td>
<td>10.62±0.71</td>
<td>16.58±0.85</td>
<td>35.19±2.13</td>
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* Data are shown as percentages of TTF-1 and α-SMA double-positive cells (mean ± SEM).

Figure 4: Correlation of the percentages of double positive cells and the mRNA expressions of IL-4, IL-13, and TGF-β1 cytokines in A/J mice (A-C) and C57BL/6 mice (D-F) treated with PDE. B6; C57BL/6.

Figure 5: Correlation of the percentages of double positive cells and the measured collagen content or fibrosis score in A/J mice (A and B) and C57BL/6 mice (C and D) treated with PDE. B6; C57BL/6.
the grade 2 and 3 specimens, the percentages of double-positive cells were significantly higher in the UIP pattern (16.58±0.85%, 35.19±2.13%) than in the fibrotic NSIP (10.20±0.96%, 18.33±2.55%) or cellular NSIP pattern (9.08±1.05%, 13.36±1.66%) (p<0.05) (Figure 8E,F). In grade 1, the differences between the UIP pattern (10.62±0.71%), fibrotic NSIP pattern (9.11±0.58%), and cellular NSIP pattern (8.62±1.27%) were not significant (Figure 8D).

Discussion

The present study was conducted to evaluate the role of EMT in chronic HP in murine models and in humans. In murine models, EMT cells were significantly more prevalent in Th2-prone mice than in Th1-prone mice, and the percentages of EMT cells correlated with the expressions of the IL-13 and TGF-β cytokines. EMT cells were also more prevalent in the UIP pattern in patients with chronic HP than in the NSIP pattern in patients at the moderate stage of fibrosis.

The process of EMT was accompanied by morphological alterations and the expression of fibroblast phenotypic markers concomitant with the downregulation of epithelial phenotypic markers in vitro. TGF-β induced the EMT process in a time- and concentration-dependent manner in human A549, a cell type with an alveolar epithelial type II cell phenotype. TGF-β plays a key role in the induction of EMT in the processes of development, carcinogenesis, and fibrosis. Different TGF-β isoforms mediate different effects, depending on the specific cellular context.
The progression of fibrosis is associated with the production of the Th2 cytokines IL-4 and IL-13 and resulting up-regulation of TGF-β and promotion of fibroblast proliferation, collagen gene expression, and collagen synthesis\(^\text{17}\). Several reports have described EMT in animal models of organ fibrosis. Zeisberg et al. reported the incidence of EMT in tubular epithelial cells in chronic renal injury\(^\text{23}\). Xia et al. reported the incidence of EMT in bile duct epithelial cells in liver fibrosis induced by bile duct ligation\(^\text{24}\). EMT has also been demonstrated in vivo using animal models. A transgenic mouse reporter system developed by Kim et al. tracked the fate of resident lung epithelial cells in a model of pulmonary fibrosis overexpressing active TGF-β. The resident epithelial cells with mesenchymal markers accumulated within 3 weeks of in vivo TGF-

Figure 7: Representative images of lung specimens from patients with various histologic patterns (A and B: cellular NSIP pattern; C and D: fibrotic NSIP pattern; E and F: UIP pattern). Cells co-expressing TTF-1 in the nucleus (blue) and α-SMA in the cytoplasm (brown) are indicated by the arrow. Scale bar = 100 μm (A, C, and E) or 50 μm (B, D, F).
EMT in chronic HP.

In chronic HP, EMT cells were significantly more prevalent in the UIP pattern than in the NSIP pattern, particularly in moderate fibrosis. In our human chronic HP subjects, EMT cells were significantly more prevalent in A/J mice than in Th1-prone B6 mice in our murine model, as assessed by immunohistochemistry and western blotting. The percentages of EMT cells in A/J mice were strongly correlated with the expression levels of the cytokines IL-13 and TGF-β1. We also found that A/J mice had more severe pulmonary fibrosis than B6 mice after 12 weeks of exposure to PDE (Figure 5A and B). These results suggest that a Th2-biased genetic background may play an important role in the fibrotic processes of EMT in mice. In our human chronic HP subjects, EMT cells were significantly more prevalent in the UIP pattern than in the NSIP pattern, particularly in moderate fibrosis. In earlier studies on chronic HP with the UIP pattern, we observed Th2-biased immune responses in chemokines similar to those seen in IPF.

Figure 8: Relationships between the percentages of double-positive cells and the grade of fibrosis in cellular NSIP pattern (A), fibrotic NSIP pattern (B) and UIP pattern (C). Relationships between the percentages of double-positive cells and the histologic pattern in grade 1 fibrosis (D), grade 2 fibrosis (E), and grade 3 fibrosis (F). Data are expressed as mean ± SEM. *P < 0.05.
in patients with the fibrotic NSIP pattern or controls (Y.M. and N.I., unpublished data). Increased expression of IL-13 is a characteristic finding in surgical lung biopsies from human patients with IPF/UIP\(^{27}\). IL-13 has been identified as the predominant effector cytokine of fibrosis in several experimental models of fibrosis\(^{28}\). As described above, the progression of fibrosis is associated with an increase of the Th2 cytokines IL-4 and IL-13 and resulting upregulation of TGF-\(\beta\) 1. These findings also suggest that the Th2-biased immune response may play an important role in the fibrotic process of EMT in humans as well as in murine models.

Another important finding of the present study is the higher frequency of EMT in lung specimens with moderate fibrosis (grade 3), particularly around the fibroblastic foci (FF) (Figure 6). We already know, from an earlier study, that FF seems to predominate at grade 3\(^{29}\). FF are generally seen as ‘new’ fibrotic lesions that represent the leading edges of ongoing lung injury\(^{29}\). Persistent fibroblast proliferation in FF plays a critical role in the pathway to end-stage pulmonary fibrosis\(^{30}\). Several reports support the notion of EMT involvement in FF. According to a report from Willis et al., EMT appears in both hyperplastic epithelial cells and cells overlaying FF, and \(\alpha\)-SMA is present in up to 80% of the alveolar epithelial cells in IPF\(^{17}\). Nuclear expression of \(\beta\)-catenin, a signaling pathway with a relevant role in the induction of a mesenchymal phenotype in epithelial cells\(^{31}\), was demonstrated in FF in IPF/UIP samples\(^{32}\). Furthermore, Cosgrove et al. found that TGF-\(\beta\), an EMT-inducing agent, was preferentially expressed in FF\(^{33}\). These results, in sum, suggest that FF may contribute to the fibrotic process of EMT.

This study has provided \textit{in vivo} data in support of the occurrence of EMT in chronic HP. This data conflicts, however, with an earlier report demonstrating an absence of double-positive cells for epithelial and mesenchymal markers in immunohistochemical staining of human IPF and NSIP\(^{34}\). The authors do not rule out the possibility of EMT, given that EMT is transient during certain stages of fibrogenesis and that this transition, a process in which cells express detectable levels of epithelial and mesenchymal markers, is too short to detect by double-immunostaining. The degree to which EMT contributes to FF in UIP may vary from one case to another. If FF plays a critical role in progression of IPF/UIP, EMT may influence the course of the disease.

In conclusion, the present study demonstrates that a Th2 bias in immune response may be relevant to EMT during the fibrotic process of chronic HP both in murine models and in humans, especially in fibrosing interstitial pneumonia of either pattern, UIP or NSIP.

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Reference


