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Lung mast cell density defines a subpopulation of patients with idiopathic pulmonary fibrosis

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Abstract

Aims—The relationship of mast cells to the pathogenesis of lung fibrosis remains undefined despite recognition of their presence in the lungs of patients with pulmonary fibrosis. This study was performed to characterize the relationship of mast cells to fibrotic lung diseases.

Methods and results—Lung tissues from patients with idiopathic pulmonary fibrosis (IPF), chronic hypersensitivity pneumonitis (HP), systemic sclerosis (SSc)-related interstitial lung disease (ILD) and normal individuals were subjected to chymase immunostaining and the mast cell density quantified. Eosinophils were quantified by immunostaining for eosinophil peroxidase. Changes in lung function were correlated with mast cell density. Lung tissue obtained from IPF patients had a higher density of chymase-immunoreactive mast cells than that from patients with HP, SSc-related ILD or normal lungs. IPF lung tissue had a higher density of eosinophils than normal lung. There was no correlation between mast cell density and eosinophil density in IPF lung. IPF patients with high mast cell density had a slower rate of decline in forced vital capacity (FVC) than IPF patients with low mast cell density.

Conclusions—Mast cell density in IPF lungs is higher than in other fibrotic lung diseases and normal lungs. Increased mast cell density in IPF may predict slower disease progression.

Keywords

chloroacetate esterase; eosinophil; hypersensitivity pneumonitis; idiopathic interstitial pneumonia; systemic sclerosis

Introduction

Idiopathic pulmonary fibrosis (IPF) is a fibrotic lung disease characterized by a chronic, progressive, clinical course with an average survival of 3 years after diagnosis.[1,2] The disease is characterized histopathologically by a pattern of usual interstitial pneumonia

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(UIP) on surgical lung biopsy and the absence of inciting exposures or systemic illness such as a connective tissue disease.[1] Key pathological features of UIP include subpleural accentuation of fibrosis, honeycomb cysts and fibroblastic foci.[2] Although there have been significant advances in understanding the pathogenesis of IPF,[3] therapies that modulate the survival of IPF patients favourably are lacking.

Traditionally, mast cells have been recognized to be central effectors of immunoglobulin (Ig)E-mediated allergic inflammation.[4] More recently, mast cells have been shown to play roles in other disease processes such as obesity,[5] cancer progression,[6] atherosclerosis[7,8] and fibrogenesis.[4] Mast cells can exert bidirectional effects on matrix turnover and therefore could potentially promote both tissue repair or fibrogenesis.[4] For example, mast cell activation of the renin–angiotensin system has been reported to promote kidney fibrosis.[9–11] In contrast, tryptase and chymase activate matrix metalloproteinase-9 and collagenase, which can accelerate matrix degradation.[12] Furthermore, models of renal fibrosis in mast cell-deficient animals have suggested that mast cells protect against fibrosis.[9,13]

Several studies have documented the presence of mast cells in the lungs of IPF patients.[14–17] Despite this association, the role mast cells play in the fibrotic process remains undefined. Several studies have suggested that human mast cells are a potential source of profibrotic factors such as transforming growth factor (TGF)-β[18] or tryptase.[14,19,20] In contrast, mast cells play no role in development of lung fibrosis following bleomycin-mediated lung injury.[21,22] Thus, whether mast cells are pro- or anti-fibrotic remains uncertain..

The aims of the present study were several. First, we investigated whether mast cell density is increased in the lungs of IPF patients compared to other fibrotic lung diseases and non-diseased normal lung. To determine whether increased mast cell numbers in IPF lung tissue correlated with other inflammatory cells, the lung mast cell density was correlated with eosinophil density in IPF lung tissues. Lastly, we correlated mast cell density with markers of disease progression.

Materials and methods

STUDY POPULATION

The study population consisted of 29 patients with IPF, 16 with chronic hypersensitivity pneumonitis (HP), nine with systemic sclerosis (SSc)-related fibrotic interstitial lung disease (ILD) and 10 non-diseased normal individuals. All ILD diagnoses were established through a multidisciplinary review of clinical data, radiology and pathology. All patients with IPF fulfilled the criteria for diagnosis defined by the consensus classification of the American Thoracic Society (ATS) and European Respiratory Society (ERS).[2] The diagnosis of SSc was based on the criteria of the American College of Rheumatology.[23] Chronic HP was diagnosed based on the diagnostic criteria suggested by Hanak *et al.*[24] Diseased lung tissues were obtained through lung transplantation or video-assisted thoracoscopic surgery. Non-diseased normal lung tissues were procured from lungs not used by the Northern California Transplant Donor Network. The UCSF Committee on Human Research approved the study protocols and all study subjects underwent informed consent.

Medical records of all ILD patients were reviewed and clinical data abstracted. Pulmonary function testing, including forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO), were performed by the standards of the ATS and ERS.[25,26]

TISSUE PREPARATION AND CHLOROACETATE ESTERASE (CAE) STAINING

After harvest, lung tissue was embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. Five-μm-thick sections of

lung were fixed in 4% paraformaldehyde (PFA) for 10 min and then washed three times for 5 min in phosphate-buffered saline (PBS). Using a syringe and needle, reaction buffer (dimethyl formamide, 0.2 M Tris-maleate pH 7.5, ethylene glycol monoethyl ether, dimethylformamide and deionized water) was added to naphthol AS-D chloroacetate (Sigma Chemical Co., St Louis, MO, USA) and withdrawn into the syringe immediately. The mixture was then added to fast blue BB salt (Sigma Chemical) and applied to lung tissue sections through a 0.22-μm syringe filter (Fisher Scientific, Pittsburgh, PA, USA). After 1 min, the sections were washed in deionized water, counterstained with eosin (Sigma-Aldrich), mounted and imaged.

IMMUNOHISTOCHEMISTRY

Five-μm tissue sections were fixed in paraformaldehyde (PFA) and endogenous peroxidase inhibited by incubating the sections in 3% hydrogen peroxide for 30 min. After washing with phosphate-buffered saline (PBS), the sections were incubated for 1 h in PBS containing 5% normal goat serum and 1% bovine serum albumin (BSA), then incubated with either mouse anti-human chymase (1:2000; Millpore, Billerica, MA, USA) or mouse anti-eosinophil peroxidase (1:400; Millpore) overnight at 4°C. The sections were washed in 0.1% PBS-Tween 20, incubated for 40 min with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the bound peroxidase activity was detected using a diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA). The sections were then washed in deionized water and counterstained with Meyer's haematoxylin (Sigma-Aldrich). For each tissue, an adjacent section was stained only with secondary antibody to address non-specific binding of the secondary antibody.

QUANTIFICATION OF MAST CELLS AND EOSINOPHILS

Five randomly selected digital images were obtained from each lung section using a ×20 objective (Nikon Eclipse TE 300; Nikon Instruments, Melville, NY, USA) with a SPOT 2.3.1 camera (Diagnostic Instruments, Sterling Heights, MI, USA) and processed using SPOT 4.0.9 software (Diagnostic Instruments). Two reviewers blinded to the diagnosis counted the numbers of CAE-positive, chymase-immunoreactive or eosinophil peroxidase-immunoreactive cells and the average numbers per square millimetre were recorded for each patient. A Bland–Altman plot was performed to ensure that the difference in cell counts between the two readers was distributed normally.[27] There was consistency in the mast cell density in the five images obtained for each individual.

STATISTICAL ANALYSIS

Disease progression was quantified by the change in FVC over a 6-month period. Categorical analysis of disease progression was based on a change of 10% or more in FVC.[1] Data are expressed as the mean ± standard deviation (SD) or the median and range for continuous variables and as percentages for categorical variables. Among four groups, the continuous variables were compared by one-way analysis of variance (ANOVA) or the Kruskal–Wallis test, as appropriate. In multiple comparisons, Scheffé's and Tukey's methods were used as a *post-hoc* test if equal variances were assumed and Dunnett's T3 method was adopted if equal variances were not assumed. Between two groups, continuous variables were compared by an independent unpaired *t*-test and Mann–Whitney *U*-test if non-normally distributed and categorical variables were compared using a χ^2 test or Fisher's exact test. The relationship between continuous variables was assessed by Pearson's correlation coefficient. Two-sided *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS software (version 12.0; SPSS Inc., Chicago, IL, USA).

Results

QUANTIFICATION OF MAST CELL DENSITY USING CHYMASE IMMUNOSTAINING

Table 1 shows the demographic characteristics and pulmonary function data at the time lung tissues were obtained. To detect the presence of mast cells, lung sections were immunostained for chymase (Figure 1A) and the number of chymase-immunoreactive cells quantified (Figure 1C). Chymase-immunoreactive cells were noted in various regions of lungs from patients with lung fibrosis, including the alveolar wall as well as areas of fibrosis, irrespective of whether the tissue was from patients with IPF or non-IPF fibrotic lung diseases. There was good agreement between readers with respect to mast cell density in tissue sections [limit of agreement (mean ± 2 SD), −1.8 to 4.2, and intraclass correlation coefficient, 0.95]. The lungs of the patients with IPF exhibited greater numbers [21.0/mm^2 (0–71.5/mm^2)] of chymase-immunoreactive cells than those with chronic HP [8.1/mm^2 (0–63.1/mm^2)], SSc-related ILD [2.8/mm^2 (0–10.5/mm^2) or normal lungs (0.7/mm^2 (0–11.2/mm^2)] (Figure 2C; $P = 0.01$, $P < 0.001$ and $P < 0.001$, respectively). The number of chymase-immunoreactive cells in lungs of the patients with chronic HP did not differ significantly compared to those obtained from the patients with SSc-related ILD or normal lungs ($P = 0.57$ and $P = 0.66$, respectively).

CHLOROACETATE ESTERASE STAINING OF LUNG TISSUE

Chloroacetate esterase stains neutrophils, basophils and mast cells in tissue sections. Lung sections were subjected to CAE staining (Figure 2A) and the number of CAE-positive cells quantified (Figure 2B). Similar to chymase-immunostained tissues, the lungs of the patients with IPF exhibited greater numbers [20.3/mm^2 (0.2–21.0/mm^2)] of CAE-positive cells than those obtained from patients with chronic HP [10.2/mm^2 (0–48.4/mm^2)], SSc-related ILD [2.8/mm^2 (0–8.4/mm^2)], or normal lungs [1.4/mm^2 (0–7.7/mm^2)] (Figure 1C; $P = 0.01$, $P = 0.001$ and $P = 0.001$, respectively). There were no significant differences in the lung density of CAE-positive cells compared to chymase-immunoreactive cells within a disease group (IPF 20.3/mm^2 versus 21.0/mm^2; HP 10.2/mm^2 versus 8.1/mm^2; SSc-related ILD 2.8/mm^2 versus 2.8/mm^2; normal 1.4/mm^2 versus 0.7/mm^2). A positive correlation ($r = 0.78$; $P < 0.001$) was found between methods, suggesting that the CAE stain was largely detecting mast cells in these tissues (Figure 2C).

EOSINOPHILS IN LUNGS OF PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS

Finding an increased density of chymase-immunoreactive mast cells in lungs of IPF patients prompted the question of whether eosinophils, another inflammatory cell found commonly in association with mast cells are increased similarly in IPF lungs. To identify tissue eosinophils in fibrotic lungs, cells immunoreactive for eosinophil peroxidase were quantified in lungtissue sections from patients with fibrotic lung disease (Figure 3A). The eosinophil density differed among the groups [IPF group, 24.9/mm^2 (2.5–80.6/mm^2); chronic HP group, 35.4/mm^2 (1.4–71.5/mm^2); SSc-related ILD group, 10.2/mm^2 (1.4–33.7/mm^2); non-diseased controls, 0.4/mm^2 (0–1.4/mm^2); $P = 0.035$]. The densities of eosinophils in the IPF and chronic HP groups were significantly higher compared to normal lungs ($P = 0.004$ and $P = 0.002$, respectively). No significant differences were noted in comparisons between the other groups (Figure 3C). There was no significant correlation between mast cell and eosinophil densities ($r = -0.074$), suggesting that these cells may be recruited through independent pathways to the lungs of IPF patients (Figure 3D).

CORRELATION OF MAST CELL DENSITY TO MARKERS OF PROGRESSION OF IPF

Mast cell density was predictive of disease progression as defined by change in FVC of 10% [odds ratio (OR) 0.835, 95% confidence interval (CI) 0.698–0.999; $P = 0.049$]. To explore

this relationship further, the IPF patients were separated into two subgroups by mast cell density above or below the median value determined by chymase immunostaining (21.0/ mm^2). The baseline characteristics for these IPF subgroups are presented in Table 2. FVC decreased more substantially in IPF patients with a low mast cell density compared to those with a high mast cell density in terms of percentage change (Figure 4; −15% versus 1%, P= 0.02). The percentage of patients with a more than 10% decline in FVC over 6 months was also greater in the low mast cell density group compared with the high mast cell density group (80% versus 22%, P= 0.02).

Discussion

The results of this study demonstrate there is a significant increase in the density of chymase-immunoreactive mast cells in the lung tissue of patients with IPF, and suggest that mast cell density is related inversely to disease progression. These findings suggest that mast cells may influence the pathogenesis of IPF by attenuating the progression of fibrosis.

Previous studies have reported an abundance of mast cells in IPF lungs.[14,15,19] However, these studies were performed prior to the reclassification of idiopathic interstitial pneumonias and did not contain diseased controls.[2] Using the new classification scheme to identify patients with IPF, the present study confirmed that the density of mast cells is increased in IPF lungs. This study also examined whether mast cell hyperplasia is unique to IPF or a general feature of fibrotic lung diseases such as chronic HP and SSc-related ILD. Remarkably, mast cell density was highest in IPF lungs and elevated in HP patients compared to controls. Furthermore, the mast cell density in fibrotic lungs from SSc patients was similar to normal controls, suggesting that mast cell recruitment to fibrotic lungs may be disease-specific and not simply a general feature of lung fibrosis.

Previous studies have evaluated the prognostic value of histopathological features of IPF.[28–34] For example, the number of fibroblastic foci predicts a worse prognosis.[29,31–33] In addition, the presence of eosinophils in bronchoalveolar fluid of patients with lung fibrosis predicts disease progression and worse survival.[35,36] The data in this manuscript are unique, because they suggest that lung mast cell density may predict disease progression in IPF patients, with higher lung mast cell density being associated with slower disease progression. The use of mast cell density as a histopathological predictor of survival will require further study.

The role played by mast cells in the pathogenesis of fibrotic lung disease remains undefined. The majority of reports suggest mast cells promote lung fibrosis.[14,19,20] In this IPF cohort, a subgroup classified as having a high mast cell density exhibited a slower rate in decline of FVC compared to a subgroup with low mast cell density. Decline in FVC over time is an accepted marker of disease progression in IPF that correlates with worse prognosis.[37–39] Therefore, these data suggest that presence of mast cells in the lungs of IPF patients may protect against, rather than contribute to, disease progression.

The underlying mechanism for the increased mast cell numbers in lungs of a subset of IPF patients remains undefined. T helper type 2 (Th2) cytokines have been reported to play a role both in allergic inflammation[40] and the pathogenesis of IPF.[41] Because mast cells and eosinophils are prominent in both pathological processes, and because eosinophils and mast cells are markers of Th2 immune responses, one potential explanation is that mediators of Th2 immune response recruit both mast cells and eosinophils to IPF lung.[36] To examine this possibility, the density of mast cells was correlated to eosinophils in the lung tissue of IPF patients. Importantly, the mast cell density in IPF lung did not correlate with eosinophil

density in IPF lungs, suggesting that mast cells and eosinophils in the lung tissue of IPF are recruited to IPF lungs by different biological processes.

Controversy regarding the role of inflammation in the pathogenesis of IPF persists,[42] with the prevailing hypothesis being that inflammation does not play a major role in the pathogenesis of IPF.[43–45] Our data are notable because they correlate the presence of mast cells, a specific type of inflammatory cell, with slower loss of lung function in IPF patients. This suggests that the relative role of inflammation in the progression of IPF should be considered more broadly, including the possibility that inflammation attenuates, rather than augments, disease progression.

This study has several limitations. First, because lung tissues were obtained randomly from the patients' lungs, the lung tissue sampled may not be representative of pathological changes present in regions of lung that were not sampled. This is probably of limited importance because all biopsies included sub-pleural tissue, the region of lung involved most commonly in IPF. Secondly, as the study was performed retrospectively, a selection bias may have occurred because the analysis of physiological parameters was limited to a subgroup of patients with available longitudinal data. Thirdly, because lung tissues were obtained only once, the stage of disease could have biased the mast cell density. Fourthly, gender could have influenced measures of disease progression, as there were proportionately more males in the low mast cell group. To overcome these limitations, a prospectively designed study including larger number of patients is necessary to confirm the protective role of mast cells against the progression of IPF.

In conclusion, mast cell density is higher in IPF lungs compared to other fibrotic ILDs and normal lung. A higher mast cell density correlates with slower disease progression in IPF patients. These results suggest that mast cells may play an important role in attenuating the fibrotic process in IPF patients.

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Abbreviations

CAE	chloroacetate esterase
HP	hypersensitivity pneumonitis
IPF	idiopathic pulmonary fibrosis
SSc	systemic sclerosis

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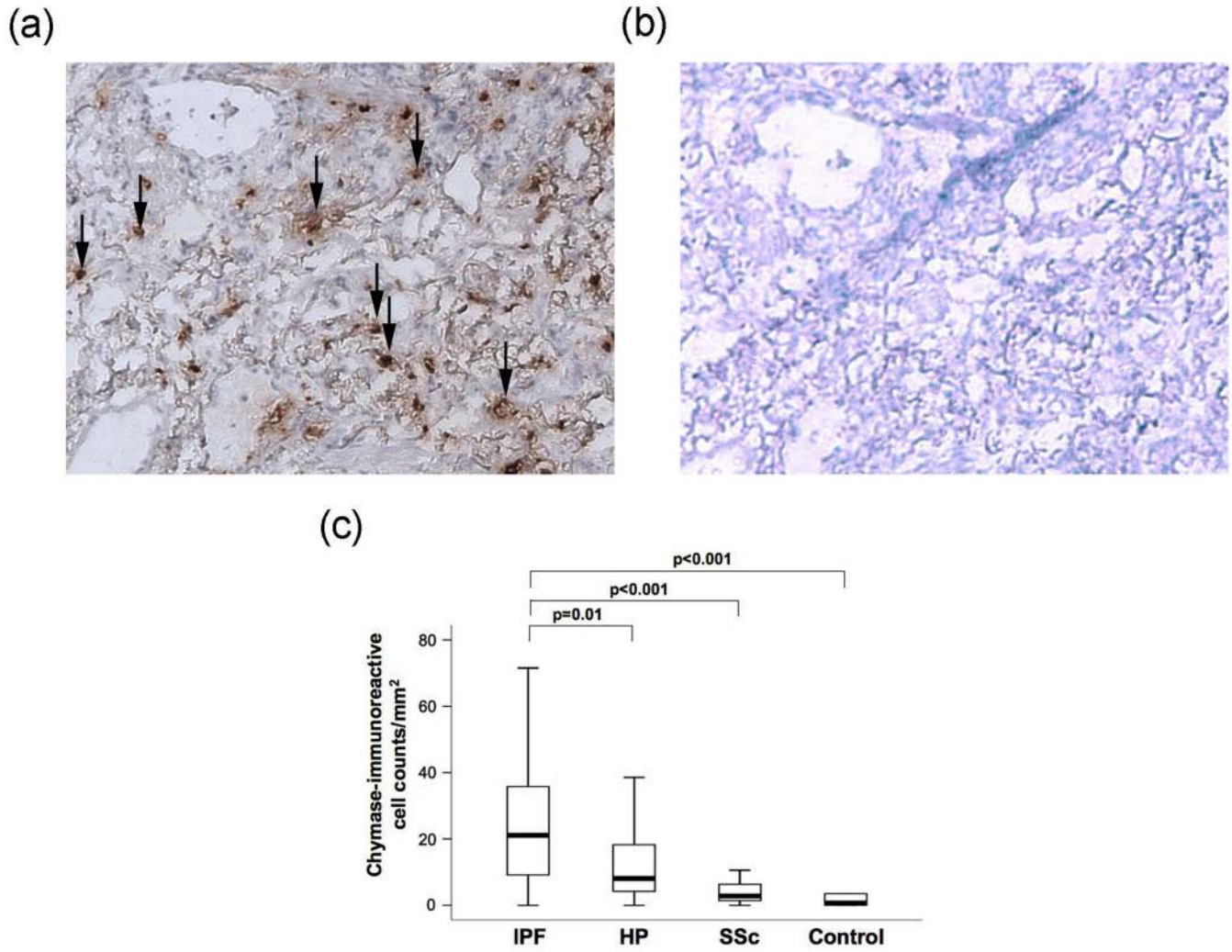

Figure 1.

Chymase immunostaining of lung tissue from patients with lung fibrosis. **A,** Sections of lung obtained from patients with idiopathic pulmonary fibrosis (IPF) were immunostained for chymase and the numbers of immunoreactive cells (arrows) counted (×20 objective). **B,** Control stain of adjacent tissue section with secondary antibody alone (×20 objective). **C,** The number of chymase-immunoreactive cells in tissue sections of IPF lungs is higher compared with chronic hypersensitivity pneumonitis (HP), systemic sclerosis (SSc)-related interstitial lung diseases or normal controls. Note: cross-bars denote median values.

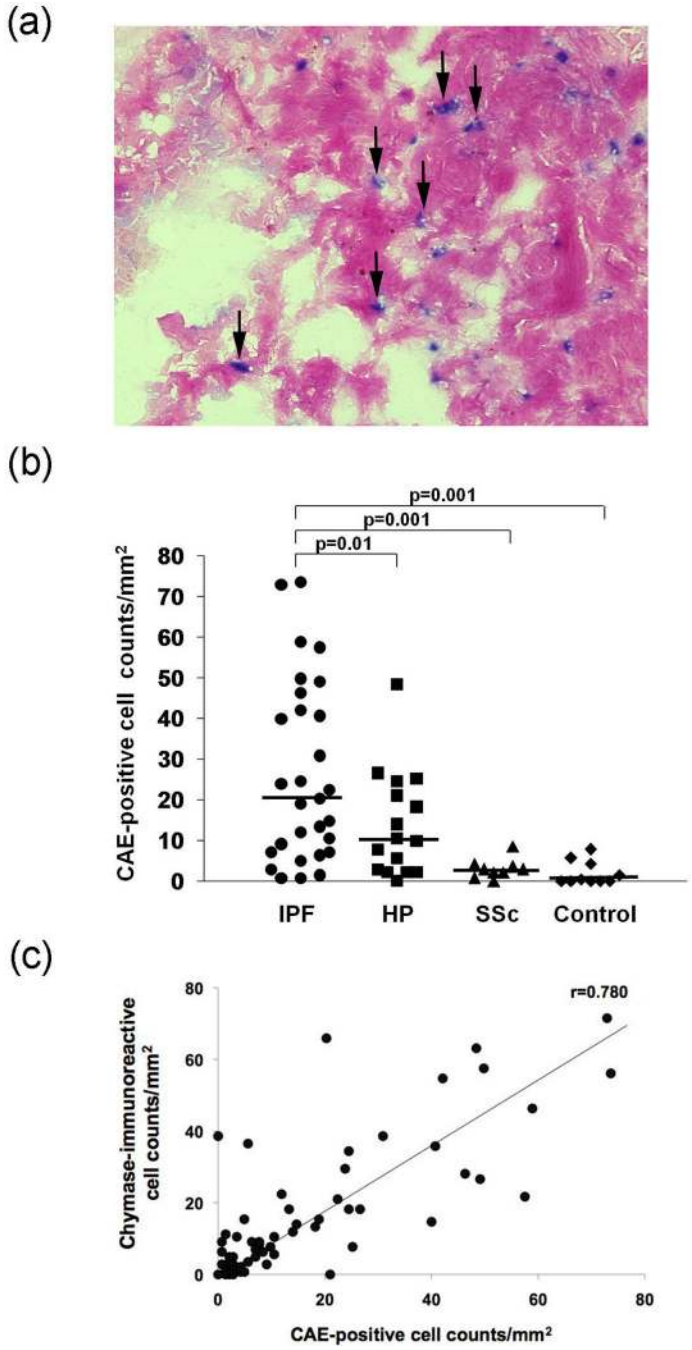

Figure 2.

Correlation of chymase-immunoreactive cell counts to chloroacetate esterase (CAE)-positive cell counts. **A,** CAE-positive cells (arrows) are located in regions of lung fibrosis (×20 objective). **B,** The number of CAE-positive cells in tissue sections of idiopathic pulmonary fibrosis (IPF) lungs are higher compared with chronic hypersensitivity pneumonitis (HP), systemic sclerosis (SSc)-related interstitial lung diseases or normal controls. Note: cross-bars denote median values. **C,** The number of chymase-immunoreactive cells was correlated to sections stained with CAE. Note the strong

correlation between chymase-immunoreactive cells and CAE-positive cells in tissues from the same patient.

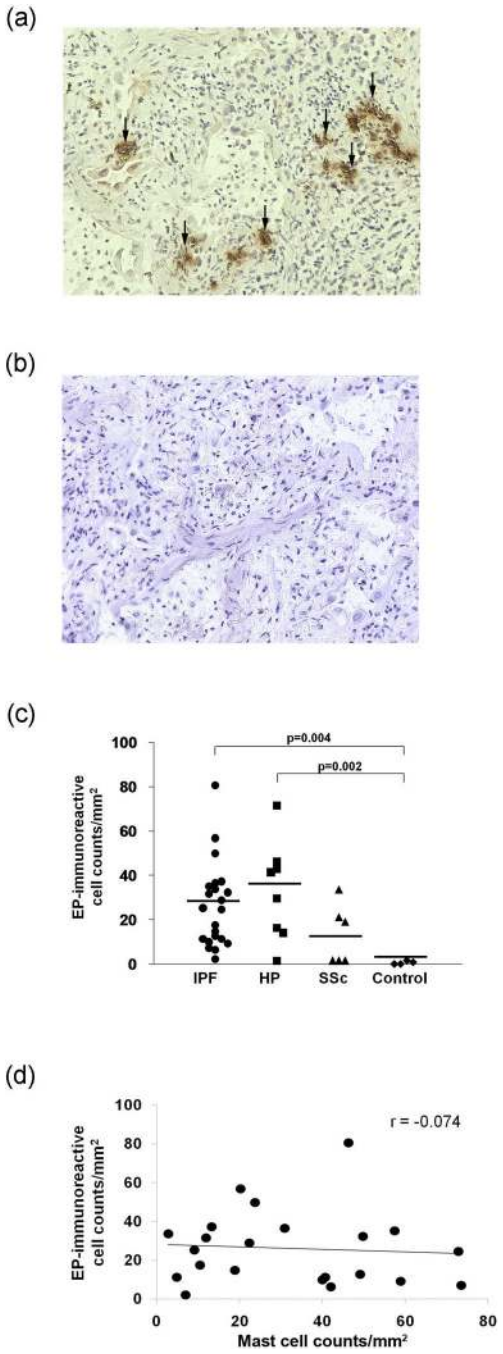

Figure 3.

Immunohistochemistry for eosinophil peroxidase (EP) in lung tissue from patients with lung fibrosis. **A,** Sections of lung obtained from a patient with idiopathic pulmonary fibrosis (IPF) were immunostained for EP. Note the brown EP-immunoreactive cells (arrows) (×20 objective). **B,** Control stain of adjacent tissue section with secondary antibody alone (×20 objective). **C,** The IPF and chronic HP groups have significantly higher eosinophil counts compared with normal lungs. **D,** The number of mast cells and eosinophils was correlated. Note the absence of correlation in tissues from the same patient.

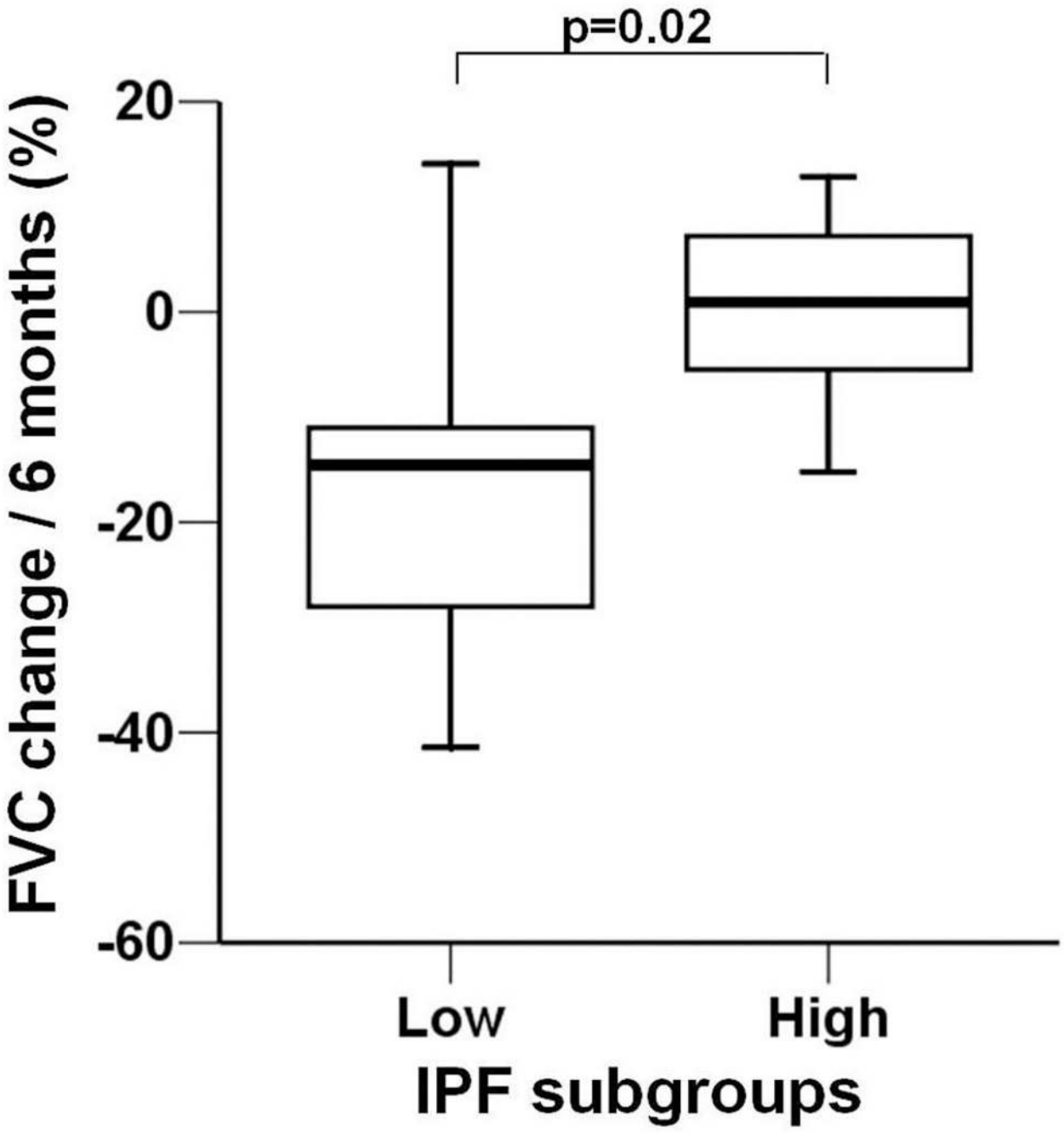

Figure 4.
Change of forced vital capacity (FVC) in low and high mast cell density subgroups of idiopathic pulmonary fibrosis (IPF). FVC declined more prominently in IPF patients with a low mast cell density compared to those with a high mast cell density.

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Table 1

Baseline characteristics of the subjects (*n*=64)

	IPF (*n*=29)	**HP (*n*=16)**	**SSc (*n*=9)**	**Normal (*n*=10)**	***P*-value**
Age, years	59±8	57±13	54±12	NA	0.45
Gender, male/female	22/7	5/11	3/6	NA	0.01
Smoking status, ever/never-smokers	21/8	7/9	2/7	NA	0.02
Use of corticosteroid *, % (total number with data)	77 (*n*=26)	57 (*n*=14)	50 (*n*=8)	NA	0.09
FVC, litres	2.0±0.7	1.8±0.9	1.9±0.6	NA	0.66
FVC% predicted, %	49±15	53±31	56±20	NA	0.71
DLCO, ml/min/mmHg	8.3±4.3	8.1±4.8	8.4±3.5	NA	0.99
DLCO% predicted, %	30±14	34±20	30±13	NA	0.79

IPF: idiopathic pulmonary fibrosis; HP: hypersensitivity pneumonitis; SSc: systemic sclerosis; FVC: forced vital capacity; DLCO: diffusing capacity for carbon monoxide; NA: not available.

*Use of corticosteroid within 3 months prior to acquisition of lung tissue.

Table 2

Baseline characteristics of idiopathic pulmonary fibrosis (IPF) mast cell density subgroups

	Low mast cell density (*n*=14)	**High mast cell density (*n*=15)**	***P*-value**
Age, years	61±7	57±9	0.49
Gender, male/female	13/1	9/6	0.08
Smoking status, ever/never-smokers	12/2	9/6	0.22
Use of corticosteroid *, % (total number with data)	67 (n=12)	86 (n=14)	0.37
FVC, litres	2.1±0.6	1.9±0.8	0.55
FVC% predicted, %	47±13	51±16	0.44
DLCO, ml/min/mmHg	9.6±5.6	7.3±2.8	0.23
DLCO% predicted, %	32±18	28±11	0.54

FVC: forced vital capacity; DLCO: diffusing capacity for carbon monoxide.

* Use of corticosteroid within 3 months prior to acquisition of lung tissue.