Histological characterization of mast cell chymase in patients with pulmonary hypertension and chronic obstructive pulmonary disease

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Abstract: Our previous findings demonstrated an increase in pulmonary mast cells (MCs) in idiopathic pulmonary arterial hypertension (IPAH). Also, literature suggests a potential role for MCs in chronic obstructive pulmonary disease (COPD). However, a comprehensive investigation of lungs from patients is still needed. We systematically investigated the presence/expression of MCs/MC chymase in the lungs of IPAH and COPD patients by (immuno)histochemistry and subsequent quantification. We found that total and perivascular chymase-positive MCs were significantly higher in IPAH patients than in donors. In addition, chymase-positive MCs were located in proximity to regions with prominent expression of big-endothelin-1 in the pulmonary vessels of IPAH patients. Total and perivascular MCs around resistant vessels were augmented and a significant majority of them were degranulated (activated) in COPD patients. While the total chymase-positive MC count tended to increase in COPD patients, the perivascular number was significantly enhanced in all vessel sizes analyzed. Surprisingly, MC and chymase-positive MC numbers positively correlated with better lung function in COPD. Our findings suggest that activated MCs, possibly by releasing chymase, may contribute to pulmonary vascular remodeling in IPAH. Pulmonary MCs/chymase may have compartment-specific (vascular vs. airway) functions in COPD. Future studies should elucidate the mechanisms of MC accumulation and the role of MC chymase in pathologies of these severe lung diseases.

Keywords: idiopathic pulmonary arterial hypertension, chronic obstructive pulmonary disease, mast cells, chymase.

Pulm Circ 2014;4(1):128-136. DOI: 10.1086/675642.

INTRODUCTION

Chronic lung diseases such as pulmonary arterial hypertension (PAH) and chronic obstructive pulmonary disease (COPD) are important health burdens of world population, and no cure is yet available for these diseases. PAH is a condition of multifactorial etiologies, characterized by an elevation of mean pulmonary arterial pressure, pulmonary vascular remodeling, and right ventricular hypertrophy. Idiopathic PAH (IPAH) is PAH of unknown etiology accompanied by a complex pulmonary vascular pathology, such as neointima formation and plexiform le-

sions at the advanced stage, and if left untreated, it leads to a fatal consequence due to right heart failure.^{1,2} COPD is an obstructive pulmonary disease characterized by air-flow limitation associated with progressive destruction of pulmonary parenchyma and remodeling of pulmonary vessels,³ as a consequence of an abnormal inflammatory response of the lungs to noxious particles or gases, primarily cigarette smoke.^{4,5} Despite the differences in the key structural alterations leading to respiratory insufficiency, the processes of inflammation and tissue remod-

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Submitted August 30, 2013; Accepted January 15, 2014; Electronically published March 14, 2014.

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eling are among the features of pathogenesis shared by these lung diseases; furthermore, the cellular and molecular culprits involved are not completely explored.

Mast cells (MCs) are tissue-resident cells of hematopoietic lineage. When activated, MCs release various mediators that may play a role in inflammatory processes in many diseases characterized by chronic inflammation and tissue remodeling.^{6,7} Among these mediators, MC chymase has been attributed a role in angiotensin II synthesis, transforming growth factor- β (TGF- β) activation, and promotion of endothelin (ET)-1 synthesis, and therefore it has been described in vascular pathophysiology.^{8,9} In addition, MC chymase is involved in activation of pro-matrix metalloproteases (MMP) 2 and 9.¹⁰ The finding that PAH patients have an elevated number of lung MCs hints at a potential role of MCs in the pathogenesis.^{11,12} The increase in epithelial MCs in the bronchioli of smokers with airflow limitation¹³ and the enhanced chymase activity detected in the lungs of hamsters experiencing chronic smoke exposure implicate MCs in the development of COPD.¹⁴ On the contrary, increased MC chymase in the peripheral airways was shown to correlate with better lung function in COPD patients.¹⁵ Clearly, an implication of MCs in COPD pathogenesis is yet unresolved. Taken together, the literature suggests a potential involvement of MCs in pathogenesis of IPAH and COPD,¹⁶⁻¹⁸ however, a comprehensive investigation of tissue samples from patients has still not been done. Therefore, in this study we systematically investigated the lungs from IPAH and COPD patients by assessing quantitatively the presence/expression of MCs/MC chymase and the activation/degranulation of MCs.

METHODS

Patient characterization

Human lung tissues were obtained from donors ($n = 9$) and patients with IPAH ($n = 9$) or COPD ($n = 9$) undergoing lung transplantation. The patients suffering from IPAH had a mean pulmonary artery pressure of ~ 73 mmHg and a mean cardiac index of ~ 1.99 L/min/m², and most of them were in the third New York Heart Association functional classification stage. COPD patients had an FEV1/FVC (forced expiratory volume in 1 s/forced vital capacity) ratio of $\sim 39\%$, and all of them were classified in the GOLD IV (Global Initiative for Chronic Obstructive Lung Disease) stage. After explantation, lung tissues were formalin fixed and paraffin embedded according to common tissue-processing protocols.¹⁹ The study protocol for tissue donation was approved by the ethics committee of the University Hospital Giessen, in accordance with national law

and international guidelines.¹⁹ Written informed consent was obtained from each individual patient or the patient's next of kin, as mentioned in previous work.¹⁹

Histology of MCs

According to the standard protocol, paraffin-embedded lung tissue sections (3 μ m thick) from COPD and IPAH patients and donors were stained with toluidine blue to identify MCs, similarly to previous descriptions.¹⁹ The tissue sections were dewaxed, rehydrated, and incubated with toluidine blue for 2–3 minutes. Perivascular MCs were counted and analyzed by categorizing vessels into three size classes (20–50-, 50–150-, and >150 - μ m diameter) by means of light microscopy and a computerized morphometric system (Qwin, Leica, Wetzlar, Germany). Granulation and degranulation of MCs were identified as described in the literature.¹⁹

Immunohistochemistry for MC chymase

Immunostaining of MC chymase was performed in 3- μ m-thick sections of paraffin-embedded lung tissues from IPAH and COPD patients and donors. Paraffin-embedded lung tissue sections were deparaffinized in xylol and rehydrated in a graded ethanol series to phosphate-buffered saline (pH 7.4).²⁰ Antigen retrieval was performed by pressure cooking in citrate buffer (pH 6.0).²⁰ Following blocking with bovine serum albumin (10%) for 1 hour and then with blocking serum (PostBlock, ZytoMed) for 5 minutes, the sections were incubated overnight at 4°C with primary antibody. Mouse monoclonal antibody against human MC chymase (CC1, Abcam) was used as the primary antibody. Development of the dye was carried out with alkaline phosphatase and substrate according to manufacturer's (Chromogen) instructions. Finally, sections were counterstained with hematoxylin and coverslipped using mounting medium. The total number of chymase-positive MCs in each section was counted by using a light microscope. In addition, the perivascular MC count was analyzed by categorizing vessels into three size classes (20–50, 50–150, and >150 μ m) with the assistance of a computerized morphometric system (Qwin).

Immunohistochemistry for chymase and big-ET-1

Double immunostaining for chymase (Abcam) and big-ET-1 (Biocompare) was performed in 3- μ m-thick sections of paraffin-embedded lung tissues from IPAH patients. The three-dimensional effect of the photomicrographs was achieved by using special prisms for the microscope (Interferenz-Kontrast, Kondensorprisma, Objectivprisma, Leica).

Data analysis

All results are shown as mean + SEM. The patient groups (IPAH and COPD) were compared with donors by using the *T* test for statistical analysis. A value of $P < 0.05$ was considered statistically significant. The Spearman and Pearson tests were used for correlation analysis.

RESULTS

Prevalence of chymase-positive MCs in the lungs of IPAH patients

Immunohistochemistry revealed that chymase-positive MCs were scattered throughout the lung tissues, including perivascular areas, in donors and IPAH patients (Fig. 1a). There was a significant increase in chymase-positive MCs in the lungs of IPAH patients, as compared with those of donors (Fig. 1b). Moreover, we investigated the perivascular chymase-positive MCs and found that

they were significantly increased in IPAH patients, as compared with donors in all vessel sizes analyzed (Fig. 1c).

Correlation between total/perivascular and total/perivascular chymase-positive MC counts in patients with IPAH

We further analyzed data to evaluate whether there was a correlation between total and perivascular MC numbers and total and perivascular chymase-positive MC numbers. As expected, there was a significant positive correlation in both cases (Fig. 2).

Prevalence of MCs and chymase-positive MCs in the lungs of COPD patients

MCs were scattered throughout the lung tissue, including perivascular areas, in donors and COPD patients (Fig. 3a). There was a significant augmentation of MCs

Figure 1. Prevalence of chymase-positive mast cells in patients with idiopathic pulmonary arterial hypertension (IPAH). Immunostaining for mast cell (MC) chymase was performed as described in "Methods." *a*, Representative photomicrographs from a donor (A) and an IPAH patient (B). Arrows indicate chymase-positive MCs; scale bars = 20 µm. *b*, Total chymase-positive MC number. *c*, Perivascular chymase-positive MC count. Bars represent mean + SEM ($n = 9$); asterisk indicates $P < 0.05$ (*T* test). A color version of this figure is available online.

Figure 2. Correlations in patients with idiopathic pulmonary arterial hypertension. *a*, Correlation between total mast cell and chymase-positive mast cell counts (Pearson $r = 0.80$, $P < 0.01$ [double asterisks]; Spearman $r = 0.70$, $P < 0.05$ [asterisk]). *b*, Correlation between perivascular mast cell and chymase-positive mast cell numbers (Pearson $r = 0.98$; Spearman $r = 0.92$; $P < 0.001$ [triple asterisks] for both tests).

in the lungs of COPD patients, compared to those of donors (Fig. 3*a*). Furthermore, the analysis of MC activation revealed that the index of granulation was significantly decreased in COPD patients, relative to that in donors, suggesting that more MCs are activated in disease condition (Fig. 3*b*). MC chymase was localized by immunohistochemistry. Chymase-positive MCs were distributed throughout the lung tissue, including perivascular areas, in donors and COPD patients (Fig. 3*c*). The chymase-positive MCs showed a strong tendency toward increased counts in the lungs of COPD patients, as compared with those of donors (Fig. 3*c*).

Prevalence of perivascular MCs and chymase-positive MCs in COPD patients

We then investigated the perivascular MCs and found that those associated with resistant vessels (20–150- μm diameter) were significantly increased in the lungs of COPD patients, as compared with those of donors (Fig. 4*a*). Furthermore, the perivascular chymase-positive MC count was determined, and the count was significantly augmented in COPD patients, as compared with that in donors, in all vessel sizes analyzed (Fig. 4*b*).

Correlation between total/perivascular and total/perivascular chymase-positive MC counts in patients with COPD

We further analyzed data to evaluate whether there was a correlation between total and perivascular MC numbers

and total and perivascular chymase-positive MC numbers. As expected, there was a significant positive correlation in both cases (Fig. 5).

Correlation between MC and chymase-positive MC counts and lung function in patients with COPD

We analyzed the data to determine whether there was a correlation between total MCs or chymase-positive MCs and the lung function (assessed by FEV1/FVC) of COPD patients. Surprisingly, there was a significant positive correlation between total MC/chymase-positive MC counts and lung function (Fig. 6).

Chymase-expressing MCs and big-ET-1 signal in the pulmonary vessels of IPAH patients

Double immunostaining revealed that MCs that produced chymase were located in the pulmonary vasculature with prominent expression of big-ET-1 (Fig. 7).

DISCUSSION

The major findings of this study are (1) total and perivascular chymase-positive MC counts were significantly higher in IPAH patients; (2) the total MC number was significantly augmented and chymase-positive MCs showed a strong tendency to increase in COPD patients; (3) a significant majority of the MCs were degranulated (activated) in COPD patients; (4) perivascular MCs and chymase-positive MCs were noticeably higher in COPD patients; and (5) MC and chymase-positive MC numbers were pos-

Figure 3. Mast cell and chymase-positive mast cell populations in patients with chronic obstructive pulmonary disease (COPD). *a*, Lung tissue sections from a donor (A) and a COPD patient (B) stained with toluidine blue as described in “Methods.” Arrows indicate mast cells (MCs). *b*, The total MC number is shown on the left. MCs were classified as granulated or degranulated, and the index of granulation (%) is shown on the right. *c*, Representative photomicrographs from a donor (A) and COPD patients (B, C). Arrows indicate chymase-positive MCs. Immunostaining for MC chymase was performed as described in “Methods.” The bar graph shows the total chymase-positive MC number. In all graphs, bars represent mean + SEM ($n = 9$); an asterisk indicates $P < 0.05$ (T test). Scale bars = 20 μ m. A color version of this figure is available online.

itively correlated with better lung function in COPD patients.

MCs are multifunctional, tissue-resident cells that, following activation, are capable of releasing a plethora of different mediators such as histamine, leukotrienes, prostanooids, cytokines/chemokines, and proteases (tryptase and chymase).^{21,22} Several studies have suggested that activated MCs, by releasing their mediators, may significantly influence inflammation and tissue remodeling.^{6,7,23} Moreover, a growing body of literature hints at a potential role for MC chymase in chronic and devastating lung diseases such as IPAH and COPD.^{12,13}

Increased MC population in the lungs of patients with pulmonary hypertension and vasculopathy was reported by early studies.^{11,12,24} In agreement with the literature, we previously found a significant increase of MCs in the lungs of IPAH patients.¹⁹ In the current study, we used other tissue samples and confirmed our previous findings that the MC number and activation (degranulation) were augmented in IPAH patients, compared to those in donors

(data not shown).¹⁹ Among the mediators, several studies implicated the serine protease chymase in angiotensin II synthesis, TGF- β activation, promotion of ET-1 synthesis, and generation of biologically active interleukin-18 (IL-18) from pro-IL-18.^{8,9,25-28} The findings thus suggested chymase as a potential candidate involved in the pulmonary vascular remodeling process. In this study, we therefore determined the total number of chymase-positive MCs and found that it was significantly increased in the lungs of IPAH patients. Furthermore, we determined the perivascular chymase-positive MC count, which was significantly augmented in IPAH patients, as compared with that in donors. Importantly, there was a positive correlation between the total/perivascular MC number and the total/perivascular chymase-positive MC count in IPAH patients. Surprisingly, there was no correlation between the MC/chymase-positive MC number and mean pulmonary artery pressure in patients with IPAH (data not shown). Finally, chymase-producing MCs were located in close proximity to regions with prominent expression of

Figure 4. Perivascular mast cell (a) and chymase-positive mast cell (b) counts for vessels of different sizes in donors and patients with chronic obstructive pulmonary disease (COPD). Bars represent mean + SEM ($n = 9$); an asterisk indicates $P < 0.05$ (T test).

big-ET-1 in the pulmonary vessels of IPAH patients, suggesting a potential role of chymase in the promotion of ET-1 synthesis by converting big-ET-1 into ET-1 (1–31), which could be further processed into biologically active ET-1.^{28–30} Our findings are in agreement with the literature and substantiate the potential role of MC chymase in the pathogenesis of pulmonary vascular remodeling.

It was previously shown that the number of epithelial MCs was increased in the bronchioli of smokers with airflow limitation, suggesting a role for MCs in the development of COPD.¹³ In the current study, we found that the MC count was significantly increased in the lungs of COPD patients. As the structural alterations include

not only emphysema but also pulmonary vascular remodeling,³¹ we further examined the perivascular MCs in COPD patients. Interestingly, there was an accumulation of perivascular MCs, as evident from the significantly increased MC count in resistant vessels of COPD patients' lungs relative to that in donors; moreover, a significant majority of the MCs were activated (degranulated). The findings suggest that the mediators released from the activated MCs might be involved in pulmonary vascular remodeling in COPD patients. Among the MC mediators, we focused on chymase. Chymase is known to be involved in synthesis of angiotensin II and ET-1 and activation of IL-18 and MMPs.^{8–10,25–28} It is noteworthy that

Figure 5. Correlations in patients with chronic obstructive pulmonary disease. a, Correlation between total mast cell and chymase-positive mast cell counts (Pearson $r = 0.94$; Spearman $r = 0.95$). b, Correlation between perivascular mast cell and chymase-positive mast cell numbers (Pearson $r = 0.96$; Spearman $r = 0.94$). Triple asterisks indicate $P < 0.001$ for both tests.

Figure 6. Correlations with lung function in patients with chronic obstructive pulmonary disease. *a*, Correlation between FEV1/FVC (ratio of forced expiratory volume in 1 second to forced vital capacity) and total mast cell count (Pearson $r = 0.86$, $P < 0.01$ [double asterisks]; Spearman $r = 0.83$, $P < 0.05$ [asterisk]). *b*, Correlation between FEV1/FVC and chymase-positive mast cell count (Pearson $r = 0.95$, $P < 0.001$ [triple asterisks]; Spearman $r = 0.91$, $P < 0.01$ [double asterisks]).

overproduction of the cytokine IL-18 and dysregulation of the MMP system were implicated in COPD pathogenesis and that enhanced angiotensin II levels were found in the lungs of smoke-exposed hamsters.^{14,32-34} We then investigated MC chymase and found that the number of chymase-positive MCs showed a strong tendency to increase in the lungs of COPD patients. Surprisingly, we found that higher MC and chymase-positive MC counts were correlated with better lung function in COPD patients. Our findings were corroborated by Gosman et al.,¹⁵

who reported that increased MC chymase in the peripheral airways correlated with better lung function in COPD patients. In this context, it is tempting to speculate that this reflects a protective association of MCs/chymase with COPD pathogenesis or that MCs might have compartment-specific functions in the lungs of COPD patients. We then determined the number of perivascular chymase-positive MCs and found that they were significantly accumulated in pulmonary vessels of COPD patients' lungs. Importantly, there was a positive correlation between the total/per-

Figure 7. Chymase-expressing mast cells and big-endothelin-1 signal in the pulmonary vessels of patients with idiopathic pulmonary arterial hypertension (IPAH). Double immunostaining for mast cell (MC) chymase and big-endothelin-1 was performed as described in "Methods." Representative photomicrographs from IPAH patients are shown. Black arrows indicate chymase-positive MCs (large, dark cells [colored red online]) and gray (red online) arrows indicate big-endothelin-1-stained structures (greenish color online). Scale bars = 20 μm. A color version of this figure is available online.

vascular MC number and the total/perivascular chymase-positive MC count in COPD patients. As a majority of the MCs were activated and were positive for chymase, MCs may be involved in vascular pathology of COPD patients. Taken together, our data suggest a compartment-specific role for MCs/chymase and their contribution to pulmonary vascular remodeling and thus that they might be detrimental to pulmonary vasculature but beneficial to airways, as evident from the positive correlation of lung MCs/chymase with better lung function in COPD patients. Nevertheless, the precise role of MCs/chymase in COPD pathogenesis is indeed enigmatic, and the focus of future research should be on elucidating their differential involvements in airways versus pulmonary vascular pathologies, by employing selective approaches at the levels of cellular (in vitro) and animal-model (in vivo) studies.

In summary, our findings suggest that activated MCs, by releasing the mediator chymase, might be involved in the pathogenesis of IPAH. Pulmonary MCs/chymase may have compartment-specific (vascular vs. airway) functions in COPD, although the role of MCs/chymase in COPD pathogenesis is still enigmatic. Future studies should focus on elucidating precisely the molecular mechanisms of pulmonary MC accumulation and the role of MC chymase on the pathogenesis of these chronic severe and yet incurable diseases.

ACKNOWLEDGMENTS

We acknowledge Ewa Bieniek for her valuable technical assistance. In addition, we thank Walter Klepetko, from University of Vienna, for providing human lung tissues.

Source of Support: Nil.

Conflict of Interest: None declared.

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