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Bronchial mucosal mast cells in asymptomatic smokers relation to structure, lung function and emphysema

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Summary The pathologic mechanisms of chronic obstructive pulmonary disease (COPD) most certainly involves neutrophil granulocytes, cytotoxic T-cells, macophages and mast cells. The aim of this study was to investigate the relation between the number of mast cells in different compartments in bronchial biopsies of central proximal airways to structural changes, lung function tests and emphysema detected by high resolution computed tomography (HRCT).

Twenty nine asymptomatic smoking and 16 never-smoking men from a population study were recruited. Central bronchial biopsies were stained to identify mast cells by immunohistochemistry. The number of mast cells in the epithelium, lamina propria and smooth muscle as well as epithelial integrity and thickness of the tenascin and laminin layer were determined.

Smokers had increased numbers of mast cells in all compartments ($P<0.001$). Structural changes were correlated to mast cell numbers with the closest associations to mast cell numbers in the smooth muscle [epithelial integrity ($R_s=-0.48$, $P=0.008$), laminin layer ($R_s=0.63$, $P=0.0002$), tenascin layer ($R_s=0.40$, $P=0.03$)]. Similar correlations between mast cells and lung function tests were seen [functional residual capacity (FRC) ($R_s=0.60$, $P=0.0006$), total lung capacity (TLC) ($R_s=0.44$, $P=0.02$) and residual volume (RV) ($R_s=0.41$, $P=0.03$)]. No correlations could be detected between mast cells and FEV_1 or to emphysema.

Smoking is associated with an increase of mast cells in all compartments of the bronchial mucosa, including smooth muscle, and this is related to altered airway structure and function.

Introduction

Chronic obstructive pulmonary disease (COPD) is globally a common cause of morbidity and

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mortality. Progressive airway obstruction and chronic airway inflammation are hallmarks of the disease. However, it is not clear how the mechanisms induced by smoking are involved in the development of COPD.

The inflammatory features of COPD are characterised by neutrophil leucocytes[1,2] in the bronchial lumen. There is a relation between activation of neutrophils and decreased lung function.[3–5] Although some groups have demonstrated the presence of an increased number of neutrophils in the bronchial mucosa in smokers with severe COPD,[6–8] it appears that the inflammatory features in the bronchial mucosa differ from those in the bronchial lumen. Several independent groups have shown a relation between increased numbers of CD3+ cells (T lymphocytes), CD8+ T cells (cytotoxic T lymphocytes) and a reduced FEV_1.[9–12] In smokers with chronic bronchitis, increased numbers of macrophages have been found in the bronchial mucosa and in the bronchial glands.[6,7] Also eosinophils were increased, although not degranulated as in asthma,[13] in the bronchial mucosa and in BAL in smokers with chronic bronchitis compared to those without this.[6,14] The number of mast cells in the bronchial mucosa were increased in smokers with COPD compared to smokers without this.[15–18] Mast cells in the airway wall may thus be important in the development of smoking related changes prior to the development of COPD. Recently, infiltration of bronchial smooth muscle by mast cells has been associated with the variable airflow obstruction and hyperresponsiveness characteristic of asthma.[19] Such a mast cell myositis was not a feature of eosinophilic bronchitis.

We showed recently that neutrophils, eosinophils, macrophages and mast cells are increased in asymptomatic smokers compared to never-smokers.[20] The smokers also had structural changes such as increased tenascin, laminin layers and significantly decreased epithelial integrity. The increased thickness of laminin and tenascin layers were correlated to the number of eosinophils and mast cells, indicating a role for these cells in the remodelling process.

The aim of this study was to further investigate the distribution of mast cells in different compartments in the bronchi of asymptomatic smokers in relation to structural changes and clinical signs. We hypothesised that, in asymptomatic smokers there are increased numbers of activated mast cells in central bronchial biopsies and that these mast cells might be localised to the smooth muscle, as earlier described in asthmatics. Further, we hypothesised that, in smokers these mast cells, mainly localised to the smooth muscle, are related to structural changes, lung function tests and emphysematous changes on high resolution computed tomography (HRCT). Our results were compared with results from a randomly selected never-smoking group recruited from the same population study.

Material and methods

Study population

The subjects were recruited from the population study "Men born 1933 in Göteborg".[21] In a previous paper the sampling procedure has been described in detail.[5] All subjects that had sought medical attention for any airway disease (including asthma), congestive heart failure, angina pectoris or any other severe disease were excluded. Bronchial biopsies were collected from 30 smokers and 18 never-smokers. Fourteen of the smokers were light smokers (<15 cigarettes/day) while the rest ($n = 15$) were heavy smokers (⩾15 cigarettes/day). The mean number of pack-years was 32 ± 14 (median 32, range 9–56). Lung function differed between smokers and never-smokers as well as emphysema score (Table 1).[22]

The study was approved by the local Ethics Committée at Göteborg University, Göteborg Sweden, and informed consent was obtained.

Fibreoptic bronchoscopy and sample collections

Premedication was given with diazepam 5 mg orally followed by 0.5 ml morphine-scopolamine intramuscularly. If the person had a history of kidney or gall bladder disease, pethidine 75 mg intramuscularly and atropine 0.5 mg subcutaneously were given instead of morphine-scopolamine. Additional diazepam (2.5–5 mg) intravenously was given during the bronchoscopic procedure in some cases.

All persons were given terbutaline 0.25 mg/dose 2 inhalations t.i.d. in a nebulizer to avoid unexpected bronchoconstriction during the procedure. Local anaesthesia was given initially with 1% tetracaine-spray in the mouth and laryngeal tract. Additional anaesthesia was applied through the bronchoscope channel for the lower respiratory tract. The bronchoscopy was performed transorally with an Olympus flexible fibreoptic bronchoscope (Tokyo, Japan). The subjects were examined in a supine position by one experienced bronchoscopist. Oxygen saturation was measured with an Ohmeda Pulse Oximeter (Louisville, USA) during the bronchoscopy and supplemental oxygen was given

Table 1 Lung function and emphysematous changes in the subjects.

	Smokers ($n = 29$)	Never-smokers ($n = 16$)	P
TLC	97 ± 14 (71–125)	99 ± 10 (80–115)	n.s.
RV	116 ± 32 (50–173)	98 ± 27 (64–170)	n.s.
FRC	106 ± 20 (74–162)	104 ± 25 (68–171)	n.s.
VC	87 ± 12 (63–113)	96 ± 11 (76–118)	0.021
FEV_1	92 ± 14 (67–134)	109 ± 14 (91–146)	0.0005
DLCO	85 ± 16 (51–135)	96 ± 15 (70–131)	0.015
Emphysematous changes	12	0	<0.0001
Emphysema score	0.74 ± 0.42 (0.17–1.63)	—	

(All values are % predicted, except for emphysematous changes where number of subjects with emphysema are expressed, P is according to Mann–Whitney's nonparametric test, except for emphysematous changes where P is according to Fishers' exact test).

at a rate of 2–3 l/min through a nasal catheter when needed.

All bronchoscopies were made between 8 AM and 10 AM. Bronchial biopsies (3–4 biopsies/subject) were taken with an alligator forceps from the main carina between the right and left main bronchus. The biopsies were gently removed from the forceps immediately placed in a sterile moistened chamber and transported to the laboratory for further processing. Twenty nine of 30 biopsies from the smokers and 16 out of 18 biopsies from the never-smokers were evaluable based on size and morphology.

Immunohistochemistry

The mast cells were identified with monoclonal antibodies on frozen sections. The anti-tryptase antibody 1, AA1 (M 7052, Dako, Glostrup, Denmark), for the identification of mast cells, was used at a final concentration of 0.00315 mg/ml, Monoclonal antibodies to Laminin (M 0638) and Tenascin (M 0636; Dako) were used on frozen sections at concentration of (0.0065 mg/ml and 0.047 mg/ml, respectively). The distinction between lamina propria and smooth muscle was confirmed by immunostaining of smooth muscles with anti-actin antibodies. Monoclonal antibodies to anti-human smooth muscle actin (M 0851; Dako) were used on frozen sections at a concentration of 3.00 mg/ml.

The bronchial biopsy specimens taken from the main carina were frozen immediately in melting propane previously cooled in liquid nitrogen. Frozen biopsies were kept in liquid nitrogen until sectioned. The samples were attached to the specimen holder of a cryostat microtome (Microm, HM 500 M, Heidelberg, Germany) in a drop of OCT compound (Tissue-Tek, Miles, Elkhart, IN, USA) and cut in sections with a thickness of 4 μm. After drying in air at room temperature, the sections were wrapped in aluminium foil and stored at −70°C until they were used for immunohistochemistry.

The sections were thawed and then fixed with undiluted Ortho Permeafix (Ortho Diagnostics, Raritan, USA) for 40 min at room temperature prior to incubation with antibodies to mast cell markers while laminin and tenascin markers fixed with aceton for 10 min at room temperature. In preliminary experiments it was found that fixation and permeabilization with the commercial reagent, Ortho Permeafix, produced comparable results in immunocytochemistry to the paraformaldehyde saponin method, but resulted in improved structural preservation. Sections were incubated with monoclonal antibodies at room temperature in a humid chamber for 1 h and the biopsies were processed as previously described.[23]

Microscopic evaluation of sections

All the specimens were coded and examined by the microscopist without any knowledge of the current smoking habits. Microscopic evaluation of two separate sections from each of two different biopsies was carried out with a Nikon ECLIPSE E800 microscope. The number of cells in the mucous membrane was counted in the whole specimen and the data were converted to an area of 1 mm^2 of tissue. Cell numbers were counted separately in three different compartments, i.e. in the epithelium, in the lamina propria and in the smooth muscle. The areas of the different compartments were outlined and the contained area

was measured using image analysis with a Bit Pad two data tablet (Summagraphics Corp., Seymour, CT, USA) with a Synoptics (Cambridge, UK) Synapse framestore and software package after calibration with the aid of a stage squared micrometer. The variation in cell counts and estimation of structural changes between the two microscopic sections varied between 3% and 5% (% coefficient of variation). Degranulating and non-degranulating mast cells were identified in the specimen. Mast cells were categorised as degranulating mast cells when extracellular deposition of anti-tryptase stained material was observed. Figure 1 shows the number of mast cells in different compartments in both smokers and never-smokers. Figure 2 shows degranulating and non-degranulating mast cells in different compartments in smokers and never-smokers.

The epithelial integrity of the bronchial wall of the subjects from the different groups was assessed by light microscopy in 4 μm thick frozen sections stained with Mayer's hematoxyline. Epithelial integrity was estimated using a × 10 objective and sidearm light microscopic attachment that allowed the image of the section to be projected onto a computer screen. The total length of the basement membrane and the length of intact epithelium in the section through each biopsy were determined.[18] The total length of intact epithelium was expressed as a percentage of the total length of basement membrane present in each biopsy.

The measurement of the thickness of the tenascin and laminin layers (in μm) was performed in 4-μm thick immunolabelled frozen sections using a × 10 objective and a computerised image analysis system after calibration with the aid of a stage micrometer as described above. The measurements were carried out on 100 randomly selected sites per section and the results expressed as the means of these measurements.

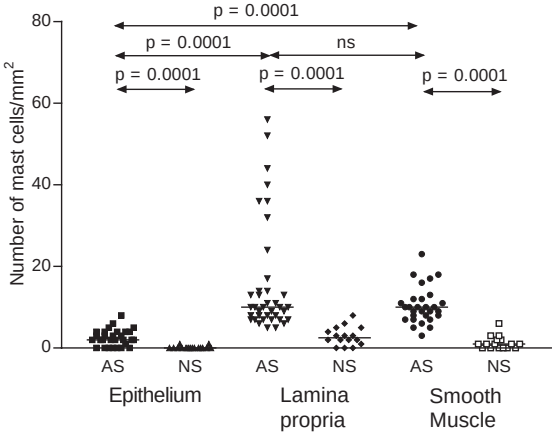

Figure 1 Number of mast cells per mm^2 in different compartments in asymptomatic smokers (AS) and never-smokers (NS) (*P* according to the Mann–Whitney's test).

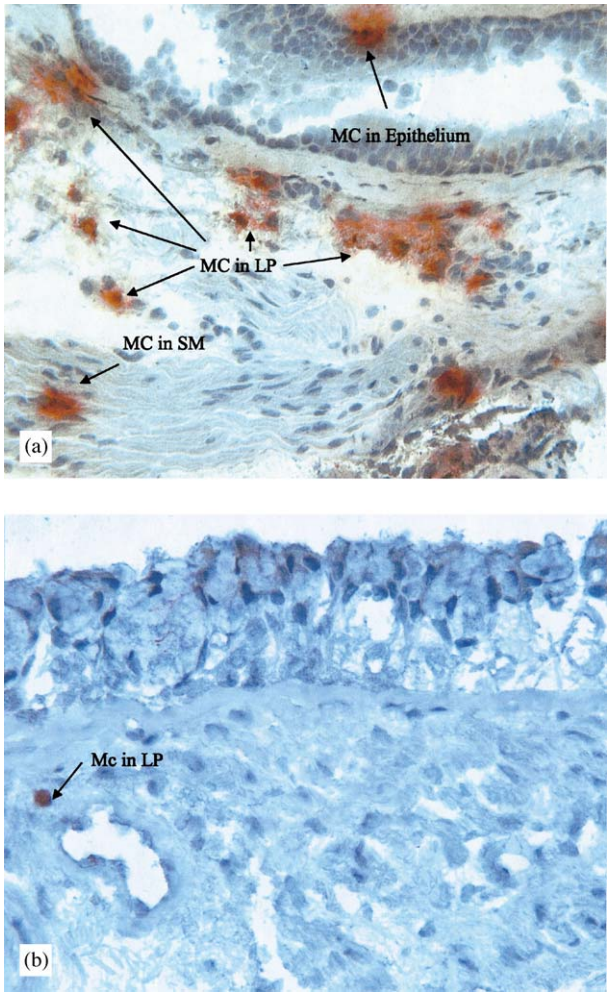

Figure 2 Cryostat sections of bronchial biopsy specimens for AA1 positive cells in (a) asymptomatic smokers and (b) never smokers. Mast cell degranulations were present in the smooth muscle (SM) and lamina propria (LP) of asymptomatic smokers. Few mast cells were found in the tissues of never smokers. Stain: Mayer's hematoxyline. Original magnification: × 340.

Lung function tests

In all subjects' lung volumes, spirometry and diffusion capacity were determined. Lung volumes were obtained by a flow displacement body pletysmograph (Body box 2800 SensorMedics Co, Bilthoven, The Netherlands). Forced expired volume in one second (FEV_1) and vital capacity (VC) were obtained on a water sealed regularly calibrated bell spirometer. Carbon monoxide transfer (DLCO) was assessed by the single breath method with standard equipment (SensorMedics 2200, SensorMedics Co, Bilthoven, The Netherlands). We

used the European Respiratory Society reference values[24] for lung volumes and spirometry and the reference values according to Salorinne[25] for DLCO.

High resolution tomography

The subjects were examined by HRCT on the same day as the investigation of lung function. The entire thorax was scanned in full inspiration with the subject supine. A Picker PQ2000 (Picker International, Cleveland, USA) was used. Slice thickness was 1.5 mm and there was 30 mm between slices. Scan time was 1 s and exposure data were 130 kV and 200 mA. The images were reconstructed with the sharp algorithm of Picker and displayed on $14'' \times 17''$ film with 6 images/film. Window width was set to 1400HU and window level to −400HU. Two experienced thoracic radiologists evaluated the images independently without knowledge of smoking history. Areas of low attenuation and/or sparse and stretched vessels without intervening thick walls were classified as emphysematous lesions. The extent of emphysematous lesion in each lung in each slice was classified on a scale from 0 to 4 (0 = no emphysematous lesions, 1 = 1–25% of lung area, 2 = 26–50%, 3 = 51–75% and 4 ⩾ 75%).[26] The scores for each lung in each slice were added and divided by the number of slices leaving a mean score for each subject. No effort was made to classify the severity of the lesions. The mean value of the radiologists' scores was used for comparison with other investigations. The details of the evaluation have been presented previously.[22]

Questionnaire

The questionnaire was a modified version of the European Community Respiratory Health Survey.[27] It was divided into three principal parts. The first section, symptoms, contained items concerned with the level of symptomatology, including wheezing, dyspnea, cough, sputum production, one non-specific question, "Do you ever have trouble with your breathing?"; if the answer to this last question was yes, the subject was asked to indicate whether this was continuously/repeatedly/only rarely. The subject was also asked if they had asthma or if any doctor had told them to have asthma. The second section contained detailed questions about smoking habits. In the third section, the subjects were asked about other diseases and concomitant medication. The subjects answered the questionnaire before any lung function test, bronchoscopy or HRCT was done. All subjects answered the questionnaire except for one smoker and one never-smoker.

Statistics

All the statistics were calculated using non-parametric tests. Comparisons between asymptomatic smokers and never-smokers were performed with the Mann–Whitney *U*-test. For correlations within a group, Spearman's rank correlation test was used. A *P*-value of <0.05 was regarded as statistically significant. Calculations were performed on a PC using the statistical package Statistica for Windows v. 5.5 ® from Statsoft (USA).

Results

Mast cells in central bronchial biopsies in different mucosal compartments

The number of mast cells were counted in different mucosal compartments in the biopsies. As shown in Fig. 1 we found significantly ($P<0.001$) elevated numbers of mast cells in biopsies from asymptomatic smokers in all three compartments (epithelium, lamina propria and smooth muscle). There was no statistically significant difference between the number of mast cells in lamina propria or smooth muscle. However, both these compartments demonstrated significantly higher numbers of mast cells than in the epithelium ($P<0.001$). The differences between the mucosal compartments were seen for both asymptomatic smokers and never-smokers. Figure 2 shows the immunostaining of mast cells in biopsies from asymptomatic smokers and never-smokers. This figure demonstrates that mast cells in asymptomatic smokers are degranulated to a larger extent than in biopsies of never-smokers. We also found that mast cells in the epithelium of asymptomatic smokers showed less signs of degranulation than the two other mucosal compartments (results not shown).

The numbers of mast cells and degranulated mast cells were similar if smokers were compared with respect to present smoking habits (heavy/light smokers) or due to smoking history (more/less than 35 pack-years). The number of mast cells did not correlate to the number of pack-years.

Relationships between numbers of mast cells in different mucosal compartments and structural changes in the mucosa

As reported before,[20] we found highly significant correlations between the total number of mast cells in central bronchial biopsies and other structural changes in the biopsies of asymptomatic

smokers. In this study, we found significant correlations between mast cells in all three compartments and structural changes (Table 2). This was seen in asymptomatic smokers but not in never-smokers. The strongest correlation was seen between mast cells in the smooth muscle compartment and the thickness of the laminin layer ($R_s = 0.63$, $P < 0.001$). Similar correlations were obtained when the number of degranulated mast cells were counted.

Mast cells in central bronchial biopsies in relation to lung function tests

In smokers, we found significant positive correlations between the total number of mast cells and FRC, RV and TLC (Fig. 3). In never-smokers, we did not find any significant correlation. In smokers, we investigated the relation between the numbers of mast cells in the three different mucosal compartments to lung function tests (Table 3). The strongest positive correlations were seen between mast cells in the smooth muscle compartment and FRC, TLC and RV, respectively. The correlations were of similar magnitude when the number of degranulated mast cells in relation to lung function tests was considered (results not shown). In smokers, we did not find any significant correlation between the number of mast cells and FEV_1.

Mast cells in central bronchial biopsies in relation to emphysema on HRCT

In smokers, we found no significant difference in the total number of mast cells among subjects with and without emphysema (22.8 ± 6.3 vs. 22.5 ± 10.6, $P =$ n.s.). The results were similar when the number

Table 2 Correlations between the number of mast cells in different compartments and structural changes in smokers ($n = 29$).

	Epithelium		Lamina propria		Smooth muscle	
	R_s	*P*-value	R_s	*P*-value	R_s	*P*-value
Tenascin layer (μm)	0.40	0.030	0.36	0.052	0.40	0.031
Laminin layer (μm)	0.35	0.059	0.39	0.037	0.63	0.0002
Epithelial integrity (μm)	−0.43	0.018	−0.46	0.011	−0.48	0.0079

(R_s = Spearman rank correlation coefficient, *P* according to the Spearman rank correlation test).

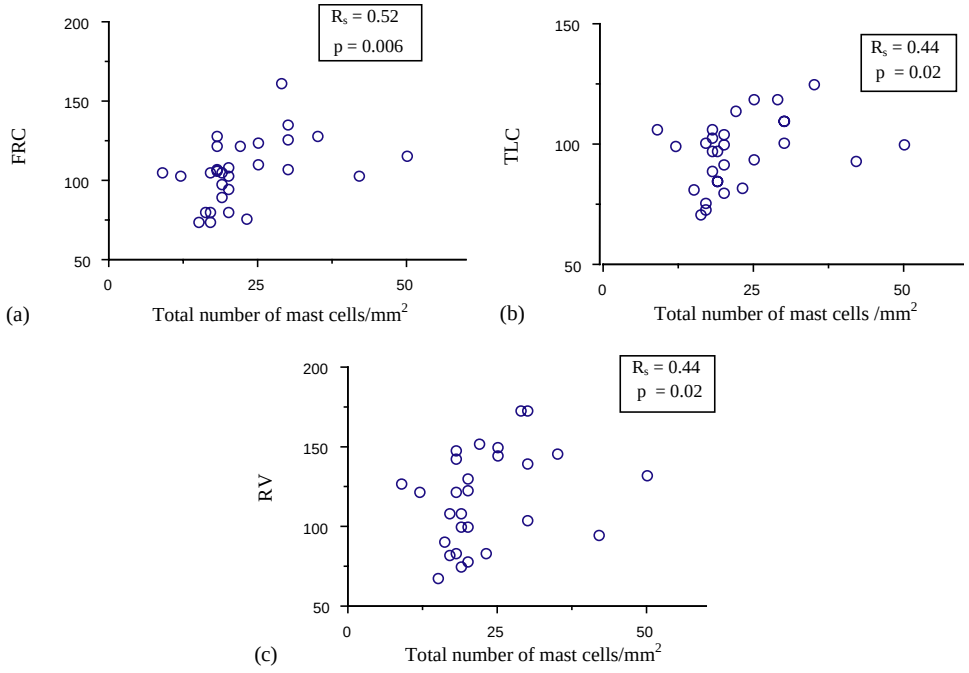

Figure 3 Correlations between (a) functional residual capacity (FRC) (% predicted); (b) total lung capacity (TLC) (% predicted); (c) residual volume (RV) (% predicted), and the total number of mast cells per mm^2 in smokers ($n = 29$) ($R_s =$ Spearman rank correlation coefficient, *P* according to the Spearman rank correlation test).

of mast cells in the three different mucosal compartments were considered (results not shown). The total number of mast cells did not relate to emphysema score.

Mast cells in central bronchial biopsies in relation to respiratory symptoms

Even if these subjects did not spontaneously report respiratory symptoms, some reported respiratory symptoms when approached with a questionnaire.[28] In smokers, we found no significant differences in the total number or in the number of mast cells in the three different mucosal compartments, in those with vs. those without different respiratory symptoms (wheeze, mucus hypersecretion and unspecific respiratory problems).

However, on the basis of the results from other groups[6,17] we also, as an ad hoc study, isolated the group of smokers that reported mucus hypersecretion when approached with the questionnaire ($n = 12$). In subjects with hypersecretion, the correlations between the total number of mast cells and lung function tests (TLC, RV), were significant (Fig. 4), whereas no correlation between mast cells and lung function were discernible in the group without hypersecretion (results not shown).

Discussion

This study aimed at further investigating our findings of mast cell occurrence and structural changes linked to these cells, in central bronchial biopsies in asymptomatic smokers.[20] On the basis of this, the numbers of mast cells in three different mucosal compartments (epithelium, lamina propria and smooth muscle) were studied in relation to structural changes, smoking habits, lung function and emphysema. Our main findings were a close relationship between mast cells in the smooth muscle compartment to structural changes and to lung functions such as FRC, TLC and RV. However, no correlation could be detected between mast cells and FEV_1 or to emphysema on HRCT. Our data suggest a mast cell myositis in smokers.

In this study we evaluated mast cells in various mucosal compartments in the biopsies, i.e. the epithelium, the lamina propria and the smooth muscle. Although we found correlations between numbers of mast cells in most compartments and structural changes, the strongest correlations were seen for the occurrence of mast cells in the smooth muscle compartment and in particular to the thickness of the laminin layer. Our data indicate that mast cell myositis could be important also for structural changes in the airways with "remodelling" indicated by the changes we see in the basal membrane. It must however be realised that our findings do not establish a cause-relationship between mast cells and the development of further tissue changes. The possibility of mast cells occurring as an epiphenomenon to, i.e. an eosinophil increase, is still possible. Interestingly, in asthma a recent report showed a relationship between smooth muscle accumulation of mast cells and bronchial hyperresponsiveness,[19] indicating a central mast cell myositis as important in that disease.

Most studies did not find differences between smokers and never-smokers or in smokers with and without COPD in the occurrence of mast cells, in peripheral bronchial[7–9,15,29] or in operative biopsies.[30] Some studies, however, showed significantly more mast cells in smokers with chronic bronchitis compared to never-smokers in bronchial biopsies[17] and in operative biopsies in smokers with COPD compared to those without COPD.[16] Our data support these last findings, especially as we also

Table 3 Correlations between number of mast cells in different mucosal compartments and lung function test in smokers ($n = 29$).

	Epithelium		Lamina propria		Smooth muscle	
	R_s	*P*-value	R_s	*P*-value	R_s	*P*-value
TLC	0.11	0.56	0.35	0.08	0.44	0.017
RV	0.24	0.21	0.31	0.10	0.41	0.03
FRC	0.12	0.53	0.43	0.02	0.60	0.0006
VC	0.015	0.93	0.29	0.12	0.32	0.09
FEV_1	−0.23	0.24	0.09	0.64	0.11	0.58

(All values are expressed as % of predicted, R_s = Spearman rank correlation coefficient, *P* according to the Spearman rank correlation test).

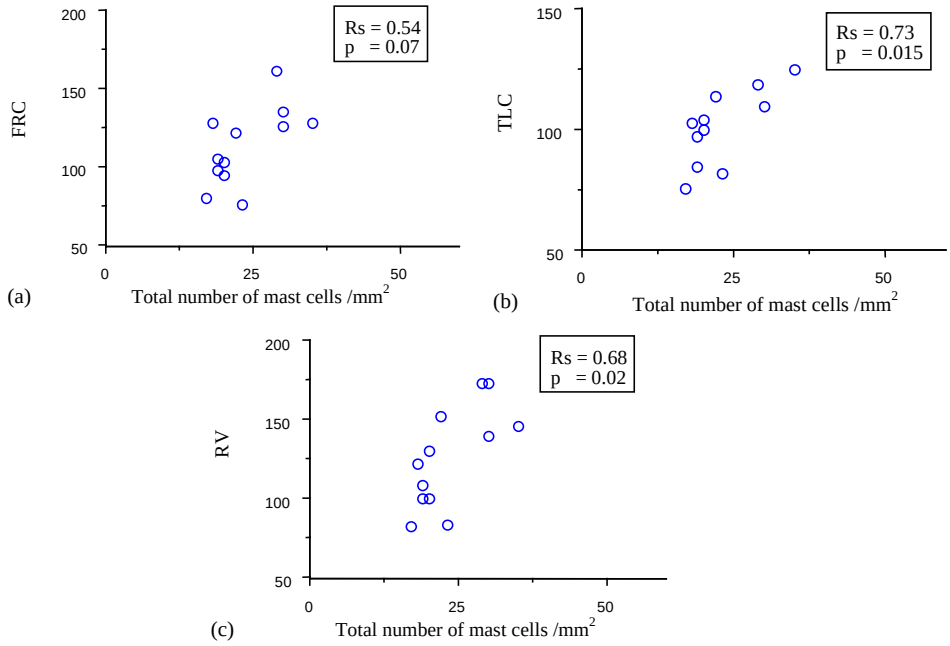

Figure 4 Correlations between (a) functional residual capacity (FRC) (% predicted); (b) total lung capacity (TLC) (% predicted); (c) residual volume (RV) (% predicted), and the total number of mast cells per mm^2 in smokers with mucus hypersecretion ($n = 12$) (R_s = Spearman rank correlation coefficient, P according to the Spearman rank correlation test).

found correlations between mast cells and lung function tests in smokers reporting hypersecretion, when approached with a questionnaire, but not in smokers without this. Further, another link between mast cells and hypersecretion is that tryptase has been found to have a secretagogue effect in the airways.[31] It is therefore possible that the occurrence of mast cells is a factor contributing to phlegm production in smokers.

Our findings of close correlations between mast cell numbers in the smooth muscle compartment of smokers and lung functions such as FRC, TLC and RV are unexpected and interesting. These findings could relate to the structural remodelling changes mentioned above. The increase in FRC could possibly be a finding indicating further development to the more severe increase in FRC seen in fully developed COPD.[32] The lack of relation in our data with emphysematous changes on HRCT may indicate that the increase of FRC occurs earlier in the development of disease than emphysematous changes as visualised by HRCT. It is generally considered that emphysema development in COPD is a release of tissue destructive products from neutrophils and macrophages. Probably this occurs later in the progression of the disease than the mast cell driven changes we have seen structurally in the tissues and physiologically as change of FRC and RV. A hypothesis for further testing could be that mucosal mast cells are involved in a pre-emphysematous stage of disease development. Another possibility is that there are different phenotypes of reactions to smoking. One early reaction could be neutrophil-macrophage dominated pathogenetic events with physiologic deterioration mainly seen as a decrease of diffusion[5] and consequent development of emphysematous changes.[5] Another route of development could be mast cell accumulation and activation and subsegment changes of FRC and RV. It would have been interesting to study any change of bronchial hyperresponsiveness related to a mast cell occurrence,[19] in the current study. However, that was not feasible in the study.

We have earlier demonstrated that several inflammatory cells, neutrophils, eosinophils, mast cells and macrophages, are increased in asymptomatic smokers compared to never-smokers.[20] In the present paper, we have shown that smoking is associated with an increase of the numbers of mast cell, which is correlated to various lung function tests measuring hyperinflation (FRC, TLC, RV). Our study also have demonstrated that these relations were most obvious for mast cells located in the smooth muscle, suggesting the mast cell myositis causally related to both "remodelling" of the bronchi and changes in lung functions. If there is any relation between this mast cell myositis and the development of COPD remains to be further studied.

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