ORIGINAL ARTICLE

Differential distribution of inflammatory cells in large and small airways in smokers

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Background: Smoking induces structural changes in the airways, and is considered a major factor in the development of airflow obstruction in chronic obstructive pulmonary disease. However, differences in inflammatory cell distribution between large airways (LA) and small airways (SA) have not been systematically explored in smokers.

Hypothesis: The content of cells infiltrating the airway wall differs between LA and SA.

Aims: To compare the content of neutrophils, macrophages, lymphocytes and mast cells infiltrating LA and SA in smokers who underwent surgery for lung cancer.

Methods: Lung tissue from 15 smokers was analysed. Inflammatory cells in the lamina propria were identified by immunohistochemical analysis, quantified by digital image analysis and expressed as number of cells per surface area.

Results: The number of neutrophils infiltrating the lamina propria of SA (median 225.3 cells/mm²) was higher than that in the lamina propria of LA (median 60.2 cells/mm²; $p < 0.001$). Similar results were observed for mast cells: 313.3 and 133.7 cells/mm² in the SA and LA, respectively ($p < 0.001$). In contrast, the number of CD4 cells was higher in LA compared with SA (median 217.8 vs 80.5 cells/mm²; $p = 0.042$).

Conclusions: These findings indicate a non-uniform distribution of neutrophils and mast cells throughout the bronchial tree, and suggest that these cells may be involved in the development of smoking-related peripheral lung injury.

Smoking induces an abnormal inflammatory response in the lungs and is the major risk factor for the development of chronic obstructive pulmonary disease (COPD). Several cell types are involved in smoking-induced airway inflammation.¹ In the large airways (LA), higher numbers of macrophages and T lymphocytes are present in asymptomatic smokers compared with non-smokers.^{2–3} In the small airways (SA) of smokers, the number of submucosal neutrophils is related to the number of pack years⁴ and is higher than that in lifelong non-smokers.⁵ Moreover, in smokers, the CD8 cells negatively correlate with the degree of airflow limitation,⁶ and the CD4/CD8 cell ratio shows a shift in favour of CD8 cells compared with non-smokers.⁵ Changes in peripheral lung tissue are thought to be the major contributors to the airflow limitation in smokers who develop COPD.^{2–7}

Bronchial biopsies (ie, LA) have been used in studies on the pathogenesis of the disease⁸ or on evaluation of the effects of new anti-inflammatory drugs.⁹ However, one of the still unresolved questions in patients with smoking-associated lung disease is whether the patterns of inflammation in central and peripheral lungs are similar. This is highly relevant in view of the role of SA in the pathogenesis of COPD.¹⁰ In asthma, there is evidence that immune cells accumulate differently within the distinct lung regions.^{11–12} To our knowledge, there are no studies that directly compare inflammatory cell density in the peripheral airways with that in the central airways in smokers. Such knowledge is certainly important for a better understanding of disease pathogenesis, for a broader interpretation of bronchial biopsy studies and for targeting of deposition of inhaled drugs. Therefore, the aim of this study was to compare the number of neutrophils, macrophages, lymphocytes and mast cells infiltrating the lamina propria of LA with that infiltrating the SA, in tissues from smokers with or without COPD.

METHODS

Additional details for this section are provided in the online supplement (available online at <http://jcp.bmj.com/supplemental>).

Subjects and study design

Consecutive patients were selected and prospectively enrolled in this cross-sectional study. Patients were smokers or ex-smokers scheduled for surgical resection for primary lung cancer. Subjects who underwent surgery for other diseases were excluded. Fifteen smokers were recruited (table 1), including seven current smokers and eight ex-smokers, who had stopped smoking at least 6 months before the lung resection.

The study was approved by the medical ethics committee of the Leiden University Medical Centre (Leiden, The Netherlands), and informed written consent was obtained for each subject.

HISTOLOGY

Tissue sampling and processing

At least two samples of peripheral parenchyma and one or two samples of central airways free of tumour were obtained.

One or two LA and all transversally cut SA, defined by an internal perimeter larger (LA) or smaller (SA) than 6 mm, were selected for each patient.^{6–13} To avoid measurements in tangentially cut airways, airways with a short/long diameter ratio < 0.33 were excluded from the study.¹⁴

Inflammatory cells

Mouse monoclonal antibodies were used for identification of neutrophils, macrophages, CD4 cells, CD8 cells and mast cells.

Abbreviations: COPD, chronic obstructive pulmonary disease; LA, large airways; SA, small airways

Table 1 Patient characteristics (n = 15)	
Age* (years)	61.1 (10.5)
Sex (F/M)	3/12
FEV ₁ %Pred*	79.3 (15.3)
FEV ₁ /FVC%	70.6 (9.3)
Pack years*	40.0 (22.9)
Ex-smokers/current smokers	8/7
Steroid inhaler (yes/no)	4/11
Oral steroid (yes/no)	1/14

F, female; FEV₁, forced expiratory volume in one second; FEV₁% Pred, forced expiratory volume in one second, expressed as a percentage of the predicated value; FVC, forced vital capacity; M, male.
*Data are given as mean (SD)

A polyclonal antibody was used for the identification of total CD3 T lymphocytes.

Morphometric analyses

Cases were coded and measurements were carried out without knowledge of the clinical data. Lamina propria (or inner wall) was defined as the zone between the epithelial basement membrane and the smooth muscle. The cellular infiltrate was quantified in the lamina propria of SA and LA using a fully automated image analysis system.¹⁵ Data were expressed as number of cells per mm².

STATISTICAL ANALYSIS

Statistical analyses were carried out using SPSS software V.13.0. Data were presented as mean (SD) or median (range), depending on whether they were normally distributed. Paired observation differences were analysed using the Wilcoxon rank test. Correlations between variables were expressed using the Spearman correlation coefficient (r_s). Differences at probability values of p<0.05 were considered to be significant.

RESULTS

Table 1 reports the characteristics of the 15 smokers included in the study.

The mean forced expiratory volume in one second, expressed as a percentage of the predicted value (FEV₁%Pred), was 79.3 (15.3)% and the mean amount of cigarettes smoked was 40.0 (22.9) pack years. The ex-smokers had quit an average of 11.2 (7.9) years (mean (SD)) before the study.

One or two LA were obtained per patient. The median internal perimeter of the LA was 22.8 mm (range 9.1–42.3) and the median analysed area of the lamina propria was 0.93 mm² (range 0.42–3.77) per airway. At least one SA suitable for

analysis was obtained from all patients, with a median of four peripheral airways (range 1–11) analysed per patient. The median internal perimeter of the SA was 1.6 mm (range 0.6–5.5), and the median analysed total area of the lamina propria was 0.023 mm² (range 0.004–0.150) per airway.

In the whole group, the number of neutrophils in the lamina propria of SA (median 225.3 cells/mm² (range 42.1–874.9)) was higher than that in LA (median 60.2 cells/mm² (range 9.0–743.3); p<0.001; figs 1 and 2).

Similar results were observed for mast cells: the median number of mast cells in the lamina propria of SA was 313.3 cells/mm² (range 112.9–507.5), whereas a median number of 133.7 cells/mm² was found in the LA (range 43.0–292.0; p<0.001; figs 1 and 3).

In contrast, the CD4 cell content was higher in the lamina propria of LA (median 217.8 cells/mm² (range 0.0–903.4)) compared with SA (median 80.5 cells/mm² (range 6.6–452.9); p = 0.042; fig 1).

Differences between the number of cells infiltrating the lamina propria of SA and of LA were not found for the other analysed cell types, including macrophages, CD3 cells and CD8 cells (fig 1). In both LA and SA, the content of neutrophils, mast cells and CD4 lymphocytes did not correlate with FEV1% Pred, the FEV₁/FVC ratio or the amount smoked, expressed in pack years (lowest p value = 0.059).

In addition to these main results, we performed a subgroup analysis by dividing the sample into current and ex-smokers, and into patients with and without COPD and non-COPD (for details, see online supplement (available online at <http://jcp.bmj.com/supplemental>).

DISCUSSION

Our main conclusion is that the neutrophil and mast cell density in the lamina propria of SA is higher than in the LA of smokers with or without COPD. This suggests that these cells may be involved in the development of smoking-related peripheral lung injury driven by inflammation in the SA. Interestingly, whereas the number of neutrophils and mast cells is higher in the peripheral airways, the number of CD4 cells is higher in the LA.

There are no studies in smokers describing the inflammatory cell distribution throughout the bronchial tree by direct comparison of LA and SA. However, in asthma, a higher density of CD45 cells and eosinophils has been reported in the lamina propria of LA compared with the SA.¹⁶

Neutrophils in the SA could play a relevant role in the pathogenesis of smoking-related diseases. A positive correlation

Figure 1 Inflammatory cell counts in the lamina propria of large airways (LA) and small airways (SA) (n = 15 subjects). Horizontal lines represent median values. AA1+, mast cells; CD68, macrophages; CD3, total T lymphocytes; CD4, CD4-positive T lymphocytes; CD8, CD8-positive T lymphocytes; CD4/CD8, CD4/CD8 cell ratio; NE+, neutrophils.

Figure 2 Neutrophils in small (A) and large (B) airways from the same patient with chronic obstructive pulmonary disease: neutrophil elastase-positive cells are in red; sections are counterstained with haematoxylin. AL, alveolar lumen; G, bronchial glands; L, airway lumen; M, smooth muscle. Arrows: example of positive cells in lamina propria. Original magnification: $\times 200$.

between relative sputum neutrophil count and high-resolution computed-tomography indicators of peripheral airway dysfunction in smokers has been demonstrated.¹⁷ Furthermore, the number of neutrophils infiltrating the smooth muscle of SA in smokers with or without COPD is higher when compared with controls,¹⁸ and higher neutrophil numbers have been reported in the submucosa of SA in smokers compared with lifelong non-smokers.⁵ However, other studies examining the inflammatory cells infiltrating the peripheral airway wall did not show statistically significant differences in neutrophils when patients with COPD were compared with smokers with normal lung function.^{4, 6, 19}

In smokers, the development of COPD is accompanied by increased numbers of mast cells and macrophages in the epithelium, but not in the remainder of the airway wall of the peripheral airways.¹⁹ In the central airways, the analysis of bronchial biopsy specimens demonstrated a higher density of mast cells in the mucosa of asymptomatic smokers compared with never smokers.²⁰ Furthermore, the number of mast cells positively correlated with the thickness of the tenascin and laminin layers, suggesting a role for these cells in tissue remodelling in smokers.²⁰

The mechanisms implicated in the non-uniform distribution through the bronchial tree of inflammatory cells are not fully understood. It is possible that a different inflammatory milieu exists in the airways, with a gradient in cytokine expression. Using an allergen-induced asthma model in rhesus monkeys, Miller *et al*¹² reported increased levels of cytokines and chemokines within the distal lung, whereas CD4 lymphocytes accumulated preferentially in the proximal to midlevel region of the lungs. The authors proposed that the distal lung may be involved in the mobilisation of effector immune cells into the

airways, and that proximal airways may serve as a reservoir for immune cells that contribute to chronic inflammation. Our results on smoking-related inflammation lend further support to this hypothesis.

The finding of a preferential distribution of CD4 cells in proximal airways, observed in the present study and by Miller *et al*,¹² is also supported by studies in rodents. In a study performed in normal rat lungs, effector T lymphocytes accumulated preferentially in the lamina propria of the bronchi and not in the alveolar region.²¹ Similarly, human lymphocytes tended to adhere more to the bronchial endothelium than to the alveolar endothelium, according to preliminary data discussed by Ainslie *et al*.²²

It has been proposed that lymphocytes (ie, CD4 cells) may preferentially reach lung compartments supplied by bronchial vessels, whereas neutrophils and mast cells may migrate preferentially into the postcapillary venules of pulmonary vessels, and, in particular, into the capillaries of the pulmonary system.^{23, 24}

Assessing expression of chemokines and other inflammatory mediators potentially involved in the recruitment of inflammatory cells may help in understanding the underlying mechanisms. However, results from a previous study from our group,²⁵ using tissue from a patient population that was in part comparable to that in the present study, indicate that this may be complex. In that study, the epithelial IL-8 expression in SA did not correlate with numbers of neutrophils and other inflammatory cells.²⁵ This finding is most likely explained by the fact that cellular influx is regulated not only by a single cytokine (such as IL-8), but probably also by a more complex mechanism involving, for example, various chemoattractants and adhesion molecules. Therefore, a study on a large range of

Figure 3 Mast cells in small (A) and large (B) airways from the same patient with chronic obstructive pulmonary disease: AA1-positive cells are in red; sections are counterstained with haematoxylin. AL, alveolar lumen; L, airway lumen; M, smooth muscle. Arrows: example of positive cells in lamina propria. Original magnification: $\times 200$.

Take-home messages

- The distribution of inflammatory cell infiltration in the lamina propria is not uniform throughout the bronchial tree in smokers.
- Neutrophil and mast cell numbers are higher in the small airways (SA) than in the large airways (LA).
- In contrast, the number of CD4 cells is higher in the LA.
- Selective recruitment of inflammatory cells to the SA may contribute to chronic inflammation and remodelling of the SA, resulting in decreased lung function.

mediators and adhesion molecules would be required. However, such an analysis was beyond the scope of the present study.

In the present study, the fully automated cell count was performed using a reproducible and previously published method developed by our group.¹⁵ The fact that the differences between SA and LA were not observed for all cell types studied suggests that these findings are not likely to be the result of a bias introduced by the methods used. Estimating cell numbers by stereological methods (3D, volume) could provide more accurate results, since—as opposed to 2D (area) measurements—the different sizes of cell profiles are taken into account.^{26–27} However, unbiased stereology requires unbiased sampling, which is difficult to achieve in studies using resected lung tissue because selection is based on, for example, diagnostic strategies for lung cancer. To date, the large majority of studies in the smokers/COPD field have used cells/area as outcome, with consistent results.²⁸ The additional benefits of using stereology in inflammatory lung diseases are a subject of ongoing discussion^{26–29} and require further investigation.

A potential weakness of this study is that tissue from an adequate control group of lifelong non-smokers was not available for analysis. Therefore, the possibility that the specific distribution observed for neutrophils and mast cells is not exclusive to smokers cannot be excluded. Indeed, some evidence suggests that normal subjects may have higher mast cell density in membranous airways than in cartilaginous airways.³⁰ Our study is limited to the analysis of the inner layer of the airway, which is also the main constituent of bronchial biopsies, and therefore comparisons between our results and those obtained using bronchial biopsies are valid. Although inflammation certainly occurs in other airway layers, Saeetta *et al*¹⁴ reported that in smokers with COPD there is a larger cell density in the “inner”, submucosal region than in the “outer”, advential layers, and suggested that airflow obstruction in COPD could be related to submucosal cellular density.

In conclusion, our results show a differential influx of neutrophils and mast cells in the lamina propria of SA and LA of smokers, suggesting that the concentration and/or functional activity of inflammatory stimuli that mediate cell recruitment may vary in different compartments of the lung. Therefore, increased cell recruitment in the periphery of the lung may contribute to chronic inflammation and remodelling at this level.

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Professor Vignola died in December 2004.

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