Patterns of Inflammatory Responses in Large and Small Airways in Smokers with and without Chronic Obstructive Pulmonary Disease

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For editorial comment see p. 359

Key Words

Chronic obstructive pulmonary disease • Large airways • Small airways • Inflammation

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is characterised by progressive and irreversible airway obstruction. Smoking causes persistent inflammation in lung tissue. However, differences in inflammatory responses between the large and small airways have not been systematically explored among smokers with and without COPD.

Objectives: The aim of our research was to characterise the expression and localisation of NF- κ Bp65 and histone deacetylase 2 (HDAC2) as well as inflammatory cell (macrophages, lymphocytes, neutrophils) distribution in large and small airways, in nonsmokers and in smokers with and without COPD.

Methods: Nineteen nonsmokers, 20 smokers with normal lung ventilation function and 20 smokers with moderate COPD, undergoing lung resection for a solitary peripheral carcinoma, were enrolled in the study. Immunohistochemical methods were used to evaluate NF- κ Bp65 and HDAC2 expression and identify inflammatory cells in airways. **Re-**

sults: COPD patients had increased NF- κ Bp65 expression compared to nonsmokers and smokers without COPD, in both large and small airways, which corresponded to increased numbers of macrophages, CD8+ T lymphocytes and neutrophils. COPD patients had more macrophages in large compared to small airways and more CD8+ T lymphocytes and neutrophils in small compared to large airways. HDAC2 expression was significantly downregulated in smokers with COPD in small compared to large airways. **Conclusions:** Our findings indicate a nonuniform distribution of inflammatory cells throughout the bronchial tree. However, in both smokers with and without COPD, similar patterns of inflammatory processes occur in both large and small airways. The difference between smokers with and without COPD is only quantitative.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by slowly progressive and irreversible airway obstruction. Cigarette smoking is implicated as a

major risk factor for the development of COPD because approximately 90% of COPD patients are smokers [1]. However, only a minority of smokers develop COPD. The basic reason for this phenomenon is still poorly understood. The pathological hallmarks of COPD are inflammation of the large and small airways and destruction of lung parenchyma, resulting in the development of pulmonary emphysema [2]. Bronchial biopsies have demonstrated infiltration by mononuclear cells, particularly CD8+ T lymphocytes, rather than neutrophils. Similar changes are found in lung parenchyma, with a predominance of macrophages and CD8+ T cells at sites of parenchymal destruction [3–6]. Additionally, in the small airways of COPD patients, increased numbers of CD8+ and CD4+ T lymphocytes and neutrophils compared to asymptomatic smokers and nonsmokers have been demonstrated [7].

It has been increasingly recognised that oxidative stress is the underlying cause of COPD [8]. Cigarette smoke contains many oxidants and free radicals, which cause sequestration and accumulation of neutrophils in the pulmonary microcirculation and accumulation of macrophages in respiratory bronchioles, with the potential release of oxidants [8, 9]. Acute exposure to cigarette smoke is associated with nuclear factor (NF)- κ B activation and interleukin (IL)-8 synthesis in alveolar macrophages [9].

NF- κ B is a redox-sensitive transcription factor that promotes the transcription of various cytokines that characteristically promote COPD, such as IL-1, IL-6, IL-8, tumour necrosis factor- α , endothelin-1, intercellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1 and inducible nitric oxide synthase [10–12].

The activated form of NF- κ B is a heterodimer composed of p50 and p65 subunits, the latter being the most frequent component in humans. Upon activation, NF- κ B translocates to the nucleus and binds to DNA. NF- κ B regulates the expression of many genes involved in inflammation [13]. NF- κ Bp65 expression is increased in the large airways of smokers with and without COPD [10].

Covalent modifications of histones, in particular deacetylation of lysine residues, play important roles in the regulation of NF- κ B activity [14]. These modifications, which regulate the accessibility of transcriptional regulatory proteins to chromatin, are controlled by histone deacetylases (HDACs). In mammals, the family of HDAC enzymes consists of 17 isoforms grouped into 3 families [15]. Several HDAC enzymes have been identified in humans and are associated with repression of gene tran-

scription. Decreased HDAC2 levels are associated with increased tumour necrosis factor- α and IL-8 levels [15].

Moreover, inhibition of HDAC2 is associated with increased inflammation in smokers in both alveolar macrophages and leucocytes [16, 17]. Cigarette smoke induces proinflammatory cytokine release by activating NF- κ B and posttranslational modifications of HDAC in macrophages [16]. Furthermore, oxidative stress and the redox status of the cells can also regulate nuclear histone modifications, such as acetylation, methylation and phosphorylation, leading to chromatin remodelling, in addition to recruitment of basal transcription factors and RNA polymerase II, leading to the induction of proinflammatory mediators [15].

However, one unresolved question in patients with COPD is whether the patterns of inflammatory responses in the large and small airways are similar. Such knowledge is important for a better understanding of the pathogenesis of COPD. To our knowledge, there is only one study that directly compared inflammatory cell infiltration in large and small airways in COPD [7]. The number of neutrophils infiltrating the lamina propria of small airways is higher than that in the lamina propria of large airways, but the number of CD4+ T lymphocytes is higher in large compared to small airways [7]. Furthermore, no studies have directly compared NF- κ Bp65 and HDAC2 expression in large and small airways in smokers with and without COPD.

Therefore, we attempted to compare NF- κ Bp65 and HDAC2 expression and inflammatory cell distribution (CD8+ T lymphocytes, macrophages and neutrophils) in large and small airways in nonsmokers, smokers with normal function of lung ventilation and patients with COPD.

Subjects and Methods

Study Design

Fifty-nine individuals undergoing lung resection for a solitary peripheral non-small cell carcinoma were enrolled in the study. There were 3 groups; 19 individuals were nonsmokers with normal lung ventilation function, 20 subjects were current smokers with normal lung function and 20 were current smokers who had moderate COPD. The diagnosis of COPD was established according to the definition of the Global Initiative for Chronic Obstructive Lung Disease guidelines [18]. COPD patients had forced expiratory volume in the first second (FEV₁)/forced vital capacity (FVC) <70% and 50% < FEV₁ < 80% of predicted values, based on demographic characteristics.

None of the subjects had suffered a recent exacerbation, defined as increased dyspnoea associated with a change in the qual-

Table 1. Subject characteristics

Study group	Age, years	Smoking history, pack-years	FEV ₁ % predicted	FEV ₁ /FVC%	Male/female ratio	Height, cm	Weight, kg
Nonsmokers (n = 19)	64 ± 10	–	93 ± 21	79 ± 7	11:8	170 ± 9	81 ± 14
Asymptomatic smokers (n = 20)	62 ± 8	27 ± 14	90 ± 17	74 ± 5	18:2	173 ± 6	74 ± 12
COPD patients (n = 20)	64 ± 6	30 ± 15	54 ± 8*	60 ± 7*	16:4	176 ± 5	78 ± 15

Data are presented as means ± SD. * $p < 0.05$ compared with control nonsmokers and asymptomatic smokers.

ity and quantity of sputum that would have led them to seek medical attention during the month preceding the study. All subjects were free of acute upper respiratory tract infections, and none had received glucocorticoids, theophylline or antibiotics within the preceding month. All subjects were nonatopic; they had negative skin tests for common allergen extracts and had no history of asthma or allergic rhinitis.

The study was approved by a local ethical committee, and it conformed to the Declaration of Helsinki; informed consent was obtained from all subjects. The clinical characteristics of the groups are presented in table 1.

Methods

The lung tissue specimens for small airway evaluation were taken from the sub-pleural parenchyma, but for the large airways, bronchial rings were taken from the segmental bronchus of the lobe obtained at surgery, as far as possible from the tumour site. Samples were fixed without inflation in 10% neutral buffered formalin, processed and embedded according to standard procedures.

Pulmonary Function Tests

Pulmonary function testing was performed on a Jaeger MasterScreen spirometer (Jaeger GmbH, Germany) in the week before surgery [18].

Immunohistochemistry

The lung tissue specimens were taken from the sub-pleural parenchyma of the lobe or the whole lung obtained at surgery, as far as possible from the tumour site. Bronchial rings were taken from the lobar bronchus. Samples were fixed without inflation in 10% neutral buffered formalin, processed and embedded according to routine procedures. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue was cut into 4-µm-thick sections. Antigen retrieval was achieved by treatment in a domestic microwave oven for 30 min in citrate buffer (pH 6.0). Sections were incubated in 0.5% H₂O₂/PBS to quench endogenous peroxidase activity and then blocked with protein block (Dako, Denmark).

The slides were then incubated overnight at 4°C with a primary antibody against NF-κBp65 (rabbit polyclonal; Abcam, UK; dilution 1:200) or HDAC2 (rabbit polyclonal; Abcam; prediluted) or for 1 h at room temperature with antibody against CD68

(mouse monoclonal; Dako; dilution 1:100), CD8 (mouse monoclonal; dilution 1:50) or neutrophil elastase (mouse monoclonal; Dako; 1:200). Polyclonal goat anti-rabbit and subsequently rabbit polyclonal anti-goat antibodies were used for visualisation of binding with the NF-κBp65 and HDAC2 antibodies, but an LSAB+ kit (Dako) was used for visualisation of CD68+ cells, CD8+ cells and neutrophils. 3,3'-Diaminobenzidine tetrahydrochloride was applied as chromogen (7 min). Sections were counterstained in haematoxylin (2 min). As a positive control, human palatine tonsil tissue was used. Negative controls were performed by omitting the primary antibody. The results are expressed as cells per square millimetre. NF-κBp65 and HDAC2 immunostaining intensity in 10 high-power fields at ×400 magnification was graded in a blinded fashion by two pathologists (S.I. and D.S.) from 0 to 3 (0 = no staining; 1 = weak; 2 = moderate; 3 = strong). Analysis of airways was performed using a light microscope connected to a video recorder linked to a computerised image system (Motic Image Advanced 3.2; Motic, Xiamen, China). The cases were coded and the measurements made in a blinded fashion by two pathologists (S.I. and D.S.) without knowledge of clinical data for a given patient specimen.

Cell Counts in Large Airways

Bronchial rings were taken from the segmental bronchus of the lobe obtained at surgery, away from the tumour site. At least 2 intact airways with a diameter of more than 2 mm were identified for each patient. Large airways were defined as cartilaginous bronchi with submucosal glands. The number of NF-κBp65-positive and HDAC2-positive cells and CD8+ T lymphocytes, macrophages and neutrophils in large airways was counted by a blinded investigator in the epithelium and subepithelium of each section, excluding areas of smooth muscle and mucous-secreting glands when such were identified. The relevant area was outlined on a captured image, and the contained area was measured using image analysis software. All cell counts are expressed as cells per square millimetre.

Cell Counts in Small Airways

Small airways were considered membranous bronchioles without cartilage or glands and with an internal diameter less than 2 mm, as previously described [2]. At least 4 intact airways with a diameter of less than 2 mm were identified for each patient. In each airway, we quantified the number of NF-κBp65-positive

and HDAC2-positive cells and CD8+ T lymphocytes, macrophages and neutrophils in the epithelium, submucosa and adventitia. The submucosa was defined as the area that extends from the distal edge of the basement membrane to the internal edge of the smooth muscle, whereas the adventitia was defined as the area that extends from the outer edge of the smooth muscle to the alveolar attachments. All cell counts are expressed as cells per square millimetre. All cell counts were performed by two pathologists (S.I. and D.S.).

Statistical Analysis

Group data are expressed as means $\pm$ SD for functional data or medians (range) for morphological data. Differences between groups were analysed using analysis of variance (ANOVA) for functional data or the Kruskal-Wallis test for morphological data. When the differences were significant, ANOVA was followed by an unpaired t test and the Kruskal-Wallis test was followed by the Mann-Whitney U test for comparison between groups. Correlation coefficients were calculated using Spearman's rank method. A p value of <0.05 was considered statistically significant. Data analysis was performed using the GraphPad Prism version 5 software.

At least 3 replicate measurements of NF- κ Bp65-positive and HDAC2-positive cells as well as CD8+ T lymphocytes, macrophages and neutrophils were performed by the same observer in 10 randomly selected slides, and the intraobserver reproducibility was assessed with the coefficient of variation and with the interclass correlation coefficient. The intraobserver coefficient of variation was 4–12%, and the intraobserver correlation coefficient was 0.82–0.88.

Results

Clinical Findings

The clinical characteristics and lung function data of the patients are presented in table 1. As expected from the selection criteria, the values of FEV₁% predicted and FEV₁%/FVC were significantly different in patients with COPD compared to both nonsmokers and smokers without COPD. All patients, except for the nonsmoker group, were current smokers. No significant differences in age, weight or height were found among the subjects. Furthermore, no differences in pack-years of smoking history were found between asymptomatic smokers and COPD patients (27 ± 14 vs. 30 ± 15 pack-years; $p > 0.05$). The nonsmoker group contained an approximately equal number of males and females, but asymptomatic smokers and COPD patients were predominantly males.

NF- κ Bp65 Expression in Large Airways

Figure 1 shows the number of NF- κ Bp65-positive cells in large airways. NF- κ Bp65 was predominantly expressed in airway subepithelium and epithelium (fig. 2a–c). The number of NF- κ Bp65 immunoreactive cells in large airways in COPD patients and smokers with normal lung

Fig. 1. NF- κ Bp65-positive cells in large and small airways of non-smokers (NS), smokers with normal lung function (S) and COPD patients (COPD). ● = Cells in large airways; ○ = cells in small airways. Each circle represents an individual patient. Horizontal bars represent medians. * $p < 0.05$: control nonsmokers compared with asymptomatic smokers, and control nonsmokers and COPD patients and asymptomatic smokers compared with COPD patients. Statistical analysis was performed by the Mann-Whitney U test for comparison between groups. Nonsmokers: $n = 19$; healthy smokers: $n = 20$; COPD patients: $n = 20$.

function was significantly higher compared to nonsmokers (fig. 1; $p < 0.0001$). COPD patients had a predominantly nuclear p65 localisation ($51 \pm 22\%$) compared to nonsmokers ($19 \pm 11\%$; $p < 0.0001$) and asymptomatic smokers ($32 \pm 19\%$; $p = 0.02$). NF- κ Bp65 was predominantly expressed in macrophages, but increased NF- κ Bp65 expression in lymphocytes in COPD patients (22% of all cells, range 6–65%) and asymptomatic smokers (16%, range 3–32%) compared to nonsmokers (7%, range 2–24%; $p < 0.001$) was observed.

Epithelial cells of COPD patients (score 2.58 ± 0.60) and asymptomatic smokers (2.21 ± 0.53) had more intensive nuclear and cytoplasmic staining for NF- κ B compared to nonsmokers (0.94 ± 0.62 ; $p < 0.0001$).

When all patients were analysed together, a negative correlation between airflow obstruction (FEV₁%) and NF- κ Bp65 expression was observed ($r = -0.51$, $p = 0.0006$). Additionally, there was a positive correlation between pack-years of smoking and NF- κ Bp65 expression ($r = 0.62$, $p = 0.001$).

NF- κ Bp65 Expression in Small Airways

Figure 1 shows the number of NF- κ Bp65-positive cells in small airways. NF- κ Bp65 was predominantly expressed in airway subepithelium and epithelium. The number of NF- κ Bp65-immunoreactive cells in small air-

Fig. 2. **a–c** NF-κBp65-positive cells stained immunohistochemically with NF-κBp65 antibody in brown (chromogen 3,3'-diaminobenzidine tetrahydrochloride) in nonsmokers (**a**), asymptomatic smokers (**b**) and COPD patients (**c**) in large airways. Magnification $\times 200$. Arrows in **a** and **b** indicate positively stained cells, and the arrow in **c** indicates strong epithelial staining in COPD patients. **d–f** HDAC2-positive cells stained immunohistochemically with HDAC2 antibody in brown (chromogen 3,3'-diaminobenzidine tetrahydrochloride) in nonsmokers (**d**), asymptomatic smokers (**e**) and COPD patients (**f**) in large airways. Magnification $\times 200$.

Fig. 3. HDAC2-positive cells in large and small airways of non-smokers (NS), smokers with normal lung function (S) and COPD patients (COPD). ● = Cells in large airways; ○ = cells in small airways. Each circle represents an individual patient. Horizontal bars represent medians. * $p < 0.05$: control nonsmokers compared with asymptomatic smokers, nonsmokers and COPD patients. Statistical analysis was performed by the Mann-Whitney U test for comparison between groups. Nonsmokers: $n = 19$; healthy smokers: $n = 20$; COPD patients: $n = 20$.

ways in COPD patients and smokers with normal lung function was significantly higher compared to nonsmokers (fig. 1; $p < 0.0001$).

NF- κ Bp65 was predominantly expressed in macrophages, but increased NF- κ Bp65 expression in lymphocytes in COPD patients (22% of all cells, range 2–56%) and asymptomatic smokers (17%, range 3–32%) compared to nonsmokers (9%, range 0–46%; $p < 0.001$) was observed.

Epithelial cells of COPD patients (2.27 ± 0.64) and asymptomatic smokers (1.95 ± 0.70) had more intensive nuclear and cytoplasmic staining for NF- κ B compared to nonsmokers (1.16 ± 0.83 ; $p < 0.0001$).

When all patients were analysed together, a negative correlation between airflow obstruction ($FEV_1\%$) and NF- κ Bp65 expression was revealed ($r = -0.32$, $p = 0.03$), just as in large airways. COPD patients had a predominantly nuclear p65 localisation ($68 \pm 21\%$) compared to nonsmokers ($24 \pm 18\%$; $p = 0.001$) and asymptomatic smokers ($43 \pm 26\%$; $p = 0.045$).

HDAC2 Expression in Large Airways

Figure 3 shows the number of HDAC2-positive cells in large airways. HDAC2 was predominantly expressed in airway subepithelium and epithelium (fig. 2d–f). COPD patients had fewer HDAC2-positive cells in large airways compared to nonsmokers ($p < 0.0001$) and asymptomatic smokers (fig. 3; $p = 0.001$).

HDAC2 expression was observed in the cells of the epithelium and subepithelium. Epithelial cells of non-smokers had more intensive nuclear and cytoplasmic staining for HDAC2 compared to asymptomatic smokers (scores 2.0 ± 0.81 vs. 1.26 ± 0.69 ; $p = 0.001$) and COPD patients (2.0 ± 0.81 vs. 0.84 ± 0.70 ; $p = 0.0007$).

When all patients were analysed together, a positive correlation between HDAC2 expression and airflow obstruction ($FEV_1\%$; $r = 0.34$, $p = 0.008$) was observed. Furthermore, a negative correlation between smoking history (pack-years of smoking) and HDAC2 expression was revealed ($r = -0.32$, $p = 0.04$).

HDAC2 Expression in Small Airways

Figure 3 shows the number of HDAC2-positive cells in small airways. HDAC2 was predominantly expressed in airway subepithelium and epithelium. COPD patients had fewer HDAC2-positive cells in small airways compared to nonsmokers ($p < 0.0001$) and asymptomatic smokers (fig. 3; $p = 0.001$).

Epithelial cells of nonsmokers had more intensive nuclear and cytoplasmic staining for HDAC2 compared to asymptomatic smokers (scores 2.4 ± 0.7 vs. 1.68 ± 0.89 ; $p = 0.002$) and COPD patients (2.4 ± 0.7 vs. 0.58 ± 0.74 ; $p < 0.0001$).

When all patients were analysed together, a statistically significant positive correlation between HDAC2-positive cells and airflow obstruction ($FEV_1\%$; $r = 0.62$, $p < 0.0001$) was observed. Furthermore, a negative correlation between smoking history (pack-years of smoking) and HDAC2 expression ($r = -0.32$, $p = 0.02$) was observed in small airways.

Inflammatory Cells in the Airways

Table 2 summarises the number of NF- κ Bp65-positive and HDAC2-positive cells and the number of CD8+ T lymphocytes, macrophages and neutrophils in large and small airways.

CD8+ T Lymphocytes

The number of CD8+ T lymphocytes in large airways was significantly higher in COPD patients (321 cells/mm^2 , range 77–543) compared to asymptomatic smokers (154 cells/mm^2 , range 12–398; $p = 0.0003$) and nonsmokers (71 cells/mm^2 , range 8–141; $p < 0.0001$). In addition, in small airways the number of CD8+ T lymphocytes was also higher in COPD patients (432 cells/mm^2 , range 245–678) compared to asymptomatic smokers (254 cells/mm^2 , range 86–432; $p = 0.0003$) and nonsmokers (56 cells/mm^2 , range 18–96; $p < 0.0001$). CD8+ T lymphocyte infiltration

Table 2. The number of NF- κ Bp65-positive, HDAC2-positive and inflammatory cells in large and small airways

Patients	NF- κ Bp65-positive cells, cells/mm ²	HDAC-2-positive cells, cells/mm ²	CD8+ T lymphocytes, cells/mm ²	Neutrophils cells/mm ²	Macrophages cells/mm ²
<i>In large airways</i>					
Nonsmokers	245 (198–310)	58 (54–168)	71 (8–141)	87 (23–518)	90 (21–269)
Asymptomatic smokers	319 (290–367) ^{b, c}	61 (2–128)	154 (12–398) ^{b, c}	234 (72–456) ^c	234 (23–564) ^{b, c}
COPD patients	490 (399–510) ^a	29 (12–144) ^{a, b}	321 (77–543) ^a	290 (90–457) ^a	590 (211–678) ^a
<i>In small airways</i>					
Nonsmokers	161 (145–197)	68 (41–102)	56 (18–196)	56 (23–104)	90 (43–234)
Asymptomatic smokers	245 (199–309) ^{b, c}	35 (8–80) ^{b, c}	254 (86–432) ^{b, c}	268 (34–904) ^{b, c}	350 (21–534) ^{b, c}
COPD patients	521 (478–579) ^a	16 (2–72) ^a	432 (245–678) ^a	433 (234–754) ^a	274 (98–430) ^a

Data are presented as medians (range). ^a $p < 0.05$ compared to nonsmokers; ^b $p < 0.05$ compared to COPD patients; ^c $p < 0.05$ compared to nonsmokers.

between the airways in COPD patients was more prominent in small compared to large airways ($p < 0.001$).

Furthermore, the increased CD8+ T lymphocyte numbers in both large and small airways correlated with airflow limitation (FEV₁%; $r = -0.43$, $p = 0.0009$, and $r = -0.51$, $p < 0.0001$, respectively) and pack-years of smoking ($r = 0.56$, $p < 0.0001$, and $r = 0.63$, $p < 0.0001$, respectively).

Macrophages

The number of macrophages in large airways was significantly higher in COPD patients (590 cells/mm², range 211–678) compared to asymptomatic smokers (234 cells/mm², range 23–564; $p = 0.009$) and nonsmokers (90 cells/mm², range 21–269; $p = 0.0003$).

In addition, in small airways the number of macrophages in COPD patients (274 cells/mm², range 98–490) and asymptomatic smokers (250 cells/mm², range 21–534) was higher compared to nonsmokers (90 cells/mm², range 43–234; $p < 0.0001$ and $p = 0.009$, respectively). There was no difference in the number of macrophages in small airways between the smokers with and without COPD.

Macrophage infiltration between the airways in COPD patients was more prominent in large compared to small airways ($p = 0.002$), but there was no difference in asymptomatic smokers or control nonsmokers.

Furthermore, the increased macrophage numbers in large and small airways correlated with airflow limitation (FEV₁%; $r = -0.49$, $p < 0.0001$, and $r = -0.28$, $p = 0.04$, respectively) and pack-years of smoking ($r = 0.56$, $p < 0.0001$, and $r = 0.29$, $p = 0.03$, respectively).

Neutrophils

The number of neutrophils in large airways was significantly higher in COPD patients (290 cells/mm², range 90–457) and asymptomatic smokers (234 cells/mm², range 72–456) compared to nonsmokers (87 cells/mm², range 23–518; $p < 0.0001$). However, there was no difference in neutrophil number between COPD patients and asymptomatic smokers.

In small airways, the number of neutrophils was also higher in COPD patients (433 cells/mm², range 234–754) compared to asymptomatic smokers (268 cells/mm², range 34–904; $p = 0.01$) and nonsmokers (56 cells/mm², range 23–104; $p < 0.0001$).

Neutrophil infiltration between the airways in COPD patients was more prominent in small compared to large airways ($p = 0.002$), but there was no difference in asymptomatic smokers or control nonsmokers.

Furthermore, the increased neutrophil numbers in large and small airways correlated with airflow limitation (FEV₁%; $r = -0.41$, $p = 0.0014$, and $r = -0.47$, $p = 0.0002$, respectively) and pack-years of smoking ($r = 0.38$, $p = 0.003$, and $r = 0.62$, $p < 0.0001$, respectively).

Discussion

Our study shows a nonuniform distribution of inflammatory cells throughout the bronchial tree in smokers with and without COPD. Even asymptomatic smokers showed signs of inflammation manifesting with increased counts of macrophages, neutrophilic leucocytes and CD8+ T cells in airway walls that corresponded to

higher expression of NF- κ Bp65 and its nuclear localisation, when compared to nonsmoking persons. COPD patients exhibited a more intensive inflammatory pattern, with more neutrophil and CD8+ T lymphocyte involvement and increased NF- κ Bp65 expression.

NF- κ B expression is reportedly increased in large airways in both smokers with and without COPD [10]. Our findings confirm these results and extend this observation by showing that the increased number of NF- κ Bp65-positive cells in large airways correlated with NF- κ B overexpression in small airways. However, in COPD patients, we demonstrated a twofold increase in NF- κ Bp65 expression in large compared to small airways, which correlated with increased numbers of macrophages. Our results support previous evidence that macrophages are considered a major source of NF- κ B [19].

To some extent, our results are in agreement with a paper by Yagi et al. [19], who showed decreased numbers of phosphorylated I κ B (NF- κ B inhibitory protein) in airway epithelial cells of asymptomatic smokers and COPD patients.

I κ B- α levels are significantly decreased in lung tissue of healthy smokers and current ex-smokers with COPD when compared to nonsmokers, with an associated increase in NF- κ B DNA binding in current smokers [20]. I κ B is an inhibitory protein whose activity correlates inversely with NF- κ Bp65 expression. Caramori et al. [21], examining p65 expression in sputum leucocytes from patients with exacerbated COPD, found increased NF- κ Bp65 expression in macrophages.

We observed that COPD patients had increased NF- κ Bp65 nuclear immunolocalisation in airway epithelial cells compared to nonsmokers and smokers without COPD. We speculate that NF- κ B activation in small airway epithelia most likely plays a triggering role in airway inflammation in smokers with COPD. In humans, epithelial cell line activation of NF- κ B results in the acetylation of specific lysine residues on histones, which is correlated with increased expression of inflammatory genes [10, 16].

Previous studies have shown that airway epithelium cells obtained from COPD patients produce larger amounts of cytokines, e.g. IL-1 β , IL-6, IL-8 and intercellular adhesion molecule-1, than epithelial cells of asymptomatic smokers and that the transcription of these cytokines at the genetic level is regulated by NF- κ B [12, 16]. These cytokines and chemokines are the key stimulatory factors for the migration of CD8+ T lymphocytes and neutrophils in the airways.

Our study shows that COPD patients had decreased HDAC2 expression in large and small airways compared to nonsmokers and smokers without COPD, which correlated with increased inflammatory cell infiltration. Furthermore, we found significant negative correlations between HDAC2 expression and airflow limitation and smoking history (pack-years of smoking). HDAC2 expression in airway epithelium of COPD patients was significantly decreased in small compared to large airways, suggesting the significance of HDAC2 in the development of bronchiolitis characteristic for COPD.

Ito et al. [17] examined the expression of HDAC genes (HDAC1 to HDAC8) in lung tissue and alveolar macrophages from patients with COPD in Global Initiative for Chronic Obstructive Lung Disease stages 0–4. They observed a significant decrease of HDAC2 expression in alveolar macrophages relative to disease severity. As HDAC2 plays an important role in the regulation of inflammation, it is evidently implicated in the dysregulation of the inflammatory process in COPD. Also, cigarette smoking decreases HDAC2 activity in alveolar macrophages [8].

Szulakowski et al. [20] found that cytoplasmic HDAC2 protein levels are significantly reduced in all patients with COPD compared with nonsmokers and smokers without COPD.

Our results support previous findings and extend them by showing that HDAC2 was downregulated in the large and small airways of both smokers with and without COPD. However, smokers with COPD had significant HDAC2 downregulation in small compared to large airways. This leads to the hypothesis that not only direct effects of cigarette components but predominantly the products of subsequent inflammatory responses derived from neutrophils and CD8+ T lymphocytes in small airways may contribute to COPD, which in susceptible smokers causes disease.

Our study demonstrates the nonuniform distribution of inflammatory cells in smokers with and without COPD. COPD patients had more macrophages in large compared to small airways and more CD8+ T lymphocytes and neutrophils in small compared to large airways. In addition, in smokers without COPD and nonsmokers, the numbers of macrophages and neutrophils did not differ between the airways. However, the numbers of CD8+ T lymphocytes in asymptomatic smokers was higher in small compared to large airways. In addition, COPD patients had increased numbers of CD8+ T lymphocytes in both large and small airways compared to asymptomatic smokers and nonsmokers.

Furthermore, COPD patients had more macrophages in large airways and more neutrophils in small airways compared to asymptomatic smokers and nonsmokers.

Battaglia et al. [7] recently showed that the number of neutrophils infiltrating the lamina propria of small airways is higher than that in the lamina propria of large airways, but that the number of CD4+ T lymphocytes is higher in large compared to small airways. Our results support these findings and extend them by showing that in COPD the number of macrophages was increased in large compared to small airways, but that the number of CD8+ T lymphocytes was increased in small compared to large airways.

In addition, our results are consistent with previous studies that demonstrated higher numbers of macrophages in large compared to small airways [22–25]. Furthermore, the number of neutrophils infiltrating the small airways in smokers with or without COPD is higher when compared with nonsmokers [22–24]. Cigarette smoking causes oxidative damage with subsequent sequestration and accumulation of macrophages in large airways in both smokers with and without COPD [8]. However, small airways disease (respiratory bronchiolitis) is more characteristic for COPD patients [26].

T lymphocytes, particularly CD8+ cells and macrophages, are the prevalent inflammatory cells in the lungs of healthy smokers and patients with mild COPD, while total and activated neutrophils predominate in severe COPD [26]. In addition, the numbers of CD4+ cells, CD8+ cells and macrophages expressing NF- κ B, signal transducer and activator of transcription-4 and interferon- γ proteins as well as endothelial adhesion molecule-1 in endothelium are increased in mild/moderate disease. In contrast, activated neutrophils (myeloperoxidase-positive cells) and increased nitrotyrosine immunoreactivity develop in severe COPD [26].

The mechanisms underlying the nonuniform distribution of inflammatory cells throughout the bronchial tree 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. [27] 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 the proximal airways may serve as a reservoir for immune cells that contribute to chronic inflammation.

Furthermore, it has been suggested that different inflammatory cell distributions could be associated with upregulation of the pro-inflammatory transcription factors NF- κ B and signal transducer and activator of transcription-4 in mild, activated epithelial and endothelial cells in more severe disease, which might contribute to this differential prevalence of infiltrating cells [26].

The nonuniform distribution of inflammatory cells could be explained by anatomical differences and different vascularisation of the large and small airways. Lymphocytes may preferentially reach lung compartments supplied by bronchial vessels, whereas neutrophils may migrate preferentially through postcapillary venules of pulmonary vessels and, in particular, into the capillaries of the pulmonary system [28, 29].

In conclusion, we demonstrated that even though COPD is a disease of the small airways, similar patterns of inflammatory processes occur in both large and small airways. So-called healthy smokers present inflammation in both large and small airways, with activated NF- κ B, infiltration with neutrophils and CD8+ cells and suppressed HDAC2 activity. The difference between smokers with and without COPD is only quantitative. Evidently, the severity of the inflammatory processes with high concentrations of aggressive enzymes and free radicals that are not counteracted by the body's defence mechanisms plays a crucial role in the development of COPD in smokers.

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