Measurement of soluble perforin, a marker of CD8\(^+\) T lymphocyte activation in epithelial lining fluid

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Summary

Background: CD8\(^+\) T lymphocytes in the peripheral airways have been suggested to be involved in the pathogenesis of COPD. However, the significance of CD8\(^+\) T lymphocyte activation in COPD is not well understood. A biomarker of CD8\(^+\) T lymphocyte activation in patients with COPD is required.

Methods: Thirty COPD patients and twenty-one healthy controls (eleven ex-smokers and ten who had never smoked or were light ex-smokers) were included in this study. We separately obtained epithelial lining fluid (ELF) from central and peripheral airways using a bronchoscopic microsampling technique. Levels of perforin in ELF were measured and we examined correlations between its values and patients characteristics including pulmonary function.

Results: Perforin levels in both the central and peripheral airways in COPD patients were significantly higher than those in the healthy control groups. In the healthy control groups, there was no significant difference in perforin levels between central and peripheral airways. However, in COPD patients, perforin levels in peripheral airways were significantly higher than those in central airways. Perforin levels in peripheral airways were significantly correlated with FEV\(_1\) (percent predicted), FEV\(_1\)/FVC, and DLco (percent predicted) in COPD patients.

Conclusion: The microsampling technique is safe and useful for separately obtaining ELF from central and peripheral airways. Levels of perforin in ELF from peripheral airways were significantly increased and correlated with the degree of pulmonary dysfunction. Perforin might reflect inflammation involving CD8\(^+\) T-lymphocytes. This novel biomarker might enable better understanding of the pathogenesis of COPD.

Abbreviations: COPD, Chronic obstructive pulmonary disease; ELF, epithelial lining fluid; FEV\(_1\), forced expiratory volume in one second; FVC, forced vital capacity; DLco, diffusing capacity for carbon monoxide; IFN, interferon; TNF, tumor necrosis factor; BALF, bronchoalveolar lavage fluid; GOLD, Global Initiative for Chronic Obstructive Lung Disease; ELISA, enzyme-linked immunosorbent assay.

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Introduction
Chronic obstructive pulmonary disease (COPD) is a major worldwide health problem that has exhibited increasing prevalence and mortality. The prevalence, morbidity, and mortality of COPD vary appreciably across countries, but in general are directly related to the prevalence of cigarette smoking. COPD is characterized by airflow limitation that is not fully reversible, usually progressive, and associated with abnormal inflammatory responses of the lung. Indeed, one important pathological feature of COPD is chronic inflammation characterized by an influx of inflammatory cells in the lumen and wall of the bronchial and bronchiolar airways and parenchyma. In this respect, there are strong correlations between lung function, airway wall area, the degree of luminal occlusion, and the degree of inflammatory infiltrates in the airways of patients with COPD. However, several studies using surgically resected lung tissue, autopsy lung specimens, and transbronchial biopsies have indicated that more severe inflammatory and structural changes occur in the small airways of COPD. Thus, the airflow limitation in COPD probably occurs as a result of small airways inflammation.

There is accumulating evidence that CD8+ T lymphocytes play an important role in the initiation and progression of COPD. In particular, a previous study reported that the number of CD8+ T lymphocytes in COPD lungs was directly related to the degree of airflow limitation. Moreover, a close correlation was found between emphysema assessed by CT and the number of tissue CD8+ lymphocytes. Furthermore, increased CD8+ T lymphocytes in BALF has indicated that more severe inflammatory and structural changes occur in the small airways of COPD. Thus, the airflow limitation in COPD probably occurs as a result of small airways inflammation.

Methods
Subjects
Thirty COPD patients and twenty-one control subjects from the outpatient clinic of our hospital were enrolled in this study. They underwent bronchoscopy to identify the cause of small peripheral nodules. Subjects who agreed to undergo ELF sampling were randomly selected for inclusion in this study. All COPD patients were ex-smokers, and satisfied the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria for the diagnosis of COPD. They had been free of acute upper respiratory tract infections, and none had received inhaled bronchodilators or corticosteroids. Control subjects were divided into two groups based on smoking history (eleven subjects were ex-smokers and ten who had never smoked or were light ex-smokers with less than a five-pack-year history). In this study, we defined the latter group as non-smokers. All ex-smokers had quit smoking at least one year before entering this study. Pulmonary function tests including the diffusing capacity of the lung for carbon monoxide (DLco) were performed in all subjects. In all study subjects, chest computed tomographic scans revealed no abnormal diffuse interstitial infiltrates, and results of arterial blood gas analyses were normal. All subjects gave written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Bronchoscopic microsampling technique
ELF was obtained using a previously described bronchoscopic microsampling technique. Using this method, we attempted to measure the concentrations of the soluble form of perforin in ELF in central or peripheral airways separately, and determined whether its levels were correlated with results of pulmonary function in patients with COPD.
rod. We obtained ELF from the seventh or eighth lower lobe bronchioles under direct vision using this thin bronchofiber scope in the same subjects (peripheral airway sample). ELF was collected in each subject from a lung field contralateral to the small peripheral nodules. The inner probe was advanced into the bronchial lumen slowly for 15 s to avoid injuring the bronchial wall. The inner probe was then withdrawn into the outer tube, and the two were withdrawn together to avoid contamination. The same procedure was repeated three times. Then the inner probe was cut 3 cm distal from its tip, and the wet probe was frozen at −80 °C freezer until use. Diluted solution for measurements of biochemical constituents was prepared by adding 1 ml of saline to the tube containing the frozen probe and vortexed for 1 min. The probe was dried and weighed to measure the ELF volume recovered, and the dilution factor was calculated.

Measurement of biochemical constituents in ELF

Using the ELF samples processed as described above, the concentration of soluble perforin was measured by enzyme-linked immunosorbent assay (ELISA) (Mabtech AB, Sweden). The assay kits for perforin have a 36 pg/ml detection limit, using antiserum that has 100% reactivity with perforin.

Statistical analysis

All values are presented as the median [range]. Comparisons between the COPD group and two healthy controls were performed using the Kruskal–Wallis test. When the comparisons were significant, nonparametric analyses of variance with the Bonferroni/Dunn’s post hoc test were performed. Comparisons between perforin levels in peripheral and central airways in each group were performed with the Wilcoxon matched-pairs signed-rank test. The significances of correlations were determined by calculation of Spearman’s rank correlation coefficients. For all tests, findings of $p<0.05$ were considered significant.

Results

The clinical characteristics of the 10 non-smokers, 11 ex-smokers, and 30 COPD patients are shown in Table 1. The three groups were well matched for gender and age.

<table>
<thead>
<tr>
<th>Table 1 Clinical characteristics of study subjects.</th>
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<tr>
<td><strong>Non-smokers</strong></td>
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<tr>
<td><strong>Subject No. (male/female)</strong></td>
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<tr>
<td><strong>Age (yr)</strong></td>
</tr>
<tr>
<td><strong>Smoking history (pack-years)</strong></td>
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<tr>
<td><strong>FEV1 (% predicted)</strong></td>
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<td><strong>FEV1/FVC (%)</strong></td>
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<td><strong>DLco (% predicted)</strong></td>
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All values are presented as mean (SD). Definition of abbreviations: FEV1 = forced expiratory volume in one second, FVC = forced vital capacity, DLco = diffusing capacity for carbon monoxide.

**$p<0.01$ compared with non-smokers.

*p $0.05$ compared with non-smokers.

#p $0.01$ compared with ex-smokers.
results of pulmonary function. There were trends that perforin levels in central airways were correlated with the degree of airflow obstruction (Fig. 3, Fig. 4) and the decline of DLco (Fig. 5). Moreover, perforin level in peripheral airways was closely correlated with \( \text{FEV}_1 \) \((r = -0.37, p = 0.04)\), \( \text{FEV}_1/\text{FVC} \) \((r = -0.47, p = 0.01)\), and DLco \((r = -0.66, p < 0.01)\).

**Discussion**

We successfully measured the concentration of the soluble form of perforin in ELF samples in central and peripheral airways in human subjects, and our microsampling technique was found to be very safe without any complications. Using our method, we found that (1) perforin levels in ELF...
in both central and peripheral airways were significantly higher in COPD patients than in non-smokers and ex-smokers. (2) these levels in peripheral airways of COPD patients were closely correlated with FEV1, FEV1/FVC, and DLco. The number of CD8⁺ T lymphocytes in the airways is known to be increased in COPD patients compared with control subjects. Since perforin is strongly expressed in activated CD8⁺ T lymphocytes, it is plausible that soluble perforin levels in ELF reflect the number of activated CD8⁺ T lymphocytes that have infiltrated in the airways. Actually, concerning the magnitude of perforin expression in CD8⁺ T lymphocytes from induced sputum and BALF, perforin expression was significantly increased in COPD.7,8 These findings thus suggest that CD8⁺ T lymphocytes infiltrate into both central and peripheral airway walls in COPD patients. Moreover, as peripheral airways inflammation has attracted much recent attention, separate observation in central and peripheral airways will be needed. In this regard, since we selectively obtained ELF from the seventh or eighth bronchioles using a thin bronchoscope with a thin probe as peripheral airways, our analysis of ELF samples from peripheral airways is considered to reflect peripheral airways inflammation. In this study, we demonstrated that perforin levels in the peripheral airways were significantly

![Figure 4: Correlation between perforin levels and FEV1/FVC(%) in COPD patients.](image)

![Figure 5: Correlation between perforin levels and %DLco in COPD patients.](image)
higher than those in the central airways in COPD patients. Activated CD8\(^+\) T lymphocytes might thus be increased in peripheral airways of COPD patients. It is known that magnitude of inflammation is higher in peripheral airways in COPD patients. Proinflammatory cytokines in small airways of COPD might activate CD8\(^+\) T lymphocytes and up-regulate the expression of perforin in these cells.

We found significant correlation between perforin levels in peripheral airways and the degree of airflow obstruction in COPD patients. The so-called peripheral airways consist of small bronchioles and are subject to airway narrowing by either inflammatory process such as thickening of airway walls or following airway remodeling. Hogg et al. previously reported an inverse correlation between the severity of inflammation in small airways and FEV\(_1\) over the full range of COPD severity. These findings suggest that higher levels of perforin in peripheral airways are associated with increased severity of airflow limitation in COPD patients. Decreases in DLco in COPD patients usually reflect the destruction of pulmonary parenchyma. In COPD airways, CD8\(^+\) T lymphocytes infiltrate and initiate apoptosis of lung constitutive cells by co-releasing perforin and granzyme B. In the alveolar region, apoptosis might induce loss of alveolar walls leading to emphysema. Thus, perforin in peripheral airways might be involved in the pathogenesis of COPD apoptosis. In this study perforin levels in central airways of COPD patients were not closely correlated with the degree of pulmonary function. However, we could see trends that those levels in central airways were correlated with such parameters. This lack of correlation might be due to a relatively small number of samples. The fact that perforin levels in central airways were significantly higher in COPD patients than in non-smokers and ex-smokers indicates activated CD8\(^+\) T lymphocytes might be increased in central airways as in peripheral airways. The number of activated CD8\(^+\) T lymphocytes in peripheral airways may be more increased than in central airways.

Exposure to noxious gas such as cigarette smoke is the main cause of COPD. There is some evidence regarding the relationship between cigarette smoke exposure and CD8\(^+\) T-cell activation. Cigarette smoke exposure might affect the expression of perforin in COPD airways via CD8\(^+\) T lymphocyte activation. However, no correlations were found between smoking indices and the perforin levels in either central or peripheral airways in COPD patients in this study. This indicates that the influence of smoking on the degree of CD8\(^+\) T lymphocyte activation in increasing perforin expression differs in each patient. It is known that, only ten to fifteen percent of smokers develop COPD. This difference in susceptibility to COPD is believed to be related to hereditary factors. The result of this study suggests that the diversity in degree of CD8\(^+\) T lymphocyte activation in response to cigarette smoke exposure might be one of those factors. In addition, perforin levels in ELF in neither central nor peripheral airways significantly differed between non-smokers and ex-smokers. There are two possible explanations of this result. One is that in ex-smokers, airway inflammation might not occur from the very beginning. Another is that airway inflammation is sustained during smoking but subsides after smoking cessation. Genetic variation might exist in whether the inflammation in airways is prolonged or not once initiated by smoking. Perhaps prolongation of chronic inflammation leads to COPD. The levels of perforin in peripheral airways may be predictive factor for risk of COPD.

In this study, we separately measured perforin levels in central and peripheral airways and found increased levels of perforin in peripheral airways of COPD patients. The microsampling technique is less invasive and more repeatable than bronchial biopsy, and repeated sampling such as pre- and post-therapeutic intervention will aid determination of the dynamics of CD8\(^+\) T-lymphocytes in the pathogenesis of COPD. Moreover, the result using epithelial lining fluid to observe airways might be replaced with induced sputum which is less invasive and more repeatable. Increased levels of perforin in peripheral airways might be an important feature of airway inflammation of COPD, and perforin has the potential for use as a novel biomarker for better understanding of the pathogenesis of COPD. Further examinations addressing the potential of perforin and its role in COPD are needed.

**Conflict of interest**

None.

**References**