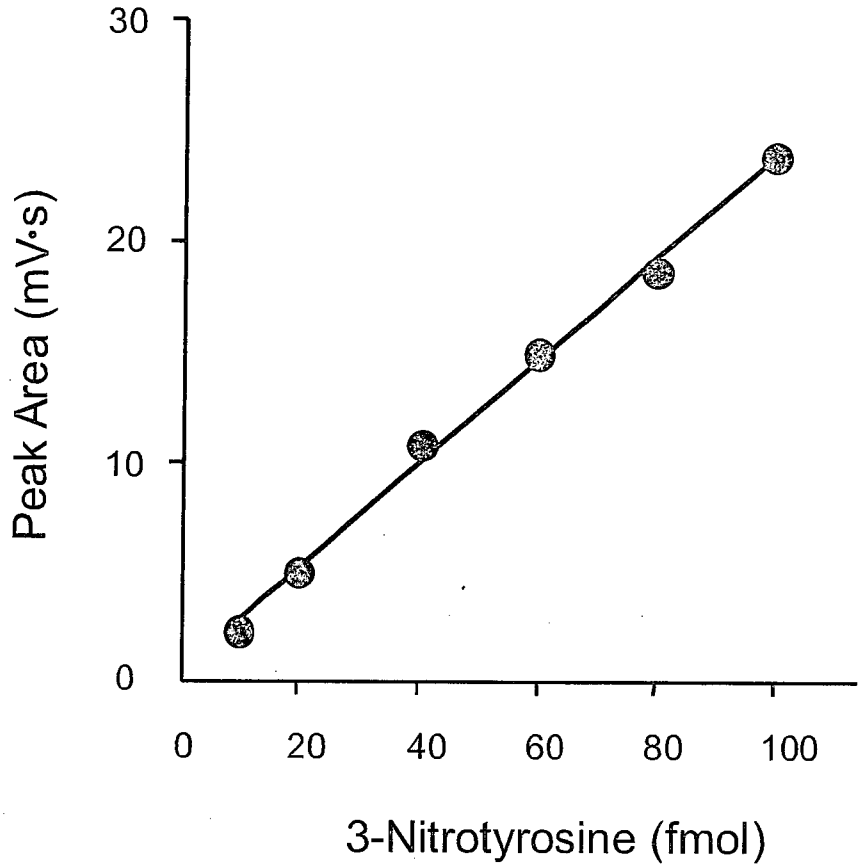

Figure 1. Standard curve for 3-nitrotyrosine by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). A linear electrochemical response was observed in a wide range of authentic 3-nitrotyrosine concentrations. The detection limit of this HPLC-ECD system was 10 fmol.

(10 kDa cut off). The amounts of protein-bound 3-NT and the free amino acid form of 3-NT when 50 μl hydrolysate were injected are shown in Fig. 2. The mean value of the amount of protein-bound 3-NT was 65.0 fmol (31.2 to 106.4 fmol), whereas the amount of the free amino acid form of 3-NT was under the detection limit (<10 fmol) with this system (Fig. 2).

Protein-bound 3-NT and tyrosine in sputum and saliva

In COPD patients, the levels of 3-NT in saliva were significantly lower than those in sputum (sputum: 0.55 ± 0.15 pmol/ml, saliva: 0.02 ± 0.01 pmol/ml; $p < 0.01$). The levels of tyrosine in saliva were also significantly lower than in sputum (sputum: 0.81 ± 0.43 micro mol/ml, saliva: 0.07 ± 0.04 micro mol/ml; $p < 0.01$) (Fig. 3). The values of 3-NT and tyrosine in saliva were about 3.1% and 9.0% of those in sputum, respectively.

Discussion

In the current study, we found that the amount of free amino acid form of 3-NT was very small and was under the detection limit in this HPLC-ECD system, though it could be present in the sputum. This suggests that the influence of the free amino acid form of 3-NT on the values of protein-bound 3-NT would be very small and could be negligible. Furthermore, we found that the values of 3-NT and tyrosine in saliva were 3.1%, 9.0% of those in sputum, respectively.

Tyrosine is one of the amino acids that compose proteins. Protein-bound 3-NT and protein-bound tyrosine are composed of various proteins, and free amino acid forms of 3-NT and of tyrosine are present in induced sputum. The free amino acid form of 3-NT might affect the measured values of protein-bound 3-NT by HPLC-ECD. Compared with the amount of protein-bound 3-NT, which was 65.0 fmol, that of the free amino acid form of 3-NT was very small and under the

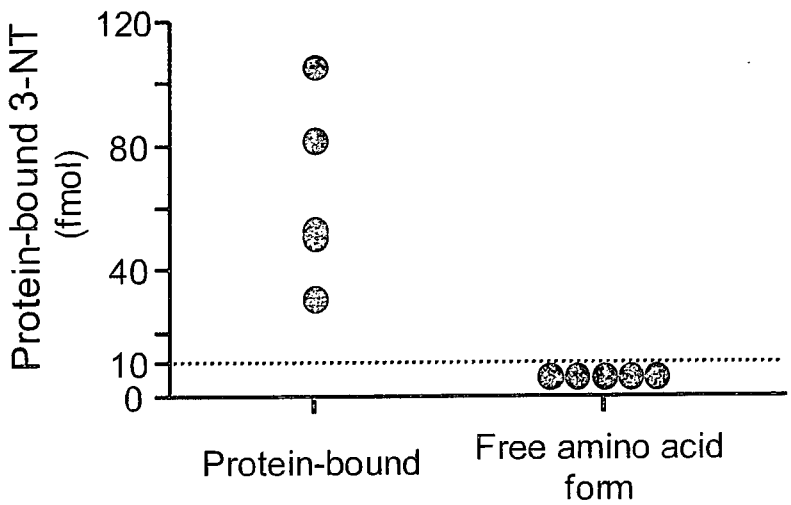

Figure 2. The levels of protein-bound and the free amino acid form of 3-nitrotyrosine in the hydrolysate from COPD patients. The amount of the free amino acid form of 3-nitrotyrosine was under the detection limit (<10 fmol) and much smaller than that of protein-bound 3-nitrotyrosine. 3-NT: 3-nitrotyrosine.

detection limit (<10 fmol). This suggests that the influence of the contamination of free 3-NT would be very small.

Salivary contamination in induced sputum cannot be avoided completely, but most can be removed by gargling and then by rolling the sputum samples on dry gauze as much as possible after the induction. To reduce the contribution of saliva to the induced sputum samples, sputum and saliva have been collected separately during sputum induction for measuring cytokines (Keatings et al. 1996) or eosinophil cationic protein (ECP) (Gershman et al. 1996). Pin et al. restricted their analyses of induced sputum to plugs of mucus extracted from the sample, and the volume of the contaminating saliva was assumed to be very small

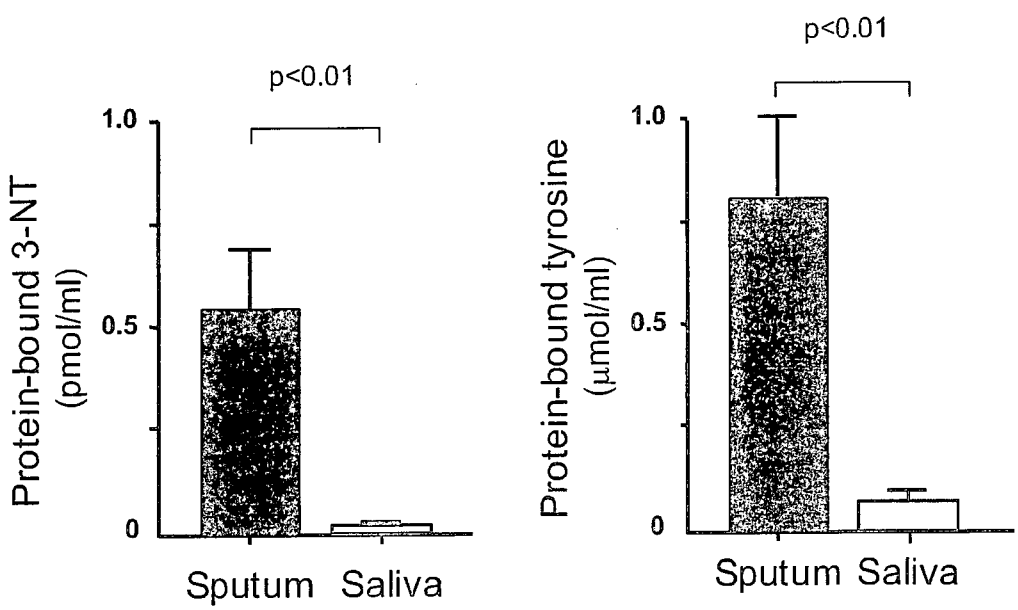

Figure 3. Concentration of protein-bound 3-nitrotyrosine and protein-bound tyrosine in sputum and saliva. Both the concentrations of protein-bound 3-nitrotyrosine and protein-bound tyrosine were significantly lower in saliva than in sputum. 3-NT: 3-nitrotyrosine. Data are presented as mean ± SD.

in comparison with the volume of sputum (Pin et al. 1992). These reports suggest that the contribution of saliva to the measurement values can be minimized by various methods.

Examinations of the influence of salivary contamination in induced sputum have been reported. Fahy et al. (Fahy et al. 1993) reported that the amount of ECP in saliva from asthmatic subjects was much lower than in paired induced sputum samples. They concluded that the principal effect of saliva in induced sputum was only that of diluting the sample. Dauletbaev et al. (Dauletbaev et al. 2001) reported that the glutathione content of the saliva was significantly lower than that in the sputum. Sagel et al. (Sagel et al. 2001) reported that the salivary IL-8 levels were one-fifteenth the levels in the induced sputum samples and were, therefore, unlikely to significantly affect the levels measured in the sputum. These reports were compatible with our data, in which the concentrations of 3-NT and tyrosine in saliva were 3.1% and 9.0% of those in induced sputum, respectively.

For the measurement of 3-NT from human samples, a large number of studies using antibody-based methods have been reported. For example, immunohistochemical staining of 3-NT has been reported in the tissue or fluid from inflamed colonic epithelium (Singer et al. 1996), chronic hepatic diseases (Cuzzocrea et al. 1998; Garcia-Monzon et al. 2000), atherosclerotic plaques (Cromheeke et al. 1999), Alzheimer's disease (Smith et al. 1997) and cystic fibrosis (Morrissey et al. 2002). We also measured the 3-NT in induced sputum from COPD patients by immunohistochemical staining (Ichinose et al. 2000; Hirano et al. 2006), but this method is semi-quantitative. Other measurements made by employing ELISA assay have been reported (Ceriello et al. 2001; Oldreive et al. 2001; ter Steege et al. 1998; Banks et al. 1998), but there was no rigorous assay validation and the reliability was poor because the peroxidase employed in the process of ELISA might have stimulated the production of 3-NT (Duncan, 2003).

HPLC combined with an electrochemical detection method is a quantitative method for measuring 3-NT. Both UV (Crow and Ischiropoulos, 1996) and fluorescence detection (Kamisaki et al. 1996) have been employed, but they were too insensitive. Consequently, HPLC-EC systems have been commonly employed to measure 3-NT in human samples (Crow, 1999; Hensley et al. 1998; Moore and Mani, 2002; Shigenaga et al. 1997; Ohshima et al. 1999). In these studies, the detection limit of 3-NT was 0.1 pmol/20 µl injection, but with our current HPLC-ECD system the detection limit was 10 fmol/10 µl injection which was 20 times more sensitive than the other reported methods. The current HPLC-ECD is thought to be the most accurate system for quantifying 3-NT in sputum.

We previously reported that the levels of 3-NT were increased in induced sputum from asthma and COPD patients by HPLC-ECD (Sugiura et al. 2004), and that theophylline reduced the levels of 3-NT in COPD patients (Hirano et al. 2006). Our present report supports the precision of the data obtained with this HPLC-ECD system.

The presence of co-morbid diseases associated with higher protein-bound 3-NT production in saliva such as periodontal disease, might influence the values of 3-NT in sputum. Further study should be necessary to clarify the influence of co-morbid diseases in oral cavity.

In conclusion, the free amino acid form of 3-NT does not affect the measurement of protein-bound 3-NT. Furthermore, the influence of salivary contamination on the measurement of protein-bound 3-NT in induced sputum by means of HPLC-ECD is very small and could be negligible.

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Overexpression of CD-11b and CXCR1 on Circulating Neutrophils*

Its Possible Role in COPD

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Background: **It has been shown that the β_2-integrin molecule is up-regulated in circulating neutrophils in COPD subjects. However, little has been reported about the expression of the cell surface molecules in such patients and their relationship with pulmonary function. The aim of the present study was to investigate the surface expression of molecules in circulating neutrophils and to clarify their possible role in the airflow limitation of COPD.**

Methods: **The surface expression of Mac-1 cells (*ie*, CD-11b and CD-18 cells) and CXC chemokine receptor (CXCR) 1 and CXCR2 of circulating neutrophils obtained from COPD patients and healthy subjects (HSs) was measured by flow cytometry analysis. The serum levels of interleukin (IL)-8 were measured by enzyme-linked immunosorbent assay.**

Results: **Both CD-11b and CXCR1 expression were significantly higher in COPD patients than in HSs (mean [± SE] CD-11b concentration: HSs, 9.7 ± 1.0; COPD patients, 14.2 ± 1.8 [$p < 0.05$]; mean CXCR1 concentration: HSs, 9.6 ± 0.5; COPD patients, 11.9 ± 0.4 [$p < 0.01$]). Although aging was positively correlated with the expression of CXCR1 ($r = 0.440$; $p < 0.01$), none of the other background factors, including smoking and body mass index, showed a correlation with the expression of the molecules. Although serum IL-8 levels were higher in patients with COPD than in HSs, no significant correlation between serum IL-8 levels and the expression of any molecule was seen. The expression of CD-11b ($r = -0.317$) and CXCR1 ($r = -0.383$) showed a significant negative correlation with the severity of airflow limitation (both $p < 0.05$).**

Conclusions: **The overexpression of CD-11b and CXCR1 in circulating neutrophils may be associated with the development of airflow limitation in COPD patients.**

(CHEST 2007; 132:890–899)

Key words: β_2-integrin; chemokine receptor; COPD; interleukin-8; neutrophil

Abbreviations: BMI = body mass index; CXCR = CXC chemokine receptor; FITC = fluoroscein isothiocyanate; HS = healthy subject; IL = interleukin; PE = phycoerythrin

COPD is characterized by an abnormal inflammatory response, mainly to tobacco smoking.[1] Although a multiplicity of cells and mediators are involved in the pathophysiology of COPD, neutrophils play a key role in the development of COPD.[2–5] It has been reported[2,6,7] that the number of neutrophils is increased in samples of induced sputum or BAL fluid from patients with COPD. In addition, there is a correlation between the number of neutrophils in bronchial biopsy specimens[8] or induced sputum samples[2,3,9] and the severity of airflow limitation.

It is thought that circulating neutrophils are recruited into the airways through adhesion to endothelial cells and migration toward various neutrophil chemotactic factors, including interleukin (IL)-8.[5] Since E-selectin is up-regulated in endothelial cells in the airways of patients with COPD,[10] the adhesion of neutrophils may be facilitated in the development of this disease. Among various neutrophil chemotactic factors, it has been also reported that levels of the potent neutrophil chemoattractant IL-8 are increased in induced sputum samples[2,11] or BAL fluid sam-

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ples[12,13] from patients with COPD, and that there is a positive correlation between the levels of IL-8 in BAL fluid and the degree of neutrophil infiltration.[12] Thus, the migration of neutrophils into the airways is also thought to be facilitated in the development of COPD.

It has been reported that there is an increase in the concentrations of granule proteins such as myeloperoxidase and human neutrophil lipocalin, or the respiratory burst response that correlates with airflow limitation.[7,11,14] Because the priming of neutrophils is an important event for degranulation and the generation of superoxide anion in neutrophils,[15] an alteration of the surface expression of Mac-1 cells or CXC chemokine receptors (CXCRs), even in neutrophils in peripheral blood, may contribute to the development of COPD.

The surface expression of the adhesion molecule Mac-1 (CD-11b/CD-18) on circulating neutrophils has been reported to be up-regulated in patients with COPD.[16–18] In addition, it has been also shown that the expression of CXCR2, but not CXCR1, is decreased in circulating neutrophils,[18] which may affect neutrophil recruitment. This issue is still controversial because another report[19] has reported no apparent difference in the expression of CXCR1 and CXCR2 in circulating neutrophils obtained from patients with COPD.

However, the precise nature of the neutrophil surface molecules has not been fully elucidated. Further, the role of the molecules in the pathophysiology of COPD is also uncertain. Therefore, the aim of the present study was to investigate the expression of surface molecules on circulating neutrophils and to evaluate the relationship between the expression of these molecules and airflow limitation in COPD patients.

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Materials and Methods

Subjects

Sixteen stable patients with mild-to-severe COPD (stage I 2, stage II 3, stage III 9, and stage IV 2), and 24 healthy subjects (HSs) [15 smokers with normal lung function and 9 life-long never-smokers] were recruited from the outpatient clinic of our institution. All COPD patients were considered to be clinically stable because none of them had required medical attention and/or a change in their regular therapy such as inhaled bronchodilators; nor had any of them had bronchial or respiratory tract infections during the 4 months preceding the study. None of them had been treated with inhaled or oral corticosteroids. Patients with bronchial asthma, pneumonia, or lung cancer were excluded from the study. All patients refrained from smoking for the 12 h before the blood sampling. This study was approved by the ethics committee of our institution. Informed written consent was obtained from all subjects. The characteristics of each group are shown in Table 1.

Lung Function Testing

Lung function was evaluated with a dry rolling-seal spirometer (System 7; Minato Medical Science; Osaka, Japan). Each measurement was performed 15 min after the inhalation of 400 μg of salbutamol via a metered-dose inhaler.

Isolation of Circulating Neutrophils

Human circulating neutrophils were isolated from whole blood by a density gradient technique using a medium designed for separating polymorphonuclear leukocytes from

Table 1—*Characteristics of Study Subjects

Characteristics	HSs (n = 24)	COPD Patients (n = 16)
Age, yr	62.3 ± 2.1	71.4 ± 2.0†
Gender		
Male	24	16
Female	0	0
Smoking status		
Nonsmoker	9	0
Ex-smoker	8	13
Current smoker	7	3
Pack-yr	54.6 ± 5.5	75.5 ± 5.3
FVC		
L	3.28 ± 0.16	3.04 ± 0.14‡
%	95.3 ± 3.8‡	91.0 ± 3.4
IC		
L	2.45 ± 0.16	2.11 ± 0.15
%	107.4 ± 4.7	108.1 ± 7.3
FEV_1		
L	2.62 ± 0.13	1.26 ± 0.13§
% predicted	100.4 ± 3.9	49.3 ± 4.7§
FEV_1/FVC ratio, %	80.0 ± 1.4	42.5 ± 3.4§

*Values are given as the mean ± SE, unless otherwise indicated. IC = inspiratory capacity.
†$p < 0.01$ vs HSs.
‡$p < 0.05$ vs HSs.
§$p < 0.0001$ vs HSs.

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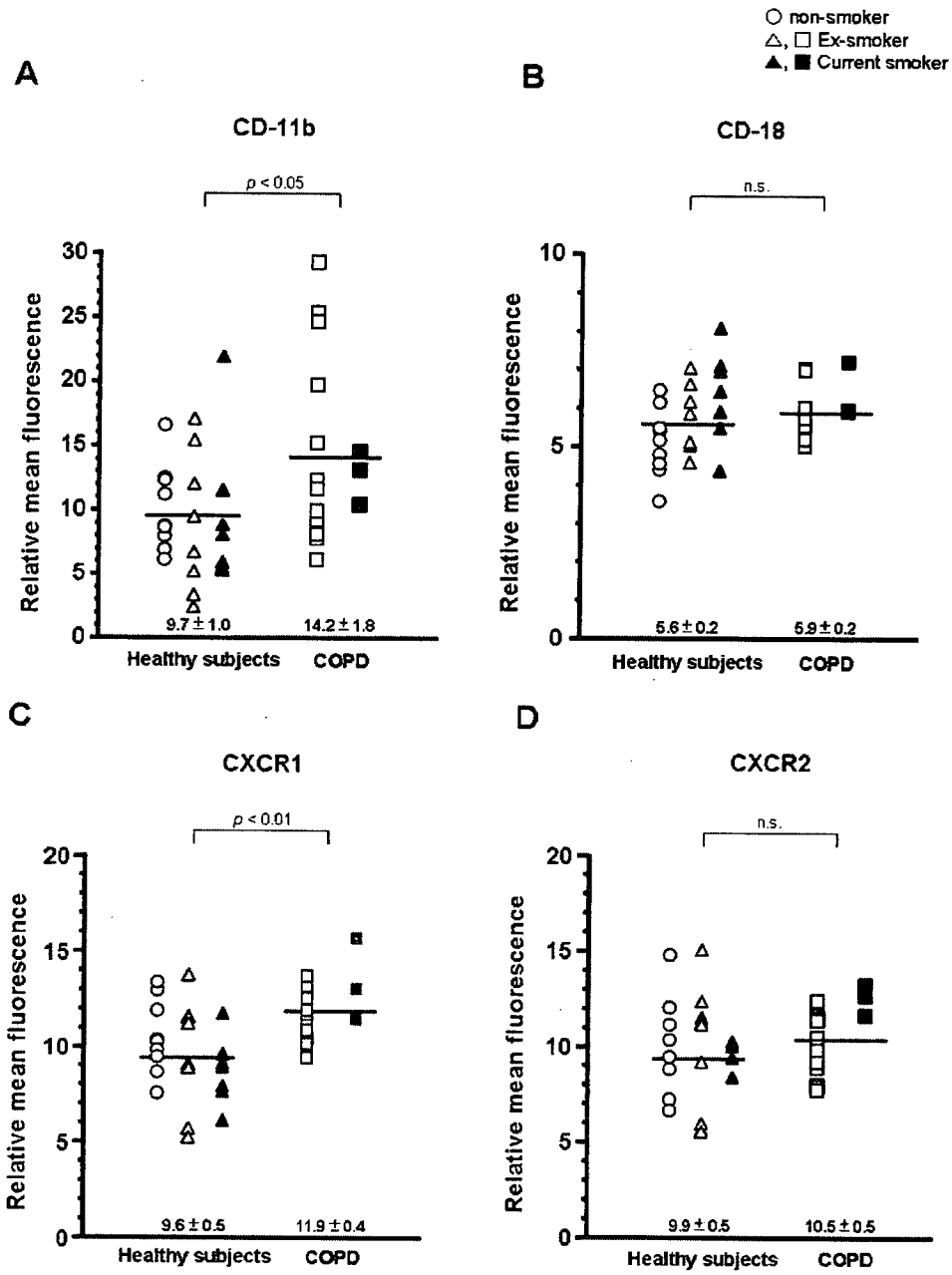

FIGURE 1. Surface expression of CD-11b, CD-18, and CXCRs on circulating neutrophils. *Top left, A*: the expression of CD-11b was significantly higher in patients with COPD than in HSs. No significant difference in CD-11b expression between nonsmokers and smokers was seen. *Top right, B*: there was no significant difference in CD-18 expression in HSs and patients with COPD. *Bottom left, C*: the expression of CXCR1 was significantly higher in patients with COPD than in HSs. No significant difference in the CXCR1 expression between nonsmokers and smokers was seen. *Bottom right, D*: there was no significant difference in CXCR2 expression between the two groups. Bars indicate mean values. n.s. = not significant.

whole blood cells (Mono-Poly Resolving Medium; Dainippon Pharmaceutical Co, Ltd, Laboratory Products; Osaka, Japan) according to the directions of the manufacturer.[20,21] Briefly, 10 mL of whole blood was collected by vein puncture into tubes containing ethylenediaminetetraacetic acid anticoagulant. Then, 5-mL blood samples were gently mounted onto 4 mL of the medium designed for separating polymorphonuclear leukocytes from whole blood cells (Mono-Poly Resolving Medium; Dainippon Pharmaceutical Co, Ltd, Laboratory Products) without mixing. The samples were then centrifuged at 400g for 20 min at room temperature. The blood was separated into the following four layers from top to bottom:

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plasma; lymphocytes/mononuclear cells; neutrophils; and RBCs. The neutrophil layer was gently collected by Pasteur pipettes without aspirating the other layers and was put into fresh 50-mL tubes. This procedure enables one to obtain neutrophils with > 90% purity. After washing in phosphate-buffered saline solution and counting the number of cells, the neutrophils were suspended in phosphate-buffered saline solution at a concentration of 5×10^6 cells/mL; then the expression of each molecule was measured by flow cytometry.

Flow Cytometry Analysis

Measurements of the surface expression of Mac-1 cells (*ie*, CD-11b/CD-18 cells), CXCR1, and CXCR2 in HS and patients with COPD were performed (FACSCalibur flow cytometer; Becton Dickinson Biosciences; San Jose, CA). Briefly, 1×10^6 neutrophils were incubated in 20 μL of each antibody solution for 40 min at 4°C. After washing, the cells in 50 mL of phosphate-buffered saline solution were fixed by 1% paraformaldehyde, and then the binding of each antibody was detected using a flow cytometer. The specific binding of each antibody was expressed as the relative fluorescence shown by the ratio of the obtained mean fluorescence intensity values of the cells stained with CD-11b, CD-18, CXCR1, or CXCR2 to the mean fluorescence values for the IgG1 or IgG2a isotype control, as previously reported.[22]

Quantification of Serum IL-8

The levels of serum IL-8 were measured using a commercially available enzyme-linked immunosorbent assay kit (Becton Dickinson Biosciences) according to the instructions provided by the manufacturer. The minimum detectable concentration of IL-8 was 0.8 pg/mL. A standard curve was obtained with serial dilution of the supplied recombinant human IL-8 by linear regression. The concentration of IL-8 in each sample was obtained by interpolation of its absorbance from a standard curve, and the mean value of the duplicate samples was then taken as a representative value.

Reagents

A medium for separating polymorphonuclear leukocytes from whole blood cells (Mono-Poly Resolving Medium; Dainippon Pharmaceutical Co, Ltd, Laboratory Products) was used. Phycoerythrin (PE)-conjugated antihuman CD-11b, PE-conjugated anti-human CXCR1 antibody, fluorescein isothiocyanate (FITC)-conjugated antihuman CD-18, FITC-conjugated antihuman CXCR2 antibody, PE-conjugated or FITC-conjugated Ig G1 isotype control, and PE-conjugated IgG2a isotype control were used (Becton Dickinson Biosciences). All other chemicals were purchased from Sigma Aldrich Japan (Tokyo, Japan).

Statistical Analysis

The data were expressed as the mean ± SE. A Mann-Whitney *U* test was used for single comparisons. For multiple comparisons, analysis of variance and Fisher protected least significant difference tests were performed. The analysis of correlations between each factor was performed by using the Spearman correlation coefficient rank test. A p value of < 0.05 was considered to be significant.

Results

The surface expression of CD-11b on circulating neutrophils from patients with COPD was significantly higher than that from HSs (HSs, 9.7 ± 1.0; COPD patients, 14.2 ± 1.8; $p < 0.05$) [Fig 1, *top left, A*]. No apparent difference in CD-11b expression was seen between nonsmokers and ex-smokers or current smokers. In the COPD group, there was also no significant difference in CD-11b expression between ex-smokers and current smokers. There was no significant difference in the expression of CD-18 cells between HSs and COPD patients (HS, 5.6 ± 0.2; COPD patients, 5.9 ± 0.2) [Fig 1, *top right, B*].

The surface expression of CXCR1 in circulating neutrophils in COPD patients was significantly higher than that in HSs (HSs, 9.6 ± 0.5; COPD, 11.9 ± 0.4; $p < 0.01$) [Fig 1, *bottom left, C*]. However, no apparent difference was seen between nonsmokers and ex-smokers or current smokers. In COPD group, there was also no significant difference in CXCR expression between ex-smokers and current smokers. Compared with CXCR1 expression, no significant difference in the surface expression of CXCR2 was seen between the two groups of subjects (HSs, 9.9 ± 0.5; COPD patients, 10.5 ± 0.5) [Fig 1, *bottom right, D*].

To determine the factors that affect the up-regulation of CD-11b and CXCR1 expression in COPD patients, the correlation between the expression of these molecules and various background characteristics including age, smoking amount, and body mass index (BMI) were evaluated. As shown in Table 2, none of the characteristic parameters such as aging, smoking amount, and BMI had an apparent correlation with the degree of CD-11b expression. As shown in Figure 2, *top left, A*, the overall expression of CXCR1 was positively correlated with aging ($r = 0.440$; $p < 0.01$). The overall expression of CXCR1 was also not correlated with smoking amount and BMI (Fig 2, *top right, B, bottom left, C*). As shown in Table 2, the expression of CD-18 and CXCR2 had no correlation with any of the background characteristics.

The correlation analysis between each surface molecule in circulating neutrophils is shown in Table 3. Among the expression of each molecule,

Table 2—*Correlation Among β_2-Integrin, CXCR Expression, and Subject Characteristics**

Variables	CD-11b	CD-18	CXCR1	CXCR2
Age	0.253	−0.092	0.440†	0.165
Pack-yr	0.230	0.238	0.262	0.007
BMI	−0.157	0.081	−0.271	0.041
IL-8 level	−0.113	0.011	0.017	0.114

*Values are given as Spearman correlation coefficients.
†$p < 0.01$.

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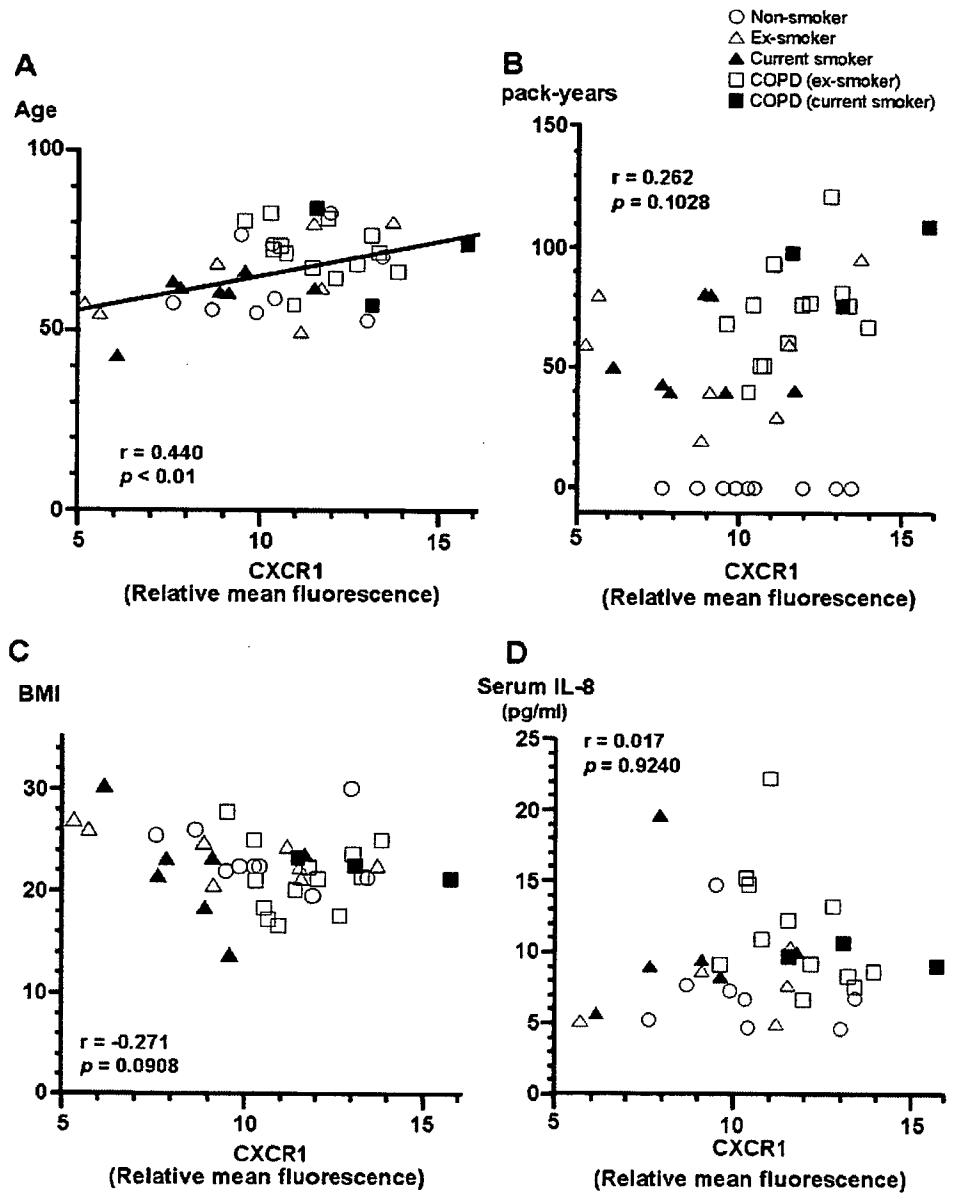

FIGURE 2. Correlation between the CXCR1 expression and background characteristics. *Top left, A*: overall expression of CXCR1 was positively correlated with age. Overall expression of CXCR1 was not correlated with the amount of smoking (*top right, B*), BMI (*bottom left, C*), and serum IL-8 levels (*bottom right, D*).

there was a significant positive correlation between the expression of CD-11b and CXCR1 ($r = 0.488$; $p < 0.01$). Therefore, we next evaluated whether the baseline serum levels of IL-8, a common ligand for CXCR1 and CXCR2, was involved in the expression of CD-11b and CXCR1. First, we measured the serum level of IL-8 in each group of subjects. As shown in Figure 3, the serum IL-8 levels in COPD patients were significantly higher than those in HSs (HS, 8.1 ± 0.8; COPD patients, 10.9 ± 1.0 pg/mL; $p < 0.05$). However, there was no apparent correlation between serum IL-8 levels and the expression of CD-11b and CXCR1 (Table 2, Fig 2, *bottom right, D*). Background characteristics such as aging and smoking status were also not correlated with the serum levels of IL-8.

To elucidate whether the up-regulation of CD-11b and CXCR1 is associated with the alteration of the physiologic parameters, the correlation between the expression of these molecules and pulmonary function was evaluated. Neither CD-18

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Table 3—*Correlation Between β_2-Integrin and CXCR Expression**

Variables	CD-18	CXCR1	CXCR2
CD-11b	−0.082	0.488†	−0.022
CD-18		−0.132	0.126
CXCR1			0.413

*Values are given as Spearman correlation coefficients.
†$p < 0.01$.

nor CXCR2 was correlated with any of the parameters of pulmonary function. Neither the surface expression of CD-11b nor that of CXCR1 was correlated with the FVC percent predicted and inspiratory capacity percent predicted (Table 4; Fig 4 *top left, A, top right, B*; Fig 5 *top left, A, top right, B*). In contrast, there was a significant negative correlation between the CD-11b expression and the severity of airflow limitation ($r = -0.317$; $p < 0.05$) [Table 4, Fig 4, *bottom, C*]. Similarly, the expression of CXCR1 also had a significant negative correlation with the severity of the airflow limitation ($r = -0.383$; $p < 0.05$) [Table 4, Fig 5, *bottom, C*].

Discussion

In the present study, we have shown that the expressions of CD-11b and CXCR1 were significantly increased on the surface of circulating neutrophils in COPD patients. Furthermore, the degree of the CD-11b and CXCR1 expression had a significant negative correlation with the severity of the airflow limitation.

CD-11b is an adhesion molecule that is expressed on the neutrophil surface and plays an important role in the adhesion and migration of neutrophils from the systemic circulation into the airways, and in the activation of neutrophils.[23,24] Therefore, an alteration of CD-11b expression may affect the migratory function of neutrophils. It has been reported[16–18] that the expression of CD-11b in circulating neutrophils is already up-regulated in COPD patients, suggesting that neutrophils in the systemic circulation are primed in COPD patients. The results in our present study are consistent with these reports and suggest that the neutrophil migratory function might be facilitated in the systemic circulation in COPD patients. In contrast, it has been recently reported[25] that the chemotactic activity of IL-8 is reduced in neutrophils from patients with severe COPD compared with those from patients with mild COPD. The precise reason for this discrepancy is unclear. However, it might be due to differences in the techniques employed or the patient characteristics.

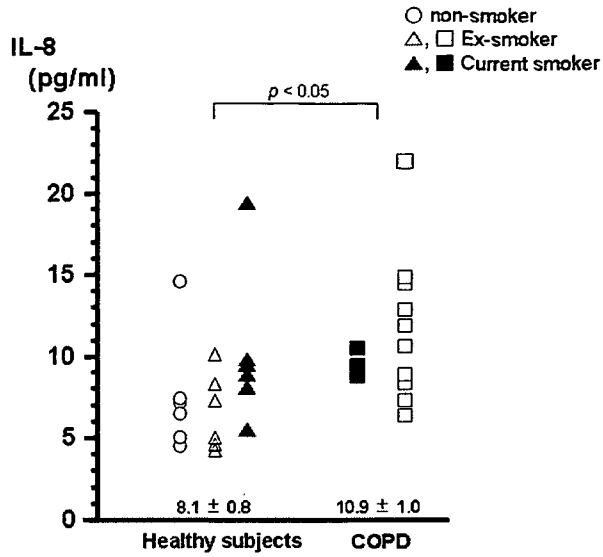

FIGURE 3. Serum levels of IL-8. The serum levels of IL-8 in the COPD patient group were significantly higher than those in the HS group.

CXCR1 activates neutrophils and mediates neutrophil functions including chemotaxis,[26] intracellular calcium changes,[27] phospholipase D activation,[27] and the respiratory burst[27,28] through the binding of specific ligands such as IL-8. Therefore, our present results show the possibility that, in patients with COPD, neutrophils are primed and susceptible to activation while in the systemic circulation, which is an issue that needs to be clarified by further investigation. With regard to the expression of CXCRs, controversial results have been reported. Pignatti et al[18] have shown that CXCR2 expression in circulating neutrophils, but not CXCR1 expression, was decreased in COPD patients, whereas Nicholson et al[19] have shown more recently that there was not difference in both CXCR1 and CXCR2 expression between COPD patients and HSs. Both results are inconsistent with those of the present study. However, the airflow limitation of the subjects in these reports was less severe than in the patients in the present study. Therefore, there is a possibility that

Table 4—*Correlation Between β_2-Integrin, Chemokine Receptor Expression, and Pulmonary Function**

Variables	CD-11b	CD-18	CXCR1	CXCR2
FVC % predicted	0.038	−0.041	−0.124	0.003
IC % predicted	−0.046	0.025	−0.107	0.072
FEV_1 % predicted	−0.317†	−0.257	−0.383†	−0.045

*Values are given as Spearman correlation coefficients.
†$p < 0.05$.

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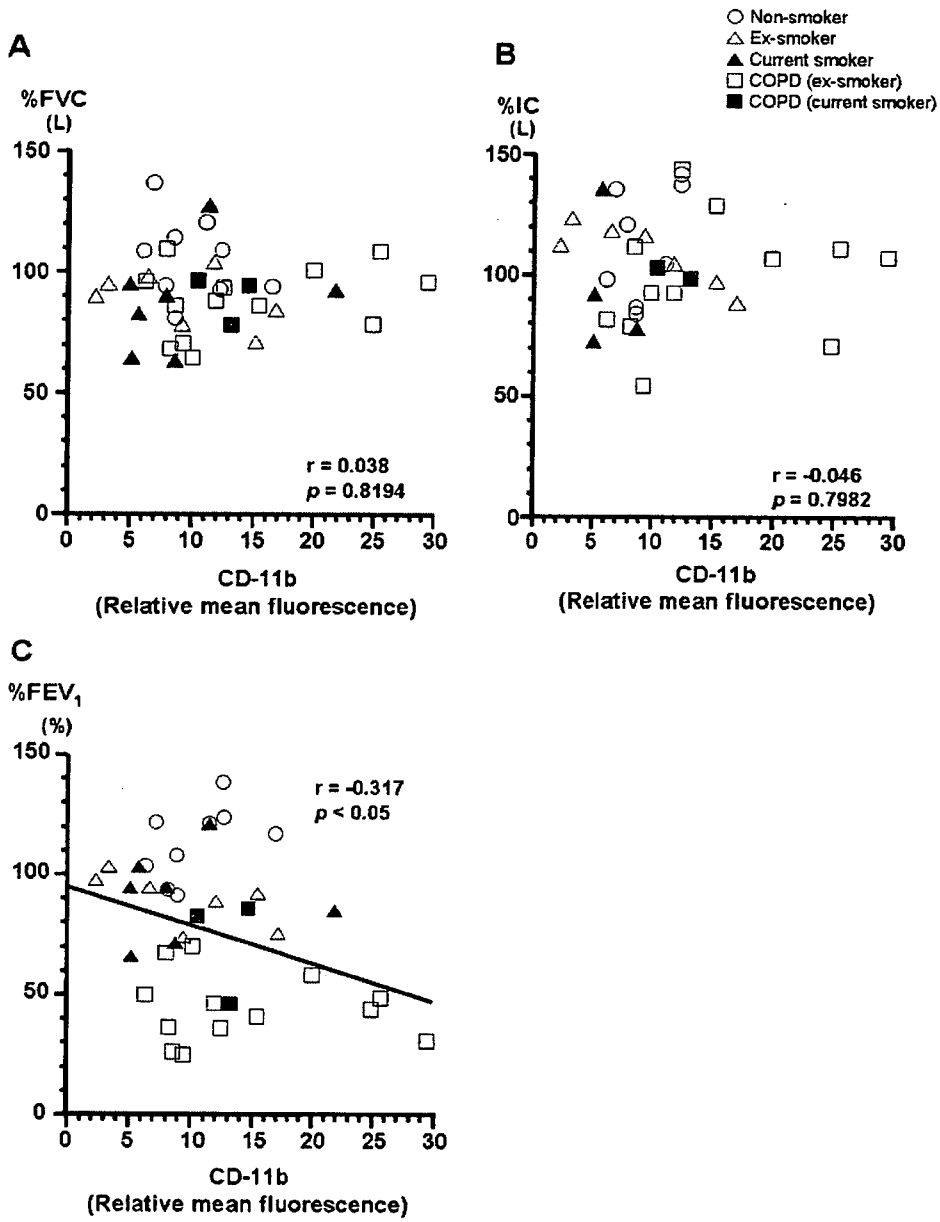

FIGURE 4. Correlation between the expression of CD-11b and pulmonary function. There was no significant correlation between the expression of CD-11b and the percent predicted FVC (*top left*, A) or the percent predicted IC (*top right*, B). *Bottom*, C: the expression of CD-11b showed a significant negative correlation with the severity of airflow limitation. %FVC = FVC percent predicted; %IC = IC percent predicted; $\%FEV_1$ = FEV_1 percent predicted.

our present results might show the expression of CXCRs in patients with more severe COPD. In addition, in the study by Nicholson et al,[19] the number of the subjects was relatively small. Thus, such differences may account for the discrepancy in the CXCR expression.

Among various background characteristics, except age in CXCR1 expression, no other background factors including smoking amount and BMI had any correlation with the expression of CD-11b, CD-18, and CXCR1. In the present study, there was a difference in age between the two subject groups. Thus, there is a possibility that the up-regulation of CXCR1 expression in COPD patients was due to the difference in age. However, there was no such correlation within the COPD group alone. Therefore, aging could be a factor in the expression of CXCR1; however, some other mechanism might also be related to the up-regulation of CXCR1 expression. It has been

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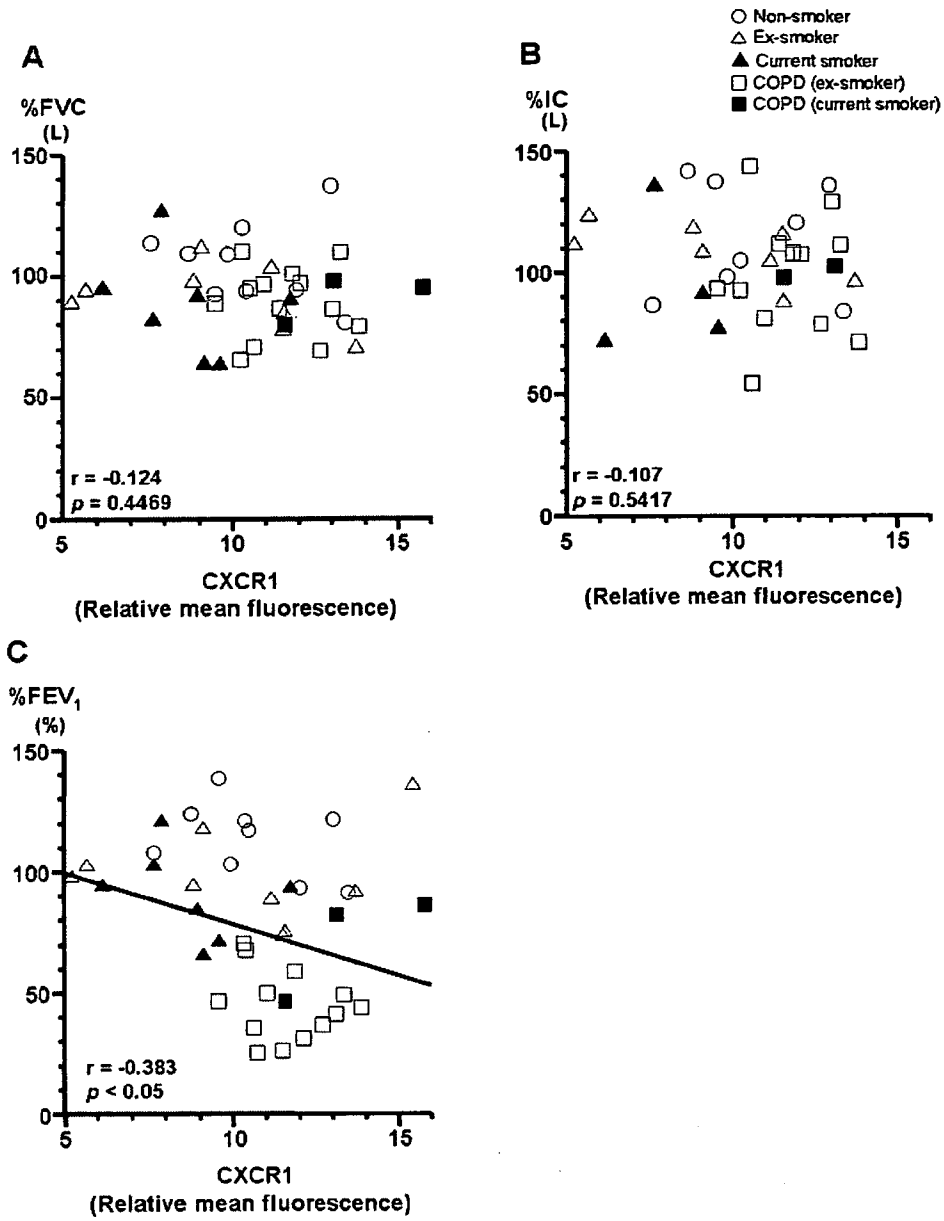

FIGURE 5. Correlation between the expression of CXCR1 and pulmonary function. There was no significant correlation between the expression of CXCR1 and %FVC (*top left, A*) or %IC (*top right, B*). *Bottom, C*: the expression of CXCR1 showed a significant negative correlation with the severity of the airflow limitation. See the legend of Figure 4 for abbreviations not used in the text.

reported[29] that smoking causes an increase in neutrophil retention in the lung and has a stimulatory effect on granulocyte-macrophage colony-stimulating factor,[30] which may affect neutrophil function. In fact, a previous study[31] showed that cigarette smoke condensate augmented CD-11b/CD-18 expression in neutrophils. Thus, there is a possibility that smoking may cause the up-regulation of each molecule in circulating neutrophils. In the present study, the amount of smoking did not correlate with the overall expression of each molecule. However, in subjects who had a history of smoking (patients with COPD and smokers), the amount of smoking correlated only with CXCR1 expression (data not shown; $r = 0.427$; $p < 0.05$). Therefore, long-term exposure to smoking may play some role in the regulation of CXCR1. In the present study, the subject number was relatively small, which may affect the present results. Further investigation with a larger number of subjects will be needed to confirm this possibility.

Recent investigations have shown that granulo-

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cyte-macrophage colony-stimulating factor or bradykinin may enhance CXCR1 expression in human polymorphonuclear leukocytes[32] or human nasal tissue,[33] respectively. In addition, it has been demonstrated that IL-4 and IL-13 strongly increased CXCR1 and CXCR2 expression in human monocytes and macrophages.[34] It has been demonstrated[35] that the numbers of IL-4 and IL-13 immunopositive cells were increased in the bronchial submucosa of patients with chronic bronchitis. Thus, there is a possibility that various other mechanisms may contribute to the up-regulation of CXCR1 expression.

In the present study, a significant correlation was seen between the expression of CD-11b and that of CXCR1. This suggests that the specific ligand of CXCR1, IL-8, may cause the up-regulation of CD-11b through the activation of CXCR1. Indeed, it has been reported that the stimulation with IL-8 up-regulates the surface expression of Mac-1 cells in human neutrophils.[36,37] Therefore, we next evaluated whether the baseline serum levels of IL-8 were involved in the expression of these molecules. As shown in Figure 3, the serum levels of IL-8 were increased in patients with COPD. However, there was no significant correlation between serum IL-8 levels and the expression of each molecule. In addition, there was no correlation between serum IL-8 levels and background characteristics. Although we never evaluated whether the increased expression of CXCR1 is associated with the increased sensitivity for IL-8, these results suggest that baseline serum IL-8 levels may have no role in the up-regulation of CD-11b and CXCR1 expression.

It has been demonstrated[2–5] that neutrophils play a key role in the development of COPD. Indeed, it has been reported[2,6,7] that the infiltration of neutrophils is increased in induced sputum or BAL fluid from COPD patients. In addition, more importantly, it has also been shown that the extent of this increase in the infiltration of neutrophils in bronchial biopsy specimens[8] or induced sputum samples[2,3,9] has a significant negative correlation with the severity of airflow limitation. In the present study, as shown in Figures 4, *bottom, C*, and 5, *bottom, C*, the up-regulation of both CD-11b and CXCR1 expression in circulating neutrophils significantly correlated with the severity of the airflow limitation. Taken together, it was considered that the up-regulation of CD-11b and CXCR1 may have a role in the development of COPD, probably through the increase in the migration of neutrophils into the airways in response to various chemotactic factors including IL-8 and the activation of neutrophils in inflamed airways, which may cause the augmentation of airway inflammation and the subsequent progression of airflow limitation.

In conclusion, the surface expression of CD-11b and CXCR1 on circulating neutrophils was up-regulated in COPD patients compared with that in HSs. This up-regulation of CD-11b and CXCR1 expression was significantly correlated with the severity of the airflow limitation, suggesting that the up-regulation of these molecules may play a role in the enhancement of the neutrophil migration into airways and in the activation in airways, and then in the development of airflow limitation in COPD. However, the precise mechanisms of the regulation of the expression of these molecules are still unclear. Further investigation will be needed to elucidate the exact mechanisms by which these molecules are up-regulated and their role in the pathophysiology of COPD.

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Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung

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Abstract

The involvement of inflammation in the pathogenesis of chronic obstructive pulmonary disease (COPD) has been investigated using samples from relatively central airways such as airway biopsies, but there have been fewer studies in the peripheral lung, which is thought to be the main site of the disease process. To determine the molecules that relate to the mechanisms underlying the pathogenesis of COPD, we evaluated the mRNA expression of inflammatory cytokines, chemokines, oxidant enzymes, antioxidant enzymes, proteinases and antiproteinases in peripheral lung tissues from 33 COPD and non-COPD subjects who were undergoing lung resection for lung cancer using an RT-PCR technique.

Among the 42 studied candidate genes, the expressions of mRNA for catalase, glutathion *S*-transferase P1 (GSTP1), glutathion *S*-transferase M1 (GSTM1), microsomal epoxide hydrolase (mEPHX) and tissue inhibitor of metalloproteinase 2 (TIMP2) were significantly decreased in COPD lung tissues compared with those in non-COPD tissues, and most of these decreases were significantly correlated with the degree of airflow limitation. On the other hand, the expressions of mRNA for interleukin 1β (IL-1β), interleukin 8 (IL-8), growth-related oncogene-α (Gro-α) and monocyte chemotactic protein-1 (MCP-1) were significantly increased in COPD lungs. Most of these changes were also associated with cigarette smoking.

These data suggest that an impairment of protective mechanisms against oxidants and xenobiotics, in addition to the upregulation of CXC- and CC-chemokines, may be associated with cigarette smoking and involved in the inflammatory process of COPD.
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Keywords: Catalase; Glutathion *S*-transferase P1; Glutathion *S*-transferase M1; Microsomal epoxide hydrolase; Tissue inhibitor of metalloproteinase 2; mRNA; RT-PCR

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a disease characterized by airway inflammation and progressive airflow limitation that is not fully reversible. The morbidity and mortality of the disease have increased in recent years and it is a serious public health problem in many countries throughout the world [1]. Abnormal inflammatory responses of the lungs against noxious gases and particles, such as cigarette smoke, are thought to cause small airway disease, namely obstructive bronchiolitis, and parenchymal destruction, leading to the pathophysiologic changes of COPD such as airflow limitation [2]. Although the precise mechanisms of these processes have not been fully clarified, several mechanisms have been suggested to contribute to the disease process.

The cellular inflammatory response in COPD is characterized by increases in neutrophils, macrophages and CD8-positive T lymphocytes in the lungs [3]. Inflammatory mediators from these cells and epithelial cells contribute to interactions among these cells and cause the

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pathophysiological changes of COPD including mucus hypersecretion, fibrosis, and parenchymal destruction [4]. Oxidative stress, which may result from excessive oxidants and/or impaired antioxidant activities, is thought to be one of the major causes of inflammation and injury in diseased lungs. Oxidative stress arises during the inflammatory process in the lung and also from the environment. Both endogenous reactive oxygen species released from inflammatory cells such as neutrophils and macrophages and oxidant compounds in cigarette smoke or air pollution cause injuries to lung tissues [5]. These harmful molecules are eliminated by antioxidant enzyme activities in normal lungs [6]. In COPD lungs, these protective mechanisms seem to be impaired. Another mechanism in the COPD pathogenesis is an imbalance of proteinase and antiproteinase. α_1-antitrypsin has been shown to be involved in the structural changes in COPD, and a number of proteinases and antiproteinases have been reported to play roles in the inflammatory process in COPD [7].

The involvement of inflammation, oxidative stress and a proteinase/antiproteinase imbalance in the pathogenesis of COPD has been investigated using samples from COPD subjects. Many studies employing samples from relatively central airways such as airway biopsies and induced sputum have revealed an alteration in the formation of inflammatory mediators, oxidant, antioxidant, proteinase and antiproteinase in COPD airways. However, there have been fewer studies in the peripheral lung, which is thought to be the main site of the disease process.

In this study, we evaluated the expressions of 42 genes for inflammatory cytokines, chemokines, oxidant enzymes, antioxidant enzymes, proteinases and antiproteinases in peripheral lung tissues from COPD and non-COPD subjects. We found decreased mRNA expressions for catalase, glutathion *S*-transferase P1 (GSTP1), glutathion *S*-transferase M1 (GSTM1), microsomal epoxide hydrolase (mEPHX) and tissue inhibitor of metalloproteinase 2 (TIMP2) and increased expressions for interleukin 1β (IL-1β), interleukin 8 (IL-8), growth-related oncogene-α (Gro-α) and monocyte chemotactic protein-1 (MCP-1) in the COPD lung. Most of these changes were associated with the degree of airflow limitation and cigarette smoking.

2. Materials and methods

2.1. Subjects

Thirty-three patients with or without COPD who were undergoing lung resection for lung cancer took part in the study after giving written informed consent. This study was approved by the Tohoku University Committee on Clinical Investigations and by the Ethics Review Board of Miyagi Prefectural Cancer Center. According to the presence or absence of COPD and a history of smoking, the subjects were divided into three groups: 10 non-COPD subjects who never smoked, 9 non-COPD smokers and 14 COPD subjects. All patients with COPD satisfied the Global Initiative for Chronic Obstructive Lung Disease guidelines [2] and were diagnosed as having pulmonary emphysema through computed tomography. The smokers were divided into current smokers and ex-smokers, defined as those who had quit smoking for at least 3 months prior to the study. The clinical characteristics of the study subjects are shown in Table 1. All patients were stable and had no respiratory tract infection during the month preceding the study. Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), maximum flow rate at 50% of vital capacity divided by measured body height (V_{50}/HT) and maximum flow rate at 25% of vital capacity divided by measured body height (V_{25}/HT) were measured using a dry rolling-seal spirometer (FUDAC-70; Fukuda Denshi Co., Ltd., Tokyo, Japan) in the week before surgery.

2.2. Lung tissue collection

Peripheral lung tissue was obtained from the subpleural parenchyma of the lobe resected at surgery, avoiding areas involved by tumor. The tissue specimen (size 5 × 5 × 10 mm) was immediately immersed in Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and kept at −80 °C until RT-PCR analysis.

2.3. Isolation of total RNA and real-time quantitative PCR

Total RNA from each sample was extracted in guanidine isothiocyanate and phenol (Isogen) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed with random hexamer and MultiScribe Reverse Transcriptase using TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA). cDNA samples corresponding to 8 ng of total RNA were measured by real-time quantitative PCR using Applied Biosystems prism 7900HT Sequence Detection System according to the manufacturer's instructions. The primers and probes were

Table 1
Characteristics of study subjects

	Non-COPD		COPD
	Never smoked	Smokers	
Number	10	9	14
Male/Female	2/8	5/4	12/2
Age (year)	66.6±2.5	57.1±5.3	72.9±1.5††
Smoking status (ex/current)	—	5/4	11/3
Smoking history (pack-year)	0	21.6±6.7**	51.6±6.0**
%FVC (%)	122.3±8.3	107.3±3.0	89.6±4.5**
%FEV_1 (%)	135.6±9.7	111.7±3.2	78.5±6.7**
FEV_1/FVC% (%)	79.8±1.4	77.2±1.0	58.6±3.0**

The data are expressed as mean±SEM.
**$P<0.01$ compared with the non-COPD never-smoked group (Mann–Whitney *U*-test).
††$P<0.01$ compared with the non-COPD smoker group (Mann–Whitney *U*-test).

obtained from TaqMan Gene Expression Assays or Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA) or designed for each of the genes according to the Primer Express 2.0 program provided by Applied Biosystems (Table 2). Reporter dyes and quencher dyes for all designed probes were 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), respectively.

The amount of target message in each sample was estimated from a threshold cycle number (CT), which is inversely correlated with the number in its initial mRNA level and used to determine gene expression. The CT-values were generated by the ABI PRISM 7900HT SDS software version 2.0. The CT value of each gene was normalized using the formula $\Delta CT = (CT \text{ of each gene}) - (CT \text{ of } \beta\text{-actin})$. The expression levels of each gene per β-actin were calculated according to the formula $2^{-(\Delta CT)}$.

2.4. Statistical analysis

All data are expressed as mean $\pm$ SEM. Statistical analysis was carried out using nonparametric analysis of variance (Kruskal–Wallis test) to evaluate variance among the three groups. If a significant variance was found, an unpaired two-group test (Mann–Whitney *U*-test) was used to determine significant differences between individual groups. For analysis within group correlations, Spearman's rank correlation test was used. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. mRNA expressions of the 42 studied candidate genes

Table 3 shows all the data of the mRNA amounts observed in this study. We found some significant differences between COPD and non-COPD subjects. The expressions of mRNA for catalase, GSTP1, mEPHX, GSTM1 and TIMP2 were significantly decreased in COPD lung tissues compared with those in non-COPD tissues. On the other hand, the mRNA expressions for IL-1β, IL-8, Gro-α and MCP-1 were significantly increased in COPD compared with non-COPD. None of the other studied genes showed significant changes in mRNA expression in COPD versus non-COPD subjects.

3.2. Decreased expression of enzymes protective against oxidants and xenobiotics

The expressions of mRNA for catalase, GSTP1 and mEPHX were significantly decreased in COPD lung tissues compared with those in non-COPD tissues (Fig. 1). The expression of mRNA for catalase was significantly decreased in lung tissues from COPD subjects (0.165 ± 0.013) compared with those from non-COPD smokers (0.233 ± 0.026, $P = 0.023$) and those who never smoked (0.251 ± 0.025, $P = 0.003$). The GSTP1 mRNA expression was significantly decreased in lung tissues from COPD subjects (0.449 ± 0.027) compared with those from non-COPD smokers (0.554 ± 0.028, $P = 0.023$) and those who never smoked (0.647 ± 0.069, $P = 0.019$). The mEPHX mRNA expression was significantly decreased in lung tissues from COPD subjects (0.153 ± 0.014) compared with those from non-COPD smokers (0.223 ± 0.014, $P = 0.003$) and those who never smoked (0.228 ± 0.028, $P = 0.022$). The expression of mRNA for GSTM1 was significantly increased in lung tissues from non-COPD smokers (0.229 ± 0.044) compared with those from non-COPD subjects who never smoked (0.136 ± 0.033, $P = 0.034$) and those from COPD subjects (0.126 ± 0.019, $P = 0.014$) (Fig. 2).

3.3. Decreased expression of antiproteinase

The expression of mRNA for TIMP2 was significantly increased in lung tissues from non-COPD smokers (1.135 ± 0.101) compared with those from non-COPD subjects who never smoked (0.873 ± 0.166, $P = 0.014$) and those from COPD subjects (0.870 ± 0.110, $P = 0.038$) (Fig. 2).

3.4. Increased expression of inflammatory cytokines and chemokines

The expressions of mRNA for IL-1β (0.203 ± 0.058 vs. 0.051 ± 0.021, $P = 0.004$), IL-8 (0.618 ± 0.188 vs. 0.071 ± 0.036, $P = 0.003$), Gro-α (0.015 ± 0.04 vs. 0.002 ± 0.001, $P = 0.005$) and MCP-1 (1.300 ± 0.316 vs. 0.286 ± 0.120, $P = 0.002$) were significantly increased in lungs from COPD subjects compared with those from subjects who never smoked. The differences in the expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 between in COPD subjects and in non-COPD smokers were not statistically significant (Fig. 3).

3.5. Correlations between gene expressions and pulmonary function and cigarette smoking habit

The expressions of mRNA for catalase, GSTP1 and mEPHX were significantly correlated with the parameters of pulmonary function: % of predicted values of forced vital capacity (%FVC), % of predicted values of forced expiratory volume in one second (%FEV_1), FEV_1/FVC%, V_{50}/HT and V_{25}/HT (Fig. 4 and Table 4). The mRNA expressions for these enzymes were negatively correlated with cigarette smoking history assessed as pack-year (Table 4).

In contrast, the expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 were negatively correlated with V_{50}/HT and were associated with cigarette smoking history (Table 4). The expressions of IL-8 and Gro-α were also negatively correlated with FEV_1/FVC% (Table 4).

When analyzed in two groups of non-COPD smokers and COPD subjects, the expression of mRNA for TIMP2

Table 2
Real-time quantitative PCR primers and probes

Gene	Accession no.	Sequence or assay ID	
XDH	NM_000379	Hs00166010_m1	
HMOX1	NM_002133	Hs00157965_m1	
GPX3	genbank:NM_002084NM_002084	TCCCTGCAACCAATTTGGA	Forward primer: 593–613
		CCATTGACATCCCCTTTCTCA	Reverse Primer: 723–703
		TCCTTCCTACCCTCAAGTATGTCCGACCA	TaqMan probe: 642–670
GSTM1	NM_000561	AGGACTTCATCTCCCGCTTTG	Forward primer: 599–619
		CCATCTTTGAGAACACAGGTCTTG	Reverse Primer: 691–668
		TCTGCCTACATGAAGTCCAGCCGCTT	TaqMan probe: 637–662
GSTP1	NM_000852	CCTCCGCTGCAAATACATCTC	Forward primer: 326–346
		CAGTGCCTTCACATAGTCATCCTT	Reverse Primer: 398–375
		CTCATCTACACCAACTATGAGGCGGG	TaqMan probe: 348–373
GSTT1	NM_000853	CATAAGGTGATGTTCCCTGTGTTC	Forward primer: 346–369
		GGAGATGTGAGGACCAGTAAGGA	Reverse Primer: 492–470
		TCGAGGACAAGTTCCTCCAGAACAAGGC	TaqMan probe: 440–467
SOD1	NM_000454	AAAACACGGTGGGCCAAA	Forward primer: 210–227
		ACATCGGCCACACCATCTTT	Reverse Primer: 293–274
		CCCAAGTCTCCAACATGCCTCTCTTCA	TaqMan probe: 257–231
SOD2	NM_000636	Hs00167309_m1	
SOD3	NM_003102	Hs00162090_m1	
Catalase	NM_001752	Hs00156308_m1	
mEPHX	NM_000120	Hs00164458_m1	
MMP1	NM_002421	Hs00233958_m1	
MMP2	NM_004530	Hs00234422_m1	
MMP9	NM_004994	Hs00234579_m1	
MMP12	NM_002426	Hs00159178_m1	
ELA2	NM_001972	Hs00357734_m1	
TIMP1	NM_003254	Hs00171558_m1	
TIMP2	NM_003255	Hs00234278_m1	
SERPINA1	NM_000295	Hs00165475_m1	
SLPI	NM_003064	Hs00268206_m1	
IL-13	NM_002188	H327046T	
TNF-α	NM_000594	H327055T	
TNF-α R	NM_001065	GCTTCAGAAAACCACCTCAGACA	Forward primer: 552–574
		ATGCCGGTACTGGTTCTTCCT	Reverse Primer: 683–663
		TCAGCTGCTCCAAATGCCGAAAGG	TaqMan probe: 580–603
IL-1β	NM_000576	4327035T	
IFN-α	NM_000619	4327052T	
IL-17	NM_002190	4327048T	
GM-CSF	NM_000758	4327057T	
IL-10	NM_000572	4327043T	
Gro-α	NM_001511	TTCTGAGGAGCCTGCAACATG	Forward primer: 754–774
		TCCCCTGCCTTCACAATGAT	Reverse Primer: 842–823
		CGGATCCAAGCAAATGGCCAATGA	TaqMan probe: 798–821
IL-8	NM_000584	CGGAAGGAACCATCTCACTGT	Forward primer: 73–93
		ATCAGGAAGGCTGCCAAGAGA	Reverse Primer: 145–125
		GTAAACATGACTTCCAAGCTGGCCGTG	TaqMan probe: 96–122
BLT1	NM_000752	CATCTGGGTGTTGTCCTTTCTG	Forward primer: 2137–2158
		GACAGCCTCGAAGATTAGATGGA	Reverse Primer: 2278–2256
		TGCCCTGGAAAACGAACATGAGCC	TaqMan probe: 2193–2216
BLT2	NM_019839	ACCTTCTCATCGGGCATCAC	Forward primer: 1624–1643
		GAAGTCTTCCAGCTCAGCAGTGT	Reverse Primer: 1767–1745
		CCACCTGTAGGCCCAGAAGGATGT	TaqMan probe: 1692–1715
VEGF	NM_003376	Hs00173626_m1	
MUC5AC	AJ298317	TACTCCACAGACTGCACCAACTG	Forward primer: 1263–1285
		CGTGTATTGCTTCCCGTCAA	Reverse Primer: 1391–1372
		TGTGCTTGGAGGTGCCCACTTCTCAA	TaqMan probe: 1343–1368
MUC5B	Z72496	ACCGACCACAGAGCTGGAGA	Forward primer: 863–882
		ATGTCAGTCCTTCTGAGAGGGTG	Reverse Primer: 1005–983
		TTCTCAACGCCGCAGCCTACGAGT	TaqMan probe: 912–935
MUC8	U14383	CGGCCATCATTCCTTTTTACTG	Forward primer: 1226–1247
		AACCCACATGACCATCAACTGA	Reverse Primer: 1315–1294
		CTGTGTGAATCCACCGCTAGAAACCCA	TaqMan probe: 1263–1289
CTSL	NM_145918	Hs00377632_m1	

Table 2 (*continued*)

Gene	Accession no.	Sequence or assay ID
MCP-1	NM_002982	4329524T
TGF-β	NM_000660	4327054T
EGF	NM_001963	Hs00153181_m1
IL-4	NM_000589	4327038T
IL-5	NM_000879	4327039T

Numbers refer to oligonucleotide position contained within the published cDNA.

was significantly correlated with V_{50}/HT ($r_s = 0.469$, $P = 0.028$), but not with other parameters including %FEV_1 ($r_s = 0.376$, $P = 0.077$) nor cigarette smoking history ($r_s = -0.342$, $P = 0.106$). The expression of mRNA for GSTM1 had no significant relationship with parameters for airflow limitation (data not shown) and cigarette smoking history ($r_s = -0.393$, $P = 0.065$).

In smokers with and without COPD, there was no association between the mRNA expressions for these molecules and duration since smoking cessation (data not shown).

4. Discussion

In this study, we observed decreased expressions of mRNA for catalase, GSTP1, GSTM1, mEPHX and TIMP2 and increased expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 in peripheral lungs from patients with COPD. Most of these changes in mRNA expressions were associated with the degree of airflow limitation and with the cigarette smoking habit.

Although the precise mechanisms of the pathogenesis of COPD have not been fully elucidated, it is thought that the inflammatory responses of the lungs induced by noxious gases and particles, such as oxidants and xenobiotics, play a major role in the onset and progression of the disease. An impairment of protective mechanisms of the lungs against such harmful molecules would also contribute to the disease process.

Oxidative stress and imbalances in the host defense mechanisms appear to be among the causes of COPD. Reactive oxygen/nitrogen species have been suggested to be involved in the pathophysiology of COPD through several studies analyzing airway samples such as induced sputum [8,9]. It also has been reported that antioxidant enzyme activities are altered in COPD. In the present study, the mRNA expression for catalase, a catalyzing enzyme for the clearance of hydrogen peroxide, was decreased in the peripheral lungs from COPD subjects. It has been reported that catalase activity is decreased in circulating red blood cells in patients with COPD [10], while the level of catalase is often increased in those from smokers, probably due to upregulation as an adaptive response against oxidative stress by cigarette smoking [11]. Recently, Ning and colleagues have reported the decreased gene expression for catalase in surgically obtained lung tissue from patients with COPD, using a serial analysis of gene expression [12]. Our results are consistent with their result and further extend the findings by showing that the decrease in catalase mRNA expression in the peripheral lung tissue was correlated to the degree of airflow limitation in COPD patients. These results suggest that impaired protective activity of catalase against oxidative stress may lead to enhanced pathophysiological changes in COPD.

Other protective mechanisms against toxic substrates involve xenobiotic-metabolyzing enzymes such as GSTP1, GSTM1 and mEPHX, the mRNA expressions of which were decreased in COPD patients in the present study. GSTP1 and GSTM1 are members of the glutathione *S*-transferase family known as antioxidative xenobiotic enzymes. GSTP1 and GSTM1 are expressed in alveoli, alveolar macrophages and respiratory bronchioli in the peripheral lung [13], and play an important role in the detoxification of xenobiotics which are contained in cigarette smoke and occupational and environmental pollutants. GSTP1 has been reported to exert a protective effect against cigarette smoke extract in human lung fibroblasts in vitro [14]. mEPHX is strongly expressed in bronchial epithelial cells in the lung [15], and is involved in the first-pass metabolism of highly reactive epoxide intermediates. Several genetic researches also have suggested that polymorphisms of GSTP1, GSTM1 and mEPHX genotypes may associate with a susceptibility to COPD or the severity of the disease in some races including Japanese [16–18].

Also, members of the TIMP family exert inhibitory activities against matrix metalloproteinases the involvement of which in the disease process of COPD has been suggested [19]. Recently, it has been reported that polymorphisms of the TIMP2 gene are associated with COPD susceptibility in a Japanese population suggesting a relationship between the decreased activity of TIMP2 and the pathogenesis of the disease [20]. It has been also reported that TIMP2 mRNA expression is decreased in the lung tissue from severer COPD subjects [12]. Our data are in line with these reports.

In the present study, decreased mRNA expressions for catalase, GSTP1 and mEPHX were evident in the peripheral lung tissue in COPD. The degree of the decrease in the mRNA expressions for these antioxidant and xenobiotic enzymes significantly correlated with the degree of airflow limitation, %FVC, %FEV_1, FEV_1/FVC%, V_{50}/HT

Table 3
mRNA expressions of the 42 studied candidate genes

	Non-COPD		COPD
	Never smoked	Smokers	
Genes that are significantly downregulated in COPD versus non-COPD			
Catalase	0.251±0.025	0.233±0.026	0.165±0.013**,†
GSTP1	0.647±0.069	0.554±0.028	0.449±0.027*,†
mEPHX	0.228±0.028	0.223±0.014	0.153±0.014*,††
GSTM1	0.136±0.033	0.229±0.044*	0.126±0.019†
TIMP2	0.873±0.166	1.135±0.101*	0.870±0.110†
Genes that are significantly upregulated in COPD versus non-COPD			
IL-1β	0.051±0.021	0.105±0.037	0.203±0.058**
IL-8	0.071±0.036	0.237±0.093	0.618±0.188**
Gro-α	0.002±0.001	0.009±0.006	0.015±0.005**
MCP-1	0.286±0.120	0.739±0.255	1.299±0.316**
Genes where no significant change is observed in COPD versus non-COPD			
XDH	0.001±0.000	0.001±0.000	0.001±0.000
HMOX1	0.133±0.020	0.211±0.040	0.333±0.083
GPX3	0.736±0.178	1.030±0.117	0.831±0.206
GSTT1	0.013±0.006	0.022±0.012	0.023±0.008
SOD1	0.058±0.008	0.047±0.010	0.045±0.007
SOD2	0.384±0.090	0.517±0.122	0.660±0.139
SOD3	0.221±0.018	0.234±0.013	0.187±0.013
MMP1	0.001±0.001	0.002±0.002	0.004±0.002
MMP2	0.649±0.113	0.867±0.161	0.816±0.091
MMP9	0.009±0.002	0.016±0.010	0.012±0.004
MMP12	0.002±0.001	0.002±0.001	0.002±0.001
ELA2	0.001±0.000	0.001±0.000	0.001±0.000
TIMP1	0.545±0.148	0.766±0.240	0.931±0.178
SERPINA1	0.529±0.062	0.685±0.084	0.804±0.085
SLPI	0.524±0.173	0.354±0.089	0.293±0.050
IL-13	0.001±0.001	0.001±0.001	0.003±0.001
TNF-α	0.019±0.003	0.034±0.005	0.034±0.006
TNF-α R	0.114±0.013	0.146±0.011	0.131±0.011
IFN-γ	0.001±0.001	0.002±0.001	0.001±0.000
IL-17	0.000±0.000	0.000±0.000	0.000±0.000
GM-CSF	0.000±0.000	0.000±0.000	0.000±0.000
IL-10	0.004±0.001	0.007±0.002	0.006±0.001
BLT1	0.004±0.000	0.004±0.000	0.003±0.000
BLT2	0.001±0.000	0.002±0.000	0.001±0.000
VEGF	0.412±0.053	0.491±0.039	0.397±0.047
MUC5AC	0.011±0.010	0.004±0.004	0.000±0.000
MUC5B	0.001±0.001	0.001±0.001	0.001±0.000
MUC8	0.001±0.000	0.001±0.000	0.001±0.000
CTSL	0.118±0.019	0.099±0.014	0.134±0.018
TGF-β	0.121±0.013	0.161±0.012*	0.159±0.012
EGF	0.000±0.000	0.000±0.000	0.001±0.000
IL-4	0.000±0.000	0.000±0.000	0.000±0.000
IL-5	0.000±0.000	0.000±0.000	0.001±0.000

The data are expressed as mean±SEM.
*$P<0.05$, **$P<0.01$ compared with the non-COPD never-smoked group (Mann–Whitney U-test).
†$P<0.05$, ††$P<0.01$ compared with the non-COPD smoker group (Mann–Whitney U-test).

and V_{25}/HT. These data suggest that impairments in the activities of these enzymes protective against oxidants and xenobiotics could contribute to the pathophysiological changes in COPD. Since reactive xenobiotics also inhibit antiproteinases and increase proteinase secretion from neutrophils, a combination of oxidative stress and an imbalance of proteinase/antiproteinases may aggravate the airway inflammation and tissue disruption in COPD. On the other hand, the expressions of mRNA for GSTM1 and TIMP2 were significantly increased in lung tissues from non-COPD smokers compared with those from non-COPD subjects who never smoked, but were not increased in lung tissues from COPD subjects. These changes may imply that GSTM1 and TIMP2 could be upregulated due to cigarette smoking as adaptive responses and that these protective mechanisms could be impaired in COPD lungs leading to the development of the disease.

Decreased mRNA expression levels of catalase, GSTP1 and mEPHX, but not GSTM1 and TIMP2, were associated with cigarette smoking history assessed as the amount of cigarettes smoked (pack-years). The relationships between cigarette smoke exposure and the expressions of these enzymes are uncertain. However, some studies have suggested that cigarette smoke exposure may decrease the activities or expressions of catalase and GSTP1. The catalase activity in erythrocytes was decreased in cigarette smokers compared with non-smokers, and was slowly increased in smokers after smoking cessation [21]. The activity of GST in lung tissue was reduced by cigarette smoking [22]. In another study, ongoing cigarette smoke exposure correlated with depressed levels of GSTP1 mRNA expression in buccal cells [23]. Also, cigarette smoke exposure to a previously tobacco-naïve subject induced a transient decline of GSTP1 mRNA expression in such cells [23]. As for mEPHX, however, its activity and mRNA expression have been reported so far to be not affected in rat tissues including lungs [24] or enhanced in the lungs of patients with lung cancer [22] by cigarette smoke exposure. In those studies, the changes of mEPHX activity might have resulted from the relatively short-term effect of cigarette smoke exposure. The negative correlation between the mEPHX mRNA expression in the peripheral lung tissues and cigarette smoking in the present study may suggest a long-term effect of cigarette smoke exposure on the mEPHX expression in the lungs. The mechanisms of the decreases in the mRNA expressions for catalase, GSTP1 and mEPHX in the peripheral lung tissue by the effects of cigarette smoke are unknown. Further investigation that addresses this issue may increase our understanding about the cigarette smoke-induced processes by which the protective mechanisms against toxic substrates in the lungs become impaired.

In the present study, we found elevated expression levels of mRNA for several inflammatory cytokines and chemokines, IL-1β, IL-8, Gro-α and MCP-1, in the lungs from COPD subjects. The cellular inflammatory response in COPD is dominated by neutrophils, macrophages and CD8-positive T lymphocytes in the lungs [3]. It has been reported that increased activities of inflammatory cytokines and chemokines related to these inflammatory cells are associated with COPD and cigarette smoke exposure