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Table 4 HRs (95% CI) for development of isolated IFG or IGT according to the quartiles of %FVC*or FEV_1%†

	I	II	III	IV	p for trend
IFG					
%FVC					
Model 1	1.0	0.85 (0.38 to 1.92)	0.81 (0.36 to 1.79	1.96 (0.71 to 5.26)	0.31
Model 2	1.0	1.07 (0.48 to 2.39)	1.35 (0.60 to 3.03)	0.54 (0.20 to 1.49)	0.32
FEV_1/FVC					
Model 1	1.0	0.96 (0.42 to 2.17)	1.20 (0.51 to 2.86)	0.98 (0.43 to 2.27)	0.95
Model 2	1.0	0.99 (0.43 to 2.31)	0.84 (0.35 to 2.00)	1.04 (0.45 to 2.47)	0.96
IGT					
%FVC					
Model 1	1.0	1.96 (1.00 to 3.85)	2.63 (1.27 to 5.56)	3.03 (1.43 to 6.67)	0.006
Model 2	1.0	2.22 (1.02 to 3.88)	2.26 (1.07 to 4.78)	2.74 (1.26 to 5.98)	0.02
FEV_1/FVC					
Model 1	1.0	2.13 (0.96 to 4.76)	1.67 (0.81 to 3.45)	1.03 (0.54 to 1.96)	0.15
Model 2	1.0	2.09 (0.92 to 4.72)	1.69 (0.81 to 3.52)	1.11 (0.57 to 2.16)	0.10
IFG or IGT					
%FVC					
Model 1	1.0	2.13 (0.93 to 3.03)	1.85 (1.03 to 3.57)	2.63 (1.43 to 4.76)	0.01
Model 2	1.0	1.48 (0.89 to 2.44)	1.38 (0.82 to 2.34)	2.40 (1.30 to 4.44)	0.04
FEV_1/FVC					
Model 1	1.0	1.47 (0.84 to 2.56)	1.47 (0.85 to 2.56)	1.01 (0.61 to 1.69)	0.32
Model 2	1.0	1.47 (0.83 to 2.61)	1.47 (0.84 to 2.56)	1.09 (0.64 to 1.84)	0.21

*%FVC quartile; I (highest group) (≥106.0%), II (96.6%≤%FVC<106.0%) III (88.1%≤%FVC<96.6%), IV (lowest group) (%FVC<88.1%).
†FEV_1/FVC quartile; I (highest group) (≥85.0%), II (80.9%≤FEV_1/FVC<85.0%), III (76.0%≤FEV_1/FVC<80.9%), IV (lowest group) (FEV_1/FVC<76.0%).
IGT, impaired glucose tolerance; IFG, increased fasting glucose.
Model 1 denotes crude model and model 2, adjusted for age, BMI, pack-year smoking and systolic BP.

prevalence.[8 9] In addition, such association between lower lung function and impaired glucose metabolism was also demonstrated in Western populations with higher BMI but lower smoking prevalence, and the association had been shown to be independent of smoking or obesity (refs. [1–6], for review ref. [7]).

The mechanisms for the association are not clarified at present. It has been suggested that IGT is caused mainly by insulin resistance in the muscle, and IFG mainly by insulin resistance in the liver.[25] Reduced lung volume is associated with reduced maximum oxygen uptake, which may lead to poorer physical fitness and physical activity, and thus result in insulin resistance and DM.[26–28] This may explain why IGT is more closely associated with lung volume. Furthermore, poorer lung function in adulthood may be due to low birth weight or early-life malnutrition,[29 30] both of which have been reported to be associated with the development of diabetes.[31] Malnutrition as a neonate may be an important early cause of cardiac and metabolic disorders in adulthood as a consequence of fetal programming.[32 33]

This study had several limitations. The study population was limited to men, owing to the fact that sufficient female subjects were not available at the institute. The occupational cohort used in this study may not be representative of Japanese men in general. For example, the prevalence rates of hypertension and hyperlipidaemia in this cohort were 13% and 7%, respectively (data not shown). The National Health and Nutrition Examination Survey in Japan showed prevalence rate of these in general Japanese men aged 40–60 years, in general, were around 30% and 35%, respectively, suggesting that our occupational cohort may be healthier. Subjects taking medications, including simvastatin, which have been shown to lower the risk of impaired glucose metabolism were not excluded, although the distributions of %FVC and the FEV_1/FVC ratio in those taking drugs for hypertension, dyslipidaemia and hyperuricaemia were not significantly different from those of subjects not on such medication.

In conclusion, this study provides evidence for a prospective relationship between lung volume and the incidence of newly diagnosed prediabetes among subjects with normal glucose metabolism at baseline. Among subjects with prediabetes, the study also suggests that lung volume may be a risk factor for the development of IGT, which is mainly caused by insulin resistance in the

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muscle, but not IFG, which is caused mainly by insulin resistance in the liver. Although there is published evidence for an association between COPD and DM, our results suggest that prediabetes is not associated with at least the early stage of COPD.

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RESEARCH ARTICLE Open Access

Positive association between the plasma levels of 5-hydroxyindoleacetic acid and the severity of depression in patients with chronic obstructive pulmonary disease

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Abstract

Background: The role of plasma monoamines in patients with chronic obstructive pulmonary disease (COPD) with depression is unclear. To investigate monoamines in 20 depressed patients with COPD, the plasma concentrations of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid, and 3-methoxy-4-hydroxyphenylglycol (MHPG) were measured and compared with those in 50 non-depressed COPD patients, and also with 23 age- and gender-matched non-smokers and 13 smokers as non-depressed healthy controls.

Methods: Diagnosis of depression was assessed using the Centre for Epidemiologic Studies Depression Scale. Plasma concentrations of monoamines were measured by high-performance liquid chromatography.

Results: None of the depressed COPD patients had suicidal ideation. The plasma 5-HIAA level [median, (25% and 75% quartiles)] in depressed COPD patients [6.8 ng/mL, (4.9 and 13.1)] was significantly higher than in non-depressed COPD patients [5.4, (4.2 and 7.5)] (p=0.022) and non-smokers [5.1 (3.8 and 7.2)] (p=0.041), but not smokers [4.7, (4.0 and 6.7)] (p>0.05). The plasma 5-HIAA level (r=0.24, p=0.049) was significantly associated with the severity of depression in patients with COPD. The plasma MHPG level was significantly higher in depressed COPD patients (p=0.043) than in smokers, but was not higher than that in non-depressed COPD patients or non-smokers, although the level of MHPG was not associated with the severity of depression.

Conclusion: The plasma 5-HIAA level is increased in depressed COPD patients. Plasma monoamines may be a good biomarker for detection of depression in patients with COPD.

Keywords: COPD, Monoamine, Depression

Background

Chronic obstructive pulmonary disease (COPD) is characterized by a chronic airflow limitation, and is recognized as a major health problem responsible for chronic morbidity and mortality worldwide [1]. Symptomatic COPD patients who have suffered previous repeated exacerbations have poor disease control and prognosis [2]. Improvement of symptoms and prevention of exacerbations may contribute to an improvement of health-related quality of life (HRQOL) and lower mortality for patients with COPD.

COPD patients often have psychological disorders, including depression, and such patients tend to have more frequent exacerbations and a poor prognosis [3-7]. Recently, we demonstrated that depressed COPD patients had a lower HRQOL and more frequent exacerbations and hospitalizations those non-depressed COPD patients [3]. The severity of depression in COPD is closely associated with suicidal ideation [8,9].

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It is well known that depressive symptoms are associated with dysfunction of brain monoaminergic neurons, and that the levels of serotonin (5-hydroxytryptamine [5-HT]) released in the brain are linked to a decrease in responsiveness to anti-depressants [10,11]. It is also well known that the functions of monoamine and monoamine oxidase are associated with smoking-related diseases [12,13]. However, the relationship between levels of plasma monoamines and their metabolites in patients with COPD-associated depression is still unclear.

In the present study, we analyzed serotonin metabolites to investigate possible biomarkers of depressed COPD patients. Plasma homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were measured in COPD patients who were depressed (depressed COPD) and COPD patients who were not depressed (non-depressed COPD), and also in age- and gender-matched non-depressed nonsmokers and smokers as controls.

Methods

Participants

The subjects of this study were outpatients or healthy volunteers. We randomly enrolled 70 patients with COPD, and recruited 36 age- and gender-matched healthy controls between September 1st 2009 and August 31st 2011 at the Chest Disease Center of Kurume University Hospital, Japan (Table 1). All of the patients analyzed had had stable COPD for at least 4 weeks prior to blood tests. None had received oral or injective corticosteroids or antibiotics for 4 weeks prior to the blood tests. Individuals with asthma, bronchiectasis, interstitial pneumonia, tuberculosis, pneumoconiosis, ischemic heart disease, chronic heart disease, renal or liver failure, active malignancies of any organs, sleep apnea syndrome, and a presence and history of psychological diseases such as major depression, bipolar disorder, or schizophrenia were excluded. Also excluded were patients who had been taking anti-depressants, and patients who had a history of lung volume reduction surgery, lung transplantation, or pneumonectomy. Patients with central nervous system disorders and cerebrovascular diseases were excluded on the basis of brain computed tomography (CT) or magnetic resonance imaging (MRI) examinations. Patients with COPD who were undertaking respiratory rehabilitation, or receiving long-term oxygen therapy and non-invasive positive pressure ventilation were excluded, because these treatments are thought to affect psychological status. We carefully excluded any subjects with renal function disorders (serum creatinine levels >1.2 mg/dL).

As reported previously [14,15], the sample sizes for the patients with COPD and healthy controls were >70 and >35, respectively, in plasma levels of monoamines, when the sample ratio was 1:2 (power = 80%; alpha error = 5%; and beta error = 80%).

Study protocol

After the patients had provided written informed consent, information on age, gender, smoking status (current-, ex- or non-smoker), cumulative smoking history (pack-yrs), body mass index (BMI; weight/height^2), comorbidities, and history of pharmacological treatments was obtained. Each subject underwent blood tests, spirometry, electrocardiography, chest radiography, chest high-resolution CT (HRCT), and brain CT or MRI. Spirometry and bronchodilator response tests were performed using an electronic spirometer (Chestgraph Jr HI-101, CHEST Ltd., Tokyo, Japan) in accordance with the American Thoracic Society (ATS) recommendations [16]. A metered-dose salbutamol (400 mcg/subject, GSK, Japan) inhaler was used as a bronchodilator, and bronchodilator response tests were performed before and 30 min after salbutamol inhalation. Predicted values of spirometry parameters were calculated according to the prediction equations of the Japanese Respiratory Society, as we have reported previously [17]. HRQOL was assessed using the validated Japanese St. George's Respiratory Questionnaire (SGRQ) [18,19]. The SGRQ contains three subscales (symptoms, activity, and impact), and the total score varies from 0 to 100 with a higher score indicating a worse health status [19]. Dyspnea was evaluated using the 5-grade (0 to 4) modified Medical Research Council (mMRC) dyspnea scale [20]. Arterial blood gas analysis was performed with each subject supine breathing room air. After assessing the SGRQ, the mMRC dyspnea scale, and the Centre for Epidemiologic Studies depression (CES-D) scale (Purchased from Saccess Bell Co., Ltd, Japan) for depression, all blood samples were taken between 9:00 and 10:00 AM following 10 minutes with the subjects supine. Samples were kept at -80°C until analysis.

The study protocols (Approval No. 08091, May 29th, 2009) were approved by the research ethics board of Kurume University and written informed consent was obtained from the internal review board and all participants.

Diagnosis and severity of COPD

Diagnosis and staging of COPD were in accordance with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [2], and included a post-bronchodilator forced expiratory volume in 1 second/forced vital capacity (FEV_1/FVC) ratio of <70%, and <200 mL and <12% reversibility of FEV_1 before and after bronchodilator administration. Patients with COPD who had a smoking history of 10 pack-yrs and over and also had emphysematous changes in the lungs were selected to carefully remove asthmatics. The emphysematous

Table 1 Profiles of the four participant groups

Parameter	Control subjects		COPD patients	
	Nonsmokers	**Smokers**	**Non-depressed**	**Depressed**
Number of subjects	23	13	50	20
Age (yr)	66.7 ± 9.0	66.5 ± 11.6	68.5 ± 7.2	68.2 ± 7.8
Gender[a] (no. of males; %)	15 (65.2)	12 (92.3)	47 (94.0)	14 (70.0)
Body mass index (kg/m^2)	22.1 ± 2.4	23.0 ± 2.8	21.9 ± 3.1	19.8 ± 3.8[†]
Smoking status[a]				
Non / Ex / Cu (no.)	23 / 0 / 0	0 / 4 / 9	0 / 33 / 17	0 / 14 / 6
Smoke index (pack-yrs)	0	40.0 ± 18.2[***]	59.3 ± 30.3[***]	52.2 ± 27.0[***]
Comorbidities[a]				
Hypertension (no; %)	7 (30.4)	4 (30.8)	13 (26.0)	4 (20.0)
Diabetes (no; %)	5 (21.7)	1 (7.7)	11 (22.0)	6 (30.0)
Duration of COPD (yr)	N/A	N/A	5.3 ± 3.9	5.5 ± 4.4
GOLD stage[a]				
I / II / III / IV (no.)	N/A	N/A	7 / 22 / 17 / 4	2 / 4 / 8 / 6
Lung function parameters				
Before bronchodilator				
FVC (L)	3.5 ± 0.9	3.6 ± 0.7	3.4 ± 0.8	2.7 ± 0.9[*†‡]
%FVC	108.5 ± 17.0	105.6 ± 19.3	98.8 ± 18.3	86.4 ± 22.2[**†]
FEV_1 (L)	2.6 ± 0.6	2.6 ± 0.5	1.5 ± 0.6[***†††]	1.1 ± 0.7[***†††]
%FEV_1	100.8 ± 14.3	94.9 ± 20.1	54.9 ± 20.8[***†††]	44.8 ± 24.9[***†††]
FEV_1/FVC (%)	77.2 ± 6.9	73.8 ± 5.2	44.4 ± 12.7[***†††]	41.0 ± 16.2[***†††]
After bronchodilator				
FVC (L)	3.4 ± 0.9	3.5 ± 0.7	3.4 ± 0.8	2.6 ± 1.0[*†‡]
%FVC	108.0 ± 17.4	104.3 ± 18.0	99.3 ± 18.5	84.9 ± 23.5[**†‡]
FEV_1 (L)	2.7 ± 0.6	2.7 ± 0.4	1.6 ± 0.6[***†††]	1.2 ± 0.7[***†††]
%FEV_1	103.0 ± 15.5	96.5 ± 19.9	56.4 ± 20.8[***†††]	45.3 ± 25.0[***†††]
FEV_1 / FVC (%)	79.2 ± 6.5	76.0 ± 5.9	45.6 ± 13.4[***†††]	42.3 ± 15.9[***†††]
Reversibility of FEV_1 (%)	2.2 ± 4.5	1.8 ± 3.8	3.3 ± 5.3	1.0 ± 5.2
Arterial blood gases				
PaO_2 (Torr)	90.3 ± 7.5	92.9 ± 5.9	76.6 ± 9.5[***†††]	71.9 ± 13.9[***†††]
$PaCO_2$ (Torr)	41.4 ± 3.2	41.7 ± 3.5	40.0 ± 4.0	44.6 ± 7.1[‡]
mMRC dyspnea scale	0.0 ± 0.0	0.2 ± 0.6	1.0 ± 1.1[***†††]	2.1 ± 1.6[***†††§]
SGRQ				
Total score (units)	8.4 ± 8.3	15.7 ± 12.0	32.6 ± 15.9[***††]	57.8 ± 20.8[***†††¶]
Symptom score (units)	19.6 ± 13.4	30.9 ± 18.9	40.8 ± 21.3[**††]	66.2 ± 16.7[***†††¶]
Activity score (units)	6.0 ± 7.1	20.9 ± 17.2	42.9 ± 24.1[***†††]	68.2 ± 29.7[***†††§]
Impact score (units)	6.2 ± 10.0	8.0 ± 9.9	21.3 ± 13.9[**††]	52.0 ± 23.2[***†††¶]
CES-D scale	1.7 ± 2.9	2.5 ± 3.1	8.5 ± 5.2[***†††]	24.5 ± 6.0[***†††¶]
Treatments for COPD[b]				
LAMA (no; %)	0	0	33 (66.0)	16 (80.0)

Table 1 Profiles of the four participant groups *(Continued)*

LABA (no; %)	0	0	21 (42.0)	10 (50.0)
ICS (no; %)	0	0	15 (30.0)	11 (55.5)
SRT (no; %)	0	0	7 (14.0)	4 (20.0)

All data were expressed as mean ± SD and compared by one-way ANOVA and Tukey-Kramer test for multiple comparisons among the four groups.
[a] Data were compared among groups by chi-squared test for trend.
[b] Data were compared between depressed and non-depressed COPD patients by Fisher's exact test. Numbers of non-depressive and depressive COPD patients who used salmeterol and fluticasone devices in combination were 13 and 10, respectively. Some patients were taking multiple medications.
* p<0.05, ** p<0.01, and *** p<0.001 vs. nonsmokers.
† p<0.05, †† p<0.01, and ††† p<0.001 vs. smokers.
‡ p<0.05, § p<0.01, and ¶ p<0.001 vs. non-depressed COPD patients.
Non, non-smokers; Ex, ex-smokers; Cu, current smokers; GOLD, Global Initiative for Chronic Obstructive Lung Disease; FVC, forced expiratory capacity; FEV_1, forced expiratory volume in 1 second; PaO_2, partial pressure of arterial oxygen; $PaCO_2$, partial pressure of arterial carbon dioxide; mMRC, modified Medical Research Council; SGRQ, St George's respiratory questionnaire; CES-D, Center for Epidemiological Studies depression scale; LAMA, long-acting muscarinic receptor antagonist; LABA, long-acting β_2 agonist; ICS, inhaled corticosteroid; SRT, slow-release theophylline; N/A, not available.

changes were visually recognized as low-attenuation areas by chest HRCT [21].

Diagnosis of depression

Diagnosis of depression was assessed using the validated Japanese CES-D scale. The cut-off point for depression was CES-D >16 [22,23] and a CES-D score was assessed for each subject. The results of the CES-D score were not opened until this study was completed. Therefore, physicians did not know the psychological conditions of each subject. In this study, patients with a CES-D score of > 16 were a subgroup of "depressed" COPD patients. For the purpose of this study, "depression" means possible "depression" as determined by the CES-D score.

Measurement of plasma monoamine levels

Plasma levels of 5-HT, 5-HIAA, HVA, and MHPG were measured by high-performance liquid chromatography (SRL Inc., Tokyo, Japan), as previously reported [15,24-26]. The lowest detection limits for 5-HT, 5-HIAA, HVA, and MHPG were 0.01 μg/mL, 1.8 ng/mL, 4.4 ng/mL, and 3.2 ng/mL, respectively, and for statistical analysis one half of the lowest value was assigned if the value was below the detection limit.

Statistical analyses

Data analyses were performed using JMP version 7 (SAS Institute, Inc., Cary, NC). Data for the subjects were expressed as the mean ± standard deviation (SD), and data for plasma monoamine levels were expressed as the median, and 25% and 75% quartiles. Statistical analyses were performed using parametric Student's *t* test for comparison between two groups, or one-way analysis of variance (ANOVA) and Tukey-Kramer test for multiple comparisons among four groups. Correlations were analyzed by parametric or non-parametric Spearman's tests. Differences between groups were evaluated using the chi-squared test for trend and Fisher's exact test. The level of significance was set at P <0.05.

Results

Subject characteristics

A total of 106 subjects participated in the study and the characteristics of the four groups, namely the 23 non-smokers and 13 smokers (as age- and gender-matched non-depressed healthy controls), and 50 non-depressed and 20 depressed COPD patients, are compared in Table 1. The depressed subjects without COPD were not enrolled in the study. None of participants, including the depressed COPD patients, had any history of attempted suicide.

The control subjects were 23 non-, 4 Ex-, and 9 current smokers, whereas the number of non-, Ex-, and current smokers were zero, 57, and 23 in patients with COPD (p<0.001). There was no significant difference in the smoke index among smokers, and non-depressed, and depressed COPD patients. There were no significant difference in the populations of subjects with hypertension and diabetes among nonsmokers, smokers, and, non-depressed, and depressed COPD patients (p>0.05). All the subjects with hypertension had been taking anti-hypertensive medications whereas the numbers of non-smokers, smokers, non-depressed, and depressed COPD patients taking anti-diabetes medications were 5, 1, 7 and 4, respectively.

In COPD, there was no significantly difference in duration of COPD between non-depressed and depressed COPD patients (p>0.05). The depressed COPD patients trended to have more progressive GOLD stages than the non-depressed patients but there was no significant difference in the populations of GOLD stage I (14% vs 10%), II (44% vs 20%), III (34% vs 40%), and IV (8% vs 30%) between two groups (p>0.05 by Chi-square test for trend).

Lung function tests showed that he depressed COPD patients had significantly lower FVC and %FVC than the non-depressed patients both before and after bronchodilator use, although both depressed and non-depressed COPD patients had significantly lower FEV_1, $\%FEV_1$ and FEV_1/FVC both before and after bronchodilator use than non-smokers and smokers, respectively (all p<0.001).

However, there was no difference in the reversibility of FEV_1 after bronchodilator use among the four groups.

Arterial blood gas analysis showed that the depressed COPD patients (p<0.05) had significantly more severe hypercapnia than the non-depressed patients, whereas both depressed (both, p<0.001) and non-depressed COPD patients (both, p<0.001) had significantly more severe hypoxia than the non-smokers and smokers, respectively.

Depressed COPD patients had significantly higher mMRC dyspnea scales (p<0.05) and lower HRQOL scores (p<0.05) than the non-depressed patients, although both the depressed (both, p<0.001) and non-depressed COPD patients (both, p<0.001) had significantly higher MRC dyspnea scales and lower HRQOL scores than the non-smokers and smokers, respectively.

In managements for COPD, all patients with COPD were receiving vaccinations for seasonal and H1N1 influenza virus and the numbers of depressed and non-depressed COPD patients who had been receiving pneumococcal vaccination within 5 yrs before recruitment were 13 and 8, respectively. There was no significant difference in the ratio of regular use of ICS, LAMA, LABA, and SRT between non-depressed and depressed COPD patients (p=0.061, p=0.387, p=0.601, and p=0.717, respectively. The effects of ICS on psychological and mood status could not be directly determined, as the study was not designed to include a period for wash-out of each controller for COPD.

Plasma monoamine levels

Plasma 5-HIAA levels [median, (25% and 75% quartiles)] in the depressed COPD patients [6.8 ng/mL, (4.9 and 13.1)] were significantly higher than in the non-depressed patients [5.4, (4.2 and 7.5)] (p=0.022) and non-smokers [5.1 (3.8 and 7.2)] (p=0.041), respectively, but were not significantly higher than in the smokers [4.7, (4.0 and 6.7)] (Figure 1).

Median (25% and 75% quartiles) plasma 5-HT levels in the non-smokers, smokers, and non-depressed and depressed COPD patients were 0.06 μg/mL (0.04 and 0.09), 0.05 (0.04 and 0.08), 0.06 (0.03 and 0.08), and 0.06 (0.02 and 0.09), respectively. The differences among the four groups were not significant (Figure 1).

Median (25% and 75% quartiles) plasma HVA levels in the non-smokers, smokers, and non-depressed and depressed COPD patients were 12.8 ng/mL (11.0 and 14.8), 11.8 (10.3 and 21.3), 14.8 (11.1 and 20.9), and 15.7 (9.9 and 22.2), respectively. The differences among the four groups were not significant (Figure 1).

Plasma MHPG level [median, (25% and 75% quartiles)] in the depressed COPD patients [6.8 ng/mL, (5.2 and 8.7)] (p=0.043) was significantly higher than in the smokers [4.6, (4.3 and 5.5)]. There was no significant difference in plasma MHPG level between the depressed COPD patients and either non-smokers [5.7 (4.7 and 7.2)] or non-depressed COPD patients [6.7, (4.1 and 8.2)] (p>0.05), respectively (Figure 1).

To investigate seasonal effects in plasma 5-HIAA levels, plasma obtained in four seasons, spring (March-May), summer (June-August), fall (September-November), and winter (December-February), were measured. Number of all subjects and COPD patients in four seasons were 17 and 10, 34 and 27, 25 and 16, and 30 and 17, respectively. There was no significant difference in median plasma 5-HIAA [6.7 ng/mL (5.1 and 7.6) in spring, 5.3 ng/mL (4.3 and 7.4) in summer, 5.9 ng/mL (3.9 and 10.6) in fall, and 5.4 ng/mL (3.7 and 8.2) in winter, respectively, p>0.05].

The plasma levels of 5-HT, 5-HIAA, HVA, and MHPG were not associated with age and there was no significant difference in those plasma levels between male and female.

Correlation between plasma 5-HIAA level and total CES-D scales in patients with COPD

Plasma level of 5-HIAA (r=0.24, p=0.049), but not that of 5-HT (r=-0.06, p>0.05), HVA (r=0.19, p>0.05), or MHPG (r=0.14, p>0.05), was significantly associated with total CES-D scales in patients with COPD (Figure 2).

Correlation between plasma 5-HIAA level and BMI, lung function, arterial blood gas parameters, and total SGRQ score in patients with COPD

There was a significant correlation between BMI and plasma MHPG (r=-0.24, p=0.041), but not 5-HIAA (r=-0.21, p>0.05), 5-HT (r=0.07, p>0.05), HVA (r=-0.09, p>0.05), and MHPG (r=-0.24, p=0.041) level in COPD patients.

Plasma 5-HIAA and MHPG level showed significant negative associations with %FVC, $\%FEV_1$, and partial pressure of arterial oxygen (PaO_2), and positively associations with partial pressure of arterial carbon dioxide ($PaCO_2$) and total SGRQ scores. There was no significant correlation between the plasma 5-HT level and lung function, arterial blood gas parameters, or total SGRQ scores. Plasma HVA level showed a significant negative association with $\%FEV_1$ and PaO_2, and a positive association with $PaCO_2$ and total SGRQ scores (Table 2).

Correlation between lung function, total SGRQ scores and total CES-D scales in patients with COPD

The $\%FEV_1$ showed a significant negative association with total SGRQ scores (r=-0.69, p<0.0001) and total CES-D scales (r=-0.27, p=0.025) in patients with COPD. The PaO_2 (r=-0.53, p<0.0001) and $PaCO_2$ (r=0.44, p=0.0002) showed a significant association with total SGRQ scores. Interestingly, there was no correlation between PaO_2 (r=-0.21, p>0.05) and $PaCO_2$ (r=0.22, p>0.05) and total CES-D scales in COPD patients.

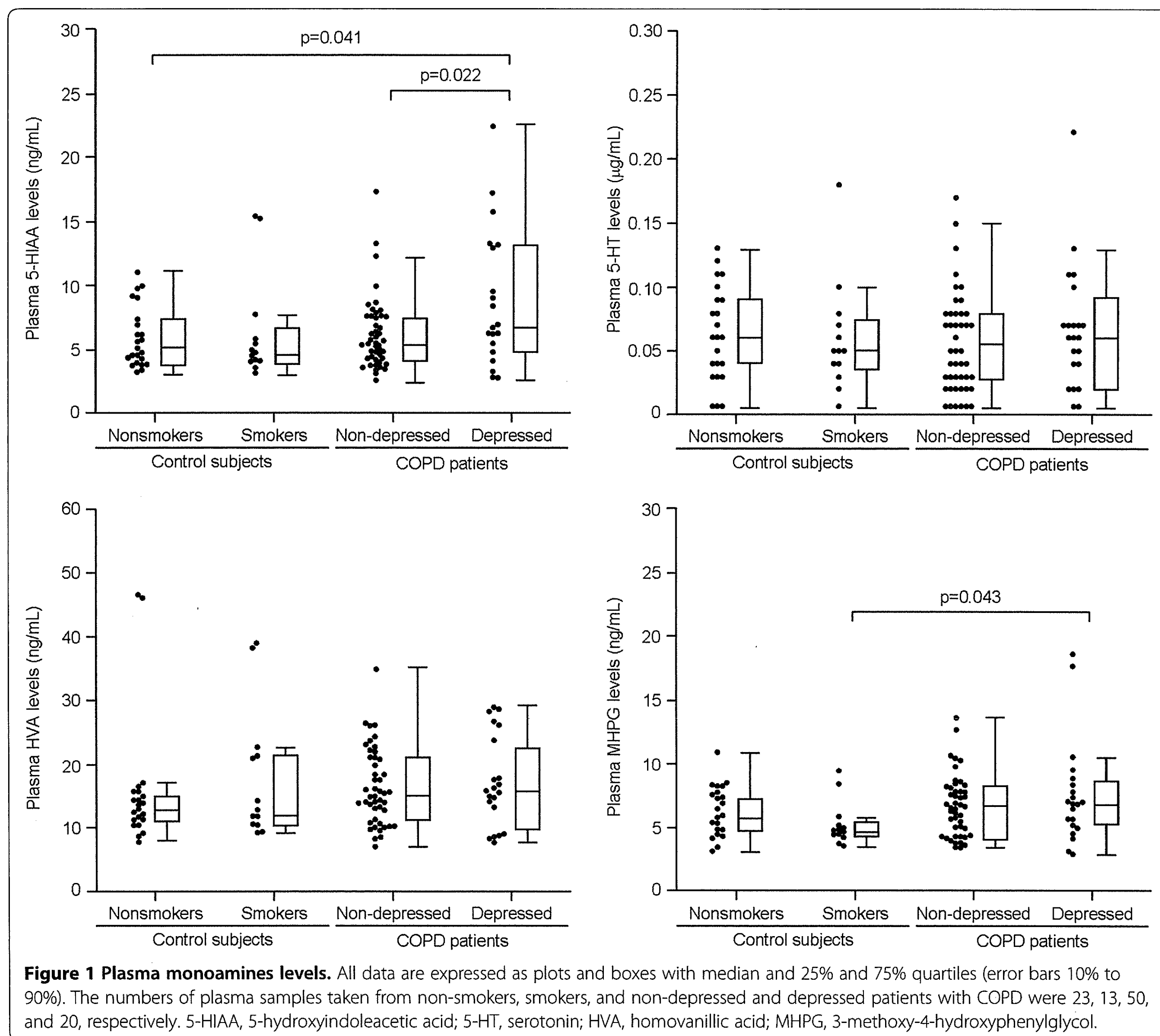

Figure 1 Plasma monoamines levels. All data are expressed as plots and boxes with median and 25% and 75% quartiles (error bars 10% to 90%). The numbers of plasma samples taken from non-smokers, smokers, and non-depressed and depressed patients with COPD were 23, 13, 50, and 20, respectively. 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol.

Discussion

To our knowledge, this is the first study to have measured the plasma levels of monoamines and their metabolites in depressed patients with COPD. Our present results demonstrated that depressed patients with COPD had significantly higher plasma 5-HIAA levels than non-depressed COPD patients and non-smokers. The plasma 5-HIAA levels also showed a significant positive correlation with the severity of depression in the patients with COPD. Our present results support those of a previous study demonstrating that the plasma 5-HIAA levels were significantly increased in patients with depression relative to control subjects, and that the plasma 5-HIAA levels were positively related to the severity of depression [14]. Previous studies have shown that the level of 5-HIAA in cerebrospinal fluid (CSF) was positively associated with the severity of depression in abstinent alcoholics, and that treatment with the antidepressant fluoxetine decreased both the CSF 5-HIAA levels and the mean Hamilton depression rating scale score [27,28]. Other studies have demonstrated that decreased CSF 5-HIAA levels were associated with attempted suicide in patients with depression, and that non-impulsive suicide attempters had higher plasma 5-HIAA levels than impulsive suicide attempters [15,29,30]. In the COPD patients we analyzed, increased plasma 5-HIAA levels were also associated with poor lung function, hypoxia and hypercapnia. Poor lung function is closely correlated with a poor HRQOL, and may result in depression. In COPD patients it has been shown that hypoxia and/or hypercapnia induces oxidative stress and results in an increase of reactive oxygen species (ROS) throughout the whole body including the lungs, brain, and muscles [1-5]. Therefore, it is possible that ROS may have a direct or

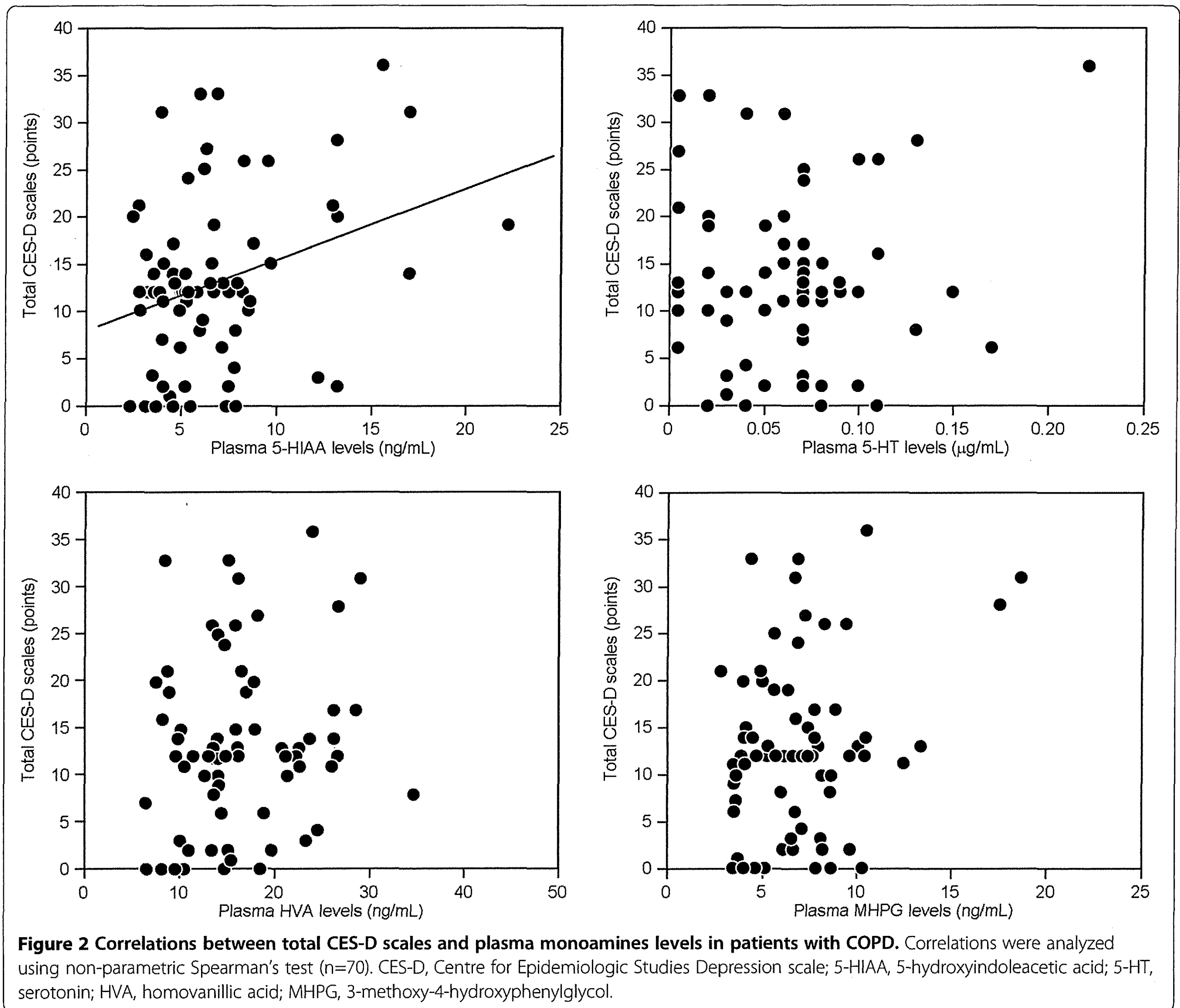

Figure 2 Correlations between total CES-D scales and plasma monoamines levels in patients with COPD. Correlations were analyzed using non-parametric Spearman's test (n=70). CES-D, Centre for Epidemiologic Studies Depression scale; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol.

indirect effect on serotonergic innervation of the lungs, and also affect brain serotonin turnover in the respiratory centres/autonomic centres of the brain. Further analysis will be needed to clarify this issue. None of the participants included in our study had previously attempted suicide. It has been reported that the severity of depression in patients with COPD is closely associated with suicidal ideation [8,9]. Therefore, it would be worthwhile to rank COPD patients in terms of current suicide risk. Taken together, the data suggest that a depressive status and the severity of COPD may be related to increased plasma 5-HIAA level in depressed COPD patients.

Table 2 Correlations between plasma monoamines levels and lung function, arterial blood gas parameters, and total SGRQ scores in the patients with COPD

	%FVC, %	%FEV_1, %	PaO_2, Torr	$PaCO_2$, Torr	Total SGRQ, units
5-HIAA, ng/mL	−0.36 (0.002)	−0.40 (<0.001)	−0.38 (<0.001)	0.26 (0.031)	0.33 (0.006)
5-HT, μg/mL	−0.09 (NS)	−0.08 (NS)	−0.17 (NS)	0.06 (NS)	0.10 (NS)
HVA, ng/mL	−0.36 (0.002)	−0.49 (<0.001)	−0.39 (<0.001)	0.09 (NS)	0.26 (0.029)
MHPG, ng/mL	−0.21 (NS)	−0.46 (<0.001)	−0.41 (<0.001)	0.25 (0.036)	0.40 (<0.001)

All correlation coefficients were expressed as r (p value).
Post-bronchodilator data for %FVC and %FEV_1 were used.
5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; FVC, forced expiratory capacity; FEV_1, forced expiratory volume in 1 second; PaO_2, partial pressure of oxygen; $PaCO_2$, partial pressure of carbon dioxide; SGRQ, St. George's Respiratory Questionnaire.

The metabolism of 5-HT is controlled exclusively via the action of monoamine oxidase and aldehyde dehydrogenase, resulting in the formation of 5-HIAA. In this study, we did not find any differences in the plasma 5-HT levels among the four groups (non-smokers, smokers, non-depressed COPD and depressed COPD). However, a previous study demonstrated that the plasma 5-HT levels were increased in patients with COPD and was also associated with aging [31]. Further analysis should be needed to verify this issue.

HVA is a dopamine metabolite. Previous studies of patients with depression have demonstrated that the levels of HVA in plasma and CSF decreased along with the levels of 3, 4-dihydroxyphenylacetic acid [14,32,33]. In the present study, plasma HVA levels showed no differences among the four groups we examined. However, the plasma level of HVA showed significant negative associations with %FVC, $\%FEV_1$, and PaO_2 levels, and a positive association with the total SGRQ scores in patients with COPD. In COPD patients, most of the HVA in plasma may be derived from precursor dopamine in sympathetic nerves rather than brain dopamine. Our results suggest that poor lung function induced ROS, and perhaps resulted in an increase of HVA derived from precursor dopamine in sympathetic nerves in the COPD patients we studied. Further analysis will be needed to verify this hypothesis.

MHPG is a metabolite of both epinephrine and norepinephrine. Depressed COPD patients had significantly higher plasma MHPG levels ($p=0.043$) than smokers. Placidi and coworkers [29] suggested that the level of MHPG in CSF might have a positive correlation with aggressive and impulsive suicide, and that selective norepinephrine reuptake inhibitors might increase the risk of suicidal acts. In the present study, however, the plasma level of MHPG was not associated with the severity of depression, but showed significant negative associations with % FEV_1 and PaO_2, and positive associations with $PaCO_2$ and the total SGRQ score in patients with COPD. Thus, the plasma levels of both HVA and MHPG may be related to the severity of COPD rather than to depression in COPD.

There were some limitations to the present study. First, we measured the levels of monoamines in plasma but not in CSF. Fluctuations in the levels of monoamines and their metabolites can differ between peripheral blood (plasma) and the brain (CSF). Second, the depressed and non-depressed COPD patients were not matched for the severity of COPD, and this parameter may be correlated with plasma monoamine levels. Third, we did not take into account the effects of treatments with antidepressants on the plasma levels of monoamines in depressed COPD patients, although previous studies have reported that antidepressants are of little benefit to patients with COPD [9,34,35]. Further analysis of these issues will be necessary.

Conclusion

In summary, the present study has shown that plasma 5-HIAA levels are significantly increased in COPD patients with depression, and also associated with the severity of depression in such patients. We also found that the plasma 5-HIAA, MHPG, and HVA levels were negatively associated with lung function, HRQOL and arterial blood gas abnormalities in patients with COPD. Plasma monoamine levels may be applicable as biomarkers for detection of depression in patients with COPD.

Abbreviations

ANOVA: Analysis of variance; ATS: American thoracic society; BMI: Body mass index; CES-D: Centre for epidemiologic studies depression; COPD: Chronic obstructive pulmonary disease; CSF: Cerebrospinal fluid; CT: Computed tomography; FEV_1: Forced expiratory volume in 1 second; FVC: Forced vital capacity; GOLD: Global strategy for diagnosis, management, and prevention of COPD; HRCT: High resolution computed tomography; HRQOL: Health-related quality of life; HVA: Homovanillic acid; ICS: Inhaled corticosteroid; LABA: Long-acting β_2 agonist; LAMA: Long-acting muscarinic receptor antagonist; MHPG: 3-methoxy-4-hydroxyphenylglycol; MRI: Magnetic resonance imaging; $PaCO_2$: Partial pressure of arterial carbon dioxide; PaO_2: Partial pressure of arterial oxygen; SD: Standard deviation; SGRQ: St. George's respiratory questionnaire; SRT: Slow-release theophylline; mMRC: Modified medical research council; $\%FEV_1$: Percent of predicted forced expiratory volume in 1 second; %FVC: Percent of predicted forced vital capacity; 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: Serotonin (5-hydroxytryptamine).

Competing interests

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Authors' contributions

TS-K contributed to protocol design, data collection, analysis, and writing of the manuscript. TK contributed to protocol design and editing of the manuscript. KI contributed to data collection. YS supervised the protocol design. KM contributed to data collection. MO contributed to data collection. NE contributed to data collection. HI contributed to data collection. NU supervised the protocol design and edited the manuscript. TH supervised the protocol design and edited the manuscript. All authors read and approved the final manuscript.

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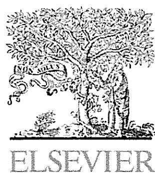

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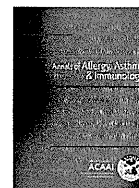

Interleukin-18 expression, CD8$^+$ T cells, and eosinophils in lungs of nonsmokers with fatal asthma

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ABSTRACT

Background: The process of airway inflammation in the lungs of nonsmokers who die of asthma (fatal asthma) has not been reported in detail.
Objective: To examine nonsmokers who had died of asthma to exclude chronic obstructive pulmonary disease and investigate pulmonary inflammatory cells and the expression of interleukin-18 (IL-18) and its receptor in lung tissues compared with those in patients with well-controlled mild asthma and nonsmokers.
Methods: Lung tissues were obtained at autopsy examination from 12 nonsmokers with fatal asthma, excluding cases of chronic obstructive pulmonary disease, and from 5 nonsmokers with well-controlled mild asthma and 10 nonsmokers who had undergone surgical resection for lung cancer. Pulmonary inflammatory cells were examined and the expression of the proinflammatory cytokine IL-18 and its receptor in the lungs was evaluated.
Results: The numbers of eosinophils and lymphocytes, but not basophils or macrophages, were significantly increased in the lungs of patients with fatal asthma compared with the other 2 groups. The lung neutrophil count did not differ significantly between the fatal and mild asthma groups but was significantly higher in the fatal asthma group than in nonsmokers. CD8$^+$ T cells, but not CD4$^+$ T cells, were significantly increased in the lungs of the fatal asthma group compared with the other 2 groups. IL-18 protein and IL-18 receptor were strongly expressed in the lungs in the fatal asthma group.
Conclusion: Caspase-1 inhibitors, anti–IL-18 antibodies, anti–IL-18 receptor antibodies, IL-18 binding protein, or inhibitors of genes downstream of the IL-18 signal transduction pathway may be of clinical benefit for the treatment of patients with severe asthma.
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Introduction

Autopsy studies of the lungs of patients with fatal asthma, conducted in the 1950s, reported that the outstanding feature was the presence of numerous mucus plugs in the airways and focal areas of collapse.[1,2] Thereafter, several series of studies of fatal asthma cases reported the presence of severe airway remodeling accompanied by mucus plugging, goblet cell hyperplasia, smooth muscle hypertrophy, submucosal gland hyperplasia, basement membrane thickening, and eosinophilic inflammation.[3–7] In addition, severe airway remodeling was found in the large and small airways of patients who had died of asthma.[8–10] These results suggested that severe airway remodeling and pulmonary inflammatory cells might be involved in the pathogenesis of death from asthma.[11]

Studies of bronchial biopsies have shown that nonsmokers with mild and stable asthma have increased numbers of activated CD4$^+$ T cells producing T-helper cell type 2 [T_H2] cytokines (eg, interleukin [IL]-4, IL-5, and IL-13) and eosinophils in the mucosa of the large airways. A previous study reported that CD8$^+$ T cells were

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increased in the airways in some fatal cases of asthma.[12] Moreover, $CD8^+$ T cells producing the T-helper cell type 1 [T_H1] cytokine interferon-γ (IFN-γ) are reportedly increased in the lungs of patients with severe asthma, including fatal asthma.[13] However, it is still unclear whether $CD8^+$ T cells and/or $CD4^+$ T cells are involved in the pathogenesis of asthma deaths. It is noteworthy that the patients analyzed in the previous studies included smokers, ex-smokers, or patients with asthma whose smoking status was unknown.[14] Moreover, in the 1950s, it was believed that the major pathologic findings of fatal asthma was plugging of the bronchial tree with mucus, eosinophilia, thickening of the basement membrane, and emphysema.[1] Therefore, some of those patients[14] and those studied in the 1940s and 1950s[1] may have had chronic obstructive pulmonary disease (COPD) with or without asthma.

Interleuin-18 is a proinflammatory cytokine, originally discovered as an IFN-γ–inducing factor. IL-18 is a member of the IL-1 family of cytokines.[15] Like IL-1, IL-33, IL-36, IL-37, and IL-38, IL-18 is produced intracellularly from a biologically inactivated precursor, pro–IL-18. Mature IL-18 is secreted after cleavage of pro–IL-18 by caspase-1, originally identified as an IL-1β converting enzyme. Activated macrophages produce large amounts of mature IL-18 after cleavage of pro–IL-18 by caspase-1.[16] IL-18 plays a well-known important role in T_H1 polarization and can act as a cofactor for T_H2 cell development and IgE production.[16–19] It also plays important roles in the pathogenesis of inflammatory and allergic diseases, such as rheumatoid arthritis, adult-onset Still disease, Sjögren syndrome, inflammatory bowel diseases including Crohn disease, allergic rhinitis, and atopic dermatitis.[16,20,21] IL-18 also is involved in the development of lung diseases, including lung injury,[22,23] idiopathic pulmonary fibrosis,[24] and COPD.[25] Recently, the authors reported that IL-18 protein was strongly expressed in airway epithelial and smooth muscle cells in airway biopsy samples from patients with allergic asthma. Moreover, the serum levels of IL-18 were significantly higher in patients with asthma than in patients without asthma with allergy or healthy controls.[26] The present study selected 12 nonsmokers who had died of asthma and excluded any with COPD and investigated pulmonary inflammatory cells and the expression of IL-18 and its receptor (IL-18R) in their lung tissues compared with controls.

Methods

Patients

This study carefully excluded smokers and ex-smokers and analyzed nonsmokers to exclude any with COPD. Twelve patients (9 male and 3 female, 5–79 years old) diagnosed as having asthma were monitored at Kurume University Hospital (Kurume, Japan) and the Fukuoka National Hospital (Fukuoka, Japan). These patients with asthma were nonsmokers and had died from 1973 to 1998. Lung tissues were obtained at autopsy examination in each case. The details of these 12 patients are listed in Table 1. As materials for comparison, lung tissues were obtained from 5 nonsmokers with mild asthma (1 male and 4 female, 58–79 years old) and normal lung tissues were obtained as controls from 10 nonsmokers (5 male and 5 female, 27–78 years old), all of whom underwent surgical resection for lung cancer at Kurume University Hospital (Table 2). Sample collection and all procedures were approved by the ethics committees of Kurume University in accordance with the ethical standards of the Declaration of Helsinki of 1975.

Pulmonary Function Tests

Japanese predicted normal values were used to calculate the predicted percentage of forced expiratory volume in 1 second, which met the Japanese Pulmonary Function Standard in the Japanese Respiratory Society Statement, as previously reported.[27] Further details of these analyses are provided in the online supplementary data.

Histology

Lung tissues were fixed with 10% formalin and embedded in paraffin wax, as reported previously.[24] One to 3 paraffin-embedded lung tissues were obtained from each patient. Sequential sections were made from each paraffin-embedded lung tissue. Sections (4 μm thick) were serially cut, placed on poly-L-lysine–coated slides, and incubated overnight at 55°C to 60°C. Deparaffinized sections were stained with hematoxylin and eosin and May-Giemsa stain. Then, digitized video images of the entire lung fields were captured by a CCD camera and were modulated by Adobe Photoshop CS (Adobe, Tokyo, Japan), as previously reported.[24,25]

Quantitative Assessment of Infiltrating Inflammatory Cells in Lung Tissues

Pulmonary inflammatory cells, such as eosinophils, basophils, neutrophils, lymphocytes, and alveolar macrophages, were counted. Briefly, in May-Giemsa–stained sections of lung tissues, 3 square fields at $\times$40 magnification (2.5 $\times$ 3.5 mm, 8.75 mm^2) were selected in which small-airway inflammation appeared most severe; within each field, 4 other individual square fields at $\times$400 magnification (0.22 $\times$ 0.32 mm, 0.0704 mm^2) were selected. These smaller square fields were defined as observation fields (OFs), and 12 different OFs were selected within 3 different square fields (3 $\times$ 8.75 mm^2) on each lung section. Eosinophils, basophils, neutrophils, lymphocytes, and alveolar macrophages were

Table 1
Characteristics of 12 nonsmokers with fatal asthma

Patient number	Age	Sex	Year of autopsy examination	Therapy						Duration from asthma attack
				Systemic corticosteroid	ICS	β_2 Agonist	Theophylline	Leukotriene receptor antagonist	Ventilator	
1	32	M	1973	+	–	–	+	–	–	75 min
2	52	M	1974	+	–	–	–	–	–	5 d
3	5	M	1977	–	–	–	–	–	+	36 h
4	67	M	1980	–	–	–	–	–	–	5 h
5	44	M	1981	+	–	–	–	–	–	<24 h
6	75	M	1982	+	–	–	–	–	–	20 min
7	16	F	1984	–	–	+	+	–	–	DOA (6 h)
8	79	F	1986	+	–	–	–	–	+	6 d
9	57	M	1986	+	–	–	–	–	–	unknown
10	14	M	1994	–	–	–	–	–	–	DOA (13 d)
11	24	M	1998	+	+	+	+	–	–	4 d
12	68	F	1998	–	+	–	–	–	+	DOA (7 d)

Abbreviations: DOA, death on arrival; F, female; ICS, inhaled corticosteroid; M, male.

Table 2
Characteristics of patients with fatal asthma, those with mild asthma, and control patients

	Asthma death	Mild asthma	Control
Patients (male/female), n	12 (9/3)	5 (1/4)	10 (5/5)
Age (y), mean ± SD	44.4 ± 7.4	66.8 ± 4.1	62.5 ± 4.9
Body mass index (kg/m²), mean ± SD	20.61 ± 1.0	25.0 ± 1.9	22.9 ± 1.8
FVC (% predicted), mean ± SD	ND	107.5 ± 6.7	107.3 ± 7.8
FEV_1 (% predicted), mean ± SD	ND	97.3 ± 11.0	103.3 ± 7.0
FEV_1/FVC (%), mean ± SD	ND	66.2 ± 6.51[a]	78.9 ± 2.8
WBC count (cells/μL), mean ± SD	ND	6233 ± 521	5530 ± 576
Eosinophils (cells/μL), mean ± SD	ND	168 ± 83[a]	90 ± 11

Abbreviations: FEV_1, forced expiration in 1 second; FVC, forced vital capacity; ND, not done; SD, standard deviation; WBC, white blood cell.
[a]$P < .05$ vs control patients.

counted within each of the 12 OFs. Results were expressed as mean ± standard error of the mean for cells per square millimeter. For example, when 20 lymphocytes were counted within 1 OF at ×400 magnification, the number of lymphocytes was 284 cells/mm². Two examiners manually counted these lung sections independently and in a blinded manner, without prior knowledge of the patients' clinical status.

Immunohistochemical Assay

Immunohistochemical analysis was performed as reported previously.[23–25] Antihuman CD4 monoclonal antibody (mAb; 4B12 [mouse IgG1]; Dako, Tokyo, Japan), antihuman CD8 mAb (C8/144B [mouse IgG1]; Dako), antihuman IL-18 mAb (1-8D [mouse IgG1][23] or clone 8 [mouse IgG2a],[24] kindly provided by Dr Do-Young Yoon, Laboratory of Cellular Biology, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea), and anti–IL-18R α-chain (IL-18Rα; H44 [mouse IgG1])[24] were applied to the sections at 4°C for 18 hours. Positive reactivity was identified by Permanent Red (Dako) using an EnVision G|2 System/AP (Dako) with rabbit/mouse (Permanent Red) or 3-3′-diaminobenzidine-4HCl (DAB) using an EnVision+ kit with horseradish peroxidase (Dako). Further details of these analyses are provided in the eMethods.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Nonparametric tests (Kruskal-Wallis test and Mann-Whitney *U* test) were used to compare differences among groups. SAS 9.1.3 (Japanese edition; SAS Institute, Cary, North Carolina) was used for statistical analysis. A *P* value less than .05 was considered statistically significant.

Results

Clinical Findings

All 12 patients with fatal asthma were nonsmokers, and none had COPD. Their ages ranged from 5 to 79 years (mean age 44.4 years). Five of the 12 patients had died within 24 hours after the onset of the asthma attack. The duration of disease in these patients with asthma covered a wide range, from 10 months to 50 years; 9 patients had had asthma for longer than 6 years. Seven patients had been treated with systemic corticosteroid, and only 2 had been receiving inhaled corticosteroid (ICS; beclomethasone dipropionate). The other 10 patients had died without treatment with systemic corticosteroid or ICS (Table 1). Lung tissues also were obtained from 5 nonsmokers with mild asthma and 10 nonsmokers, all of whom had undergone surgical resection for lung cancer. The 5 patients with mild asthma showed a significantly decreased ratio of the percentage of forced expiratory volume in 1 second to forced vital capacity and a significant increase of the peripheral eosinophil count compared with the control patients (Table 2). Three patients with mild asthma had been treated with ICS at the time of surgery: 2 had received 400 μg of fluticasone propionate and 1 had received 400 μg of fluticasone propionate. Another 2 patients with mild asthma had not received any form of medication.

Histopathologic Characteristics of the Lungs in Fatal Cases of Asthma

Representative examples of the histology of the lung tissues obtained from 2 patients with fatal asthma are shown in Figure 1A. Hematoxylin and eosin staining showed severe airway remodeling, and pulmonary inflammation was evident in the lung tissues obtained from 2 men who had been 32 and 52 years old (patients 1 and 2 in Table 1). Severe airway remodeling accompanied by hypertrophy of smooth muscle, airway wall edema, hyperplasia and hypertrophy of goblet cells, mucous plugs, and massive pulmonary inflammation were observed in all 12 patients with fatal asthma, including a 5-year-old boy (patient 3). Emphysematous change was not observed in the lungs of any of the 12 patients with fatal asthma. In contrast to the fatal asthma group, airway remodeling was barely evident in the lung tissues obtained from the 5 patients with mild asthma and the 10 nonsmokers, as reported previously (data not shown).[26]

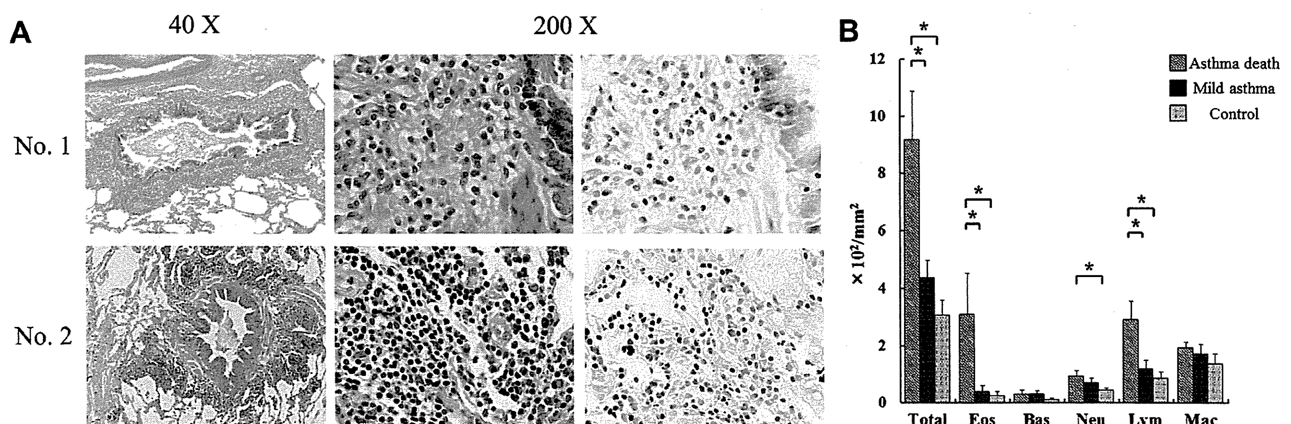

Figure 1. Histologic analysis of lungs obtained from patients who died of asthma. *(A)* Hematoxylin and eosin (×40 and ×200) and May-Giemsa staining of lung tissue samples (×200). *(B)* Quantitative analysis showed that counts of eosinophils (Eos) and lymphocytes (Lym) were significantly larger in the airways of patients who died of asthma (n = 12) than in those of patients with mild asthma (n = 5) or controls (n = 10). *$P < .05$. Bas, basophils; Mac, macrophages; Neu, neutrophils.

Examination of May-Giemsa–stained lung sections from the fatal asthma group showed massive accumulation of eosinophils and lymphocytes in the airways, especially the submucosa and basal membrane, whereas basophils, alveolar macrophages, and neutrophils were scarcely evident (*right panels* in Fig 1A). Quantitative analysis showed that the airway counts of total cells, namely eosinophils and lymphocytes, but not alveolar macrophages or basophils, were significantly ($P < .05$) higher in the fatal asthma group than in the mild asthma or control group. Neutrophils were not increased significantly in the fatal asthma group compared with the mild asthma group, although the neutrophil count was significantly larger in the former group than in the control group (Fig 1B).

Increase of CD8⁺ T Cells in Airways of Patients with Fatal Asthma

Because lymphocytes were greatly increased in the lungs of patients with fatal asthma, the authors investigated which T-cell population was increased in this group. Sequential sections were prepared from paraffin-embedded lung tissues obtained from all 3 groups of patients and subjected to immunohistochemical analysis using anti-CD4 and anti-CD8 mAbs. This showed the presence of $CD4^+$ T cells in the lungs of the fatal asthma group, and $CD8^+$ T cells were especially increased in the airways of this group. Figure 2A shows representative examples of immunohistochemical analysis of $CD4^+$ T cells and $CD8^+$ T cells in the lungs obtained from a 16-year-old girl (patient 7). Quantitative analysis showed that the counts of $CD4^+$ T cells in the lungs of the fatal asthma group, mild asthma group, and control group were 65.9 ± 11.5, 49.0 ± 10.0, and 14.8 ± 2.9 cells/mm^2, respectively. $CD4^+$ T cells were significantly ($P < .05$) more numerous in the lungs in the fatal asthma and mild asthma groups than in the control group; the difference between the former 2 groups was not significant. The counts of $CD8^+$ T cells in the airways of the 3 groups were 170.9 ± 25.2, 73.5 ± 24.1, and 54.5 ± 14.1 cells/mm^2, respectively, being significantly ($P < .05$) increased in the fatal asthma group, but not in the other 2 groups. Moreover, the count of $CD8^+$ T cells was significantly ($P < .005$) larger than that of $CD4^+$ T cells in the lungs of patients who had died of asthma (Fig 2B).

Expression of IL-18 and IL-18Rα in the Lungs of Patients with Fatal Asthma

We examined whether IL-18 protein was expressed in the lungs of patients who had died of asthma. As reported previously,[24,25] IL-18 was expressed constitutively, but weakly, in the bronchoalveolar epithelium, alveolar macrophages, and endothelium of small vessels in control patients. In contrast, IL-18 protein and IL-18Rα were strongly expressed in inflammatory cells, airway epithelial cells, and smooth muscle in the lungs of patients with fatal asthma (Fig 3).

Discussion

In this study, the numbers of eosinophils and lymphocytes, but not neutrophils, were significantly increased in the lungs of patients with fatal asthma compared with patients with mild asthma and control nonsmokers. Moreover, $CD8^+$ T cells, but not $CD4^+$ T cells, were significantly increased in the lungs of the fatal asthma group compared with the other 2 groups. It has been proposed that fatal asthma can be differentiated according to the time from the onset of an acute attack to the time of death.[28,29] A previous study has shown that eosinophils exceed neutrophils in the airway submucosa of patients with slow-onset fatal asthma, whereas neutrophils exceed eosinophils in patients with sudden-onset fatal asthma.[28] In addition, patients in whom the duration of a terminal asthma attack exceeded 24 hours have been shown to have a significantly larger count of activated eosinophils than patients who died suddenly.[30] In contrast, severe asthma exacerbations from noninfective causes are characterized by increased eosinophil activation and IL-5 in sputum.[31] In the present study, 12 patients who died of asthma and were nonsmokers were examined. Five of 12 patients died within 24 hours and were classified as having sudden-onset fatal asthma. In the present study, there was no significant difference in eosinophil counts in patients with fatal asthma who died within 24 hours vs longer than 24 hours after the onset of the asthma attack (data not shown). These results suggest that eosinophils were chronically increased and/or activated in the lungs of the patients before the fatal attack. Neutrophils were not significantly increased in the fatal asthma group compared with the mild asthma group, although neutrophils were significantly increased in the former compared with the control group. Moreover, none of the patients in the fatal asthma group showed an increase in the neutrophil count compared with eosinophils or lymphocytes (data not shown). It is well known that $CD8^+$ T cells, neutrophils, eosinophils, and/or macrophages are increased in the lungs of patients with severe COPD and in those with exacerbation of COPD.[15] Moreover, viral infections

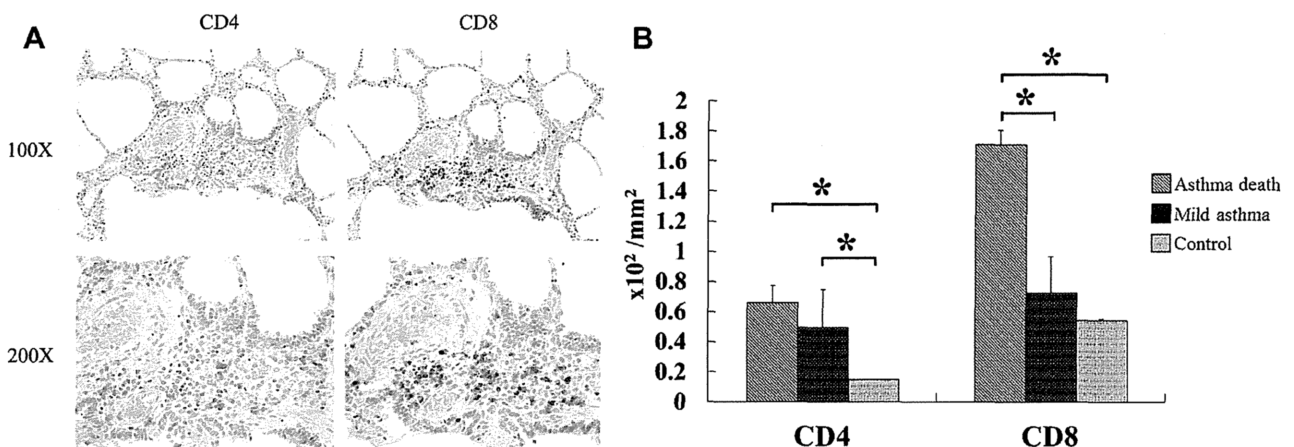

Figure 2. Immunostaining of lung tissue samples from patients who died of asthma using mouse anti-CD4 and mouse anti-CD8 monoclonal antibodies. *(A)* Positive staining for CD4 and CD8 is evident in lung tissue samples from patients who died of asthma (×100 and ×200). Positive reactivity was identified by Permanent Red (Dako). *(B)* Quantitative analysis showed that $CD8^+$ T cells were significantly increased in the airways of patients who died of asthma (n = 12), but not in those of patients with mild asthma (n = 5) or controls (n = 10). $^*P < .05$.

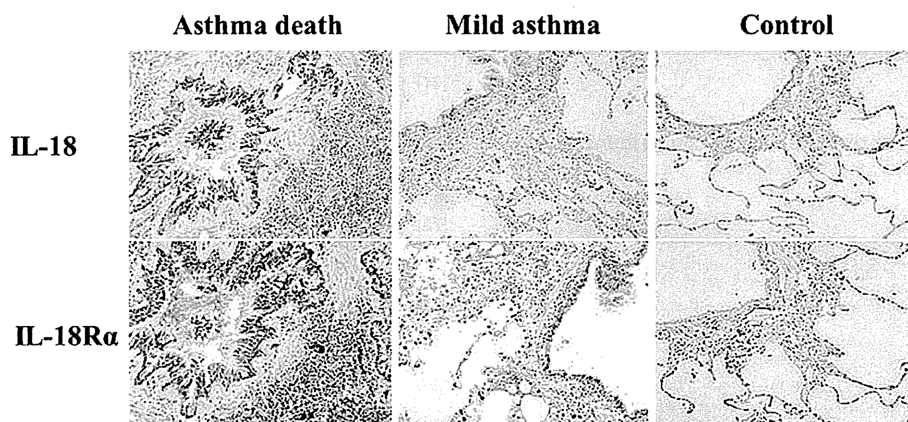

Figure 3. Immunostaining of lung tissue samples with mouse anti–interleukin-18 (IL-18) and interleukin-18 receptor α (IL-18Rα) from patients who died of asthma, patients with mild asthma, and control nonsmokers (×200). IL-18 and IL-18Rα positive reactivities were identified by 3-3′-diaminobenzidine-4HCl treatment (*brown*).

characteristically elicit strong $CD8^+$ T cells predominated by cytotoxic IFN-γ–secreting cells.[29] Therefore, the present findings suggest that previous studies may have included patients with COPD, patients with COPD and asthma, or patients with viral infection.[28,29] The present study showed that neutrophils may not be dramatically increased in the lungs of patients with sudden-onset fatal asthma or life-threatening asthma. Treatment with systemic corticosteroids was compared with nontreatment in fatal asthma. The number of neutrophils was not significantly different between the 2 groups. These results suggest that systemic corticosteroids may not affect neutrophil inflammation in the lungs of patients who succumbed to the effects of asthma.

Interleukin-18 strongly induces IFN-γ production upon exposure to various stimuli and can induce T_H2 responses.[16–19] Patients with acute asthma have higher serum IL-18 levels than normal control subjects. The IL-18 level has a tendency to correlate inversely with peak expiratory flow,[32,33] and the secretion of IL-18 from mononuclear cells of patients with bronchial asthma and atopic dermatitis is significantly higher than that in nonallergic controls.[34] It has been reported that the IL-18 gene polymorphism is significantly associated with the disease severity of asthma, with the re5744247 variant reflecting higher transcriptional activity and higher expression of IL-18 in lipopolysaccharide-stimulated monocytes and higher serum IL-18 levels.[35] IL-18R is a Toll-like receptor superfamily. Patients with fatal asthma were reported to have higher Toll-like receptor 2, 3, and 4 expression in the epithelial and airway compared with deceased control subjects.[36] Recently, the authors found that overexpression of IL-18 in the lungs can induce airway hyper-responsiveness and pulmonary inflammation through upregulating $CD4^+$ T cells and IL-13.[37] These findings suggest that IL-18 and IL-18R may be related to the severity of asthma. In the present study, the authors found that bronchoalveolar epithelial, smooth muscle, and pulmonary inflammatory cells strongly expressed IL-18 protein and IL-18Rα in the lungs of patients who died of asthma, whereas this was not the case in patients with mild asthma or normal controls. These results suggest that IL-18 released from bronchoalveolar epithelial cells and inflammatory cells, such as alveolar macrophages, can activate $CD8^+$ T cells in the airways of patients with severe asthma. Thereafter, IL-18 may induce T_H1 and T_H2 responses (eg, IL-4, IL-5, IL-13, and IFN-γ production) under conditions of severe allergic inflammation, with a potentially fatal outcome. A previous study showed that 12 younger (mean age 32 years) patients with fatal asthma who did not smoke had thicker basement membrane, airway smooth muscle, and outer wall areas in the small and large airways compared with 14 patients with fatal COPD who were longtime smokers (mean age 71 years).[38] The present study showed that severe airway remodeling accompanied by hypertrophy of smooth muscle, airway wall edema, and massive pulmonary inflammation was observed in all 12 patients with fatal asthma, including the 5-year-old boy. These data and a previous study suggest that fatal asthma produces severe airway remodeling regardless of age. It is known that the fungal allergens, especially *Alternaria alternate*, cause fatal asthma exacerbations. Sensitization and exposure to the spores of *A alternate* induce a natural helper cell-mediated rapid burst of T_H2 cytokine (IL-5 and IL-13) production and eosinophilic inflammation.[39] In this study, the authors do not know whether the allergens caused the fatal asthma exacerbations. Some patients with fatal asthma analyzed in this study may have been sensitized and challenged by *A alternate*. Further analysis is needed to test this issue.

The preferred treatment for severe persistent asthma is high-dose ICS plus a long-acting inhaled β agonist. In addition, for patients whose asthma is inadequately controlled on high-dose ICS and long-acting inhaled β agonist, a low dose of oral corticosteroids can be added. The anti-IgE humanized mAb (omalizumab) is also available. However, approximately 5% of patients with asthma are thought to have very severe disease that is resistant to high-dose ICS, oral corticosteroids, and/or anti-IgE antibody treatment. Such patients are thought to be at greatest risk of death from asthma. Therefore, new anti-inflammatory treatments are needed for severe asthma, especially asthma that is resistant to high-dose ICS and/or systemic steroid. The present study has shown the overproduction of IL-18 in the lungs of patients who succumbed to asthma. These results raise the possibility that blockade of IL-18 may be a feasible treatment for very severe asthma. Caspase-1 inhibitors, anti–IL-18 antibodies, anti–IL-18R antibodies, IL-18 binding protein, or inhibitors of genes downstream of the IL-18 signal transduction pathway, such as myeloid differentiation primary response gene 88, interleukin receptor-associated kinase, tumor necrosis factor receptor-associated factor 6, nuclear factor-κB, c-Jun N-terminal kinase, and p38 microtubule-associated protein kinase, may be of clinical benefit for the treatment of patients with severe asthma.

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Supplementary Data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.anai.2013.09.004.

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eMethods

Pulmonary Function Tests

Pulmonary function tests, including measurement of vital capacity (VC), forced expiratory volume in 1 second (FEV_1), and forced VC (FVC), were performed by spirometry. To assess the reversibility of the airway obstruction in all patients with a ratio (percentage) of FEV_1 to FVC lower than 70%, the FEV_1 measurement was repeated 20 minutes after the inhalation of 200 μg of salbutamol. Japanese predicted normal values were used to calculate the percentages of predicted VC, FVC, and FEV_1; those predicted values met the Japanese Pulmonary Function Standard in the Japanese Respiratory Society Statement.[1]

Immunohistochemical Assay

Immunohistochemical analysis was performed as reported previously.[2–4] Immunohistochemical analysis for $CD4^+$ and $CD8^+$ cells was performed as follows. Slides were stripped of paraffin wax, autoclaved at 125°C for 5 minutes in 0.01 mol/L of sodium citrate buffer (pH 6.0; for CD4 mAb) or in Tris-EDTA buffer (1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH9.0; for CD8 mAb), and treated with 0.3% H_2O_2 methanol for 10 minutes at room temperature. Blocking solution (Protein Block; Dako) was used at room temperature for 30 minutes. Antihuman CD4 (4B12 [mouse IgG1]; Dako) and antihuman CD8 (C8/144B [mouse IgG1]; Dako) were used at room temperature for 60 minutes. Positive reactivity was identified by Permanent Red (Dako) using an EnVision G|2 System/AP for rabbit/mouse (Permanent Red; Dako).

The immunohistochemical analysis for IL-18Rα^+ cells was performed as follows. Slides were stripped of paraffin wax, autoclaved at 125°C for 5 minutes in Tris-EDTA buffer (1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH9.0), and treated with 0.3% H_2O_2 methanol for 10 minutes at room temperature. Blocking solution was used at room temperature for 30 minutes. Anti–IL-18Rα (H44 [mouse IgG1], 4 μg/mL) mAb was used at 4°C for 18 hours. Positive reactivity was identified by DAB using an EnVision Kit with horseradish peroxidase (Dako).

The immunohistochemical analysis for IL-18^+ cells was performed as follows. Slides were stripped of paraffin wax, autoclaved in 0.01 mol/L of sodium citrate buffer (pH 6.0; Dako REAL; Dako, Kyoto, Japan) at 125°C for 5 minutes, and treated with 0.3% H_2O_2 methanol for 10 minutes at room temperature. Then, the slides were washed 3 times with 0.05 mol/L of Tris buffered saline (pH 7.6; Dako) at room temperature. Blocking solution (Protein Block Serum-Free; Dako) was used at room temperature for 30 minutes to prevent nonspecific staining. Antihuman IL-18 (clone 8 [mouse IgG2a][2] and clone 1-8D [mouse IgG1])[5] mAbs were used at 4°C for 18 hours to detect human IL-18. Then, the slides were washed 3 times with Tris buffered saline (pH 7.6). Positive reactivity was identified by DAB using an EnVision Kit with horseradish peroxidase (Dako). As negative controls, mouse-purified IgG2a and IgG1 Abs (Caltag Laboratories, Burlingame, California) were used for immunohistochemical assay.

Quantitative Assessment of Cells Producing IL-18

Percentages of $CD4^+$ or $CD8^+$ cells were assessed as previously reported.[2] Nine different OFs (9×1.4 mm^2) were selected at $\times 100$ magnification within 3 different square fields (3×8.75 mm^2), as described previously. The OFs were scanned under a microscope at $\times 400$ magnification. The percentages of cells that expressed CD4 or CD8 were counted within 3 different areas on each OF at $\times 400$ magnification. Then, the mean percentage of cells that expressed CD4 or CD8 within 9 OFs was calculated. Therefore, the mean number of $CD4^+$ or $CD8^+$ cells per square millimeter in inflammatory cells was calculated as: (total number of cells per square millimeter) $\times$ (mean percentage of cells expressing CD4 or CD8). Two pathologists examined these sections independently, without prior knowledge of the patients' clinical status and in a blinded manner.

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ORIGINAL RESEARCH

Tulobuterol inhibits rhinovirus infection in primary cultures of human tracheal epithelial cells

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Abstract

A transdermal patch preparation of the β_2 agonist tulobuterol has been designed to yield sustained β_2 agonistic effects and has been used as a long-acting β_2 agonist (LABA) in Japan. LABAs reduce the frequency of exacerbations of chronic obstructive pulmonary disease and bronchial asthma. However, inhibitory effects of LABAs on the replication of rhinovirus (RV), the major cause of exacerbations, have not been demonstrated. To examine the effects of tulobuterol on RV replication and on the production of the replication-induced pro-inflammatory cytokines, human tracheal epithelial cells were infected with a major group RV, type 14 rhinovirus (RV14). Tulobuterol reduced the RV14 titers and RNA levels; the concentrations of cytokines, including interleukin (IL)-1β, IL-6, and IL-8, in the supernatants; and susceptibility to RV14 infection. Tulobuterol reduced the expression of intercellular adhesion molecule-1 (ICAM-1), the receptor for RV14, and the number of acidic endosomes in the cells in which RV14 RNA enters the cytoplasm. Tulobuterol inhibited the activation of nuclear factor kappa B (NF-κB) proteins in nuclear extracts. A selective β_2-adrenergic receptor antagonist, ICI 118551 [erythro-dl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol], reversed the inhibitory effects of tulobuterol on the RV14 titers and RNA levels, the susceptibility to RV14 infection, cytokine production, and ICAM-1 expression. Tulobuterol may inhibit RV replication by reducing ICAM-1 expression and acidic endosomes and modulate airway inflammation during RV replication.

Introduction

Rhinoviruses (RVs) are the major cause of the common cold as well as the most common acute infection illnesses in humans (Turner and Couch 2006). RVs are also associated with exacerbations of inflammatory chronic pulmonary diseases such as chronic obstructive pulmonary disease (COPD) (Seemungal et al. 2000) and bronchial asthma (Johnston et al. 1995). Several mechanisms of RV-induced exacerbations of these diseases have been proposed, including virus-induced mucus hypersecretion, airway inflammation (Pizzichini et al. 1998; Seemungal et al. 2000), mast cell activation, and smooth muscle contraction.

Short-acting and long-acting β_2 agonists (LABAs) improve the symptoms and lung function in patients with bronchial asthma and COPD. Furthermore, LABAs by themselves, or in combination with inhaled corticosteroids reduce the frequency of exacerbations in patients with COPD (Calverley et al. 2007) and bronchial asthma (Pauwels et al. 1997). It has been suggested that these clinical benefits of β_2 agonists are related to the various effects of the agents, including bronchodilation and anti-inflammatory effects (Johnson 1991), improvement of mucociliary clearance and mucosal edema, and inhibition of mucus hypersecretion (Rogers and Barnes 2006).

RV infection induces the production of cytokines and monokines including interleukin (IL)-1, IL-6, and IL-8 (Subauste et al. 1995; Zhu et al. 1996). These cytokines and monokines have pro-inflammatory effects (Akira et al. 1990) and may also be involved in the pathogenesis

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