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Original Articles

An increase of soluble Fas, an inhibitor of apoptosis, associated with progression of COPD

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In chronic obstructive pulmonary disease (COPD) which consists of emphysema and chronic bronchitis, alveolar tissue and/or bronchiolar walls are progressively destroyed. This suggests cell death by necrosis and/or apoptosis although no direct evidence of apoptosis has been reported. It was speculated that the apoptosis-related factors are associated with the progression of COPD. Fas/Apo-1 receptor (Fas), Fas ligand (Fas-L) and soluble Fas ligand (sFas-L) are inducers, while soluble Fas (sFas) is an inhibitor of apoptosis. In this study, plasma sFas and sFas-L were measured in 19 COPD patients receiving supplemental O_2 (severe COPD) and 20 COPD patients not receiving supplemental O_2 (mild/moderate COPD). Twenty-two age- and sex-matched healthy volunteers (healthy controls) and 20 patients receiving supplemental O_2 and with levels of hypoxaemia similar to severe COPD due to other pulmonary diseases (disease controls) were also examined. Plasma sFas-L was within normal limits in all groups. Plasma sFas levels were similar among healthy controls, disease controls, and mild/moderate COPD patients, but significantly increased in severe COPD ($2{\cdot}6 \pm 1{\cdot}1$, $2{\cdot}6 \pm 0{\cdot}2$, $2{\cdot}8 \pm 0{\cdot}2$ and $4{\cdot}8 \pm 1{\cdot}0$ ng ml^{-1}, respectively). Although PaO_2 was lower in severe COPD than in mild/moderate COPD, and $PaCO_2$ was higher in severe COPD than in mild/moderate COPD, they were close between severe COPD and disease controls. Tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and C-reactive protein (CRP) were increased in patients with COPD, but were similar in both severe and mild/moderate COPD patients. We conclude that increased plasma sFas, which is independent of hypoxaemia, and increases in $PaCO_2$, TNF-α, IL-6 and inflammation, may be associated with progression of COPD.

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Introduction

Apoptosis differs from necrosis in terms of ultrastructural and biochemical features that include cytoplasmic and nuclear condensation, subsequent formation of membrane-bound apoptotic bodies (segmentation of the nucleus), and extensive degeneration of chromosomal DNA into oligomers of about 180 bp caused by activation of endogenous endonuclease (1). Apoptosis is not characteristically associated with inflammatory cell infiltration, unlike necrosis. Recent evidence suggests that apoptosis contributes to the pathophysiology of cancer, [including lung cancer (2,3)], viral infections, autoimmune diseases, and neurodegenerative disorders (4). Tsuyuki *et al.* showed that activation of the Fas/Apo-1 receptor (Fas) on lung eosinophils induces apoptosis and reduces eosinophilic inflammation of airways (5). Chronic obstructive pulmonary disease (COPD) is defined as a disease state characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema, the two usually occurring together (6). At present, there is no direct evidence that apoptosis occurs in the lungs of patients with COPD. However, it is well-known that a turnover is observed in alveolar cells or bronchial cells in the physiological state (7,8). COPD is a destructive disease of the lungs and causes progressive loss of alveoli and/or bronchioles (9–11). These findings suggest that cell death in alveoli and/or bronchioles may occur during COPD and may be at least in part due to apoptosis.

To date, several inducers and inhibitors of apoptosis have been reported. The Fas–Fas ligand (Fas-L) system is a potential inducer of apoptosis. Fas (designated as CD95) is a cell surface protein consisting of 319 amino acids with a single transmembrane domain, and belongs to the nerve

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growth factor receptor – tumour necrosis factor (TNF) receptor family (type I membrane protein) (12–15). Molecular cloning and nucleotide sequence analysis have revealed a human Fas mRNA variant that encodes a soluble Fas (sFas) molecule lacking the transmembrane domain because of a deletion of the exon encoding this region. sFas is found in culture supernatants of human B- and T-cell lines. Fas-L is a type II membrane protein that belongs to the TNF family (16). The membrane-bound human Fas-L is converted to soluble form (sFas-L) by matrix metalloproteinase-like enzyme (17). Human sFas-L, a 26 kDa glycoprotein, consists of the extracellular region of Fas-L. In mice, Fas-L is predominantly expressed in activated T cells, whereas Fas is expressed in various tissues including the thymus, heart, lungs, liver, and ovaries but not in the brain or spleen (15,18). Binding of Fas-L, sFas-L or agonistic anti-Fas antibody to Fas induces apoptosis (16,18,19). However, sFas blocks apoptosis, by inhibiting binding between Fas and Fas-L or sFas-L on the cell membrane. Serum sFas levels have been reported to be elevated in patients with systemic lupus erythematosus and B- and T-cell leukaemias, as are serum sFas-L levels in patients with large granular lymphocytic leukaemia and natural killer cell lymphoma (20–22). However, whether plasma levels of sFas and sFas-L are elevated or not in patients with COPD remains to be elucidated. In addition, TNF-α, the cytolytic activity of which is mediated by Fas, and interleukin 6 (IL-6), a cytokine inducing transcription of Fas gene, are Fas–Fas-L system related factors (16,23,24), and TNF-α, and C-reactive protein (CRP) are increased in the plasma of patients with COPD (25,26).

Therefore, the present study assessed (1) whether the plasma concentrations of sFas-L, an inducer of apoptosis, and sFas, an inhibitor of apoptosis, are increased in mild, moderate, or severe COPD patients; and (2) whether the concentrations of these proteins are related to other clinical parameters, such as blood gas analysis, pulmonary function test, and CRP, TNF-α, or IL-6.

Methods

SUBJECTS

Thirty-nine patients with COPD diagnosed by history, physical examination, roentgenographic examination and pulmonary function tests, according to the *Guidelines of the American Thoracic Society* (6), were included in the present study. The following criteria were used for patient selection: 1, clinically stable condition; 2, no recent change of drugs; 3, normal left ventricular ejection fraction (LVEF) in echocardiography (LVEF >0·55); 4, normal plasma creatinine concentration; and 5, absence of other pathological conditions (e.g. bronchial asthma, cancer, tuberculosis, neurodegenerative disorder, viral infection, liver disease, cardiac disease, collagen disease, sarcoidosis, and diabetes mellitus). The 39 patients with COPD were classified into 19 patients receiving supplemental O_2 (severe COPD) and 20 patients no receiving supplemental O_2 (mild/moderate COPD). Age and sex (male/female) were similar in severe COPD (66 ± 2, 13/6) and mild/moderate COPD (66 ± 3, 14/6). The controls (22 in total) were healthy age- and sex-matched volunteers without any disease. In addition, 20 age- and sex-matched patients receiving supplemental O_2 and with levels of hypoxaemia similar to severe COPD including 12 with old tuberculosis, six with idiopathic pulmonary fibrosis, and two with pneumoconiosis were used as disease controls. Supplemental O_2 was given to the severe COPD and disease control patients with PaO_2 levels below 55 mmHg at rest during hospitalization. Healthy and disease controls were not matched on smoking history. Bronchodilator agents including beta-agonists, anticholinergic agents and theophylline, antitussives, and expectorants were administered in COPD patients. However, there were no significant differences in these drugs between severe and mild/moderate COPD patients and disease controls. Informed consent was obtained from all subjects. The study was approved by the Research and Ethics Committee of Gifu University.

BLOOD SAMPLING

Peripheral venous blood samples were taken slowly from an antecubital vein, and transferred to chilled disposable tubes containing 2 sodium-ethylenediamine tetraacetic acid (1·5 mg ml^{-1}). Then, the disposable tubes were promptly centrifuged at 4°C, and aliquots of plasma immediately stored at −80°C until analysis for plasma levels of sFas, sFas-L, and the cytokines.

Arterial blood gas samples were taken from a femoral artery and were transferred to chilled disposable tubes containing heparin, and pH, $PaCO_2$, and PaO_2 levels were measured.

SFAS AND SFAS-L LEVELS BY ELISA

We have developed a highly sensitive enzyme-linked immunosorbent assay (ELISA) for sFas in plasma (27). Polystyrene plates were precoated with rabbit IgG for anti-human Fas synthetic peptide. The detection limit was 0·01 ng ml^{-1}. The average within-run and between-run coefficients of variation were 3·4% and 5·7%, respectively. The recovery of added sFas to serum ranged from 93% to 118%.

Plasma sFas-L was also quantitated by sandwich ELISA using NOK-1 (mouse IgG1, k) and NOK-3 (mouse IgM, k), monoclonal antibodies purified from mice immunized by intraperitoneal injection of human Fas-L (mouse T lymphoma cell lines) (28). Serial dilution of purified sFas-L, which was affinity purified from human Fas-L supernatant on a NOK-1 column, was used as the standard. The detection limit of this assay was 0·2 ng ml^{-1}.

For eight patients with COPD, a second plasma sampling for the measurement of the reproducibilities of plasma sFas and sFas-L levels was performed 1 day after the first plasma sampling. The reproducibilities of the assay of sFas and sFas-L for the same plasma and paired samples taken at intervals of 1 day by this method were $3·4 \pm 3·9\%$ and

TABLE 1. Patient characteristics

Group	Severe COPD	Mild/moderate COPD	Disease control	Healthy control
n	19	20	20	22
Gender (M/F)	13/6	14/6	14/6	15/7
Age (years)	66 ± 2	66 ± 3	67 ± 3	66 ± 1
Hugh–Jones				
I	0	6	0	22
II	0	6	3	0
III	7	2	4	0
IV	5	6	6	0
V	7	0	7	0
Smoking				
Never smoked (%)	1 (5)	1 (5)	11 (55)	9 (41)
Formerly smoked (%)	11 (58)	10 (50)	6 (30)	6 (27)
Current smoker (%)	7 (37)	9 (45)	3 (15)	7 (32)
Brinkman smoking index	1109 ± 123*,**	1045 ± 201*,**	588 ± 151	587 ± 129
pH	7·372 ± 0·016	7·395 ± 0·010	7·374 ± 0·019	7·401 ± 0·005
$PaCO_2$ (mmHg)	48·4 ± 1·8*,***	42·2 ± 2·8**	49·4 ± 2·2*	38·0 ± 0·8
PaO_2 (mmHg)	51·7 ± 0·9,*** [65·2 ± 2·3]	76·3 ± 2·4*,**	51·3 ± 1·1* [65·9 ± 2·8]	86·9 ± 0·9
VC (% pred)	70·5 ± 2·6*,**,***	81·9 ± 3·3*,**	46·2 ± 3·0*	92·6 ± 0·8
FEV_1/FVC (%)	42·0 ± 2·3*,**	48·0 ± 1·9*,**	75·9 ± 4·0*	88·9 ± 1·1
CRP† (mg dl^{-1})	2·33 ± 0·52*	1·52 ± 0·24*	2·41 ± 0·59	0·09 ± 0·06

Values are expressed as means ± SEM. pH, $PaCO_2$, and PaO_2 are the data before patients received supplemental O_2. [ ], The data after patients received supplemental O_2. †, C-reactive protein.
*$P<0·05$ *vs* healthy control; **$P<0·05$ *vs* disease control; ***$P<0·05$, *vs* mild/moderate COPD.

4·9 ± 2·9%, 7·0 ± 2·2% and 4·3 ± 2·0%, respectively. These results were satisfactorily small and reliable.

MEASUREMENTS OF PLASMA LEVELS OF TNF-*α*, IL-6, AND CRP

The plasma level of TNF-*α* was measured using a sandwich ELISA kit with two monoclonal anti-human TNF-*α* antibodies (No. 1121, IMMUNOTECH, Marseille, France). The plasma level of IL-6 was also measured using a two-step sandwich ELISA kit with two monoclonal anti-human IL-6 antibodies and rIL-6 standards (Human Il-6 ELISA Kit Fujirebio, Fujirebio Inc., Japan). The normal plasma level of IL-6 is less than 3 pg ml^{-1}. CRP was measured by latex nephelometric immunoassay with a detection limit of 0·03 mg dl^{-1}.

STATISTICAL METHODS

Statistical analysis was performed on the data obtained from the 81 subjects. Plasma levels of sFas, sFas-L, and the cytokines, and the parameters obtained from blood gas analysis and pulmonary function test were compared among groups using one-way analysis of variance (ANOVA) (Bonferroni/Dunn). This method is recommended for general use since it is easier to apply, has a wider range of applications, and gives lower critical values than other procedures (29). If the difference was significant, a modified unpaired *t*-test was used. Correlations of the plasma levels of sFas and sFas-L with cytokines were examined using simple linear regression analysis by the least-squares method.

All values are expressed as the mean ± standard error of the mean (SEM). A *P* value of less than 0·05 was regarded as significant.

Results

PATIENT CHARACTERISTICS AND BLOOD GAS OR PULMONARY FUNCTIONAL DATA

The characteristics of the patients are summarized in Table 1. There were no significant differences in age and gender among severe COPD, mild/moderate COPD, and the healthy and disease controls. Brinkman smoking index was significantly higher in severe and mild/moderate COPD patients than in healthy and disease controls. The percentage of smokers was also significantly higher in severe and mild/moderate COPD groups than in the two control groups. However, there were no significant differences between severe and mild/moderate COPD patients. There were no significant differences in the age at which smoking started among the four groups. The $PaCO_2$ concentration was significantly higher in severe COPD and disease

TABLE 2. Plasma concentrations of sFas, sFas-L, TNF-α, and IL-6

Group	Severe COPD	Mild/moderate COPD	Disease control	Healthy control
sFas (ng ml^{-1})	4·8 ± 1·0*,**,***	2·8 ± 0·2	2·6 ± 0·2	2·6 ± 0·1
sFas-L (ng ml^{-1})	0·58 ± 0·01	0·55 ± 0·02	0·54 ± 0·02	0·56 ± 0·02
TNF-α (pg ml^{-1})	23·2 ± 8·4*,**	20·6 ± 6·9*,**	9·4 ± 2·7	3·9 ± 0·5
IL-6 (pg ml^{-1})	13·9 ± 2·2*	16·5 ± 5·4*	15·4 ± 5·2*	2·1 ± 0·3

Values are means ± SEM.
*P<0·05 *vs* healthy control; **P<0·05 *vs* disease control; ***P<0·05 *vs* mild/moderate COPD.

controls than in mild/moderate COPD and healthy controls. PaO_2 was significantly lower in severe COPD and disease controls than in mild/moderate COPD. This value was also significantly lower in mild/moderate COPD than in the healthy controls. However, PaO_2, $PaCO_2$, and pH were similar between severe COPD and disease controls. VC (vital capacity) was decreased markedly in disease controls and slightly in severe and mild/moderate COPD. FEV_1/FVC was decreased markedly in severe and mild/moderate COPD and slightly in disease controls. The amount of CRP was significantly higher in severe and mild/moderate COPD patients and disease controls than in healthy controls. However, there was no significant difference among severe COPD, mild/moderate COPD, and disease controls.

PLASMA LEVELS OF SFAS AND SFAS-L

Plasma levels of sFas-L were similar among the four groups (Table 2). Plasma levels of sFas were significantly increased in severe COPD, compared to mild/moderate COPD and the healthy controls and disease controls. There was no significant difference among mild/moderate COPD, the healthy controls, and disease controls (Table 2, Fig. 1). The plasma levels of sFas in the Grade V patients (the Hugh–Jones classification) (30) were increased significantly in COPD patients (Fig. 2), but not in disease control (Grade V: 3·2 ± 0·2 ng ml^{-1}). There was also no significant difference in sFas levels between patients with low CRP (CRP<1·0 mg dl^{-1}) and high CRP (CRP≥1·0 mg dl^{-1}) (Fig. 3).

PLASMA LEVELS OF TNF-α AND IL-6

Although plasma TNF-α concentrations increased in both severe COPD and mild/moderate COPD, compared to the healthy and disease controls, there was no significant difference between the two COPD groups. Plasma IL-6 concentrations increased in severe and mild/moderate COPD, and disease controls, compared to the healthy controls, however, there was no significant difference among severe and mild/moderate COPD, and disease controls (Table 2). In severe and mild/moderate COPD patients, neither TNF-α nor IL-6 correlated with sFAS levels. Plasma IL-6 concentrations were significantly higher in the high CRP group than in the low CRP group, in severe and mild/moderate COPD patients (Fig. 3).

Discussion

The serum level of sFas-L was 0·56 ± 0·02 ng ml^{-1} in healthy controls. There was no evidence of an increase in sFas-L levels in patients with COPD.

The plasma sFas level for a group of 155 healthy subjects was 2·3 ± 0·6 ng ml^{-1}, and was higher in males than females and increased with age (27), but was similar to the present data in the healthy subjects. However, the plasma

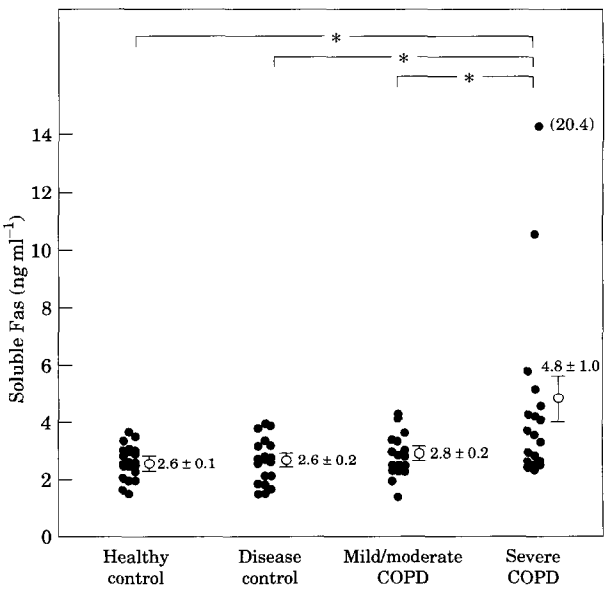

FIG. 1. Comparison of plasma levels of soluble Fas among four groups. A group of chronic obstructive pulmonary disease patients receiving supplemental O_2 (severe COPD): n=19; male/female=13/6, age=66 ± 2 years. A group of chronic obstructive pulmonary disease patients not receiving supplemental O_2 (mild or moderate COPD; mild/moderate COPD): n=20; male/female=14/6, age=66 ± 3 years. A group consisting old tuberculosis, idiopathic pulmonary fibrosis and pneumoconiosis as disease controls receiving supplemental O_2 (disease control): n=20; male/female=14/6, age=67 ± 3 years. Healthy control group: n=22; male/female=15/7, age=66 ± 1 years. Values are means ± SEM. *P<0·05.

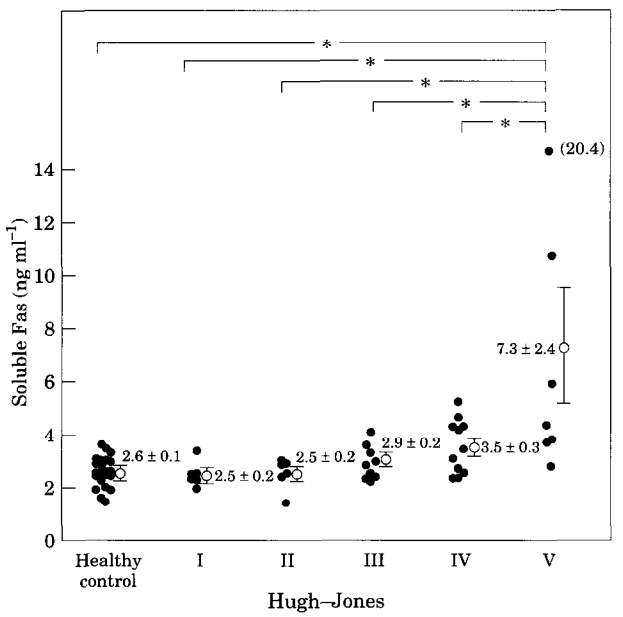

FIG. 2. Comparison of plasma levels of soluble Fas among five grades (the Hugh–Jones classification) of chronic obstructive pulmonary disease. I: $n=6$, male/female=5/1, age=70 ± 3 years. II: $n=6$, male/female=3/3, age=62 ± 7 years. III: $n=9$, male/female=8/1, age=66 ± 3 years. IV: $n=11$, male/female=8/3, age=64 ± 4 years. V: $n=7$, male/female=3/4, age=68 ± 3 years. Values are means ± SEM. *$P<0.05$.

sFas levels were much lower than those of a previous study by Cheng *et al.* (below 30 ng ml^{-1}) (20), although the rate of elevation in severe COPD patients (approximately two-fold) was similar to that in patients with systemic lupus erhythematosus, B- and T-cell leukaemias and lymphoma reported by them (20,21). Although the reason for this difference is unknown, there may be differences in the standard and the specificity of antibody to sFas used. In addition their study was of a small scale ($n=10$), and they did not define sex or age differences in healthy subjects.

Plasma sFas levels were not increased in mild/moderate COPD and disease controls, but were in severe COPD. PaO_2 and $PaCO_2$ were similar between severe COPD and disease controls. Of the patients with Grade V in the Hugh–Jones classification (30), the degree of dyspnoea, which is related with pulmonary function despite large variations within each degree (31–33), plasma sFas was increased in COPD patients, but not in disease controls. No relationship between plasma sFas and CRP, an indicator of inflammation, was seen. It is well-known that Fas mRNA is IL-6 dependent in the KT-3 cell line; IL-6 is a potent mediator of inflammation (34). Wada *et al.* reported that the nuclear factor needed for IL-6 expression is also an inducer of Fas mRNA in influenza virus-infected Hela cells of human (23). However, plasma IL-6 levels were similar among severe COPD, mild/moderate COPD, and disease controls, although significantly elevated relative to healthy controls. These findings suggest that (1) plasma sFas is an indicator of severity of COPD; and (2) the increase of

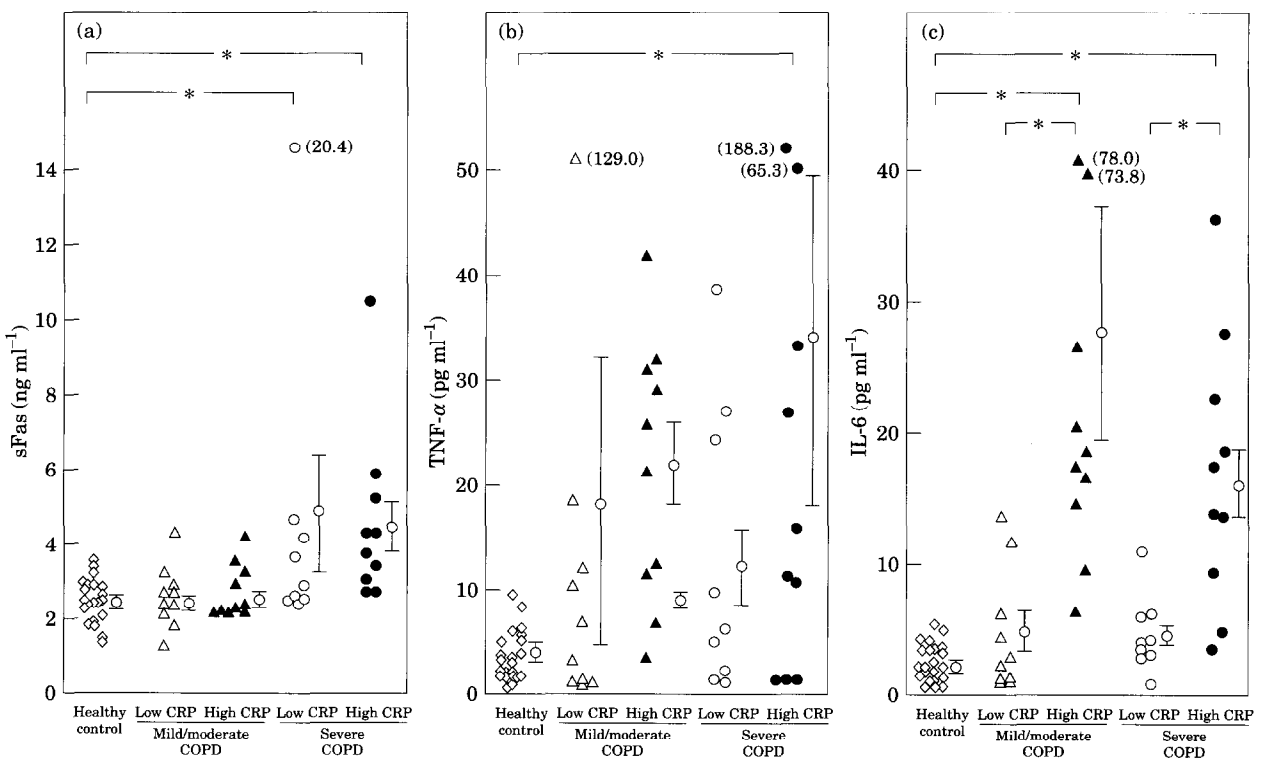

FIG. 3. Comparison of plasma levels of soluble Fas (sFas) (a), tumour necrosis factor-α (TNF-α); (b), and interleukin-6 (IL-6) (c) between low and high C-reactive protein (CRP) groups (low CRP; CRP<1·0 mg dl^{-1}, high CRP; CRP≧1·0 mg dl^{-1}). *$P<0.05$.

plasma sFas is not due to hypoxaemia, decrease of VC and FEV_1/FVC, inflammation or an increase of plasma IL-6.

Plasma sFas levels in severe COPD patients receiving supplemental O_2 and with Hugh–Jones Grade V varied from normal to very high. Significant elevation of the plasma sFas level over two standard deviations was seen in eight of 19 severe COPD patients and four of seven COPD patients with Hugh-Jones Grade V. The mechanism of the variability remains unknown. However, plasma sFas may be related to the pathophysiology of the progression of COPD, at least, in patients with high plasma levels.

Abnormality of various apoptosis-related factors has been reported in patients with COPD. Levels of cytokines such as plasma TNF-α, an inducer of apoptosis, are elevated in COPD (25). The cytolytic activity of TNF is mediated by Fas in animal models (24). Fas and Fas-L exhibited homology with TNF receptor and TNF, respectively. Fas is abundantly expressed in activated lymphocytes and in the primary cells from various organs, including the lungs. The activation of Fas induces apoptosis of inflammatory cells. Recently, studies using bronchial biopsies in patients with COPD have shown an increased number of $CD3^+$ (35). In another study, the authors found that more severely ill COPD patients had higher concentrations of T lymphocytes, suggesting that the latter are related to the bronchial hyperreactivity found in some COPD patients (35). In the present study, we found that plasma sFas levels were increased approximately two-fold in severe COPD patients. The elevation of plasma sFas was similar to that in patients with systemic lupus erythematosus, B- and T-cell leukaemias and lymphoma by Cheng *et al.* (20). In addition, these investigators described that sFas when injected at approximately two-fold the control level in mice exhibited activity *in vivo* and inhibited apoptosis, by inhibiting the binding of Fas-L or sFas-L to Fas on the cell membrane (20). Therefore, the increased plasma sFas in severe COPD patients may have an inhibiting effect on apoptosis of the infiltrated T lymphocytes in the lungs of patients with COPD, although plasma sFas-L, an inducer of apoptosis, was not increased in the present study. There has been no report on direct evidence that apoptosis, Fas and Fas-L in lungs were increased in COPD patients. Further investigation on the cytotoxic properties of lymphocytes and the identification, localization and quantitation of cellular death in lungs is warranted.

We conclude that elevation of plasma sFas may play an important role in the pathophysiology of COPD progression.

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