variables were analyzed using Pearson's correlation coefficient, and Student's *t*-test was performed to compare mean values. Stepwise forward and backward multiple regression analyses were performed to examine relative contributions of comorbidities to the CAT score. In these analyses, comorbidities were included as a categorical variable. χ^2 analysis was conducted to compare the frequencies between two groups. P values less than 0.05 were considered significant. All data were analyzed using the JMP version 9.0.2 software for Windows.

Results

Clinical features of the study population

The clinical characteristics of the study subjects are shown in Table 1. Among the 403 subjects in this study, 67 were excluded from the COPD group based on spirometry results. For the 336 COPD patients, all spirometric GOLD stages were represented, with 73 (21.7%) patients in GOLD I, 155 (46.1%) patients in GOLD II, 84 (25.0%) patients in GOLD III, and 24 (7.1%) patients in GOLD IV. The average age of the COPD patients was 72.4 ± 8.0 years, which was older than the non-COPD subjects (66.1 ± 11.7 years; $p < 0.001$). The COPD patients included 15 never smokers. The mean CAT score and the SGRQ total score were significantly higher in COPD patients than in non-COPD subjects (Table 1).

Correlations of CAT, SGRQ, and SF-36 scores

To validate the CAT in a Japanese COPD population, the correlations between the CAT and SGRQ or SF-36 were examined. The CAT score was significantly correlated to the SGRQ total score and to each SGRQ component score in COPD patients ($p < 0.001$) (Figure 1). These coefficients (Pearson's r) ranged from 0.649 (activity score) to 0.810 (total score). When the SGRQ total score was divided into quartiles (0≦and < 30, 30≦and < 45, 45≦and < 60, 60≦) as shown in a previous study [11], the mean CAT score corresponded to 8.4 ± 5.0, 13.5 ± 5.0, 19.9 ± 6.7, and 27.0 ± 5.5, respectively, and clearly distinguished between the categories ($p < 0.001$). The CAT score was also significantly correlated ($p < 0.001$) with all SF-36 component scores; these ranged from r = −0.363 (bodily pain) to r = −0578 (general health) (Figure 2).

Table 1 Clinical characteristics of the study groups

	Non-COPD (n = 67)	COPD (n = 336)	p value
Male, n (%)	63 (94)	307 (91)	NS
Age, years	66.1 ± 11.7	72.4 ± 8.0	<0.001
Smoking, pack-years	49.3 ± 27.9	56.7 ± 30.3	NS
Never smoker, n (%)	0 (0)	15 (4)	NS
Ex-smoker, n (%)	50 (75)	282 (84)	NS
Current smoker, n (%)	17 (25)	39 (12)	<0.01
BMI, kg/m^2	22.8 ± 4.1	22.5 ± 3.3	NS
%VC, %	94.5 ± 16.5	94.7 ± 18.6	NS
%FEV$_1$, %	89.6 ± 16.4	61.6 ± 21.6	<0.001
CAT score	9.4 ± 6.6	12.4 ± 8.3	<0.01
SGRQ total	20.7 ± 16.4	29.4 ± 19.6	<0.001

Data are presented as means ± SD.
COPD Chronic obstructive pulmonary disease, *NS* Not significant, *BMI* Body mass index, *VC* Vital capacity, *FEV$_1$* Forced expiratory volume in one second, *CAT* COPD assessment test, *SGRQ* the St. Georges Respiratory Questionnaire.

Prevalence of comorbidities and association with CAT

The percentage of patients with each comorbidity is listed in Table 2. Hypertension, GERD, and osteoporosis were the three most prevalent comorbidities of the COPD patients. The prevalence of osteopenia was 33% besides osteoporosis. The prevalence of anxiety and depression were 7% and 10%, respectively, and the concurrent prevalence of both anxiety and depression was 3%. There was a significant correlation between the HAD-A score and the HAD-D score (data not shown, R = 0.625, $p < 0.001$) among the COPD patients. The univariate analysis indicated that COPD subjects with GERD, anxiety, depression, osteoporosis, or arrhythmia had a significantly higher CAT score than those without (Table 2).

Patients with GERD were treated with H_2 blocker (9%) or proton pump inhibitor (20%), while those with osteoporosis were treated with bisphosphonate only in 11%. Depression and anxiety were also overlooked and treated with medications only in 20 and 9%, respectively. In contrast more patients with arrhythmia were treated with anticoagulant (33%), antiplatelet (18%), and other medications.

Prevalence of other comorbidities and the relationships with the CAT score is presented as Additional file 1: Table S1 including infrequent comorbidities (<5%) and local comorbidities potentially associated with treatment of COPD (cataract, glaucoma, prostatic hypertrophy).

Multivariate comorbid determinants of the CAT score

Using the results of the univariate analysis, a stepwise multiple regression for the CAT score was then performed including GERD, anxiety, depression, osteoporosis, arrhythmia, age, pack-years, body mass index (BMI), and %FEV$_1$ as variables. The significant comorbid associations with the CAT score were GERD, depression, %FEV$_1$, arrhythmia, and anxiety although each contribution was modest (as indicated by low R^2 values) (Table 3). Taken together, these chief comorbidities accounted for about 20% of the variance in the CAT score. Osteoporosis, age, pack-years, and BMI were not significantly associated with a higher CAT score on multivariate analysis.

The association of the CAT score with %FEV$_1$ was modest on univariate analysis (r = −0.258, $p < 0.001$). BMI was also correlated with the CAT score (r = −0.167, $p < 0.01$), whereas age and pack-years were not related to

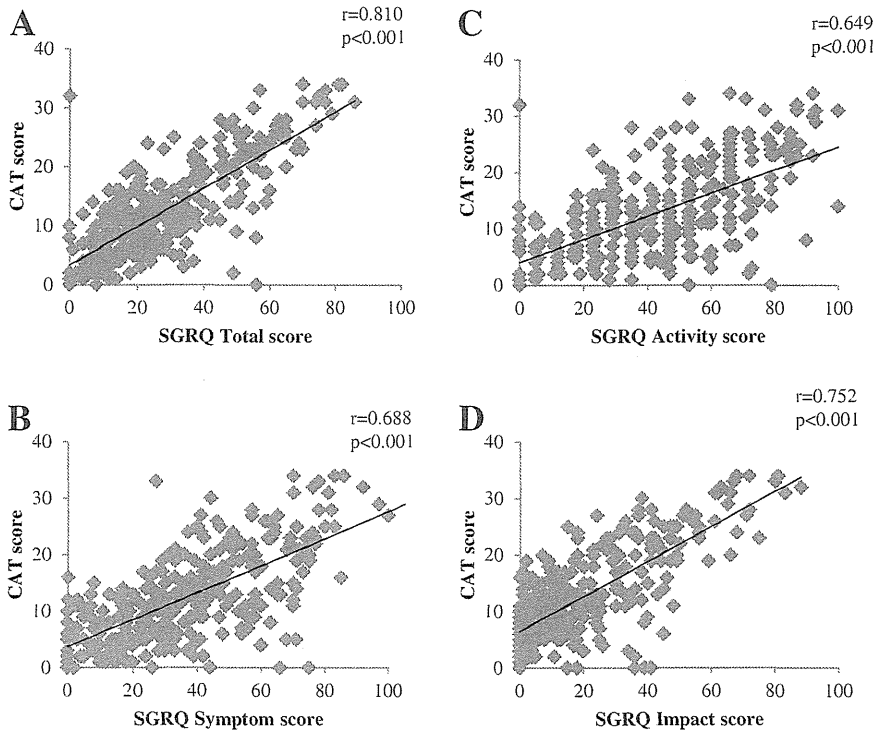

Figure 1 Correlation between CAT score and SGRQ scores in COPD patients (n = 336). **A**: SGRQ Total score, **B**: SGRQ Symptom score, **C**: SGRQ Activity score, **D**: SGRQ Impact score.

that (p = 0.17 and 0.62, respectively). In addition the CAT score in the underweight patients (16.9 ± 9.4) (13%, BMI < 18.5) was higher than those in the normal (12.0 ± 8.1) (65%, 18.5≦BMI<25) and overweight patients (11.1 ± 7.2) (22%, 25≦BMI). Although weight loss is associated with various comorbidities and BMI was not related to the CAT score on multivariate analysis, these observations may imply certain association of low BMI with impaired QOL in COPD patients.

Relationships between each CAT item and comorbidities

The CAT consists of eight items, each presented as a 6-point semantic differential scale (Additional file 2: Table S2). Thus, the multivariate comorbid determinants of each CAT item were examined using stepwise multiple regression analysis. As shown in Table 4, no comorbidities were associated with worsened cough or phlegm, but the presence of GERD was associated with higher scores of the other CAT items. The presence of depression was also significantly associated with increased scores of all CAT items except for cough, phlegm, and breathlessness.

Discussion

Comorbidities are frequent in subjects with COPD, but the study of their contribution to COPD-related QOL impairment has been limited [2,12,24]. In this study, the validity of the Japanese version of the CAT was first confirmed in a well-characterized cohort of COPD patients, since previous studies demonstrated that the SGRQ scores also tended to be lower in Japanese COPD patients than in patients in Western countries [15]. It was found that the CAT performed in a very similar manner to that reported recently in other Asian countries [25]. The mean score of the COPD patients was slightly lower than in studies in other countries [8,11], but the present population had a higher mean $\%FEV_1$ compared to those studies, so they were likely to have had milder disease. It should be noted that there were some discrepancies between the SGRQ and CAT scores in individual patients despite good correlation between these scores at the population level. The discrepancy in the total score appeared to be derived from the differences in the activity and symptom scores, but was within the same range as previously reported (11). In addition the CAT scores clearly distinguished between the quartiles SGRQ score categories. The present study provides good evidence for the validity of the CAT in a Japanese population. To the best of our knowledge, this was also the first comparison between the CAT (a disease-specific measure) and the SF-36, which is a generic measure of health (Figure 2). We therefore believe that our data are generalizable to other languages and countries.

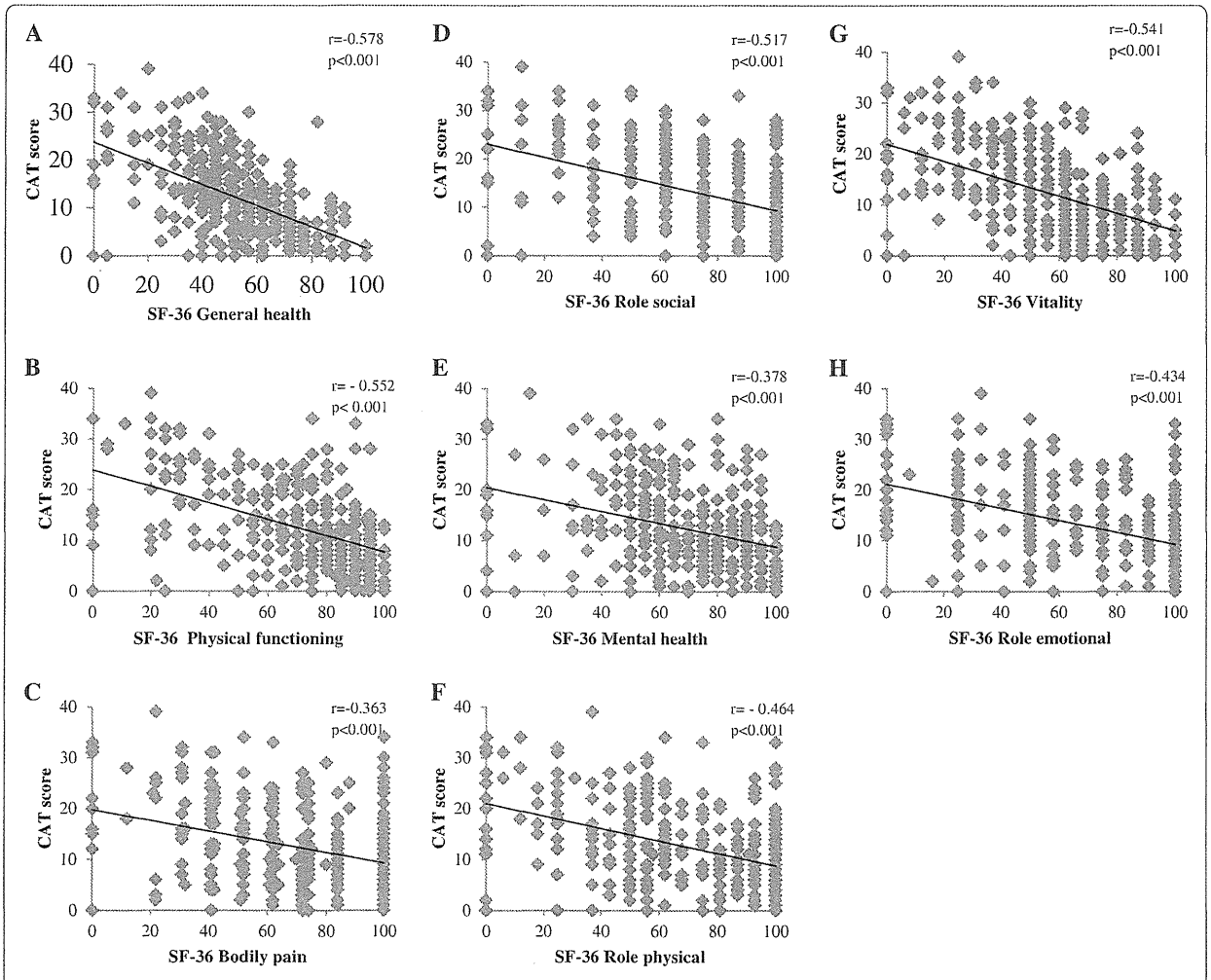

Figure 2 Correlations between the CAT score and SF-36 scores in COPD patients (n = 336). **A**: SF-36 General health, **B**: SF-36 Physical functioning, **C**: SF-36 Bodily pain, **D**: Role social, **E**: SF-36 Mental health, **F**: SF-36 Role physical, **G**: SF-36 Vitality, **H**: SF-36 Role emotional.

The prevalence of comorbidities was then comprehensively examined, and their relationships with the CAT score were assessed among the COPD patients. Among a variety of comorbidities examined, the prevalence of GERD and depression was positively related to the total CAT score. In addition, GERD and depression were associated with 6 and 5 of the 8 CAT items, respectively, worsening overall health status in COPD patients. This finding is particularly important, because those diseases may co-exist unrecognized and untreated.

The prevalence of GERD symptoms in the present COPD population was higher than that reported previously in Western and Japanese studies [3,26]. The significant relationships between GERD symptoms and the CAT draw attention to the possibility that comorbid GERD may worsen the symptoms of COPD, even though GERD may have little impact on mortality [3]. On the other hand, no association was found between GERD symptoms and $\%FEV_1$ (data not shown); Mokhlesi et al. reported a high prevalence of GERD symptoms in patients with COPD, with a trend toward it being higher in those with severe COPD [27]. GERD is a digestive disorder in which the mechanisms that keep stomach contents inside the stomach malfunction, releasing acidic stomach contents into the esophagus. Cough can then be induced by the acid stimulus-derived vagal reflex. However, analysis of the relationships between each CAT item and GERD symptoms did not suggest a correlation between GERD symptoms and the extent of cough or phlegm; other CAT items were more closely correlated with GERD in this study (Table 4). We speculate that the occurrence of acid reflux, as reflected by a high FSSG score, might further worsen the health status of COPD patients, in addition to any effect of airflow limitation.

Depression and anxiety are well known comorbidities and are independently associated with a higher risk of

Miyazaki et al. Respiratory Research 2014, 15:13
http://respiratory-research.com/content/15/1/13

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Analysis of comorbid factors that increase the COPD assessment test scores

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Abstract

Background: The chronic obstructive pulmonary disease (COPD) Assessment Test (CAT) is a concise health status measure for COPD. COPD patients have a variety of comorbidities, but little is known about their impact on quality of life. This study was designed to investigate comorbid factors that may contribute to high CAT scores.

Methods: An observational study at Keio University and affiliated hospitals enrolled 336 COPD patients and 67 non-COPD subjects. Health status was assessed by the CAT, the St. Georges Respiratory Questionnaire (SGRQ), and all components of the Medical Outcomes Study Short-Form 36-Item (SF-36) version 2, which is a generic measure of health. Comorbidities were identified based on patients' reports, physicians' records, and questionnaires, including the Frequency Scale for the Symptoms of Gastro-esophageal reflux disease (GERD) and the Hospital Anxiety and Depression Scale. Dual X-ray absorptiometry measurements of bone mineral density were performed.

Results: The CAT showed moderate-good correlations with the SGRQ and all components of the SF-36. The presence of GERD, depression, arrhythmia, and anxiety was significantly associated with a high CAT score in the COPD patients.

Conclusions: Symptomatic COPD patients have a high prevalence of comorbidities. A high CAT score should alert the clinician to a higher likelihood of certain comorbidities such as GERD and depression, because these diseases may co-exist unrecognized.

Trial registration: Clinical trial registered with UMIN (UMIN000003470).

Keywords: Chronic obstructive pulmonary disease, Health status, Depression, Gastro-esophageal reflux, Comorbidity, Osteoporosis

Background

Chronic obstructive pulmonary disease (COPD) is characterized by progressive and partially reversible airflow limitation, and it is among the leading causes of mortality worldwide [1]. COPD patients manifest a range of comorbidities, some of which may worsen quality of life (QOL) [2], and others may increase the risk of death [3].

According to the latest version of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guideline, assessment of COPD should be based on the patient's level of symptoms, future risk of exacerbations, and the severity of spirometric abnormalities [4]. A number of questionnaires are available that assess COPD-specific health status, including the St. Georges Respiratory Questionnaire (SGRQ) [5] and the Chronic Respiratory Questionnaire [6]. These are validated and widely used for clinical trials, but they are complex and require special software or licenses to use, limiting their routine applicability in clinical practice. A newly developed questionnaire, the COPD Assessment Test (CAT), offers an alternative to those complex tools [7]. It consists of eight items, each presented as a 6-point semantic differential scale, providing a score out of 40, indicating the impact of the disease.

The usefulness of CAT has recently been reported in a variety of clinical settings, such as for evaluating the

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severity of COPD exacerbations and the effects of rehabilitation [8-10]. However, relatively little is known about the impact of comorbidities on the CAT score of individuals with COPD. A recent study reported that the presence of cardiovascular comorbidity did not significantly affect the CAT score [11]. In another study, the CAT score appeared unaffected by potentially confounding comorbidities including renal failure, obesity, and sleep disorder [8]. A recent study has shown that metabolic and cardiovascular comorbidities may increase in frequency in worse GOLD groups [12]. However, the differential impact of other major comorbidities remains to be investigated.

We hypothesized that symptomatic COPD patients exhibiting high CAT scores have unrecognized comorbidities. Therefore, comorbid factors that might have an impact on increasing the CAT score in COPD patients enrolled in a well-characterized cohort study, called the Keio COPD Comorbidity Research (K-CCR), were evaluated.

Materials and methods

Study populations

Keio University and affiliated hospitals have established an observational COPD cohort designed to prospectively investigate the management of COPD comorbidities. A total of 572 subjects were enrolled between April 2010 and December 2012, including patients who had been diagnosed as having COPD and at risk for COPD (non-COPD) by pulmonary physicians. Inclusion criteria consisted of (1) age≧40 years old, (2) forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) < 0.7, (3) presence of emphysematous changes on chest computed tomography (CT) scans, and (4) chronic respiratory symptoms with significant smoking history (≧30 pack-years). Pulmonary function tests and chest CT scan were performed in all participants, and the COPD group fulfilled the criteria (1) and (2), while the non-COPD group met the criteria (1) and either (3) or (4) without airflow limitation (FEV_1/FVC≧0.7). Excluded were patients who had a history of lung resection surgery or serious complications such as unstable cardiovascular or cerebral diseases and malignant tumors under treatment. For the purpose of this study, only subjects with complete data available for comorbidities (n = 403) were enrolled. All patients were clinically stable and without exacerbations for at least one month prior to recruitment. The protocol was approved by the ethics committees of Keio University and the affiliated hospitals, and written, informed consent was obtained from each patient.

Assessment of clinical parameters

Spirometry was performed in all patients in a stable condition using an electronic spirometer in accordance with the guidelines of the American Thoracic Society [13]. Predicted values of spirometric measurements were derived from the guidelines for pulmonary function tests issued by the Japanese Respiratory Society [14]. Regular treatment was not changed prior to spirometric testing.

At enrollment, a full medical and smoking history and information about current pharmacological treatment were obtained, and clinical examinations were performed. Comorbid diagnoses were established using clinical history and examination findings, supported by a review of available medical records. All of the following questionnaires were completed by the patients themselves at home, when in the stable state.

Questionnaires on QOL

The Japanese version of the CAT was applied for the assessment of COPD-specific health status, together with the SGRQ in Japanese [5,15,16]. The Medical Outcomes Study Short-Form 36-Item (SF-36) version 2 was used to assess general health status [17].

Evaluation of gastro-esophageal reflux disease (GERD)

GERD symptoms were evaluated using a self-reported Frequency Scale for the Symptoms of GERD (FSSG) questionnaire, consisting of 12 items. This is known to reflect the severity of the endoscopic findings of GERD [18], with a cut-off score of 8 points for GERD [19].

Evaluation of anxiety and depression

Depression and anxiety were assessed at baseline using the Hospital Anxiety and Depression Scale (HADS) [20]. This is a validated screening tool for cases of depression and anxiety in both hospitalized and primary care patients with chronic diseases, including COPD [21]. The HADS consists of seven items for anxiety (HAD-A) and seven items for depression (HAD-D). The scores range from 0 to 21 for each subscale, with a score of 0–7 denoting a non-case, 8–10 a possible case, and 11 or higher a probable case, which may guide referral for psychological support [20].

Dual X-Ray Absorptiometry (DXA)

DXA measurements of bone mineral density (BMD) were performed at the hip and lumbar spine using a Hologic 4500A Discovery bone densitometer (Hologic, Bedford, MA) for 248 of the 336 COPD patients. The T-score was used for the evaluation of osteoporosis, in which a T-score greater than −1 is considered normal, -1 to −2.5 osteopenia, and less than −2.5 is diagnostic of osteoporosis [22,23].

Statistical analyses

Data are presented as means ± standard deviation (SD). Univariate associations between CAT scores and other

variables were analyzed using Pearson's correlation coefficient, and Student's *t*-test was performed to compare mean values. Stepwise forward and backward multiple regression analyses were performed to examine relative contributions of comorbidities to the CAT score. In these analyses, comorbidities were included as a categorical variable. χ^2 analysis was conducted to compare the frequencies between two groups. P values less than 0.05 were considered significant. All data were analyzed using the JMP version 9.0.2 software for Windows.

Results

Clinical features of the study population

The clinical characteristics of the study subjects are shown in Table 1. Among the 403 subjects in this study, 67 were excluded from the COPD group based on spirometry results. For the 336 COPD patients, all spirometric GOLD stages were represented, with 73 (21.7%) patients in GOLD I, 155 (46.1%) patients in GOLD II, 84 (25.0%) patients in GOLD III, and 24 (7.1%) patients in GOLD IV. The average age of the COPD patients was 72.4 ± 8.0 years, which was older than the non-COPD subjects (66.1 ± 11.7 years; $p < 0.001$). The COPD patients included 15 never smokers. The mean CAT score and the SGRQ total score were significantly higher in COPD patients than in non-COPD subjects (Table 1).

Correlations of CAT, SGRQ, and SF-36 scores

To validate the CAT in a Japanese COPD population, the correlations between the CAT and SGRQ or SF-36 were examined. The CAT score was significantly correlated to the SGRQ total score and to each SGRQ component score in COPD patients ($p < 0.001$) (Figure 1). These coefficients (Pearson's r) ranged from 0.649 (activity score) to 0.810 (total score). When the SGRQ total score was divided into quartiles (0≦and < 30, 30≦and < 45, 45≦and < 60, 60≦) as shown in a previous study [11], the mean CAT score corresponded to 8.4 ± 5.0, 13.5 ± 5.0, 19.9 ± 6.7, and 27.0 ± 5.5, respectively, and clearly distinguished between the categories ($p < 0.001$). The CAT score was also significantly correlated ($p < 0.001$) with all SF-36 component scores; these ranged from r = −0.363 (bodily pain) to r = −0578 (general health) (Figure 2).

Table 1 Clinical characteristics of the study groups

	Non-COPD (n = 67)	COPD (n = 336)	p value
Male, n (%)	63 (94)	307 (91)	NS
Age, years	66.1 ± 11.7	72.4 ± 8.0	<0.001
Smoking, pack-years	49.3 ± 27.9	56.7 ± 30.3	NS
Never smoker, n (%)	0 (0)	15 (4)	NS
Ex-smoker, n (%)	50 (75)	282 (84)	NS
Current smoker, n (%)	17 (25)	39 (12)	<0.01
BMI, kg/m^2	22.8 ± 4.1	22.5 ± 3.3	NS
%VC, %	94.5 ± 16.5	94.7 ± 18.6	NS
%FEV$_1$, %	89.6 ± 16.4	61.6 ± 21.6	<0.001
CAT score	9.4 ± 6.6	12.4 ± 8.3	<0.01
SGRQ total	20.7 ± 16.4	29.4 ± 19.6	<0.001

Data are presented as means ± SD.
COPD Chronic obstructive pulmonary disease, *NS* Not significant, *BMI* Body mass index, *VC* Vital capacity, *FEV$_1$* Forced expiratory volume in one second, *CAT* COPD assessment test, *SGRQ* the St. Georges Respiratory Questionnaire.

Prevalence of comorbidities and association with CAT

The percentage of patients with each comorbidity is listed in Table 2. Hypertension, GERD, and osteoporosis were the three most prevalent comorbidities of the COPD patients. The prevalence of osteopenia was 33% besides osteoporosis. The prevalence of anxiety and depression were 7% and 10%, respectively, and the concurrent prevalence of both anxiety and depression was 3%. There was a significant correlation between the HAD-A score and the HAD-D score (data not shown, R = 0.625, $p < 0.001$) among the COPD patients. The univariate analysis indicated that COPD subjects with GERD, anxiety, depression, osteoporosis, or arrhythmia had a significantly higher CAT score than those without (Table 2).

Patients with GERD were treated with H_2 blocker (9%) or proton pump inhibitor (20%), while those with osteoporosis were treated with bisphosphonate only in 11%. Depression and anxiety were also overlooked and treated with medications only in 20 and 9%, respectively. In contrast more patients with arrhythmia were treated with anticoagulant (33%), antiplatelet (18%), and other medications.

Prevalence of other comorbidities and the relationships with the CAT score is presented as Additional file 1: Table S1 including infrequent comorbidities (<5%) and local comorbidities potentially associated with treatment of COPD (cataract, glaucoma, prostatic hypertrophy).

Multivariate comorbid determinants of the CAT score

Using the results of the univariate analysis, a stepwise multiple regression for the CAT score was then performed including GERD, anxiety, depression, osteoporosis, arrhythmia, age, pack-years, body mass index (BMI), and %FEV$_1$ as variables. The significant comorbid associations with the CAT score were GERD, depression, %FEV$_1$, arrhythmia, and anxiety although each contribution was modest (as indicated by low R^2 values) (Table 3). Taken together, these chief comorbidities accounted for about 20% of the variance in the CAT score. Osteoporosis, age, pack-years, and BMI were not significantly associated with a higher CAT score on multivariate analysis.

The association of the CAT score with %FEV$_1$ was modest on univariate analysis (r = −0.258, $p < 0.001$). BMI was also correlated with the CAT score (r = −0.167, $p < 0.01$), whereas age and pack-years were not related to

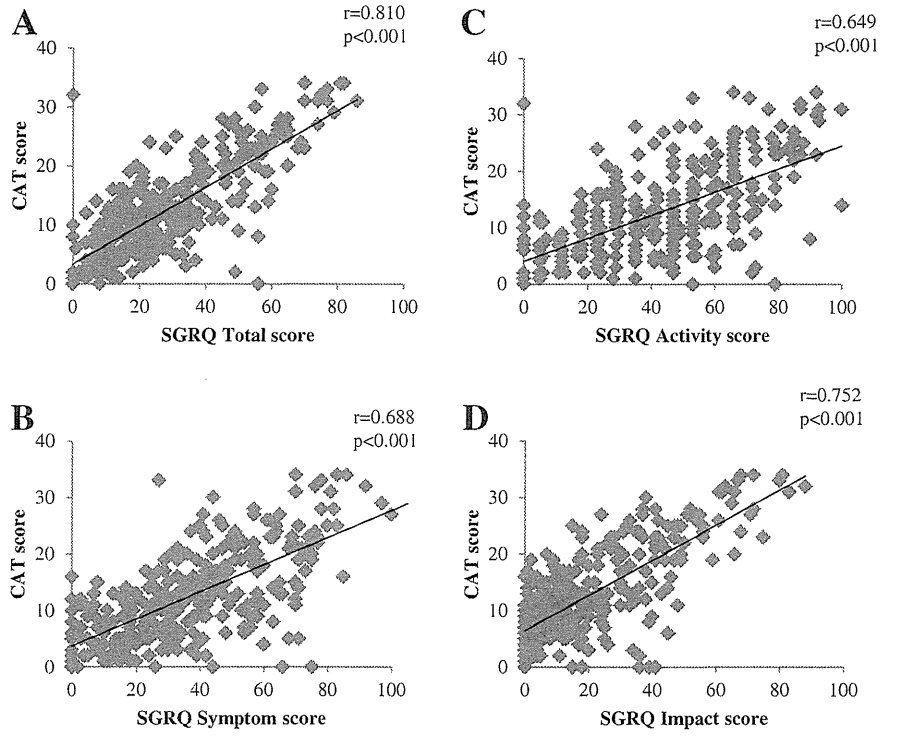


Figure 1 Correlation between CAT score and SGRQ scores in COPD patients (n = 336). **A**: SGRQ Total score, **B**: SGRQ Symptom score, **C**: SGRQ Activity score, **D**: SGRQ Impact score.

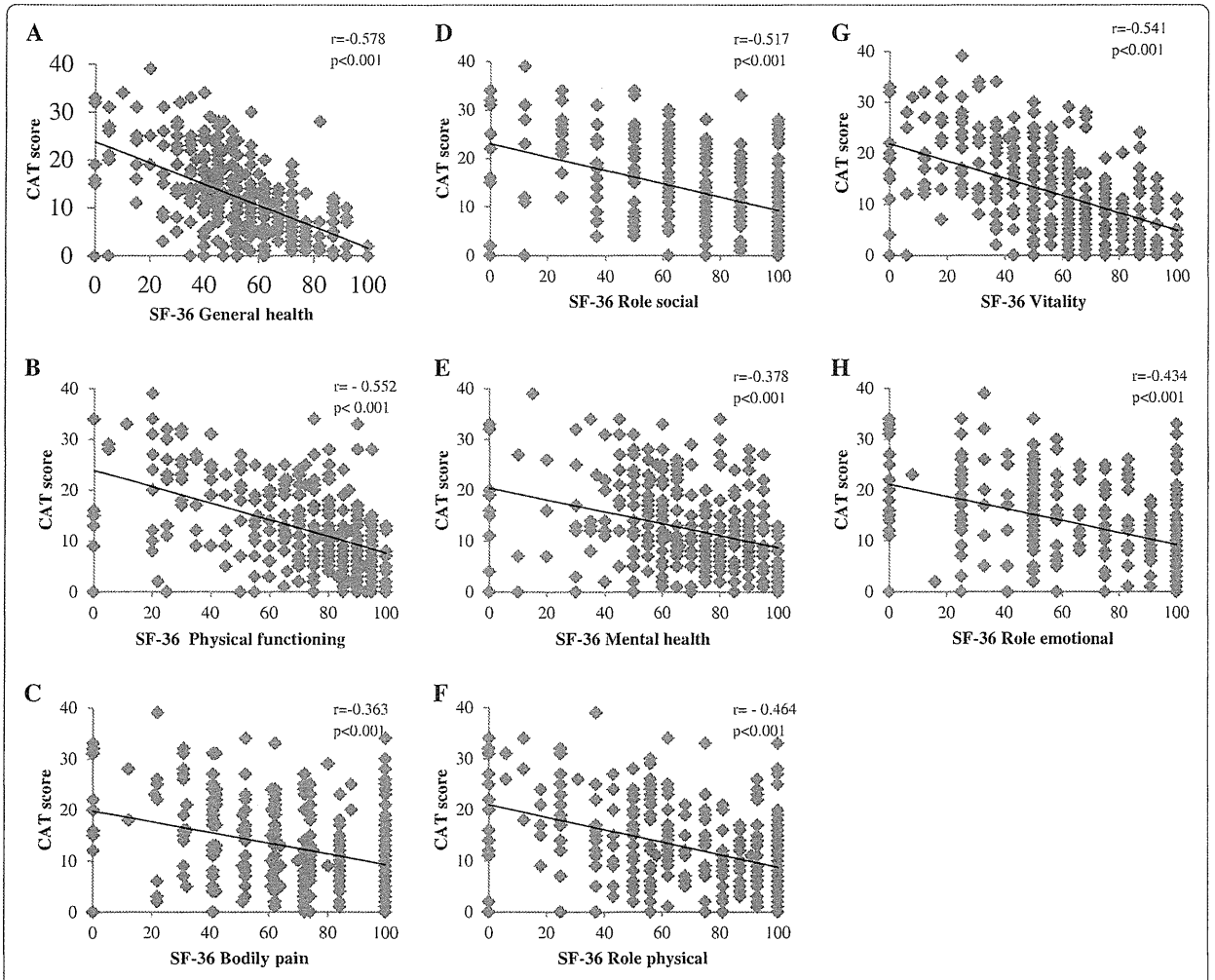
that ($p = 0.17$ and 0.62, respectively). In addition the CAT score in the underweight patients (16.9 ± 9.4) (13%, BMI < 18.5) was higher than those in the normal (12.0 ± 8.1) (65%, 18.5≦BMI<25) and overweight patients (11.1 ± 7.2) (22%, 25≦BMI). Although weight loss is associated with various comorbidities and BMI was not related to the CAT score on multivariate analysis, these observations may imply certain association of low BMI with impaired QOL in COPD patients.

Relationships between each CAT item and comorbidities

The CAT consists of eight items, each presented as a 6-point semantic differential scale (Additional file 2: Table S2). Thus, the multivariate comorbid determinants of each CAT item were examined using stepwise multiple regression analysis. As shown in Table 4, no comorbidities were associated with worsened cough or phlegm, but the presence of GERD was associated with higher scores of the other CAT items. The presence of depression was also significantly associated with increased scores of all CAT items except for cough, phlegm, and breathlessness.

Discussion

Comorbidities are frequent in subjects with COPD, but the study of their contribution to COPD-related QOL impairment has been limited [2,12,24]. In this study, the validity of the Japanese version of the CAT was first confirmed in a well-characterized cohort of COPD patients, since previous studies demonstrated that the SGRQ scores also tended to be lower in Japanese COPD patients than in patients in Western countries [15]. It was found that the CAT performed in a very similar manner to that reported recently in other Asian countries [25]. The mean score of the COPD patients was slightly lower than in studies in other countries [8,11], but the present population had a higher mean $\%FEV_1$ compared to those studies, so they were likely to have had milder disease. It should be noted that there were some discrepancies between the SGRQ and CAT scores in individual patients despite good correlation between these scores at the population level. The discrepancy in the total score appeared to be derived from the differences in the activity and symptom scores, but was within the same range as previously reported (11). In addition the CAT scores clearly distinguished between the quartiles SGRQ score categories. The present study provides good evidence for the validity of the CAT in a Japanese population. To the best of our knowledge, this was also the first comparison between the CAT (a disease-specific measure) and the SF-36, which is a generic measure of health (Figure 2). We therefore believe that our data are generalizable to other languages and countries.



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The prevalence of comorbidities was then comprehensively examined, and their relationships with the CAT score were assessed among the COPD patients. Among a variety of comorbidities examined, the prevalence of GERD and depression was positively related to the total CAT score. In addition, GERD and depression were associated with 6 and 5 of the 8 CAT items, respectively, worsening overall health status in COPD patients. This finding is particularly important, because those diseases may co-exist unrecognized and untreated.

The prevalence of GERD symptoms in the present COPD population was higher than that reported previously in Western and Japanese studies [3,26]. The significant relationships between GERD symptoms and the CAT draw attention to the possibility that comorbid GERD may worsen the symptoms of COPD, even though GERD may have little impact on mortality [3]. On the other hand, no association was found between GERD symptoms and $\%FEV_1$ (data not shown); Mokhlesi et al. reported a high prevalence of GERD symptoms in patients with COPD, with a trend toward it being higher in those with severe COPD [27]. GERD is a digestive disorder in which the mechanisms that keep stomach contents inside the stomach malfunction, releasing acidic stomach contents into the esophagus. Cough can then be induced by the acid stimulus-derived vagal reflex. However, analysis of the relationships between each CAT item and GERD symptoms did not suggest a correlation between GERD symptoms and the extent of cough or phlegm; other CAT items were more closely correlated with GERD in this study (Table 4). We speculate that the occurrence of acid reflux, as reflected by a high FSSG score, might further worsen the health status of COPD patients, in addition to any effect of airflow limitation.

Depression and anxiety are well known comorbidities and are independently associated with a higher risk of

Table 2 Prevalence of comorbidities and relationships with the CAT score

Comorbidity	Prevalence (%)	CAT score		p value
		Comorbidity (+)	Comorbidity (−)	
GERD(FSSG ≥ 8)	34	16.3 ± 9.1	10.5 ± 7.2	<.0001
Anxiety (HAD-A ≥ 11)	7	20.0 ± 11.9	12.1 ± 7.7	<.0001
Depression (HAD-D ≥ 11)	10	19.8 ± 10.2	11.8 ± 7.6	<.0001
Osteoporosis	18	14.9 ± 9.6	11.5 ± 7.6	0.013
Hypertension	36	12.4 ± 8.7	12.6 ± 8.0	NS
Diabetes mellitus	15	13.0 ± 8.3	12.4 ± 8.3	NS
Dyslipidemia	17	11.3 ± 8.9	12.7 ± 8.2	NS
Hyperuricemia	8	13.4 ± 9.4	12.4 ± 8.2	NS
Coronary artery disease	13	12.1 ± 8.0	12.5 ± 8.4	NS
Chronic heart failure	8	13.8 ± 7.1	12.4 ± 8.4	NS
Arrhythmia	11	15.2 ± 9.8	12.1 ± 8.0	0.031
Cerebral infarction	7	12.9 ± 8.5	12.5 ± 8.3	NS
Peptic ulcer	8	14.0 ± 8.9	12.3 ± 8.2	NS
Lung cancer	6	12.9 ± 7.8	12.5 ± 8.3	NS
Other malignancies	19	13.1 ± 8.2	12.4 ± 8.3	NS

Data are presented as means ± SD.
NS Not significant, *CAT* COPD assessment test, *GERD* Gastro-esophageal reflux disease, *FSSG* Frequency Scale for the Symptoms of GERD, *HAD-A* The seven items for anxiety of Hospital Anxiety and Depression Scale, *HAD-D* The seven items for depression of Hospital Anxiety and Depression Scale.

exacerbations and hospitalizations for patients with stable COPD [20], as well as a higher risk of mortality [3]. Burgel et al. have recently reported that the presence of depression (HAD-D ≥ 10) was the most important contributor to the SGRQ total score in COPD [24], but the impact on the CAT score has not yet been clarified. In the present study, depression was found to be a greater cofactor than anxiety for raising the CAT score (Table 3), extending and reinforcing the importance of psychiatric comorbidity in COPD, as previously reported

Table 3 Comorbidities associated with the CAT score on stepwise multiple regression analysis

Comorbidity	p value	Cumulative R^2
GERD (FSSG ≥ 8)	<0.001	0.0986
Depression (HAD-D ≥ 11)	<0.001	0.1520
%FEV_1	<0.001	0.2032
Arrhythmia	0.0030	0.2326
Anxiety (HAD-A ≥ 11)	0.0188	0.2521
Osteoporosis	0.1372	0.2595
Age	0.3540	0.2623
BMI	0.4910	0.2639
Pack-year	0.6585	0.2645

CAT COPD assessment test, *GERD* Gastro-esophageal reflux disease, *FSSG* Frequency Scale for the Symptoms of GERD, *HAD-D* The seven items for depression of Hospital Anxiety and Depression Scale. *HAD-A* The seven items for anxiety of Hospital Anxiety and Depression Scale, *BMI* Body mass index.

Table 4 Comorbidities associated with the score of each CAT item on stepwise multivariate regression analysis

CAT item	Comorbidity	p value	Cumulative R^2
Cough			
	None		
Phlegm			
	None		
Chest tightness			
	GERD (FSSG ≥ 8)	<0.001	0.0901
	Depression (HAD-D ≥ 11)	0.0033	0.123
Breathlessness			
	GERD (FSSG ≥ 8)	<0.001	0.0705
	Osteoporosis	0.0355	0.0876
Activity limitation			
	GERD (FSSG ≥ 8)	<0.001	0.0926
	Depression (HAD-D ≥ 11)	<0.001	0.1452
	Arrhythmia	0.0026	0.1773
	Osteoporosis	0.0222	0.1954
Confidence to leave home			
	GERD (FSSG ≥ 8)	<0.001	0.0631
	Depression (HAD-D ≥ 11)	<0.001	0.107
	Osteoporosis	<0.001	0.1337
	Hypertension	0.0109	0.1571
	Arrhythmia	0.0445	0.1714
Sleep			
	Depression (HAD-D ≥ 11)	<0.001	0.0807
	GERD (FSSG ≥ 8)	0.0033	0.1134
	Anxiety (HAD-A ≥ 11)	0.01	0.1378
	Arrhythmia	0.0404	0.153
Energy			
	GERD (FSSG ≥ 8)	<0.001	0.1191
	Depression (HAD-D ≥ 11)	<0.001	0.1972
	Arrhythmia	<0.001	0.2487
	Anxiety (HAD-A ≥ 11)	0.0333	0.263

Abbreviations are the same as those in Table 3.

[21,24]. It should be noted that the prevalence of anxiety and depression was only 7% and 10%, respectively, in the present study, and both are lower compared to a previous study performed in China using the same HADS cut-off [21] or another study using other measures of depression [2]. The reason for these differences is not clear, but as shown by the CAT score and FEV_1, the present population had relatively mild disease.

Although the objective measurement of BMD on DXA was performed only in 248 of 336 COPD patients, more than half of the COPD patients showed reduced BMD, with a T-score < −1.0 (51%). The prevalence of osteoporosis (18%, T-score < −2.5) was similar to the most recent analysis of the Towards a Revolution in COPD Health cohort, with 18% in men and 30% in women [28]. Although

osteoporosis did not contribute to the CAT score, it appeared to be related to the individual items concerning breathlessness, activity limitation, and confidence to leave home. This is compatible with the awareness that physical activity is a determinant of osteoporosis. The current problem in clinical practice is that many patients remain undiagnosed, because patients are generally asymptomatic until they experience a fracture [29].

The presence of other comorbidities including metabolic diseases, cardiovascular diseases, peptic ulcer, and cancers did not obviously contribute to raising the CAT score in the present study. Although it has been reported that cardiovascular disease is a major comorbidity associated with prognosis in COPD, the prevalence of comorbid heart disease was lower compared to previous studies [30]. It is not clear whether this is caused by ethnic or genetic differences, or by environmental differences, including socioeconomic factors. It is possible that the recruitment of older patients may have resulted in a selection bias by eliminating patients who had previously suffered from severe cardiovascular disorders when they were younger. Another possibility is that lower levels of current smokers in this study (12%) may account for low rates of cardiovascular diseases since smoking cessation is known to decrease the risk of these disorders.

There are also several limitations in this study. Dyspnea was not separately assessed using modified British Medical Research Council breathlessness scale, although it is one of the most important determinants of QOL. Exacerbations are also among important determinants of QOL in COPD patients. However, exacerbation frequencies were not included as factors raising CAT scores in the present study. Roles of undiagnosed comorbidities in QOL of the patients should be considered since this study analyzed only diagnosed comorbidities. Rutten et al. have suggested the importance of undiagnosed left heart failure in COPD [31]. In addition COPD patients mostly consisted of men (91%) in this study, and the results may not apply to women.

Conclusions

Comorbidities are common in COPD patients and are often overlooked. This study suggests that poorer health status, as indicated by a high CAT score, may indicate the presence of certain comorbidities, but the overall picture suggests that COPD-specific measures such as FEV_1 and CAT do not reliably suggest the presence of comorbidities, which should be specifically sought.

Additional files

Additional file 1: Table S1. Prevalence of other comorbidities and relationships with the CAT score.

Additional file 2: Table S2. COPD Assessment Test questionnaire.

Abbreviations

COPD: Chronic obstructive pulmonary disease; CAT: COPD Assessment test; SGRQ: St. Georges respiratory questionnaire; SF-36: Medical outcomes study short-form 36-Item; GERD: Gastro-esophageal reflux disease; GOLD: Global initiative for chronic obstructive lung disease; QOL: Quality of life; K-CCR: Keio COPD Comorbidity research; FEV_1: Forced expiratory volume in one second; FVC: Forced vital capacity; CT: Computed tomography; FSSG: Frequency scale for the symptoms of GERD; HADS: Hospital anxiety and depression scale; HAD-A: The seven items for anxiety; HAD-D: The seven items for depression; DXA: Dual X-Ray absorptiometry; BMD: Bone mineral density; SD: Standard deviation; BMI: Body mass index; NS: Not significant; VC: Vital capacity; FEV_1: Forced expiratory volume in one second.

Competing interests

TB discloses having received honoraria/paid expert testimony and her university having received research grants from GlaxoSmithKline. PWJ discloses that his university has received honoraria and research grants from GlaxoSmithKline. The other authors declare that they have no competing interests.

Authors' contributions

MM participated in the design of the study and performed the statistical analyses, and was a major contributor in writing the manuscript. HN planned the study design, and contributed to interpretation of results. NM, KA, and TB conceived the study, participated in its design and coordination, and helped to draft the manuscript. SC, MS, MH, SY, KT, TS, ST, HK, MN, FS, TT, and KS contributed to collection of data and interpretation of results. PWJ contributed to the data analysis, interpretation of data, and editing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to acknowledge Chiyomi Uemura for her contribution to collection of data, and the following members for their participation in the K-CCR Group: Takashi Inoue at Sano Public Welfare General Hospital, Naoki Miyao at Nihon Kokan Hospital, Kazumi Nishio at Kawasaki City Ida Hospital, Hiroki Tateno at Saitama City Hospital, Yoshitaka Oyamada at Tokyo Medical Center, Tatsu Matsuzaki at Tokyo Dental College Ichikawa General Hospital, and Akira Umeda at International Medical Welfare College Shioya Hospital. The authors would all like to acknowledge the late Professor Akitoshi Ishizaka for administrative support for this study.

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Received: 1 October 2013 Accepted: 3 February 2014
Published: 6 February 2014

References

1. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, Van Weel C, Zielinski J: **Global initiative for chronic obstructive lung disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary.** *Am J Respir Crit Care Med* 2007, 176:532–555.
2. Vanfleteren LE, Spruit MA, Groenen M, Gaffron S, Van Empel VP, Bruijnzeel PL, Rutten EP, Op't Roodt J, Wouters EF, Franssen FM: **Clusters of comorbidities based on validated objective measurements and systemic inflammation in patients with chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 2013, 187:728–735.
3. Divo M, Cote C, de Torres JP, Casanova C, Marin JM, Pinto-Plata V, Zulueta J, Cabrera C, Zagaceta J, Hunninghake G, Celli B, BODE Collaborative Group: **Comorbidities and risk of mortality in patients with chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 2012, 186:155–161.

4. Vestbo J, Hurd SS, Agustí AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, Stockley RA, Sin DD, Rodriguez-Roisin R: **Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary.** *Am J Respir Crit Care Med* 2013, **187**:347–365.
5. Jones PW, Quirk FH, Baveystock CM, Littlejohns P: **A self-complete measure of health status for chronic airflow limitation. The St. George's respiratory questionnaire.** *Am Rev Respir Dis* 1992, **145**:1321–1327.
6. Guyatt GH, Berman LB, Townsend M, Pugsley SO, Chambers LW: **A measure of quality of life for clinical trials in chronic lung disease.** *Thorax* 1987, **42**:773–778.
7. Jones PW, Harding G, Berry P, Wiklund I, Chen WH, Kline Leidy N: **Development and first validation of the COPD Assessment Test.** *Eur Respir J* 2009, **34**:648–654.
8. Mackay AJ, Donaldson GC, Patel AR, Jones PW, Hurst JR, Wedzicha JA: **Usefulness of the chronic obstructive pulmonary disease assessment test to evaluate severity of COPD exacerbations.** *Am J Respir Crit Care Med* 2012, **185**:1218–1224.
9. Dodd JW, Hogg L, Nolan J, Jefford H, Grant A, Lord VM, Falzon C, Garrod R, Lee C, Polkey MI, Jones PW, Man WD, Hopkinson NS: **The COPD assessment test (CAT): response to pulmonary rehabilitation. A multicentre, prospective study.** *Thorax* 2011, **66**:425–429.
10. Jones PW, Harding G, Wiklund I, Berry P, Tabberer M, Yu R, Leidy NK: **Tests of the responsiveness of the COPD assessment test following acute exacerbation and pulmonary rehabilitation.** *Chest* 2012, **142**:134–140.
11. Jones PW, Brusselle G, Dal Negro RW, Ferrer M, Kardos P, Levy ML, Perez T, Soler Cataluña JJ, van der Molen T, Adamek L, Banik N: **Properties of the COPD assessment test in a cross-sectional European study.** *Eur Respir J* 2011, **38**:29–35.
12. Jones PW, Nadeau G, Small M, Adamek L: **Characteristics of a COPD population categorised using the GOLD framework by health status and exacerbations.** *Respir Med* 2014, **108**:129–135.
13. The American Thoracic Society board of directors: **Standardization of spirometry, 1994 update.** *Am J Respir Crit Care Med* 1995, **152**:1107–1136.
14. Committee of Pulmonary Physiology, the Japanese Respiratory Society: *The Japanese Respiratory Society Guidelines for pulmonary function tests: spirometry, flow-volume curve, diffusion capacity of the lung (in Japanese).* Tokyo; 2004.
15. Hajiro T, Nishimura K, Tsukino M, Ikeda A, Koyama H, Izumi T: **Comparison of discriminative properties among disease-specific questionnaires for measuring health-related quality of life in patients with chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 1998, **157**:785–790.
16. Hajiro T, Nishimura K, Tsukino M, Ikeda A, Koyama H, Izumi T: **Analysis of clinical methods used to evaluate dyspnea in patients with chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 1998, **158**:1185–1189.
17. Fukuhara S, Bito S, Green J, Hsiao A, Kurokawa K: **Translation, adaptation, and validation of the SF-36 Health Survey for use in Japan.** *J Clin Epidemiol* 1998, **51**:1037–1044.
18. Danjo A, Yamaguchi K, Fujimoto K, Saitoh T, Inamori M, Ando T, Shimatani T, Adachi K, Kinjo F, Kuribayashi S, Mitsufuji S, Fujiwara Y, Koyama S, Akiyama J, Takagi A, Manabe N, Miwa H, Shimoyama Y, Kusano M: **Comparison of endoscopic findings with symptom assessment systems (FSSG and QUEST) for gastroesophageal reflux disease in Japanese centres.** *J Gastroenterol Hepatol* 2009, **24**:633–638.
19. Kusano M, Shimoyama Y, Sugimoto S, Kawamura O, Maeda M, Minashi K, Kuribayashi S, Higuchi T, Zai H, Ino K, Horikoshi T, Sugiyama T, Toki M, Ohwada T, Mori M: **Development and evaluation of FSSG: frequency scale for the symptoms of GERD.** *J Gastroenterol* 2004, **39**:888–891.
20. Zigmond AS, Snaith RP: **The hospital anxiety and depression scale.** *Acta Psychiatr Scand* 1983, **67**:361–370.
21. Xu W, Collet JP, Shapiro S, Lin Y, Yang T, Platt RW, Wang C, Bourbeau J: **Independent effect of depression and anxiety on chronic obstructive pulmonary disease exacerbations and hospitalizations.** *Am J Respir Crit Care Med* 2008, **178**:913–920.
22. Biskobing DM: **COPD and osteoporosis.** *Chest* 2002, **121**:609–620.
23. Report of a WHO Study Group: **Assessment of fracture risk and its application to screening for postmenopausal osteoporosis.** *World Health Organ Tech Rep Ser* 1994, **843**:1–129.
24. Burgel PR, Escamilla R, Perez T, Carré P, Caillaud D, Chanez P, Pinet C, Jebrak G, Brinchault G, Court-Fortune I, Paillasseur JL, Roche N: **INITIATIVES BPCO scientific committee: impact of comorbidities on COPD-specific health-related quality of life.** *Respir Med* 2013, **107**:233–241.
25. Kwon N, Amin M, Hui DS, Jung KS, Lim SY, Ta HD, Thai TT, Jones PW: **Validity of the COPD assessment test translated into local languages for Asian patients.** *Chest* 2013, **143**:703–710.
26. Terada K, Muro S, Sato S, Ohara T, Haruna A, Marumo S, Kinose D, Ogawa E, Hoshino Y, Niimi A, Terada T, Mishima M: **Impact of gastro-oesophageal reflux disease symptoms on COPD exacerbation.** *Thorax* 2008, **63**:951–955.
27. Mokhlesi B, Morris AL, Huang CF, Curcio AJ, Barrett TA, Kamp DW: **Increased prevalence of gastroesophageal reflux symptoms in patients with COPD.** *Chest* 2001, **119**:1043–1048.
28. Ferguson GT, Calverley PM, Anderson JA, Jenkins CR, Jones PW, Willits LR, Yates JC, Vestbo J, Celli B: **Prevalence and progression of osteoporosis in patients with COPD: results from the TOwards a Revolution in COPD Health study.** *Chest* 2009, **136**:1456–1465.
29. Ogura-Tomomatsu H, Asano K, Tomomatsu K, Miyata J, Ohmori N, Kodama M, Ueda S, Takihara T, Tanaka K, Kamiishi N, Suzuki Y, Fukunaga K, Oguma T, Sayama K, Betsuyaku T: **Predictors of osteoporosis and vertebral fractures in patients presenting with moderate-to-severe chronic obstructive lung disease.** *COPD* 2012, **9**:332–337.
30. Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, Crim C, Willits LR, Yates JC, Vestbo J: **TORCH Investigators: Cardiovascular events in patients with COPD: TORCH study results.** *Thorax* 2010, **65**:719–725.
31. Rutten FH, Moons KG, Cramer MJ, Grobbee DE, Zuithoff NP, Lammers JW, Hoes AW: **Recognising heart failure in elderly patients with stable chronic obstructive pulmonary disease in primary care: cross sectional diagnostic study.** *BMJ* 2005, **331**:1379–1382.

doi:10.1186/1465-9921-15-13
Cite this article as: Miyazaki *et al.*: Analysis of comorbid factors that increase the COPD assessment test scores. *Respiratory Research* 2014 15:13.

Molecular Immunology 59 (2014) 217–225

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

A new technique of bronchial microsampling and proteomic analysis of epithelial lining fluid in a rat model of acute lung injury

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ARTICLE INFO

Article history:
Received 3 December 2012
Received in revised form 9 February 2014
Accepted 25 February 2014

Keywords:
Rat bronchial microsampling
Epithelial lining fluid
Bronchoalveolar lavage
Lipopolysaccharide stimulation
Proteomic analysis

ABSTRACT

The standard technique to evaluate the proteins present in epithelial lining fluid (ELF) is bronchoalveolar lavage (BAL). Bronchoscopic microsampling (BMS) method has been developed for humans as a less invasive alternative. We establish the usefulness of a rat bronchial microsampling (rBMS) to evaluate various proteins in ELF in lipopolysaccharide (LPS)-induced lung injury models in rats. In the first experiment of this study, we validate that whether the rBMS can obtain information from ELF in place of BAL. Tumor necrosis factor (TNF)-α concentrations were increased in the rBMS samples similar to BAL 1 and 3 h after LPS instillation. In the second part of this study, a proteomic analysis of the rBMS, using the Protein Chip[R] system, revealed the presence of proteins whose molecular weights corresponded to TNF-related proteins in the LPS-treated rats. In rats treated with a TNF-α converting enzyme inhibitor, the concentrations of these proteins in rBMS decreased or disappeared. In the third experiment, rBMS was performed without tracheostomy at 6 and 24 h after instillation of LPS, and a rat multiple cytokines assay system detected heterogeneous variations in the concentrations of interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-αand interferon (IFN)-γ in ELF. The cytokine profile was significantly modified by pre-treatment with dexamethasone. This new rBMS technique could be used to measure TNF-α in LPS-induced acute lung injury (ALI) as well as for proteomic analysis, without sacrificing the rats. Furthermore, this procedure enables the serial collection of ELF, which would allow the examination of time-dependent cytokine variations in rat ALI model.

1. Introduction

Bronchoalveolar lavage (BAL) is used to diagnose or monitor the status of various respiratory disorders. However, since it may cause lung injury and adverse cardio-pulmonary effects, it is a challenging procedure in patients whose lung function is impaired (Klech and Hutter, 1990; Montravers et al., 1993). We have developed a bronchoscopic microsampling (BMS) method, which allows the collection of epithelial lining fluid (ELF), using probes with polyurethane adsorptive tips, without the risk of lung injury associated with conventional BAL (Ishizaka et al., 2001). Our BMS method has been used to analyze the ELF of patients suffering from acute respiratory distress syndrome, asthma, or lung cancer (Durairaj et al., 2006; Ishizaka et al., 2004; Kamiya et al., 2011; Komaki et al., 2005; Sasabayashi et al., 2007; Watanabe et al., 2003; Yamazaki et al., 2003; Yanagi et al., 2005). We have recently developed a rat bronchial microsampling (rBMS), which is thinner than the probe used in humans. Since, unlike the standard BAL method, our new method allows the collection of ELF without causing lung injury, serial examinations can be performed without sacrificing the rats.

The purpose of this experiment is establishment and validation of this technique on small animals. We plan to use new probes to collect ELF serially on various rat lung injury models to elucidate the pathophysiology in future. The present study is the first step of our plan to obtain basic data. Pathognomonic new molecules or time-dependent changes of cytokines could be found by proteomic analysis or multiple cytokine assays.

In the first part of this study, we examined whether our new rBMS method allows the detection of changes in tumor necrosis factor (TNF)-α in an experimental rat model of acute lung injury (ALI).

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http://dx.doi.org/10.1016/j.molimm.2014.02.013
0161-5890/

The rBMS and BAL were performed 1 and 3 h after the endotracheal administration of lipopolysaccharide (LPS) and the concentrations of TNF-α measured in the two procedures were compared.

The rBMS method also has to dilute ELF because of its small amount. It cannot deny to be developed to technical/statistical error. Then we tried to obtain ELF directly with centrifugation. In the second part of this study, the rBMS material underwent proteomic analysis, using the Protein Chip^R system which can measure it in small amount of specimens, without dilution.

In the third experiment, we examined the contribution made by rBMS instrumentation to the serial profiling of inflammatory cytokines, in a LPS-induced rat model of ALI, using a multiple cytokine assay system. We measured the concentrations of eight rat cytokines in ELF at 6 and 24 h after the intratracheal instillation of LPS. Standard BAL was also compared with the new rBMS method.

2. Materials and methods

This study was reviewed and approved by the Animal Experimentation Committee of Keio University, and was performed in compliance with its Animal Experimental Guidelines.

2.1. Concentrations of TNF-α in epithelial lining fluid

2.1.1. Animal preparation

We used specific pathogen-free, male, 10 to 12-week-old Lewis rats, weighing 250–300 g. After the induction of anesthesia with pentobarbital, 40 mg/kg, i.m., a tracheostomy was performed under aseptic conditions, and the animal was intubated with a 14G angiocatheter, and mechanically ventilated with a SN-480-7 ventilator (Shinano Seisakusho Co., Tokyo, Japan), using a 10 ml/kg tidal volume at a rate of 90 cycles per min. Anesthesia was maintained by inhalation of sevoflurane.

The animals were evenly assigned to a LPS and a control group. The five rats in the LPS group received 200 μg of *Escherichia coli* 0111: B4 (Wako Pure Chemical Industries, Tokyo, Japan) dissolved in 100 μl of normal saline, and the five control rats received 100 μl of normal saline, each administered endotracheally. Because TNF-α is released from inflammatory immune cells in the early phase after stimulation of bacterial components such as LPS, we chose the time point 1 and 3 h for TNF-α measurement.

2.1.2. Bronchial microsampling

The rBMS probe developed for rats (Olympus Corporation, Tokyo, Japan), has an outer polyethylene sheath and a 1.0-mm in diameter and 10-mm long inner probe, with a polyurethane adsorptive tip at its distal extremity, capable of absorbing up to 15 μl of liquid (Fig. 1A). After disconnection of the ventilator, ELF was collected by introducing an rBMS probe through the tracheostomy tube, and advancing it into the distal airway until we perceived the slightest resistance (Fig. 1C). The inner probe was left in place for 10 s, before its withdrawal through the outer sheath. Since it collects only 1–5 μl of ELF at a time, rBMS was repeated three times. After its withdrawal (1) each wet probe tip was weighed, (2) the ELF collected from the three consecutive probes was extracted with 1 ml of saline, and (3) each probe was dried and weighed again. The amount of ELF collected was calculated as the difference between wet and dry weights. The extracted sample was centrifuged at 3000 rpm for 15 min, and the supernatant was used for enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

2.1.3. Bronchoalveolar lavage

Rats were sacrificed 1 or 3 h after the administration of LPS or saline. BAL samples were collected 30 s after the intratracheal injection of 5 ml of saline, twice consecutively. The BAL fluid was centrifuged at 3000 rpm for 15 min, and the supernatant stored at −80 °C until analysis.

2.1.4. Measurements of tumor necrosis factor-α and rat albumin

The concentrations of TNF-α in BAL fluid and ELF were measured by enzyme-linked immunosorbent assay (ELISA). A colorimetric assay kit (Beckman, Fullerton, CA) was used for the measurements of rat albumin.

Assuming that 1 ml = 1000 mg, the weights of the rBMS samples were converted to volumes and the concentration of TNF-α in ELF (in pg/ml) was calculated as the concentration of TNF-α in 1 ml of saline × [1 mg/(wet weight (mg) − dry weight (mg))]. The concentration of rat albumin (in mg/ml) was calculated similarly.

2.2. Proteomic analysis

To directly obtain rBMS samples for the proteomic analysis, we centrifuged the probe in a tube (Fisher Scientific, Tokyo, Japan) at 5000 × *g* for 2 min. The undiluted ELF samples were analyzed on the Protein Chip^R system (Bio-Rad Laboratories, Tokyo, Japan). Using the "surface-enhanced laser desorption/ionization, time of flight mass spectrometry", the proteins immobilized onto the chip were separated from its surface and sent into flight by a pulse laser, and their molecular weight was calculated from the time of flight. The samples were placed on the chip, using the SAX2 (anionic exchange) and WCX2 (cationic exchange) protocols. For SAX2, 50 mM Tris–HCl (pH 8.5), and for WCX2, 20 mM Na phosphate (at pH 6.5), were used as binding/washing buffers, of which 2 μl were added to each spot on the chip array, and twice fixed for 5 min. A 2 μl sample was then added to each spot, incubated for 20 min and, after the addition of 2 μl of binding/washing buffer to each spot for 5 min, the chip was twice washed with distilled water. A mixture of 5 mg of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), 150 μl of 30% acetonitryl, and 350 μl of 0.1% trifluoroacetic acid was prepared as an energy absorbing molecule, of which 1 μl was added to each spot and air-dried twice. The chip with its immobilized sample was placed in the protein chip reader at a Hardshot increase setting of 10, Laser intensity of 300, and Detector sensitivity of 7, and the molecular weights of the proteins in ELF were measured.

The proteins were assumed from the peaks observed, using the molecular weights of candidate proteins forming similar peaks in the Swiss-Prot protein knowledgebase, posted at http://au.expasy.org/tools/tagident.html. This analysis was performed on BMS fluid collected from two rats treated with LPS, and compared with the analyses of fluid collected from two control rats. To determine whether these peaks were related to TNF-α, the same analysis was performed in two other rats treated with a 3-mg/kg bolus of Y-41654, a TNF-α converting enzyme (TACE) inhibitor (Mitsubishi Pharma Corp., Osaka, Japan), administered via the jugular venous catheter, before the administration of LPS, followed by a continuous 3-mg/kg/h infusion.

2.3. Serial analysis of multiple cytokines in the epithelial lining fluid

2.3.1. Animal preparation

In a separate study, we measured the serial concentrations of inflammatory cytokines in ELF collected by the rBMS method. We used specific, 10-week-old, pathogen-free, Sprague–Drawly rats, (CLEA Japan, Inc., Tokyo, Japan) weighing 300–350 g. Under anesthesia with pentobarbital, the animals were orally intubated with a 14-gauge intravenous catheter. The rats were randomly assigned to (1) a control group, in which 200 μl of saline was instilled into the lungs through an intratracheal tube, (2) a LPS group, which received

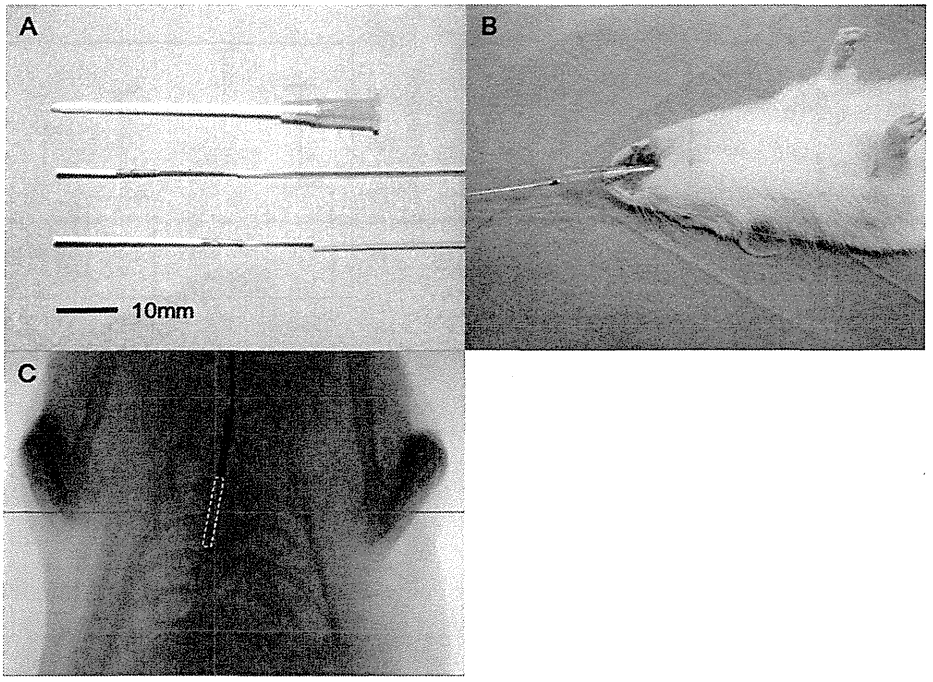

Fig. 1. A: from top to bottom: 14G angiocatheter used for the endotracheal intubation of rats; 1.0 mm in diameter and 10 mm long BMS probe for rat; 1.5 mm in diameter and 30 mm long BMS probes for human use.
B. BMS introduced through an oro-tracheal tube in a rat.
C. Fluoroscopic image of BMS probe inserted into a rat's right main bronchus. A radiolucent, polyurethane adsorptive tip is outlined by a dotted rectangle.

200 μg of LPS (*E. coli* O55: B5; Sigma, St. Louis, MO) suspended in 200 μl of saline instilled into the lungs through an intratracheal tube, to create lung injury, and (3) a dexamethasone (DEX) + LPS pre-treatment group, which received 200 μg of LPS intratracheally, 2 h after the intraperitoneal delivery of 200 μg/kg of DEX. After the instillation of LPS or saline, the rats were extubated and allowed to wake up.

2.3.2. Serial bronchial microsampling and bronchoalveolar lavage

At 6 and 24 h after the administration of LPS or saline, the rats were anesthetized and re-intubated. Multiple cytokine assays include both early-phase cytokines such as IL-1 and late-phase cytokines such as IFN-γ and IL-10. We set the time point to 6 and 24 h for multiple cytokine assay so that we could estimate the change of each cytokine level. Oro-tracheal rBMS was repeated three times at each time point in each rat (Fig. 1B and C). The tips of the probes were weighed and stored at −80 °C until measurements of the cytokines. The animals were sacrificed at 24 h, immediately after the collection of ELF. The left lung was lavaged with 5 ml of saline, the BAL fluid was centrifuged for 15 min at 3000 rpm and 4 °C, and the supernatant was stored at −80 °C until analyses.

2.3.3. Cytokines assay

We used a Bio-Plex™ rat cytokine assay (Bio-Rad Laboratories, Hercules, CA) for simultaneous measurements of interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α and interferon (IFN)-γ in ELF and BAL fluid. The ELF absorbed in three separate probes was eluted in 200 μl of normal saline. The probes were weighed after drying to measure the amount of absorbed ELF. The ELF concentrations of different cytokines were corrected by each dilution factor in the same way as the TNF-α measurement described above. The BAL fluid was used without modification. When a cytokine concentration was below in the lower limit of the assay, it was assigned a value of 0.

2.4. Statistical analyses

The data are expressed as means ± SD. In the first part of this study, differences in measurements between time points or groups were examined by Student's unpaired *t*-test. In the second part, ANOVA followed by Scheffe's *post-hoc* test was used to determine the levels of significance of the differences among groups, and the differences in measurements between time points were examined by Student's paired *t*-test. The histological scores were compared by Kruskal–Wallis non-parametric ANOVA for factorial experiments, followed by Dunn's procedure for *post-hoc* multiple comparisons. A P value <0.05 was considered statistically significant. The analyses were performed with the StatView-J software, version 5.0 (Abacus Concepts Inc., Berkeley, CA).

3. Results

3.1. TNF-α measurement and albumin in ELF

The concentrations of TNF-α in ELF were $3.7 \pm 0.5 \times 10^3$ pg/ml at 1 h, and $3.2 \pm 0.4 \times 10^3$ pg/ml at 3 h in the control group, and $10.4 \pm 4.0 \times 10^3$ pg/ml at 1 h and $12.4 \pm 2.9 \times 10^3$ pg/ml at 3 h ($P < 0.005$ versus control) in the LPS group (Fig. 2A). The concentrations of albumin in ELF at 1 and 3 h in the control (109 ± 29 and 100 ± 27 mg/ml, respectively) versus the LPS group (146 ± 27 and 94 ± 21 mg/ml, respectively) were similar (Fig. 2B). The volumes of BAL fluid collected in the control group were 7.6 ± 1.2 and 7.3 ± 1.0 ml at 1 and 3 h, respectively, versus 7.4 ± 1.5 and 7.3 ± 0.9 ml, respectively, in the LPS group (ns). The concentrations of TNF-α in BAL fluid were significantly higher in the LPS-treated than in the control group (Fig. 2C) at 1 h ($4.7 \pm 1.3 \times 10^3$ versus 90 ± 45 pg/ml; $P < 0.01$) and at 3 h ($8.8 \pm 2.0 \times 10^3$ versus 87 ± 20 pg/ml; $P < 0.005$). The concentrations of albumin in BAL fluid at 1 and 3 h (0.33 ± 0.06 and 0.55 ± 0.03 mg/ml, respectively

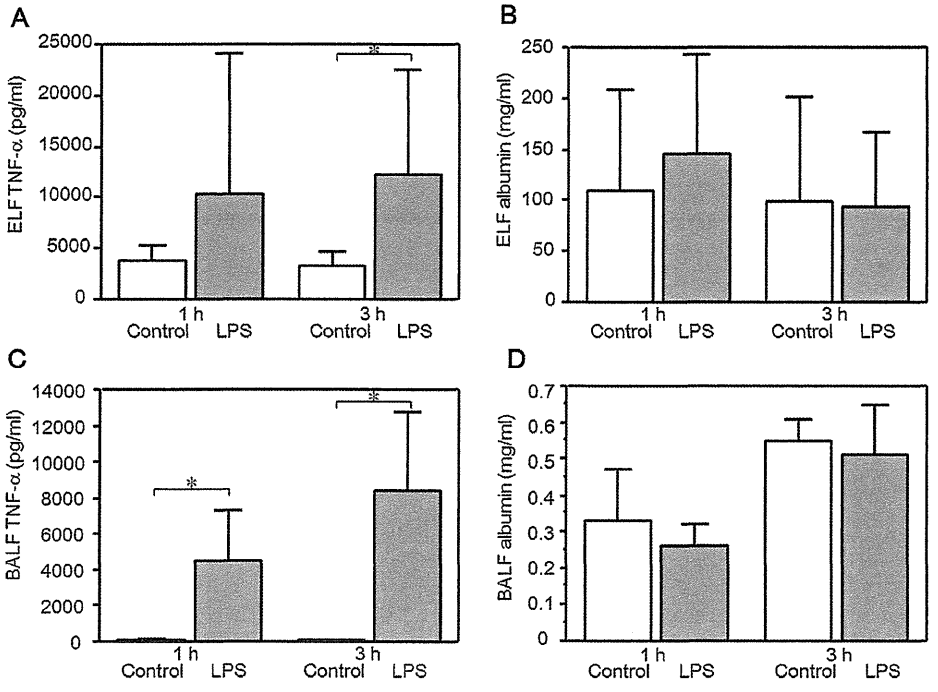

Fig. 2. Concentrations of TNF-α and albumin in ELF (A and B), and in BAL fluid (C and D), collected by rat BMS 1 and 3 h after the instillation of normal saline or LPS. $n = 5$ in each group. $^*P < 0.05$.

in controls, versus 0.26 ± 0.03 and 0.51 ± 0.06 mg/ml respectively in the LPS group) were similar (Fig. 2D).

3.2. Proteomes in epithelial lining fluid

The proteomic analysis of the rBMS samples collected 3 h after treatment with LPS showed peaks of molecular weights at 31,000, 47,000 and 63,000 Da, corresponding to respective TNF-related proteins (Fig. 3B). In contrast, in the rats treated with LPS and the TACE inhibitor, the proteomic analysis showed a lower peak at 31,000 Da and the absence of peaks at 47,000 and the 63,000 Da (Fig. 3C). The peaks were similar in both animals of each group.

We used the TagIdent software http://au.expasy.org/tools/tagident.html to identify the proteins matching the peaks observed in the LPS-stimulated rats. The values for each variable entered in the Internet-based tool are shown in the appendix. The results were as follows: (1) TNF, membrane form (MW 25,806) p*I* = 5.13; (2) TNF ligand superfamily member 6 (MW 31,140) p*I* = 9.32; (3) TNF receptor superfamily member 11B (MW 43,670) p*I* = 8.89; (4) fractalkine (MW 39,350) p*I* = 6.59; (5) glucose-6-phospate isomerase (MW 62,696) p*I* = 7.50; and (6) macrophage colony stimulating factor-1 (MW 58,854) p*I* = 5.20, etc., confirming that these proteins were TNF-α related.

3.3. Serial analysis of multiple cytokines in the epithelial lining fluid

Fig. 4 shows the total volume of ELF collected by rBMS. The volume of ELF eluted from the probes in the control group was significantly smaller ($P < 0.05$) at 24 (9.4 ± 6.6 μl) than at 6 h (13.9 ± 4.6 μl). In the LPS group, however, the volume of ELF eluted from the probes at 6 and 24 h were 13.3 ± 6.4 and 13.6 ± 7.7 μl, respectively (ns). The volume eluted at 24 h was significantly larger ($P < 0.05$) than in the control group. In the DEX + LPS-treated group, the 6-h volume (5.0 ± 3.4 μl) was significantly smaller ($P < 0.0001$) than in both the control and the LPS-treated groups. Finally, in the DEX + LPS-treated group, the 24-h volume eluted (4.6 ± 5.1 μl) was significantly smaller ($P < 0.0005$) than in the LPS group.

Fig. 5 compares the changes in ELF cytokines concentrations between 6 and 24 h in the control, LPS-treated and DEX + LPS-treated groups, by Student's paired *t*-test. In the control group the ELF concentrations of the eight cytokines remained unchanged between 6 and 24 h (Fig. 5A–H). In the LPS-treated group, the concentrations of IL-2 (Fig. 5C) and IL-10 (Fig. 5F) were significantly higher at 24 than at 6 h, whereas the concentrations of IL-6 decreased significantly (Fig. 5E) between 6 and 24 h. In the DEX + LPS group, the concentrations of IL-1α, IL-1β, IL-2, and IL-10 increased significantly between 6 and 24 h (Fig. 5A, B, C and F), whereas the concentration of IL-6 decreased significantly (Fig. 5E).

We compared the differences in cytokine concentrations in ELF among the groups at each time point by ANOVA, followed by Scheffe's *post-hoc* test. All cytokines concentrations in ELF were significantly higher in the LPS-treated than in the control group, except IFN-γ at 6 h (Fig. 5A–G). The 6-h cytokines concentrations in ELF were significantly higher in the DEX + LPS-treated than in the control group, except IL-1α, IL-4 and IFN-γ (Fig. 5B, C, E–G). The ELF concentrations of IL-1α and IL-4 in the DEX + LPS group at 6 h were significantly lower than in the LPS group (Fig. 5A and D). The ELF concentrations of IL-1α, IL-4 and IL-6 at 24 h were significantly higher in the LPS than in the control group (Fig. 5A, D, and E). Likewise, the ELF concentrations of IL-1β at 24 h were significantly higher (Fig. 5B) in the DEX + LPS than in the LPS group, whereas the ELF concentrations of IL-4 at 24 h were significantly lower (Fig. 5D) in the DEX + LPS than in the LPS group.

The cytokine concentrations in BAL fluid were measured 6 and 24 h in the control, LPS-treated and DEX + LPS-treated groups. In the control group, the BAL fluid concentration of IFN-γ was significantly higher at 24 h than 6 h (Fig. 6H), whereas the others remained unchanged between 6 and 24 h (Fig. 6A–G). In the LPS-treated group, the concentration of IL-4 was significantly higher

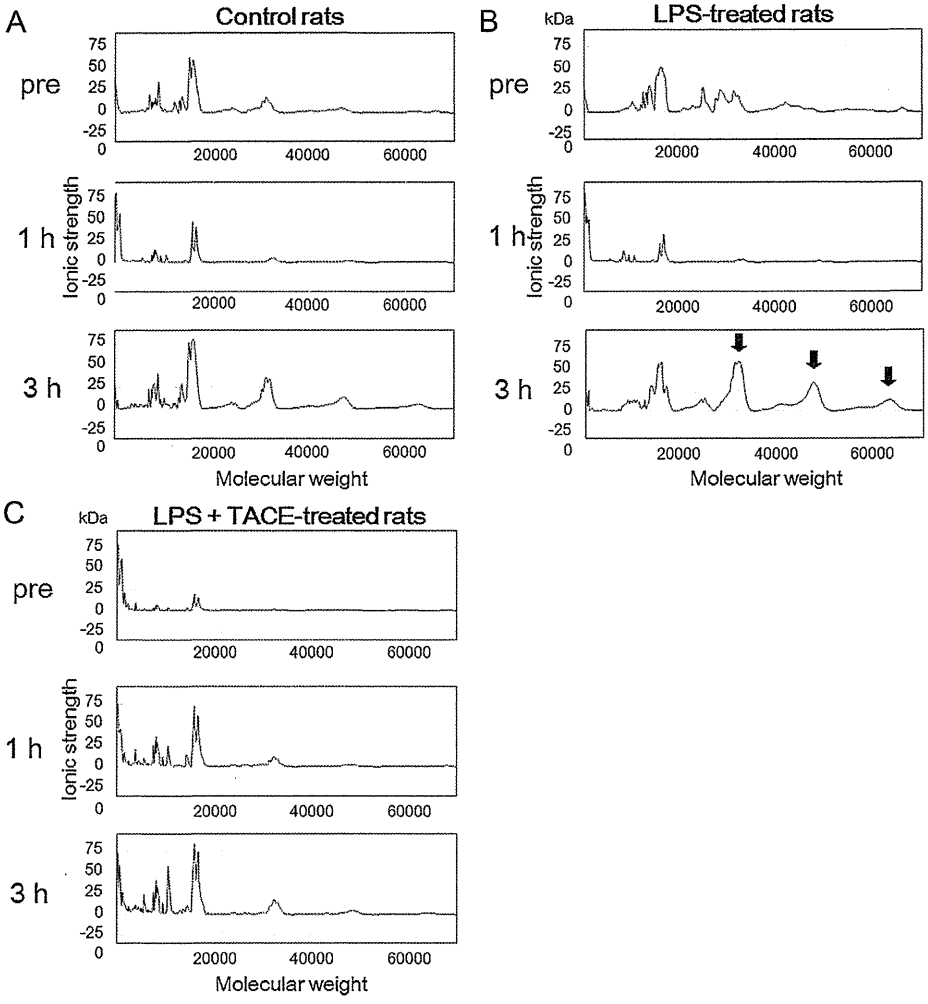

Fig. 3. Proteomic analysis of ELF collected by the rat BMS method 3 h after the instillation of normal saline or LPS.
A: Controls ($n=2$ rats).
B: LPS-treated ($n=2$ rats). Discrete peaks have developed at 3 h, at molecular weights of 31,000, 47,000 and 63,000 Da (arrows).
C: LPS+TACE inhibitor-treated ($n=2$ rats). At 3 h, the peak in the 31,000 Da regions is lower than in the LPS-treated rats, and no peak is present in the 47,000 and 63,000 Da regions. The range of molecular weights is shown on the horizontal axis. The ionic strength is shown on the vertical axis.

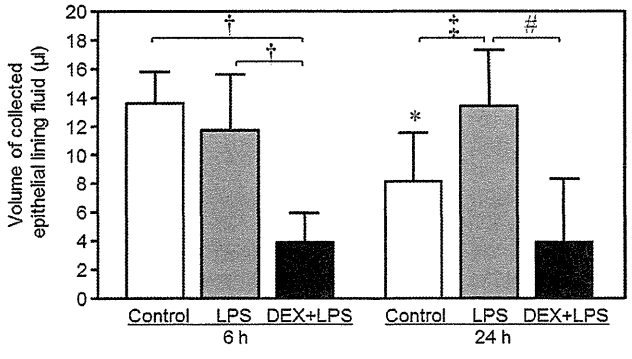

Fig. 4. Total amount of ELF eluted from three rats BMS probes at 6 and 24 h after instillation of normal saline or LPS, with or without pre-treatment with DEX. $n=8$ in each group. $^{*}P<0.05$: difference between 6 and 24 h in controls. $^{\dagger}P<0.05$: control versus DEX+LPS and LPS versus DEX+LPS at 6 h; $^{\ddagger}P<0.05$: difference between control and LPS-treated at 24 h; $^{\#}P<0.05$: LPS versus DEX+LPS at 24 h.

at 24 h than 6 h, whereas the concentrations of IL-1α, IL-1β, IL-6 and TNF-α decreased significantly between 6 and 24 h (Fig. 6A, B, D, E, and G). In the DEX+LPS-treated group, the concentrations of IL-1α, IL-1β, IL-4, IL-10 and IFN-γ increased significantly between 6 and 24 h (Fig. 6A, B, D, F, H), whereas the concentration of TNF-α decreased significantly (Fig. 6G).

The differences in cytokine concentrations in BAL fluid among the groups at each time points were compared by ANOVA, followed by Scheffe's *post-hoc* test. At 6 h, the concentrations of IL-1α, IL-1β, IL-6, IL-10 and TNF-α were significantly higher in the LPS group than in the control group (Fig. 6A, B, E–G). Only the concentrations of TNF-α were significantly higher in the DEX+LPS group than in the control group (Fig. 6G). The concentrations of IL-1α, IL-1β, IL-6 and IL-10 in the DEX+LPS group were significantly lower than in the LPS group (Fig. 6A, B, E, and F). At 24 h, the BAL concentrations of TNF-α were significantly higher in the LPS group than in control group (Fig. 6G). In the DEX+LPS group, IL-1α, IL-1β, IL-2, IL-6, IL-10 and TNF-α were significantly higher in the control group (Fig. 6A–C and E–G). And IL-1α, IL-1β, IL-2 were significantly higher than in the LPS group (Fig. 6A–C).

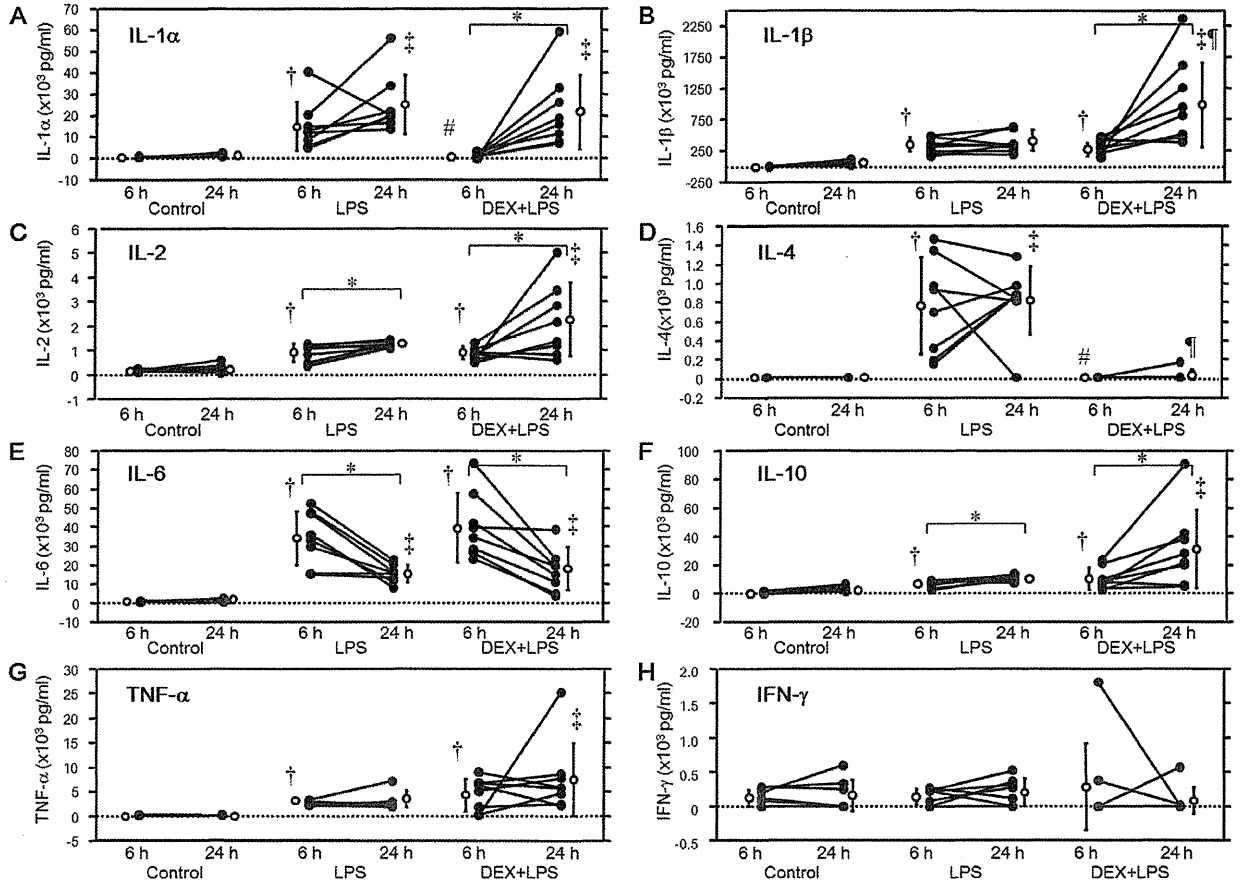

Fig. 5. Changes in cytokine concentration between 6 and 24 h after normal saline or LPS instillation, with or without pre-treatment with DEX in ELF collected by rat BMS in individual rats. The mean concentrations of cytokines at each time point are shown as open circles with standard deviations. $n = 8$ in each group. $^{*}P < 0.05$: difference between 6 and 24 h; $^{\dagger}P < 0.05$; differences between control and LPS, or control and DEX + LPS at 6 h; $^{\ddagger}P < 0.05$: differences between control and LPS, or control and DEX + LPS at 24 h; $^{\#}P < 0.05$: difference between LPS and DEX+LPS at 6 h; $^{\P}P < 0.05$: difference between LPS and DEX + LPS at 24 h.

4. Discussion

The main observations made in this first study of rBMS in a rat model, are:

(1) This method can be used to analyze ELF in a rat model of ALI, and (2) the ELF collected by rBMS could be used for the proteomic analysis of TNF-α-related proteins without dilution, and (3) for the simultaneous and serial measurements of multiple cytokines of bronchial area. We plan to use this method to collect ELF serially on the various rat lung injury models, especially in which serial sampling is important such as rat lung transplantation model. New molecules or important kinetics of cytokines which may cause lung injury could be found out in future.

We made our measurements in BAL fluid within 3 h after the administration of LPS, i.e. before the development of epithelial inflammation, though found significantly increased concentrations of TNF-α at 1 and 3 h compared with the control group. The absence of an increase in the concentration of albumin confirmed that the measurements were made before an increase in epithelial permeability. Furthermore, the rBMS samples collected at 3 h contained significantly higher concentrations of TNF-α in the LPS-treated than in the control rats, confirming that our new rBMS method, like the BAL fluid analysis, is capable of measuring changes in concentrations of TNF-α in ELF. The difference in TNF-α concentration between LPS and control groups did not, however, reach significance in the rBMS sample at 1 h. We assume that these samples were obtained from the proximal airways, while the BAL samples consisted mainly of alveolar fluids. These observations suggest that the rBMS method was not able to detect the alveolar TNF-α produced by macrophages at 1 h. The differences in absolute values between the measurements made by rBMS and BAL were due to different dilution ratios between the two methods. And the rBMS has another fault compared with BAL that it cannot receive cytological information from airway. Despite this limitation, the rBMS method allows the serial collection of ELF samples without sacrificing the animal, unlike standard BAL, which requires sacrificing the rats for each sampling. And the rBMS could detect pathophysiological changes secondary to inflammatory disruptions of alveolar barrio function (Ishizaka et al., 2001).

We presume that the peaks observed in our proteomic analyses corresponded to TNF-related proteins and inflammatory cytokines. While we need to confirm that these peaks coincide with the various molecules identified in our Swiss-Prot protein database-matching search, the experiments in rats treated with a TACE inhibitor showed prominent blunting or elimination of the peaks, indicating that the production of TNF-related proteins and inflammatory cytokines was suppressed. This rBMS with centrifugation method is the only way to obtain undiluted ELF. This method hereafter might lead up to new pathophysiological molecules in comprehensive analyses.

Using the Bio-Plex assay, we were able to analyze multiple cytokines in serial ELF samples collected by oro-tracheal rBMS, in our rat model of LPS-induced ALI, and we observed dynamic changes in the concentrations of cytokines in ELF. Since these

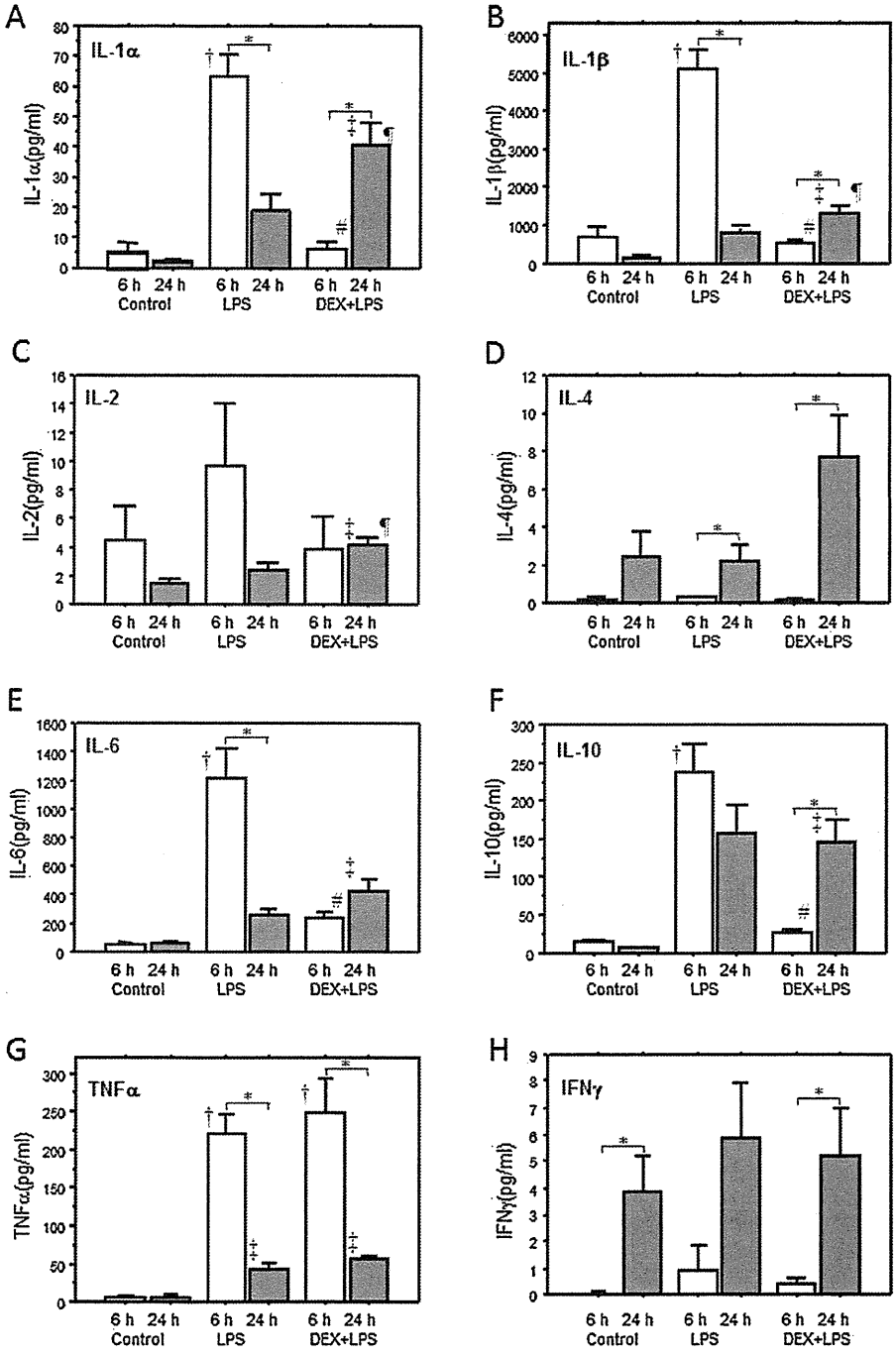

Fig. 6. Cytokine concentrations in BAL fluid 6 and 24 h after instillation of normal saline or LPS, with or without pre-treatment with DEX. $n=8$ in each group. $^{*}P<0.05$: difference between 6 and 24 h; $^{\dagger}P<0.05$; differences between control and LPS, or control and DEX + LPS at 6 h; $^{\ddagger}P<0.05$: differences between control and LPS, or control and DEX + LPS at 24 h; $^{\#}P<0.05$: difference between LPS and DEX + LPS at 6 h; $^{\P}P<0.05$: difference between LPS and DEX + LPS at 24 h.

molecular biological analyses are essential to understand the mechanisms of various lung injuries, our rBMS technique is likely to make important research contributions.

Our results suggest that anesthesia, intubation and manipulation of the rBMS probes had minimal effects on the measurements of cytokine concentrations in ELF. At 6 h, all cytokine concentrations in ELF in the control group were very low, and nearly all below the detection limit. If one considers that BAL washes out the cytokines and alveolar surfactant, as well as removes inflammatory cells such as macrophages and neutrophils, which secrete inflammatory mediators, causing additional alveolar injury and inflammation, our rBMS technique is less invasive and might be optimal to make serial molecular biological measurements.

The amount of ELF collected by rBMS varied among groups (Fig. 4). The volume of ELF collected at 24 h in the control group was significantly smaller than at 6 h, whereas it increased in the LPS-treated group, though the difference between 6 and 24 h was not statistically significant. It might be because the anesthetized

rats were dehydrated at 24 h, while, in the LPS group, the increased pulmonary vascular permeability increased the production of ELF by the bronchial epithelium. The amount of ELF collected in the DEX + LPS group was significantly smaller than in the LPS group at both 6 and 24 h, probably because DEX stabilized the pulmonary endothelial and epithelial membranes (Mafra de Lima et al., 2010). Differences in volume of water in ELF can affect the concentration of cytokines. To judge the gross quantity of cytokines secreted in ELF, values that crossed concentration and quantity of ELF collected by rBMS might be useful, though we cannot collect the entire ELF in a rat.

Our rBMS technique detected heterogeneities in the concentrations of eight cytokines in ELF in this rat model of LPS-induced ALI (Fig. 5). It is noteworthy that the concentrations of IL-2 and IL-10 in the LPS-treated group increased significantly between 6 and 24 h, suggesting that they peaked beyond 24 h after the administration of LPS. In contrast, the concentrations of IL-6 in the LPS-treated group decreased significantly between 6 and 24 h. IL-6 is an early-phase inflammatory cytokine, which probably peaked before 6 h after the administration of LPS (Wu et al., 2009). Other cytokines concentrations, including of TNF-α and IFN-γ, remained unchanged between 6 and 24 h in the LPS-treated group. TNF-α, an early-phase cytokine, might peak before 6 h, while IFN-γ, a late-phase cytokine, might peak beyond 24 h after the administration of LPS. Wu et al. have reported that, in LPS-treated mice, the concentrations of TNF-α, IL-6, IL-10 and IFN-γ in BAL fluid, reached a maximum at 8, 16, 24 and 48 h, respectively (Wu et al., 2009). The detailed chronological changes of individual cytokines warrant further studies. In preliminary experiments, rats with LPS-induced ALI survived long after twice undergoing rBMS. We have also confirmed, in another study, that they can undergo five oro-tracheal BMS in 5 days. Therefore, we believe that our rat BMS could be used to examine the time-dependent changes in cytokine concentrations.

A single pre-treatment with DEX modified the cytokine concentrations in ELF. IL-1α and IL-4 were significantly decreased by DEX at 6 h, while other cytokines remained unchanged. The effect of DEX on IL-1α was no longer detectable at 24 h. Moreover, the concentration of IL-1β in ELF was significantly higher in the DEX + LPS, than in the LPS only-treated group at 24 h. On the other hand, the concentration of IL-4 remained low in the DEX + LPS-treated group, 24 h after the administration of LPS.

The evolution of cytokine concentrations was not always similar between ELF and BAL fluid (Figs. 5 and 6). The concentrations of IL-1α, IL-1β, IL-2 and IL-10 increased at 6 h after LPS stimulation, suppressed by DEX pre-treatment and then rose again at 24 h both in ELF and BAL fluid. However the concentrations of IL-1α, IL-1β, IL-2 and IL-10 at 24 h in the LPS group kept high levels in ELF, but decreased in BAL fluid. The concentration of IL-6 and TNF-α were also elevated at 6 h both in ELF and BAL fluid, though they showed different changes at 24 h and different responses to pre-treatment with DEX. The time-dependent changes and responses to DEX pre-treatment of IL-4 and IFN-γ concentration were also different from other cytokines. These differences in cytokine measurement might be caused by the fact that BAL reflects the condition of alveolar area on the other hand ELF reflects that in bronchial area. The lesion of cytokine production, the metabolic rate of cytokines and the speed of the drainage through respiratory tract can participate, too.

5. Conclusions

Our rBMS method can be used to measure cytokines in rat ALI model, and was also available for proteomic analysis despite their small sample volumes. In addition, this method, which enabled serial measurements of multiple cytokines in single surviving animals, revealed the existence of heterogeneous dynamic changes among cytokines. This study confirmed the applicability of experimental rBMS, particularly in rats suffering from ALI and impaired pulmonary function. It will limit the need to sacrifice the animals used to study the mechanisms of various types of lung injury. Since the characteristics of rBMS are different from those of BAL, it is important to use each properly.

Grants

This study was supported, in part, by a grant-in-aid for Fundamental Scientific Research 14,770,684 (I.K.) from the Japanese Ministry of Education.

Authors' contributions

IK, MK, HN, Koichi K, and MW designed the study; IK, MK, Kazunori K, HN, and MW carried out the study; IK, MK, HN, MS, and MW analyzed the data; IK, MK, HN, and MW composed the manuscript.

Disclosures

The authors have no conflict of interest to disclose.

Acknowledgements

The authors dedicate this article to Akitoshi Ishizaka, MD, the late Professor of the Department of Internal Medicine, Keio University School of Medicine.

The authors thank Mitsubishi Pharma Corporation, Osaka, Japan, for supplying the TACE inhibitor and pertinent information regarding the drug.

Appendix A.

Values entered in the protein-identifying TagIdent tool:

(1) isoelectric point (p*I*) = min 5, max 10 (p*I* unknown); (2) Mw = 31,000, 47,000, 63,000 Da; (3) Mw-range 20%; (4) organism species "*Rattus norvegicus*"; (5) keyword = cytokine.

References

Durairaj, L., Neelakantan, S., Launspach, J., Watt, J.L., Allaman, M.M., Kearney, W.R., Veng-Pedersen, P., Zabner, J., 2006. Bronchoscopic assessment of airway retention time of aerosolized xylitol. Respir. Res. 7, 27.

Ishizaka, A., Matsuda, T., Albertine, K.H., Koh, H., Tasaka, S., Hasegawa, N., Kohno, N., Kotani, T., Morisaki, H., Takeda, J., Nakamura, M., Fang, X., Martin, T.R., Matthay, M.A., Hashimoto, S., 2004. Elevation of KL-6, a lung epithelial cell marker, in plasma and epithelial lining fluid in acute respiratory distress syndrome. Am. J. Physiol. Lung Cell. Mol. Physiol. 286, L1088–L1094.

Ishizaka, A., Watanabe, M., Yamashita, T., Ogawa, Y., Koh, H., Hasegawa, N., Nakamura, H., Asano, K., Yamaguchi, K., Kotani, M., Kotani, T., Morisaki, H., Takeda, J., Kobayashi, K., Ogawa, S., 2001. New bronchoscopic microsample probe to measure the biochemical constituents in epithelial lining fluid of patients with acute respiratory distress syndrome. Crit. Care Med. 29, 896–898.

Kamiya, K., Watanabe, M., Kohno, M., Izumi, Y., Horinouchi, H., Kawamura, M., Shimada, N., Nomori, H., 2011. KL-6 and CEA levels in epithelial lining fluid microsamples predict response to gefitinib in patients with advanced non-small cell lung cancer. Respirology 16, 976–982.

Klech, H., Hutter, C., 1990. Side-effects and safety of BAL. Eur. Respir. J. 3, 939–940, pp. 961–969.

Komaki, Y., Sugiura, H., Koarai, A., Tomaki, M., Ogawa, H., Akita, T., Hattori, T., Ichinose, M., 2005. Cytokine-mediated xanthine oxidase upregulation in chronic obstructive pulmonary disease's airways. Pulm. Pharmacol. Ther. 18, 297–302.

Mafra de Lima, F.V.A., Salgado, M.A., Castro-Faria-Neto, H.C., Munin, E., Albertini, R., Aimbire, F., 2010. Low intensity laser therapy (LILT) in vivo acts on the neutrophils recruitment and chemokines/cytokines levels in a model of acute pulmonary inflammation induced by aerosol of lipopolysaccharide from *Escherichia coli* in rat. J. Photochem. Photobiol. B 101, 271–278.

Montravers, P., Gauzit, R., Dombret, M.C., Blanchet, F., Desmonts, J.M., 1993. Cardiopulmonary effects of bronchoalveolar lavage in critically ill patients. Chest 104, 1541–1547.
Sasabayashi, M., Yamazaki, Y., Tsushima, K., Hatayama, O., Okabe, T., 2007. Usefulness of bronchoscopic microsampling to detect the pathogenic bacteria of respiratory infection. Chest 131, 474–479.
Watanabe, M., Ishizaka, A., Ikeda, E., Ohashi, A., Kobayashi, K., 2003. Contributions of bronchoscopic microsampling in the supplemental diagnosis of small peripheral lung carcinoma. Ann. Thorac. Surg. 76, 1668–1672, discussion 1673.
Wu, C.L., Lin, L.Y., Yang, J.S., Chan, M.C., Hsueh, C.M., 2009. Attenuation of lipopolysaccharide-induced acute lung injury by treatment with IL-10. Respirology 14, 511–521.
Yamazaki, K., Ogura, S., Ishizaka, A., Oh-hara, T., Nishimura, M., 2003. Bronchoscopic microsampling method for measuring drug concentration in epithelial lining fluid. Am. J. Respir. Crit. Care Med. 168, 1304–1307.
Yanagi, S., Ashitani, J., Ishimoto, H., Date, Y., Mukae, H., Chino, N., Nakazato, M., 2005. Isolation of human beta-defensin-4 in lung tissue and its increase in lower respiratory tract infection. Respir. Res. 6, 130.

Glossary

ALI: acute lung injury
BAL: bronchoalveolar lavage
BMS: bronchoscopic microsampling
DEX: dexamethasone
ELF: epithelial lining fluid
rBMS: rat bronchial microsampling
TACE: tumor necrosis factor-α converting enzyme