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Change in serum marker of oxidative stress in the progression of idiopathic pulmonary fibrosis

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ABSTRACT

Background: Increased oxidative stress is supposed to be involved in the etiology of idiopathic pulmonary fibrosis (IPF). It was reported that oxidative stress values measured by a spectrophotometric technique (d-ROMs test) were significantly higher in IPF patients than in controls, and were negatively correlated with Forced Vital Capacity (FVC) and Carbon Monoxide Diffusing Capacity (DLCO). However, the relationship between progression of IPF over time and change in serum oxidative stress marker remains unclarified.

Aims: This study aimed to investigate the change in serum oxidative stress marker during progression of IPF.

Subjects and methods: The levels of oxidative stress in blood samples of 43 treatment-naïve IPF patients were measured by the d-ROMs test. FVC and DLCO were measured concurrently. The changes in oxidative stress and pulmonary function were evaluated in 27 untreated patients 6 months later. Oxidative stress levels of 13 patients with acute exacerbation of IPF (AE-IPF) and 30 healthy controls were also evaluated.

Results: Oxidative stress values [median, interquartile range (IQR); Carratelli units (U.CARR)] were significantly higher in 43 IPF patients than in controls (366, 339–443 vs. 289, 257–329, $p < 0.01$) and were significantly increased 6 months later in 27 untreated patients (353, 311–398 at baseline to 385, 345–417 at follow-up, $p < 0.01$). The increase in oxidative stress values (24.0, 6.0–49.0 U.CARR/6 months) was negatively correlated with baseline DLCO ($r_s = -0.44$, $p < 0.05$) and FVC changes after 6 months ($r_s = -0.54$, $p < 0.01$). Oxidative stress values were significantly higher in IPF patients with acute exacerbation than in those with stable disease (587, 523–667 vs. 366, 339–443 U.CARR, respectively; $p < 0.01$).

Conclusions: Serum oxidative stress values increased with disease progression in IPF patients.

1. Introduction

Idiopathic pulmonary fibrosis (IPF), a progressive disease with an as yet unidentified cause, is often fatal and characterized by irreversible change in alveolar structure by fibroblast growth and remodeling of extracellular matrix [1].

IPF is a disease with poor prognosis, and the median survival time is 2.5–3.5 years. The clinical course of individual patients varies from slow to rapid progression [2]. Unpredicted acute exacerbation that develops in some patients is often fatal [3].

Pathohistologically, IPF exhibits usual interstitial pneumonia (UIP), which is characterized by various levels of fibrosis in time and space, scarring and honeycombing along with areas of unaffected parenchyma, and by a paucity of inflammatory findings. The centers of growth of fibroblasts are called “fibroblastic foci” which seem to appear at sites of alveolar injury [4].

It has been long believed that pulmonary fibrosis begins with alveolar inflammation and that chronic inflammation modulates fibrogenesis [5]. However, the clinical degree of inflammation showed no correlation with disease severity or clinical course, and treatment with anti-inflammatory drugs failed to improve the

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prognosis. Recent findings suggest that inflammation does not play a major role in the etiology and that abnormal wound healing in response to alveolar epithelial injury results in lung fibrosis [6].

It has been found that oxidative stress is increased in IPF and is involved in its pathogenesis. Cantin et al. demonstrated that cells in the bronchoalveolar lavage fluid (BALF) produced oxidants and myeloperoxidase at higher concentrations in IPF patients than in control patients. They also showed that increased peroxidase activity was involved in the epithelial injury in IPF [7]. Jackson et al. reported that free radical activity was increased based on the change in serum markers [8], while Rahman et al. showed an imbalance between oxidative stress and anti-oxidative potency in serum and BALF in IPF patients [9].

The Diacron Reactive Oxygen Metabolites test (the d-ROMs test) is a method to evaluate oxidative stress of the whole body by measurement of the total content of hydroperoxide in the blood [10]. Daniil et al. reported that serum oxidative stress values measured by the d-ROMs test were significantly higher in 21 treatment-naïve IPF patients than in controls, and were negatively correlated with Forced Vital Capacity (FVC) and Carbon Monoxide Diffusing Capacity (DLCO) [11].

However, the relationship between progression of IPF over time and change in serum oxidative stress values remains unclarified. The relationship between acute exacerbation (AE) of IPF (AE-IPF) and oxidative stress also remains unclear. The aim of this study was to investigate how serum oxidative stress values would change as IPF deteriorated over time.

2. Subjects and methods

2.1. Subjects and methods

Among all IPF patients who visited our department between January 1, 2009 and September 30, 2012, 47 of them who were not treated with oxygen, steroids, immunosuppressants, pirfenidone, or N-acetylcysteine were subjected to measurement of serum oxidative stress values. IPF was diagnosed according to the criteria of ATS/ERS/JRS/ALAT statement for IPF [1]. After exclusion of pulmonary fibrosis due to collagen disease, professional exposure, hypersensitivity pneumonitis, and drug-induced lung disease, high-resolution CT (HRCT) images were evaluated by more than one respiratory specialist and experienced radiologists for confirmation of the usual interstitial pneumonia (UIP) pattern. No surgical biopsy was carried out. After exclusion of two cases each of suspected chronic airway infection and confirmed concurrence of a malignant tumor, the relationship between oxidative stress values and pulmonary function was retrospectively examined in the remaining 43 cases.

Oxidative stress values were also measured in 30 healthy control subjects (46–81 years old) who were matched with the 43 IPF patients in gender and smoking history. The 43 IPF patients comprised of 33 males and 10 females with an average age of 69.4 ± 8.9 years. Meanwhile, the healthy control subjects were composed of 23 males and 7 females with an average age of 65.0 ± 11.5 years. In both groups, no smoker was included at the time of the examination (Table 1).

Of the 43 IPF patients, 27 untreated patients who did not develop any complications such as malignant tumors and infectious disease and had been followed-up for six months were subjected to retrospective analyses on changes in serum oxidative stress values and respiratory function. 27 patients (24 men and 3 women) were aged 67.7 ± 8.4 years, and 23 were ex-smokers while 4 were nonsmokers.

Oxidative stress values were additionally measured in a total of 13 cases (9 men and 4 women) with acute exacerbation of IPF (AE-IPF) before treatments (other than oxygen administration) were initiated. 13 cases were consisted of 5 cases (among our 43 IPF cases) that occurred during follow-up at our hospital, and 8 additional cases who were diagnosed with IPF at another hospital and transferred to our hospital at the time of AE-IPF. They were aged 67.4 ± 8.3 years, and 9 were ex-smokers while 4 were nonsmokers.

Table 1
Characteristics of patients with idiopathic pulmonary fibrosis (IPF) and controls.

	IPF	Control
N	43	30
Sex, m/f	33/10	23/7
Age, yr	69.4 ± 8.9	65.0 ± 11.5
Smoker/ex-smoker/nonsmoker	0/32/11	0/23/7

AE-IPF was diagnosed according to the following criteria [3]: IPF was diagnosed previously or at the same time; unpredicted acute worsening of dyspnea within 30 days; development of new ground-glass opacity or consolidation with the background findings of reticular shadows or honeycomb lungs with the UIP pattern; and exclusion of pulmonary infection, left heart failure, pulmonary embolism, and acute lung injury with an already identified cause.

This study was approved by the ethical committee of our institute, and written informed consent was obtained from the patients and control subjects after detailed explanation.

2.2. d-ROMs test

Oxidative stress was evaluated by measurement of serum hydroperoxide by the d-ROMs test. Free Radical Analytical System 4 (FRAS 4, Wismerll Co. Ltd., Tokyo, Japan) was used for the test. The principle of the test is based on the concept that the serum organic hydroperoxide content reflects the free radical content that produces it. Standard test procedures are as follows. Blood samples collected from peripheral vein of the patients were centrifuged at 4 °C at 1500 × *g* for 15 min, and 20 μl of serum was mixed with acid buffer solution (pH 4.8, R1 reagent of the kit) in a cuvette, which was then supplemented with 20 μl of the chromogen (R2 reagent of the kit). Serum hydroperoxide, reacting with the transition metal ion released from the protein in the blood under acidic environments, changed to alkoxy or peroxy radicals. These newly produced radicals oxidized the chromogen and yielded a purple product.

The concentration of the stable product was easily measured with a spectrophotometer (absorbance at 505 nm). The normal range of the test results was 250–300 U.CARR (Carratelli Units), where 1 U.CARR corresponded to 0.8 mg/l of hydrogen peroxide. Results exceeding 300 U.CARR indicated increased oxidative stress.

2.3. Serum markers and pulmonary function tests

Examination of serum markers and pulmonary function tests were performed at the same time as the d-ROMs test. Biochemical tests were done with JOEL BM-6050 (JOEL Ltd., Tokyo, Japan), while serum KL-6 and SP-D were measured with an electrochemical luminescence immunoassay kit (EIDIA, Tokyo, Japan; normal range<500 U/ml) and an enzyme immunoassay kit (Yamasa Corporation, Chiba, Japan; normal range<110 U/ml), respectively. For assessing respiratory function, the CHESTAC-9800 pulmonary function test (Chest, Tokyo, Japan) was employed. FVC was measured at least three times, and the highest value was recorded. DLCO was measured by the helium diffusion method with one-time breath holding.

2.4. Statistical analysis

In cases with no annotation, statistics were expressed as average ± tandard deviation. For comparison of oxidative stress

Table 2
Oxidative stress, pulmonary function, and serum marker levels of patients with IPF.

	Normal value	IPF (n = 43)
d-ROMs (U.CARR) mean ± SD		383 ± 76
median, interquartile range		366, 339–443
FVC (ml)		2390 ± 890
FVC (% predicted)		76.9 ± 22.3
%Dlco (% predicted)		64.7 ± 22.4
PaO_2 (mmHg)		71.9 ± 13.4
LDH (IU/L)	120–240	221 ± 40.4
KL6 (U/ml)	<500	1259 ± 1030
SP-D (ng/ml)	<110	292 ± 197

d-ROMs, Diacron Reactive Oxygen Metabolites; FVC, forced vital capacity; DLCO, diffusing capacity; KL6, sialylated carbohydrate antigen; SP-D, surfactant protein D.

values between two groups, Mann–Whitney's U test was employed, while Wilcoxon's rank sum test was used for the comparison between the baseline and follow-up values. For analysis of a correlation between different parameters, Spearman's rank correlation coefficient was used. A p-value less than 0.05 was considered statistically significant. Statistical analysis software SPSS 12.0 was employed.

3. Results

3.1. IPF patients and controls

The pulmonary function tests and evaluation of serum biomarkers performed in the 43 IPF patients revealed that FVC was 2390 ± 890 (ml) and 76.9 ± 22.3 (% predicted), DLCO (% predicted) was 64.7 ± 22.4 (%), serum marker LDH was 221 ± 40.4 (IU/l), KL-6 was 1259 ± 1030 (U/ml), and SP-D was 292 ± 197 (ng/ml) (Table 2).

Oxidative stress values in the 43 IPF patients were 383 ± 76 (U.CARR) (Table 2), and were significantly higher than in controls [median, interquartile range (IQR): 366, 339–443 vs. 289, 257–329 U.CARR, respectively; $p < 0.01$] (Fig.1). Oxidative stress values showed no significant correlation with DLCO (% predicted) ($p = 0.58$, $r_s = -0.1$) or FVC (% predicted) ($p = 0.06$, $r_s = -0.295$). In addition, oxidative stress values had no significant correlation with LDH, KL6, or SP-D (Table 3).

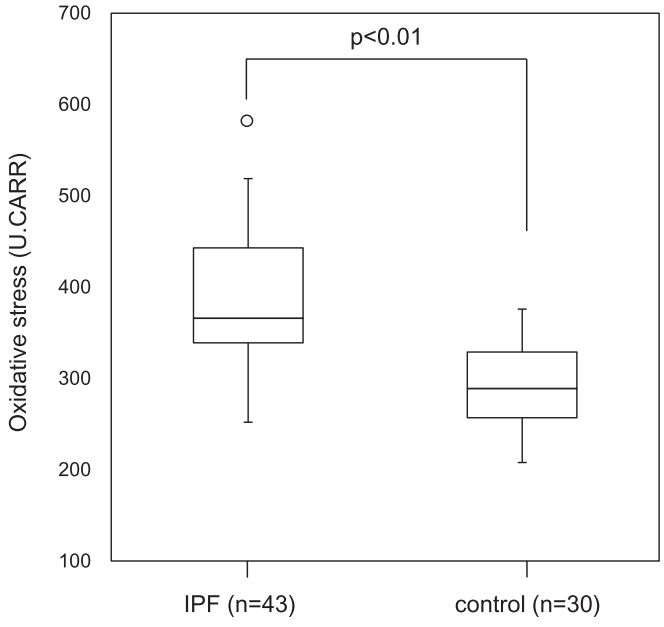

Fig. 1. Oxidative stress levels in patients with IPF and controls. The two groups differ significantly as indicated.

Table 3
Correlation of oxidative stress with age, pulmonary function tests, PaO_2, and serum markers in 43 patients with IPF.

	Oxidative stress	
	p	rs
Age	0.95	−0.01
FVC (% predicted)	0.06	−0.295
Dlco (% predicted)	0.582	−0.1
PaO_2 (mmHg)	0.12	−0.19
LDH (IU/L)	0.08	0.28
KL-6 (U/ml)	0.51	0.11
SP-D (ng/ml)	0.85	0.001

3.2. Changes over time

Changes in the different parameters were evaluated at the six month follow-up in 27 untreated patients. FVC (ml) and DLCO (% predicted) were significantly decreased compared with the baselines, by 131 ± 174 ml and 9.1 ± 9.4%, respectively, after 6 months.

There was no significant change in LDH, KL-6, or SP-D during the follow-up period. Oxidative stress values were significantly increased in 22 of 27 patients as compared to baseline values (median, IQR: 353, 311–398 at baseline to 385, 345–417 at follow-up U.CARR, $p < 0.01$) (Table 4).

The median (IQR) elevation in the oxidative stress value after 6 months was 24.0 (6.0–49.0) U.CARR/year, which was negatively correlated with FVC changes after 6 months ($r_s = -0.60$, $p < 0.01$) and baseline DLCO ($r_s = -0.44$, $p < 0.05$) (Figs. 2 and 3).

As the decrease in FVC vary from rapid to slow, the cases were classified into “rapid progressor” as the cases which showed a 5% or more decrease in FVC (ml) after 6 months, and “slow progressor” as the cases which showed a less than 5% decrease in FVC (ml). There was no significant difference in the baseline oxidative stress value between rapid progressor (n = 13), and slow progressor (n = 14), but the oxidative stress value increased significantly more in “rapid progressor” after 6 months (median, IQR: 31.3, 21.6–52.6 vs. 14.3, −7.1–25.0 U.CARR, respectively, $p < 0.01$) (Fig. 4).

3.3. Acute exacerbation of IPF

In the 13 patients with acute exacerbation of IPF, the PaO_2 to FiO_2 (P/F) ratio was 177.8 ± 54.3, while LDH was 382 ± 125 IU/l, KL-6 was 1815 ± 1030 IU/l, and SP-D was 505 ± 467 ng/ml (Table 5).

The oxidative stress values in these patients with acute exacerbation were significantly higher as compared to that in patients with stabilized IPF (median, IQR: 587, 523–667 vs. 366, 339–443 U.CARR, $p < 0.01$) (Fig. 5). In all five AE-IPF cases who were part of

Table 4
Change in pulmonary function, serum marker levels, and oxidative stress in 27 patients with IPF.

	Baseline	After 6 months	p-value
N	27		
Sex (m/f)	24/3		
Smoker/ex-smoker/non-smoker	0/23/4		
Age	67.7 ± 8.4		
FVC (ml)	2680 ± 900	2550 ± 904	P < 0.01
FVC(% predicted)	82.6 ± 23.6	78.9 ± 24.4	P < 0.01
Dlco (% predicted)	68.3 ± 19.3	59.8 ± 17.3	P < 0.05
LDH (IU/L)	217 ± 37	216 ± 37	N.S
KL6 (U/ml)	1150 ± 886	1065 ± 889	N.S
SP-D (ng/ml)	315 ± 208	269 ± 160	N.S
d-ROMs (U.CARR) mean ± SD	359 ± 66	385 ± 65	P < 0.01
median, interquartile range	353,311–398	385,345–417	

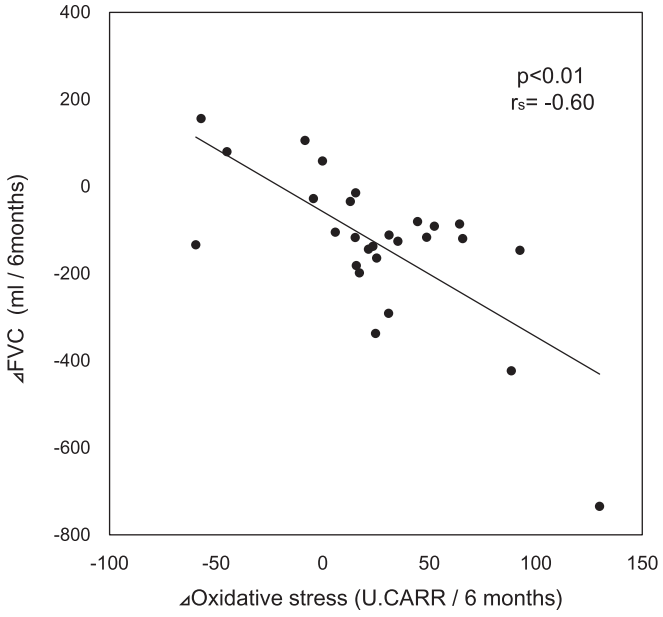

Fig. 2. Change in oxidative stress correlates negatively with change in FVC.

our original cohort, the oxidative stress level increased after exacerbation (data not shown).

Among these 13 patients, the oxidative stress value range was 401–724 in 9 patients who were inhaled oxygen during measurement, while 543–711 in 4 patients without oxygen administration during measurement.

4. Discussion

The oxidative stress level in IPF patients was significantly higher as compared to normal controls in the present study, consistent with the report by Daniil et al. [11]. Increased oxidative stress in the lungs *in situ* leads to influx of hydroperoxide into the blood, which is measured by the d-ROMs test and is a more stable substance

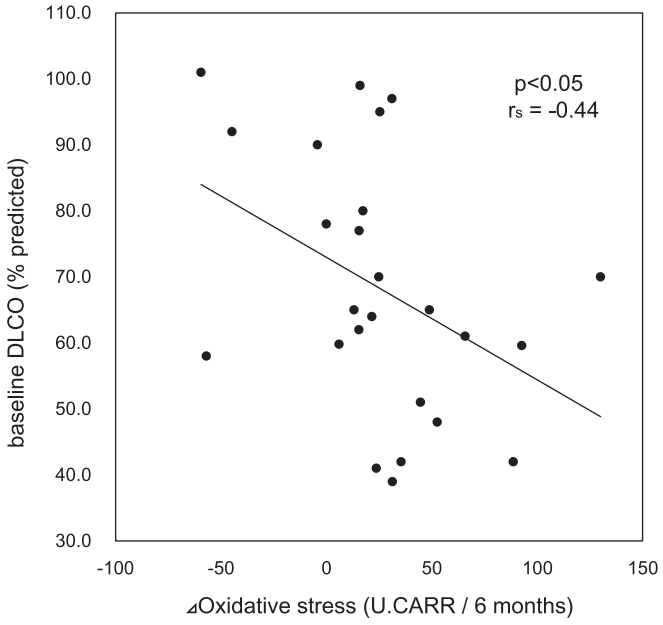

Fig. 3. Change in oxidative stress correlates negatively with baseline DLCO.

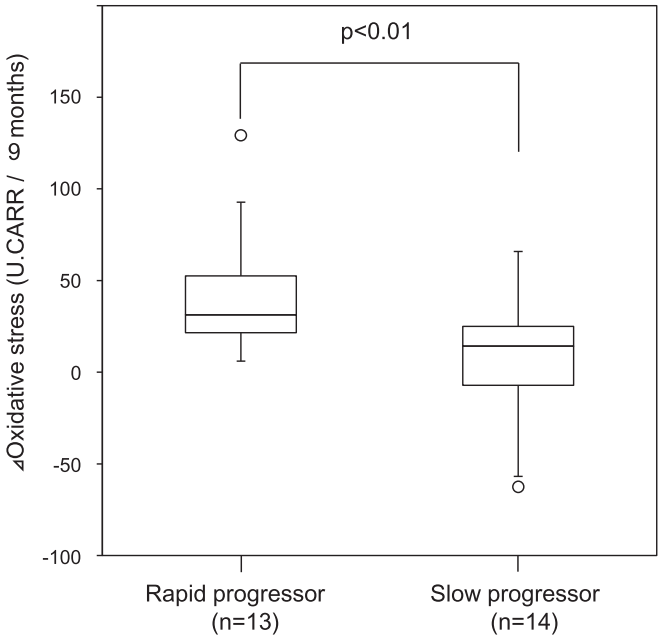

Fig. 4. Change in oxidative stress was significantly higher in "rapid progressor" than in "slow progressor". rapid progressor: 5% or more decrease in FVC after 6 months; slow progressor: less than 5% decrease in FVC after 6 months.

compared with reactive oxygen intermediate (ROI), and is expected to increase d-ROMs values.

In contrast to the previous findings by Daniil et al., our data showed that the baseline oxidative stress value had no significant correlation with DLCO or FVC.

The serum oxidative stress value reflects systemic oxidative stress, and it is understandable that it is not always correlated with the baseline severity of IPF. On the other hand, serum oxidative stress values significantly increased after six months in the follow-up study of 27 cases. During this period, there was no disease development or treatment intervention, and the increase of serum stress value was considered a reflection of a time-course elevation in oxidative stress in the lungs.

It has been suggested that time-course change in respiratory function rather than the baseline value is more important in the evaluation of IPF. Collard et al. reported that the degree of pulmonary dysfunction at baseline had no correlation with the speed of aggravation of pulmonary function in IPF, and change in pulmonary function over a period of 6–12 months rather than baseline values was more reflective of prognosis of IPF [15]. Jegal et al. followed-up 179 cases with fibrotic interstitial pneumonia, and found that FVC change, baseline DLCO, and gender were three independent prognostic factors based on multivariate analyses [16]. In particular, FVC change was underscored as a sensitive marker [17] and has been employed as a major evaluation variable in recent clinical trials [18–20].

Baseline FVC (% predicted) is influenced by not only the severity of IPF, but also muscle strength and body build. Meanwhile, change in FVC is unlikely to be influenced by factors other than IPF *per se*. It is reasonable that change in FVC rather than baseline FVC is a prognostic factor. In this study, the change in oxidative stress after six months correlated with a decrease in FVC, which was considered the most important prognostic factor for IPF.

Unlike FVC, DLCO correlated with the baseline value. Jegal et al. reported that baseline DLCO value and not FVC was a prognostic factor. These results are attributable to the fact that DLCO decreases before FVC decreases and DLCO, unlike FVC, is not associated with

Table 5
Characteristics of patients with acute exacerbation of IPF (AE-IPF). P/F ratio; PaO_2 to FiO_2 ratio.

	AE-IPF (n = 13)
Sex, m/f	9/4
Age (yr)	67.4 ± 8.3
Smoker/ex-smoker/nonsmoker	0/9/4
P/F ratio	177.8 ± 54.3
LDH (IU/L)	382 ± 125
KL-6 (U/ml)	1815 ± 1030
SP-D (ng/ml)	505 ± 467
d-ROMs (U.CARR) mean ± SD	583 ± 98
median interquartile range	587, 523–667

any other factor except progression of lung fibrosis. Meanwhile, change in DLCO had no correlation with the change in oxidative stress. As Jegal et al. suggested, DLCO measurements tend to fluctuate, which may explain these results [16].

Although a number of studies have investigated whether the prognosis of IPF can be predicted by serum biomarkers, it remains inconclusive. Yokoyama et al. reported that an increase in KL-6 levels was a poor prognostic factor for IPF, while Takahashi et al. reported that increased SP-D was a poor prognostic factor for IPF [21,22]. Meanwhile, Song et al. reported that neither KL-6 nor SP-D alone was a poor prognostic factor for IPF [23]. The results of our present study showed that pulmonary function at baseline had no significant correlation with KL-6 or SP-D, and there was no significant correlation between the change over time in these markers and pulmonary function change. Taken together, no established serum marker has been definitely established as a prognostic factor for IPF, but our results show that change in the oxidative stress value could be a reliable serum marker to predict the prognosis of IPF.

As lung fibrosis advances, vital capacity decreases in IPF, which correlates with an increase in oxidative stress. Does increased oxidative stress advance fibrosis in IPF or does advancement of fibrosis increase oxidative stress? With regard to the relationship between fibrosis and oxidative stress in IPF, reactive oxygen species (ROS) accelerate the production of transforming growth factor (TGF)-β, a cytokine involved in fibrosis, in epithelial cells [12,13], while TGF-β promotes ROS production in fibroblasts [14]. These results suggest that a positive feedback may exist between pulmonary fibrosis and oxidative stress. Increased oxidative stress accelerates pulmonary fibrosis following alveolar cell injury, and progression of pulmonary fibrosis may potentially increase oxidative stress over time.

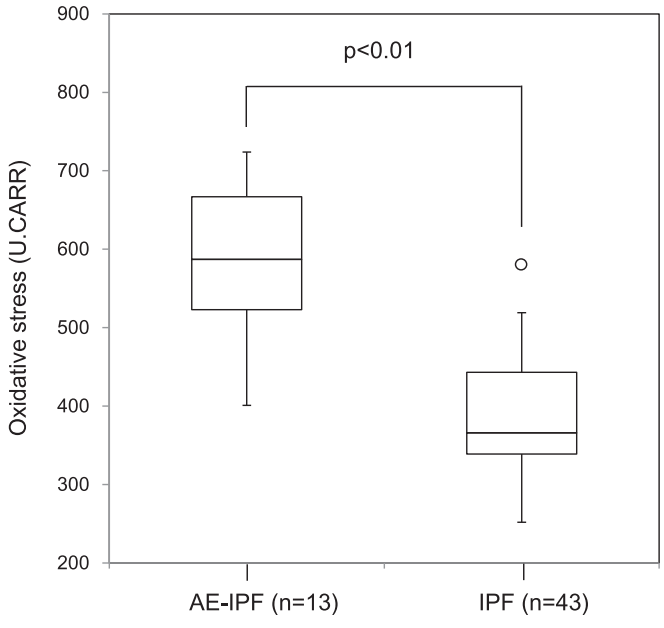

Fig. 5. Oxidative stress levels in patients with acute exacerbation of IPF (AE-IPF) and in stable IPF patients. The two groups differ significantly as indicated.

Baseline oxidative stress values showed no correlation with the severity of IPF. Oxidative stress is increased even in mild IPF cases with normal respiratory function. Is oxidative stress increased prior to the onset of IPF?

Recently, it has been elucidated that excessive iron load in the lower airway is involved in oxidative stress in IPF [24]. In addition, it was demonstrated that an HFE genetic polymorphism was involved in the mechanism [25]. Increased oxidative stress in IPF may potentially be determined by the background genetic polymorphism profile before the onset of IPF.

No report has investigated the relationship between AE-IPF and oxidative stress, which remains to be elucidated. In this study, oxidative stress values were significantly higher in the 13 AE-IPF cases than in the stable IPF cases, and were increased before the occurrence of AE in all five cases in which oxidative stress values were measured before AE. Although oxygen administration during AE may influence oxidative stress values, these values ranged from 543 to 711 in 4 cases without oxygen administration during measurement and 401 to 724 in 9 cases with oxygen administration during measurement, which suggested that there is little association between the increase in oxidative stress values and oxygen administration. In our present study, oxidative stress values were increased not only in the chronic stage of IPF, but also during AE. Nevertheless, how oxidative stress is increased in AE-IPF remains unclear.

There are limitations to this study; the study was retrospective and selection bias could not be excluded, and oxidative stress in the lungs was measured in serum which may not be the most appropriate specimen. For evaluation of oxidative stress in the lungs *in situ*, d-ROMs test using BALF samples may be more appropriate. Rahman et al. measured oxidative stress values in both serum and BALF, and found that both were higher in IPF patients than in controls [9]. However, measurement with BALF is too invasive to examine change over a period of time. Another sample type to examine oxidative stress in the lungs of IPF patients is exhaled breath condensate which can be evaluated to obtain information on the lungs *in situ* and for a variety of respiratory diseases on a trial basis [26]. It was also reported that oxidative stress measurement with exhaled breath condensate was unaffected by systemic diseases [27]. However, like the report by Daniil et al. [11], the above studies performed single measurement in each patient. Serial investigation of oxidative stress markers in expired breath condensate may be more important.

Since an imbalance between oxidative stress and anti-oxidative potency is involved in fibrosis in IPF, “anti-oxidative therapy” to suppress oxidative stress may potentially be useful for treatment of IPF. In bleomycin-induced lung injury, an animal model of pulmonary fibrosis, PC-SOD (lecithinized superoxide dismutase) suppressed the disease by eliminating cytotoxicity of superoxide [28]. In addition, we reported that administration of PC-SOD was effective for treating steroid-resistant interstitial pneumonia [29]. N-acetylcysteine has been reported to be effective for IPF, and the mechanism of action is probably related to its anti-oxidant effect [18]. Pirfenidone, which has been confirmed to suppress the progression of IPF, is thought to have anti-oxidative activity as well as anti-fibrotic activity [30].

In addition to anti-inflammatory and anti-fibrotic therapies, anti-oxidant treatment may also be used for IPF, and lecithinized

superoxide dismutase is a strong candidate. Measuring oxidative stress markers before and after anti-oxidant therapy for IPF is expected to be useful.

5. Conclusions

As IPF progressed, oxidative stress was significantly increased which was significantly correlated with a reduction in FVC. In AE-IPF cases, oxidative stress was remarkably increased. These results suggested that oxidative stress may not only be the cause of IPF, but may also play an important role in its deterioration.

Conflict of interest

The authors declared that they have no conflicts of interests.

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