Significance of Elevated Procollagen-III-Peptide and Transforming Growth Factor-β Levels of Bronchoalveolar Lavage Fluids from Idiopathic Pulmonary Fibrosis Patients

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Hiwatari, N., Shimura, S., Yamauchi, K., Nara, M., Hida, W. and Shirato, K. *Significance of Elevated Procollagen-III-Peptide and Transforming Growth Factor-β Levels of Bronchoalveolar Lavage Fluids from Idiopathic Pulmonary Fibrosis Patients.* Tohoku J. Exp. Med., 1997, **181** (2), 285-295 —— Although both procollagen III aminopeptide (P-III-P) and transforming growth factor-β (TGF-β) are reported to be present in lung tissue and/or elevated in bronchoalveolar lavage fluid (BALF) from idiopathic pulmonary fibrosis (IPF) patients, we have little knowledge concerning the clinical significance of elevated P-III-P and TGF-β levels in BALF. Using a radioimmunoassay, we measured P-III-P and TGF-β in BALF from 48 IPF patients (16F and 32M, 59 ± 2 years, mean $\pm$ s.e.) who received BAL in our clinic over the past 13 years before glucocorticosteroid treatment. Among them, we could detect a significant amount of P-III-P (2.2 ± 1.0 U/ml; range 0.03 to 16.5 U/ml) in BALF in 18 of the patients (5F and 13M, 58 ± 3 years) (group B), but not (0.03 U/ml or less) in the other 30 patients (11F and 19M, 59 ± 2 years) (group A). Lymphocyte (%) and basophil (%) in BALF from group B was much larger than that from group A (33% vs. 8%, $p < 0.01$). Group B showed a longer duration of onset to BAL (36 months vs. 23 months, $p < 0.05$). TGF-β levels were obtained using an ELISA system kit from the same BALF samples. TGF-β was not detected in 10 patients (100 pg/ml or less) (3F and 7M, 59 ± 4 years) (group I), while the remaining 38 patients showed a significant amount of TGF-β (329 ± 44 pg/ml, range 100 to 1,360 pg/ml). The latter patients were further divided into two groups; group II 100 to 300 pg/ml (10F and 14M, 56 ± 3 years) and group III 350 or more (3F and 11M, 63 ± 2 years). Group III showed significantly better values in PaO_2, $Aa-DO_2$, %VC and %DLco, and smaller percentage of basophils in BALF than did groups I and/or II, whereas survival after BAL in group III was significantly shorter than in group I (31 vs. 19 months, $p < 0.05$). There was no significant relationship between P-III-P and TGF-β levels in BALF. These findings suggest that elevated P-III-P level is

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accompanied by an increase in lymphocyte population in BALF from IPF patients, resulting in a longer duration of the disease, while elevated TGF-β level reflects alveolar inflammation at an earlier stage of the disease which induces a progression of the disease, resulting in a shorter survival in IPF patients. ————— idiopathic pulmonary fibrosis; BALF ; P-III-P and TGF-β

Idiopathic pulmonary fibrosis (IPF) is characterized by a recurrent or chronic inflammation in the alveolar regions which progresses to interstitial fibrosis or increased collagen deposits in the alveolar walls. Namely, it is characterized by varying degrees of both chronic inflammation and fibrosis that results in derangement of the gas-exchanging units of the lungs. Much attention has been paid to collagen deposits by chronic inflammation in alveolar or peripheral lung regions when studying the pathophysiology of IPF. The deposition of collagen is a multistep process involving intracellular synthesis by connective tissues such as fibroblasts, secretion into the extracellular space, extracellular enzymatic processing, and subsequent cross-linking. Part of the extracellular processing of collagen involves the cleavage of procollagen peptides from the C-terminal and N-terminal ends of the molecule. This results in the release of discrete C-terminal procollagen pepetides that can be measured to identify changes in collagen synthesis (Risteli and Risteli 1986; Kuhn et al. 1989). Procollagen III amino-terminal peptide (P-III-P) is released from fibroblasts prior to the process of collagen fibril formation and is known to be a marker of activity of fibroblasts (Risteli and Risteli 1986). Transforming growth factor-β (TGF-β) is a potent profibrotic cytokine that stimulates fibroblast collagen and fibronectin biosynthesis (Ignotz and Massague 1986; Varga et al. 1987), and can also stimulate fibroblast growth (Hill et al. 1986). TGF-β is present in bronchoalveloar lavage fluid (BALF) in normal human subjects (Yamauchi et al. 1988). Further, TGF-β levels are increased in the lung in animal models of pulmonary fibrosis (Raghow et al. 1989) and the cytokine is present at sites of extracellular matrix deposition in IPF patients (Broekelmann et al. 1991; Khalil et al. 1991). A noticeable and consistent increase in TGF-β production was observed in epithelial cells and macrophages in sections of lung from IPF patients (Khalil et al. 1989). The genes producing TGF-β are also reported to be elevated in human pulmonary fibrosis (Broekelmann et al. 1991) and bleomycin-induced pulmonary fibrosis in hamster (Raghow et al. 1989). Recently, Giri et al. (1993) provided further evidence implicating TGF-β in the pathogenesis of pulmonary fibrosis, showing that antibodies to TGF-β significantly reduced lung collagen accumulation in bleomycin induced pulmonary fibrosis in mice. Thus, TGF-β seems to play a central role in the etiology of pulmonary fibrosis although a number of cytokines have been known to upregulate the inflammatory and fibroproliferative response of lungs leading to increased extracellular matrix production. Although P-III-P is reported to be elevated in BALF from IPF patients (Cantin et al. 1988; Low

et al. 1992; Behr et al. 1993), we have little knowledge concerning the clinical significance of P-III-P and TGF-β levels in BALF. This study was performed to determine the relationship between the P-III-P and TGF-β values in BALF and various clinical features of IPF patients.

SUBJECTS AND METHODS

Subjects

Forty-eight IPF patients were selected from the medical and pathological records at Tohoku University Hospital during 1981 to 1993 for the present study. They consisted of 16 females and 32 males with a mean age of 59$\pm$2 years, and 29 smokers and 19 non-smokers. They were all chronic type patients with IPF who survived one year. The diagnosis of IPF was made from a combination of medical records, clinical and laboratory data, and histological findings of autopsied lungs, according to the previous criteria of IPF (Crystal et al. 1976; Winterbauer et al. 1978). The other previously reported diseases and causes of pulmonary fibrosis were all excluded by histological findings of autopsied lungs using serial sectioning in addition to clinical, lung biopsy (transbronchial and/or open chest lung biopsy) and laboratory findings. They included collagen diseases, sarcoidosis, pneumoconiosis, fungus diseases, viral infections, tuberculosis, aspiration pneumonia and hypersensitive pneumonia. All subjects except two patients died of cor pulmonale and respiratory failure, and the other two patients died of fungus infections.

Pulmonary function tests

Laboratory, radiological and pulmonary function data were obtained within one month before BAL were used for data analysis.

Pulmonary function tests were performed as described previously (Aoki et al. 1984). The vital capacity (VC) and FEV1 were obtained with a Benedict-Roth spirometer. The pulmonary resistance (Rl) and static lung compliance (Cst) were measured in a pressure-compensated volume displacement body plethysmograph using an esophageal balloon (Macklem et al. 1974). Transpulmonary pressure (the difference between mouth and esophageal pressure) was obtained using an esophageal-ballon catheter, and the esophageal pressure at total lung capacity (TLC) was defined as the maximal esophageal pressure (Pes max). TLC, functional residual capacity (FRC), and residual volume (RV) were determined by the gas dilation method (Rodenstein and Stanescu 1982). Carbon monoxide diffusion capacity was measured by the single-breath method (Ogilvie et al. 1957). The predicted normal values of Cotes were used (Cotes 1993). Arterial gas tension and pH were measured with a pH-blood gas analyzer (Instrumentation Laboratories 1302, Lexington, MA, USA).

Bronchoalveolar lavage (BAL)

All patients were clinically stable and no patients were treated with glucocorticoid steroids.

Fiberoptic bronchoscopy was performed with lidocaine topical anesthesia using a 5.2 mm bronchoscope (Olmypus Co., Tokyo). A subsegment of the right middle lobe or lingula was lavaged by the instillation and immediate withdrawal by gentle suction of three 50-ml boluses of 0.9% sterile saline by a low negative pressure (-100 mmHg) to prevent collapse of the bronchus. All subjects tolerated lavage without difficulty. The recovered lavage fluids were passed through a sterile gauze. BAL cell spreads were prepared by cytocentrifugation, and 300 or more cells were differentially counted to determine macrophages, lymphoctes, neutrophils, eosinophils and basophils by means of Wright-Giemsa and nonspecific esterase stain. Cells and debris were removed by centrifugation at $400 \times g$ for 15 to 20 min and fluids were stored at 70°C until the following analysis.

P-III-P and TGF-β assays

BALF samples were concentrated 10-fold on an Amicon PM-10 membrane (retained proteins and pepetides $>10,000$ daltons), and type III procollagen aminoterminal pepetide-related antigens were assayed using a radioimmnunoassay kit (Behringwerke AG, Marburg, Germany), according to methods described previously (Low et al. 1992; Behr et al. 1993), measuring procollagen III N-terminal pepetide during the processing of newly synthesized type III collagen. A P-III-P level of 0.03 U/ml or less is not detected by this assay. For TGF-β assay, an ELISA kit (Amersham Japan Co., Tokyo) was used to measure the total amount of TGF-β_1, including all active TGF-β_1. A TGFβ-level of 100 pg/ml or less is not detected by this assay. Lavage fluid albumin was determined by laser nephlometric analysis (Behring LN modular system, Marburg, Germany) using immunochemical method. By determining the urea concentrations in both plasma and BALF, the total volume of recovered epithelial lining fluid (ELF) was calculated by simple dilution principles (Rennard et al. 1986).

Data analysis

Data are expressed as mean$\pm$S.E. For mean comparisons, Students two-tailed paired or unpaired t-test was used. The regression coefficient and one-way analysis of variance were also used for statistical analysis. Significance was accepted at $p < 0.05$.

RESULTS

The following results on P-III-P and TGF-β of BALF are all expressed as their concentrations in recovered BALF, except where otherwise stated, since almost all and/or main results did not changed when expressed by ELF from urea

and albumin.

P-III-P assay

BALF samples from 18 IPF patients showed significant amounts of P-III-P (0.37±0.12 U/ml, mean±s.e., range; 0.03 to 1.65 U/ml) (group B), as shown in Table 1, and P-III-P levels of BALF samples from the remaining 30 patients showed undetectable or less than 0.03 U/ml, as shown by group A in Table 1. Further, in five normal volunteers (all male, 32±6 years), P-III-P was not detected in BALF. Basophil percentage significantly correlated with P-III-P/ ELFurea (r=0.60, $p < 0.05$), whereas no other parameters did. Then, we examined differences in clinical and laboratory data between these two groups, group A and group B. As shown in Table 1, we could find no differences in sex, age, survival after BAL, dyspnea or smoking habit between groups A and B. However, concerning the duration from onset to BAL examination, there was a

TABLE 1. *Clinical and laboratory data in each group divided by P-III-P level in BALF*

	Group A (undertectable level)	Group B (0.03 U/ml or more)
Number of patients (sex)	30 (11F and 19M)	18 (5F and 13M)
Age (years) (range)	58.9±2.2 (38 to 82)	57.8±3.0 (40 to 81)
Duration of onset to BAL (mo.)	33.2±4.2	36.3±3.7*
Survival after BAL (mo.)	23.8±6.2	25.5±6.2
Grade of dyspnea[a]	2.0±0.2	2.5±0.2
Smoking habit (pack-year)	27.9±5.3	29.1±6.3
Serum LDH (IU/liter)	489±34	558±60
PaO$_2$ (mmHg)	75.8±2.0	73.0±2.2
A-aDO$_2$ (mmHg)	26.7±2.9	30.7±2.8
%VC	87.6±3.6	75.5±5.9
%DLco	55.0±4.4	48.0±3.8
DLco/V$_A$ (ml/min/mmHg/liter)	4.3±0.3	4.8±3.8
Pes max (cmH$_2$O)	−38.9±3.3	−45.1±2.4
BAL		
Recovery rate (%)	46.1±3.2	44.4±3.7
Macrophage (%)	87.1±2.0	59.0±7.4
Lymphocyte (%)	7.7±1.6	33.3±8.0**
Neutrophil (%)	2.9±0.7	4.4±1.6
Eosinophil (%)	2.3±0.6	3.0±0.8
Basophil (%)	0.07±0.03	0.3±0.1*

Mean±s.e. *$p < 0.05$; **$p < 0.01$, compared to group A. [a]Based on the classification of Hugh-Jones (1952).

significant difference between the two groups; i.e., larger values in group B compared to group A (36 months vs. 23 months). The onset of disease was defined by symptoms or abnormal chest radiogram findings. As seen in Table 1, there was no significant difference in pulmonary function data although group B showed worse values compared to group A. In the cell differentials of BALF, both lymphocyte and basophil percentages in group B were significantly larger than those in group A (Table 1). A dominant difference was observed in the lymphocyte population, i.e., a marked increase (33%) was seen in the lymphocyte from group B.

TGF-β assay

TGF-β data were obtained from the same BALF of IPF patients.

TGF-β was not detected in 10 patients, while BALF from the remaining 38

TABLE 2. *Clinical and laboratory data in each group divided by TGF-β level in BALF*

	Group I (undetectable level)	Group I (100 to 300 pg/ml)	Grtoup III (350 pg/ml or more)
Number of patients (sex)	10 (3F and 7M)	24 (10F and 14M)	14 (3F and 11M)
Age (years) (range)	59.2±4.3 (39 to 71)	59.9±2.6 (38 to 81)	62.5±2.4 (50 to 82)
Duration of onset to BAL (mo.)	24.0±10.2	28.8±4.9	26.4±8.0
Survival after BAL (mo.)	31.4±12.0	24.7±5.8	19.2±3.7*
Grade of dyspnea[a]	2.6±0.9	2.3±1.1	1.7±1.7*†
Smoking habit (pack-year)	19.0±21.1	20.7±5.1	36.1±8.5
Serum LDH (IU/liter)	543±64	538±50	446±21
PaO_2 (mmHg)	65.8±2.9	75.1±1.9*	80.6±24**
$A\text{-}aDO_2$ (mmHg)	43.6±2.7	27.9±2.4**	18.1±2.4**††
%VC	70.3±6.9	78.2±4.6	92.3±3.2**†
%DLco	43.0±4.1	49.8±4.7	61.4±3.8**
$DLco/V_A$ (ml/min/mmHg/liter)	4.3±0.3	4.7±0.3	4.3±0.3
Pes max (cmH_2O)	−40.3±3.8	−42.7±2.6	−38.9±4.2
BAL			
Recovery rate (%)	50.4±4.2	42.64±3.3	47.5±4.4
Macrophage (%)	77.5±7.3	74.3±4.9	79.7±7.8
Lymphocyte (%)	16.4±7.1	18.7±4.9	15.4±7.8
Neutrophil (%)	3.6±1.1	3.3±0.8	3.6±2.0
Eosinophil (%)	2.6±0.8	3.3±0.8	1.3±0.4††
Basophil (%)	0.3±0.1	0.1±0.08	0.1±0.1

Mean±s.e. *$p<0.05$; **$p<0.01$, compared to group I. †$p<0.05$; ††$p<0.01$, compared to group II. [a]Based on the classification of Hugh-Jones (1952).
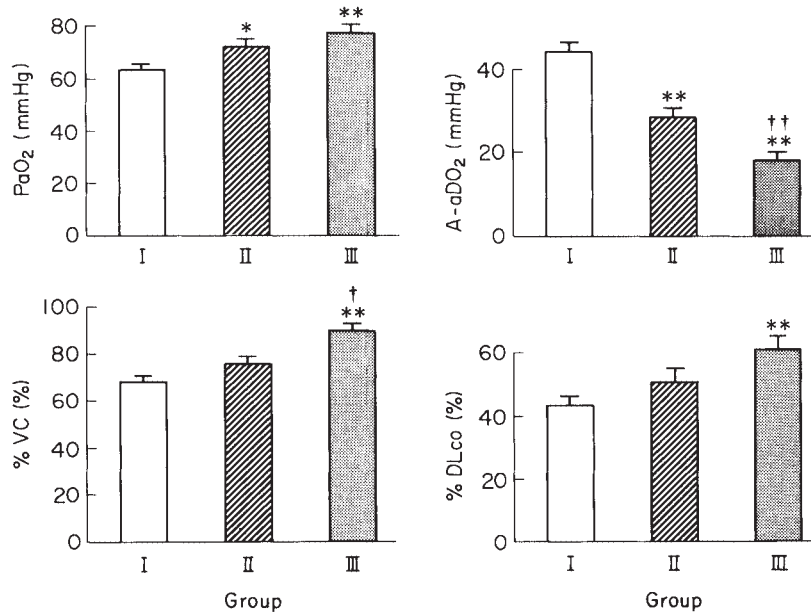
Fig. 1. Pulmonary function data in groups I, II and III divided by TGF-β level.
All pulmonary function data in group III showed better values than did group
I and/or II.   Mean±s.e. $*p<$ 0.05, $**p<0.01$, compared to group I. $^\dagger p<$
0.05, $^{\dagger\dagger}p<0.01$, compared to group II.

patients showed significant amounts of TGF-β, ranging 109 to 1,360 pg/ml and
the mean was 329±44 pg/ml.   TGF-β in BALF significantly correlated with %
VC values in each patient (r=0.37, $p<0.05$). Further, TGF-β/ELFalb
significantly correlated with lymphocyte percentage in BALF in each patient
(r=0.41, $p<0.05$).   However, it did not correlate with any other parameters.
According to the TGF-β level in BAL fluids, therefore, we divided our IPF
patients into three groups.   Group I was undetectable or 100 pg/ml or less ($n=$
10), group II from 100 to 300 pg/ml ($n=24$, range; 109 to 298 pg/ml, 220±12 pg/
ml) and group III 350 pg/ml or more ($n=14$, 350 to 1,360 pg/ml), as shown in
Table 2.   Meanwhile, in five normal healthy volunteers, the TGF-β in BALF was
undetectable.   As shown in Table 2, there were no significant differences among
the three groups in sex, age, duration of disease after BAL, or smoking habit.
Grade of dyspnea in group III was significantly slighter than that in groups I and
II (Table 2).   Eosinophil percentage in BALF in group III was significantly
smaller than group I (Table 2).   Fig. 1 shows the comparison of pulmonary func-
tion data between the three groups.   PaO₂, A-aDO₂, %VC and %DLco from
group III showed significantly better values than groups I and/or II (Fig. 1),
whereas survival after BAL in group III was significantly shorter than in group I
(Table 2).

TGF-β values as a whole did not correlated with P-III-P values in BALF in
the present study and further, there was no significant difference between groups
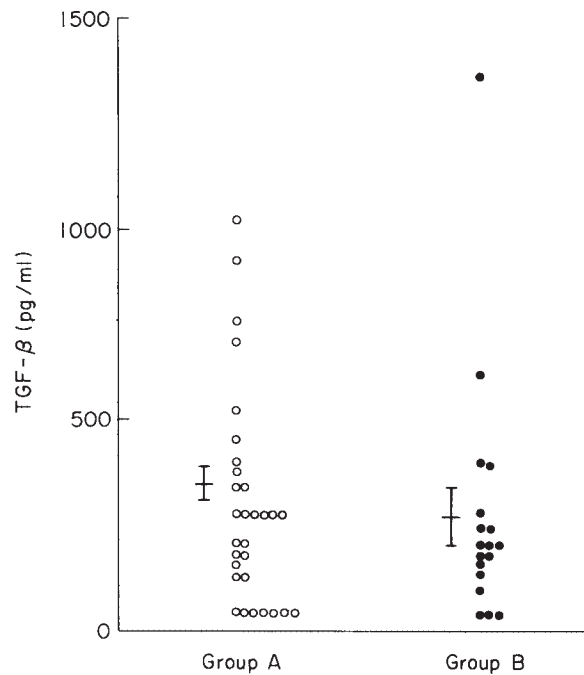A and B, divided by P-III-P level of BALF, as shown in Fig 2.

Fig. 2.   TGF-β levels of BALF in groups A and B divided by P-III-P level.   No
difference in TGF-β values was observed between groups A and B.   Bars
indicates mean$\pm$s.e.

Discussion

Our present study showed that elevated P-III-P levels of BALF are seen in
IPF patients, confirming previous studies (Cantin et al. 1988; Low et al. 1992;
Behr et al. 1993).   Low et al. (1992) have reported that the alveolar atrial oxygen
gradient during exercise significantly correlated with P-III-P concentration of
BALF but our study lacked this parameter because of the retrospective study.
Although it was not significant, our study also showed severe clinical and labora-
tory findings, as a whole, in patients with elevated P-III-P levels, compared to
those in patients with undetectable P-III-P levels.   Further, patients with ele-
vated P-III-P levels in BALF showed significantly longer durations of from onset
to BAL, compared to patients with undetectable levels.   However, survival after
BAL did not show a significant difference between patients with elevated and
non-elevated P-III-P levels, suggesting that the elevation of the P-III-P level in
the alveolar region does not directly contribute to the progression of disease.   In
the present study, a marked increase in lymphocytes of BALF was seen in IPF
patients with elevated P-III-P levels.   Previous studies (Rudd et al. 1981;
Watters et al. 1987) suggested that a high lymphocyte count reflects a good
response to therapy and predicts a longer survival.   An elevated P-III-P level
may reflect a repair or healing process of alveloar damage rather than progression
of disease.   In farmer's lung and in sarcoidosis, increased concentration of P-III-P
seems to predict the ongoing inflammation and not necessarily a fibrotic process
(Eklund et al. 1992; Larsson et al. 1992; Teschler et al. 1993).   Further, Behr et

al. (1993) found that the P-III-P level in BALF and fibroblast chemoattractant activity were not significantly correlated in IPF patients.

To our best knowledge, there have been no reports concerning the clinical significance of an elevated TGF-β level in BALF from IPF patients, as compared to the P-III-P level in BALF. Patients with an elevated TGF-β level in BALF showed significantly shorter survival after BAL, compared to patients with undetectable levels. In spite of the shorter survival, patients with elevated TGF-β levels showed slighter dyspnea and better pulmonary functions, suggesting the earlier stage or slighter aspects of the disease. These findings suggest that elevation of TGF-β in BALF is an indicator of the poor prognosis or rapid progression of the disease. Although the elevation of TGF-β is speculated to be followed by that of P-III-P patients, our present study revealed no significant correlations between P-III-P and TGF-β levels of BALF in IPF patients. The lack of a correlation between P-III-P and TGF-β in BALF from IPF patients may be explained by a feedback action of TGF-β since TGF-β is known to suppress T- and B-lymphocyte cell proliferation (Kehrl et al. 1986a, b) and also to have an inhibitory action to macrophages (Tsunawaki et al. 1988) which are supposed to play a role in the initiation and development of pulmonary fibrosis. Whatever the mechanism is, our present study indicates that the elevations of P-III-P and TGF-β in BALF reflect different aspects of clinical features in IPF patients. Namely, an elevated P-III-P level in BALF is accompanied by an increase in lymphocyte population and does not alter the survival (or is related to a rather longer duration of disease), while the TGF-β level in BALF reflects the alveolar inflammation at an earlier stage of disease than does P-III-P, which progresses to fibrosis and results in shorter survival in IPF patients.

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