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 radiomunoassay, we measured P-III-P and TGF-β in BALF from 48 IPF patients (16F and 32M, 59±2 years, mean±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 PaO₂, Aa-DO₂, %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
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 peptides 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 bronchoalveolar 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**

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 ± 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 dilution 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 (Olympus 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, lymphocytes, neutrophils, eosinophils, and basophils by means of Wright-Giemsa and nonspecific esterase stain. Cells and debris were removed by centrifugation at 400×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 peptides >10,000 daltons), and type III procollagen aminoterminal peptide-related antigens were assayed using a radioimmunoassay kit (Behringwerke AG, Marburg, Germany), according to methods described previously (Low et al. 1992; Behr et al. 1993), measuring procollagen III N-terminal peptide 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, including all active TGF-β. A TGF-β-level of 100 pg/ml or less is not detected by this assay. Lavage fluid albumin was determined by laser nephelometric 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±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>
<thead>
<tr>
<th>Table 1. Clinical and laboratory data in each group divided by P-III-P level in BALF</th>
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<tbody>
<tr>
<td>Group A (undetectable level)</td>
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<td>-------------------------------</td>
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<tr>
<td>Number of patients (sex)</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Duration of onset to BAL (mo.)</td>
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<tr>
<td>Survival after BAL (mo.)</td>
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<tr>
<td>Grade of dyspnea*</td>
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<tr>
<td>Smoking habit (pack-year)</td>
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<tr>
<td>Serum LDH (IU/liter)</td>
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<tr>
<td>PaO₂ (mmHg)</td>
</tr>
<tr>
<td>A-aDO₂ (mmHg)</td>
</tr>
<tr>
<td>%VC</td>
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<tr>
<td>%DLco</td>
</tr>
<tr>
<td>DLco/Vₐ (ml/min/mmHg/liter)</td>
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<td>Pes max (cmH₂O)</td>
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**Notes:** Mean ± s.e. *p < 0.05; **p < 0.01, compared to group 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**

<table>
<thead>
<tr>
<th></th>
<th>Group I (undetectable level)</th>
<th>Group I (100 to 300 pg/ml)</th>
<th>Group III (350 pg/ml or more)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (sex)</td>
<td>10 (3F and 7M)</td>
<td>24 (10F and 14M)</td>
<td>14 (3F and 11M)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.2 ± 4.3 (range 39 to 71)</td>
<td>59.9 ± 2.6 (range 38 to 81)</td>
<td>62.5 ± 2.4 (range 50 to 82)</td>
</tr>
<tr>
<td>Duration of onset to BAL (mo.)</td>
<td>24.0 ± 10.2</td>
<td>28.8 ± 4.9</td>
<td>26.4 ± 8.0</td>
</tr>
<tr>
<td>Survival after BAL (mo.)</td>
<td>31.4 ± 12.0</td>
<td>24.7 ± 5.8</td>
<td>19.2 ± 3.7</td>
</tr>
<tr>
<td>Grade of dyspnea*</td>
<td>2.6 ± 0.9</td>
<td>2.3 ± 1.1</td>
<td>1.7 ± 1.7 **</td>
</tr>
<tr>
<td>Smoking habit (pack-year)</td>
<td>19.0 ± 21.1</td>
<td>20.7 ± 5.1</td>
<td>36.1 ± 8.5</td>
</tr>
<tr>
<td>Serum LDH (IU/liter)</td>
<td>543 ± 64</td>
<td>538 ± 50</td>
<td>446 ± 21</td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>65.8 ± 2.9</td>
<td>75.1 ± 1.9*</td>
<td>80.6 ± 24**</td>
</tr>
<tr>
<td>A-aDO₂ (mmHg)</td>
<td>43.6 ± 2.7</td>
<td>27.9 ± 2.4**</td>
<td>18.1 ± 2.4***</td>
</tr>
<tr>
<td>%VC</td>
<td>70.3 ± 6.9</td>
<td>78.2 ± 4.6</td>
<td>92.3 ± 3.2** *</td>
</tr>
<tr>
<td>%Dlco</td>
<td>43.0 ± 4.1</td>
<td>49.8 ± 4.7</td>
<td>61.4 ± 3.8* *</td>
</tr>
<tr>
<td>DLCO/Vₐ (ml/min/mmHg/liter)</td>
<td>4.3 ± 0.3</td>
<td>4.7 ± 0.3</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Pes max (cmH₂O)</td>
<td>-40.3 ± 3.8</td>
<td>-42.7 ± 2.6</td>
<td>-38.9 ± 4.2</td>
</tr>
<tr>
<td>BAL</td>
<td></td>
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<tr>
<td>Recovery rate (%)</td>
<td>50.4 ± 4.2</td>
<td>42.64 ± 3.3</td>
<td>47.5 ± 4.4</td>
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<tr>
<td>Macrophage (%)</td>
<td>77.5 ± 7.3</td>
<td>74.3 ± 4.9</td>
<td>79.7 ± 7.8</td>
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<tr>
<td>Lymphocyte (%)</td>
<td>16.4 ± 7.1</td>
<td>18.7 ± 4.9</td>
<td>15.4 ± 7.8</td>
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<tr>
<td>Neutrophil (%)</td>
<td>3.6 ± 1.1</td>
<td>3.3 ± 0.8</td>
<td>3.6 ± 2.0</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>2.6 ± 0.8</td>
<td>3.3 ± 0.8</td>
<td>1.3 ± 0.4 *</td>
</tr>
<tr>
<td>Basophil (%)</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.08</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Mean ± s.e. *p < 0.05; **p < 0.01, compared to group I. *p < 0.05; **p < 0.01, compared to group II. \*Based on the classification of Hugh-Jones (1952).
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/ELFαβ 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 lighter 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 function data between the three groups. PaO₂, A-aO₂, %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 ± 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 laboratory 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 elevated 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 alveolar 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 chemotactic
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.

Acknowledgment

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References


