Proliferative Characteristics of Fibroblast Lines Derived from Open Lung Biopsy Specimens of Patients with IPF (UIP)*

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We compared the doubling time of fibroblasts derived from idiopathic pulmonary fibrosis (usual interstitial pneumonia) (IPF [UIP]) lung tissues and control fibroblasts, cultured in usual growth medium, and examined the response of these fibroblasts to platelet-derived growth factor (PDGF) and prostaglandin E\(_2\) (PGE\(_2\)). Ten fibroblast lines from open lung biopsy specimens of patients with IPF (UIP) and ten control fibroblast lines from surgically resected lung tissues of patients with limited lung diseases were established. The average doubling time of fibroblast lines was 32.0±6.0 h (mean±SD) in UIP and 33.2±10.4 h in controls, showing no difference between the two groups. To examine the responses of fibroblasts to PDGF and PGE\(_2\), the differences between fibroblasts derived from fibrotic tissues with different intensity of fibrosis, lung specimens from five patients with IPF were subdivided into two groups, higher-intensity fibrotic lesions (H) and lower-intensity fibrotic lesions (L). The fibroblast lines were established separately. \(^{3}\)H-thymidine uptake with or without PDGF or PGE\(_2\) was examined. Results were expressed as the index of thymidine incorporation into the fibroblasts. There were no differences in the doubling times and the responses to PDGF and PGE\(_2\) between H and L. There were no differences between control and H regarding their response to PDGF. In response to PGE\(_2\), the growth inhibition for H was significantly decreased compared with the control (p<0.05). There was no difference in growth inhibition between H and L. The finding that PGE\(_2\) inhibits fibroblast proliferation less in UIP lung tissue suggests that fibroblasts from UIP were functionally altered cells or, to some extent, out of normal regulation. These results suggest an abnormal proliferation of fibroblasts observed in IPF (UIP).

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The most common pathologic form of idiopathic pulmonary fibrosis (IPF), usual interstitial pneumonia (UIP), is characterized by patchily distributed fibrotic areas in normal parenchyma.\(^1\)\(^2\) Excessive accumulation and qualitative change of collagen and proliferation of fibroblasts are shown in a fibrotic lesion.\(^3\)\(^4\) Fibroblast proliferation is generally evaluated as an excessive repair process following alveolitis.\(^3\)\(^5\)\(^6\) Unknown stimuli cause the influx of immune and inflammatory cells into alveoli. These cells, especially alveolar macrophages, synthesize many kinds of cytokines and growth factors. The proliferation of fibroblasts is promoted by these cytokines. Among cytokines and growth factors, IGF-I and fibronectin secreted by alveolar macrophages seem to play an important role in fibrotic processes.\(^7\)\(^8\)

However, an alveolitis with increased immune and inflammatory cells was not frequently observed in open lung biopsy specimens derived from our patients with UIP. Fibrotic lesions are intermingled with apparently normal alveoli. In most fibrotic lesions, an accumulation of fibroblasts and extracellular matrix was observed, but the accumulation of immune and inflammatory cells in alveoli was not commonly found.\(^2\)\(^9\) From these findings, it appears that an excessive proliferation of fibroblasts is not promoted by continuous stimulation of growth factors secreted by these cells. The proliferation of fibroblasts may not require the continuous presence of growth factors, but fibroblasts of IPF (UIP) themselves may be functionally altered cells, as can be seen in pulmonary fibrosis. Growth characteristics of fibroblasts derived from fibrotic lung tissues have been investigated.\(^10\)\(^13\) The presence of fast-growing fibroblasts in fibrotic lungs has been reported,\(^11\)\(^12\) but it is not conclusive whether fibroblasts in patients with IPF (UIP) are different from others in terms of proliferative characteristics. To evaluate the possibility that fibroblasts in UIP are functionally altered cells, proliferative characteristics of lung fibroblast lines obtained from UIP lungs cultured with usual growth medium, platelet-derived growth factor (PDGF) and prostaglandin E\(_2\) (PGE\(_2\)) were investigated. PDGF is a well-known growth factor for fibroblasts and many other kinds of cells, and may act as an autocrine growth factor for fibroblasts.\(^14\)\(^15\) PGE\(_2\) is an inhibitory substance for fibroblast proliferation. Alveolar macrophages are known to release PGE\(_2\) and are a factor in stimulating PGE\(_2\) production in fibroblasts.\(^16\)\(^17\)

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MATERIALS AND METHODS

Fibroblast lines were established from open lung biopsy specimens obtained from ten patients with IPF. The patients, eight men and two women, had a mean age of 62.2 ± 5.8 years. None had a history of dust exposure or findings suggesting fibrotic lung diseases such as collagen vascular disease, hypersensitivity pneumonitis, or sarcoidosis. All patients were histologically diagnosed as having UIP and clinically were in a stable state at the time of biopsy. None was given any immunosuppressive drug or corticosteroids before open lung biopsy. Eight patients had a smoking history, but at the time of the biopsy, all had quit smoking.

The fibroblast reactivity to PDGF and PGE_2 was investigated in five cases. According to both macroscopic appearance and microscopic examination, lung specimens from each patient were subdivided into two groups: specimens obtained from less fibrotic lesions (L) and specimens obtained from higher-intensity fibrotic lesions (H). Group L showed histologic features of less fibrotic and mild cellular infiltration. Group H showed a yellowish color and loss of elasticity and histologic features of highly fibrotic areas. Fibroblasts from both specimens were cultured separately. No specimen was taken from honeycombing, end-stage fibrotic lesions.

Control fibroblast lines were established from macroscopically normal areas of surgical lung specimens of ten patients undergoing resective surgery for lung cancer (n = 8), arteriovenous malformation (n = 1), and empyema (n = 1). The patients, eight men and two women, had a mean age of 57.0 ± 4.5 years. The reactivity of fibroblasts to PDGF and PGE_2 was investigated in five cases.

Open lung biopsy was performed to make a pathologic diagnosis. All patients were informed of our study purpose and lung specimens were used for study with patients' permission.

Culture of Fibroblast Cell Line

Sterile lung specimens were washed with phosphate-buffered saline solution, and pleural tissue and bronchi were removed after resection. Specimens were chopped in pieces <1 cu mm and washed three times with Dulbecco's minimal essential medium (DMEM; Nissui, Tokyo). The minced pieces were centrifuged (1,000 rpm × 1 min) and resuspended in regular growth medium (RGM). As RGM, DMEM supplemented with 10 percent fetal bovine serum (FBS) (FBS, MAB, Maryland) and 1 percent nonessential amino acid solution (Gibco, NY), kanamycin 60 mg/L, and penicillin G 50 U/ml (Meiji, Tokyo) was used. Suspensions of specimens were added to culture flasks (25 sq cm, Corning), and cultured at 37°C in humidified air containing 5 percent CO_2. The medium was changed twice weekly. When culture bottles were coated with a layer of fibroblasts at near confluence, usually three to five weeks later, cells were trypsinized for 2 min, resuspended in RGM, and further cultured. Subsequently, when cells reached confluence, they were split 1:2. On average this was done once a week. Four to seven passages of cells were used for assay. Cultured cells were microscopically spindle shaped and had morphologic features of fibroblasts. To prevent mycoplasma infection, fibroblasts were incubated with MC-210 (Dainihon Seiyaku, Osaka) for at least one week, and checked (with Hoechst Dye 33258, Wako Jyunyaku, Tokyo) before the assay. The karyotype examination was performed with cells the shape and size of which had changed during culture or the doubling time of which was much different from that of other cells.

In Vitro Proliferative Characteristics of Fibroblasts Cultured with RGM

Fibroblasts from confluent bottles were trypsinized and resuspended in RGM (2 × 10^5 cells per milliliter); 500 µl of suspended cells was added into 24 well plates (Corning, NY). Cultures were incubated for 24 to 144 h at 37°C in humidified air containing 5 percent CO_2. Cells of four wells were trypsinized every 24 h and a differential cell count was performed. Numbers were plotted on a semilog graph, and the doubling time of fibroblast lines was calculated from the linear portion of the time-growth curve by means of least-squares linear regression. Tests on the regression parameters were performed. When p values were more than 0.05, measurements of doubling times were restudied.

As one of the morphologic measurements, the mean size of each fibroblast was calculated from the number of cells at confluence.

The Response of Fibroblasts to PDGF or PGE_2

To study the response of fibroblasts to exogenous PDGF or PGE_2, fibroblasts from confluent culture bottles were trypsinized and resuspended at 10^5 cells per milliliter of RGM, and 100 µl of suspension was added into flat-bottomed microtiter plates (96-well multiwell plate, Corning, NY). Cells were incubated until confluence, which usually took 48 h. Thereafter, to render the cells in a quiescent state, RGM was removed and each well was washed twice with DMEM. Two hundred microliters of DMEM with 0.2 percent FBS was added to each well, and cells were cultured for another 48 h, after which the medium was changed into assay medium. To study the promotive effect of PDGF on fibroblast proliferation, DMEM with 1 percent FCS was used. To study the inhibitory effect of PGE_2, DMEM containing 10 percent FCS was used. Various concentrations of PDGF and PGE_2 were added to the test medium. The highest concentration of PDGF was 1 U/ml and of PGE_2, it was 1 µg/ml. After another 24-h incubation, cultures were pulsed with 1.85 kBq of 3H-thymidine for the final 12 h. Trypsinized cells were harvested, and the 3H-thymidine uptake was measured by a scintillation counter. Results were expressed as stimulation index, which is the ratio of 3H-thymidine uptake between PDGF and PGE_2 added groups and controls.

Analysis of Results

Student's t test was used to compare the doubling times or mean areas of fibroblasts between control and IPF, and responses of fibroblasts derived from H and L to PDGF and PGE_2. The paired t test was used to compare the responses of fibroblasts to PDGF and PGE_2 between H and L.

RESULTS

Doubling Time of Fibroblasts Derived from Lung Specimens of Patients with IPF

The average doubling time of fibroblasts cultured in RGM was 32.0 ± 5.6 h (mean ± SD) in patients with IPF and 33.2 ± 10.4 h in controls. There was no statistically significant difference (Fig 1). The doubling time of 3H-thymidine uptake per cell of H and L fibroblast lines were investigated separately, but there was no difference between L and H. Areas of fibroblasts were 1.04 ± 0.60 × 10^-3 sq mm in patients with IPF and 1.25 ± 0.92 × 10^-3 sq mm in controls (Fig 2). There was no statistically significant difference.

Effect of PDGF and PGE_2 on the Proliferation of Fibroblasts

The addition of PDGF induced a dose-dependent promotion of proliferation of fibroblasts from both IPF and controls. The response of fibroblasts to 1 U/ml PDGF was 1.89 ± 0.78 in H, 1.97 ± 1.19 in L, and 2.29 ± 0.55 in control. There was no statistically significant difference among these groups (Fig 3). The
addition of PGE$_2$ induced a dose-dependent inhibition of the proliferation of the control fibroblasts, but the inhibition of the proliferation of fibroblasts derived from patients with IPF did not behave in a dose-dependent manner (Fig 5). The response to 1 µg/ml PGE$_2$ was 0.88±0.24 in H, 0.69±0.49 in L, and 0.44±0.33 in control. There was no difference between H and L. There was a statistically significant difference between H and C (p<0.05) (Fig 4).
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![Graph](image_url)

**Figure 5.** Dose-dependent inhibition of thymidine incorporation by PGE₂. The crosses represent H, the circles L, and the squares control fibroblasts. Each point and bar represents mean ± SE. Control fibroblast lines showed dose-dependent inhibition by PGE₂. In contrast, dose dependency of inhibition was not clear in H and L fibroblast lines.

**Discussion**

It is generally accepted that pulmonary fibrosis is the end result of a repairing process following alveolitis. Excessive collagen deposition and fibroblast accumulation in parenchyma are histologic features of pulmonary fibrosis. Fibroblasts are thought to be a major cell type responsible for collagen synthesis in the lung. An increased B-cell activity and presence of circulating and BALF immune complexes are reported to be found in patients with IPF. These immune complexes stimulate alveolar macrophages to release neutrophil chemotactic factors, which cause accumulation and activation of neutrophils within the lungs. Alveolar macrophages also secrete growth factors and cytokines, which promote proliferation of fibroblasts and synthesis of collagen by fibroblasts. The interaction between alveolar macrophages and fibroblasts has been widely investigated, but little is known about fibroblasts in the lungs of patients with IPF. Recently, Jordan et al., Raghu et al., and Mio et al. have reported faster growth characteristics of established fibroblast lines from lung tissue of patients with IPF. However, it is not conclusive whether fibroblasts in patients with IPF (UIP) have different growth characteristics than control fibroblasts.

In this study cultured fibroblast lines were investigated. Characteristics of fibroblasts in *vitro* are different from those of *in vitro* cultured cells. Culture methods and conditions may affect some characteristics of cells. A selection of subpopulations, which adapt to the culture condition, may occur. On the other hand, some characteristics of fibroblast cell lines derived from different tissues remain unchanged during culture *in vitro* while others change. For example, the growth rate of a fibroblast line decreased over 25 generations, but the collagen synthesis rate was constant through the sixth to 30th generations. Korn reported that fibroblasts once stimulated by mononuclear cell-conditioned medium showed elevated PGE₂ synthesis over 19 generations. Although a comparative study using cultured cells derived from different origins is difficult to interpret, it is valuable to investigate characteristics of fibroblasts derived from IPF and control lung tissues. The number of passages is one of the factors influencing cell characteristics. In our study, the number of cells seeded initially was unknown, leaving the accurate number of cell divisions unclear. However, the initial culture period from seeding to the first subculture was similar between IPF and control, and both groups had a similar doubling time, suggesting that the substantive number of cell divisions may be about the same.

In contrast to previous reports about cultured fibroblasts derived from fibrotic lung tissue, we did not detect faster proliferation characteristics of fibroblasts derived from patients with IPF. Different results may be due to different characteristics of subjects from whom the lung specimens were obtained, including the diagnosis of disease, age, and clinical status of the subjects. The pathologic patterns of our cases revealed UIP and the clinical diagnoses were IPF.

In contrast to our study, subjects of previously reported studies included patients with diseases other than IPF. The mean age of the patients in this study was 62.2 ± 5.8 years, which is higher than the mean ages of subjects from both Jordan and Raghu et al. However, the mean age of our patients is more compatible with the patient characteristics of IPF than those of previous reports. Our patients were clinically in a stable state, and the disease was progressing slowly. Open lung biopsy specimens were obtained from regions that were expected to represent the disease, usually one from the upper lobe and another from the upper area of the lower lobe. As end-stage fibrotic lesions were evaluated as a common terminal lesion of fibrotic diseases and did not have features to make a diagnosis of UIP, they were usually avoided. Remarkable alveolitis was not frequently found in the specimens used for this study. If only the presence of a faster proliferation rate of fibroblasts is essential for the pathogenesis of IPF, even in not seriously ill or stable patients, there may be a difference in fibroblast proliferative characteristics between IPF and controls. As many factors affect the characteristics of fibroblast proliferation in *vitro*, the possibility still remains that some factors affecting the fibroblast proliferation concealed the difference in IPF and control fibroblast proliferations in our study. Because fibroblasts are a heterogeneous cell group, selection of some peculiar subpopulations of fibroblasts, which fit our culture conditions or which have faster growing characteris-
tics, may occur during in vitro culture.

The report of Raghu et al. showed that the proliferative characteristics of fibroblasts derived from patients with early fibrosis with alveolitis were different from those of patients with dense fibrosis without alveolitis. To examine whether this result is applicable to UIP, we examined the proliferative characteristics of fibroblasts derived from fibrotic tissues with different intensity of fibrosis. There were no differences in doubling times and response to PDGF and PGE_2.

Recently, findings from open lung biopsy specimens revealed that a pathologic feature of UIP is a patchy distribution of fibrotic lesions. In our series of IPF cases, an extensive accumulation of immune and inflammatory cells in alveoli was not observed. This finding is consistent with current reports on IPF. Thus, fibroblasts are not stimulated continuously by cytokines from coexisting immune inflammatory cells. One possible explanation is that immune and inflammatory cell accumulation transiently occurs in turn in the alveoli, and once stimulated by cytokines, fibroblasts proliferate and continue to synthesize extracellular matrix after the immune and inflammatory cells have disappeared. Another possible explanation is that fibroblasts of patients with IPF are essentially out of normal regulation, including the autocrine one, and make fibrotic lesions without paracrine stimulation.

From the finding that the proliferation of fibroblasts from IPF is less inhibited by PGE_2, there is a possibility that some functional alterations occur in fibroblasts of IPF. It is not sure whether this alteration is an essential change related to the pathogenesis of IPF or a result of secondary clonal selection in inflammatory tissues. In inflammatory tissues, PGE_2 may be synthesized more by inflammatory cells than in normal tissues, and clones less sensitive to PGE_2 may be selected. If this means that the fibroblasts of IPF lung tissues are inhibited with difficulty by normal regulation systems, such fibroblasts would be expected to proliferate more rapidly than normal fibroblasts. As a result of this situation, fibroblast accumulation in alveoli occurs without findings of immune and inflammatory cell infiltration. Additional experiments will be required to evaluate this possibility and to determine how the results obtained in vitro using fibroblast cultures reflect the pathophysiologic processes occurring in vivo.

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REFERENCES
13 Mio T, Nagai S, Iizumi T. Proliferative characteristics of fibroblast lines derived from open lung biopsy specimens of patients with IPF (UIP) [abstract]. Am Rev Respir Dis 1990; 141(suppl): A495
18 Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER. Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis 1982; 126:332-37


