

FIBROBLAST migration, proliferation, extracellular matrix protein synthesis and degradation, all of which play important roles in inflammation, are themselves induced by various growth factors and cytokines. Less is known about the interaction of these substances on lung fibroblast function in pulmonary fibrosis.

The goal of this study was to investigate the effects of PDGF alone and in combination with IL-1 β and TNF- α on the production of human lung fibroblast matrix metalloproteinases, proliferation, and the chemotactic response. The assay for MMPs activity against FITC labeled type I and IV collagen was based on the specificity of the enzyme cleavage of collagen. Caseinolytic and gelatinolytic activities of secreted proteinases were analyzed by zymography. Fibronectin in conditioned media was measured using human lung fibronectin enzyme immunoassay. Cell proliferation was measured by ³H-Thymidine incorporation assay. Cell culture supernatants were tested for PGE2 content by ELISA. Chemotactic activity was measured using the modified Boyden chamber.

Matrix metalloproteinase assay indicated that IL-1 β , TNF- α and PDGF induced interstitial collagenase (MMP-1) production. MMP assay also indicated that IL-1 β and TNF- α had inhibitory effects on MMP-2,9 (gelatinase A,B) production. Casein zymography confirmed that IL-1 β stimulated stromelysin (matrix metalloproteinase 3; MMP-3) and gelatin zymography demonstrated that TNF- α induced MMP-9 production in human lung fibroblast, whereas PDGF alone did not. PDGF in combination with IL-1 β and TNF- α induced MMP-3 and MMP-9 activity, as demonstrated by zymography. PDGF stimulated lung fibroblast proliferation in a concentration-dependent manner, whereas IL-1 β and TNF- α alone had no effect. In contrast, the proliferation of human lung fibroblasts by PDGF was inhibited in the presence of IL-1 β and TNF- α , and this inhibition was not a consequence of any elevation of PGE2. PDGF stimulated fibroblast chemotaxis in a concentration-dependent manner, and this stimulation was augmented by combining PDGF with IL-1 β and TNF- α .

These findings suggested that PDGF differentially regulated MMPs production in combination with cytokines, and further that MMP assay and zymography had differential sensitivity for detecting MMPs. The presence of cytokines with PDGF appears to modulate the proliferation and chemotaxis of human lung fibroblasts.

Key words: Metalloproteinase, PDGF, IL-1 β , TNF- α , Lung fibroblast, Human

Differential regulation of metalloproteinase production, proliferation and chemotaxis of human lung fibroblasts by PDGF, Interleukin-1 β and TNF- α

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Introduction

Pulmonary fibrosis and other fibrotic lung diseases are characterized by diffuse interstitial inflammation and exaggerated collagen accumulation. This in turn leads to destruction of alveolar structures and remodeling.¹

Fibroblast migration, proliferation, extracellular matrix protein synthesis and degradation, all of which play important roles in inflammation, are themselves induced by various cytokines, such as IL-1 β and TNF- α , and growth factors, such as TGF and PDGF, as well as by the combined use of these agents.²⁻⁸ Less is known about the interaction of

these substances on lung fibroblast function in pulmonary fibrosis.

Recent studies have shown that some extracellular matrix components, including collagen, fibronectin and cytokines (e.g. PDGF and TGF) have an effect on fibroblast chemotactic responses.^{3,8} The role of PDGF in combination with cytokines in fibroblast collagen production or degradation is unclear. By using a Boyden chamber to assay chemotaxis, the migration of fibroblasts across collagen-coated filters was shown to be a response to proteinase products of fibroblasts as well as to the products of malignant cells.^{9,10} Stromelysin (matrix metalloproteinase-3, MMP-3) and the type IV procollagenase (matrix metalloproteinase-9, gelatinase B) are known to degrade a wide range of extracellular matrix molecules, including proteoglycan, gelatin, type IV and V collagen, and fibronectin.

Recent evidence suggests that matrix metalloproteinases (MMPs), including MMP-2 (gelatinase A), MMP-3 and MMP-9 play major roles in the degradation of the extracellular matrix in tumor invasion.¹⁰⁻¹³ Interestingly, cytokines when applied in combination can stimulate the release of these proteinases from fibroblasts, which degrade to a number of structural proteins of the extracellular matrix, in addition to interstitial collagen.^{14,15}

The present study investigated the effect of PDGF alone and in combination with IL-1 β and TNF- α on MMPs production, proliferation and the chemotactic response of human lung fibroblast *in vitro*.

Materials and methods

Materials

Recombinant human PDGF-BB was purchased from Genzyme (MA, USA). IL-1 β was purchased from Otsuka Pharmaceutical Co (Tokushima, Japan) and TNF- α were from Hayashibara Biochemical Laboratories Inc (Okayama, Japan).

Cell culture

Human lung fibroblasts CCL-153 obtained from the American Type Culture Collection (ATCC) were cultured in a cell culture flask, in modified Ham's F12 (SIGMA) medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO₂ and 95% air and plated at a density of 1.0 \times 10⁵ cell in a 24-well plate until confluence.

Metalloproteinases (MMP) assay

The assay for MMPs activity against FITC labeled type I and IV collagen was based on the specificity of the enzyme cleavage of collagen. One unit of collagenase activity was defined as the amount of enzyme

degrading 1 μ g of collagen per minute at 37°C. The specificity of the assay was confirmed by identification of collagenase reaction products by immunofluorescence electrophotometry, as described previously.¹⁶

Zymographic analysis of metalloproteinases

Caseinolytic and gelatinolytic activities of secreted proteinases were analyzed by zymography according to the methods of Chin *et al.*¹⁴ with some modifications. SDS-polyacrylamid gels contain 1.25 mg/ml casein or 1 mg/ml gelatin. Samples to be tested were mixed with an equal volume of the concentrated SDS sample buffer and then electrophoresed on casein or gelatin-containing gels. After electrophoresis of the gels, the separated proteinases were renatured by gentle shaking of the gel in 2.5% Triton X-100 containing 50 mM Tris-HCl and 0.1 M NaCl at room temperature for 1 h to remove SDS, followed by incubation in 250 ml of 50 mM Tris-HCl containing 10 mM CaCl₂ and 0.02 M NaN₃ at 37°C for approximately 18 h. The resultant gels were stained with Coomassie brilliant blue.

Fibronectin assay

Fibroblasts were incubated in serum-free F12 medium or with growth factor and cytokines for 24 h. Fibronectin in conditioned media was measured using human lung fibronectin enzyme immunoassay according to the manufacturer's instructions, purchased from Paesel (Frankfurt, Germany).

³H-Thymidine incorporation assay

Cells were incubated in serum-free F12 medium, or with growth factor and cytokines for 24 h and then pulsed with ³H-Thymidine (0.04 μ Ci/ml). Incorporation of ³H-Thymidine was measured 24 h later by detaching cells with trypsin solution and harvesting the cells on filters using a cell harvester. Radioactivity on the filters was determined using scintillation counting.

Prostaglandin E₂ assay

Cell culture supernatants were tested for PGE₂ content by ELISA kits according to the manufacturer's instructions (purchased from Neogen Corporation).

Chemotaxis assay

Chemotactic activity was measured using the modified Boyden chamber as described previously.⁹ The chemotactic agent was diluted in Ham's F12 medium and placed in the lower well chamber. The lower well was then covered with a collagen-coated polycarbo-

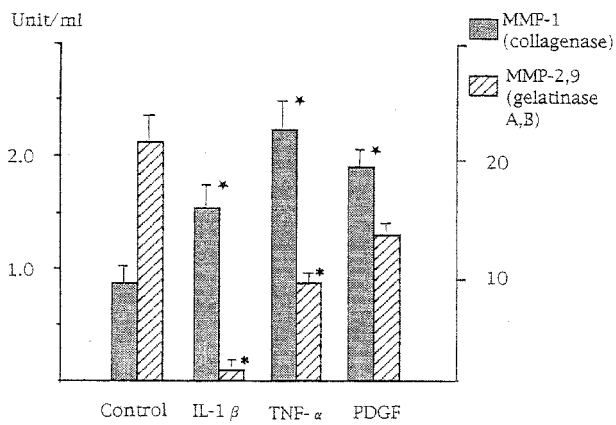


FIG. 1. Matrix metalloproteinases activity in human lung fibroblasts stimulated for 24 h with IL-1 β (10 ng/ml), TNF- α (10 ng/ml) and PDGF (5 ng/ml). Bar graph representing the activity of MMP-1, light-shaded column and MMP-2,9, oblique-lined column.

nate filter. Human lung fibroblasts (5×10^4 cells) were added to the upper well of the chamber containing Ham's F12 medium. After 8 h incubation at 37°C in 95% air and 5% CO₂, the filters were removed and cells were fixed and stained using Diff-Quik stain. The chemotactic response was analyzed by counting the number of cells/microscopic field on the lower surface of the filter. The stimulation index (SI) was expressed as follows: SI = number of migrating cells with PDGF or cytokines/number of migrating cells without PDGF.

Data analysis

A comparison of proliferation and chemotaxis within experiments was performed using Student's *t*-test. Statistical significance was accepted at $p < 0.05$.

Results

Matrix metalloproteinase production

IL-1 β , TNF- α and PDGF caused a significant increase in matrix metalloproteinase activity (MMP-1; Fig. 1). In contrast, MMP-2,9 activity was significantly reduced by IL-1 β , TNF- α and PDGF (Fig. 1). TNF- α alone and PDGF in combination with IL-1 β and TNF- α stimulated MMP-9 production in lung fibroblasts, whereas neither PDGF alone nor IL-1 β alone did so (Fig. 2a). Various combinations of PDGF with IL-1 β or TNF- α also induced MMP-3 activity (Fig. 2b).

Fibronectin production

IL-1 β and TNF- α , either alone or in combination, both failed to significantly alter basal fibronectin

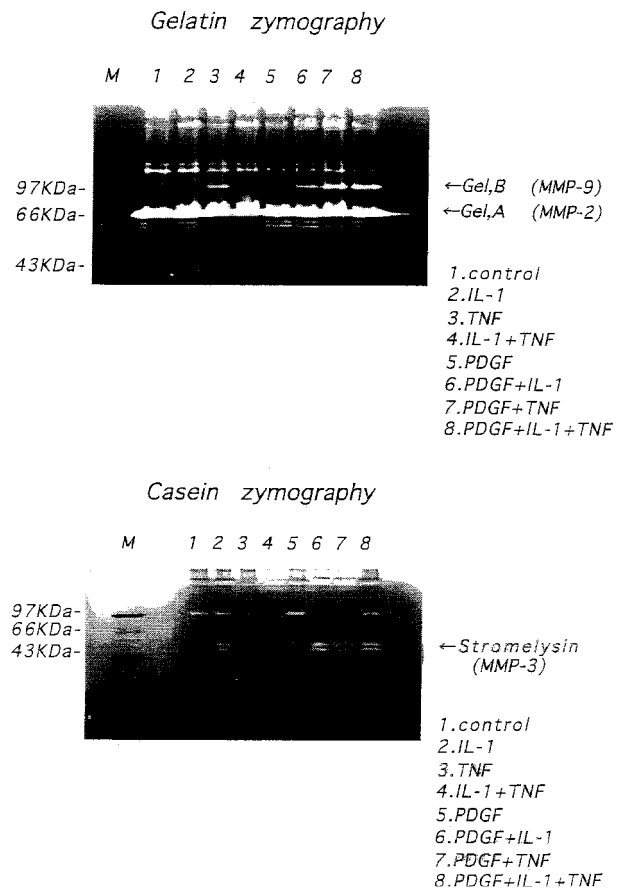


FIG. 2. Zymographic analysis of metalloproteinase expression by fibroblasts treated with PDGF, IL-1 β and TNF- α . (a) Supernatants from untreated fibroblasts (lane 1) or fibroblasts treated (lanes 2–8) for 24 h with PDGF (5 ng/ml), IL-1 β (10 ng/ml), or TNF- α (10 ng/ml) or in combination were analyzed by gelatin zymography for detection of MMP-2 and MMP-9. (b) Supernatants from untreated fibroblasts (lane 1) or fibroblasts treated (lanes 2–8) for 24 h with PDGF (5 ng/ml), IL-1 β (10 ng/ml), or TNF- α (10 ng/ml) or in combination were analyzed by casein zymography for detection of MMP-3.

production in human fibroblasts in culture in the absence of PDGF (Fig. 3, $p > 0.05$). In contrast, PDGF alone (10 ng/ml) significantly stimulated fibronectin production (control 2.2 ± 0.4 μ g/ml vs PDGF 10 ng/ml 4.3 ± 0.7 μ g/ml, $p < 0.05$; Fig. 3). However, both IL-1 β and TNF- α , as well as the combination of these cytokines, significantly inhibited the increase in fibronectin production induced by PDGF (10 ng/ml) ($p < 0.05$; Fig. 3).

Fibroblast proliferation

PDGF stimulated proliferation as assessed by 3H-Thymidine incorporation in fibroblasts (control 2050 ± 298 dpm, PDGF 1 ng/ml 3490 ± 350 dpm, PDGF 10 ng/ml 8728 ± 294 dpm, $p < 0.05$). Either IL-1 β or TNF- α alone failed to significantly alter basal proliferation in human fibroblasts cultured with PDGF (data not shown). The proliferation of human lung fibro-

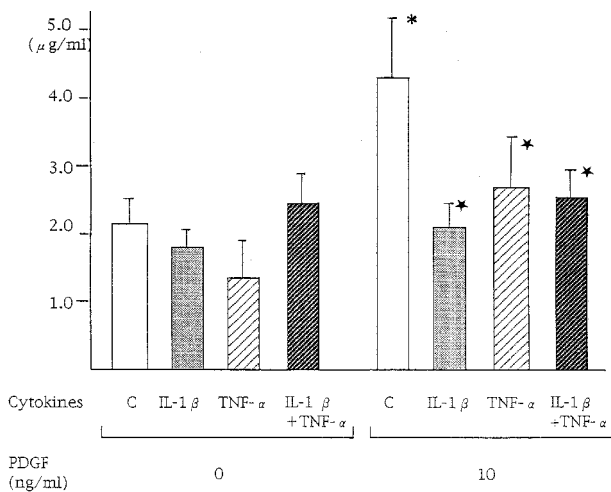


FIG. 3. Fibronectin production in human lung fibroblasts stimulated for 24 h with PDGF (5 ng/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml). Bar graph representing the fibronectin production stimulated by IL-1β, light-shaded column, TNF-α, oblique-lined column and in combination, dark-shaded column.

blasts by PDGF was significantly inhibited in the presence of IL-1β and TNF-α in combination but this effect only achieved statistical significance with PDGF (10 ng/ml; $p < 0.05$, Fig. 4).

Prostaglandin E₂ assay

Under basal conditions, human lung fibroblasts in culture produced very little PGE₂ (<0.5 ng/ml). Incubation of human lung fibroblasts with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) alone or in combination for 24 h did not significantly increase PGE₂ production (Table 1). Incubation of human lung fibroblasts with IL-1β (10 ng/ml) or IL-1β (10 ng/ml) in combination with TNF-α (10 ng/ml) for 72 h increased PGE₂ production, which inhibited fibroblast proliferation (Table 1). PDGF did not augment the PGE₂ production induced by IL-1β (10 ng/ml) or TNF-α (10 ng/ml) alone or in combination for 24 h.

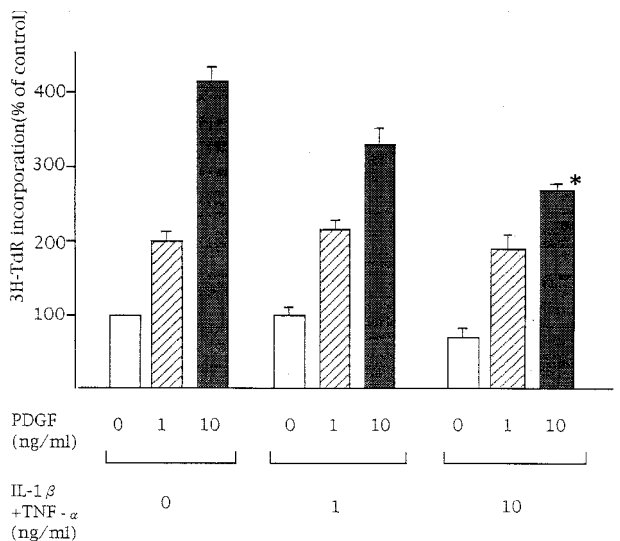


FIG. 4. Effects of PDGF, IL-1β and TNF-α in combination on 3H-Thymidine incorporation by fibroblasts. The average cpm incorporated by untreated fibroblasts was defined as 100. Values give incorporation of specified radiolabeled substances as percentage incorporation. Bars indicate means with SEM derived from one of three experiments with six replicates for each mean. $p < 0.05$ compared with untreated fibroblasts.

Fibroblast chemotaxis

Neither IL-1β, TNF-α nor the combination of these two cytokines significantly stimulated fibroblast chemotaxis in the absence of PDGF (Fig. 5). In contrast, PDGF (10 ng/ml) significantly increased fibroblast chemotaxis (control 3.8 ± 0.5 cells vs PDGF 8.5 ± 0.5 cells, $p < 0.05$, SI = 2.3 ± 0.2), whereas IL-1β ($p < 0.05$ cf. control) and TNF-α ($p < 0.05$ cf. control) significantly inhibited chemotaxis induced by PDGF (10 ng/ml). However, no significant inhibition was observed when both cytokines were present (Fig. 5).

Discussion

In pulmonary fibrosis, the fibrotic process is thought to be initiated by a variety of events following cell migration including extracellular matrix degradation,

Table 1. The effect of IL-1β and TNF-α either alone or in combination on fibroblast PGE₂ production. PGE₂ was measured by ELISA as described in Materials and Methods

Cytokines	Level of PGE ₂ (ng/ml)	
	24 h	72 h
IL-1β (10 ng/ml)	< 0.5	3.65±3.0
TNF-α (10 ng/ml)	< 0.5	0.84±0.6
IL-1β + TNF-α	< 0.5	8.06±4.5
IL-1β (10 ng/ml) + PDGF(5 ng/ml)	< 0.5	
TNF-α (10 ng/ml) + PDGF(5 ng/ml)	< 0.5	
IL-1β + TNF-α + PDGF	< 0.5	

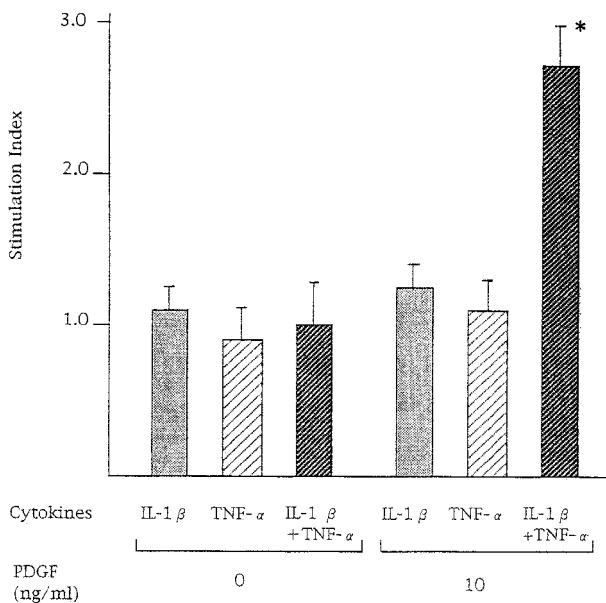


FIG. 5. Fibroblast chemotaxis treated with PDGF, IL-1 β , or TNF- α for 8 h. Responses are expressed as SI. The SI was calculated as described in Materials and Methods. Each bar is the average of triplicate values. Four experiments were performed. $p < 0.05$ compared with PDGF.

cell proliferation and remodeling. These phenomena are induced by growth factors and cytokines produced by infiltrating platelets, macrophages, fibroblasts and residential cells. Of particular interest is the role of PDGF in this process given the recently reported finding that PDGF-like proteins are localized in macrophages and epithelial cells of patients with idiopathic pulmonary fibrosis.¹⁷⁻²¹ Lung fibroblasts are also reported to be capable of synthesizing growth factors known to stimulate fibroblast proliferation *in vivo*, which in turn suggests an autocrine pathway for proliferation of these cells.²¹⁻²⁴

Cytokines such as TNF- α have also been reported to stimulate PDGF secretion-dependent fibroblast proliferation.^{25,26} Several experiments have demonstrated that lung fibroblasts have different potentials to produce collagenase during the development of pulmonary fibrosis.²⁷⁻²⁹ However, little is known concerning the interaction between PDGF, IL-1 β and TNF- α during pulmonary inflammation. We have demonstrated that PDGF in combination with IL-1 β and TNF- α stimulated the lung fibroblast production of MMP-3 and MMP-9. Under similar conditions, the proliferation, but not the migration, of lung fibroblasts was inhibited.

Bronchoalveolar lavage fluid from patients with idiopathic fibrosis contain a high level of active collagenase.³⁰ Matrix metalloproteinases, such as MMP-1, MMP-3 and MMP-9, are important in catalyzing the destruction of collagen, fibronectin, gelatin and other extracellular matrix proteins in inflammation. Tumor cells in culture with metastatic

potential have been demonstrated to secrete MMPs.^{14,15} Cytokines including TGF- α and IL-1 β augmented the synthesis of MMP-9 in murine skin fibroblasts.³¹ Our experiments suggested that MMP-1 and MMP-2 were spontaneously released, whereas MMP-3 and MMP-9 were not from human lung fibroblasts. In addition, MMP-3 and MMP-9 were also not induced by PDGF alone. In contrast, IL-1 β stimulates MMP-3 production while TNF- α stimulates MMP-9 production. Furthermore, we provided evidence for the coordination of the effects of IL-1 β and TNF- α in reducing MMP-3 and MMP-9 production. However, the major finding of the present study was the synergistic production of MMP-3 and MMP-9 induced by PDGF combined with IL-1 β , TNF- α and both cytokines. This finding suggested that PDGF has a complex impact on MMP production in human lung fibroblasts, with the mechanism underlying this effect remaining to be established. A possible mechanism may involve PDGF acting on the transcription pathway.

IL-1 β and TNF- α have been reported to enhance and inhibit respective collagen production in lung fibroblasts.²⁸ We have shown that IL-1 β and TNF- α had no effect of fibronectin production. PDGF had an effect on fibronectin production, which was not augmented by IL-1 β and TNF- α . Under these conditions, PDGF may play the main role in human lung proliferation and contribute to cell migration in the early stages of inflammation. PDGF is released from α -granules found in platelets and is not only a mitogen for connective tissue cells *in vitro*, but is also a potent chemotactic agent for fibroblasts and smooth muscle cells.³²⁻³⁴ IL-1 β alone and TNF- α alone had no synergistic effect on PDGF-induced fibroblast proliferation in this experiment (data not shown). In the presence of IL-1 β and TNF- α , the proliferation induced by PDGF was inhibited, while the chemotactic response to PDGF was increased.

It is known that IL-1 β stimulates the release of PGE₂, which in turn inhibits fibroblast proliferation.^{35,36} Our experiment suggested that under our experimental conditions, PGE₂ does not play a role in fibroblast proliferation. Indomethacine did not reverse the inhibitory effects of IL-1 β and TNF- α on fibroblast proliferation (data not shown). These results suggest that the inhibition caused by the combination of these cytokines is unrelated to fibroblast prostaglandine synthesis. Transmodulation of the PDGF receptor by IL-1 β or the transmodulation of the FGF receptor by PDGF have been shown to occur in other systems.^{37,38} The possibility exists that PDGF modulates IL-1 β and TNF- α receptor expression.

The present results indicated that PDGF in combination with IL-1 β and TNF- α differentially regulated metalloproteinase production, proliferation activity and the chemotactic response in lung fibroblasts.

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