Expression of a 2.8-kb PDGF-B/c-sis Transcript and Synthesis of PDGF-Like Protein by Human Lung Fibroblasts*

Luz Rojas-Valencia, MSc; Fernando Montiel, PhD; Martha Montaño, MSc; Moisés Selman, MD, FCCP; and Annie Pardo, PhD

The replication of fibroblasts is thought to be controlled by exogenous growth factors mainly secreted by macrophages and epithelial cells. However, under standard culture conditions, lung fibroblasts are able to produce several growth factors, suggesting an autocrine pathway of proliferation. In this work, we examined the expression of platelet-derived growth factor (PDGF-A) and PDGF-B messenger RNA (mRNAs) by fibroblasts derived from four human adult normal lungs and from two fibrotic lungs. Northern blot analysis showed that both normal and idiopathic pulmonary fibrosis (IPF)-derived fibroblasts expressed a 2.8 PDGF-B/c-sis mRNA. This transcript was also observed as a minor form in human osteosarcoma cell line, used as control, which predominantly expressed a 4.0-kb PDGF-B mRNA. In two fibroblast cell lines, one fibrotic and one normal, the 4.0-kb transcript was also observed but was always weaker than the 2.8-kb mRNA. PDGF-A mRNA was not detected. By immunofluorescence, lung fibroblasts exhibited intracytoplasmic PDGF-like protein. Likewise, conditioned media from normal and IPF lung fibroblasts stimulated 3H-thymidine incorporation in BALB/c-3T3 cells that was significantly inhibited by anti-PDGF antibody. These results show that in vitro, some human lung fibroblasts express PDGF-B/c-sis mRNA, mainly an alternate 2.8-kb transcript, and produce PDGF-like protein.

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**Key words:** growth factors; lung fibroblasts; PDGF; pulmonary fibrosis

Fibroblasts represent the most abundant cell population in lung parenchyma, and the control of their proliferation is a cardinal element in the maintenance of the structure-function relationships.\(^1\)\(^,\)\(^2\) Several growth factors are believed to be implicated through paracrine and autocrine pathways in the regulation of the lung fibroblast proliferative response.\(^3\) Among them, the platelet-derived growth factor (PDGF), a potent mitogenic agent, appears to play an important role.\(^4\) PDGF is a cationic dimeric protein composed of two polypeptide chains termed A and B;\(^5\) the genes encoding for both chains are in different chromosomes and their expression is independently regulated. Active PDGF can exist in three different molecular forms: AA, BB, and AB.\(^4\)\(^,\)\(^5\)

PDGF can be synthesized by a variety of lung cells, such as alveolar macrophages and epithelial cells which under certain stimuli may secrete this growth factor thus controlling fibroblast duplication.\(^6\)\(^,\)\(^7\) Studies concerning the potential capability of fibroblasts themselves to produce PDGF are scanty. Paulsson et al\(^8\) showed that when normal human foreskin fibroblasts were exposed to PDGF or epidermal growth factor (EGF), a transient expression of PDGF-A messenger RNA (mRNA) with a concomitant synthesis of functional PDGF-AA homodimers occurred. Antoniades et al\(^9\) illustrated by in situ hybridization a reversible expression of PDGF-B in fibroblasts following cutaneous injury in pigs. It has been reported that adult rat lung fibroblasts spontaneously produced in vitro PDGF-AA,\(^10\) and recently the expression of both chains was demonstrated in fetal rat lung fibroblasts.\(^11\) Until now, however, constitutive expression of PDGF-A or PDGF-B by human lung fibroblast has not been demonstrated to our knowledge. Nevertheless, in the course of an experiment with fibroblast's conditioned media derived from fibrotic lungs, we observed PDGF-like mitogenic activity over BALB/c-3T3 cells. To further explore this finding, we examined the PDGF-A and B mRNAs expression by fibroblasts obtained from four human adult normal lungs and from two lungs with idiopathic pulmonary fibrosis (IPF).
Materials and Methods

Cell Lines

Cultures of human diploid fibroblast-like cells were established from pulmonary parenchyma obtained by thoracotomy of adult patients undergoing surgery for clinically relevant reasons. Fibroblast-like cells were derived from lung specimens of one patient with histologic evidence of IPF (HIPF-13) and from two other patients with normal lung parenchyma (N2, NA). The patients fulfill all the diagnostic criteria for IPF. Controls were selected from individuals having lobectomy for removal of a primary lung tumor; no morphologic evidence of disease was found in the tissue samples used for fibroblasts isolation. In addition, two adult normal human lung fibroblast cell lines (CCD11-Lu and LL-24) and one derived from IPF (LL-29) were obtained from the American Type Culture Collection (ATCC). As positive control for PDGF expression, a human osteosarcoma (U-2 OS) cell line was used. For mitogenic assays, BALB/c 3T3 fibroblasts were employed.

Lung fibroblasts were isolated by trypsin dispersion and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). Cell strains were vimentin positive, cytokeratin negative. For primary cultures, early passage cell cultures were used. The cells were grown in flasks (T-75 Falcon) at 37°C in 95% air, 5% CO2 atmosphere until early confluence. Fibroblasts were incubated for 24 h in serum-free media and the conditioned media (CM) were collected and stored at −20°C until used. The ATCC cell lines were grown as recommended.

RNA Isolation and Northern Blot

Total cellular RNA was isolated by the acid guanidinium thiocyanate/phenol chloroform extraction method. Total RNA (20 μg per lane) was fractionated on a 1% agarose gel containing 0.66M formaldehyde. Ribosomal RNA was visualized with ethidium bromide and the fractionated RNA was transferred onto transfer membranes (Nytran, Schleicher & Schuell, Keene, NH) by capillary blotting overnight. The membranes were air-dried and baked at 80°C for 2 h. The membranes were prehybridized at 42°C for 16 h in 5X standard saline citrate (SSC), 50% formamide, 5X Denhardt’s solution, and 0.5% SDS, containing 100 μg/mL of denatured salmon sperm DNA. Hybridization was carried out at 42°C for 16 h in hybridization buffer containing 50% dextran sulfate plus heat denatured 32P-labeled probe. The membranes were washed in 2X SSC 0.1% SDS at room temperature three times for 5 min, followed by 0.25X SSC 0.1% SDS at 55°C twice for 15 min and twice with 0.1X SSC 0.1% SDS at 65°C for 15 min. After drying, the membranes were exposed to film (Kodak X-AR) at −70°C with an intensifying screen. Loading of RNA samples was monitored by assessing the mRNA level of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). cDNA clones for human PDGF-B chain and GAPDH were obtained (from the ATCC). PDGF-A13 was kindly provided (Mark W. Majesky). The probes were radiolabeled with 32P-deoxyctydine triphosphate (dCTP) to specific activity of 200X106 dpm/μg using a multiprime DNA labeling kit (DuPont NEP-105).

In Situ Hybridization

Cells grown on glass coverslips were washed with warmed DMEM and fixed at room temperature for 20 min in 4% paraformaldehyde, 5 mM MgCl2, in phosphate-buffered saline solution (PBS) at a pH of 7.4. Coverslips were stored in 70% ethanol at 4°C until used. Coverslips were rehydrated for 10 min in 2X SSC. Slides were incubated for 10 min at room temperature in 0.25% acetic anhydride, 0.1 M threitolomaline at a pH of 8.0. After, incubation slides were washed with 2X SSC. Hybridization utilizing 35S-labeled cRNA probes were performed as described elsewhere.

Immunochemistry

Cells were grown into four-chamber slides (Miles Lab, Naperville, Ill) in DMEM, 10% fetal calf serum (FCS), until early confluence and then in serum-free medium for additional 24 h. Cells were fixed in 2% paraformaldehyde at room temperature for 20 min. The slides were washed with PBS and incubated with rabbit antiserum to human PDGF (IgG, R&D Systems) at 1:500 dilution. Normal rabbit serum at the same dilution was used as control. The slides were incubated at room temperature for 30 min, washed with PBS, and a second antibody (antirabbit IgG) conjugated with fluorescein isothiocyanate (Cappel, Malvern, Pa) was added at 1:500 dilution and the slides were incubated in the dark at room temperature for 30 min. The slides were thoroughly washed, coated with mounting solution (90% glycerol, 10% PBS), and covered with glass coverslips. Immunofluorescence staining was examined under epifluorescence illumination on a standard microscope (Zeiss 16).

Assay for Mitogenic Activity

To evaluate the presence of PDGF-like protein activity in the fibroblast supernatants, the mitogenic assay as described by An-
toniades and Pantazis\textsuperscript{16} was used. Serum-free media conditioned for 24 h by confluent cell cultures were collected and brought to 0.2% bovine serum albumin, clarified by centrifugation, and lyophilized. The samples were dissolved in 0.5 mL of distilled water and extensively dialyzed against PBS at 4°C.

BALB/c 3T3 cells were plated in 0.3 cm\textsuperscript{2} microtiter wells in DMEM with 10% calf serum and incubated at 37°C. At confluence, the cells were carefully washed with PBS and the medium was replaced with DMEM supplemented with 5% human platelet poor plasma (PPP). To determine if the conditioned media had PDGF-like activity, 10, 25, and 50 μL per well were added per triplicate. Aliquots of the experimental media were incubated with PDGF antisera. After overnight incubation, the samples were assayed for mitogenic activity. Positive control samples

\[\text{Figure 2. Evaluation of fibroblasts PDGF-B expression by in situ hybridization. Panels A and B exhibit autoradiographs of preparations of IPF lung fibroblasts (HIPF-13) and panel C illustrates human normal lung fibroblasts (CCD-11 Lu) after hybridization using }^{35}\text{S-labeled PDGF-B RNA antisense. Panel D shows HIPF-13 with sense probe.}\]

\[\text{Figure 3. Immunofluorescence staining for PDGF-like proteins in human lung fibroblasts. Cells were grown on culture slides and subjected to indirect immunofluorescence with rabbit antiserum to human PDGF at 1:500 dilution. Panel A, CCD-11 Lu; panel B, HIPF-13.}\]
Table 1—Effect of Conditioned Media (CM) From Various Lung Fibroblast Cell Lines on $^3H$-Thymidine Incorporation by BALB/c 3T3 Cells (cpm×10^{-3})*

<table>
<thead>
<tr>
<th></th>
<th>CM, μL</th>
<th>Anti-PDGF, μg/mL</th>
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<tr>
<td></td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>5% PPP</td>
<td>38.8±16</td>
<td></td>
</tr>
<tr>
<td>PDGF-BB, 5 ng/mL</td>
<td>58.7±12.6</td>
<td></td>
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<tr>
<td>CCD-11Lu</td>
<td>47.9±8.8</td>
<td>64.1±10.8\dagger</td>
</tr>
<tr>
<td>LL-29</td>
<td>42.8±5.0</td>
<td>79.6±10\dagger</td>
</tr>
<tr>
<td>HIPF-13</td>
<td>43.9±2.4</td>
<td>52.9±8.6</td>
</tr>
<tr>
<td>U2-OS</td>
<td>50.4±9.7</td>
<td>39.2±7.1\dagger</td>
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*Data represent mean±SE determined from triplicate samples for each point. ND=not done.
\dagger Statistical significant differences from 5% PPP alone (p<0.01).

containing 5 ng of recombinant PDGF BB, 1, 5, and 10% calf serum, and negative controls consisting of serum-free media were also used. Cells were incubated for 18 h and then pulse-labeled for 6 h with $^3H$-thymidine (5 μCi/mL). At the end of the incubation, the cells were rinsed with cold PBS, fixed in 200 μL of 10% trichloroacetic acid, and then rinsed with distilled water. The cells were solubilized in 200 μL of 1% SDS and the radioactivity was measured in a liquid scintillation counter.

**Statistical Analysis**

Statistical significance of the metabolic activity assay was determined by an analysis of variance (ANOVA) with a post hoc Tukey test. For comparison of two groups, the Student's t test for independent groups was used.

**RESULTS**

Northern blot analysis for PDGF B-chain mRNA consistently revealed that some human adult lung fibroblasts expressed low levels of a transcript of approximately 2.8 kb (Fig 1). No differences were observed between cell strains derived from normal or fibrotic lungs. This 2.8-kb PDGF-B/c-sis mRNA was also observed as a minor form in human U-2 OS cells that predominantly expressed a 4.0-kb transcript. In two fibroblast cell lines, one normal and one from IPF, a 3.8 to 4.0-kb mRNA was also observed. Rehybridization of the same filters with a cDNA for GAPDH showed that the differences in PDGF-B expression were not attributable to variations in the RNA levels. PDGF-A gene expression was below the limits of detection by northern blot analysis.

Fibroblasts from one normal (CCD-11 Lu) and one IPF lung (HIPF-13) were hybridized in situ with PDGF-B c/sis antisense RNA to identify cells expressing PDGF-B mRNA. In parallel, coverslips were hybridized with sense probe. Hybridization with complementary antisense RNA displayed numerous autoradiographic grains in both examined cell lines in contrast to sense RNA, as illustrated in Figure 2.

Indirect immunofluorescence performed in fibroblasts derived from normal (CCD-11 Lu) and interstitial fibrotic (HIPF-13) lungs is illustrated in Figure 3. Using polyclonal antibody against PDGF, cytoplasmic immunoreactive PDGF-like protein was detected in the examined cell strains. No differences in cell staining were observed among normal and fibrotic-derived fibroblasts. Control samples using non-immune sera did not show any specific reaction (not shown).

We further analyzed the presence of growth factor bioactivity in the 24-h serum-free CM of lung fibroblasts. For this purpose, three fibroblast cell strains, one from normal lung (CCD-11 Lu) and two from IPF lungs (LL-29 and HIPF-13), were tested. Conditioned medium from U-2 OS cells, known to secrete PDGF, was used as positive control. The CMs obtained from all the cell lines examined were capable of stimulating $^3H$-thymidine incorporation in BALB/c-3T3 fibroblasts. Usually, 25 and 50 μL of 10-fold concentrated conditioned media induced a significant increase in $^3H$-thymidine incorporation in the target cells compared with that obtained with 5% PPP (Table 1). The growth-promoting activity of 50 μL of CM derived from CCD-11-Lu, LL-29, and HIPF-13 fibroblasts was 145%, 115%, and 101% higher than the response induced by 10% FCS. BALB/c 3T3 induced proliferation was assayed in the presence of several concentrations of anti-PDGF-AB antiserum and 50 μL of CM of one normal lung and one IPF lung fibroblasts. Two hundred micrometers per milliliter of PDGF antibody inhibited between 60 and 70% of the induced mitogenic activity. In U2-OS cells, 55% inhibition was achieved (Table 1).

**DISCUSSION**

Replication of fibroblasts is a finely regulated event, where suppressive and stimulating growth factors through both autocrine and paracrine pathways should be operating. Among them, PDGF appears to be one of the most important promoting growth factors. This potent mitogen initiates the transition of nonreplicating cells into the G1 phase of the cell cycle and is considered a prototype of a competence growth factor triggering early events in cell proliferation.17
Much evidence supports that PDGF derived from macrophages, endothelial cells, and epithelial cells plays a role in the paracrine stimulation of fibroblast replication.6,7,16 Concerning autocrine synthesis, several studies have demonstrated the expression of PDGF-A chain by mesenchymal cells, but evidence of PDGF-B autocrine production has been more elusive. Two kinds of evidence support the autocrine production of PDGF-A by fibroblasts. On one hand, the spontaneous expression of PDGF-A chain gene has been shown in rat lung fibroblasts.10 On the other hand, several cytokines such as transforming growth factor beta (TGFβ), PDGF, tumor necrosis factor, and interleukin 1 are able to induce fibroblast PDGF-A chain gene expression and the production of PDGF-A-like proteins.8,19,20 These findings suggest that fibroblast-derived PDGF may play a role in the autocrine regulation and/or paracrine regulation of fibroblast function.8,19,21 Concerning the PDGF-B chain, it was found that c-sis mRNA is expressed by fibroblasts following cutaneous injury in pigs.9 In addition, treatment of quiescent cultures of a mouse embryo-derived fibroblast cell line with TGFβ-induced PDGF-B expression through a putative autocrine loop.22 Until now, however, the expression of c-sis/PDGF-B by human fibroblasts had not been demonstrated.

Our results showed that human adult fibroblasts, derived from both normal and fibrotic lungs, expressed a 2.8-kb transcript PDGF-B c/sis, and occasionally a 3.8 to 4.0-kb mRNA, PDGF-B. This finding was observed in diverse culture passages and without apparent differences between normal and fibrotic-derived fibroblasts. Since almost all cell lines examined expressed the 2.8-kb transcript under basal conditions, our finding suggests that it could be playing a role in the regulation of physiologic fibroblast proliferation. Lung fibroblast expression of PDGF-B mRNA was also detected by in situ hybridization. By contrast, we were unable to reveal PDGF-A mRNA that was below the detection limit of northern blot analysis.

Although cells expressing PDGF-B chain generally show a prominent 3.8 to 4.2-kb mRNA transcript, it has been described that some cells display a diffuse minor band of 2.8 kb that is similar to that found in our study.23-25 In addition, it has recently been reported that normal rat lungs express a 4.0-kb transcript and a minor 2.7-kb PDGF-B mRNA. Furthermore, during the development of hypoxic pulmonary hypertension, both species increased in the lungs and reached their maximum level at day 1 of hypoxic exposure.26 Therefore, both types of PDGF-B chain mRNA are constitutively expressed in rat lungs, although the cellular source was not determined.

The size heterogeneity of PDGF-B/c-sis transcripts derives from differences in position of 5′ termini within untranslated first exon sequence that contains elements that inhibit translation efficiency.24,25 In this sense, Rao et al25 have shown that deletion of 985 nucleotides of PDGF-B 5′ untranslated first exon sequence increased translation of downstream autologous and heterologous coding sequences by up to 40-fold. Considering that the 2.8-kb PDGF-B transcript lacks most of this 5′ untranslated sequence, it has been suggested that this transcript should be more efficiently transcribed than the larger and more abundant form.23 In our study, lung fibroblasts usually expressed a weak signal of 2.8-kb PDGF-B mRNA. Interestingly, however, it has been speculated that in the absence of other regulatory mechanisms, levels of 5′ truncated PDGF-B/c-sis mRNA as low as 2 to 3% that of full-length message may account for equivalent levels of PDGF-B protein translation.24 Here, together with the generally faint mRNA signal, PDGF-like protein was detected by immunofluorescence in the cytoplasm of fibroblasts. Likewise, PDGF-like activity inducing mitogenic activity on BALB/c 3T3 cells was found in the conditioned media of lung fibroblasts.

The expression of PDGF-B/c-sis mRNA and the synthesis of PDGF-like molecules by human lung fibroblasts raise the inquest about possible functions of this cytokine under physiologic and pathologic conditions. Besides a putative autocrine role as a growth factor, PDGF might be playing other functions in a paracrine way, thus contributing to the role of fibroblasts as effector cells.2 It is known that PDGF influence, besides mesenchymal cells, the behavior of different types of cells. In the case of the immune response, for example, it has been found that PDGF exerts a profound effect on T lymphocytes, enhancing IL-2 and depressing the production of IL-4, IL-5, and IFN-γ. Interestingly, the PDGF-BB isoform is the most active in the regulation of the T-cell function.27 Likewise, PDGF-BB upregulates the number of IL-1 receptors in articular chondrocytes and potentiates some actions of this cytokine.28 Concerning inflammatory cells, several studies have suggested that PDGF may promote the activation of monocytes and neutrophils.29,30

In summary, our results show that human lung fibroblasts constitutively express in vitro a 2.8-kb PDGF-B transcript, and occasionally a 3.8 to 4.0-kb mRNA. Additionally, they are able to produce PDGF-like proteins. Further studies should help to determine the in vivo role of this fibroblast-produced growth factor in the lung parenchyma under physiologic and pathologic conditions. A better understanding of the regulation of pulmonary fibroblasts may provide a basis for future therapies of lung fibrosis.
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REFERENCES


