

Acute Lung Injury

Pathogenesis of Intraalveolar Fibrosis

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Abstract

In patients dying with acute lung injury, interstitial mesenchymal cells migrate into the airspace where they replicate and deposit connective tissue. We therefore hypothesized that peptides capable of promoting mesenchymal cell migration and replication would be present in the alveolar airspace. To examine this hypothesis, patients with severe acute diffuse lung injury ($n = 26$) underwent bronchoalveolar lavage. Acutely ill patients without lung injury served as controls ($n = 12$). Recovered effluent was examined for mesenchymal cell growth-promoting and migration-promoting activity. Lavage cell supernates from both patients and controls were devoid of bioactivity. However, substantial growth-promoting and migration-promoting activity was present in lavage fluid from nearly every patient, whereas little or none was present in fluid from controls. Characterization of the bioactivity indicated a significant proportion consisted of three peptides related to PDGF: (a) a 14-kD peptide that shared with PDGF several biophysical, biochemical, receptor-binding, and antigenic properties; (b) a 29-kD peptide that appeared identical to PDGF of platelet origin; and (c) a 38-kD peptide that was biophysically and antigenically similar to PDGF. These data indicate that peptide moieties are present in the airspace of patients after acute lung injury that can signal mesenchymal cell migration and replication. (*J. Clin. Invest.* 1991. 88:663–673.) **Key words:** acute lung injury • growth control • growth factors • platelet-derived growth factor • pulmonary fibrosis

Introduction

Acute lung injury results when the alveolar wall is damaged after exposure to noxious environmental or endogenous agents (1–4). In its most severe form, the adult respiratory distress syndrome (ARDS),¹ lung injury leads to a profound impair-

ment of the gas exchange apparatus which exacts a mortality in excess of 50% (5). The disease process begins when the causative agent directly interacts with critical lung parenchymal cell macromolecules. In addition, there is a concomitant explosive transmural inflammatory response within the alveolar wall in which neutrophils and platelets degranulate releasing toxic, pro-inflammatory mediators (6, 7). The resultant lung parenchymal cell dysfunction and death manifests in the patient as severe respiratory insufficiency. Despite the advanced state of contemporary critical care approaches to patients with acute lung injury, as the mortality data suggest, outcome varies from functionally complete repair of the gas exchange apparatus, to failure of the repair process and death (8, 9).

Detailed morphological studies both in patients and model systems have defined the sequence of events during lung repair. When the repair process is effective, the alveolar microcirculation, interstitium and air–lung interface are reconstituted by coordinated parenchymal cell migration, replication, and connective tissue deposition (10–12). In contrast, in those patients dying with acute lung injury, an exuberant fibroproliferative response ensues. Interstitial mesenchymal cells migrate through gaps in the epithelial basement membrane into the airspace where they replicate, eventually obliterating the gas exchange surface (13, 14).

Whereas the anatomy of the mesenchymal cell response after acute lung injury has been precisely detailed, the growth factors involved in directing this response remain largely undefined. Therefore, this investigation was designed to examine the alveolar microenvironment from patients with acute lung injury for growth factors capable of influencing mesenchymal cell migration and replication. The results indicate the presence of biological activity on the alveolar epithelial surface that potently stimulates mesenchymal cell migration and replication. Characterization of the biological activity indicates that a significant portion consists of three peptides that are closely related to platelet-derived growth factor (PDGF).

Methods

Study population

Approval for this study was obtained from the University of Minnesota Committee for the Use of Human Subjects in Research. Two groups of individuals were examined for the presence of mesenchymal cell growth factors on their alveolar epithelial surface.

Group 1: acute lung injury ($n = 26$). Patients with severe acute diffuse lung injury who met established criteria for ARDS constituted this group (5). In each case there was an appropriate antecedent history, severe hypoxemia despite high concentrations of supplemental oxygen, chest radiograph showing diffuse infiltrates, and no other explanation for respiratory distress. In each patient the precipitating event was either sepsis or trauma, and only patients who were previously in good health before the acute illness were studied. In addition, in each patient, the clinical problem precipitating lung injury was controlled and lung

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1. *Abbreviations used in this paper:* A- aDO_2 , alveolar-to-arterial oxygen gradient; ARDS, adult respiratory distress syndrome; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; T_D , population doubling time; TGF, transforming growth factor.

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injury did not manifest in the context of the multiple organ failure syndrome. Patients were studied within 3 d of the onset of lung injury (Table I).

Group 2: critically ill controls (n = 12). Patients being cared for in the critical care unit who were receiving mechanical ventilatory support because of hypermetabolism (oxygen consumption $\geq 135 \text{ ml m}^{-2} \text{ min}^{-1}$; mean 171 ± 23) and decreased level of consciousness ($n = 7$) or congestive heart failure ($n = 5$) comprised this group. In the former group the alveolar-to-arterial oxygen gradient (A-aDO₂) was ≤ 3 -fold normal (normal $\leq 12 \text{ torr at } 0.21 \text{ atm O}_2$), and in the latter, the A-aDO₂ was ≥ 10 -fold normal.

Bronchoalveolar lavage

In order to obtain samples from the alveolar epithelial surface, bronchoalveolar lavage was performed on all patients after obtaining appropriate informed consent from the patients or their families. Bronchoscopy with bronchoalveolar lavage was performed as previously described (15). Five 20-ml aliquots of 0.9% saline were instilled with each aliquot gently suctioned before instillation of the next. Up to three separate anatomic sites were sampled in each patient. In patients receiving mechanical ventilatory support, bronchoscopy was carried out through an adaptor (Bodai) attached to the endotracheal tube. To ensure patient safety, several additional precautions were taken: 100% O₂ for 15 min before and during the entire lavage procedure; continuously monitored hemoglobin O₂ saturation (with the procedure terminated if values $< 90\%$ occurred for $> 1 \text{ min}$); no more than three anatomic

locations were lavaged; and the procedure did not exceed a total of seven minutes. Utilizing this approach no durable adverse effects were encountered. Two patients with acute lung injury experienced a transient decrease in oxygen saturation $< 90\%$ (80% and 88%), which rapidly resolved when the bronchoscope was removed from the endotracheal tube.

Processing of bronchoalveolar lavage specimens

Routine analytical examination of the effluent fluid was performed as described (16), including measurement of volume of recovered fluid, cell count, and differential. Recovered cells were separated from lavage effluent by centrifugation (600 g for 15 min) and resuspended in Dulbecco's modified Eagle's medium (DME) at 2×10^6 nucleated cells/ml. Recovered cells were cultured in polypropylene tubes (37°C, 10% CO₂, 90% air) in that medium for varying times (4 or 24 h) and centrifuged (2,000 g for 5 min) to generate cell supernates. Positive controls assuring cell viability and ability to produce growth factors when stimulated were as previously described (15). Cell-free lavage effluent ("fluid") and cell-free culture supernatant fluid ("supernates") were aliquoted, frozen on dry ice, and stored at -70° until assayed for growth factor content and migration-promoting activity. In addition, total protein concentration of fluid was assessed using a colorimetric assay (17), and albumin concentration was quantified utilizing an enzyme-linked immunoassay, as described (18).

Biological assay of lavage fluid and cell supernates

Each specimen was analyzed for its ability to influence the two key elements of the mesenchymal cell response in the fibroproliferative process: replication and migration. Replication was assessed by the ability of patient samples to signal lung fibroblast division in defined medium. Migration was assayed using skin fibroblasts in a standard modified Boyden chamber system (Nucleopore, Pleasanton, CA).

Replication. The cell division assay utilized lung fibroblasts (CCL 190, American Type Culture Collection, Rockville, MD) cultivated in defined medium, thus avoiding the complexities introduced by the presence of the numerous growth modulators in serum or plasma. Cells were routinely cultivated (37°C, 10% CO₂, 90% air) in DME + 10% calf serum, medium was replenished every 2–3 d, and subcultivated weekly as described (19). All experiments were performed before the 12th subcultivation. Population doubling time (T_D) during this time period ranged from 19 to 21 h.

The bioassay was designed to take advantage of the known growth factor requirements of diploid fibroblasts (19–26). As a good first approximation, three separate cell cycle specific signals are needed for optimal growth in defined medium: (a) one signaling fibroblasts to exit G₀ and enter G₁ (e.g., PDGF); (b) a second, midway through G₁ (e.g., epidermal growth factor [EGF] or transforming growth factor- α [TGF- α]); (c) a third, facilitating the remainder of G₁ traverse (e.g., an insulin-like growth factor [IGF]).

An optimized "basal medium" (F12 + Heparin 25 mM, pH 7.4 + bovine serum albumin, 0.1 mg/ml + Fe-transferrin, 10 $\mu\text{g/ml}$ + selenium, 10^{-8} M + linoleic acid, $3 \times 10^{-6} \text{ M}$) was devised that permitted maximum fibroblast division (T_D 26 h) in the lowest concentration of the three required growth factors: PDGF 100 pM, EGF 100 pM, insulin 100 nM (Collaborative Research, Inc., Bedford, MA). Omission of any of the required factors from this "optimal medium" increased the T_D to $> 84 \text{ h}$. Increasing the concentration of any factor did not significantly alter the rate of division. The basic approach of the assay was to selectively omit one of the three required growth factors from the optimal media and replace it with a defined dilution of test fluid or supernate. This permitted detection of biological activities that replaced the omitted factor. To quantify the amount of bioactivity, the reciprocal of the dilution of test fluid that resulted in 50% of the fibroblast division observed in optimal medium (i.e., containing all three growth factors) was defined as the number of growth factor units present (27). As a control, to detect putative growth inhibitors in the test fluid, a parallel assay was carried out in which the omitted factor was added to the test fluid.

Table I. Study Population: Acute Lung Injury

Patient No.	Age	Sex	Diagnosis	Day*	Outcome†
	yr				
1	76	F	Sepsis	2	D
2	48	F	Sepsis	2	D
3	30	M	Sepsis	1	D
4	69	F	Sepsis	3	D
5	40	F	Sepsis	1	D
6	23	F	Sepsis	3	A
7	34	F	Sepsis	2	A
8	37	F	Sepsis	1	A
9	58	M	Sepsis	1	A
10	79	F	Sepsis	2	D
11	59	F	Trauma	3	A
12	59	M	Sepsis	3	D
13	59	M	Sepsis	3	D
14	21	M	Trauma	3	D
15	54	M	Sepsis	1	D
16	43	M	Sepsis	3	D
17	66	M	Sepsis	1	A
18	18	F	Sepsis	1	D
19	23	M	Trauma	3	D
20	31	F	Sepsis	1	A
21	67	M	Sepsis	1	A
22	36	F	Sepsis	2	D
23	54	F	Sepsis	1	A
24	33	F	Trauma	3	A
25	34	F	Sepsis	1	A
26	75	M	Sepsis	2	A
Mean \pm SD	48 \pm 18			1.9 \pm 0.9	

* Time after disease onset that bronchoalveolar lavage was performed.

† D, died; A, alive.

To initiate the replication assay, fibroblasts were removed from culture dishes with trypsin (0.25%, 25°C, < 5 min; Gibco Laboratories, Grand Island, NY), washed in medium F12 containing 0.4% calf serum to inactivate the trypsin, centrifuged (600 g for 10 min), and seeded in that medium on day 0 at 30,000 cells/35-mm well (six-well cluster, Falcon Labware, Oxnard, CA). On day 3 the fibroblasts were rinsed three times in preequilibrated (37°C, 90% air, 10% CO₂) basal medium and cultured in this medium (37°C, 1 d). Under these conditions the fibroblasts remain viable and fully responsive to growth factors but have a very low rate of replication (< 6% increase in cell number/day). On day 4, the nonreplicating fibroblasts were rinsed once in basal medium and cultured in basal medium containing either none or two of the three required growth factors ("assay medium") and a defined dilution of the test fluid. On days 6 and 8, growth was assessed by direct cell counts utilizing an automated particle counter (Coulter Corp., Hialeah, FL) after detachment of cells from culture dishes with trypsin/EDTA. The cell division induced by the test fluid or supernate was compared to that observed in optimal medium (all three growth factors present, i.e., positive control), as well as to the division observed in assay medium (one factor omitted, i.e., negative control).

In order to assay large numbers of column fractions during the biochemical characterization of the bioactivity, these samples were assessed for their ability to induce DNA synthesis in the lung fibroblasts. To accomplish this, measurement of [³H]thymidine incorporation into acid-insoluble counts was carried out as previously described (19). Briefly, 10⁴ cells were seeded into microtiter wells (96-well plate). All subsequent steps were carried out exactly as described above, except at the time of test fluid or supernate addition, 0.1 μCi/ml of [³H]thymidine was added (DuPont/New England Nuclear, Boston, MA, 5 Ci/mmol) and the cultures were continued for 24 h. Cell layers were washed, and acid-insoluble counts were collected onto nylon fiber filters using an automated harvester (Skatron, Inc., Sterling, VA). Controls and quantitation were as above. All positive results were confirmed using the cell division assay.

Migration. Migration was assessed in a modified Boyden chamber apparatus, as previously described (28, 29), except the target cells for the assay were human foreskin fibroblasts (CRL 1475, American Type Tissue Collection). Routine culture of the target cells was similar to that for the lung fibroblasts except the culture medium used was α-minimum essential medium (MEM) + 22% fetal calf serum. To achieve maximum migration, cells were used before the 10th subcultivation.

To initiate a migration assay, fibroblasts were removed from culture dishes (0.05% trypsin, 0.53 mM EDTA; 22°C, < 5 min), washed twice with PBS, and resuspended in medium 199 + 0.2% lactalbumin at 10⁶ cells/ml. 45 μl of cell suspension was added to the upper chamber, which was separated from the lower chamber by a type I collagen-coated (100 μg/ml, 30 min each side) porous polycarbonate filter (8-μm pores, Nucleopore). Varying concentrations of the test fluid were placed in the lower chamber, and the apparatus was incubated (37°C, 4 h; 90% air, 10% CO₂) to permit cell migration to occur. The filters were removed, and the upper side was wiped free of cells using a rubber policeman, fixed in methanol, and stained (Diff Quik, American Scientific Products, McGaw Park, IL). Migration was quantified as the number of cells on the lower side of the filter per ×100 field. PDGF (4–165 pM) served as a positive control and medium 199 + 0.2% lactalbumin as a negative control. To detect putative inhibitors of migration, PDGF (165 pM) was added in a parallel assay to each patient sample and migration quantified. Sufficient fluid was available to perform this assay in only a subset of patients with ARDS (*n* = 9).

Characterization of the bioactivities stimulating cell division and migration

The replication bioassay indicated the presence of all three classes of growth factors in the lavage fluid, but not the cell supernates of patients with acute lung injury. Our approach was to formally characterize the molecule(s) in lavage fluid replacing PDGF in the replication bioassay. In four patients, sufficient fluid obtained at postmortem by whole-lung lavage (performed within 2 h of death) was available to complete all the

analyses indicated. The remainder of the data was derived from each patient's fluid as availability permitted. Bioactivity was detected using the replication bioassay and confirmed in positive fractions using the migration assay.

Biophysical analysis was performed as previously described (29). Properties assessed included: (a) extractability into nonpolar solvents (1:1 ethyl acetate); (b) heat stability (100°, 15 min); (c) protease sensitivity (trypsin 5 × 10⁻⁶ M, 30 min, 37°C followed by a twofold molar excess of soybean trypsin inhibitor); (d) acid stability (1 M acetic acid, 30 min); and (e) sensitivity to reduction (2 mM β-mercaptoethanol, 30 min). Biochemical analysis included several chromatographic procedures: ion exchange, gel filtration, reverse phase, and immunoaffinity.

Ion exchange chromatography was carried out by applying lavage fluid (50 ml to 1.5 liter, depending on the quantity available from the patient samples examined) that had been diluted 1:1 with 100 mM Tris, pH 7.4, to an anion exchange resin (DEAE-Sephacel, Pharmacia LKB Biotechnology, Piscataway, NJ), which had been equilibrated with starting buffer (75 mM NaCl, 50 mM Tris, pH 7.4). The column was washed free of unbound protein with starting buffer, and developed in a stepwise fashion with buffered NaCl (50 mM Tris, pH 7.4) of increasing ionic strength (0.25 M NaCl, 0.5 M NaCl, 1 M NaCl). Each fraction was dialyzed against Dulbecco's PBS, filter-sterilized (0.22 μm filter, Millipore Corp., Bedford, MA), and assessed for its ability to induce fibroblast replication. The fraction with biological activity was applied to a cation exchange resin (Sulfapropyl-Sephadex, Pharmacia) in starting buffer, washed, and developed as above for the anion exchange column. Fractions were dialyzed, filter-sterilized, and assayed as above. The fraction eluting with 0.5 M NaCl contained > 80% of the bioactivity and will be termed "cationic lavage proteins."

To perform gel filtration, biologically active fractions were pooled and dialyzed against acetic acid (0.1 M) and lyophilized to dryness. The bioactivity was dissolved in a small volume of acetic acid (1.0 M) and applied to a gel filtration column (Superose 12, 1.0 × 30 cm, Pharmacia) that had been preequilibrated with aqueous buffer containing 10% acetonitrile, 0.1% trifluoroacetic acid. The column was developed with that buffer at a flow rate of 0.5 ml/min. Fractions were evaporated to dryness (Speed Vac concentrator, Savant Instruments, Inc., Farmingdale, NY), redissolved in a small volume of 0.1 M acetic acid, neutralized with 0.1 M sodium hydroxide, and assayed for biological activity as above.

Bioactive fractions were applied to a reversed-phase HPLC column (Pro RPC HR 5/10, Pharmacia) in aqueous buffer containing 0.1% trifluoroacetic acid. The column was developed with a gradient of from 0% to 60% organic buffer (acetonitrile + 0.1% trifluoroacetic acid). Fractions were evaporated to dryness and handled the same as the gel filtration fractions prior to biological assay.

The moieties responsible for the majority of the replication as well as migration bioactivity shared many properties with PDGF. To further examine this apparent similarity, immunoaffinity chromatography, Western blot analysis and radioligand binding analysis were carried out.

An immunoaffinity column was prepared by coupling a goat polyclonal anti-human PDGF antibody (Collaborative Research, Inc.) to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Antibody was dissolved in coupling buffer (0.5 M NaCl, 0.1 M NaHCO₃, pH 8.3) at 5 mg/ml and added to the gel (300 mg) which had been preequilibrated with coupling buffer. The solution was gently agitated (1 h, 22°C), and the coupling reaction was stopped by addition of blocking buffer (coupling buffer + 0.2 M glycine). The antibody-coupled gel was washed, packed into a column (1-ml bed volume) and stored (4°C) in PBS + Na azide (0.05%) until use.

Immediately before use, the column was washed with PBS, and the test fluid applied (2–40 ml). Nonspecifically bound material was removed with sequential 10 bed volume washes of PBS (22°C), phosphate-buffered 0.6 M NaCl (22°C, pH 7.4), PBS + 0.5% sodium deoxycholate (30°C), and PBS (22°C). The column was developed with 1 M acetic acid. Specifically bound material eluted in this manner was evap-

orated to dryness and subjected to further biochemical or biological analysis as indicated.

To examine the antigenic similarity of the bioactivity identified in lavage fluid to one of the defined isoforms of PDGF (i.e., AA, AB, BB), Western blot analysis of cationic lavage proteins was performed. PDGF AB purified from platelet α -granules (30), as well as recombinant PDGF AA and BB (Collaborative Research, Inc.) served as standards. Samples and standards were subjected to SDS-PAGE (15%), and electrophoretically transferred to 0.2- μ m nitrocellulose paper. Blots were incubated with primary antibody (anti-PDGF AB, 20 μ g/ml; anti-PDGF B, 50 μ g/ml; or anti-PDGF A, 50 μ g/ml; all obtained from Collaborative Research, Inc.), horseradish peroxidase-coupled anti-goat IgG(Fc) (dissolved according to the manufacturer's directions, diluted 1:250; Accurate Chemical and Scientific Corp., Westbury, NY), and developed by addition of diaminobenzidine (2.2 μ M; see immunoblot assay below). Of note, despite satisfactory results detecting AB and BB with either anti-AB or anti-B antibodies, reproducible immunodetection of PDGF AA could not be achieved.

The radioligand binding assay for PDGF was carried out as previously described with minor modifications (29). Dermal fibroblasts (CRL 1475, American Type Culture Collection) were cultivated in 24-well dishes until density arrested ($\sim 10^5$ fibroblasts per well). Cell layers were rinsed twice with binding medium (DME + 0.5 mg/ml BSA + 50 mM Hepes/50 mM Tris, pH 7.4) and cultured (16 h, 37°C; 95% air, 5% CO₂) in that medium. Before assay, cells were rinsed an additional time with binding medium, and 500 μ l of either PDGF standard (100–5,000 pM; BB homodimer, Collaborative Research, Inc.) or test fluid in binding medium was added. The assay was initiated by the addition of 5×10^4 dpm of ¹²⁵I-PDGF (11.1×10^5 dpm/pmol) prepared according to the procedure of Hunter and Greenwood (31). After incubation (2 h, 22°C), cells were washed in isotonic solution twice (PBS containing BSA, 1 mg/ml; CaCl₂, 2 mM; MgSO₄, 1 mM; pH 7.4, 4°) and in high ionic strength buffer one additional time (phosphate-buffered 1 M NaCl, pH 7.4, 4°C) to remove nonspecifically bound counts. Specifically bound counts were recovered by enzymatic removal of cells from the culture dish (trypsin 0.05%, EDTA 0.53 mM) and quantified in a γ counter. Approximately 4% of added counts bound specifically in the absence of additional exogenous PDGF. The standard curve was carried out in quadruplicate and all test fluids were assayed in duplicate at a minimum of three different concentrations.

Quantitation of PDGF in patient samples

Two assays were utilized to quantify PDGF in lavage fluid: a radioligand-binding assay (above) and immunoblot analysis. Preliminary studies with the radioligand-binding assay indicated that the PDGF concentration of lavage fluid was too low to permit direct assay (radioligand binding detects ≥ 100 pM). In addition, similar evaluation of the immunoblot assay indicated that the fluid contained too much protein (100–1,000 μ g/ml) to reproducibly load > 50 μ l onto each region of the nitrocellulose paper used for the assay. This volume was inadequate for reproducible quantitation of PDGF in lavage fluid (lower limit of detection > 30 pmol). Therefore, for both assays, additional preparation of the samples was required.

Sample preparation. To prepare fluid for radioligand-binding assays, ~ 20 ml was lyophilized to dryness, resuspended in 1 ml of 0.1 M acetic acid, and dialyzed against that buffer (4°C, two changes, 500 vol). Dialyzed samples were frozen, lyophilized, and resuspended in binding buffer. Three concentrations of each sample were assayed in duplicate, with quantitation carried out by graphic comparison to a standard curve. Losses of PDGF during the entire handling procedure were formally assessed using ¹²⁵I-PDGF as tracer and ranged from 29% to 36%.

The strategy to prepare samples for the immunoblot assay was based on the high degree of PDGF binding to cation exchange resins at low ionic strength compared to the other proteins in lavage fluid. 10 ml of fluid was applied to a 200- μ l bed volume column packed with the strong cation exchange resin Sulfapropyl-Sephadex preequilibrated

with buffer (50 mM NaCl, 10 mM Na phosphate, pH 6.5). The column was washed free of unbound material with 3 ml of equilibration buffer, and developed with 500 μ l of elution buffer (1 M NaCl, 10 mM Na phosphate, pH 6.5). Using this procedure, 78–88% of ¹²⁵I-PDGF added to lavage fluid (fluid from two normals and two ARDS patients were used) bound to the resin while only 5–8% of total applied protein bound. Development of the cation exchange column eluted 87–93% of bound counts. Thus 68–82% of the PDGF in a test sample was recovered for application to the nitrocellulose paper. Formal quantitation of the efficiency of the application procedure indicated that binding of PDGF to nitrocellulose is essentially complete if the amount of protein applied is ≤ 70 μ g. After elution from the ion exchange column, this was the case for all samples in the study.

Immunoblot assay. PDGF in lavage fluid was quantified by immunoblotting using a 96-well vacuum blotter (Minifold, Schleicher & Schuell, Inc., Keene, NH), which transfers the proteins from up to 500 μ l of sample onto 20-mm² of nitrocellulose paper. All transfers and dilutions were performed using pipettes and containers that had been siliconized, incubated in a solution of nonfat dry milk (1 mg/ml), and rinsed in distilled water before use. After transfer of a standard or test solution to the nitrocellulose paper, remaining binding sites on the paper were blocked by incubation (1 h, 22°C) in T-TBS (150 mM NaCl, 100 mM Tris, 0.05% Tween 20, pH 7.4) containing 1 mg/ml nonfat dry milk. Blocking buffer was decanted and the blot was incubated (1 h, 22°C) in a solution of primary antibody (goat anti-human PDGF, 20 μ g/ml; Collaborative Research, Inc.). The blot was rinsed (four times with T-TBS) and incubated (3 h, 22°C) in a solution of secondary antibody (rabbit anti-goat IgG [Fc] coupled to horseradish peroxidase [Dako Corp., Santa Barbara, CA], 4 μ l/ml in T-TBS). Since variable, but considerable, cross-reactivity occurred between the secondary antibody preparations and human IgG (which is abundant in lavage fluid), a preadsorption step was necessary before using the secondary antibody. To accomplish this, the secondary antibody was preadsorbed (1 h, 22°C) with T-TBS containing nonfat dry milk (1 mg/ml), BSA (1 mg/ml), and human IgG (1 mg/ml). The blot was rinsed (four times with T-TBS) and color developed by addition of diaminobenzidine (0.8 mg/ml) for ~ 30 –45 s. The reaction was stopped by flooding the blot with water. The intensity of color was quantified using a video densitometer (32). The quantity of PDGF in a test fluid was calculated as the geometric mean of the signal corresponding to more than or equal to three dilutions of that fluid that fell within the boundaries of the standard curve.

Quantitation of other platelet α -granule contents

β -Thromboglobulin was quantified by radioimmunoassay according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL). Lavage fluid was concentrated ~ 10 -fold by speed evaporation. The lower limit of detection in the assay was 0.5 ng/ml lavage fluid.

Statistical analysis

In general, the distributions of data elements within patient groups were not Gaussian. Therefore comparisons were carried out using non-parametric statistics (Wilcoxon rank order test).

Results

Bronchoalveolar lavage. All but two patients in the study population underwent bronchoalveolar lavage without difficulty. In two patients with acute lung injury, a transient decrease in oxygen saturation below 90% occurred (80% and 88%, respectively) during the procedure. This rapidly resolved when the bronchoscope was removed from the endotracheal tube. In both patients, because the procedure was clinically necessary, bronchoalveolar lavage was carried out later that day without problems. The amount of instilled fluid recovered was $58 \pm 9\%$.

Nucleated cell counts varied considerably (range = 56–3,860 cells/ μ l; mean \pm SD = 469 \pm 1,124 cells/ μ l) in patients with acute lung injury, and there were 20-fold more RBC on average than nucleated cells (10,300 \pm 13,400 cells/ μ l). As expected (33), the nucleated cell differential count revealed a preponderance of neutrophils (\geq 63%; 87 \pm 11%), with macrophages representing nearly all of the remaining cells (13 \pm 11%). Total protein (718 \pm 460 μ g/ml) and albumin (339 \pm 286 μ g/ml) concentrations were in the range previously reported by other investigators (33, 34). Overall mortality for the patients with lung injury was 54%. Taken together, these data indicate that the degree of lung injury in our study group was comparable to that observed in other reports (33, 34). Similarly the bronchoalveolar lavage cell counts, differentials and protein concentrations in the critically ill controls and patient controls fell within the expected range for these populations (33–35).

Growth of lung fibroblasts in response to bronchoalveolar lavage fluid and cell supernates. When nonreplicating fibroblasts were cultured with cell-free lavage fluid from patients with ARDS, there was a marked increase in replication rate (Fig. 1). The bioassay utilized took advantage of the known growth factor requirements of fibroblasts, and was designed to assess the ability of a test sample to replace one or more of the required factors. Fibroblast replication slowed greatly after cultivation in medium F12 + 0.4% calf serum for 3 d and serum-free medium F12 (i.e., basal medium, see Methods) for 1 additional day (< 6% increase in cell number/day). The addition of basal medium containing appropriate concentrations of the three required factors (PDGF 100 pM, EGF 100 pM, insulin 100 nM; i.e., "optimal medium") resulted in a growth rate that was 70–80% of that observed in medium F12 + 10% calf serum. Omission of any one of the required factors from the

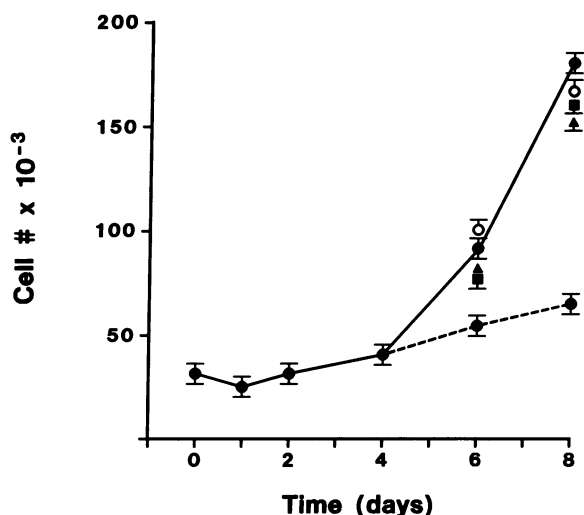


Figure 1. Growth of lung fibroblasts in response to bronchoalveolar lavage fluid from a patient with acute lung injury. Nonreplicating fibroblasts were incubated with a test fluid in basal medium containing two of the three growth factors (i.e., EGF + IGF, PDGF + IGF, or PDGF + EGF) required for optimal growth. Shown is fibroblast cell number as a function of time when the omitted factor was PDGF (○); EGF (▲), or IGF (■). Also shown is fibroblast growth in optimal medium with no added test fluid (all three growth factors added; solid line), and in basal medium containing any two of the three required growth factors (dashed line).

optimal medium dropped the rate of replication four- to five-fold. In the example shown (Fig. 1), addition of lavage fluid from a patient with ARDS significantly augmented growth in each of the basal media supplemented with any two of the three required growth factors (i.e., EGF + IGF, PDGF + IGF, PDGF + EGF) ($P < 0.01$, all comparisons). These findings indicated the presence of bioactive moieties capable of replacing each of the three required factors. The increased rate of replication observed when this fluid was added to fibroblasts cultured in basal medium in the absence of additional growth factors (14 of 16 lavage fluid samples tested in this way were positive; mean increase in cell number was 43 \pm 11% above control) corroborated this result (data not shown). In contrast to the stimulatory influence of lavage fluid from patients with ARDS on fibroblast replication, lavage cell supernates from these patients (mainly RBC and neutrophils) induced minimal or no increase in fibroblast replication (data not shown). For that reason, further experiments focused on the bioactivity present in lavage fluid.

While lavage fluid from nearly all patients with ARDS contained substantial amounts of growth promoting bioactivity, fluid samples from controls contained little or no activity (Fig. 2). The quantity of bioactivity in each test sample was defined as the reciprocal of the dilution inducing 50% of the maximum growth response observed in optimal medium. Among the positive lavage samples from patients with ARDS, values for the quantity of bioactivity replacing PDGF ranged from 60 to 160 U/ml (Fig. 2 A); the quantity replacing EGF, from 45 to 125 U/ml (Fig. 2 B); and the quantity replacing insulin, from 60 to 130 U/ml (Fig. 2 C). The highest value observed in any of the controls was 5 U/ml ($P < 0.01$, all comparisons).

In addition to growth promoting activity, inhibitors of fibroblast replication were detected in each lavage fluid from both patients and controls (data not shown). Addition of growth factors (PDGF 100 pM, EGF 100 pM, insulin 100 nM) to each of the fluids to be assayed indicated the presence of inhibitory activity that was undetectable in all but two ARDS samples after a 10-fold dilution of the fluid. Of note, it required a 150-fold dilution to eliminate the inhibitory activity from the two ARDS lavage fluids that were negative in the fibroblast growth bioassay. Despite the complex interplay of forces modulating fibroblast replication within the alveolar microenvironment, a striking net growth-promoting activity characterized the lavage fluid of nearly all patients with ARDS.

Fibroblast migration in response to bronchoalveolar lavage fluid and cell supernates. Cell-free lavage fluid from patients with ARDS significantly stimulated fibroblast migration (Fig. 3). In contrast, none of the specimens obtained from controls induced significant fibroblast migration. Consistent with the observations made for growth promoting activity, only the fluid and not the cell supernates contained the bioactivity (data not shown). In the assay utilized, fibroblast migration in the presence of buffer alone was 53 \pm 14 cells per \times 100 field. The addition of PDGF (positive control), 4–165 pM, resulted in values ranging from 75 \pm 21 to 350 \pm 34 per \times 100 field, encompassing the range of migration observed in response to lavage fluid from ARDS patients. Similar to the findings in the replication bioassay, inhibitors of cellular migration were found when exogenous PDGF (165 pM) was added to the lavage samples (not shown). In all cases, a 1:8 dilution eliminated this activity. While small amounts of bioactivity stimulating fibroblast mi-

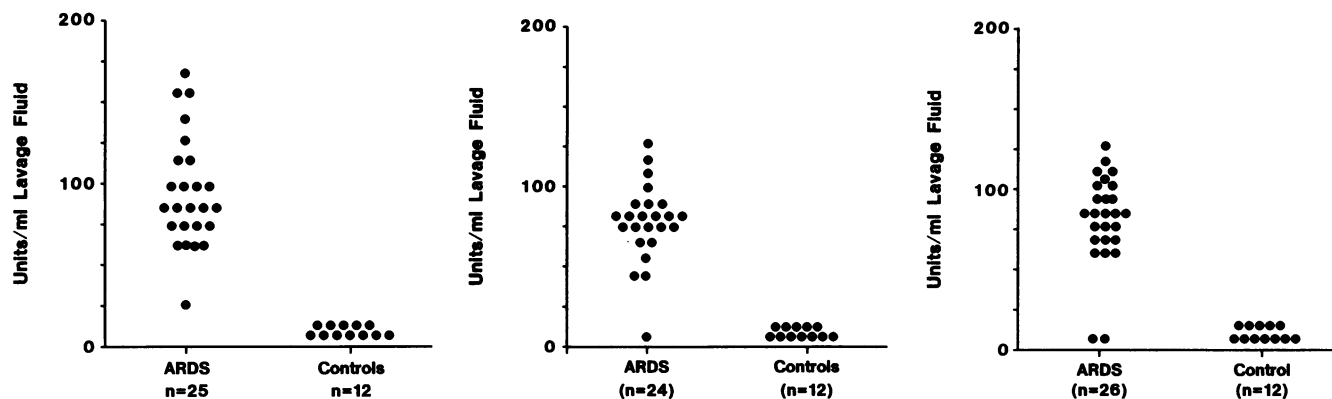


Figure 2. Quantitation of growth-promoting bioactivity in bronchoalveolar lavage fluid. For each sample tested the number of growth factor units present is expressed as the reciprocal of the dilution of test fluid resulting in 50% of the maximum growth response observed in optimal medium. Shown are data obtained in medium lacking (left) PDGF, (middle) EGF, (right) IGF.

gration could have been obscured by the inhibitors detected, significant net migration promoting activity was observed in the lavage fluid from all patients with ARDS, and in none of the controls ($P < 0.01$).

Characterization of the growth-promoting activity replacing PDGF in the fibroblast growth bioassay. Efforts to characterize the identified biological activities were focused on the moieties replacing PDGF in the fibroblast growth assay. This approach had the advantage of likely isolating at least one of the principal fibroblast migration promoting activities as well. The strategy for biophysical and biochemical characterization took advantage of the known properties of the defined isoforms of PDGF.

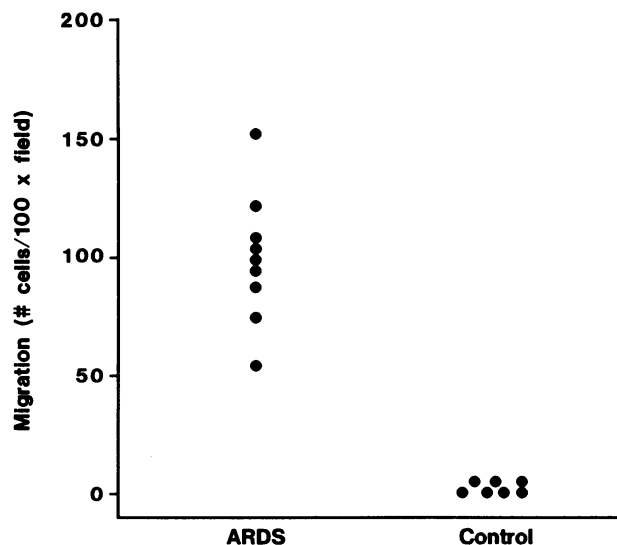


Figure 3. Fibroblast migration in response to bronchoalveolar lavage fluid. Fibroblasts were placed in the upper chamber of a modified Boyden chamber apparatus. They were separated from the test fluid placed in the bottom well by a collagen-coated polycarbonate filter. Shown is fibroblast migration expressed as the number of cells migrating per $\times 100$ field in response to bronchoalveolar lavage fluid from patients with acute lung injury as well as patient controls. For each data point the number of cells migrating toward the negative control fluid = 53 ± 14 (medium 199 + 0.2% lactalbumin) was subtracted from the total number of migrating cells.

Similar to PDGF, the majority of the growth and migration promoting activity was heat stable (100°C , 15 min) and acid resistant (pH 2.0), was extractable into polar but not nonpolar solvents, and was sensitive to incubation with reducing agents (β -mercaptoethanol, 2 mM) as well as proteolytic enzymes (trypsin, 5×10^{-6} M) (Table II).

While $< 15\%$ of the bioactivity bound to an anion exchange resin (DEAE), $> 80\%$ of the bioactivity bound to the cation exchange resin, Sulfapropyl-Sepharose. Gel filtration of the bioactivity eluted from Sulfapropyl-Sepharose (0.5 M NaCl, pH 7.4) resolved it into three fractions (Fig. 4). One comigrated with an authentic PDGF standard (AB), at an apparent M_r of 29 kD. Of the other two moieties one was larger than PDGF, with an apparent M_r of 38 kD, and the second was smaller at 14 kD. Each of the three bioactive fractions reacted positively in an immunoblot assay using a polyclonal anti-PDGF antibody (data not shown).

In an effort to more quantitatively examine the different molecular mass forms of the bioactivity present in recovered lavage fluid and examine their antigenic relationship to PDGF, Western blot analysis of all lavage proteins was attempted. Owing to the high concentration of total protein, this was not technically feasible. To circumvent this problem, two separate procedures were utilized: (a) Western blot analysis of cationic lavage proteins (i.e., those binding to Sulfapropyl-Sepharose in 0.075 M NaCl, eluted with 0.5 M NaCl); and (b) immunoaffin-

Table II. Biophysical Properties of the Growth-promoting Activity Replacing PDGF in the Bioassay

Treatment	Percentage of activity remaining
Heat (100°C , 15 min)	69
Acid (pH 2.0, 30 min)	74
Lipid solubility (ethyl acetate)	0
Reduction (β -mercaptoethanol 2 mM, 30 min)	11
Protease (trypsin, 5×10^{-6} M, 37°C , 30 min)	< 5

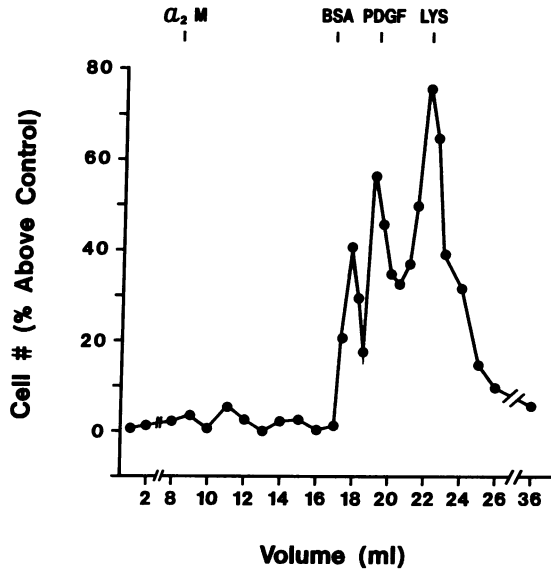


Figure 4. Gel filtration chromatography of bronchoalveolar lavage cationic proteins. Bronchoalveolar lavage fluid from patients with acute lung injury was allowed to bind to sulfapropyl Sepharose and eluted with 0.5 M NaCl. The resultant eluted proteins were chromatographed on a Superose-12 column. Shown is fibroblast growth (expressed as percent increase in cell number above control) in response to each column fraction. Also shown are the positions of the molecular mass standards (α_2M = alpha-2 macroglobulin, 850 kD; BSA = bovine serum albumin, 66 kD; PDGF = ^{125}I -PDGF AB, 29 kD; LYS = lysozyme, 14 kD).

ity chromatography of total lavage fluid protein using a solid-phase polyclonal anti-PDGF antibody. Consistent with the results of the gel filtration analysis, Western blot analysis of lavage cationic proteins indicated the presence of three molecular mass forms of PDGF immunoreactive material. Of note, while the 38- and 29-kD peptides were variably present and represented a minority of the PDGF immunoreactivity, the 14-kD peptide was the predominant form (Fig. 5). Similar results were obtained by immunoaffinity chromatography (not shown).

Further purification of the 14-kD peptide was carried out by subjecting cationic lavage fluid proteins to sequential immunoaffinity, gel filtration, and reversed-phase HPLC with each fraction examined for PDGF by immunoblot. Densitometric analysis of a silver-stained PAGE indicated that the 14-kD peptide was > 95% pure, with a very small amount of 29-kD peptide as well as other higher molecular mass peptides present (Fig. 6). Examination of the growth-promoting, migration-promoting, and receptor-binding properties of the 14-kD peptide confirmed that it was biologically active, and not inactive PDGF monomer which has a similar electrophoretic mobility (Fig. 7).

Although insufficient quantities of the 38-kD peptide were available for additional characterization, further analysis of the 29-kD peptide by reversed-phase HPLC demonstrated coelution with a PDGF standard (purified from platelets) at 30% organic phase (data not shown). Strong evidence that some of the 29-kD PDGF identified resulted from platelet degranulation was provided by the detection of another platelet α -granule product, β -thromboglobulin, in the epithelial lining fluid of

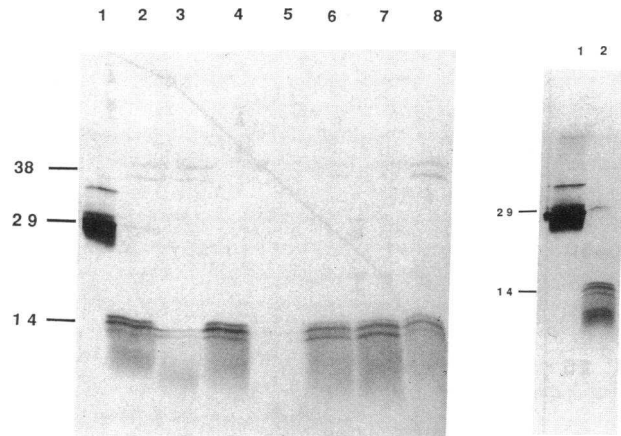


Figure 5. Western blot analysis of cationic lavage proteins. Lavage proteins eluted from sulfapropyl Sepharose were subjected to SDS-PAGE (15%) and transferred electrophoretically onto nitrocellulose paper. Proteins were allowed to bind to an anti-PDGF polyclonal antibody, the primary antibody identified with HPO-conjugated secondary antibody, with color developed by adding H_2O_2 and diaminobenzidine. (A) Shown are the patterns corresponding to six different patients with acute lung injury (lanes 2-4 and 6-8), and one patient control (lane 5). Also shown is the signal generated by 0.33 pmol of PDGF (BB homodimer) used as a standard (lane 1). The 38-kD peptide when present appeared as a doublet (lanes 2, 3, and 8) and the 14-kD peptide generally appeared as a doublet or triplet. (B) Shown is the pattern corresponding to a patient who demonstrated the presence of 29-kD PDGF as well as 14-kD PDGF (lane 2). PDGF standard (0.33 pmol; lane 1) is also shown.

patients with acute lung injury. Of note, in patients with acute lung injury, β -thromboglobulin concentrations ranged from 2.1 to 153 ng/ml (27.7 ± 43.9 ng/ml), while in control patients β -thromboglobulin was uniformly < 0.5 ng/ml; $P < 0.01$. Therefore, whereas at least a portion of the 29 kD bioactivity was likely PDGF originating from platelet α -granules, the origin of the 14- and 38-kD bioactivities remained an unresolved issue.

Quantitation of PDGF in bronchoalveolar lavage fluid from the study population: immunoblot and radioligand-binding assay. Biologically significant concentrations of PDGF were detected both by immunoassay and radioligand binding assay in



Figure 6. Purification of the 14-kD peptide. The biological activity after immunoaffinity and gel filtration chromatography corresponding to the 14-kD peptide was further purified using reversed-phase HPLC. The resultant peak of biological activity was subjected to SDS-PAGE (15%). Shown are the proteins identified by silver staining of the gel.

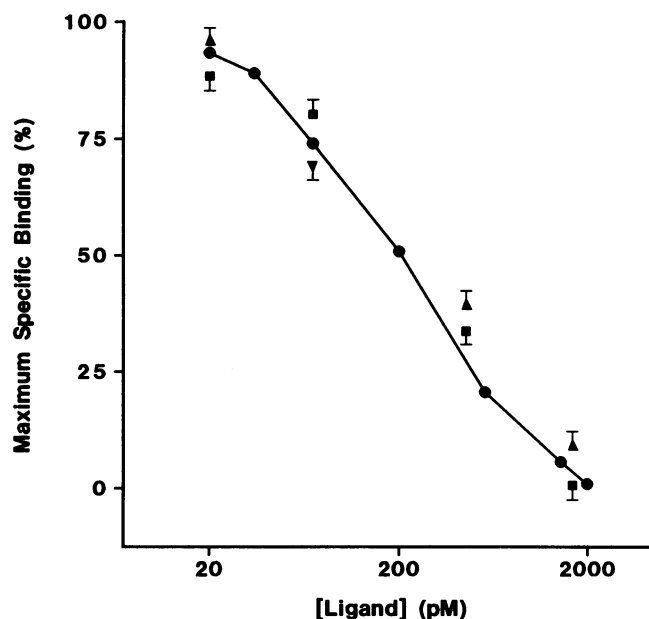
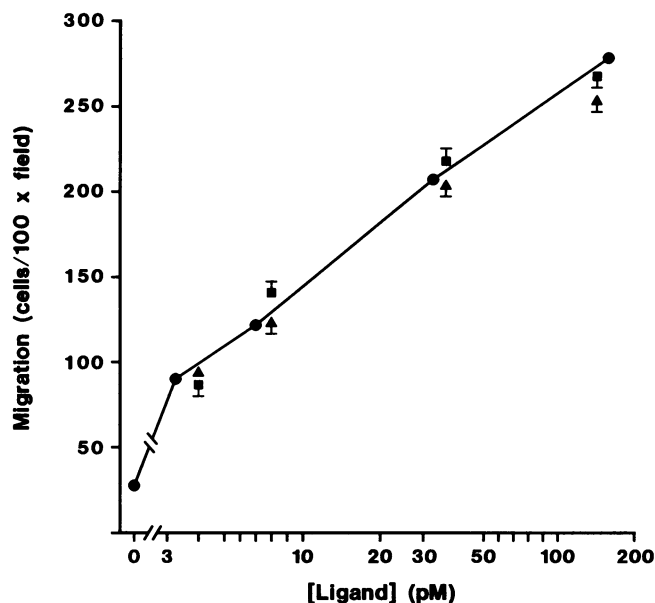
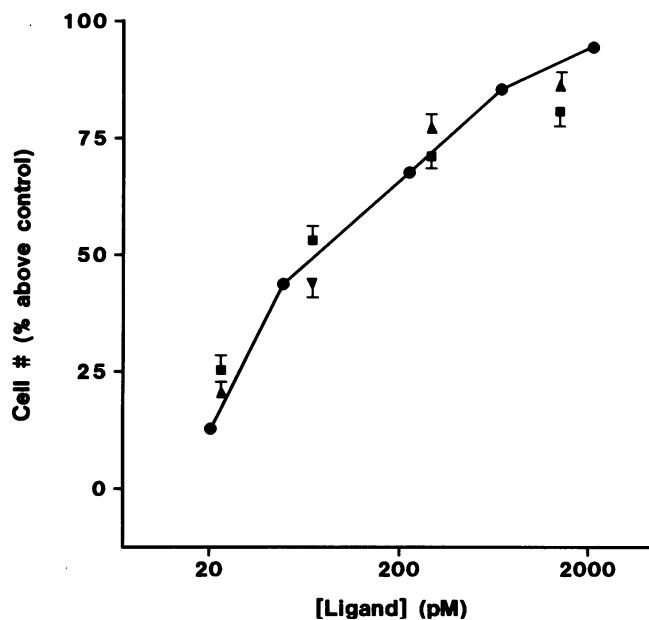


Figure 7. Biological characterization of the 14-kD peptide. After purification, the 14-kD peptide (see Fig. 6 above) was examined for its growth-promoting, migration-promoting, and receptor-binding properties. (Upper left) Shown is fibroblast growth (expressed as the percent increase in cell number above control) in response to increasing amounts of the 14 kD peptide (▲). (Upper right) Fibroblast migration is displayed as number of cells per $\times 100$ field in response to increasing concentrations of the 14-kD peptide. (Lower left) The ability of increasing concentrations of the 14-kD peptide to displace radiolabeled PDGF BB from its receptor on skin fibroblasts is shown. Also shown in each panel are the results using PDGF purified from platelet α granules (■) and recombinant PDGF BB homodimer (●).

lavage fluid from patients with acute lung injury (Fig. 8). In lung injured patients, concentrations of PDGF ranged from 60 to 410 pM by receptor assay (Fig. 8, top) and from 80 to 420 pM by immunoassay (Fig. 8, bottom). As expected from the bioassay data, little or no PDGF was detected in the lavage fluid obtained from normals or patient controls ($P < 0.01$, both comparisons). Although precise quantitation of PDGF concentrations in the epithelial lining fluid in our study population cannot be made owing to the variable dilution of this lining fluid during the lavage procedure, the values obtained in patients with acute lung injury are in a range consistent with a pathogenetic role in vivo.

Discussion

In patients dying with acute lung injury, a fibroproliferative response characterized by mesenchymal cell migration and rep-

lication results in obliteration of the airspace. The present study has identified growth regulatory signals on the surface of the airspace which may be instrumental in directing this response. Among these were three peptides related to PDGF. The most ubiquitous and abundant was a 14-kD peptide that shared with PDGF its growth- and migration-promoting properties for cells as well as several biophysical, biochemical, receptor-binding, and antigenic properties. The second appeared identical to PDGF of platelet origin. The third was a 38-kD peptide which was biologically, biophysically and antigenically similar to PDGF.

Granulation of the injured airspace. Available morphological information in patients after lung injury as well as in model systems indicates that the intraalveolar fibroproliferative response is anatomically similar to granulation of a wound space (13, 14, 36). In particular, both processes feature a rapid inflam-

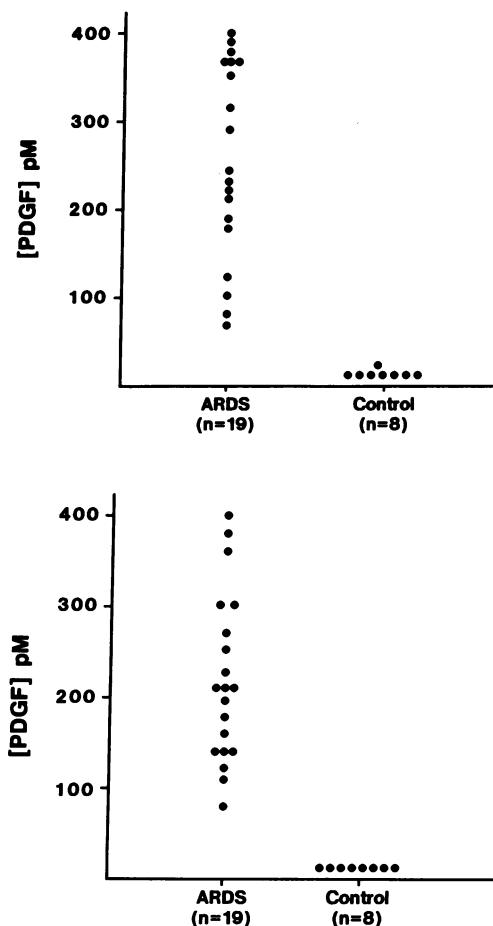


Figure 8. Quantitation of PDGF in bronchoalveolar lavage fluid. (Top) Radioligand-binding assay. Bronchoalveolar lavage fluid concentrated by lyophilization (8–20-fold) was dialyzed against 0.1 M acetic acid, speed evaporated to dryness, and reconstituted with assay medium. Shown is the quantity of PDGF receptor-binding activity using PDGF BB as a standard. Values are corrected for the extent of concentration. (Bottom) Cationic lavage proteins were applied to nitrocellulose paper and color developed by sequential addition of polyclonal anti-PDGF, secondary antibody coupled with HPO, and diaminobenzidine. Shown are the results of videodensitometric quantification of color compared to a BB-PDGF standard for patients with acute lung injury and controls.

matory response and the subsequent deposition of a provisional matrix in the wound space. Prompt ingrowth of capillaries and myofibroblasts ensues, followed by deposition of matrix macromolecules. Although the events and anatomy are nearly identical, the outcomes are diametric. Obliteration of the airspace by granulation tissue is maladaptive, precluding effective gas exchange, whereas prompt granulation of a wound space is a highly adaptive response leading to restoration of an intact integument. Despite the difference in outcome, it is likely that the molecular mechanisms governing the process of wound healing will also pertain to intraalveolar fibrosis. It is therefore important to examine the results of the present study within the framework of current knowledge regarding wound healing.

When Matsuoka and Grotendorst (37) examined wound fluid for fibroblast migration-promoting activity, the major

bioactivity was an ~ 16-kD peptide recognized by a polyclonal anti-PDGF antibody. It is noteworthy that in lung injured patients, a 14-kD biologically active PDGF-like peptide was invariably recovered from the surface of the airspace. Without amino acid composition or sequence data it is not possible to know if these molecules are identical. Nonetheless, their striking similarity indicates that in two anatomic locations after injury, the most abundant form of PDGF recovered is a 14-kD peptide, and not the 29-kD peptide purified from platelet α -granules.

The origin and cellular source of the 14-kD peptide remains an unresolved issue. First, it is unclear whether the 14-kD peptide is proteolytically cleaved from a 29-kD parent peptide, or whether it is released from cells in that form. Based on its reactivity with anti-B and polyclonal anti-PDGF antibody (which detects BB, AB, but not AA), the 14-kD peptide probably contains β -chain amino acid sequences. However, the actual chain composition is uncertain. Candidate cellular sources include platelets (38–40), mononuclear phagocytes (29, 41–44), endothelial cells (45–47), and autocrine production by mesenchymal cells themselves (48, 49). Excluding platelets, each of these cell types has been observed to release PDGF-like peptides distinct from the 29-kD form (defined biologically, by receptor-binding properties and/or antigenically) with molecular masses ranging from 13 to 67 kD. Defining the amino acid sequence of the bioactive peptides and their corresponding cellular sources will be an important step in assigning a role for these growth factors in the pathogenesis of intraalveolar fibrosis.

A surprising aspect of the present study was the relative paucity of the 29-kD form of PDGF in the lavage fluid of patients after acute lung injury. The unambiguous ultrastructural evidence of degranulated platelets in the lung, coupled with the clear demonstration of β -thromboglobulin in lavage fluid in the present study, suggests that the 29-kD form of PDGF should be present. However, while detectable biologically in all lavage samples handled preparatively, Western blot analysis of cationic proteins demonstrated its presence in only a small proportion of the patients, and in all cases it appeared to be a minor portion of the total immunoreactive material. One possibility is that 29-kD PDGF is released and rapidly processed to the 14-kD form. Alternatively, 29-kD PDGF may be bound to a noncationic plasma protein which is overly represented in the airspace by virtue of the severe epithelial injury present. In this regard, 29-kD PDGF is known to bind avidly to α_2 -macroglobulin (50) (and probably other plasma proteins as well), which is present in the lavage fluid of lung injured patients in high concentrations (~ 25 μ g/ml) (34). Not only does α_2 -macroglobulin fail to bind to cation exchangers, but also PDGF bound to α_2 -macroglobulin is not detected by the polyclonal anti-PDGF antibody utilized in the current study (51). The potential bioavailability of α_2 -macroglobulin-bound PDGF is an issue that has received limited attention, but based on the enigmatic lack of 29-kD PDGF in this study and studies of wound healing, clearly requires further examination.

There is biological precedent for larger molecular mass forms of PDGF. Although the data from the present study must be considered preliminary, based on the limited characterization of this peptide, PDGF produced by simian sarcoma virus transformed cells is a dimer of p^{28} sis, *i.e.*, 56 kD (52). In addition, higher molecular mass forms have been identified among macrophage secretory products as well as from other

cell types (43, 53–55). Moreover, wound fluid was also noted to contain an ~ 38-kD PDGF-related peptide (37). Discerning the relationship of the 38-kD lavage peptide to these other forms of PDGF awaits further purification and characterization of the relevant moieties.

Clinical implications. Based on the results of the present study, it is attractive to hypothesize that intraalveolar PDGF contributes to the evolution of intraalveolar fibrosis in patients after acute lung injury. However, there is a fundamental problem with this simple formulation. Although nearly every patient studied had markedly elevated concentrations of PDGF and the other yet uncharacterized bioactivities in their airspace, only half of the patients die with alveolar fibrosis. It remains unclear whether the survivors never develop extensive intraalveolar fibrosis, or whether in this patient population the process develops and then regresses. This raises the important question as to whether persistently elevated levels of trophic factors for mesenchymal cells are required for progressive intraalveolar fibrosis and whether it is those patients who go on to obliterate their airspaces. Follow-up studies in which patient outcome is correlated with serial measurements of growth factor concentration may help clarify this issue.

An alternative pathogenetic sequence supported by both morphological data in patients as well as by study of model systems is that nearly all patients with severe lung injury do develop significant intraalveolar fibrosis (10–12). As a corollary therefore, it is only those patients capable of resolving the intraalveolar fibrosis and repairing the damaged alveolar wall that survive. Clearly the body has mechanisms to terminate a fibroproliferative response, as evidenced by the anatomic precision of integumentary wound healing. In contrast, regression of established fibrosis is less well established as a biological process. One paradigm for resolution of fibrosis derives from events during ontogeny, where regression of entire structures is routinely accomplished by the developing fetus. Viewed within this framework, regression of the granulation tissue within the airspace could, in principle, occur.

Acute lung injury is a devastating clinical syndrome, with an exceptionally high mortality. Survival occurs only if a rapid coordinated restoration of the alveolar wall ensues, permitting effective gas exchange to resume. The present study provides some insight into the elements of the alveolar microenvironment that may lead to intraalveolar granulation rather than alveolar repair. Demonstrating high concentrations of the potent mesenchymal cell growth factor and chemoattractant PDGF in the lower respiratory tract of patients with acute lung injury represents one important step toward establishing a causal role for this growth factor in the fibroproliferative process subverting effective lung repair.

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