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Obliterative bronchiolitis after lung transplantation: A fibroproliferative disorder associated with platelet-derived growth factor

(organ transplantation/*in situ* hybridization)

MARSHALL I. HERTZ*†, CRAIG A. HENKE*, RAOUF E. NAKHLEH‡, KEITH R. HARMON*, WILLIAM A. MARINELLI*, JOAN M. K. FOX*, SPENCER H. KUBO*, SARA J. SHUMWAY§, R. MORTON BOLMAN III§, AND PETER B. BITTERMAN*

Departments of *Internal Medicine, ‡Laboratory Medicine and Pathology, and §Surgery, University of Minnesota Medical School and the University of Minnesota Heart and Lung Institute, Minneapolis, MN 55455

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ABSTRACT **Fibroproliferative disorders are characterized by accumulations of mesenchymal cells and connective tissue in critical locations, leading to organ dysfunction. We examined the role of platelet-derived growth factor (PDGF) in the pathogenesis of obliterative bronchiolitis, a fibroproliferative process that occurs after lung transplantation and results in small airway occlusion. Bronchoalveolar lavage fluid from obliterative bronchiolitis patients significantly stimulated fibroblast migration, whereas fluid from patient controls did not. Quantitation by radioligand binding assay demonstrated increased concentrations of PDGF in lavage fluid from obliterative bronchiolitis patients (patients, 104 ± 26.9 pM; controls, 8.4 ± 6.9 pM; $P < 0.01$). Heparin affinity, gel filtration, and Western blot analysis confirmed the presence of PDGF in lavage fluid. Immunohistochemical and *in situ* hybridization studies of histologic sections and bronchoalveolar lavage cells suggest that alveolar macrophages are one cellular source. Prospective evaluation of sequential bronchoalveolar lavage samples from a patient who developed obliterative bronchiolitis demonstrated markedly increased PDGF concentrations before the onset of irreversible airflow obstruction. These findings are consistent with a role for PDGF in the fibroproliferative changes observed in obliterative bronchiolitis.**

Fibroproliferative disorders, which include atherosclerosis, hepatic cirrhosis, and pulmonary fibrosis, are a major cause of death worldwide. For example, in the United States and Poland between 1984 and 1989, fibroproliferative disorders accounted for 45% of annual mortality, representing in the aggregate the single most frequent cause of death (1). These disorders are all characterized by accumulations of mesenchymal cells and their connective tissue products in critical anatomic locations, leading to organ dysfunction. This anatomic kinship has stimulated several related investigations of the molecular pathogenesis of the fibroproliferative process. Among these are studies examining atherosclerosis and pulmonary fibrosis, which indicate that the potent modulator of mesenchymal cell function, platelet-derived growth factor (PDGF), is closely associated with the presence of established disease (2–14). However, the disorders studied to date have protracted kinetics, which span years to decades. This has limited investigators to examination of the fibroproliferative disease process long after the characteristic anatomic changes have occurred, making inferences about the pathogenetic role of PDGF less certain. Our research has focused on a fibroproliferative process occurring in lung transplant recipients, obliterative bronchiolitis, which results in the occlusion of small airways (15–17). It affects up to 30% of recipients and is frequently fatal. This disease process represents a very useful model for examining the molecular pathogenesis of fibroproliferative disorders. It evolves over a dramatically compressed time period (i.e., weeks to months) and the site of disease can be safely and repetitively sampled. To exploit these advantages, we examined lower respiratory specimens obtained from lung transplant recipients with evolving and established obliterative bronchiolitis and from patient controls for the presence of PDGF protein product and mRNA.

METHODS

Patient Characteristics. Five heart–lung recipients and two lung recipients with obliterative bronchiolitis were studied (Table 1). Six of these patients have since died of the disease. In each case, the diagnosis of obliterative bronchiolitis was confirmed by biopsy and/or autopsy. Nine healthy lung transplant recipients (two lung only, seven heart–lung) undergoing routine surveillance bronchoalveolar lavage (BAL) and transbronchial biopsies served as controls. All controls were clinically well 2–40 months posttransplant, with stable pulmonary function tests [including FEV_1, FVC, and midexpiratory flow rate (FEF_{25-75})], no pathogenic microorganisms recovered from BAL fluid, and no evidence of rejection on transbronchial biopsies.

BAL and Processing of Lavage Effluent. To obtain samples from the distal lower respiratory tract, BAL was performed as described (18). Briefly, the flexible fiberoptic bronchoscope (Olympus BF P10) was wedged in a segmental bronchus. For each anatomic site studied, five 20-ml aliquots of 0.9% saline were instilled, with each aliquot gently suctioned prior to instillation of the next. Two or three anatomic sites were sampled in each patient. Lavage effluent volume was 40–70% of the instilled volume in each instance. Recovered cells were separated from lavage fluid by centrifugation (200 × *g*; 10 min), and cell-free lavage fluid not immediately used was aliquotted and stored at −70°C until used.

Identification and Quantitation of PDGF in BAL Fluid. To identify and quantify PDGF in BAL fluid, three independent assay systems were used; a bioassay to detect fibroblast migration-promoting activity, a radioligand binding assay, and an immunoassay. In addition, Western blot analysis was carried out to confirm the presence of PDGF in BAL fluid.

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Abbreviations: PDGF, platelet-derived growth factor; BAL, bronchoalveolar lavage.

†To whom reprint requests should be addressed at: Division of Pulmonary and Critical Care Medicine, University of Minnesota Hospital, Box 276, 420 Delaware Street S.E., Minneapolis, MN 55455.

Table 1. Characteristics of patients with obliterative bronchiolitis

Patient	Sex	Age, yr	Pretransplant diagnosis	Time from transplant to onset of OB	Confirmation of diagnosis	Outcome
1	M	30	PPH	14 months	Transbronchial biopsy; autopsy	Died of OB
2	F	33	CHD	24 months	Transbronchial biopsy; autopsy	Died of OB
3	F	9	PPH	14 months	Transbronchial biopsy; autopsy	Died after retransplantation
4	M	34	PPH	5 months	Open lung biopsy	Died of OB while waiting for retransplantation
5	F	54	AAT	8 months	Open lung biopsy; autopsy	Died of OB while waiting for retransplantation
6	F	28	HX	5 months	Transbronchial biopsy; autopsy	Died of RSV pneumonia while waiting for retransplantation
7	F	48	PPH	19 months	Transbronchial biopsy	Alive

PPH, primary pulmonary hypertension; CHD, congenital heart disease; AAT, α_1-antitrypsin deficiency; OB, obliterative bronchiolitis; HX, histiocytosis X; RSV, respiratory syncytial virus.

Fibroblast migration assay. To detect fibroblast migration-promoting bioactivity in lavage fluid, a modified Boyden chamber apparatus (Nuclepore) was used as described (19). Data are expressed as chemotactic units (1 unit = reciprocal of the dilution giving 50% of the fibroblast migration in response to 165 pM PDGF). For studies of anti-PDGF antibody neutralization, a polyclonal goat anti-human PDGF AB antibody (Collaborative Research) was used exactly as described by the manufacturer. For every 5 ng of PDGF per ml of fluid, 5 mg of antibody was used to neutralize bioactivity.

Radioligand binding assay. A radioligand binding assay to detect PDGF was carried out as described (20, 21). To concentrate and desalt the lavage fluid before assay, ≈20 ml from each individual was frozen, lyophilized, dissolved in acetic acid (0.1 M), and dialyzed against that solution. Dialyzed samples were lyophilized and resuspended in binding buffer. Losses of PDGF during this handling procedure ranged from 29% to 36%. Approximately 3% 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.

Immunoblot analysis. To analyze BAL fluid for the presence of PDGF-related peptides, immunoblot analysis was carried out as described (20). The intensity of color was quantified by videodensitometry (22), and the quantity of PDGF in a test fluid was calculated by comparison to a standard curve (BB homodimer, 30–530 pM; Collaborative Research).

Biochemical analysis of PDGF. To analyze lavage fluid for PDGF, 150 ml obtained from patient 3 was applied to a heparin-Sepharose column (Pharmacia) preequilibrated with buffer (0.05 M NaCl/0.01 M Tris·HCl, pH 7.4). The column was developed with 1.0 M NaCl. Eluted proteins were applied to a gel-filtration column (Superose 12; 10 × 300 mm; Pharmacia) preequilibrated with buffer [phosphate-buffered saline (pH 7.4) containing 10% acetonitrile and 0.1% trifluoroacetic acid], and developed with that buffer at 0.5 ml/min. Fractions were dialyzed against 0.1 M acetic acid, evaporated to dryness (Speed Vac concentrator; Savant), redissolved in 0.1 M acetic acid, and neutralized with 1 M NaOH before detection of PDGF by immunoblot analysis.

Western blot analysis. PDGF-related peptides were identified immunologically by Western blot analysis as described (20) with minor modifications. Primary antibody was goat anti-human PDGF AB (Collaborative Research) and secondary antibody was biotinylated donkey anti-goat IgG. Blots were incubated with streptavidin conjugated to alkaline phosphatase (GIBCO/BRL) and color was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (GIBCO/BRL).

Localization of PDGF mRNA and Peptide in Histologic and Cytologic Specimens. *Immunohistochemical staining for PDGF.* Immunohistologic staining was performed according to the method of Hsu *et al.* (23). Anti-PDGF antibody (anti-AB; Collaborative Research) was applied to formalin-fixed, paraffin-embedded tissues or to cytospin preparations of BAL cells (Shandon; 150,000 cells per slide). Appropriate positive and negative controls were stained simultaneously with the test specimens. Slides were counterstained with hematoxylin.

In situ *hybridization. In situ* hybridization of deparaffinized histologic sections and cytospin preparations was carried out using minor modifications of the methods of Haase (24) and Lawrence and Singer (25). To better preserve cellular morphology and allow for appropriate noncomplementary controls (i.e., sense), oligonucleotides were used. Both sense and antisense oligonucleotide probes from exon 2 of the B chain of PDGF were synthesized (TAT GAG ATG CTG AGT GAC CAC TCG ATC CGC) and end-labeled with ^{35}S-labeled nucleotide precursors to 5–10 × 10^9 dpm per μg of DNA. Pretreatment used 0.2 M HCl for 30 min, followed by 0.2 M

Table 2. BAL findings

Patients	Months posttransplant	RBC per μl	WBC per μl	% PMN	% AM
OB					
1	14	46	56	51	48
2	24	59	65	2	97
3	14	38	505	34	57
4	3.5	38	440	14	69
	4.0	NA	NA	NA	NA
	4.5	8	212	NA	NA
5	8.5	43	420	71	26
6	6.5	1730	1410	40	60
Mean ± SD		280 ± 592	444 ± 463	35 ± 32*	
Median		43	420	37	
Controls					
Mean ± SD		84 ± 94.4	220 ± 146	1.1 ± 1.5	
Median		30.5	169	1	

OB, obliterative bronchiolitis; RBC, erythrocytes; WBC, leukocytes; PMN, polymorphonuclear cells; AM, alveolar macrophages; NA, not available.
*$P < 0.01$ compared to controls.

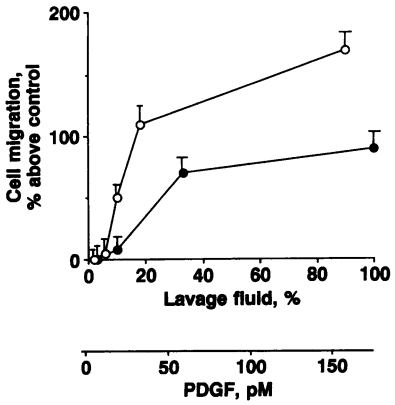

FIG. 1. Fibroblast migration assay of BAL fluid shown as cell migration in response to serial dilutions of lavage fluid (●). Also shown is fibroblast migration in response to the positive control, PDGF (BB homodimer) (3–165 pM) (○). Fibroblast migration toward medium alone (negative control) was 108 ± 63 cells per field ×45.

Tris·HCl in 0.1 M glycine solution for 15 min. Slides were hybridized (24 h; 42°C), treated with S1 nuclease (1 h; 22°C), washed, and stained with hematoxylin and eosin.

Statistical Analysis. Descriptive statistics included determination of means ± SD and medians. Comparison of data from obliterative bronchiolitis patients and patient controls was carried out using a nonparametric test of significant differences (Mann–Whitney test for independent samples), as the small sample sizes raised questions regarding the normality of data distribution.

RESULTS

BAL Findings. BAL fluid nucleated cell counts and leukocyte differentials in control subjects were similar to those reported in normal individuals (26). BAL samples from patients with obliterative bronchiolitis tended to contain more nucleated cells than those obtained from control subjects (median, 420 for obliterative bronchiolitis patients and 169 for controls; not significant) (Table 2). Strikingly, leukocyte differential counts from patients with obliterative bronchiolitis contained markedly increased proportions of neutrophils (mean ± SD, 35% ± 32% for obliterative bronchiolitis patients and 1.1% ± 1.5% for controls; $P < 0.01$).

Fibroblast Chemoattractant Bioactivity. Cell-free lavage fluid from patients with obliterative bronchiolitis ($n = 3$) significantly stimulated fibroblast migration (Fig. 1). The bioactivity was markedly reduced (>50%) by the addition of a polyclonal neutralizing anti-PDGF antibody. In contrast, similar to results reported in normal individuals (27), specimens obtained from controls ($n = 2$) did not induce significant fibroblast migration.

Quantitation of PDGF in BAL Fluid. Biologically significant concentrations of PDGF were detected by radioligand binding assay of BAL fluid from patients with obliterative bronchiolitis (Fig. 2). In affected patients ($n = 5$; Table 1), PDGF concentrations ranged from 66 to 130 pM (mean, 104 ± 26.9 pM), while in control samples ($n = 9$) little or no PDGF was detected (mean, 8.4 ± 6.9 pM; $P < 0.01$). Immunoblot analysis of lavage fluid was carried out to confirm the radioligand binding data. Consistent with the binding studies, no PDGF was detected in lavage fluid from controls. However, analysis of the lavage fluid from obliterative bronchiolitis patients revealed a marked discrepancy between the concentrations of PDGF estimated by the two assays; values obtained by immunoassay were as much as 10-fold lower than those obtained by the radioligand binding assay. One possible explanation was that the recovered PDGF was complexed to

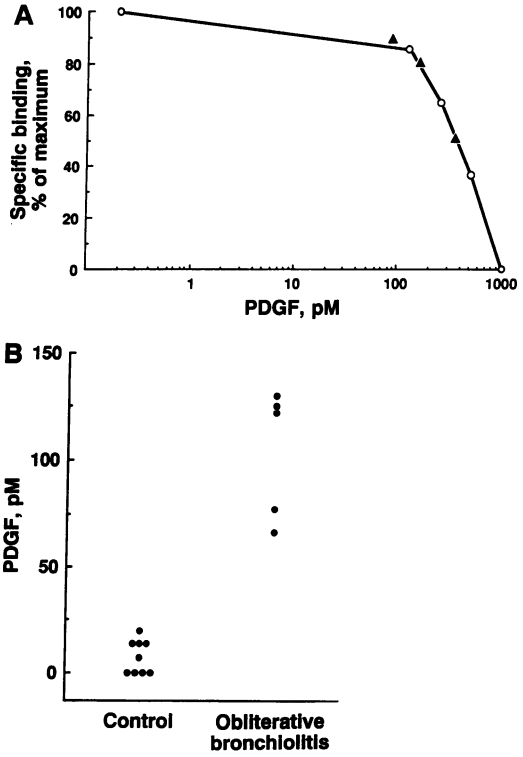

FIG. 2. Radioligand binding assay of BAL fluid for PDGF. To obtain samples from lower respiratory tract, BAL was performed as described (18). Recovered cells were separated from lavage fluid by centrifugation (200 × *g*; 10 min), and cell-free lavage fluid was stored at −70°C. (*A*) Shown is specific binding of ^{125}I-labeled PDGF to skin fibroblasts after incubation with decreasing concentrations of lavage fluid from a patient with obliterative bronchiolitis (▲) compared to specific binding after incubation with PDGF (BB homodimer; Collaborative Research) (○). (*B*) Shown are lavage fluid PDGF concentrations for healthy patient controls ($n = 9$) and for patients with obliterative bronchiolitis ($n = 5$).

a protein in lavage fluid, preventing high-affinity binding to the anti-PDGF antibody. Therefore, we used chromatographic methods to separate PDGF from potentially interfering proteins prior to immunoassay.

Biochemical Analysis of BAL Fluid for PDGF. Sufficient lavage fluid was available from patient 5 to analyze biochemically for PDGF. The procedure used took advantage of the high affinity of PDGF for heparin (28) and the ability of acid conditions to dissociate PDGF from putative binding proteins (29, 30). Heparin-Sepharose affinity chromatography fol-

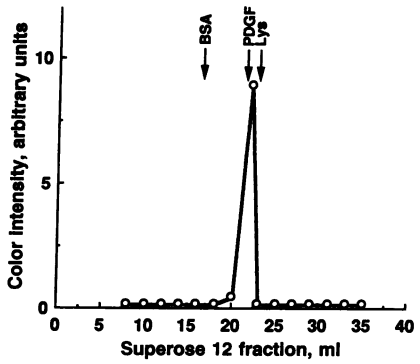

FIG. 3. Biochemical analysis of BAL fluid for PDGF. Shown is immunoblot reactivity of each column fraction quantified by videodensitometry. Also shown are elution positions of molecular mass standards [bovine serum albumin (BSA), 65 kDa; PDGF (BB homodimer), 29 kDa; lysozyme (Lys), 15 kDa].

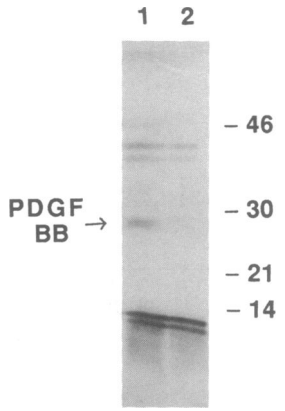

FIG. 4. Western blot analysis of heparin-binding lavage proteins. Shown are immunoreactive peptides in lavage fluid from patient 7 that bound to heparin-Sepharose. The 14- and 38-kDa peptides appear as doublets in both lanes. To be certain that 29- to 30-kDa dimeric PDGF could be detected in lavage fluid if present, 10 ng of PDGF BB homodimer was added to the lavage sample (lane 1) and the sample was processed in parallel with unaltered lavage fluid (lane 2).

lowed by acid gel filtration revealed immunodetectable PDGF-related material, eluting between a radiolabeled PDGF standard (29 kDa) and lysozyme (15 kDa) (Fig. 3). Quantitative analysis revealed close agreement between the immunoblot and radioligand binding assays.

Western Blot Analysis. To further characterize the PDGF-related peptides in lavage fluid, Western blot analysis of heparin-binding lavage proteins was carried out. Two major bands of immunoreactivity were identified (Fig. 4). The majority of the PDGF immunoreactive material was found at 14 kDa. In addition, a second band of immunoreactivity was identified at 38 kDa. Immunoreactivity at 29 kDa, typically associated with PDGF originating from platelet α granules, was not observed but could be detected when added to lavage fluid (lane 1).

Localization of PDGF mRNA and Protein Product. *Immunohistochemical staining for PDGF.* Immunohistochemical evaluation of lung tissue from patients with obliterative bronchiolitis identified two regions in which PDGF was present (Fig. 5). First, mononuclear cells within and adjacent to inflamed bronchiolar walls contained PDGF. Second, PDGF was observed along the bronchiolar epithelial brush border in several areas. This signal appeared to be localized mainly over the apical surface of bronchiolar epithelial cells. No signal was observed in biopsies from patient controls.

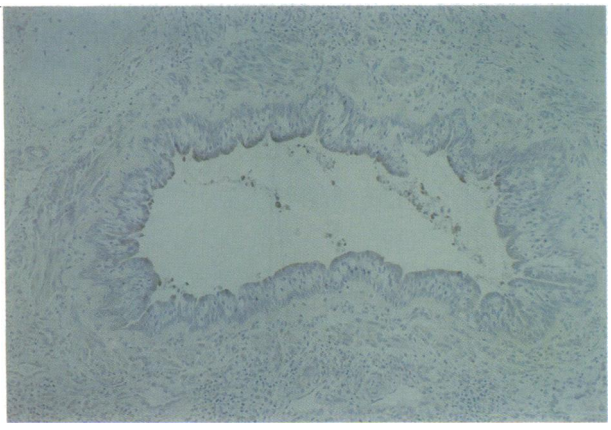

FIG. 5. Immunohistochemical staining of a representative section from an autopsy specimen. Shown is low-power view of bronchiole demonstrating staining in the apical portion of bronchiolar epithelial cells. Staining is also seen in mononuclear cells within the bronchiolar lumen. (×80.)

In situ *hybridization.* PDGF B-chain mRNA was identified in cells with the appearance of mononuclear phagocytes within alveolar septae (Fig. 6*A*). No signal was observed in biopsies from patient controls. Sequential histologic sections probed with sense oligonucleotide probes failed to demonstrate a positive signal, confirming the specificity of the signal observed (Fig. 6*B*). Due to the intensity of the signal observed in the patients with obliterative bronchiolitis, definitive identification of the positive cells as mononuclear phagocytes *in situ* was not possible. To address this issue, two additional approaches were used. First, examination of sequential histologic sections confirmed that most positive cells had the morphologic characteristics of mononuclear phagocytes (data not shown). Second, *in situ* hybridization of recovered BAL cells from a patient in whom >95% of cells were alveolar macrophages confirmed the presence of PDGF B-chain mRNA (Fig. 6 *C* and *D*). No signal was observed in bronchiolar epithelium of obliterative bronchiolitis patients or controls.

Clinical Correlation. To be plausibly involved in the pathogenesis of obliterative bronchiolitis, PDGF concentrations should increase before deterioration of pulmonary function. To examine this relationship, we compared PDGF concentrations in serial BAL samples from one patient (patient 4) before and after the onset of obliterative bronchiolitis. Before the onset of obliterative bronchiolitis, pulmonary function improved and remained stable with minor fluctuations for 4

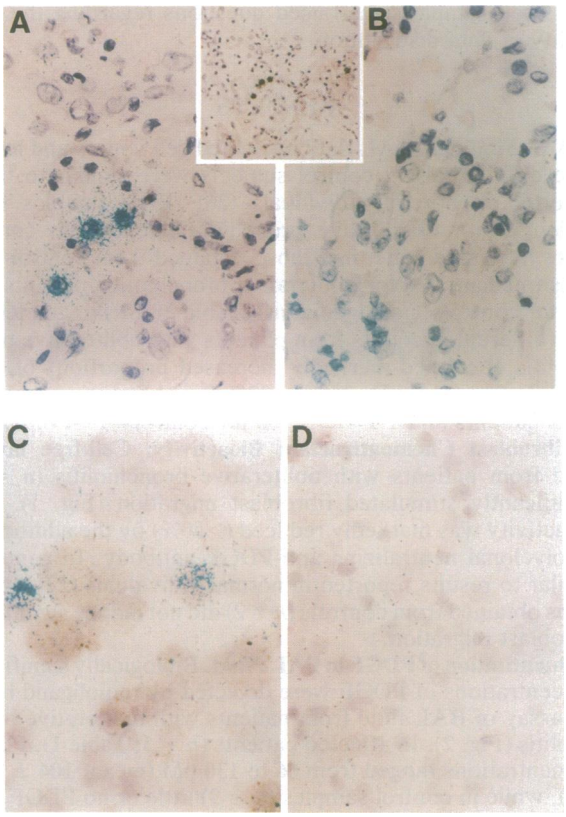

FIG. 6. *In situ* hybridization of autopsy specimen. (*A*) PDGF B-chain mRNA is observed in mononuclear phagocytes in an inflamed area within an alveolar septum. (*B*) Control of the same area using a sense oligonucleotide probe. (*Inset*) Low-power view of the same area. (*C*) Hybridization of recovered BAL cells from patient 4 (lavage differential, 86% macrophages) demonstrates PDGF mRNA. (*D*) Cells from the same lavage using a sense oligonucleotide probe show the absence of signal. Specimens were photographed by a combination of polarized light epiluminescence and bright-field illumination. (*A* and *B*, ×180; *Inset*, ×35; *C* and *D*, ×270.)

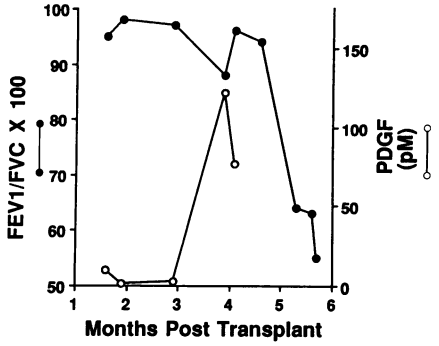

FIG. 7. Temporal relationship between lavage fluid PDGF concentration and physiological dysfunction. Irreversible airflow obstruction due to obliterative bronchiolitis developed 5 months after transplantation. Shown are serial pulmonary function test results ($FEV_1/FVC \times 100$; ●) and BAL fluid PDGF concentrations (pM) (○) after transplantation.

months (Fig. 7). PDGF concentrations in three serial surveillance BAL specimens during this time period remained in the range of controls. When symptoms of cough and exertional breathlessness occurred, two additional BAL specimens were obtained. Consistent with the hypothesis, these specimens, obtained 1 month before the development of irreversible airflow obstruction, demonstrated marked increases in PDGF concentration (122 and 77 pM).

DISCUSSION

Obliterative bronchiolitis results from a poorly controlled fibroproliferative response in which mesenchymal cells and their connective tissue products accumulate in the bronchiolar lumina, resulting in progressive airflow obstruction and graft failure. Our results indicate that biologically significant quantities of PDGF can be recovered from the airways of affected patients but not from their healthy counterparts. Biochemically, the PDGF-related peptides observed were similar to those identified after acute lung injury and during wound healing (20, 31). In addition, immunohistochemical and *in situ* hybridization studies have demonstrated PDGF-related peptides and mRNA in the lower respiratory tract. Moreover, PDGF appeared at the site of disease before the onset of permanent physiologic derangements. These findings are consistent with a role for PDGF in the pathogenesis of obliterative bronchiolitis.

Our findings support the concept that macrophages are one source of PDGF in obliterative bronchiolitis in accord with findings in other fibroproliferative processes (4, 31–33). Immunohistochemical staining also demonstrated PDGF at the apical surface of bronchiolar epithelial cells in some regions. This finding is consistent with results demonstrating PDGF mRNA in alveolar epithelial cells in specimens from patients with idiopathic pulmonary fibrosis (33), suggesting that epithelial cells are capable of producing a PDGF-related peptide. Although we did not observe PDGF mRNA in bronchiolar epithelial cells, additional *in situ* hybridization studies are being carried out to examine the possibility that PDGF gene expression by epithelial cells occurs earlier in the temporal evolution of the obliterative bronchiolitis lesion. Identifying the cellular sources of PDGF in obliterative bronchiolitis will be necessary for development of preclinical models and effective therapeutic strategies.

PDGF has been implicated in the pathogenesis of several nonmalignant fibroproliferative diseases including atherosclerosis, idiopathic pulmonary fibrosis, and acute lung injury (2–4, 20). Studies of obliterative bronchiolitis provide a unique opportunity to help clarify the role of PDGF in a fibroproliferative disease that is amenable to safe, repeated sampling of the affected structures before the onset of manifest disease. These results also provide a strong rationale for the development of preclinical model systems to examine the possibility that inhibition of PDGF release/action will interdict the fibroproliferative process in obliterative bronchiolitis and perhaps other disorders as well.

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