Emphysematous Lesions, Inflammation, and Fibrosis in the Lungs of Transgenic Mice Overexpressing Platelet-Derived Growth Factor

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Because of its expression pattern and its potent effects on mesenchymal cells, platelet-derived growth factor (PDGF) has been implicated as an important factor in epithelial-mesenchymal cell interactions during normal lung development and in the pathogenesis of fibrotic lung disease. To further explore the role of PDGF in these processes, we have developed transgenic mice that express the PDGF-B gene from the lung-specific surfactant protein C (SPC) promoter. Adult SPC-PDGFB transgenic mice exhibited lung pathology characterized by enlarged airspaces, inflammation, and fibrosis. Emphysematous changes frequently occurred throughout the lung, but inflammation and fibrotic lesions were usually confined to focal areas. The severity of this phenotype varied significantly among individual mice within the same SPC-PDGFB transgenic lineage. A pathology similar to that observed in adult mice was noted in lungs from transgenic mice as young as 1 week of age. Neonatal transgenic mice exhibited enlarged saccules and thickened primary septa. Results of these studies indicated that overexpression of PDGF-B induced distinct abnormalities in the developing and adult lung and led to a complex phenotype that encompassed aspects of both emphysema and fibrotic lung disease. (*Am J Pathol* 1999, 154:1763-1775)

Platelet-derived growth factor (PDGF) is a polypeptide growth factor that has pleiotropic effects on a number of cell types, particularly those of mesenchymal origin. PDGF was originally characterized as the predominant mitogen in serum.¹ Serum-derived PDGF originates in megakaryocytes and is stored in the α granules of platelets. PDGF was subsequently found to be produced by a variety of cell types, including macrophages and epithelial, endothelial, mesenchymal, and neuronal cells.²

PDGF has been demonstrated to be essential for normal development^{3,4} and has been implicated in disease processes such as atherosclerosis and lung fibrosis.^{5,6} Active PDGF consists of two homologous subunits, A and B, which can form three dimeric PDGF isoforms (AA, AB, and BB).⁷⁻⁹ PDGF dimers bind to specific receptors that themselves are dimers formed from two types of subunits, α and β , that differentially bind PDGF isoforms.^{10,11} The $\beta\beta$ receptor dimer binds only PDGF-BB, the $\alpha\beta$ receptor dimer binds PDGF-BB and PDGF-AB, and the $\alpha\alpha$ receptor dimer binds all three types of PDGF dimers. PDGF receptors are found primarily on mesenchymal cells.¹² Normally, PDGF is produced at low levels in adult tissues *in vivo*, but its expression increases after injury or when cells are removed for culture.¹²

PDGF is expressed at low levels in normal adult lung but is up-regulated in human interstitial lung disease and in animal models of lung fibrosis.¹³ Macrophages from patients with idiopathic pulmonary fibrosis expressed higher levels of PDGF message and secreted increased amounts of PDGF compared to macrophages from normal lung.^{14,15} Elevated levels of PDGF-B message and immunoreactivity have been found in epithelial cells and macrophages of patients with idiopathic pulmonary fibrosis.⁵ In another study, PDGF-B chain mRNA was detected in epithelium and macrophages in lung sections from patients with idiopathic pulmonary fibrosis, but PDGF-B immunoreactivity was detected only in macrophages.¹⁶ Elevated PDGF-related peptides have been detected in lavage fluid from patients with Hermansky-Pudlak syndrome, a condition characterized by severe pulmonary fibrosis.¹⁷ In an animal model of pulmonary fibrosis, asbestos inhalation rapidly induced up-regulation of PDGF-A and -B genes in epithelial cells and macrophages at sites of asbestos fiber deposition.¹⁸

Because of its documented effects on mesenchymal cell proliferation and extracellular matrix production and its expression in human interstitial lung disease and ro-

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dent models of lung fibrosis, PDGF is likely to play an important role in fibrogenic lung injury. Numerous cytokines and growth factors are elaborated during the development of fibroproliferative lung disease. To determine whether fibrotic lung disease can be initiated by PDGF alone, we generated transgenic mice that express the PDGF-B gene from the lung-specific surfactant protein C (SPC) promoter. Here we report that SPC-PDGFB transgenic mice develop lung disease characterized by enlarged airspaces, which in some animals were distributed throughout the lung, as well as inflammation and fibrosis, which were generally more focal in nature. Our results indicate that PDGF has potent effects on lung growth and development and that its expression can cause lung pathology characterized by features of two distinct diseases, emphysema and lung fibrosis.

Materials and Methods

Transgenic Mice

The SPC-PDGFB DNA construct contained promoter sequences from the human SPC gene, 5' untranslated sequences from the rat insulin gene, and coding sequences from the human PDGF-B (*c-sis*) cDNA. To obtain human SPC promoter sequences, oligonucleotides were synthesized based on the published human SPC sequence¹⁹ and used to amplify a 550-bp fragment from the first exon of the SPC gene by polymerase chain reaction. The 550-bp SPC fragment was used as a probe to screen a human DNA library in the vector λ EMBL3, and clones that hybridized to the SPC probe were isolated. The 3.7-kb *HindIII-PstI* fragment that confers lung-specific expression in transgenic mice^{20,21} was isolated and cloned into a plasmid vector for further manipulation. The identity of this fragment was confirmed by restriction mapping and partial DNA sequencing. An intron-containing fragment from the rat insulin II gene was included in the SPC-PDGFB construct upstream from the PDGF-B cDNA. This fragment contains bases +6 to +177 relative to the transcription initiation site and encodes the transcription start site, the first exon and intron, and a portion of the second exon.²² The PDGF-B (*c-sis*) cDNA (clone pSM-1) was obtained from Dr. Lee Ratner (Washington University, St. Louis, MO). The PDGF-B cDNA was excised from pSM-1 with *PstI* and *XhoI* and cloned downstream of the SPC and insulin fragments. This 2.5-kb PDGF-B fragment contains from base +890 (relative to the transcription start site) to the polyadenylation sequence and poly A tail and includes the entire PDGF-B open reading frame.²³

Transgenic mice were generated by microinjection of the linear 6.9-kb SPC-PDGFB construct into fertilized B6SJLF2 one-cell mouse embryos as described.²⁴ SPC-PDGFB transgenic mice were identified by hybridization of tail biopsy DNA with a probe derived from human SPC promoter sequences. Transgenic mice were generated on a mixed genetic background of the C57BL/6 and SJL inbred strains, and transgenic lineages were maintained by breeding transgenic individuals to nontransgenic

B6SJLF1 hybrid mice. Mice were housed under specific pathogen-free conditions in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. No evidence of viruses or respiratory pathogens has been found in SPC-PDGFB mice or other mice from the facility in which the transgenic mice were housed. We have observed no inflammation in the lungs of nontransgenic mice or in mice carrying transgenes unrelated to PDGF.

RNA Analysis

RNA was prepared by ultracentrifugation of tissue homogenates through cesium chloride.²⁵ RNA was subjected to Northern blot analysis²⁶ with a probe from the human PDGF-B gene that hybridizes with both human and mouse PDGF-B RNA. Blots were subsequently hybridized with 36B4, a human cDNA encoding the ribosomal protein P0, as a loading control.^{27,28}

PDGF Enzyme Immunoassay

PDGF was measured in lung homogenates using a previously described enzyme immunoassay that detects isoforms containing the PDGF-B chain.^{29,30} Lungs were perfused with PBS (10 mmol/L sodium phosphate, pH 7.4, containing 140 mmol/L NaCl and 3 mmol/L KCl) via the right ventricle and then homogenized in 1 ml of PBS containing 1 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L EDTA, and 7 μ g/ml aprotinin. Homogenates were rapidly frozen and thawed and then centrifuged at $16,000 \times g$ for 10 minutes at 4°C. PDGF was measured in aliquots of supernatants by enzyme immunoassay. PDGF levels in lung homogenates were normalized to protein content as determined by Bradford assay (BioRad, Hercules, CA) with bovine serum albumin as a standard.

Histological and Immunohistochemical Analyses

For histological analysis, lungs were fixed by intratracheal instillation of 10% neutral buffered formalin for 10–30 minutes at room temperature, followed by fixation by immersion overnight at 4°C. Lungs were embedded in paraffin and sectioned at 5 μ m. Elastin staining was performed by the method of Miller, but Van Gieson counterstaining was omitted.^{31,32} Eosinophils were detected in tissue sections by the method of Luna.³³ Immunohistochemistry was performed by an immunoperoxidase technique similar to that recently described.³⁴ Briefly, lung sections were stained with PGF-007 (Mochida Pharmaceutical Co., Tokyo), a mouse monoclonal antibody directed against a peptide from the PDGF-B chain,^{6,35} at a concentration of 5 μ g/ml. Bound antibody was detected by sequential incubation of the sections with biotinylated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:4000, streptavidin-conjugated horseradish peroxidase (Jackson ImmunoResearch) diluted 1:2000, and diaminobenzidine chromagen.

Morphometric Analysis

Measurements were performed by an investigator who was unaware of the identity of the samples. For each mouse, one section from each of four lobes was analyzed to determine the percentage of each lung section that exhibited abnormal histology. Areas were measured using V150 software³⁶ (Oncor Imaging, Gaithersburg, MD) by tracing the perimeter of each section (total area) or by tracing histologically abnormal regions (abnormal area). Total area measurements were performed on images captured from an Olympus SZH dissecting microscope, whereas abnormal area measurements were performed on images captured from an Olympus BH-2 microscope using a 4X objective. For each mouse, the abnormal and total areas respectively from the four lobes were summed, and the percentage of the section areas that was abnormal was calculated.

Two parameters were measured in sections from neonatal transgenic and nontransgenic mice. Airspace area was measured to compare the size of the airspaces between the two groups of mice. A second parameter, which we have called septal chord length, was measured as an indication of the thickness of the septa. This is identical to the parameter called "airspace wall thickness" by previous investigators³⁷ and is derived by overlaying a grid of parallel horizontal lines over the image, measuring the lengths of all intersections between the grid lines and airspace walls, and repeating these steps with a vertical grid. Septal chord length increases with increasing septal thickness. For measurement of airspace area and septal chord length, neonatal lung sections were viewed with a 20X objective, and images from the lungs were digitized, converted to tagged image format file, and analyzed using public domain NIH Image software (downloaded from <http://rsb.info.nih.gov/nih-image/Default.html>). For each lung, five fields lacking large airways or blood vessels were analyzed. For measurement of airspace area, images were edited to remove airways and blood vessels, thresholded manually, made binary, and inverted. A procedure was performed to retain the airspaces while eliminating the remainder of the image (Onion, written by Robert Homer and available from <ftp://codon.nih.gov/pub/nih-image/contrib/ChordLength.SurfaceArea>). Airspace areas were then measured using the "Analyze Particles" function of NIH Image. Areas from five fields were pooled and averaged to generate a mean airspace area for each mouse. Values for five transgenic and five nontransgenic mice were then averaged to evaluate the influence of the transgene on airspace area. Septal chord length was used as an indication of the thickness of the primary septa. Septal chord length was measured similarly to alveolar chord length as described previously,³⁸ but the images were not inverted before analysis, so that the operations were performed on the airspace walls instead of the airspaces. Lengths from five fields were pooled and averaged to generate a mean septal chord length for each mouse. Values for five transgenic and five nontransgenic mice were averaged to evaluate the influence of the transgene on septal chord length.

Figure 1. SPC-PDGFB DNA construct. The SPC-PDGFB construct contains a 3.7-kb *HindIII-PstI* fragment from the 5' region of the human SPC gene (shaded) (see Ref. 20). Insulin sequences (solid) are derived from the rat insulin II gene and consist of bases +6 to +177.²² Thin line indicates intronic sequences that are removed during splicing. The PDGFB coding region (open) is contained on a *PstI-BamHI* fragment derived from human c-sis cDNA clone pSM-1 (see Ref. 58). This fragment contains the entire translated and 3' untranslated sequences, but lacks 5' untranslated sequences that were found to inhibit translation (see Ref. 23).

Results

Generation of Transgenic Mice Overexpressing PDGF

Expression of the PDGF-B gene was directed to the lung by placing PDGF-B coding sequences downstream from a 3.7-kb DNA fragment from the 5' region of the human SPC gene (Figure 1).¹⁹ This fragment has been previously demonstrated to direct expression of transgenes to distal lung epithelium, including type II alveolar epithelial cells and Clara cells of the terminal bronchioles.^{20,21} The PDGF-B cDNA fragment contains the entire PDGF-B open reading frame, 3' untranslated sequences, and polyadenylation site. An intron-containing fragment from the rat insulin II gene was placed upstream of the PDGF-B coding sequences because this fragment has been shown to increase the efficiency of expression of cDNA fragments in transgenic mice.^{22,39} Five founder mice that carried the SPC-PDGFB transgene shown in Figure 1 were generated. Three of the five founder mice transmitted the transgene to progeny; one founder mouse sired offspring but did not transmit the transgene to progeny; and one female founder, designated 49-6, became sick and was euthanized before it bore offspring.

Expression of the SPC-PDGFB transgene was analyzed in four of the transgenic lineages by Northern blot analysis of lung RNA with a human PDGF-B probe (Figure 2). The probe hybridizes with the endogenous mouse PDGF-B RNA by virtue of the close homology between the human and mouse PDGF-B genes. The size of the mouse PDGF-B mRNA is 3.5 kb, whereas the SPC-PDGFB transgene message is predicted to be approximately 2.7 kb because much of the 5' untranslated region of the human PDGF-B cDNA is not present in the transgene. Two of the lineages examined did not express detectable transgene message (lanes 1 and 3); they yielded results identical to those for nontransgenic mice (lanes 5 and 6). One founder mouse that did not produce any transgenic offspring exhibited hybridizing RNA species of higher molecular weight but no hybridizing band at the predicted size (lane 4). These larger RNAs may be produced by improper RNA processing or by integration of a truncated transgene that can form a hybrid RNA with sequences at the integration site. Because this founder mouse did not sire transgenic offspring, it was not possible to determine whether PDGF protein was produced

Figure 2. Expression of transgene message in lungs of SPC-PDGFB mice. Mouse lung RNA (20 μ g total RNA) was subjected to Northern blot analysis with a probe derived from the human PDGF-B gene (top) or 36B4 gene (bottom) to serve as a loading control. Arrow indicates the size of the endogenous mouse PDGF message that also hybridizes with the probe. Arrowhead indicates the expected size of the transgene message. Lanes are as follows: 1: 46-3 lineage, 2: 47-5 lineage, 3: 47-6 lineage, 4: 47-1 founder, 5: nontransgenic mouse, 6: nontransgenic mouse.

from these aberrant RNA species. In one lineage, designated 47-5, Northern blot analysis revealed a band of the predicted size that hybridized with the human PDGF-B probe (lane 2, arrowhead). Mice of this lineage also exhibited bands that were larger than the endogenous PDGF-B RNA; the identities of these RNA species are not known. Analysis of RNA from nine other organs in the 47-5 lineage indicated that lung was the only organ in which transgene message could be detected (not shown). In summary, Northern blot analysis revealed that one of four SPC-PDGFB lineages produced a PDGF-B RNA of the predicted size specifically in the lung. RNA from the 49-6 founder mouse, which became sick and was euthanized, was not subjected to Northern blot analysis.

Production of the PDGF-B polypeptide was examined in the 47-5 lineage, which expressed a full-length transgene message as judged by Northern blot analysis. PDGF levels in lung homogenates from SPC-PDGFB transgenic mice and nontransgenic littermates were measured by enzyme immunoassay.^{29,30} This assay measures PDGF isoforms containing PDGF-B chains of human or rodent origin. The results of the enzyme immunoassay indicated that PDGF was overexpressed approximately 20-fold in the lungs of SPC-PDGFB mice relative to nontransgenic mice (Figure 3). The concentration of PDGF in plasma was not different between SPC-PDGFB and nontransgenic mice, indicating that systemic PDGF levels were not increased in the transgenic mice (not shown).

Figure 3. PDGF levels in lungs of SPC-PDGFB mice. Lungs from SPC-PDGFB transgenic mice and nontransgenic control mice ($n = 4$ for each group) were homogenized, and PDGF in the homogenates was measured by an enzyme immunoassay that detects PDGF isoforms containing the B chain as described in Materials and Methods. The data are illustrated as means $\pm$ SEM. The amount of PDGF in transgenic lungs detected by this assay was significantly larger than that in nontransgenic lungs ($P < 0.05$, Student's *t*-test).

Lung Histology in SPC-PDGFB Mice

One of the SPC-PDGFB founder mice, 49-6, became cachectic, lethargic, and cyanotic starting at approximately 7 weeks of age. This mouse was euthanized at 8 weeks of age and its lungs were fixed for histological analysis. On gross examination, the lungs appeared larger than normal and had airspaces that were obviously enlarged. Compared to normal lungs (Figure 4A), histological sections of the transgenic mouse revealed a severe disruption in the architecture of the lung characterized by enlarged airspaces, inflammation, and focal areas of fibrosis (Figure 4, B-D). Normal alveoli were not present; instead, large emphysematous air spaces were observed throughout the lung. These large airspaces were bounded by septa that appeared thickened compared to normal alveolar septa. The lungs showed a prominent inflammatory process that was dominated by macrophages and eosinophils (Figure 4C; also shown in greater detail in Figure 5). Many airspaces were filled with macrophages containing phagocytosed red blood cells and cell debris (Figure 4C). Focal areas of fibrosis were also observed (Figure 4D).

Mice from the 47-5 lineage developed a similar pattern of lung disease, but in general were not as severely affected as the 49-6 founder mouse (Figure 5). Some mice exhibited pathology throughout the lung that was characterized by enlarged airspaces, inflammation, and fibrosis. (Figure 5A). Other mice developed fibrotic lesions with less pronounced airspace enlargement but more inflammation and fibrosis (Figure 5B). Inflammation of the lungs by macrophages and eosinophils was observed (Figure 5C and 5D). In many mice a less severe pathology was observed that was characterized by focal airspace enlargement surrounded by normal lung tissue

Figure 4. Lung pathology in the 49-6 founder transgenic mouse. All photographs are at the same magnification. **A:** Section of lung from a nontransgenic mouse showing normal alveolar structure. **B, C, and D:** Sections of lung from the 49-6 SPC-PDGFB founder mouse (2 months of age). **B** shows emphysematous airspaces with thickened septa. **C** shows airspaces filled with aggregates of inflammatory cells that include macrophages (arrows) and eosinophils (arrowhead) (see also Figure 5). **D** shows focal fibrotic lesions (arrows). Scale bars, 50 μ m.

(Figure 5E). These lesions were never observed in non-transgenic littermates. Lung sections of some transgenic mice exhibited no sign of the PDGF-induced pathology and appeared normal by light microscopy (Figure 5F). The most common phenotype observed in the 47-5 lineage was enlarged airspaces in a variable proportion of the lung. Inflammation and fibrotic lesions were less frequently visible. Although some mice exhibited severely diseased lungs histologically, all of the animals appeared outwardly healthy. We have observed no excess mortality in the 47-5 lineage through 12 months of age. These results indicated that constitutive expression of PDGF could induce lung pathology, but there was incomplete penetration of the phenotype induced by the SPC-PDGFB transgene in the 47-5 lineage.

A histological analysis of lungs from mice of different ages was performed to determine whether the transgene-induced lung disease progressed with age. Figure 6 shows the extent of disease in lung sections from mice of different ages. Significant lung pathology was found in a fraction of transgenic mice of most ages but not in non-transgenic mice. Transgenic mice ≤ 3 months of age appeared to fall into two groups: one in which the majority

of the lung was affected by PDGF-induced pathology, and one in which the lung was normal or mildly affected. In transgenic mice ≥ 6 months of age, all mice exhibited moderate to severe pathology, and no animals with normal lung histology were observed. These observations are consistent with the existence of two populations of mice that vary in the degree of PDGF-induced lung disease that develops. One population is susceptible to the effects of the transgene at an early age. The second population is resistant to transgene-induced disease at young ages, but the disease eventually develops in older mice.

Because some young transgenic mice did not develop lung pathology, immunohistochemical staining of PDGF-B was performed to determine whether the transgene was expressed throughout SPC-PDGFB mouse lungs. No staining was observed in sections from non-transgenic mice (Figure 7A) or in transgenic lungs if the anti-PDGF-B monoclonal antibody was replaced with the same concentration of normal mouse IgG (Figure 7B). In areas of transgenic lung that appeared histologically normal, PDGF-B immunostaining was detected in type II

Figure 5. Lung pathology in adult SPC-PDGFB mice from the 47-5 lineage. All sections are from the lungs of SPC-PDGFB transgenic mice of the 47-5 lineage. **A:** Section from a 3-month-old mouse with severe lung pathology. Enlarged airspaces, inflammation, and fibrosis can be seen. **B:** Fibrotic lesions (arrows) in a 5-month-old mouse with less pronounced airspace enlargement. **C:** Inflammatory infiltrate consisting primarily of macrophages (arrows) and eosinophils (arrowheads) in a 1-month-old mouse. **D:** Eosinophils (arrows) visualized by Luna's stain in same mouse as **C**. Nuclei stain blue and cytoplasm of eosinophils and erythrocytes stains red. **E:** Focal airspace enlargement in a 3-month-old mouse. This area of abnormal airspaces was surrounded by normal lung structure, some of which can be seen at bottom left. **F:** Two-month-old transgenic mouse in which the lung appeared normal by light microscopy. Scale bars, 50 μ m (**A**, **B**, **E**, and **F**); 12.5 μ m (**C** and **D**).

alveolar epithelial cells and more weakly in macrophages (Figure 7C). In areas of airspace enlargement, PDGF-B staining was observed in epithelial cells in thickened septa (Figure 7D). In fibrotic areas of diseased lung, PDGF-B staining was observed in epithelial cells, macro-

phages, and in cells within the thickened interstitium (Figure 7E). The staining observed in macrophages could represent endogenous PDGF-B expressed by activated macrophages or uptake of transgene-derived PDGF released from type II cells.

Figure 6. Extent of lung pathology in SPC-PDGFB mice from the 47-5 lineage as a function of age. Lung sections from SPC-PDGFB and nontransgenic littermates were analyzed as described in Materials and Methods to determine the percentage of area of the sections that was abnormal. Each symbol corresponds to measurements from an individual mouse. Solid squares represent transgenic mice; open circles represent nontransgenic mice. Abnormalities in the transgenic mice were predominantly emphysematous and fibrotic lesions. Abnormalities in nontransgenic mice were attributed to fixation or sectioning artifacts and did not resemble the lesions in the transgenic mice. The data at each age were analyzed by the Mann-Whitney *U* test to determine whether the transgenic mice had a higher percentage of lung section area that exhibited abnormal histology. *P* values for transgenic versus nontransgenic mice for the different ages were: 2 weeks, *P* = 0.13; 1 month, *P* = 0.06; 2 months, *P* = 0.35; 3 months, *P* = 0.1; 6 months, *P* = 0.05; >10 months, *P* = 0.01. The lack of diseased lungs in 2-month-old transgenic mice is due to sampling error; we have observed other mice of this age (not included in the quantitative analysis) that had severe lung pathology.

Developmental Abnormalities in SPC-PDGFB Mouse Lungs

In our initial histological analysis, we observed a transgenic mouse that developed lung pathology by 2 weeks of age (Figure 6). This observation suggested that mice with early-onset PDGF-induced lung disease would exhibit abnormalities in lung development. We therefore analyzed lung tissue from 1-week-old and neonatal mice. Lungs from four of seven 1-week-old transgenic mice were abnormal and exhibited a pathology similar to that observed in adult mice, including enlarged airspaces, inflammation, and fibrosis (Figure 8B). Lungs from five of six neonatal SPC-PDGFB mice were abnormal, but the pathology differed from that in older mice. Lungs of neonatal mice are in the saccular stage of development, with the air sacs divided by primary septa. During postnatal days 4–14, secondary septal growth occurs to divide the saccules into adult-type alveoli. SPC-PDGFB mice appeared to have enlarged saccules and thickened primary septa but no inflammation was observed (Figure 8D). In contrast to older mice, in which airspace enlargement was heterogeneously distributed in the lung, neonatal mice appeared to have uniformly enlarged airspaces. To examine these changes quantitatively, morphometric measurements were made to compare abnormal lungs from neonatal SPC-PDGFB mice with lungs from nontransgenic littermates. Airspace area and septal chord length, a parameter that increases with septal thickness, were significantly increased in the transgenic mouse lungs (Figure 9). These results indicated that in transgenic mice that were susceptible to PDGF-induced disease, transgene expression affected lung development so that an abnormal lung structure was present at the time of birth.

Other transgenic models that develop enlarged airspaces have been generated.^{3,40} The defect in some of these mice was found to derive from a failure in secondary septal growth so that normal alveoli did not develop. Because elastin fibers appear to be crucial to this process and are disrupted in PDGF-A knockout mice³ and in emphysematous lungs, elastin staining was performed. This staining revealed the normal appearance of elastin fibers at the tips of secondary crests in both nontransgenic (Figure 8E) and transgenic (Figure 8F) mice. There did not appear to be a generalized disruption of alveolarization as has been described in PDGF-A knockout mice³ and in transgenic mice expressing transforming growth factor- α .⁴⁰ These results indicated that the process of alveolarization can occur in SPC-PDGFB mice and that inhibition of this process is not likely to explain the emphysematous lesions observed in the older transgenic mice.

Discussion

A correlation between PDGF expression and lung fibrosis has been established both in human disease and in animal models. Our strategy in the present study was to manipulate the expression of PDGF in the lung to determine the potential of this factor to produce pathology associated with pulmonary fibrosis, because PDGF could be a potent mitogen for lung fibroblasts that proliferate during fibrogenesis. We therefore hypothesized that one effect of PDGF overexpression in distal lung epithelium would be lung fibrosis. Indeed, our SPC-PDGFB mice developed localized areas of fibrosis that appeared to exhibit increased cellularity and extracellular matrix deposition. However, the focal nature of the fibrosis suggested that PDGF overexpression by itself could not produce fibrosis, but that additional events were required to trigger the fibrotic phenotype. In SPC-PDGFB mice, inflammation was always observed within or adjacent to fibrotic regions. This observation is consistent with findings from human interstitial lung disease and in other animals models, in which inflammation appears to be a necessary event in the development of pulmonary fibrosis. Previous studies in which different strategies were used to increase PDGF levels in the lungs of rats reported apparently more severe fibrosis than we observed in SPC-PDGFB transgenic mice.^{41,42} Expression of PDGF-B from an adenoviral vector delivered intratracheally was reported to induce fibrosing alveolitis.⁴² Intratracheal instillation of recombinant human PDGF-BB into rats produced prominent fibrosis that was concentrated around larger airways and blood vessels.⁴¹ The anatomical distribution of this fibrosis is likely to be caused by the method of delivery of the protein. The difference between these results and ours may be explained by the route of administration, the dose of PDGF to which the lungs were exposed, or compensatory changes in PDGF receptor expression or signaling that may have occurred in our transgenic mice because of constitutive PDGF overexpression.

Figure 7. Immunohistochemical analysis of PDGF-B expression. Lung sections were stained with the PGF-007 anti-PDGF-B monoclonal antibody as described in Materials and Methods. **A:** Section of lung from a nontransgenic mouse stained with anti-PDGF-B. No staining is present. **B:** Section of lung from a 1-month-old SPC-PDGF-B transgenic mouse stained with normal mouse IgG. No staining is present. **C, D, and E:** Sections of the same mouse as in **B** stained with anti-PDGF-B. **C:** Area of normal lung histology showing staining in type II cells (arrows) and macrophages (arrowhead). **D:** Emphysematous area showing staining in septal epithelial cells (arrows). **E:** Fibrotic area showing staining in epithelial cells (large arrow), macrophages (small arrow), and cells within the thickened interstitium (arrowhead). Scale bars, 12.5 μ m.

In addition to fibrosis, SPC-PDGF-B mice developed enlarged airspaces. This phenotype was observed in both neonatal and adult mice but was different in appearance at these two stages. At birth, the lungs of some SPC-PDGF-B mice exhibited enlarged saccules and thickened primary septa. Airspace area in these mice was increased 62% over that in nontransgenic mice, and the increase appeared to be uniformly distributed through the lung. In adult SPC-PDGF-B mice, emphysematous areas containing extremely enlarged airspaces

were observed, but in most mice these were heterogeneously distributed and were interspersed with areas of normal lung architecture. The mechanisms by which PDGF-B expression produced this emphysema-like phenotype are not known. PDGF-B expression did not appear to affect the process of alveolarization, in that septal crests with elastin fibers concentrated at their tips were readily observed in lungs from 1-week-old SPC-PDGF-B mice. Emphysema in human lungs appears to arise from disturbances in the balance between proteases and pro-

Figure 8. Developmental lung pathology. **A:** One-week-old nontransgenic mouse. **B:** One-week-old SPC-PDGFB transgenic mouse. **C:** Neonatal nontransgenic mouse. **D:** Neonatal transgenic mouse. **E:** One-week-old nontransgenic mouse, elastin stain. Septal crests with concentrated elastin staining are indicated by arrows. **F:** One-week-old transgenic mouse. Septal crests with concentrated elastin staining are indicated by arrows. Scale bars, 25 μ m (**A** and **B**); 50 μ m (**C** and **D**); 12.5 μ m (**E** and **F**).

tease inhibitors. An imbalance leading to the proteolysis of extracellular matrix, particularly elastin fibers, causes a destruction of alveolar tissue and results in emphysema. These observations raise the possibility that proteolysis of elastin fibers may contribute to the emphysematous phenotype in SPC-PDGFB mice. This possibility is consistent with studies *in vitro* that have demonstrated the up-regu-

lation of protease expression by PDGF-BB in some cell types.⁴³⁻⁴⁵

Airspace enlargement has been a commonly observed phenotype in transgenic mice with pulmonary manipulation of cytokine and growth factor expression. Mice deficient in PDGF-A chain,³ mice overexpressing TGF- α from the SPC promoter (SPC-TGF- α mice),^{40,46} and mice over-

Figure 9. Morphometric measurements from SPC-PDGFB neonatal lungs. Mean airspace area (A) and septal chord length (B) were measured in neonatal lungs from transgenic and nontransgenic mice ($n = 5$ for each) as described in Materials and Methods. The data are illustrated as means $\pm$ SEM. Both airspace area and septal chord length were significantly increased in the lungs from the transgenic mice (Student's t -test).

expressing interleukin 11 from the Clara cell secretory protein promoter (CCSP-IL-11 mice)^{38,47} all developed enlarged airspaces as a result of these genetic manipulations. In all of these cases, the enlarged airspaces have been documented to occur as a result of abnormalities in lung development. The lungs of PDGF-A knockout mice were normal at birth but developed enlarged airspaces over the first 2 postnatal weeks as a result of failed secondary septation.³ Neonatal lungs from SPC-TGF- α transgenic mice appeared to have slightly enlarged sacculi and developed dramatically enlarged airspaces

because the growth of secondary septa did not occur.⁴⁰ CCSP-IL-11 mice in which the transgene was expressed throughout lung development exhibited airspace enlargement, but mice in which IL-11 expression was not induced until adulthood did not develop this phenotype.³⁸ These observations suggest that there is a delicate balance of factors throughout development that controls alveolarization and airspace size. Disruption of this balance in any of a number of ways, including altered production of a growth factor that controls mesenchymal cell proliferation, may result in the histopathological manifestation of enlarged airspaces.

Our results indicate, in accordance with previous studies, that PDGF is an important growth factor in lung development. Expression of PDGF-A and -B genes and PDGF- α and - β receptors has been documented in the developing lung. In fetal lung, PDGF-A expression has been detected in epithelium^{48–50} and PDGF-B expression has been detected in epithelium and endothelium.^{48,49} PDGF- α receptor is expressed in mesenchymal cells underlying airway and distal epithelium^{49,50} and PDGF- β receptor is expressed in mesenchymal cells surrounding blood vessels.⁴⁹ These expression patterns are consistent with PDGF isoforms functioning as signaling molecules that control lung mesenchymal cell proliferation and development. PDGF-A has been shown to be required for normal lung development; disruption of the PDGF-A gene by homologous recombination caused an emphysema-like lung disease.³ The lungs of PDGF-A knockout mice were normal at birth, but developed enlarged airspaces within the first 2 postnatal weeks. The defect in these mice was found to be the lack of elastin-producing mesenchymal cells that are required for secondary septal growth and the formation of alveoli.⁴⁹ In contrast, our SPC-PDGFB mice exhibited an abnormal lung phenotype on the day of birth. Based on the distribution of PDGF receptors, this phenotype is likely to arise from the action of PDGF-B that is released by type II alveolar epithelial cells and binds to PDGF receptors on mesenchymal cells in the lung interstitium. We speculate that the thickened airspace walls in neonatal SPC-PDGFB mice arise from excess mesenchymal cell proliferation mediated by PDGFB, and that the enlarged airspaces develop as a result of an improper ratio between epithelial and mesenchymal cell populations.

An inflammatory influx containing macrophages and eosinophils was observed in the lungs of SPC-PDGFB transgenic mice. Inflammation was generally not distributed throughout the lung but was confined to focal areas. These focal areas of inflammation were usually within or adjacent to areas of fibrosis or severe emphysema. This observation indicated an association between inflammation and other manifestations of the PDGF-induced phenotype, but whether these events were causally related was not determined. Within the areas of inflammation, macrophages were predominantly confined to the air spaces. Eosinophils were observed both within the interstitium and in the airspaces. It is not clear whether the cells are actively migrating into the airspaces or whether they appear there as a consequence of the tissue destruction that leads to the emphysematous lesions. The

presence of eosinophils was of interest, because this cell type has recently been implicated as having a role in the pathogenesis of lung fibrosis.⁵¹ The expression of interleukin 5 and the influx of eosinophils correlated with fibrosis in a bleomycin model of lung fibrosis.⁵¹ Similarly, we observed a correlation between eosinophilic inflammation and fibrosis in SPC-PDGFB mice. Further studies will be required to establish a causal relationship between eosinophil influx and the pathogenesis of fibrosis in SPC-PDGFB mice.

Multiple potential mechanisms exist by which PDGF expression might induce an inflammatory response. The most straightforward mechanism would be a direct effect of PDGF on inflammatory cells. Expression of PDGF receptors on macrophages or eosinophils has not been demonstrated directly, but there is evidence that PDGF can act as a chemotactic factor for eosinophils.⁵² A second mechanism by which PDGF may promote inflammation is the induction of chemokines in mesenchymal cells that can respond to PDGF. Evidence that this process can occur comes from studies in cultured fibroblasts, in which treatment with PDGF caused up-regulation of expression of chemokines including the macrophage chemoattractant MCP-1 (JE).⁵³ Finally, if PDGF can promote proteolysis of lung tissue as discussed above, the process of tissue destruction may induce a pulmonary inflammatory response.

The extent of lung disease that was observed in SPC-PDGFB mice varied significantly among individual mice within the same transgenic lineage. At birth, some mice exhibited enlarged saccules and thickened septa, but the lungs of other transgenic mice appeared normal. Among young adults (up to 3 months of age) some mice displayed normal lung histology or were mildly affected by airspace enlargement, whereas other mice were severely affected by airspace enlargement and also had focal areas of inflammation and fibrosis. All SPC-PDGFB mice aged 6 months and older had some degree of histological abnormality in the lung, but still varied in the extent of the lung that was affected. These results are consistent with the existence of two populations of SPC-PDGFB mice. One population is susceptible to PDGF-B overexpression during lung development. These mice have abnormal lungs at birth and develop severe lung disease as young adults. The second population is resistant to the developmental effects of PDGF-B overexpression. These mice have normal lungs at birth, have normal or mildly affected lungs as young adults, and do not develop significant lung disease until they are 6 months of age. One potential mechanism that would explain this variation in phenotype is a possible difference in transgene expression among individual animals or regional variation in expression within a single lung. Immunostaining of transgenic lungs for PDGF-B indicated that, in both normal and abnormal areas of the lung, the transgene was expressed at similar levels in type II alveolar epithelial cells. In areas of inflammation, strong PDGF-B immunostaining was observed in macrophages, but it was not clear whether this represented endogenous PDGF expressed by macrophages or transgene-derived PDGF that had been taken up by macrophages. Thus, the im-

munostaining results did not exclude variation in PDGF expression as a causative factor for differences in PDGF-induced lung disease. However, this explanation does not account for the existence of two discrete populations of mice with differing disease susceptibilities. Variation in PDGF expression would be most likely to result in a continuum of the disease phenotype rather than the observed results with two distinct populations. Another possibility is that genetic variation among mice within the 47-5 lineage is responsible for the variation in phenotype. The SPC-PDGFB transgenic mice were generated and maintained on a genetic background that is a mixture of two inbred strains, C57BL/6 and SJL. If these strains vary in the response of the lung to PDGF, then mice with a hybrid B6/SJL background could exhibit disease-sensitive and disease-resistant phenotypes. These phenotypes could result from the differential expression of secondary genes that control the response to PDGF, such as PDGF receptors, signaling molecules, or downstream genes induced by PDGF. Strain differences in susceptibility to lung injury and disease produced by environmental agents such as bleomycin^{54,55} and asbestos⁵⁶ have been described. In these models, the production of and response to cytokines and growth factors in the lung appear to be important determinants in controlling the susceptibility to fibrotic agents.⁵⁷ Thus, there is precedence for postulating that the C57BL/6 and SJL inbred strains could respond differently to PDGF overexpression.

In summary, PDGF overexpression in distal lung epithelium resulted in enlarged airspaces, inflammation, and fibrosis. In susceptible mice, the airspace enlargement appeared to result from developmental abnormalities that occurred both before and after birth. Variation in the severity of PDGF-induced lung disease suggested that the genetic background of the mice may be important in determining the response of the lung to PDGF expression. These results highlight the importance of PDGF in lung development and disease pathogenesis. Future studies may determine whether PDGF overexpression renders mice more susceptible to lung fibrosis induced by exogenous agents such as asbestos or bleomycin.

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