Specific Inhibitors of Platelet-Derived Growth Factor or Epidermal Growth Factor Receptor Tyrosine Kinase Reduce Pulmonary Fibrosis in Rats

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The proliferation of myofibroblasts is a central feature of pulmonary fibrosis. In this study we have used tyrosine kinase inhibitors of the tyrphostin class to specifically block autophosphorylation of the platelet-derived growth factor receptor (PDGF-R) or epidermal growth factor receptor (EGF-R). AG1296 specifically inhibited autophosphorylation of PDGF-R and blocked PDGF-stimulated [³H]thymidine uptake by rat lung myofibroblasts *in vitro*. AG1478 was demonstrated as a selective blocker of EGF-R autophosphorylation and inhibited EGF-stimulated DNA synthesis *in vitro*. In a rat model of pulmonary fibrosis caused by intratracheal instillation of vanadium pentoxide (V₂O₅), intraperitoneal delivery of 50 mg/kg AG1296 or AG1478 in dimethylsulfoxide 1 hour before V₂O₅ instillation and again 2 days after instillation reduced the number of epithelial and mesenchymal cells incorporating bromodeoxyuridine (BrdU) by ~50% at 3 and 6 days after instillation. V₂O₅ instillation increased lung hydroxyproline fivefold 15 days after instillation, and AG1296 was more than 90% effective in preventing the increase in hydroxyproline, whereas AG1478 caused a 50% to 60% decrease in V₂O₅-stimulated hydroxyproline accumulation. These data provide evidence that PDGF and EGF receptor ligands are potent mitogens for collagen-producing mesenchymal cells during pulmonary fibrogenesis, and targeting tyrosine kinase receptors could offer a strategy for the treatment of fibrotic lung diseases. (*Am J Pathol* 1999, 155:213–221)

Pulmonary fibrosis is a proliferative disease that may be initiated by a variety of factors, including chemotherapeutic drugs such as bleomycin,¹ man-made fibers such as asbestos,² and metals such as cadmium and vanadium.^{3,4} The disease is characterized by the excessive proliferation of mesenchymal cells (fibroblasts, myofibroblasts, and smooth muscle cells) and the subsequent deposition of extracellular matrix proteins by these cell

types.^{5,6} Myofibroblasts, contractile interstitial cells that possess characteristics of smooth muscle cells and fibroblasts, are the principal mesenchymal cell type that contributes to collagen deposition or scarring during a fibrogenic response.^{1,7} The factors that initiate and perpetuate the myofibroblast growth response during fibrosis have not been fully clarified. However, several polypeptide mesenchymal cell mitogens are up-regulated during the early stages of fibrogenesis in humans and rodents, including platelet-derived growth factor (PDGF)^{8–10} and transforming growth factor (TGF)- α .^{11–13} The deposition of extracellular matrix proteins such as collagen, mediated largely by TGF- β family members, generally follows the proliferative phase of the disease.^{5,6}

Receptor tyrosine kinases that mediate lung myofibroblast proliferation include the PDGF receptors (PDGF-Rs)¹⁴ and the epidermal growth factor receptor (EGF-R).¹⁵ Ligand binding to the extracellular domain of the receptor triggers phosphorylation on tyrosine residues within the intracellular domain. Several ligands bind to the EGF-R with high affinity, including EGF,¹⁶ TGF- α ,¹⁷ and heparin-binding EGF (HB-EGF).¹⁸ The three isoforms of PDGF (PDGF-AA, -AB, and -BB) bind differentially to two PDGF-Rs termed PDGF-R α and PDGF-R β , resulting in three possible receptor dimers; $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$.^{14,19} PDGF-AA binds only PDGF-R α , whereas PDGF-AB and PDGF-BB bind both PDGF-R α and PDGF-R β . Ligand-induced autophosphorylation of EGF-R and PDGF-Rs allows for the docking of numerous signaling molecules that initiate the intracellular signaling cascades leading to cell division and chemotaxis.²⁰

Although several studies have documented the expression of PDGF-R, EGF-R, and their respective ligands during fibroproliferative diseases, the relative contribution of the EGF-R and the PDGF-R system to pulmonary fibrosis in relation to other growth factor receptors remains unknown. Inhibitors of PDGF receptor and EGF receptor tyrosine kinase activity have been reported for low molecular weight compounds of the staurosporine,²¹ tyrphostin,^{22,23} and 2-phenylaminopyrimidine^{24,25} classes of compounds. Specific tyrphostin analogs have been reported to block a variety of pathogenic effects.

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For example, tyrphostin AG126 blocks lipopolysaccharide-induced septic shock in mice via inhibition of tyrosine phosphorylation of p42 mitogen-activated protein kinase.²⁶ Furthermore, EGF-R- and PDGF-R-specific compounds of the 2-phenylaminopyrimidine class possess antitumor activity *in vivo*.^{24,25} In this study we have investigated two tyrphostins, AG1296 and AG1478, that specifically block autophosphorylation of the PDGF-R and EGF-R, respectively. Using a rat model of metal-induced pulmonary fibrosis, we show that both AG1296 and AG1478 inhibit the proliferative response of epithelial and mesenchymal cells within fibrotic lesions, and this results in reduced lung collagen accumulation. This study provides strong evidence that both EGF-R and PDGF-R are important to the progression of pulmonary fibrosis and suggests that targeting autophosphorylation of receptor tyrosine kinases could have potential therapeutic value in treating fibroproliferative lung diseases.

Materials and Methods

Reagents

Tyrphostins AG1296 and AG1478 were purchased from Calbiochem (La Jolla, CA). Vanadium pentoxide was from Aldrich Chemical Co. (Milwaukee, WI). Recombinant human PDGF-BB, EGF, and fibroblast growth factor (FGF)-2 were purchased from Upstate Biotechnologies (Lake Placid, NY). [³H]Thymidine was from Amersham Corp. (Arlington Heights, IL). Anti-PDGF-R α , anti-PDGF-R β , and EGF-R antibodies were from Upstate Biotechnologies. Anti-phosphotyrosine (PY20) antibody and horseradish peroxidase (HRP)-labeled mouse IgG were from Transduction Laboratories (Lexington, KY). HRP-labeled swine anti-rabbit and rabbit anti-sheep antibodies were purchased from DakoPatts (Carpinteria, CA).

Isolation of Rat Lung Myofibroblasts

Early-passage rat lung myofibroblasts were isolated and characterized as described previously from male Sprague-Dawley rats.⁴ Cell isolates at passage 1 or 2 were plated onto 3-amino-propyl-triethoxysilane (APTS)-coated glass chamber slides and grown to confluence, then fixed briefly in ice-cold acetone. Fixed cells were then subjected to overnight incubation with a murine monoclonal antibody to the antigen of interest, followed by a biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA), avidin-biotin immunoperoxidase (Vector), and 3,3'-diaminobenzidine chromogen (Vector). An irrelevant monoclonal antibody (anti-5-bromo-2'-deoxyuridine, Becton Dickinson, San Jose, CA) at equivalent IgG concentration served as control for non-specific immunoreactivity. Concentrations of primary antibodies were set by titration on appropriate rat-derived positive control cells. Cells stained positively for vimentin, desmin, and α -smooth muscle actin, which indicated a myofibroblast phenotype. In addition, examination of glutaraldehyde-fixed cell pellets by transmission electron microscopy showed ultrastructural features consistent

with a myofibroblast phenotype (abundant intermediate filaments and rough endoplasmic reticulum and lack of Weibel-Palade bodies characteristic of endothelial cells). Cells were grown to confluence in 10% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM) before being seeded for the assays described below.

Western Blotting

Myofibroblasts grown to confluency with 10% FBS/DMEM in 75-cm² tissue culture flasks were rendered quiescent for 24 hours with serum-free defined medium (SFDM) consisting of Ham's F-12 medium supplemented with 0.25% bovine serum albumin and an insulin/transferrin/selenium mixture (Boehringer Mannheim, Indianapolis, IN). Cultures were incubated with an increasing concentration of AG1296 or AG1478 (1 to 100 μ mol/L) diluted in SFDM for 24 hours to block receptor tyrosine kinase activity. Autophosphorylation was then stimulated by the addition of 50 ng/ml PDGF-AB, PDGF-BB, EGF, or TGF- α for 10 minutes. The cultures were then washed with ice-cold PBS, and cell lysates were collected by incubation with 250 μ l of lysis buffer consisting of 50 mmol/L Tris/HCl (pH 7.4), 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 0.25% sodium deoxycholate, and 20 μ g/ml of each of the following proteinase inhibitors: aprotinin, leupeptin, and pepstatin. Twenty microliters of each sample was mixed with 5 μ l of sample buffer (0.5 mol/L Tris/HCl, pH 6.8, 10% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 20% glycerol, and 50 mmol/L 2-mercaptoethanol) and separated by SDS-polyacrylamide gel electrophoresis in a 2% to 15% Tris-glycine gel (Integrated Separation Systems, Hyde Park, MA). The proteins were transferred to Hybond nitrocellulose membrane (Amersham), and the membrane was blocked with 3% milk in PBS for 1 hour before overnight incubation at 4°C with a 1:500 dilution of rabbit anti-mouse PDGF-R α antibody, rabbit anti-human PDGF-R β antibody, a sheep anti-human EGF-R antibody (Upstate Biotechnologies), or a monoclonal phosphotyrosine (PY20) antibody (Transduction Laboratories). The membranes were washed three times with PBS/Tween before a 90-minute incubation with a 1:2000 dilution of the appropriate secondary HRP-conjugated antibody: HRP-swine anti-rabbit for PDGF-R α and PDGF-R β (DakoPatts), HRP-rabbit anti-sheep for EGF-R (DakoPatts), or HRP-mouse IgG (Transduction Laboratories). After thoroughly washing in PBS/Tween, the HRP-labeled proteins were visualized with an ECL kit (Amersham).

[³H]Thymidine Incorporation Assay

Rat lung myofibroblasts were grown to confluence with 10% FBS/DMEM in 24-well tissue culture plates (2-cm² wells) and then rendered quiescent for 24 hours with SFDM containing 0.5% FBS. The cells were then treated with fresh 0.5% FBS/SFDM containing 100 μ mol/L AG1296 or AG1478 in vehicle (dimethylsulfoxide (DMSO)) or vehicle alone for 24 hours. The cultures were

rinsed and treated with an increasing concentration of PDGF-AB, EGF, or FGF-2 (0.5 to 100 ng/ml) along with 5 μ Ci/ml [^{3} H]thymidine (Amersham) for 36 hours. The cells were washed with Ham's F-12 at 25°C, placed on ice, and incubated with 0.5 ml/well 5% trichloroacetic acid for 10 minutes. After washing three times with ice-cold distilled water, solubilization was performed with 0.5 ml/well 0.2 N NaOH containing 0.1% SDS for 30 minutes on an oscillating platform. A 100- μ l aliquot of each sample was added to 1 ml of Ecolume (Costa Mesa, CA), and radioactivity was measured on a liquid scintillation counter.

Administration of Tyrphostins in Vivo and Intratracheal Instillation of V₂O₅

Sprague-Dawley rats (Charles River, Raleigh, NC) weighing ~200 g were injected intraperitoneally (i.p.) with 50 mg/kg AG1296 or AG1478 in vehicle (DMSO) or vehicle alone 1 hour before intratracheal instillation. V₂O₅ suspensions were vortexed thoroughly, then bath sonicated for 30 minutes at 25°C before instillation. The animals were instilled with saline alone or 1 mg/kg V₂O₅ in saline as has been described previously.⁴ Two days after instillation, a second i.p. injection of tyrphostin (50 mg/kg) or vehicle was administered. Groups of rats were sacrificed 3, 6, and 15 days after instillation by overdosing with an i.p. injection of Nembutol. Some groups received a single i.p. injection of 50 mg/kg bromodeoxyuridine (BrdU) 1 hour before sacrifice. The lungs were removed *en bloc* and inflated with formalin for immunohistochemistry or digested for total lung hydroxyproline as described below.

Bromodeoxyuridine Immunohistochemistry

Lung tissue was fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemistry was performed using the avidin-biotin peroxidase method. Tissue sections (6 μ m) were dehydrated through a series of graded alcohol solutions to 1X automation buffer (AB) consisting of 5% NaCl and 2% HCl (Biomedex Corp., Foster City, CA), treated with 2 N HCl at 37°C for 30 minutes and 1 mol/L borate buffer for 1 minute, and then incubated in 0.01% trypsin and 1% calcium chloride for 3 minutes at 37°C. Endogenous peroxidase was blocked in 3% (v/v) H₂O₂ for 15 minutes. After a 1X AB wash, blocking was performed for 20 minutes, and a monoclonal mouse anti-BrdU antibody (Becton Dickinson, Mountain View, CA) 1:50 was applied for 30 minutes at room temperature. Sections were washed twice with AB, then incubated for 30 minutes with the Elite avidin-biotin complex (Vector) for 30 minutes. Visualization of the antibody complex was done using a diaminobenzidine tablet (10 mg) (Sigma Chemical Co., St. Louis, MO) dissolved in 20 ml of 1X automation buffer containing 12 μ l of 30% H₂O₂ for 6 minutes in the dark. Slides were then rinsed in running tap water, counterstained with Harris hematoxylin (Harelico, Gibbstown, NJ), dehydrated through a series of graded alcohols to xylene, and coverslipped with Permount (Fisher Scientific, Fair Lawn,

NJ). Some paraffin-embedded sections were immunostained for desmin as a smooth muscle cell phenotypic marker or vimentin (clone LN6, Accurate Antibodies, Westbury, NY) as a marker of fibroblast phenotype. Mature collagen was detected by Masson's trichrome stain.

Hydroxyproline Assay

The procedure for quantitation of lung hydroxyproline has been described elsewhere.²⁷ For hydrolysis, whole lung tissue was washed in PBS and hydrolyzed for 18 hours in 6 N HCl at 110°C (~40 ml/6 g of tissue). One drop of 1% phenolphthalein in ethanol was added to each sample, and the pH was adjusted to 6.0 with NaOH titration. Two milliliters from each sample was centrifuged 5 minutes at 1500 rpm, and the pellet was oxidized with 1 ml of 0.6 mol/L chloramine-T for 30 minutes. Each sample then received 1 ml of 7.5% *p*-dimethylaminobenzaldehyde and was incubated at 65°C for 15 minutes. The absorbance was measured at 560 nm on a spectrophotometer. Hydroxyproline content was proportional to an increase in absorbance and was evident by a characteristic color change from yellow to red. Lung hydroxyproline was quantitated against a standard curve set up with purified hydroxyproline (Sigma), and values were corrected for total lung wet weight.

Statistical Analysis

The Systat (Evanston, IL) statistical package was used for all analyses. For analysis of [^{3} H]thymidine uptake data, one-way analysis of variance was performed to determine an overall effect of tyrphostin *versus* control. Two-sample *t*-tests were performed on BrdU data and hydroxyproline data to determine significant differences among control *versus* treatment groups.

Results

Autophosphorylation of PDGF-R and EGF-R in Vitro Are Specifically Blocked by AG1296 and AG1478, Respectively

AG1296 has been reported to specifically inhibit activation of PDGF receptors,²³ whereas AG1478 has been described as a selective blocker of EGF-R autophosphorylation.²² To confirm the specificity of these tyrosine kinase inhibitors in our system, we stimulated rat lung myofibroblasts with PDGF-R ligands or EGF-R ligands after pretreating the cells with AG1296 or AG1478 to block autophosphorylation on tyrosine residues. Phosphotyrosine immunoblotting showed that autophosphorylation was stimulated in a time-dependent manner that peaked 5 to 20 minutes after stimulation with PDGF-R ligands (PDGF-AB and PDGF-BB) or EGF-R ligands (EGF and TGF- α) and declined to undetectable levels by 1 hour after stimulation (data not shown). Autophosphorylation stimulated by PDGF-AB or PDGF-BB was completely blocked by 100 μ mol/L AG1296, yet autophos-

Figure 1. Tyrphostin AG1296 blocks autophosphorylation of PDGF receptor without affecting autophosphorylation of the EGF receptor. Confluent, quiescent rat lung myofibroblasts were treated for 24 hours with AG1296 (1 to 100 μ M), and then autophosphorylation was induced by treatment with growth factors for 10 minutes. Western blot using anti-phosphotyrosine (p-Tyr) antibody showing that PDGF-AB- or PDGF-BB-stimulated autophosphorylation of the PDGF-R was completely inhibited by pretreatment with 100 μ M AG1296 (left top panel). The protein levels of PDGF-R α or PDGF-R β were not affected by AG1296 treatment (left middle and lower panels, respectively). Autophosphorylation of EGF-R in response to EGF or TGF- α treatment was not inhibited by AG1296 pretreatment as measured by Western blotting for p-Tyr (right top panel), nor were levels of EGF-R affected by AG1296 (right bottom panel).

phorylation stimulated by the addition of either EGF or TGF- α was not affected by AG1296 (Figure 1). Conversely, 100 μ M AG1478 completely inhibited EGF- or TGF- α -induced autophosphorylation without affecting PDGF-stimulated autophosphorylation (Figure 2). The levels of PDGF-R α , PDGF-R β , and EGF-R were not affected by the addition of either AG1296 or AG1478 (Figures 1 and 2).

Selective Inhibition of PDGF- and EGF-Stimulated Mitogenesis by AG1296 and AG1478 in Vitro

[^{3} H]Thymidine incorporation assays were undertaken to establish that the inhibition of autophosphorylation

Figure 2. Tyrphostin AG1478 blocks autophosphorylation of EGF receptor without affecting phosphorylation of the PDGF receptor. Confluent, quiescent rat lung myofibroblasts were treated for 24 hours with AG1478 (1 to 100 μ M), and then autophosphorylation was induced by treatment with growth factors for 10 minutes. Western blot using anti-phosphotyrosine (p-Tyr) antibody showing that PDGF-AB- or PDGF-BB-stimulated autophosphorylation of the PDGF-R was not affected by pretreatment with concentrations of AG1478 as high as 100 μ M (left top panel), nor was PDGF-R expression affected by AG1478 as detected by Western blotting using antibodies specific for PDGF-R α or PDGF-R β (left middle and lower panels, respectively). Autophosphorylation of EGF-R in response to EGF or TGF- α treatment was completely blocked by AG1478 (10 to 100 μ M) pretreatment as measured by the p-Tyr Western blot (right top panel), yet levels of EGF-R were not affected (right bottom panel).

Figure 3. [^{3} H]Thymidine uptake showing the specificity of AG1296 and AG1478 for inhibition of PDGF- and EGF-mediated myofibroblast mitogenesis *in vitro*. Confluent, quiescent cultures of rat lung myofibroblasts were pretreated with 100 μ M tyrphostin (AG1296 or AG1478) for 24 hours, and then stimulated with an increasing concentration of growth factors (PDGF-AB, EGF, or FGF-2) in the presence of [^{3} H]thymidine. PDGF-stimulated mitogenesis was inhibited only by AG1296 (left panel), EGF-stimulated mitogenesis was blocked by AG1478 only (center panel), and neither tyrphostin inhibited FGF-2-stimulated mitogenesis (right panel). Data are typical of four to five experiments (SEM < 10% of the mean) and are expressed as fold increase in [^{3} H]thymidine uptake above that obtained with serum-free defined medium (SDFM) containing 0.5% FBS.

caused by AG1296 and AG1478 shown in Figures 1 and 2 resulted in inhibition of mitogenesis *in vitro*. AG1296 selectively blocked PDGF-stimulated [^{3} H]thymidine uptake but did not affect EGF-induced DNA synthesis, whereas AG1478 inhibited EGF-stimulated mitogenesis without affecting PDGF-induced DNA synthesis (Figure 3). [^{3} H]Thymidine uptake stimulated by FGF-2 was not affected by either AG1296 or AG1478.

Inhibition of Mitogenesis in Vivo by AG1296 and AG1478 during Lung Fibrogenesis

We previously described the progression of V₂O₅-induced pulmonary fibrogenesis wherein an acute inflammatory response was observed by 24 hours after instillation, a proliferative response of mesenchymal cells staining positively for vimentin and desmin occurred 3 to 6 days after instillation, and collagen deposition was evident by day 15 after instillation as determined by trichrome staining.⁴ In the present study, proliferative lesions induced by V₂O₅ intratracheal instillation at day 3 after instillation contained numerous Brdu-positive cells (Figure 4B). Day 3 was the peak of Brdu incorporation, as the number of Brdu-positive cells then declined by >50% by day 6 after instillation and were reduced by >90% by day 15 after instillation (data not shown). Cells incorporating Brdu after V₂O₅ instillation in rats treated with AG1296 were confined mainly to the corners of the alveolar walls, suggesting proliferating type II epithelial cells (Figure 4D). In contrast, Brdu-positive cells after V₂O₅ in rats treated with AG1478 were present in alveolar walls as well as in intra-alveolar spaces, suggesting the presence of some proliferating mesenchymal cells (Figure 4F). Quantification of Brdu-labeled nuclei showed that treatment with either AG1296 or AG1478 significantly reduced the number of Brdu-positive cells at day 3 by ~50% (Figure 5).

Figure 4. Bromodeoxyuridine (BrdU) immunohistochemistry in the lungs of rats that have been instilled with vanadium pentoxide (V_2O_5) with or without pretreatment with AG1296 or AG1478. Rats were pretreated with an i.p. injection of 200 μ l of DMSO vehicle containing 50 mg/kg AG1296 or AG1478 1 hour before intratracheal instillation of V_2O_5 (1 mg/kg) suspended in saline or saline alone. A second i.p. injection of tyrphostin was given 2 days after instillation. Rats were sacrificed 3 days after instillation, and the lungs were formalin fixed and paraffin embedded. BrdU immunohistochemistry was performed as described in Materials and Methods. **A:** DMSO vehicle i.p. injection with saline instillation. **B:** DMSO i.p. with V_2O_5 instillation. **C:** AG1296 i.p. injection with saline instillation. **D:** AG1296 i.p. with V_2O_5 instillation. **E:** AG1478 i.p. with saline instillation. **F:** AG1478 i.p. with V_2O_5 instillation. Numerous BrdU-positive cells were observed after V_2O_5 instillation (arrow). Quantitation of BrdU-labeled cells in each group showed that AG1296 or AG1478 significantly reduced BrdU labeling after V_2O_5 instillation (see Figure 5). Original magnification, $\times 200$.

Figure 5. Quantitation of BrdU-labeled nuclei in lungs of rats instilled with V₂O₅ that were pretreated with or without AG1296 or AG1478. Rats were treated as described in Figure 4 and in Materials and Methods. The lungs from five V₂O₅-instilled animals per group and three saline-instilled animals per group were harvested 3 days after instillation and evaluated for BrdU immunohistochemistry wherein 20 random photomicrographs of the lung were taken from each animal at $\times 150$ (one field). BrdU-labeled nuclei in V₂O₅-instilled animals were significantly reduced by treatment with AG1296 (** $P < 0.01$) or AG1478 (* $P < 0.05$).

Inhibition of Lung Collagen Accumulation by AG1296 and AG1478

Treatment of rats with tyrphostins AG1296 or AG1478 reduced the severity of V₂O₅-induced fibrotic lesions in the lung at day 15 after instillation as assessed by trichrome staining for collagen (Figure 6). V₂O₅ caused a fivefold increase in total lung hydroxyproline content 15 days after instillation (Figure 7). In preliminary studies, a single i.p. injection of AG1296 (50 mg/kg) 1 hour before instillation did not prevent an increase in hydroxyproline caused by V₂O₅-induced lung injury. However, a double injection of AG1296, 50 mg/kg 1 hour before instillation and again 2 days after instillation, prevented collagen accumulation in V₂O₅-instilled animals, resulting in hydroxyproline measurements that were nearly equivalent to the saline-instilled group. AG1478 reduced collagen accumulation in V₂O₅-instilled animals by ~50%, but hydroxyproline content was significantly elevated above the saline control group.

Discussion

In this study we have shown that PDGF-R-specific and EGF-R-specific tyrosine kinase inhibitors of the tyrphostin class block rat lung myofibroblast mitogenesis *in vitro* and inhibit the progression of pulmonary fibrosis *in vivo*. AG1296 specifically inhibited PDGF-induced autophosphorylation and PDGF-stimulated DNA synthesis, whereas AG1478 was demonstrated to be a selective inhibitor of EGF- and TGF- α -induced autophosphorylation and mitogenesis. Both AG1296 and AG1478 pre-

vented an increase in total lung hydroxyproline (ie, collagen) after lung injury with V₂O₅. These data provide strong evidence that both PDGF-R and EGF-R contribute to the proliferative phase of the fibrogenic response and that replicating PDGF- and EGF-responsive mesenchymal cells are the major source of collagen deposited during the subsequent extracellular matrix deposition phase of fibrogenesis.

Several studies have documented the up-regulation of PDGF isoforms and EGF receptor ligands during pulmonary fibrogenesis in humans and in rodent models. PDGF and TGF- α are both elevated in alveolar macrophages or bronchoalveolar lavage fluid from patients with pulmonary fibrosis.^{8,9,11,28} In rat models of fibrosis, PDGF and the PDGF α -receptor subtype are increased 2 to 3 days after lung injury by bleomycin or metals.^{4,29} EGF and TGF- α are elevated 2 to 4 days in the rat bleomycin model. Asbestos fibers also stimulate the secretion of PDGF isoforms and TGF- α *in vitro* and *in vivo* in rodent models.^{10,12,30} Due to the potent mitogenic effect of these growth factors, these studies suggest a role for PDGF and EGF receptor ligands in causing a fibrotic response. However, they do not clearly establish a link between PDGF- or EGF-mediated cell proliferation and the subsequent deposition of collagen, which defines the fibrotic lesion. Our data in the present study show that blocking PDGF- or EGF-receptor-mediated events prevents an increase in total lung collagen, and this is likely achieved by preventing the proliferation of mesenchymal cells that have the potential to produce collagen once stimulated by other growth factors, such as TGF- β . The delivery of tyrphostins at day 2 after instillation of V₂O₅ was necessary to reduce DNA synthesis and collagen accumulation *in vivo*. This time point coincides with the temporal induction of PDGF and PDGF-R α ^{4,29} and TGF- α and the EGF-R¹³ 2 to 4 days after injury.

PDGF-R-specific tyrosine kinase inhibitors have been shown to inhibit intimal hyperplasia after arterial injury *in vivo*.^{31–33} Banai and co-workers reported that *in vivo* delivery of the PDGF-R-specific tyrphostin AG1295 caused a significant reduction (>80%) in the numbers of smooth muscle cells in the intima/media area of porcine femoral arteries after balloon injury.³¹ Other investigators reported that administration of the PDGF-R-specific 2-phenylaminopyrimidine, CGP53716, inhibited an increase in intimal vascular smooth muscle cell number after balloon injury in rats by inhibiting both the migration and proliferation of smooth muscle cells.³³ At least one other blocking strategy that does not involve targeting tyrosine kinases has been reported. Fern and co-workers showed that delivery of a neutralizing antibody to PDGF blocks neointimal smooth muscle accumulation after angioplasty.³⁴ Thus, there is abundance evidence to support a role for PDGF in nonmalignant proliferative diseases such as atherosclerosis. Although the data presented in this study are the first to demonstrate reduction of lung fibrosis through inhibition of PDGF-R or EGF-R tyrosine kinase activity, several other investigators have overexpressed PDGF in the lung and observed a fibrotic response. Instillation of exogenous, recombinant PDGF in rats causes obliterative bronchiolitis characterized by the excessive

Figure 6. Trichrome staining for collagen demonstrating the effect of tyrphostins AG1296 or AG1478 on the formation of fibrotic lesions at day 15 after instillation of vanadium pentoxide (V_2O_5). Rats were pretreated with an i.p. injection of 200 μ l of DMSO vehicle containing 50 mg/kg AG1296 or AG1478 1 hour before intratracheal instillation of V_2O_5 (1 mg/kg) suspended in saline or saline alone. A second i.p. injection of tyrphostin was given 2 days after instillation. Rats were sacrificed 15 days after instillation, and the lungs were formalin fixed/paraffin embedded and stained with Masson's trichrome. **A:** DMSO vehicle i.p. injection with saline instillation. **B:** DMSO i.p. with V_2O_5 instillation. **C:** AG1296 i.p. injection with saline instillation. **D:** AG1296 i.p. with V_2O_5 instillation. **E:** AG1478 i.p. with saline instillation. **F:** AG1478 i.p. with V_2O_5 instillation. Blue-staining collagen fibrils (indicated at **arrows**) were observed in lungs from V_2O_5 -instilled rats, but such lesions were rarely observed in V_2O_5 -instilled rats that received AG1296 or AG1478 pretreatment. Quantitation of total lung collagen by hydroxyproline assay showed that AG1296 or AG1478 significantly reduced collagen deposition after V_2O_5 instillation at day 15 (see Figure 7). Original magnification, $\times 100$.

Figure 7. Hydroxyproline content in lungs of rats instilled with V₂O₅ that were pretreated with or without AG1296 or AG1478. Rats were treated with tyrphostins and V₂O₅ as described in Figure 4. The lungs from five V₂O₅-instilled animals per group and three saline-instilled animals per group were harvested 15 days after instillation and digested for hydroxyproline content using the colorimetric assay described in Materials and Methods. Lung hydroxyproline was increased fivefold in the V₂O₅-instilled group and was significantly reduced in V₂O₅-instilled animals that were treated with AG1296 (**P < 0.01) or AG1478 (*P < 0.05).

accumulation and proliferation of myofibroblasts in the airways.³⁵ Yoshida and co-workers demonstrated that overexpression of the genes encoding both PDGF-B chain and TGF- β 1 in rats resulted in interstitial lung fibrosis.³⁶ Furthermore, it is noteworthy that PDGF-AA is essential to the development of myofibroblasts in the embryonic lung.³⁷ Collectively, these studies provide strong evidence that the PDGF system is pivotal to mesenchymal cell growth in the lung.

Our study provides additional evidence that PDGF is a key mediator of the pulmonary fibrotic response and suggests that the EGF-R also plays an important role in the proliferative phase of lung fibrogenesis. However, the use of the available tyrosine kinase inhibitors has some limitations toward dissecting the relative importance of growth factor isoforms and specific receptor subtypes. For example, as AG1296 inhibits both PDGF-R α and PDGF-R β , this inhibitor does not allow us to define the relative importance of the two PDGF receptors to the progression of lung fibrosis. Previous studies from our laboratory have shown that both PDGF-R α and PDGF-R β are required for maximal proliferation of myofibroblasts and that the PDGF-R α is induced by fibrogenic agents *in vitro* and during pulmonary fibrogenesis *in vivo*.^{4,38} In addition, although the AG1478 data indicate that EGF-R activation is important to the progression of fibrosis, it does not indicate which EGF-R ligand (EGF, TGF- α , or HB-EGF) is more important to the proliferation of mesenchymal cells during fibrosis, as all of these ligands bind and activate a common EGF-R. Finally, although the specificity of AG1296 and AG1478 for PDGF-R- and EGF-

R-induced mitogenesis were clearly demonstrated *in vitro*, we cannot rule out the possibility that these tyrosine kinase inhibitors could have inhibitory effects on other tyrosine kinases *in vivo* that could contribute to a reduction in the severity of lung fibrosis. However, the doses of tyrphostins in this study were within the dose range used by other investigators investigating tyrosine kinase inhibitors under similar conditions.^{25,26,33}

Phosphorylation of the EGF-R may also be initiated by oxidant-generating environmental agents in the absence of EGF-R ligands, and in such cases it is important to consider the potential effects of AG1478 on this type of ligand-independent EGF-R activation. For example, asbestos fibers cause stimulation of a mitogen-activated protein (MAP) kinase signaling cascade after phosphorylation of the EGF-R, and asbestos-induced MAP kinase activation is blocked by AG1478.³⁹ Similarly, vanadium compounds cause MAP kinase activation and rapid tyrosine phosphorylation of other key cellular proteins.⁴⁰ This is likely related to the well known ability of vanadium compounds to inhibit protein tyrosine phosphatases.⁴⁰ Interestingly, V₂O₅-induced MAP kinase activation in cultured rat lung myofibroblasts is blocked by pretreatment with AG1478, suggesting a requirement for EGF-R (Y.-Z. Wang and J.C. Bonner, unpublished observation). Therefore, it is conceivable that the beneficial effect of AG1478 could be due in part to blocking the activation of EGF-R by V₂O₅ in the absence of EGF-R ligands.

In summary, we have shown that tyrosine kinase inhibitors specific for the PDGF-R or EGF-R reduce the proliferative response of lung myofibroblasts *in vitro* and reduce cell proliferation and collagen deposition during pulmonary fibrogenesis *in vivo* after injury with V₂O₅. These findings provide additional evidence that these growth factor receptors play a pivotal role in the proliferation of collagen-producing myofibroblasts in the lung. Additional studies are necessary to define the relative importance of the specific EGF and PDGF ligands to the disease process.

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