Cardiovascular, Pulmonary and Renal Pathology

Modulation of Prosurvival Signaling in Fibroblasts by a Protein Kinase Inhibitor Protects against Fibrotic Tissue Injury

Ragini Vittal, Jeffrey C. Horowitz,
Bethany B. Moore, Hengmin Zhang,
Fernando J. Martinez, Galen B. Toews,
Theodore J. Standiford, and Victor J. Thannickal

*From the Department of Internal Medicine, Division of
Pulmonary and Critical Care Medicine, University of Michigan
Medical Center, Ann Arbor, Michigan*

Progressive fibrotic diseases involving diverse organ systems are associated with the persistence of fibroblasts/myofibroblasts in injured tissues. Activation of focal adhesion kinase (FAK) and protein kinase B (PKB/Akt) by transforming growth factor- β 1 mediate stable induction of myofibroblast differentiation and survival. In this report, we demonstrate that transforming growth factor- β 1-induced activation of both PKB/Akt and FAK are dose dependently inhibited by the protein kinase inhibitor, AG1879, in cultured human lung fibroblasts. In a murine model of intratracheal bleomycin-induced lung fibrosis, regions of active fibrogenesis demonstrate elevated expression of PKB/Akt and FAK phosphorylation *in vivo*, effects that are attenuated in mice receiving daily intraperitoneal injections of AG1879 (bleomycin-AG1879) versus a chemically inactive analog (bleomycin-control). PKB/Akt and FAK phosphorylation are elevated in fibroblasts isolated from lungs of bleomycin-injured mice, effects that are inhibited in bleomycin-AG1879 mice. Accumulation of α -smooth muscle actin-expressing myofibroblasts is markedly reduced in lungs of bleomycin-AG1879 mice. The numbers of recruited inflammatory cells were not significantly different between these groups. Bleomycin-AG1879 mice are protected from lung fibrosis as evidenced by histopathology, trichrome staining, and biochemical analysis for collagen. Thus, targeting of prosurvival signaling pathways in fibroblasts/myofibroblasts may provide a novel and effective strategy for anti-fibrotic therapy of treatment-unresponsive fibrotic disorders. (*Am J Pathol* 2005, 166:367–375)

Inflammation and repair are stereotypical host responses to injury of adult mammalian tissues. Dysregulation of either of these processes may lead to pathological outcomes resulting in varying degrees of chronic inflammation and fibrosis.^{1,2} Regulatory mechanisms of inflammatory responses are better understood than are tissue repair/regenerative responses. Epithelial/endothelial regeneration to restore barrier functions and maintain tissue architecture is facilitated by connective tissue cells. Fibroblasts, in particular, represent a versatile and phenotypically heterogeneous population of connective tissue cells that transiently appear in response to injury and normally disappear after repair.³ Recruitment of fibroblasts to sites of tissue injury likely occurs in diverse organ systems in which epithelial/endothelial cells and the underlying basement membrane(s) are disrupted.^{4,5} Elaboration of a provisional matrix by fibroblasts is an early repair function that aids epithelial/epidermal cell migration.⁶ Phenotypic transition of fibroblasts to myofibroblasts further facilitates re-epithelialization by contracting wounds and bringing epithelial margins into closer apposition.³ Resolution of the repair response is associated with successful re-epithelialization and the eventual removal of myofibroblasts by a mechanism that likely involves apoptosis.^{7,8} The persistence of myofibroblasts at sites of tissue injury is a consistent finding in most, if not all, human fibrotic diseases.^{1,9–12}

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional cytokine that plays a central role in fibrotic diseases.^{13,14} TGF- β 1 induces myofibroblast differentiation both *in vitro*¹⁵ and *in vivo*.¹⁶ Studies from our laboratory demonstrate that this phenotypic transition is critically dependent on cell adhesive events and integrin signaling via focal adhesion kinase (FAK).¹⁷ Moreover, Hinz and colleagues¹⁸ showed that mechanical tension is crucial

Supported by the National Institutes of Health (grants HL-67967, P50 HL-56402, and P50 HL-74024).

Accepted for publication November 2, 2004.

Address reprint requests to Victor J. Thannickal, M.D., Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical Center, 6301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109. E-mail: vjt@umich.edu.

for myofibroblast modulation and for the maintenance of their contractile activity. In addition to inducing differentiation, there is evidence that TGF- β 1 may also inhibit myofibroblast apoptosis.^{19,20} Kim and colleagues²¹ demonstrated that activation of the phosphatidylinositol 3-kinases (PI3K)-protein kinase B (PKB/Akt) pathway by TGF- β 1 was, at least in part, necessary for its growth and anti-apoptotic effects. Recent studies from our laboratory demonstrate that TGF- β 1 promotes this prosurvival/anti-apoptotic phenotype by p38 MAPK-dependent induction of a secreted growth factor(s) that activates the PI3K-PKB/Akt pathway in an autocrine manner.²²

The PI3K-PKB/Akt pathway is known to regulate a number of cellular processes including cell cycle progression, glucose metabolism, angiogenesis, cell motility, and survival.²³ Activation of PKB/Akt plays a central role in cell survival/anti-apoptotic signaling by targeting multiple substrates.²⁴ Integrin-FAK signaling is also important in promoting cell survival by PI3K-dependent and -independent pathways.^{25,26} In our studies of TGF- β 1 signaling in cultured human lung fibroblasts, activation of PKB/Akt and FAK were mediated by autocrine mechanisms involving secreted soluble (growth factor) and insoluble (extracellular matrix) factors, respectively.^{17,22} Thus, both redundant and nonredundant pathways are activated by TGF- β 1 to induce myofibroblast differentiation and to promote sustained survival of this profibrotic phenotype.

Idiopathic pulmonary fibrosis (IPF) is a progressive, fibrosing disease of the distal alveolar air spaces of the lung that culminates in death, usually within 3 to 5 years of diagnosis.²⁷ There are currently no effective therapies for IPF. Anti-inflammatory agents and potent immunosuppressive regimens have not shown significant benefit. Mortality in IPF is increased in patients with greater profusion of so-called "fibroblastic foci" on lung histopathology,^{28,29} suggesting that the activation and persistence of fibroblasts/myofibroblasts may be a critical regulatory event in IPF pathogenesis.

Protein kinase inhibitors (PKIs) have recently been shown to be effective modulators of cell phenotype by targeting signal transduction pathways in human cancers.³⁰ There has been increasing interest in PKIs for certain nononcological diseases, primarily to treat inflammatory conditions such as rheumatoid arthritis.³¹ Few studies have examined the role of PKIs in fibrotic diseases. PKIs that target p38 MAPK appears to be protective in animal models of lung injury, inflammation, and fibrosis primarily by blocking the antecedent inflammatory phase.^{32,33} PKIs that target platelet-derived growth factor and epidermal growth factor receptor tyrosine kinases have been shown to inhibit myofibroblast proliferation and tissue fibrosis in other animal models.³⁴ However, inhibition of receptor tyrosine kinases, including epidermal growth factor receptor, may induce profibrotic effects by interfering with epithelial cell regeneration.³⁵ Potential efficacy of nonreceptor tyrosine kinases inhibitors that specifically targets profibrotic signaling pathways in fibroblasts/myofibroblasts *in vivo* is not known. The main purpose of this study was to determine whether the nonreceptor tyrosine kinase inhibitor, AG1879, which

inhibits TGF- β 1-activated FAK and PKB/Akt protein kinases *in vitro*, attenuates fibrotic responses to lung injury by modulating prosurvival signaling/phenotype of fibroblasts/myofibroblasts *in vivo*.

Materials and Methods

Source and Culture of Normal Human Lung Fibroblasts

Normal human fetal lung fibroblasts (IMR-90) were obtained from the Institute for Medical Research, Camden, NJ. IMR-90 cells were cultured in medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 U/ml penicillin/streptomycin (Sigma), and fungizone (Life Technologies, Inc.); medium was changed every 2 days. Passage 3 to 5 IMR-90 cells were plated on 60-mm cell culture dishes at a density of 5×10^5 cells/dish and incubated at 37°C in 5% CO₂-95% air. When cells reached 80% confluence, they were growth-arrested for 48 hours in Dulbecco's modified Eagle's medium with 0.01% fetal bovine serum before treatment with or without TGF- β 1.

Isolation and Culture of Murine Lung Fibroblasts

Mice were euthanized by CO₂ asphyxiation and perfused via the heart with 5 ml of normal saline. Whole lungs were sterily removed and cut into small 2- to 3-mm slices and allowed to adhere on tissue culture plastic. Lung tissue explants were maintained in medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and fungizone (Life Technologies, Inc.). Fibroblasts were purified by repeat trypsinization and passaging to achieve a homogeneous population of spindle cells that uniformly expressed the collagen cross-linking enzyme, prolyl 4-hydroxylase.³⁶ Cell lysates were obtained for Western blot analyses at passage 2 to 3 and confluency of 90 to 100%.

Reagents and Drugs

Porcine-derived TGF- β 1 was obtained from R&D Systems, Minneapolis, MN. All other cell culture reagents were from Sigma (St. Louis, MO). AG1879 [PP2: 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo{3,4-*d*}pyrimidine] and its inactive analog, AG/inactive [PP3: 4-amino-7-phenylpyrazolo{3,4-*d*}pyrimidine] were purchased from Calbiochem, La Jolla, CA. Drugs were initially solubilized in dimethyl sulfoxide to make a 30 mmol/L stock concentration. Cell culture experiments were performed at final concentrations ranging from 1 to 10 μ mol/L by diluting in cell culture medium. For animal experiments, the same stock concentration of AG1879 was further diluted in normal saline to make a final concentration of 0.7 mg/ml. Daily intraperitoneal injections of mice were with 0.25 ml of this final mixture (or, 0.175 mg/mouse) using a 26-

gauge sterile intradermal needle. This dose was based on the observed efficacy of the drug at concentrations $\geq 3 \mu\text{mol/L}$ in cell culture systems. The calculated dose (0.175 mg/injection) would achieve $3.0 \mu\text{mol/L}$ concentration of the drug in a volume (ml) of distribution equivalent to the weight (mg) of the mice, assuming 100% bioavailability of the drug and average weight of mice of 19 gm. Dose of AG/inactive (control) drug was calculated to achieve the same concentration as active AG1879. In early pilot experiments, bleomycin-injured mice tolerated this dose without evidence of toxicity and, in fact, AG1879-treated mice appeared to be more active and gained more weight than control drug-treated and untreated animals.

Western Immunoblotting and Antibodies

Cultured cells were washed in cold phosphate-buffered saline and lysed in ice-cold RIPA lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 mol/L NaCl, 0.01 mol/L NaH_2PO_4 , 2 mmol/L ethylenediaminetetraacetic acid, 0.5 mmol/L NaF) containing 2 mmol/L sodium orthovanadate and 1:100 dilution of protease inhibitor cocktail III (Calbiochem). Cell lysates were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses performed as previously described.¹⁷ Rabbit polyclonal antibodies to phospho-Akt (Ser473) and total Akt were from Cell Signaling Technology, Beverly, MA. Phosphorylation-specific antibody to tyrosine-397 FAK was from Biosource Int., Carmillo, CA. Antibody to total FAK was from Santa Cruz Biotechnology, Santa Cruz, CA. Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Pierce, Rockford, IL.

Mice and Bleomycin Injury Model

C57BL/6J mice were purchased from Jackson Laboratories, Bar Harbor, ME, and housed under specific pathogen-free conditions in enclosed filter top cages. Clean food and water was given *ad libitum*. The mice were handled and maintained using microisolation techniques with daily veterinarian monitoring. The University of Michigan Committee on the Use and Care of Animals approved these experiments. Intratracheal bleomycin was administered to mice as previously described^{37,38} with minor modifications. A single 30- μl aliquot containing 0.025 U of bleomycin (Sigma) diluted in normal saline was intratracheally injected using a Tridak stepper (Brookfield, CT) and a 30-gauge needle.

Sircol Assay for Collagen

Mice were euthanized by CO_2 asphyxiation and perfused via the heart with 5 ml of normal saline. Whole lungs were removed, taking care to avoid the large conducting airways, and homogenized in 1 ml of 0.5% Triton X-100. After centrifugation, 100 μl of supernatant was mixed with 1 ml of Sircol collagen assay dye reagent (30 minutes at

room temperature). After centrifugation, the pellet was resuspended in 1 ml of alkali reagent, vortexed to release the dye into solution, 100 μl was transferred to a microplate, and the absorbance measured at 540 nm. Values for experimental samples were calculated based on a standard curve of known concentrations of purified rat tail collagen.

Lung Histology

Animals were euthanized and perfused via the right ventricle with 3 ml of normal saline. Lungs were inflated with 1 ml of 10% neutral buffered formalin, removed, and fixed overnight in formalin before being dehydrated in 70% ethanol. Lungs were processed using standard procedures and embedded in paraffin. Sections (3 to 5 μm) were cut, mounted on slides, and stained with hematoxylin and eosin (H&E) or Masson's trichrome blue for collagen.

Immunohistochemical Staining

Sections from paraffin-embedded tissues for all of the treatment groups were processed for immunohistochemical localization of α -smooth muscle actin (α -SMA) to identify myofibroblasts. The slides were also immunostained with the same phospho-specific antibodies to the phosphorylated (activated) isoforms of PKB/Akt and FAK. Briefly, the tissue sections were dewaxed and were exposed to heat-induced antigen retrieval treatment using a Tendercook pressure cooker and heat antigen unmasking solution (Biogenex, San Ramon, CA) in the microwave for 13.5 minutes. Subsequently, the slides were stained using a sensitive avidin-streptavidin-peroxidase in an automated cell staining system (GenoMx model i6000; Biogenex, San Ramon, CA). The sections were then counterstained with hematoxylin and mounted. Photomicrographs were taken at $\times 200$ magnification.

Collagenase Digestions of Whole Lung

Collagenase digestions can be used to analyze both resident and recruited populations of lung cells found both in the alveolar space and interstitium. This procedure has been optimized to purify lung leukocytes.³⁹ Lungs were excised, minced, and enzymatically digested for 30 minutes using 15 ml/lung of digestion buffer [RPMI, 5% FCS, antibiotics, 1 mg/ml collagenase (Boehringer Mannheim Corp., Chicago, IL) and 30 $\mu\text{g/ml}$ DNase (Sigma)]. The cell suspension and undigested fragments were further dispersed by repeated passage through the bore of a 10-ml syringe without a needle. The total cell suspension was pelleted, and any contaminating erythrocytes were eliminated by lysis in ice-cold NH_4Cl buffer (0.829% NH_4Cl , 0.1% KHCO_3 , and 0.0372% Na_2 ethylenediaminetetraacetic acid, pH 7.4). The pellet was resuspended in 5 ml of complete medium (RPMI, 5% fetal bovine serum, 1% penicillin/streptomycin) and dispersed by 20 passages through a 5-ml syringe. The dispersed cells were filtered through a Nytex filter (Tetko, Inc., Kansas City, MO) to remove clumps. The total volume

was brought up to 10 ml with complete media. An equal volume of 40% Percoll (Sigma) was added and the cells were centrifuged at 3000 rpm for 30 minutes (room temperature) without a brake. The cell pellets were resuspended in complete media, and leukocytes were counted on a hemocytometer in the presence of trypan blue. Cells were greater than 90% viable by trypan blue exclusion. Cytospins of recovered cells were prepared for differential staining as described below.

Differential Staining

Cytospins of collagenase digests were made by centrifugation of 50,000 cells on microscope slides using a Shandon Cytospin 3 (Astmoore, UK). The slides were allowed to air-dry and were stained using a modified Wright-Giemsa stain. For Wright-Giemsa staining, the slides were fixed/prestained for 2 minutes with a one-step methanol-based Wright-Giemsa stain (Harleco; EM Diagnostics, Gibbstown, NJ), followed by steps 2 and 3 of the Diff-Quick whole blood stain (Diff-Quick; Baxter Scientific, Miami, FL). This modification of the Diff-Quick stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 300 cells were counted from randomly chosen high-power microscope fields for each sample. The differential percentage of each cell type was multiplied by the total leukocyte count to derive an absolute number of monocytes/macrophages, neutrophils, and eosinophils per sample.

Statistics

Statistical significance was analyzed using Graphpad Software: InStat version 2.01. Student's *t*-tests were run to determine *P* values when comparing two groups. When comparing three or more groups, analysis of variance was performed with a posthoc Bonferroni test to determine which groups showed significant differences; *P* < 0.05 was considered significant.

Results

The PKI, AG1879, Inhibits TGF- β 1-Induced Activation of PKB/Akt and FAK

AG1879 is a pyrazolopyrimidine compound that potently inhibits the Src family kinases and integrin-dependent FAK activation.^{17,40} Moreover, the AG1879 compound has been shown to also exert inhibitory effects on PKB/Akt.⁴¹ We examined the effect of AG1879 on TGF- β 1-induced activation of these protein kinases in normal human lung fibroblasts (IMR-90). AG1879, at varying doses of 0.1, 1, and 10 μ mol/L, were co-treated with TGF- β 1 (2 ng/ml) for 16 hours before cell lysis and Western blotting with phospho-specific antibodies to the activation state of PKB/Akt and FAK. AG1879 dose dependently inhibited TGF- β 1-induced PKB/Akt and FAK phosphorylation in these cells with almost complete inhi-

Figure 1. Effects of the PKI, AG1879, on TGF- β 1-induced phosphorylation of Akt and FAK in normal human lung fibroblasts (IMR-90). Normal fibroblasts were treated with TGF- β 1 (2 ng/ml) in the presence or absence of active AG1879 or inactive analog at the doses for 16 hours before cell lysis. Cell lysates were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with specific antibodies against phospho-S⁴⁷³ Akt and phospho-Y³⁹⁷ FAK; blots were stripped and reprobed for total Akt and FAK, respectively.

bition at 10 μ mol/L AG1879; whereas, the inactive analog of AG1879 (control, c/AG) had no effect (Figure 1).

FAK and PKB/Akt Protein Kinases Are Activated in Fibrotic Foci of Bleomycin-Injured Murine Lung and Are Inhibited by Systemic Administration of AG1879

Intratracheal instillation of bleomycin in mice induces an acute lung injury followed by well-defined inflammatory and fibrotic phases.⁴² This animal model of pulmonary fibrosis does not replicate all of the features of human IPF,⁴³ but is useful in studying certain pathophysiological mechanisms. Importantly, fibrosis in this model is associated with enhanced TGF- β 1 expression/activation and the emergence of myofibroblasts,¹⁰ typical of human fibrotic disorders.^{1,12} It is not known if fibrotic regions of bleomycin-injured animals contain cells expressing activated (phosphorylated) PKB/Akt and FAK; moreover, whether such responses may be attenuated by systemic administration of PKI has not been examined.

Daily intraperitoneal injections of AG1879 (175 μ g/mouse; ~10 mg/kg) or its inactive analog (AG 1879/inactive; same dose) were administered starting a week after bleomycin injury. This time point was selected based on relative decline in inflammation and activation of fibrogenic responses including myofibroblast emergence and persistence; doses were calculated as described in Materials and Methods. After 7 days of AG1879 treatment, lungs were harvested and tissue sections examined for the activation state of PKB/Akt and FAK by IHC staining with phospho-specific antibodies against the activated forms of these protein kinases; representative sections were also immunostained for α -SMA. As previously reported in bleomycin-induced pulmonary fibrosis,¹⁰ focal areas of dense cellularity and fibrosis contain cells that express α -SMA, a marker of myofibroblasts (Figure 2). Cells in these areas of active tissue fibrosis strongly express phosphorylated (activated) PKB/Akt and FAK (Figure 2). Systemic administration of AG1879 to injured mice attenuates the activation of these protein kinases *in vivo* in association with markedly reduced fibrotic responses (Figure 2).

Figure 2. *In vivo* effects of the PKI, AG1879, on myofibroblast differentiation and the phosphorylation states of PKB/Akt and FAK in lungs of bleomycin-injured mice. C57BL/6J mice were given intratracheal (IT) saline or bleomycin on day 1. Bleomycin-injured mice were given intraperitoneal (IP) injections of saline, active AG1879, or an inactive analog of AG1879 starting on day 8. Lungs were harvested on day 15 after bleomycin injury, formalin-fixed, and paraffin-embedded. Tissue sections were subsequently immunohistochemically (IHC) stained with antibodies against α -SMA (a marker of myofibroblast differentiation), phospho-S⁴⁷³ Akt, and phospho-Y³⁹⁷ FAK. Control staining was with biotinylated secondary IgG antibody. Streptavidin-conjugated horseradish peroxidase was used with 3,3'-diaminobenzidine as substrate (brown staining) and counterstained with hematoxylin (nuclei stain blue). Original magnifications, $\times 200$.

Systemic Administration of AG1879 Induces Stable Changes in the Profibrotic Signaling/Phenotype of Lung Fibroblasts

To determine whether systemic administration of AG1879 results in stable modulation of signaling/phenotype of fibroblasts and myofibroblasts *in vivo*, we isolated and analyzed fibroblasts isolated from lungs of bleomycin-injured mice. Fibroblasts were isolated on day 15 after lung injury by explant cultures and adherence purification; studies were performed on relatively pure (>99% by staining for the collagen crosslinking-enzyme, prolyl-4-hydroxylase³⁶ and by cell morphology) fibroblast populations at passage 2. AG1879 was administered (intraperitoneally) for 7 days, starting a week after initial bleomycin injury. Constitutive expression of activated/phosphorylated FAK and PKB/Akt as well as expression of α -SMA were assessed by Western immunoblots using

whole cell (RIPA) lysates. Fibroblasts isolated from bleomycin-injured mice showed elevated levels of α -SMA, suggesting enhanced myofibroblast differentiation; this effect was partially inhibited by AG1879 treatment (Figure 3). Bleomycin injury also induces stable up-regulation of PKB/Akt and FAK phosphorylation in fibroblasts isolated at the time (day 15) of active *in vivo* fibrogenesis; this effect is attenuated in lung fibroblasts of mice treated with AG1879 (Figure 3).

Systemic Administration of AG1879 Protects against Bleomycin-Induced Lung Fibrosis in Mice

The ability of AG1879 to modulate profibrotic signaling and phenotype of fibroblasts and myofibroblasts *in vivo* suggested the potential that this PKI may protect against

Figure 3. Modulation of lung fibroblast signaling/phenotype in bleomycin-injured mice treated with the PKI, AG1879. C57BL/6J mice were given intratracheal (IT) saline or bleomycin on day 1. Bleomycin-injured mice were given intraperitoneal (IP) injections of saline, active AG1879, or an inactive analog of AG1879 starting on day 8. Fibroblasts were isolated from lung explants on day 14 and expanded in *in vitro* cell culture. Cells were lysed in RIPA buffer at passage 2 and 90% confluency. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antibodies against α -SMA (a marker of myofibroblast differentiation), phospho-S⁴⁷³ Akt, and phospho-Y³⁹⁷ FAK. Blots are representative of three separate experiments that demonstrated similar results.

bleomycin-induced fibrosis. Daily intraperitoneal injections of AG1879 or its inactive analog were administered starting either on the day of injury (day 1) or a week later (day 8). Mice receiving AG1879 developed less fibrosis than mice receiving control drug as assessed by accumulation of total lung collagen at day 15 (Figure 4A). Anti-fibrotic effects of AG1879 administered at early time points (day 1) were similar to that observed when drug therapy was delayed (day 8) after bleomycin injury. Lung histology (H&E staining; Figure 4B, top) showed significantly less fibrotic tissue reaction in AG1879-treated mice than those receiving control drug. Masson's trichrome staining for collagen also demonstrated marked attenuation in accumulated collagen in mice receiving active versus inactive drug (Figure 4B, bottom). These results demonstrate that systemic administration of the PKI, AG1879, protects against the development of fibrotic lung injury *in vivo*. Importantly, significant protection is observed when AG1879 is administered later in the course of injury/repair, when inflammatory responses had subsided.

Protection against Fibrotic Lung Injury by AG1879 Is Not Associated with Alterations in the Inflammatory Response

The early inflammatory response after bleomycin-induced lung injury in rodents typically peaks at 3 days and persists for up to 7 days. To determine whether systemic administration of AG1879 altered inflammatory responses to injury, total numbers of inflammatory cells recruited/resident in the lung on day 7 were assessed by collagenase digest. Bleomycin-injured mice receiving inactive drug (AG/analog) developed typical increases in monocytes/macrophages and lymphocytes, and lesser increases in polymorphonuclear cells compared to mice receiving saline (control). Administration of the active

Figure 4. Anti-fibrotic effects of systemically administered AG1879 in the murine model of bleomycin-induced pulmonary fibrosis. Mice were given intratracheal bleomycin or saline (control). Bleomycin-injured mice received intraperitoneal injections of saline (bleomycin + saline), an inactive analog of AG1879 (bleomycin + AG/analog), or the active PKI, AG1879 (bleomycin + AG1879), started on day of injury (day 1) or a week after initial injury (day 8). **A:** Lungs were harvested on day 14 after bleomycin injury and total lung collagen determined as described in Materials and Methods. Values are expressed as mean $\pm$ SEM, $n = 6$ per group. *, $P < 0.05$ for bleomycin + AG1879 versus bleomycin + saline or AG/analog. This is one of three separate experiments demonstrating similar results. **B:** Representative histopathology by H&E staining (**top**); Masson's trichrome blue staining for collagen (**bottom**) of the lungs of mice intratracheally instilled with saline (control) or bleomycin. Bleomycin-injured mice were administered intraperitoneal injections of inactive drug analog (BL + AG/analog) or the active PKI, AG1879 (BL + AG1879), starting on day 8 after bleomycin injury. Lungs were examined at day 15 after bleomycin injury.

PKI, AG1879, starting on the initial day of bleomycin injury did not significantly alter the number of recruited/resident inflammatory cells of any of the subpopulations examined (Figure 5).

Figure 5. Effect of AG1879 on the inflammatory response in the lungs of bleomycin-injured mice. Mice given intratracheal saline (control) or bleomycin (BL) were administered either inactive drug analog (AG/analog) or active PKI (AG1879) on the day of initial injury. Collagenase digests of lung were performed on day 7 and differential counts made of subpopulations of monocytes/macrophages, lymphocytes, and neutrophils as described in Materials and Methods. Values represent mean $\pm$ SEM, $n = 6$ per group.

Discussion

Fibroblast/myofibroblast recruitment and activation represent normal repair responses to tissue injury.^{1,44,45} Apoptosis of these mesenchymal cells, after re-epithelialization, is essential for normal repair. Desmouliere and colleagues⁷ showed that apoptosis of granulation tissue-myofibroblasts follows effective wound closure and is required for normal cutaneous wound healing. The physiological signal(s) mediating myofibroblast apoptosis are not well defined,¹² but likely involve loss of biophysical matrix-generated tension and biochemical cues from the surrounding cellular microenvironment.^{46,47} Persistence of fibroblasts/myofibroblasts leads to extensive tissue architectural remodeling and progressive fibrosis, characteristic of human fibrotic diseases.¹²

Recent studies from our laboratory have defined the integrin-FAK and PI3K-PKB/Akt pathways as critical regulators of myofibroblast differentiation and survival.^{17,22} In this study, we demonstrate activation of these protein kinase pathways *in vivo* in response to bleomycin-induced fibrotic lung injury in mice. Increased expression of phosphorylated (activated) PKB/Akt and FAK appear to be strongest in areas of dense fibrosis and fibroblast/myofibroblast accumulation. Moreover, both PKB/Akt and FAK are constitutively activated in fibroblasts isolated *ex vivo* from bleomycin-injured lung. This is the first report implicating altered signaling involving these protein kinases in fibroblasts isolated from fibrotic tissues. The importance of these signaling pathways to disease pathogenesis is further supported by the observed activation of both FAK and PKB/Akt in response to exogenous TGF- β 1, a potent fibrogenic cytokine.^{13,14}

The *in vivo* phosphorylation/activation of both FAK and PKB/Akt were attenuated by the systemic administration of the PKI, AG1879, to bleomycin-injured mice. AG1879 is a pyrazolopyrimidine compound that is known to inhibit the Src family kinases and integrin-dependent FAK activation.^{17,40} Our studies demonstrate that AG1879, in addition to Src/FAK kinase(s) inhibition, effectively inhibits TGF- β 1-induced activation of PKB/Akt in human lung fibroblasts. Inhibitory effects of AG1879 may be because of its blockade of upstream FAK activation²⁵ or more direct actions on PKB/Akt itself.⁴¹ Importantly, the observed *in vivo* modulation of these protein kinases by systemic AG1879 therapy is accompanied by a marked reduction in tissue accumulation of myofibroblasts and attenuation of fibrotic responses to lung injury. The reduced accumulation of α -SMA-positive myofibroblasts in injured tissues is likely related to the ability of AG1879 to inhibit FAK-dependent myofibroblast differentiation¹⁷ and/or promote apoptosis of these cells by blocking pro-survival PI3K-PKB/Akt signaling.²²

The murine model of bleomycin-lung injury is a well-established model of pulmonary fibrosis.^{37,38,48} Key pathogenic mechanisms in human disease such as the expression/activation of TGF- β 1 and myofibroblasts during active fibrogenesis are important features of this model.^{10,49} A notable difference between the bleomycin animal model and human disease is the relative paucity of inflammation (whether or not it was present in earlier

disease) in usual interstitial pneumonia, the predominant histopathological pattern in human IPF. Thus, when using an inflammation-dependent fibrosis model (as in the murine bleomycin injury model) to test the efficacy of therapeutic agents, one must be careful to differentiate drug effects on the inflammatory versus fibrotic pathways. A therapeutic strategy that protects against fibrosis by directly targeting fibrogenic pathways would be more beneficial for the treatment of human IPF.⁵⁰

Several lines of evidence suggest that the protective effects of AG1879 in this model are primarily mediated by its actions on fibroblasts/myofibroblasts, preferentially to effects on other cell types. First, significant changes in the number of resident/recruited inflammatory cells in the lung were not observed when AG1879 was administered at the time of initial injury, suggesting drug effects on the inflammatory response to be minimal or inconsequential to its anti-fibrotic effects. Secondly, AG1879 attenuated fibrosis to similar degrees when drug therapy was initiated early (on the day of injury) or delayed (day 8, when inflammatory responses typically decline) after bleomycin injury. Thirdly, we have demonstrated an inhibition of the stable induction of profibrotic signaling (PKB/Akt and FAK) and phenotype (α -SMA-positive myofibroblasts) in fibroblasts isolated from AG1879-treated mice. Finally, based on the observed improvement in histopathology indicative of effective re-epithelialization with reduced fibrosis in AG1879-treated mice, potential detrimental effects of this PKI on regenerating alveolar epithelial cells appear to be minimal. Regenerating epithelial cells may use alternative signaling pathways, such as the MAPK pathways,^{51,52} for proliferation; moreover, AG1879 would be expected to exert greater effects on fibroblasts/myofibroblasts because the relevant protein kinases are markedly activated in these cells. Moreover, AG1879-treated mice were more active, less emaciated (increased body weight) with a trend toward improved survival in comparison to control mice. Overall, the beneficial effects of AG1879 far outweigh any potential detrimental effects on other cell types or nonspecific inhibitory effects of this PKI on other signaling pathways.

Cumulatively, our results suggest that targeting of myofibroblast differentiation and/or survival may be an effective strategy for fibrotic responses to tissue injury. Myofibroblasts in fibrotic tissues may acquire an autoactivated, self-sustaining phenotype that is resistant to apoptosis.¹² Prosurvival/anti-apoptotic signaling in mesenchymal cells is likely to be perpetuated by autocrine effects of secreted insoluble matrix and soluble growth factors;^{17,22} ultimately, these extracellular cues are integrated into the sustained activation of FAK and PKB/Akt. Targeting these prosurvival/anti-apoptotic pathways by PKIs may break the self-perpetuating positive-feedback cycle in fibroblasts/myofibroblasts. This study provides proof of concept that such an anti-fibrotic strategy is highly efficacious and safe in rodents, providing hope for similar therapeutic strategies for a group of otherwise treatment-unresponsive, progressive, and fatal disorders in humans.

Acknowledgment

We thank Carol Wilke for technical assistance with the murine model of bleomycin injury/fibrosis.

References

- Thannickal VJ, Toews GB, White ES, Lynch III JP, Martinez FJ: Mechanisms of pulmonary fibrosis. *Annu Rev Med* 2004, 55:395–417
- Nathan C: Points of control in inflammation. *Nature* 2002, 420:846–852
- Singer AJ, Clark RA: Cutaneous wound healing. *N Engl J Med* 1999, 341:738–746
- Svee K, White J, Vaillant P, Jessurun J, Roongta U, Krumwiede M, Johnson D, Henke C: Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. *J Clin Invest* 1996, 98:1713–1727
- Stahl PJ, Felsen D: Transforming growth factor-beta, basement membrane, and epithelial-mesenchymal transdifferentiation: implications for fibrosis in kidney disease. *Am J Pathol* 2001, 159:1187–1192
- Clark RA, Mason RJ, Folkvord JM, McDonald JA: Fibronectin mediates adherence of rat alveolar type II epithelial cells via the fibroblastic cell-attachment domain. *J Clin Invest* 1986, 77:1831–1840
- Desmouliere A, Redard M, Darby I, Gabbiani G: Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 1995, 146:56–66
- Arora PD, McCulloch CA: The deletion of transforming growth factor-beta-induced myofibroblasts depends on growth conditions and actin organization. *Am J Pathol* 1999, 155:2087–2099
- Kuhn C, McDonald JA: The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* 1991, 138:1257–1265
- Zhang K, Rekhter MD, Gordon D, Phan SH: Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. *Am J Pathol* 1994, 145:114–125
- Haber PS, Keogh GW, Apte MV, Moran CS, Stewart NL, Crawford DH, Pirola RC, McCaughan GW, Ramm GA, Wilson JS: Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. *Am J Pathol* 1999, 155:1087–1095
- Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002, 3:349–363
- Border WA, Ruoslahti E: Transforming growth factor-beta in disease: the dark side of tissue repair. *J Clin Invest* 1992, 90:1–7
- Border WA, Noble NA: Fibrosis linked to TGF-beta in yet another disease. *J Clin Invest* 1995, 96:655–656
- Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G: Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993, 122:103–111
- Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J: Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997, 100:768–776
- Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM, Thomas PE: Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem* 2003, 278:12384–12389
- Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G: Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am J Pathol* 2001, 159:1009–1020
- Zhang HY, Phan SH: Inhibition of myofibroblast apoptosis by transforming growth factor beta(1). *Am J Respir Cell Mol Biol* 1999, 21:658–665
- Jelaska A, Korn JH: Role of apoptosis and transforming growth factor beta1 in fibroblast selection and activation in systemic sclerosis. *Arthritis Rheum* 2000, 43:2230–2239
- Kim G, Jun JB, Elkon KB: Necessary role of phosphatidylinositol 3-kinase in transforming growth factor beta-mediated activation of Akt in normal and rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2002, 46:1504–1511
- Horowitz JC, Lee DY, Waghray M, Keshamouni VG, Thomas PE, Zhang H, Cui Z, Thannickal VJ: Activation of the pro-survival phosphatidylinositol 3-kinase/AKT pathway by transforming growth factor-beta1 in mesenchymal cells is mediated by p38 MAPK-dependent induction of an autocrine growth factor. *J Biol Chem* 2004, 279:1359–1367
- Cantley LC: The phosphoinositide 3-kinase pathway. *Science* 2002, 296:1655–1657
- Datta SR, Brunet A, Greenberg ME: Cellular survival: a play in three acts. *Genes Dev* 1999, 13:2905–2927
- Chen HC, Appeddu PA, Isoda H, Guan JL: Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem* 1996, 271:26329–26334
- Ilic D, Almeida EA, Schlaepfer DD, Dazin P, Aizawa S, Damsky CH: Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 1998, 143:547–560
- American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am J Respir Crit Care Med* 2000, 161:646–664
- King Jr TE, Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron Jr JA, Flint A, Thurlbeck W, Cherniack RM: Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. *Am J Respir Crit Care Med* 2001, 164:1025–1032
- Flaherty KR, Colby TV, Travis WD, Toews GB, Mumford J, Murray S, Thannickal VJ, Kazerooni EA, Gross BH, Lynch III JP, Martinez FJ: Fibroblastic foci in usual interstitial pneumonia: idiopathic versus collagen vascular disease. *Am J Respir Crit Care Med* 2003, 167:1410–1415
- Dancey J, Sausville EA: Issues and progress with protein kinase inhibitors for cancer treatment. *Nat Rev Drug Discov* 2003, 2:296–313
- Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, Firestein GS: c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J Clin Invest* 2001, 108:73–81
- Underwood DC, Osborn RR, Bochnowicz S, Webb EF, Rieman DJ, Lee JC, Romanic AM, Adams JL, Hay DW, Griswold DE: SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. *Am J Physiol* 2000, 279:L895–L902
- Matsuoka H, Arai T, Mori M, Goya S, Kida H, Morishita H, Fujiwara H, Tachibana I, Osaki T, Hayashi S: A p38 MAPK inhibitor, FR-167653, ameliorates murine bleomycin-induced pulmonary fibrosis. *Am J Physiol* 2002, 283:L103–L112
- Rice AB, Moomaw CR, Morgan DL, Bonner JC: Specific inhibitors of platelet-derived growth factor or epidermal growth factor receptor tyrosine kinase reduce pulmonary fibrosis in rats. *Am J Pathol* 1999, 155:213–221
- Suzuki H, Aoshiba K, Yokohori N, Nagai A: Epidermal growth factor receptor tyrosine kinase inhibition augments a murine model of pulmonary fibrosis. *Cancer Res* 2003, 63:5054–5059
- Konttinen YT, Nykanen P, Nordstrom D, Saari H, Sandelin J, Santavirta S, Kouri T: DNA synthesis in prolyl 4-hydroxylase positive fibroblasts in situ in synovial tissue. An autoradiography-immunoperoxidase double labeling study. *J Rheumatol* 1989, 16:339–345
- Hattori N, Degen JL, Sisson TH, Liu H, Moore BB, Pandrangi RG, Simon RH, Drew AF: Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice. *J Clin Invest* 2000, 106:1341–1350
- Kuwano K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, Suda T, Kunitake R, Maeyama T, Miyazaki H, Hara N: Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. *J Clin Invest* 1999, 104:13–19
- Huffnagle GB, Strieter RM, Standiford TJ, McDonald RA, Burdick MD, Kunkel SL, Toews GB: The role of monocyte chemoattractant protein-1 (MCP-1) in the recruitment of monocytes and CD4+ T cells during a pulmonary *Cryptococcus neoformans* infection. *J Immunol* 1995, 155:4790–4797
- Salazar EP, Rozengurt E: Src family kinases are required for integrin-mediated but not for G protein-coupled receptor stimulation of focal adhesion kinase autophosphorylation at Tyr-397. *J Biol Chem* 2001, 276:17788–17795

41. Bain J, McLauchlan H, Elliott M, Cohen P: The specificities of protein kinase inhibitors: an update. *Biochem J* 2003, 371:199–204
42. Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R: Time course of bleomycin-induced lung fibrosis. *Int J Exp Pathol* 2002, 83:111–119
43. Borzone G, Moreno R, Urrea R, Meneses M, Oyarzun M, Lisboa C: Bleomycin-induced chronic lung damage does not resemble human idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2001, 163:1648–1653
44. Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH: Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004, 113:243–252
45. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM: Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004, 114:438–446
46. Grinnell F, Zhu M, Carlson MA, Abrams JM: Release of mechanical tension triggers apoptosis of human fibroblasts in a model of regressing granulation tissue. *Exp Cell Res* 1999, 248:608–619
47. D'Addario M, Arora PD, Ellen RP, McCulloch CA: Regulation of tension-induced mechanotranscriptional signals by the microtubule network in fibroblasts. *J Biol Chem* 2003, 278:53090–53097
48. Nakao A, Fujii M, Matsumura R, Kumano K, Saito Y, Miyazono K, Iwamoto I: Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J Clin Invest* 1999, 104:5–11
49. Zhang HY, Gharaee-Kermani M, Zhang K, Karmiol S, Phan SH: Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1996, 148:527–537
50. Selman M, King TE, Pardo A: Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001, 134:136–151
51. Yoshida K, Kuwano K, Hagimoto N, Watanabe K, Matsuba T, Fujita M, Inoshima I, Hara N: MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. *J Pathol* 2002, 198:388–396
52. Cummins AB, Palmer C, Mossman BT, Taatjes DJ: Persistent localization of activated extracellular signal-regulated kinases (ERK1/2) is epithelial cell-specific in an inhalation model of asbestosis. *Am J Pathol* 2003, 162:713–720