Effects of the Protein Kinase Inhibitor, Imatinib Mesylate, on Epithelial/Mesenchymal Phenotypes: Implications for Treatment of Fibrotic Diseases

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Abbreviations:

SMAD = mammalian homolog of the Mad (mothers against decapentaplegic) gene in Drosophila and the related Sma genes in Caenorhabditis Elegans
PKI = protein kinase inhibitor
c-ABL = proto-oncogene ABL originally identified in the Abelson murine leukemia virus
BCR-ABL = translation products of a fusion mRNA derived from the breakpoint cluster region (BCR) gene and a cellular ABL (c-ABL) gene translocated to chromosome 22
c-KIT = protein-tyrosine kinase receptor for stem cell factor
PDGFR = platelet-derived growth factor receptor
CML = chronic myelogenous leukemia
GIST = gastrointestinal stromal tumors
EMT = epithelial mesenchymal transition
α-SMA = α-smooth muscle actin

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ABSTRACT

Tissue injury in mammals triggers both inflammatory and repair responses that, in some contexts, results in fibrosis. Fibrosis is characterized by the persistence of activated myofibroblasts, ineffective re-epithelialization, and variable degrees of inflammation within injured tissues. The protein kinase inhibitor (PKI), imatinib mesylate, has been proposed as a potential anti-fibrotic therapeutic agent. In this study, the efficacy of imatinib mesylate to modulate fibrogenic responses, both in-vitro and in-vivo, were examined. In an in-vitro fibroblast culture model, imatinib inhibits platelet-derived growth factor receptor activation and fibroblast proliferation, but not the stably differentiated myofibroblast phenotype. Additionally, imatinib inhibits lung epithelial cell proliferation and survival, but not the induction of epithelial-mesenchymal transition. Imatinib does not alter transforming growth factor-β/SMAD3 signaling in either cell type. In a murine model of lung fibrosis, bleomycin-induced injury to the pulmonary epithelium provokes an early inflammatory response with more delayed fibrosis during the late reparative phase of lung injury. Imatinib mesylate (10 mg/kg/day, by intraperitoneal injection or oral gavage), administered during the post-injury repair phase, failed to significantly alter fibrogenic responses assessed by histopathology, collagen content, and the accumulation of myofibroblasts within the injured lung. These studies indicate that the capacity of a PKI to inhibit fibroblast proliferation may be insufficient to mediate significant anti-fibrotic effects in late stages of tissue injury-repair. Pharmacologic agents that modulate the activities and fate of differentiated (myo)fibroblasts, while not interfering with the regenerative capacity of epithelial cells, are likely to be more effective for treatment of nonresolving, progressive fibrotic disorders.
INTRODUCTION

Tissue fibrosis may result from diverse forms of severe or recurrent injury, of known or unknown cause, that often culminates in organ dysfunction in association with significant morbidity and mortality in humans. An evolving paradigm in the pathogenesis of fibrotic diseases is the paradoxical activation and persistence of mesenchymal cells, in particular myofibroblasts, while epithelial cells within the same tissue microenvironment fail to regenerate and re-epithelialize the damaged organ (Selman et al., 2001; Thannickal, 2004). Treatment of fibrotic disorders of the lung and other organ systems with anti-inflammatory/immunosuppressive agents have been largely disappointing and emerging strategies have shifted to the development of novel anti-fibrotic agents (Thannickal et al., 2005).

Protein kinase inhibitors (PKIs) are a relatively new class of therapeutic agents that have the potential to modulate cellular phenotypes in oncologic diseases and in chronic inflammatory/fibrotic diseases (Druker et al., 1996; Druker et al., 2001; Cohen, 2002). Imatinib mesylate, (Gleevec™, Novartis; STI571), a phenylaminopyrimidine derivative, is the prototypical tyrosine kinase inhibitor with activity against the non-receptor tyrosine kinases, c-ABL and BCR-ABL (Druker et al., 1996), and the receptor tyrosine kinases, platelet-derived growth factor receptor (PDGFR) (Buchdunger et al., 2000) and c-KIT (Wang et al., 2000). Imatinib mesylate has been approved for treatment of chronic myeloid leukemia (CML) (Druker et al., 2001) and gastrointestinal stromal tumors (GIST) (Tuveson et al., 2001).

There has been recent interest in the potential efficacy of imatinib in the treatment of the most common and progressive form of human fibrotic lung disease, idiopathic pulmonary fibrosis (IPF). Both PDGFR and c-ABL tyrosine kinases have been implicated in fibrogenic pathways in the lung (Liu et al., 1997; Daniels et al., 2004). However, it is currently unclear
whether imatinib is capable of mediating significant anti-fibrotic effects and animal models of pulmonary fibrosis have, thus far, produced mixed results, likely related to differences in experimental design and drug administration (Daniels et al., 2004; Aono et al., 2005; Chaudhary et al., 2006). In this study, effects of imatinib mesylate on epithelial and mesenchymal cell signaling and associated phenotypes/fates relevant to tissue injury and repair were studied. Additionally, we evaluated the in-vivo effects of imatinib mesylate, at doses comparable to that approved for human subjects with CML and GIST, administered via two different routes (intra-peritoneal and oral) during the post-injury reparative/fibrotic phase of bleomycin-induced lung injury in mice.
METHODS

Reagents and Drugs

Porcine platelet-derived transforming growth factor-β1 (TGF-β1) was purchased from R&D systems, Minneapolis, MN. Imatinib mesylate (Gleevec; Novartis) was obtained from the University of Michigan Health System Pharmacy Services, Ann Arbor, MI. Imatinib mesylate capsules were initially solubilized in sterile water to make a 10 mM stock concentration of active drug. Cell culture experiments were performed at final concentrations ranging from 1-20 µM of imatinib mesylate by serial dilutions of this 10 mM stock solution directly in cell culture medium. For animal studies, the same 10 mM stock solution was diluted in sterile saline to 0.170 mg in 0.25 ml aliquots and administered by daily intra-peritoneal injection or oral gavage (10 mg/kg; for mice with an average weight of 17 g). All other reagents for cell culture and animal studies were from Sigma, St. Louis, MO, unless otherwise stated.

Animal Studies

The University of Michigan Committee on the Use and Care of Animals approved the animal protocol used in this study. Eight-week old female C57BL/6 mice weighing 15-20 g were obtained from Jackson Laboratories, (Bar Harbor, ME, USA). Mice were housed under specific pathogen-free conditions in plastic enclosed filter-top cages with hardwood shavings, 8-10 animals per cage. A 12 h light/dark cycle was maintained and the mice had access to water and rodent laboratory chow ad libitum until they were euthanized. The C57BL/6 mice were assigned to four weight-matched experimental groups of 8-10 each: (i) intra-tracheal (IT) saline and intra-peritoneal (IP) saline; (ii) IT-bleomycin and IP-saline; (iii) IT-bleomycin and IP-imatinib mesylate (10 mg/kg); and (iv) IT-bleomycin and imatinib mesylate (10 mg/kg) by oral gavage (PO). IP injections of imatinib (or saline) and oral gavage of imatinib were initiated on
day 7 and continued daily for two weeks. On day 21 after bleomycin administration, mice were euthanized by CO2 asphyxiation. Lungs were harvested and processed for immunohistochemical (IHC) staining, ex-vivo fibroblast isolation/culture, and tissue homogenates were collected and stored at -20°C.

**Bleomycin Instillation**

Bleomycin Sulfate (H. Lundberg A/S, Copenhagen, Denmark) was dissolved in sterile 0.9% saline on the day of IT instillation. The animals were anesthetized with a mixture of 1 ml of ketamine (100 µg/ml) and 1 ml of xylazine (100 µg/ml) and 4.6 ml of sterile saline. A single 30 µl aliquot containing 0.025 U of bleomycin (Sigma, St. Louis, MO) diluted in normal saline was injected intra-tracheally using a Tridak-stepper (Brookfield, CT) and a 30-gauge needle.

**Isolation and Culture of Mouse Lung Fibroblasts**

Whole lungs were sterilely removed and cut into small 2-3 mm slices and allowed to adhere on tissue culture plastic. These lung tissue explants were maintained in medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 U/ml penicillin/streptomycin (Sigma, St. Louis, MO), and fungizone (GIBCO, Grand Island, New York). Fibroblasts grown from explant tissues were isolated by trypsinization and passaging (typically 7-10 days after initial explant) to achieve a homogenous population of spindle cells that uniformly expressed the collagen cross-linking enzyme, prolyl 4-hydroxylase. Cell lysates were obtained for Western blot analyses at passage 2 and at a confluency of 90-100%.

**Lung Histology**

Mice were euthanized and perfused via the right ventricle with 5 ml of normal saline. Lungs were inflated with 10% neutral buffered formalin injected via the trachea. Following
overnight formalin fixation, lung tissues were dehydrated and stored in 70% ethanol. Fixed tissues were embedded in paraffin using standard procedure; 3-5 µm sections 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 the treatment groups were processed for immunohistochemical (IHC) staining for α-smooth muscle actin (α-SMA), as previously described (Vittal et al., 2005). Micrographs were captured at an original magnification of x20 or x40. Length bars were added using SPOT windows version 4.0.8, University of New South Wales, Australia.

**Sircol Assay for Collagen**

Mice were euthanized and perfused via the right ventricle with 5 ml of normal saline. Whole lungs were removed free of hilar structures and other extraneous tissue. The tissues were homogenized in 1 ml of 0.5% Triton-X 100. After centrifugation, 100 µl of supernatant was mixed with 1 ml of Sircol assay dye reagent for 30 min at room temperature. Following centrifugation, the pellet was suspended in 1 ml of alkali reagent, vortexed to release the dye into solution, and 100 µl transferred into a microplate and absorbance measured at 540 nm. Values for experimental samples were based on a standard curve of known concentrations of purified rat-tail collagen.

**Cell Culture**

Experiments were performed on normal human fetal lung fibroblasts (IMR-90; Institute for Medical Research, Camden, NJ). Cells were cultured in medium consisting of Dulbecco’s Modified Eagle’s medium (DMEM; GIBCO, Grand Island, New York) supplemented with 10%
fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 U/ml penicillin/streptomycin (Sigma, St. Louis MO), and fungizone (GIBCO, Grand Island, New York); medium was changed every two days. Passage 3-5 fibroblasts were plated on 35 mm dishes at a density of 5 x 10^5 cells/dish and incubated at 37°C in 5% CO₂-95% air.

Rat alveolar type II epithelial cells (RLE-6TN, ATCC, Bethesda, MD) were cultured in Ham’s F-12 media supplemented with growth factors, 10% FBS (Sigma, St. Louis, MO), 100 U/ml penicillin-streptomycin (Sigma, St. Louis MO), and fungizone (GIBCO, Grand Island, New York); medium was changed every two days. RLE-6TN cells were plated on 35 mm cell culture dishes at a density of 5 x 10^5 cells/dish and incubated at 37°C in 5% CO₂-95% air.

**Western Immunoblotting and Antibodies**

Cells were rinsed with ice-cold PBS and the lysates were prepared in RIPA cell lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaH₂PO₄, 2 mM EDTA, 0.5 mM NaF) containing 2 mM sodium orthovanadate and 1:100 dilution of protease inhibitor cocktail III (Calbiochem, La Jolla, CA). The cell lysates were then subjected to SDS-PAGE and Western blot analyses performed as previously described (Vittal et al., 2005).

Antibodies to PDGFR-β, phospho-PDGFR-β and phospho-SMAD3 were from Cell Signaling Technology. Antibody to total SMAD3 was from Zymed Laboratories Inc., San Francisco, CA. Mouse monoclonal antibody to α-smooth muscle actin (clone 1A4) was from Dako Automation, Carpentaria, CA. Rabbit polyclonal antibody to GAPDH (ab9485) was from Abcam Inc., Cambridge, MA. Mouse monoclonal antibody to α-tubulin (clone B-5-1-2) was from Sigma. Secondary horseradish peroxidase (HRP)-conjugated anti-goat, anti-mouse and anti-rabbit antibodies were from Pierce Biotechnology, Rockford, IL. The blots were developed using an ECL western blot detection kit (Super Signal, Pierce Biotechnology, Rockford, IL).
Densitometric analyses of Western blots were performed using the public domain NIH image program, www.rsb.info.nih.gov/nih-image.

**Assays for Cell Proliferation**

*Cell number:* Cell numbers were measured using a model ZM Coulter counter (Coulter Electronics, Hialeah, FL). Briefly, the 35 mm culture dishes were washed with Hank’s balanced salt solution (HBSS; GIBCO, Grand Island, New York). The dishes were incubated with 1 ml of trypsin-EDTA for 2-3 min and rapidly suspended in solution by repeated pipetting. A 0.2 ml aliquot of cell suspension was diluted in isotonic saline solution and cell counts assessed in the Coulter counter.

*BrdU incorporation:* Cells were plated at a known density in 96-well ELISA plates and BrdU labeling protocol was followed according to manufacturer’s instructions (Calbiochem, La Jolla, CA). Briefly, the cells were labeled with BrdU for 24 h. The medium was aspirated and the cells were fixed with fixative/denaturing solution for 30 min. The fixative was aspirated and 100 µl of anti-BrdU antibody was added for 1 h. After several washes, 100 µl of HRP-conjugated goat-anti-mouse secondary antibody was added for 15 min. The washes were repeated, 100 µl of substrate solution was added and the plate was incubated in the dark for 15 min. This was followed by 100 µl of “stop solution” provided by the manufacturer. Absorbance was read on an ELISA plate reader at dual wavelengths of 450 and 540 nm. The "background" absorbance of cells receiving no primary antibody was subtracted, and a relative proliferation index was calculated by dividing the corrected absorbance by cell counts (measured by Coulter counter) which was obtained prior to fixing the cells.
Assays for Apoptosis

**ELISA for ssDNA:** Apoptosis was quantitated with the use of an ELISA-based assay for single-stranded DNA (ssDNA; Apoptosis ELISA Kit, Chemicon International, Temecula, CA) according to the manufacturer's instructions. Cells were seeded directly into 96-well cell culture plates, grown to 60-70% confluence prior to treatment with/without TGF-β1 and assays performed as previously described (Horowitz et al., 2004).

**Caspase-3 activation:** Detection of activated (cleaved) caspase-3 was by Western blot analysis as described above. Antibody to cleaved caspase-3 was from Chemicon, Temecula, CA. Loading control was with a rabbit polyclonal antibody to GAPDH (ab9485) from Abcam Inc., Cambridge, MA.

**Statistics**

Statistical significance was analyzed using the GraphPad Prism, version 3.0; GraphPad Software, Inc., San Diego, CA. Student’s t-test was run to determine p values when comparing two groups. When comparing three or more groups, ANOVA was performed with a post-hoc Bonferroni test to determine which two groups showed significant differences; p < 0.05 was considered significant.
RESULTS

Effects of Imatinib on Fibroblast Proliferation and Myofibroblast Differentiation

Fibroblast proliferation and myofibroblast differentiation are generally considered to be key events in the initiation and/or maintenance of fibrogenic tissue responses (Tomasek et al., 2002; Thannickal, 2004). To study the effects of imatinib mesylate on proliferation of fibroblasts, quiescent normal human fetal lung fibroblasts (IMR-90) were treated with increasing concentrations of imatinib (1, 5, 10 and 20 µM) and cell proliferation in the presence of 10% FBS assessed by Coulter counting (Fig. 1A) and by BrdU incorporation (Fig. 1B). Imatinib induces a dose-dependent inhibition of cell proliferation by both methods, with almost complete inhibition noted at a 20 µM concentration of imatinib (Fig. 1A, B). This demonstrates that imatinib mesylate is an effective and potent inhibitor of fibroblast proliferation.

Myofibroblasts are key effector cells in tissue fibrosis and connective tissue remodeling in-vivo (Tomasek et al., 2002). TGF-β1 is a central mediator of myofibroblast differentiation and activation both in-vivo and in-vitro (Desmouliere et al., 1993; Thannickal et al., 2003). In IMR-90 fibroblasts, imatinib “co-treatment” inhibits TGF-β1-induced upregulation of α-SMA, a marker of myofibroblast differentiation, (Fig. 1C, top panels; 1D). However, when imatinib mesylate is added to differentiated myofibroblasts (48 h after TGF-β1 stimulation; “post-treatment”), there is a further upregulation in steady-state levels of α-SMA expression (Fig. 1C, bottom panels; 1E). These results indicate that although imatinib inhibits the induction of myofibroblast differentiation by TGF-β1, this PKI may augment the α-SMA-expressing phenotype of stably differentiated myofibroblasts.
Effects of Imatinib on Lung Epithelial Cell Proliferation, Apoptosis and Epithelial-Mesenchymal Transition

Resolution of repair responses to lung injury requires re-epithelialization of the alveolar epithelium to restore barrier function, an event that may be critical for termination of persistent fibrogenesis (Thannickal, 2004). To determine potential modulation by imatinib mesylate of epithelial cell proliferation, apoptosis and epithelial-mesenchymal transition (EMT), effects of imatinib on epithelial cell phenotypes were examined in a cell culture model of rat alveolar type II epithelial cells (RLE-6TN) (Willis et al., 2005). First, the effect of imatinib on epithelial cell proliferation was evaluated. Imatinib mesylate (1-20 µM) induced dose-dependent inhibition of proliferation of RLE-6TN cells grown in Ham’s F-12 media supplemented with growth factors over a period of 48 h (Fig. 2A). Similar effects of imatinib were also noted in primary human small airway epithelial cells (SAECs) and normal human bronchial epithelial cells (NHBECs; data not shown).

Apoptosis of alveolar epithelial cells may represent another important mechanism for the initiation and/or persistence of fibrogenic tissue responses (Thannickal and Horowitz, 2006). TGF-β1, itself, promotes epithelial cell apoptosis, either directly (Havrilesky et al., 1995) or indirectly by paracrine signaling from activated myofibroblasts stimulated with TGF-β1 (Waghray et al., 2005). To determine the effects of imatinib on apoptosis, RLE-6TN cells were treated ± imatinib mesylate (10 µM) ± TGF-β1 (2 ng/ml) for 48 h and apoptotic rates measured using an ELISA for ssDNA (Fig. 2B) and by Western immunoblotting for activated (cleaved) caspase-3 (Fig. 2C; top panel). While imatinib alone induces apoptosis of RLE-6TN cells, the combination of imatinib and TGF-β1 further augments the apoptotic response (Fig. 2B; 2C, upper panel; 2D). Lung epithelial cells during injury/repair processes may adopt alternative cell
fates, such as EMT, a process that may further impair alveolar re-epithelialization and promote pulmonary fibrosis (Willis et al., 2005; Kim et al., 2006). Previous studies have shown that RLE-6TN cells undergo EMT by expression of α-SMA in response to TGF-β1 treatment (Willis et al., 2005). Using this cell culture model of EMT, we determined if imatinib is capable of modulating TGF-β1-mediated EMT. At a dose of imatinib (10 µM) that markedly induces apoptotic responses and potently suppresses proliferation of RLE-6TN cells, minimal effects of imatinib on TGF-β1-induced α-SMA were observed (Fig. 2C, bottom panels; 2E), suggesting that EMT is not amenable to modulation by imatinib. Together, these results indicate that imatinib mesylate potently inhibits proliferation and promotes apoptosis of lung epithelial cells, but does inhibit TGF-β1-mediated EMT in the same cells.

**Imatinib Inhibits PDGFR-β Activation But Does Not Alter TGF-β/SMAD3 Signaling in Both Lung Fibroblasts and Epithelial Cells**

Imatinib mesylate is a known inhibitor of PDGFR tyrosine kinase(s) (Pietras et al., 2002), a mechanism that may account for its anti-proliferative effects on specific cell types (Gilbert et al., 2001). However, potential effects of imatinib on TGF-β signaling via the canonical SMAD pathway are less well defined. TGF-β/SMAD3 signaling is critical for the induction of myofibroblast differentiation (Flanders et al., 2002; Hu et al., 2003) and EMT (Saika et al., 2004). We first examined the ability of imatinib to interfere with early signaling of PDGF and TGF-β1 in human lung fibroblasts (IMR-90). Quiescent IMR-90 fibroblasts were stimulated with TGF-β1 (2 ng/ml) or PDGF-BB (50 ng/ml) for 30 min in cells pre-treated with/without imatinib mesylate (10 µM x 15 min) prior to cell lysis and western blot analyses. PDGF-BB, but not TGF-β1, induced rapid phosphorylation/activation of the PDGF-β receptor, an effect that is completely blocked by imatinib pre-treatment (Fig. 3A; top panels). Under the same conditions,
TGF-β1 (but not PDGF-BB) stimulates SMAD3 phosphorylation, an effect that is not altered by imatinib (Fig. 3A; bottom panels). Similar studies on the effects of imatinib in PDGF and TGF-β1 signaling were conducted in RLE-6TN alveolar epithelial cells. Although signaling via PDGF-BB/PDGFR-β was less robust in RLE-6TN cells than that observed in IMR-90 cells, the effect of imatinib to block phosphorylation of PDGFR-β without altering TGF-β/SMAD3 signaling was evident in both cell types (Fig. 3B). These results indicate that imatinib mesylate, at a concentration that effectively inhibits the PDGFR-β tyrosine kinase, fails to block TGF-β/SMAD3 signaling in both epithelial cells and fibroblasts.

**Imatinib Administration during the Post-Injury Reparative Phase Does Not Protect Against Bleomycin-Induced Pulmonary Fibrosis**

Imatinib mesylate has been proposed as a potential anti-fibrotic agent (Daniels et al., 2004; Aono et al., 2005). To evaluate the effects of imatinib on fibrogenic tissue responses, we examined the effects of administering the drug at later time-points when acute inflammatory responses are relatively minimal and repair/fibrotic responses are activated in the murine model of bleomycin-induced pulmonary fibrosis (Thrall et al., 1982; Zhang et al., 1994). C57BL/6 mice were subjected to intra-tracheal (IT) instillation of bleomycin or saline on day 0. Imatinib mesylate (10 mg/kg/day) was administered by intra-peritoneal injection (IP) or by oral gavage (PO) starting on day 7 for a period of two weeks. The selected dose of 10 mg/kg/day is similar to that used for other PKIs in this mouse model of pulmonary fibrosis (Vittal et al., 2005), but higher than that administered to patients with CML or GIST on a per-kilogram body weight basis. Bioavailability and activity of the drug given by the oral route in lung tissue was confirmed by demonstrating inhibition of PDGFR-β phosphorylation in imatinib-treated mice on day 21 post-lung injury (data not shown). Histopathological examination of the lung on day 21
demonstrated significant lung architectural remodeling in bleomycin-treated animals by H&E-staining (Fig. 4A; top panels) and increased deposition of collagen by trichrome staining (Fig. 4A; bottom panels). These effects of bleomycin-induced pulmonary fibrosis were not altered by the systemic administration of imatinib by either the IP or PO route (Fig. 4A). To quantitatively assess the fibrotic responses in these mice, whole lung homogenates were analyzed on day 21 for soluble collagen content using the Sircol assay, as previously described (Vittal et al., 2005). Bleomycin induced significant increases in lung collagen accumulation, an effect that was not significantly altered by imatinib administration (Fig. 4B). These studies indicate that systemic administration of imatinib mesylate does not attenuate fibrogenic tissue responses during the reparative phase of lung injury in this murine model.

**In-Vivo Accumulation of Lung Myofibroblasts in Bleomycin-Induced Pulmonary Fibrosis is not Inhibited by Imatinib Treatment**

Myofibroblasts are key effector cells in the fibrogenic response to lung injury in-vivo, both in human fibrotic lung diseases as well as in the bleomycin animal model (Zhang et al., 1994; Thannickal, 2004). We determined if the systemic administration of imatinib alters the presence and extent of myofibroblast accumulation in lungs of bleomycin-injured mice. Fixed, paraffin-embedded lung tissue of mice injured by intra-tracheal bleomycin and treated with saline or imatinib (10 mg/kg orally or by intra-peritoneal injection; day 7-21) were stained for the expression of \( \alpha \)-SMA, a marker for myofibroblast differentiation, by IHC on day 21 following bleomycin injury. Myofibroblasts were identified in areas of dense fibrosis in lungs of bleomycin-injured mice; however, the accumulation of myofibroblasts in fibrotic regions of the lung was not significantly modulated by imatinib treatment (Fig. 5A).
In parallel studies, fibroblasts were isolated from the lungs of these mice on day 21. Fibroblasts were isolated by explant culture and adherence purification; studies were performed on relatively pure fibroblast populations (>99% by staining for prolyl-4-hydroxylase and by cell morphology) (Vittal et al., 2005). Expression of α-SMA was assessed by Western immunoblots of whole cell lysates. Fibroblasts isolated from bleomycin-injured mice showed constitutively elevated levels of α-SMA expression, an effect that was not inhibited by imatinib treatment (Fig. 5B, C). Interestingly, there was a trend toward further enhancement of the myofibroblast phenotype in imatinib-treated groups, although this did not reach statistical significance when compared to bleomycin alone (Fig. 5C). Together, these studies indicate that imatinib administrated during the post-injury repair phase of bleomycin-induced lung injury does not mitigate the persistent expression of the myofibroblast phenotype or tissue fibrosis in late stages of lung injury repair and remodeling.
DISCUSSION

Fibrotic disorders are characterized by complex and dynamic interactions between regenerating/reparative epithelial cells and activated (myo)fibroblasts (Selman et al., 2001; Thannickal, 2004). The acquisition of dysregulated epithelial and mesenchymal phenotypes in chronic fibrotic diseases, such as IPF, makes PKIs a potentially effective class of therapeutic agents (Thannickal et al., 2005). In this study, we evaluated the effects of imatinib mesylate on specific epithelial/mesenchymal phenotypes that are likely to be important in fibrogenic tissue responses. Additionally, we tested the efficacy of imatinib as a potential “anti-fibrotic” agent in the murine model of bleomycin-induced pulmonary fibrosis. Although currently available animal models of pulmonary fibrosis do not precisely replicate the human disease, the murine model of bleomycin injury captures several essential, but not all, features of human IPF (Borzone et al., 2001). To test the efficacy of imatinib specifically on post-injury fibrogenesis in this animal model, drug treatment was begun a week after initial lung injury when inflammatory responses are relatively minimal and fibrogenic pathways are activated (Thrall et al., 1982; Zhang et al., 1994).

Our studies demonstrate that imatinib mesylate is a potent inhibitor of PDGFR-β signaling and of fibroblast proliferation. In contrast, imatinib fails to inhibit TGF-β/SMAD signaling and appears to promote the α-SMA-expressing phenotype of stably differentiated myofibroblasts (“post-treated” with imatinib). The effect of imatinib (“co-treatment”) to suppress TGF-β1-induced myofibroblast differentiation is, thus, likely due to inhibition of SMAD-independent pathways (Daniels et al., 2004). In lung epithelial cells, imatinib mediates potent anti-proliferative and pro-apoptotic effects, phenotypic alterations that would predictably impair alveolar epithelial cell regeneration and re-epithelialization in-vivo. Additionally,
imatinib does not inhibit TGF-β1-activated signaling via SMAD3 or the induction of EMT in lung epithelial cells. Together, these effects of imatinib on epithelial and mesenchymal cell phenotypes would favor an in-vivo tissue remodeling response characterized by impaired epithelial repair/regeneration and persistence of myofibroblasts. In agreement with this prediction, administration of imatinib during the post-injury reparative phase of lung injury did not protect against fibrogenic responses to bleomycin-induced lung injury in mice. This lack of therapeutic efficacy was associated with the persistence in fibrotic lung of myofibroblasts, key effector cells in tissue fibrogenesis (Tomasek et al., 2002). No significant differences in fibrotic responses between oral vs. IP administration were noted; however, there was a trend towards higher expression of the myofibroblast phenotype with oral imatinib therapy. Since serum concentrations of imatinib were not directly measured in this study, it is currently not known if this trend is attributable to differences in bioactivity of the drug with oral vs. IP route of imatinib administration. Possible mechanisms by which imatinib may promote myofibroblast accumulation within the injured lung include indirect effects of imatinib on epithelial cells that impair normal repair/regeneration of the epithelium or direct effects on myofibroblast stabilization/survival. Our in-vitro studies support both possibilities – while suppressive effects of imatinib on epithelial cell proliferation/survival were observed, steady state levels of α-SMA in differentiated myofibroblasts were found to further increase with imatinib treatment.

These observations suggest that the combined pro-fibrotic effects of imatinib mesylate on epithelial and mesenchymal signaling/phenotype may negate potential anti-fibrotic effects of the drug on fibroblast proliferation in-vivo. Alternatively, effects on fibroblast proliferation may be relatively less important during later stages of the tissue injury-repair process. Fibroblast recruitment and proliferation are perhaps more likely to occur early in the repair phase, while
their differentiation to myofibroblasts and eventual apoptosis (or the lack thereof) likely occurs later in the course of lung repair/fibrosis (Thannickal and Horowitz, 2006). During late phases of tissue repair, the acquisition of an apoptosis-resistant mesenchymal cell/myofibroblast phenotype may be critical in persistent or progressive fibrotic responses (Vittal et al., 2005; Horowitz et al., 2006a). Our previous studies with another PKI, AG1879 (4-amino-5-[4-chlorophenyl]-7-[t-butyl]pyrazolo-[3,4-D]pyrimidine), that inhibits TGF-β-induced pro-survival signaling pathways involving focal adhesion kinase (FAK) and protein kinase B (PKB/AKT) in lung myofibroblasts, proved to be an effective anti-fibrotic therapeutic approach (Vittal et al., 2005). Importantly, in this prior study, the AG1879-PKI administration was begun at the same time (following the first week of injury) as in the current study and continued for a shorter duration (one week) (Vittal et al., 2005). This supports the feasibility of targeting these pro-survival pathways in the late, reparative/fibrotic phase in this animal model. Previous studies have shown that both FAK and AKT are required for the stable induction and survival of the myofibroblast phenotype in response to TGF-β1 (Thannickal et al., 2003; Horowitz et al., 2004; Horowitz et al., 2006b). Intriguingly, unlike AG1879, imatinib was not able to inhibit TGF-β-induced FAK and AKT at doses that potently inhibit fibroblast proliferation (data not shown).

An essential role for myofibroblast deactivation and apoptosis in the termination of the repair response and subsequent healing without scar formation is better appreciated in models of cutaneous wound healing (Desmouliere et al., 1995); this may also be an important mechanism for persistent fibrosis of the lung.

Previous reports of imatinib mesylate as an anti-fibrotic agent in animal models of bleomycin-induced lung fibrosis have produced mixed results (Daniels et al., 2004; Aono et al., 2005; Chaudhary et al., 2006). Anti-fibrotic effects of imatinib have also been reported in other
organ systems such as the liver (Yoshiji et al., 2006) and kidney (Wang et al., 2005). Daniels et al. studied the effects of imatinib in female 129tsvems mice subjected to intra-tracheal bleomycin; imatinib at 50 mg/kg/day administered on day 1 following bleomycin-injury for a period of 28 days mediated significant protection from fibrosis (Daniels et al., 2004). Chaudhary et al. utilized a similar bleomycin injury model in male Wistar rats and compared the effects of administering imatinib at three different doses (10, 30, and 50 mg/kg/day); a dose of 50 mg/kg/day was observed to be the most efficacious dose in this study (Chaudhary et al., 2006). Significant protection from fibrosis was noted when 50 mg/kg/day of imatinib was given either on days 1-21 or on days 10-21 (Chaudhary et al., 2006). Similarly, Aono et al. studied female C57BL/6 mice subjected to bleomycin injury and found significant protection, assessed by lung collagen content, but only when a higher dose of imatinib (50 mg/kg/day vs. 25 mg/kg/day) was used (Aono et al., 2005). However, even with the higher dose (50 mg/kg/day), no benefit was observed with late treatment (days 14-28) when compared with early treatment (days 0-14) (Aono et al., 2005). Thus, variations in dose and timing of drug delivery as well as the genetic background of the mice may account for the observed differences in this animal model of pulmonary fibrosis.

In the current study, we selected a dose of imatinib that is comparable to that used in clinical practice for cancer therapy (Druker et al., 2001) and drug administration was delayed until a week after lung injury, a time-point at which inflammatory responses are minimal and fibrogenic pathways are activated (Thrall et al., 1982; Zhang et al., 1994). Additionally, a dose of 10 mg/kg/day in the murine fibrosis model is similar to that used for AG1879, a PKI that exerts biological effects at comparable concentrations in-vitro (Vittal et al., 2005). The recommended dose of imatinib mesylate in the chronic phase of CML and GIST is 400 to 600
mg/day or, ~5.7 to 8.6 mg/kg, respectively, in an average 70 kg human. Enrollment in a Phase II, randomized, double blind, placebo-controlled study of the clinical effects of Gleevec (imatinib mesylate; 600 mg/kg) administered orally to patients with IPF for a period of up to two years has been completed (www.clinicaltrials.gov/ct/show/NCT00131274); however, results of this trial have not been released or published.

Similar to recognized toxic effects of the EGFR kinase inhibitor, Iressa, there are an increasing number of reports documenting imatinib-induced interstitial pneumonitis (Bergeron et al., 2002; Ma et al., 2003; Yokoyama et al., 2004; Ohnishi et al., 2006). It is possible that such untoward effects of these PKIs relate to their effects on epithelial/mesenchymal cell phenotype and fates. The ideal “anti-fibrotic” PKI may be one that modulates pro-survival signaling in apoptosis-resistant myofibroblasts, while not interfering with the regenerative capacity of the epithelium. The ability of a therapeutic agent to inhibit fibroblast proliferation may be relatively less important in late stages of tissue repair and fibrosis - the clinically more relevant context in human fibrotic disorders.
ACKNOWLEDGMENTS

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REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1: Effects of imatinib on fibroblast proliferation and myofibroblast differentiation. A, Quiescent normal fetal lung fibroblasts (IMR-90) were treated without/with (1, 5, 10 and 20 µM) imatinib for 72 h in the presence of serum (10% FBS). Cell numbers were assessed using a Coulter counter. Values represent mean ± SEM; n = 4 per group; *p < 0.05 compared to untreated control. B, Quiescent IMR-90 cells were treated with (1, 5, 10 and 20 µM) imatinib for 72 h in the presence of serum (10% FBS). BrdU incorporation was measured over a 24 h period as described in “Methods”. Values represent mean ± SEM, n = 3 per group; *p < 0.05 compared to untreated control. C, Quiescent IMR-90 cells were treated ± imatinib (with doses indicated) at the same time as TGF-β1 (upper panels: “Co-treatment with Imatinib”) or 48 h after TGF-β1 treatment (lower panels: “Post-treatment with Imatinib”) for 16 h prior to cell lysis. Cell lysates were subjected to SDS-PAGE and immunoblotted for α-SMA; blots were stripped and probed for GAPDH (loading control). D, Densitometric measurements of Western blot analyses from three independent experiments represented in the upper panel of (C). Values represent mean ± SEM, n = 3 per group; *p < 0.05 compared to TGF-β1. E, Densitometric measurements of Western blot analyses from three independent experiments represented in the lower panel of (C). Values represent mean ± SEM, n = 3 per group; *p < 0.05 compared to TGF-β1.

Figure 2: Effects of imatinib on lung epithelial cell proliferation, apoptosis and epithelial-mesenchymal transition. A, Rat lung epithelial cells (RLE-6TN) were treated with the indicated doses of imatinib for 48 h in Ham’s F-12 medium containing growth factors. Cell numbers were assessed using a Coulter counter. Values represent mean ± SEM, n = 4 for each group; *p < 0.05 compared to untreated control. B, RLE-6TN cells were treated ± TGF-β1 (2
ng/ml) ± imatinib (10 µM) for 48 h. Apoptosis was determined with an ELISA for ssDNA as described in “Methods”. Values are expressed as mean ± SEM, n = 3; *p < 0.05 compared to imatinib or TGF-β1 alone. C, RLE-6TN cells were treated with/without imatinib (10 µM) in the presence/absence of TGF-β1 (2 ng/ml) for 48 h. Cell lysates were obtained and subjected to SDS-PAGE with immunoblotting for cleaved caspase-3, α-SMA and GAPDH. D, Relative densitometric analyses of Western blots obtained from three independent experiments described in (C) and represented as ratio of cleaved caspase 3 to GAPDH. Values represent mean ± SEM, n = 3; *p < 0.05 compared to imatinib or TGF-β1 alone. E, Relative densitometric analyses of Western blots obtained from three independent experiments described in (C) and represented as ratio of α-SMA to GAPDH. Values represent mean ± SEM, n = 3.

Figure 3: Imatinib inhibits PDGFR-β activation but does not alter TGF-β/SMAD3 signaling in lung fibroblasts and epithelial cells. A, Quiescent IMR-90 fibroblasts were pre-treated ± imatinib (10 µM x 15 min) and then stimulated with/without TGF-β1 (2 ng/ml x 30 min) or PDGF (50 ng/ml x 30 min) prior to cell lysis. Cell lysates were subjected to SDS-PAGE and immunoblotted for phospho-Y751 PDGFR-β and phospho-S433/435 SMAD3; membranes were stripped and re-probed for total PDGFR-β, total SMAD3 and GAPDH. B, RLE-6TN cells were pre-treated ± imatinib (10 µM x 15 min) and then stimulated with/without TGF-β1 (2 ng/ml x 30 min) or PDGF (50 ng/ml x 30 min) prior to cell lysis. Cell lysates were subjected to SDS-PAGE and immunoblotted for phospho-Y751 PDGFR-β and phospho-S433/435 SMAD3; membranes were stripped and re-probed for total PDGFR-β, total SMAD3 and GAPDH.
Figure 4: Imatinib administration during the post-injury reparative phase does not protect against bleomycin-induced pulmonary fibrosis. C57BL/6 mice were subjected to intratracheal (IT) instillation of bleomycin or saline (control) on day 0. Bleomycin-injured mice were administered intra-peritoneal (IP) injections of saline (control), IP injections of imatinib, or imatinib by oral gavage (PO) starting on day 7 for a period of 14 days. A, Representative histopathology on day 21 by hematoxylin and eosin staining (top panels) and Masson’s trichrome blue staining for collagen (bottom panels) of the lungs of mice injured with intratracheal instillation of bleomycin. B, Soluble collagen in lung homogenates on day 21 following bleomycin injury was determined using the Sircol assay as described in “Methods”. Values are expressed as mean ± SEM, n = 6 mice per group; \*p < 0.05 compared to IT-saline/IP-saline control. This is one of three independent experiments demonstrating similar results.

Figure 5: In-vivo accumulation of lung myofibroblasts in bleomycin-induced pulmonary fibrosis is not inhibited by imatinib treatment. C57BL/6 mice were subjected to intratracheal (IT) bleomycin or saline (control) on day 0. Bleomycin-injured mice were administered intra-peritoneal (IP) injections of saline (control), IP injections of imatinib, or imatinib by oral gavage (PO) starting on day 7 for a period of 14 days. A, Whole lungs on day 21 following bleomycin injury were stained by immunohistochemistry (IHC) using antibodies against \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA, a marker of myofibroblast differentiation). Control staining was with biotinylated secondary IgG antibody. Streptavidin-conjugated HRP was used with 3,3’-diaminobenzidine as substrate (brown staining) and counterstained with hematoxylin (nuclei stain blue). Photomicrographs were taken at 40x magnification. B, Whole lungs were harvested.
on day 21 following lung injury. Fibroblasts were isolated from the lung explants at passage 2 and at 90-100% confluence. Cell lysates were subjected to SDS-PAGE and immunoblotted for α-SMA; the membrane was stripped and re-probed for α-tubulin. C, Densitometric analysis of western immunoblots (shown in B) represented as ratio of α-SMA:α-tubulin. Values represent mean ± SEM, n = 3; *p < 0.05 compared to IT-saline/IP-saline control.
FIGURE 1

(A) Cell Proliferation (% increase in cell number) vs. Concentration of Imatinib (μM)

(B) BrdU Incorporation (absorbance units/million cells) vs. Concentration of Imatinib (μM)

(C) TGF-β1 (2 ng/ml)

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(D) Co-treatment with Imatinib (μM) + TGF-β1

(E) Post-treatment with Imatinib (μM) + TGF-β1
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Lung Fibroblasts (IMR-90)

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B

Alveolar Epithelial Cells (RLE-6TN)

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**Intra-tracheal: Treatment:**

- **Saline (IP)**
- **Bleomycin (IP)**
- **Bleomycin Imatinib (10 mg/kg - IP)**
- **Bleomycin Imatinib (10 mg/kg - PO)**

**Hematoxylin & Eosin (20x)**

**Masson's Trichrome (40x)**

B

**Collagen (μg/ml)**

- **Saline (IP)**
- **Bleomycin (IP)**
- **Bleomycin Imatinib (10 mg/kg - IP)**
- **Bleomycin Imatinib (10 mg/kg - PO)**