Epidermal Growth Factor Receptor Tyrosine Kinase Inhibition Augments a Murine Model of Pulmonary Fibrosis

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ABSTRACT

The inappropriate regeneration of sequentially injured epithelium is an important process leading to pulmonary fibrosis. Previous studies have shown that the epithelial expression of epidermal growth factor receptor (EGFR) is increased in fibrotic lung tissue, compared with normal lung tissue, suggesting that EGFR-mediated signaling is involved in epithelial regeneration in fibrotic lung diseases. We examined the effect of EGFR inhibition using ZD1839, a selective EGFR tyrosine kinase inhibitor (TKI), on bleomycin-induced pulmonary fibrosis in mice. ICR mice were administered a single intratracheal injection of bleomycin (5 units/kg) on day 1. ZD1839 (200 mg/kg) or vehicle alone were administered p.o. 1 h before this injection and on days 1–5 each week for 3 weeks. Lung tissue was harvested on day 21. Lung histology and collagen analysis performed on day 21 showed more severe fibrosis in the mice receiving both bleomycin and the EGFR-TKI than in the mice receiving bleomycin and the vehicle. An immunohistochemistry analysis showed that phosphorylated EGFR and proliferation cell nuclear antigen were highly expressed by the regenerated epithelial cells in the mice treated with bleomycin and the vehicle. In contrast, the expression of these antigens was attenuated in the mice treated with bleomycin and the EGFR-TKI. In vitro studies also demonstrated that the addition of ZD1839 at a concentration of ≤1 μM suppressed the proliferation of type II-like epithelial cells (A549) but not that of lung fibroblasts (IMR90). These results suggest that the inhibition of EGFR phosphorylation augments bleomycin-induced pulmonary fibrosis by reducing regenerative epithelial proliferation. Our data suggest that EGFR-TKIs should be used with caution in cancer patients with pulmonary fibrosis.

INTRODUCTION

EGFR is a M, 170,000 transmembrane glycoprotein with intrinsic tyrosine kinase activity that regulates cell proliferation and differentiation (1, 2). Four EGFR family members have been identified in mammals, erbB1 (also termed EGFR), erbB2, erbB3, and erbB4. Six different ligands, including epidermal growth factor and TGF-α, are also known to exist (3, 4). EGFR is expressed by many types of cells, particularly epithelial cells that commence cell cycle progression upon binding to a ligand (2). Thus, interactions between EGFR and its ligands are thought to play an important role in the regeneration of injured epithelium (5).

EGFR is also expressed by various epithelial tumors; high levels of EGFR expression in these cells indicates a poor prognosis or a late stage of disease (6, 7). In this context, EGFR has become a novel molecular target of cancer therapies (8). In particular, ZD1839, an p.o. active selective EGFR-TKI, has been shown to be effective against lung cancer and epithelial tumors of other origins. Phase I and II clinical studies demonstrated that ZD1839 monotherapy provides clinically significant antitumor activity and symptom relief in patients with advanced NSCLC who have previously received prior treatment with cytotoxic chemotherapy (9, 10). Furthermore, ZD1839 was well tolerated in these studies; the most common adverse events were mild diarrhea and skin rashes (9, 10). Since July 2002, ZD1839 (Gefitinib, Iressa; AstraZeneca, Osaka, Japan) has been available in Japan for patients with inoperable or recurrent NSCLC.

Pulmonary fibrosis is the most common disease that predisposes an individual to lung cancer (11). It is estimated that 9.8–38.0% of patients with IPF will develop lung cancer at some point in their life (12). IPF itself is a progressive, irreversible, and fatal lung disease. In fact, patients with IPF have a mean survival of only 2–4 years; patients with IPF and lung cancer have a much poorer prognosis (13). Given the absence of proven therapies for IPF, current clinical management for IPF is largely supportive and is aimed at the avoidance of exacerbating factors, such as cigarette smoke, viral infections, and lung toxins.

A recent hypothesis postulates that pulmonary fibrosis results from sequential alveolar epithelial injury by unidentified stimuli (14, 15). Inappropriate re-epithelization of the injured area is proposed to elicit abnormal wound healing by the fibroblasts, resulting in irreversible parenchymal fibrosis and severe lung dysfunction (14, 15). In IPF, the alveolar epithelium exhibits focal hyperplasia as a result of alveolar type II cells that are expressing EGFR at high levels, suggesting that the type II cells are attempting to proliferate to regenerate the injured epithelium (14, 16). In fact, several studies have documented the up-regulation of EGFR and EGFR ligands during the pulmonary fibrogenesis in humans and rodents (16, 17). Because the proliferation of type II cells is largely dependent on EGFR (18, 19), drug therapies for IPF patients that inhibit EGFR should be used with great caution.

In the present study, we hypothesized that the use of EGFR inhibitors for cancer therapies in patients with pulmonary fibrosis may promote fibrogenesis. To test this hypothesis, we studied the effects of ZD1839 on murine bleomycin-induced pulmonary fibrosis, a representative model of pulmonary fibrosis (20).

MATERIALS AND METHODS

Animal Treatment. The animal protocol was approved by the Animal Care and Use Committee of Tokyo Women’s Medical University. An animal model for bleomycin-induced pulmonary fibrosis was generated as described previously (21, 22). Briefly, 6-week-old male ICR mice were anesthetized by an i.p. injection of sodium pentobarbital and then intratracheally given 50 μl of a saline solution containing bleomycin hydrochloride (5 units/kg; Nippon Kayaku, Co., Tokyo, Japan). The mice were p.o. given 300 μl of a 0.5% carboxymethylcellulose (Sigma Chemical Co., St. Louis, MO) and 0.1% polyoxymethylene sorbitan mono-oleate (Tween 80; Sigma) solution containing or lacking ZD1839 (200 mg/kg body) 1 h before and on days 1–5 each week for 3 weeks after the bleomycin treatment. On day 21 after the bleomycin treatment, the animals were sacrificed by terminal anesthesia, and the lungs and heart were removed en bloc.

Tissue Processing. For the histological analysis, the lungs were inflated and fixed by an intratracheal instillation of 10% formalin at a constant pressure of 25 cm H2O. Bilateral lungs were then embedded in paraffin, sectioned sagittally (3 μm), and stained with a H&E solution or Masson’s trichrome solution. The remaining sections were processed for immunohistochemistry.
For the collagen assay, unfixed fresh lungs were snap frozen in liquid nitrogen and saved at −70°C until use.

**Histological Analysis for Pulmonary Fibrosis.** Two sagittal sections from each lung, i.e., four sections per animal, were stained with a H&E solution, and the severity of the pulmonary fibrosis was assessed. All of the specimens were numbered randomly and interpreted using a blind protocol. Visual grading of the pulmonary fibrosis was performed by determining the Ashcroft’s score (23). Briefly, the entire fields of each lung section were scanned under an Olympus BX60 microscope (Olympus Optical Co., Ltd.) at a magnification of ×100, and each field was visually graded from 0 (normal lung) to 8 (total fibrotic obliteration of the field; Ref. 23). The mean value of the grades obtained for all of the fields was then used as the visual fibrotic score (23).

The fraction of lung parenchyma was estimated using computer-assisted imaging analysis. Digitized video images of the entire fields of each section were captured using an Olympus BX60 microscope and an Olympus DP50 CCD camera at a magnification of ×40. Fields containing nonalveolated structures, such as bronchovascular bundles, were discarded. The video output of the camera was sent to an Olympus imaging microscopic workstation (CUSL2G40) equipped with a computer running Microsoft Windows 98 and a computerized color image analysis software system (Win Roof Version 3.5; Mitani Corporation, Fukuji, Japan). Each image was then subjected to computer operations that established a threshold of pixel densities to discriminate the lung parenchyma from the airspaces. The fraction of lung parenchyma on each image was regarded as the severity of pulmonary fibrosis. Finally, the fractions obtained for all of the images were averaged.

**Immunohistochemistry.** For the immunodetection of phosphorylated EGFR, tissue sections were autoclaved in a 0.1 M sodium citrate buffer (pH 6.0) for 5 min at 120°C to retrieve epitopes and then immersed in an Auto-Blocker solution (Research Genetics, Huntsville, AL) for 2 min at 40°C to inhibit endogenous peroxidase activity. After blocking the nonspecific binding sites with 3% BSA and 5% normal goat serum, the sections were incubated with polyclonal anti-phospho-EGFR (Tyr1068; 1:20 dilution; Cell Signaling Technology, Inc., Beverly, MA) for 30 min at 40°C. The primary antibody was then reacted with antirabbit IgG conjugated with horseradish peroxidase-labeled polymer (Envision+ peroxidase; DAKO Japan, Kyoto, Japan) for 15 min at 40°C. The immunoreaction was amplified using a tyramide-based signal amplification method (Tyramide Signal Amplification Biotin System; NEN Life Science Products, Inc., Boston, MA) according to the manufacturer’s instructions. Immunoreactants were then visualized using 3,3′-diaminobenzidine as a substrate and counterstained with a hematoxylin solution.

For the double staining of proliferating cell antigen and epithelial antigen, tissue sections were autoclaved in a 0.1 M sodium citrate buffer (pH 6.0) for 5 min at 120°C and then immersed in an AutoBlocker solution for 2 min at 40°C. After blocking the nonspecific binding sites with 3% BSA and 5% normal goat serum, the sections were incubated with monoclonal anti-pancytokeratin (1:100 dilution; Sigma) for 30 min at 40°C followed by a reaction with antimonos Envision+ peroxidase (DAKO Japan) and a 3,3′-diaminobenzidine solution. The slides were then immersed in 0.1 M glycine-HCl buffer (pH 2.2) for 2 h to remove the primary antibody complex. Next, the slides were incubated with monoclonal anti-PCNA (1:100 dilution; Santa Cruz) for 30 min at 40°C. The antibody was reacted with antimonos IgG conjugated with an alkaline phosphatase-labeled polymer (EnVision+ alkaline phosphatase; DAKO Japan) for 15 min at 40°C. Immunoreactants were visualized using a solution containing 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride solution (BCIP/NBT; DAKO) and 1 μM levamisole (Vector DAKO Japan) for 15 min at 40°C. The antibody was reacted with antimouse Envision polymer (EnVision+ polymer; DAKO Japan) for 15 min at 40°C. Immunoreactants were visualized using a tyramide-based signal amplification method (Tyramide Signal Amplification Biotin System; NEN Life Science Products, Inc., Boston, MA) according to the manufacturer’s instructions. Immunoreactants were then visualized using 3,3′-diaminobenzidine as a substrate and counterstained with a hematoxylin solution.

**RESULTS**

**ZD1839 Inhibits the Proliferation of Alveolar Type II-like Cells in Vitro.** First, we examined whether ZD1839, an p.o. active, specific EGFR-TKI, is capable of inhibiting the proliferation of alveolar type II-like cells (A549) in vitro. Previous studies showed that an oral administration of 50–700 mg of ZD1839 in humans results in a peak plasma concentration of 0.1–1.9 μM (25). As shown in Fig. 1, ZD1839 at concentrations of 0.05–1 μM inhibited the proliferation of A549 stimulated with FCS. These results are consistent with previous findings showing that ZD1839 at similar concentrations (0.032 to 0.8 μM) inhibited the phosphorylation of EGFR in A549 cells (26). Unlike the A549 cells, the proliferation of lung fibroblasts (IMR90) was not inhibited by treatment with ZD1839 (Fig. 1), probably because lung fibroblasts express a very low level of EGFR (17). These results suggest that ZD1839 selectively inhibits the proliferation of alveolar type II-like cells in vitro.

**ZD1839 Augments Bleomycin-induced Pulmonary Fibrosis.** Because previous studies have shown that fibrotic lung tissues express high levels of EGFR (16, 17), we next examined whether the inhibition of EGFR by ZD1839 affects murine bleomycin-induced pulmonary fibrosis, a representative model of pulmonary fibrosis (20). In accordance with previous studies (20), mice receiving a single intratracheal instillation of bleomycin developed pulmonary fibrosis after 21 days (Fig. 2). We found that mice treated with bleomycin and ZD1839 exhibited a severer degree of fibrosis than mice treated with bleomycin and the vehicle alone (Fig. 2). This notion was confirmed by two different, semiquantitative, histological analyses for the severity of pulmonary fibrosis (Fig. 3). Furthermore, the total lung collagen content was determined using the Sircol Collagen Assay kit (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer’s instructions. This assay kit is based on the specific binding of Sirius Red dye with the [Gly-X-Y] helical structure found in collagen. Collagen-bound dye was quantitatively analyzed using a spectrophotometer set at a wavelength of 540 nm.

**In Vivo Effects of ZD1839 on Alveolar Epithelial Cells and Fibroblasts.** Alveolar type II-like cells (A549; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) or fetal lung fibroblasts (IMR90; Dainippon Pharmaceutical Co., Ltd.) were plated onto a 96-well tissue culture plate in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FCS. When the cell densities reached 50% confluence, the medium was replaced with DMEM lacking FCS. After 48 h of starvation, the cells were then stimulated to proliferate by refeeding them with DMEM containing 10% FCS with or without 0.05–1 μM of ZD1839. Cell proliferation after 24 h was assessed using the Hoechst33342 DNA staining and spectrofluorimetry, as described previously (24).

**Statistical Analysis.** All of the data are expressed as the mean ± SE. Significant differences were determined using an ANOVA and a Mann-Whitney U test or a Tukey-Kramer’s procedure post hoc analysis, as appropriate. A value of P < 0.05 was considered to be significant. All of the statistical analyses were performed using StatView software, version 5.0, for the Macintosh (Abacus Concept, Inc.).
These photomicrographs are representative of the results obtained for 5 animals in each group. The panel (C) shows a photomicrograph of mice not treated with bleomycin or ZD1839. Original magnification: ×100. These photomicrographs are representative of the results obtained for 5 animals in each group.

The degree of fibrosis than mice treated with bleomycin plus vehicle (A) 1 h before and on days 1–5 each week for 3 weeks after an intratracheal injection of bleomycin (5 unit/kg). Mice treated with bleomycin and ZD1839 (B) exhibited a more severe content was significantly greater in mice treated with bleomycin and ZD1839 than in the mice treated with bleomycin and the vehicle (Fig. 4). Collectively, these results indicate that ZD1839 augments bleomycin-induced pulmonary fibrosis in mice.

**ZD1839 Inhibits EGFR Phosphorylation and Regenerative Epithelial Proliferation in Bleomycin-induced Pulmonary Fibrosis.**

Third, we evaluated the effect of ZD1839 on EGFR phosphorylation in the lungs of mice treated with bleomycin. Immunohistochemistry for phospho-EGFR using lung tissue sections obtained from mice 21 days after treatment with bleomycin and the vehicle revealed negative staining in cuboidal, regenerative epithelial cells covering the surface of the fibrotic lesions (Fig. 5A). However, the epithelial cells present in areas of normal lung tissue were negative for phospho-EGFR, suggesting that EGFR phosphorylation is required for the regeneration of injured epithelium but not for the maintenance of normal epithelium. In contrast, lung tissue sections from mice treated with bleomycin and ZD1839 showed negative staining for phospho-EGFR in all of the epithelial cells, even in those present in fibrotic areas (Fig. 5B), confirming that treatment with ZD1839 inhibited the phosphorylation of EGFR in the lungs of mice treated with bleomycin.

Lastly, we examined whether the inhibition of EGFR by ZD1839 is associated with alterations in the ability of epithelial cells to proliferate in response to bleomycin-induced lung injury. In mice treated with bleomycin and the vehicle, PCNA was frequently expressed by both cytokeratin-negative interstitial cells and cytokeratin-positive epithelial cells covering the surface of the fibrotic lesions, suggesting that these epithelial cells attempt to proliferate and regenerate the injured epithelium (Fig. 6A). However, in mice treated with bleomycin and ZD1839, PCNA was frequently expressed by the cytokeratin-negative interstitial cells but rarely by the cytokeratin-positive epithelial cells, suggesting that treatment with ZD1839 inhibits the ability of epithelial cells to proliferate (Fig. 6B). A semiquantitative analysis for epithelial proliferation in fibrotic lung areas showed that the epithelial proliferation index was significantly lower in mice treated with bleomycin and ZD1839 than in mice treated with bleomycin and the vehicle (Fig. 7). Collectively, these results suggest that treatment with ZD1839 inhibits EGFR phosphorylation and regenerative epithelial proliferation in bleomycin-induced pulmonary fibrosis.

**DISCUSSION**

In the present study, we demonstrated that the treatment of mice with ZD1839 augments bleomycin-induced pulmonary fibrosis. This augmentation of pulmonary fibrosis was associated with a decrease in the level of EGFR phosphorylation and regenerative epithelial proliferation. These results suggest that EGFR-TKIs may not be suitable for cancer patients with pulmonary fibrosis.

ZD1839, a p.o. active, selective EGFR-TKI, has been shown to be effective for the treatment of epithelial tumors, including NSCLC (9), with a favorable adverse event profile. In Phase I and II trials for NSCLC, treatment with ZD1839 was associated with low-grade side effects including skin rashes and diarrhea (9, 10). Although our results show that ZD1839 augments bleomycin-induced pulmonary fibrosis in mice, caution may be needed in interpreting these results with regard to the clinical use of ZD1839 in cancer patients. First, the mice in this study were given a larger dose (200 mg/kg/day) than that used in previous clinical trials in humans (1–4 mg/kg/day (50–700 mg/body/day); Ref. 9). Larger doses of ZD1839 may be required to inhibit the proliferation of normal epithelial cells than those required to inhibit cancer cells. Second, the murine bleomycin-induced pulmonary fibrosis model used in this study may not be predictive of the human disease. Further studies are needed to determine the clinical relevance of these findings.

**Fig. 3.** Semiquantitative analyses for the severity of pulmonary fibrosis in mice sacrificed 21 days after treatment with either bleomycin and the vehicle or bleomycin and ZD1839. H&E-stained lung tissue sections were assessed for the severity of pulmonary fibrosis by a visual grading of fibrosis (Ashcroft’s score; A) or a computer-assisted analysis of the fraction of lung parenchyma (B). Data are represented as mean obtained for 5 animals in each group, bars, ±SE, *P < 0.05 versus mice treated with bleomycin and the vehicle.

**Fig. 4.** Quantitative analysis of collagen content in the lungs of untreated mice or in the lungs of mice sacrificed 21 days after treatment with either bleomycin and the vehicle or bleomycin and ZD1839. Data are represented as the mean obtained for 5 animals in each group, bars, ±SE, *P < 0.05 versus untreated mice, †, P < 0.05 versus mice treated with bleomycin and the vehicle.
study is not an exact replica of human pulmonary fibrosis. The histology of murine bleomycin-induced pulmonary fibrosis is not identical to that of human IPF, the most common pulmonary form of fibrosis (27). Thus, the effect of ZD1839 on human IPF may be different from that on our murine model of pulmonary fibrosis. However, as there is an increased incidence of lung cancer among patients with pulmonary fibrosis (12), the use of ZD1839 in this group of patients requires additional evaluation.

Our results also provide a deeper insight into the mechanism of pulmonary fibrosis. The original hypothesis for the pathogenesis of pulmonary fibrosis is that chronic inflammation stimulates the ability of fibroblasts to migrate, proliferate, and produce the extracellular matrix, thereby resulting in parenchymal fibrosis (28). However, this original hypothesis has been challenged recently by a new hypothesis suggesting that the inappropriate regeneration of the sequentially injured epithelium is sufficient to stimulate fibroblasts, without the need of ongoing inflammation (14). This hypothesis implies that alterations in the epithelial cells act as a trigger for fibrogenesis. Our results support this view by showing that the blockage of EGFR-dependent epithelial proliferation augments pulmonary fibrosis. In our study, regenerative, but not normal, epithelial cells in mice treated with bleomycin and the vehicle were positively immunostained for phospho-EGFR and PCNA, suggesting that activation of EGFR is important for regeneration of epithelial cells. In mice treated with bleomycin and ZD1839, the immunopositive signals for phospho-EGFR and PCNA in regenerative epithelial cells were suppressed. These results suggest that ZD1839 inhibits both the activation of EGFR and the ability of regenerative epithelial cells to proliferate, leading to pulmonary fibrogenesis.

Besides the blocking of EGFR-dependent epithelial proliferation, treatment with ZD1839 may also augment fibrosis by stimulating apoptosis in alveolar epithelial cells (29, 30), by inhibiting epithelial differentiation (31), or by inhibiting pulmonary angiogenesis (32). Our results, which show that ZD1839 did not affect the proliferation of fibroblasts in vitro, also suggest that this drug allows fibroblasts to proliferate in response to profibrotic stimuli, and that leads to pulmonary fibrosis. This notion is additionally supported by our in vivo results where PCNA was expressed by cytokeratin-negative interstitial cells in mice treated with bleomycin and ZD1839.

In contrast with our findings, Rice et al. (33) showed that an EGFR-TKI, AG1478, alleviated pulmonary fibrosis induced by the treatment of rats with vanadium pentoxide. The inconsistency be-
between their results and ours may be explained in several ways. First, we used bleomycin as a fibrogenic stimulus instead of vanadium pentoxide. Vanadium pentoxide is a tyrosine phosphatase inhibitor that causes the tyrosine phosphorylation of various proteins, including EGFR and mitogen-activated protein kinase (34). Thus, the preventative effect of AG1478 on pulmonary fibrosis may have been because of the inhibition of some pharmacological activity of vanadium pentoxide, rather than the inhibition of fibrogenesis. Second, the mice in our study were treated with ZD1839 for a much longer period (3 weeks) after the bleomycin injection than the mice treated with AG1487 (for 3 days) in the vanadium study. The chronic inhibition of EGFR after lung injury may be required for an enhancement in fibrogenesis to occur.

Our results lead us to assume that the blockage of EGFR ligands would augment pulmonary fibrosis. However, a previous study has demonstrated that mice deficient in TGF-β, a potent ligand of EGFR, become less susceptible to bleomycin-induced pulmonary fibrosis than wild-type mice (35). These unexpected results may be explained by the fact that EGFR is activated by many different ligands, as well as nonligand mechanisms (2), and also that these individual ligands have multiple effects on various types of cells. For example, TGF-β has been shown to stimulate the proliferation of fibroblasts as well as epithelial cells; TGF-α also stimulates the expression of several fibrogenic genes, such as TGF-β, matrix metalloproteinase-3, and the tissue inhibitor of metalloproteinases-1 (36, 37). Thus, the blockage of a single EGFR ligand may not be sufficient to simulate the effect of EGFR inhibitors.

In July, 2002, ZD1839 was first been approved in Japan for the treatment of inoperable and recurrent NSCLC. However, the Japanese Ministry of Health, Labor, and Welfare reported that up to November 25, 2002, when 17,500 patients with NSCLC had been given ZD1839 in Japan, 291 patients (including 81 deaths) were suspected to have acquired acute lung injury (38). The results of our study may help to understand the mechanism of action of this drug in relation to the adverse effect of lung injury.

In conclusion, we have demonstrated that the treatment of mice with ZD1839 augments bleomycin-induced pulmonary fibrosis by reducing regenerative epithelial proliferation. Our results suggest that EGFR inhibitors may adversely affect patients with pulmonary fibrosis and, therefore, additional investigation is warranted.

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