Gefitinib, but Not Erlotinib, is a Possible Inducer of Fra-1-mediated Interstitial Lung Disease

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Gefitinib is an anticancer drug developed to inhibit the tyrosine kinase activity of the epidermal growth factor receptor (EGFR). Two structurally-related EGFR tyrosine kinase inhibitors, gefitinib (Iressa) and erlotinib (Tarceva), are used as oral chemotherapy by patients with non-small-cell lung cancer. Immediately after introduction of gefitinib to clinical practice, interstitial lung disease was identified as a life-threatening adverse effect, although this condition can be well managed. It is still unclear whether gefitinib and other EGFR inhibitors induce similar adverse effects in lung. We previously established mouse models of interstitial lung disease in which gefitinib induces expression of Fosl1 (which encodes the AP-1 transcription factor Fra-1) in the presence of exogenous or endogenous Toll-like receptor ligands, leading to abnormal cytokine and chemokine expression. Here, we compared and monitored the effects of EGFR inhibitors gefitinib, erlotinib and AG1517 (PD153035) on the mRNA expression levels of Fosl1, Tnf and Ccl2. Unexpectedly, gefitinib, but not the other tyrosine kinase inhibitors, elicited the Fosl1 expression profile proposed to be predictive of interstitial lung disease, suggesting that gefitinib-induced interstitial lung disease is an off-target effect not elicited by erlotinib. (doi: 10.2302/kjm.2011-0009-OA; Keio J Med 61 (4) : 120–127, December 2012)

Keywords: gefitinib, erlotinib, EGFR inhibitor, interstitial lung disease

Introduction

Gefitinib (Iressa, ZD1839) was developed as a selective inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and has been used to treat patients with advanced non-small-cell lung cancer. Other EGFR inhibitors, such as erlotinib (Tarceva), are also widely used. Like other chemotherapeutic agents, gefitinib treatment can cause adverse effects, most frequently skin rash and aminotransferase elevation, but occasionally life-threatening interstitial lung disease can occur. The incidence of interstitial lung disease in patients treated with gefitinib is currently about 1% (0.64% in regions outside Japan). Since the initial reports of gefitinib-associated fatalities appeared, gefitinib-induced interstitial lung disease has been reduced in Japan (from 5.8% in 2003–2004 to 1.28% in 2010), highlighting the importance of appropriate patient selection based on risk factors such as preexisting pulmonary fibrosis. Little is known about the molecular target underlying gefitinib-mediated interstitial lung disease, although there are reports that gefitinib has other targets in addition to EGFR. Tumor tissue destruction mediated by gefitinib and other chemotherapeutic agents likely produces endogenous Toll-like receptor (TLR) ligands, including hyaluronan and heparan sulfate. We recently reported that gefitinib administration to mice along with the exogenous TLR ligand lipopolysaccharide (LPS) or various endogenous ligands induces interstitial lung disease. Mice treated with gefitinib and LPS showed elevated expression of the transcription factor Fos-related antigen-1 (Fra-1, encoded by Fosl1) in lung tissues. Fra-1, a member of the activator protein 1 (AP-1) (Fos/Jun) transcription factor family, activates and/or represses various target genes...
by heterodimerizing with a Jun family protein, such as c-Jun, JunB or JunD. In the lungs of mice treated with gefitinib and LPS, Fra-1 upregulation inhibits expression of the genes of proinflammatory cytokines such as Tnfa [encoding tumor necrosis factor-α (TNF-α)] and induces that of cytokines such as Ccl2 [encoding monocyte chemoattractant protein 1 (MCP-1)]. In Fra-1 transgenic mice, which show preexisting pulmonary fibrosis, LPS treatment alone results in severe lung injury associated with reduced inflammatory cytokine production and increased chemokine production. The observation of increased chemokine production in Fra-1 transgenic mice was not anticipated, since these mice show reduced inflammatory responses in bone and the intestinal tract. That Fra-1 differentially modulates Tnfa and Ccl2 expression is further supported by the observation that mice lacking Fra-1 do not show skewed expression of cytokines and chemokines and are resistant to gefitinib/LPS-induced lung injury. In addition, inhibition of MCP-1 by neutralizing antibody or a small-molecule inhibitor against its receptor partially prevents lung injury. These and other observations suggest that gefitinib, in the presence of chemotherapy-induced tissue destruction, induces lung injury through transcriptional activation of Fra-1 and its downstream targets.

In this study, we used both cultured macrophages and bleomycin-induced lung injury experiments to determine whether EGFR inhibitors such as gefitinib and erlotinib act similarly to induce interstitial lung disease. We report that gefitinib is distinct from other EGFR inhibitors in that it activates the interstitial lung disease-causing signaling cascade by an off-target effect.

**Materials and Methods**

**EGFR inhibitors**

For mouse studies, gefitinib (N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy) quinazolin-4-amine; AstraZeneca, London, UK) or erlotinib (N-(3-ethylphenyl) [6,7-bis (2-methoxyethoxy) quinazolin-4-yl]amine; Roche, Basel, Switzerland) tablets were ground into powder using a mortar and pestle and suspended in water. For cell culture studies, gefitinib, erlotinib and AG1517 (4-(3-bromoanilino)-6,7-dimethoxyquinazoline; PD153035, Calbiochem, La Jolla, CA, USA) were suspended in dimethyl sulphoxide (DMSO) to make stock solutions (Fig. 1).

**Cell culture**

Mouse primary bone marrow macrophages were prepared as previously described. Briefly, tibiae and femora from adult wild-type C57BL/6 J mice were harvested by flushing with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin–streptomycin. After overnight culture in 10-cm tissue culture dishes (Falcon, Franklin Lakes, NJ, USA), floating cells were collected and expanded in the presence of 10 ng/ml macrophage-colony stimulating factor (R&D, Minneapolis, MN, USA). Cells were re-plated and treated with 10 μM of gefitinib, erlotinib or AG1517 in DMSO or vehicle for 30 min, and then 0.1 μg/ml LPS (S. minnesota Re595; Sigma-Aldrich, St. Louis, MO, USA) was added to the medium for 12 h. Where indicated, each inhibitor was added to the culture medium at 30 min before, 30 min after or 180 min after the start of LPS stimulation.

**Chemotherapy-induced lung disease models**

Six-week-old female ICR mice (a strain initiated at the Institute for Cancer Research and also known as CD-1) purchased from Clea Japan (Tokyo, Japan) were anesthetized, intratracheally administered 5 mg/kg bleomycin (Nippon Kayaku, Tokyo, Japan) and treated with gefitinib as described. Briefly, 250 mg/kg gefitinib or 100 mg/kg erlotinib was given orally 1 h before bleomycin admin-
istration, and then was administered 6 days a week for 3 weeks. The lung was isolated and fixed with 4% paraformaldehyde and embedded in paraffin. Sections 7 μm thick were prepared and stained with hematoxylin and eosin. All animal experiments were conducted in accordance with institutional review board-approved protocols.

**Quantitative reverse transcription polymerase chain reaction**

Total RNA was isolated using ISOGEN (NIPPON GENE, Tokyo, Japan). The reverse transcriptase reaction was performed with 1 μg of total RNA using PrimeScript (TAKARA BIO, Tokyo, Japan). cDNA was subjected to real-time polymerase chain reaction (PCR) using ABI 7500 Fast (Life Technologies, Grand Island, NY, USA). TaqMan PCR was performed using Premix Ex Taq (TAKARA BIO). TaqMan probes specific for GAPDH (Mm03302249_g1), Tnfa (Mm00443258_m1), Ccl2 (Mm00441242_m1), Fosl1 (Mm00487429_m1), Fos (Mm00487425_m1) and Fosl2 (Mm00484442_m1) were purchased from TaqMan Assays-on-Demand Gene Expression Products (Life Technologies). All PCR assays were performed in triplicate. Gene expression levels were normalized to that of GAPDH.

**Statistical analysis**

Statistical significance was determined using an unpaired two-tailed Student’s t test.

**Results**

**Gefitinib-dependent modulation of gene expression changes seen following LPS challenge**

Our previous studies suggested a pathogenesis model of gene expression changes associated with gefitinib-induced interstitial lung disease (Fig. 2). These findings prompted us to ask whether the EGFR inhibitors erlotinib and AG1517 could alter LPS-induced changes in gene expression in a similar way to that of gefitinib. Bone marrow macrophages from mice were exposed to gefitinib, erlotinib or AG1517 from 30 min before 12-h LPS treatment. Unexpectedly, LPS-stimulated Tnfa upregulation was blocked only in gefitinib-pretreated cells (Fig. 3A). Likewise, Ccl2 expression following LPS treatment was enhanced only in gefitinib-pretreated cells (Fig. 3B). Most importantly, Fosl1 expression, which is mildly upregulated in untreated cells stimulated with LPS, was significantly elevated by gefitinib pretreatment but not by treatment with erlotinib or AG1517 (Fig. 3B). Ectopic transgenic expression of Fosl2 in various organs reportedly promotes generalized fibrosis, especially in the lung. Therefore we investigated whether other Fos family genes, such as Fos or Fosl2, were induced by gefitinib, erlotinib or AG1517 treatment. None of the three EGFR inhibitors tested induced Fos or Fosl2 in macrophages in the presence or absence of LPS stimulation (Fig. 3D, E). These results suggest that a gefitinib-specific mechanism differing from that of other EGFR inhibitors underlies altered Fosl1 and cytokine–chemokine expression in LPS-treated cells.

Next, we determined whether and how changing the time frame of EGFR inhibitor treatment altered Tnfa, Ccl2 and Fosl1 expression. Each inhibitor was added 30 min before LPS addition or 30 or 180 min after LPS addition. When EGFR inhibitors were added after the
start of LPS treatment, gefitinib administration had no significant effect on Tnfa, Ccl2 or Fosl1 expression following LPS administration (Fig. 4A, B, C). These data show that gefitinib must be added to the culture before LPS treatment to alter the expression of these genes beyond the effect of LPS alone.

**The effect of gefitinib in bleomycin-induced lung injury**

Ccl2 and Fosl1 expression levels are upregulated in the lungs of wild-type mice treated with gefitinib and bleomycin. We therefore examined whether erlotinib combined with bleomycin could modulate Ccl2 and Fosl1 mRNA levels in the lungs of wild-type mice in a similar way to that of gefitinib. Three weeks after bleomycin administration, neither erlotinib nor gefitinib treatments significantly affected Tnf expression, and both increased Ccl2 expression (Fig. 5A, B). However, importantly, gefitinib induced Fosl1 expression, whereas erlotinib did not (Fig. 5C). Thus, gefitinib treatment, but not erlotinib treatment, enhanced bleomycin-induced lung injury, which was characterized by accumulation of infiltrating

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*Fig. 3* The effect of gefitinib on Tnfa, Ccl2, and Fosl1 expression *in vitro.* Mouse primary bone marrow macrophages were pretreated for 30 min with 10 μM of gefitinib (Gef), erlotinib (Erl), or AG1517 (AG), and then stimulated with 0.1 μg/ml LPS. Expression levels of Tnfa (A), Ccl2 (B), Fosl1 (C), Fos (D) and Fosl2 (E) were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) after 12 h. Results are shown relative to the expression of GAPDH as means ±SEM of triplicate cultures. *p < 0.05.
cells and thickened alveolar septa (Fig. 5D). These data confirm that gefitinib functions specifically in inducing the pivotal transcription factor Fosl1 in a mouse model of interstitial lung disease.

Discussion

We showed that gefitinib, but not erlotinib or AG1517, induced Fosl1 expression in macrophages treated with LPS. Furthermore, in a bleomycin-induced lung injury model, gefitinib, but not erlotinib, induced Fosl1 expression in the lung. Since Fosl1 induction is crucial for interstitial lung disease in mouse models, our data suggest that the mechanism underlying interstitial lung disease is a gefitinib-specific off-target effect.

We found that gefitinib altered LPS-induced gene expression only when cells were treated before LPS stimulation. Currently, it is unclear why pretreatment is necessary to observe the off-target effect of gefitinib reported in this study. We previously reported that gefitinib treatment and endogenous TLR ligands, such as hyaluronan and heparan sulfate, together induce interstitial lung disease. It has recently been proposed that intracellular proteins such as heat shock proteins, extracellular matrix components such as fibrinogen, and oxidized low-density lipoprotein released from damaged tissues may act as PAMP-binding molecules (PBMs) or PAMP-sensitizing molecules (PSMs), rather than genuine TLR ligands. Further studies are needed to determine the role of endogenous TLR4 ligands or PBMs/PSMs in the gefitinib-induced lung fibrosis seen in humans.

In the bleomycin-induced lung injury model, we observed that gefitinib enhanced Ccl2 and Fosl1 expression. In contrast, erlotinib did not induce Fosl1 expression. Although erlotinib induced Ccl2, which can contribute to lung injury, histological analysis revealed no enhancement of bleomycin-induced lung injury by erlotinib. Since gefitinib induces Ccl2 and several other chemokines, such as Ccl6, Ccl8 and Cxcl5, through Fosl1 upregulation in lung injury models, these chemokines likely modulate gefitinib-induced lung injury.

The EGFR inhibitors used in this study—gefitinib, erlotinib and AG1517—share the 4-anilinoquinazoline structure (Fig. 1). Both gefitinib and erlotinib bind near the EGFR ATP binding site, as revealed by crystallographic analysis. Gefitinib has chloro and fluoro moieties, whereas erlotinib and AG1517 have space-filling ethynyl and bromo groups, respectively. In addition, gefitinib has a morpholino group (a saturated nitrogen-containing cycloalkyl group), which neither erlotinib nor AG1517 has (Fig. 1). It is currently unclear whether chloro/fluoro or morpholino groups confer gefitinib’s potential to induce Fosl1 gene expression.

In addition to EGFR, several protein kinases are bound by and inhibited by gefitinib. The gefitinib analogue AX14596 has an amino group at the position of the ge-
When the amino group was used to covalently immobilize AX14596 to beads for binding analysis, multiple kinases in addition to EGFR were identified, among them BRK (breast tumor kinase, or protein-tyrosine kinase 6), GAK (cyclin-G-associated kinase) and RICK/Rip2 (receptor-interacting caspase-like apoptosis-regulatory kinase). Gefitinib inhibited kinase activities of all three kinases with a 50% inhibitory concentration of less than 90 nmol.

Recently, the kinase domain of the potential gefitinib target GAK, encoded by exons 2, 3, and 4, was genetically deleted in mice. Whereas disruption of exon 1 of the GAK gene (homozygous null mutations) results in embryonic lethality, mice expressing a smaller GAK protein lacking kinase activity (kinase-dead form) died perinatally from pulmonary dysfunction marked by an increased number of alveolar compartments and reduced thickness of the alveolar septum. In these mice, the distribution of surfactant protein A, E-cadherin and EGFR was abnormal. Although the lung histology observed in kinase-dead GAK mice is not identical to that of interstitial lung disease in humans, GAK inhibition by gefitinib may contribute to the pathogenesis of interstitial lung disease in humans. It will be interesting to discover whether

![Fig. 5](image-url)
expression in LPS-treated cultured cells or bleomycin-treated cultures of mouse models, and here we found that, of the EGFR tyrosine kinase inhibitors tested, only gefitinib induced Fosl1 expression in LPS-treated cultured cells or bleomycin-treated mice (Fig. 2). We conclude that gefitinib-dependent activation of Fosl1 may be a major determinant of the induction of interstitial lung disease. Further work is needed to identify the direct target protein of gefitinib.

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