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Effects of Gefitinib on Radiation-induced Lung Injury in Mice

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Abstract

Clinical studies have demonstrated that gefitinib, an epidermal growth factor receptor inhibitor, is an effective treatment for some patients with advanced non-small cell lung cancer and is generally well-tolerated. However, several reports have also suggested that gefitinib is associated with acute lung injury and subsequent fibrosis. One hypothesis is that gefitinib exacerbates lung injury induced by radiation therapy. It is important to confirm the safety of gefitinib in radiotherapy for patients with lung cancer. In this preclinical study we aimed to clarify the effect of gefitinib on thoracic radiotherapy. Six-week-old female C57BL/6 mice were immobilized in a plastic frame, and the thorax was irradiated once with a dose of 12 Gy on day 0. Gefitinib (20, 90 and 200 mg/kg/day) was administered on days 0 to 5 (acute phase) or days 14 to 19 (late phase) postirradiation. Thoracic irradiation induced lung injury and subsequent fibrosis 5 months later. Gefitinib, administered in the acute phase, had no effect on lung fibrosis or collagen levels induced by irradiation. A high dose of gefitinib (200 mg/kg/day) administered during the late phase significantly reduced fibrosis scores and collagen levels. These results suggest that gefitinib does not exacerbate radiation-induced lung injury and fibrosis in this strain of mice. Therefore, thoracic irradiation is unlikely to be a risk factor for lung injury associated with gefitinib treatment.
(J Nippon Med Sch 2008; 75: 96–105)

Key words: gefitinib, epidermal growth factor receptor, radiation-induced lung injury

Introduction

Gefitinib, an epidermal growth factor receptor (EGFR) inhibitor, is a novel molecularly targeted agent and an effective treatment for some patients with advanced non-small cell lung cancer (NSCLC)[1,2]. Gefitinib is generally well tolerated and is not typically associated with the cytotoxic side-effects commonly seen with chemotherapy[3]. However, gefitinib has been reported to be associated with interstitial pneumonia in Japan, with an incidence of 1.8% to 5.4%. This rate is much higher than that in non-Japanese patients treated with gefitinib[4–6]. The mechanism of interstitial pneumonia associated with gefitinib treatment remains unknown. Lung specimens obtained at autopsy have shown diffuse alveolar damage associated with gefitinib treatment[4]. Radiation therapy for NSCLC might be a risk factor for interstitial pneumonia associated with gefitinib. In this study, we developed an experimental model of radiation-induced lung injury and subsequent

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fibrosis in mice. We investigated the effect of gefitinib at different doses and phases using this model.

Materials and Methods

Animal Model of Lung Fibrosis by Thoracic Irradiation

The experimental protocol was approved by the Animal Care Committee of Nippon Medical School. Six-week-old female C57BL/6 mice were purchased from Nippon CLEA (Tokyo, Japan). A model for radiation-induced lung fibrosis was generated as described previously[7]. A plastic frame was used to hold nonanesthetized mice, and lead strips were placed to shield all parts of the body except the thoracic region. The thoraxes were irradiated with a single dose of 12 Gy on Day 0 using X-ray equipment (MBR-1505R2, Hitachi Medico Technology, Tokyo, Japan). The irradiation parameters were as follows: a dose-rate at 2.4 Gy/minute at 150 kV, and a 0.4-m source-to-surface distance as described previously with minor modifications[7].

Mice Treatment

Gefitinib (Iressa, 20, 90 and 200 mg/kg/day; donated by Astra Zeneca, Macclesfield, UK) was suspended in 0.1% Tween 80 solution (Sigma-Aldrich, St. Louis, MO) and given by gastrogavage once a day from day 0 to day 5 (acute phase) or day 14 to day 19 (late phase) postirradiation. Each experimental group comprised 6 mice. For the control group, a daily dose of vehicle (0.1% Tween 80) was given by gastrogavage. Animals were allocated to 8 groups as follows:

1) gefitinib (20, 90, or 200 mg/kg/day) alone (days 0–5)
2) irradiation (12 Gy) + vehicle (days 0–5)
3) irradiation (12 Gy) + 20 mg/kg of gefitinib during the acute phase (day 0–5)
4) irradiation (12 Gy) + 90 mg/kg of gefitinib during the acute phase (days 0–5)
5) irradiation (12 Gy) + 200 mg/kg of gefitinib during the acute phase (days 0–5)
6) irradiation (12 Gy) + 20 mg/kg of gefitinib during the late phase (days 14–19)
7) irradiation (12 Gy) + 90 mg/kg of gefitinib during the late phase (days 14–19)
8) irradiation (12 Gy) + 200 mg/kg of gefitinib during the late phase (days 14–19)

Six mice in each group were killed at 1, 2, 4, and 5 months postirradiation in both the acute-phase and late-phase experiments. Mice were killed with inhalation of ether.

In the irradiation model used in this study, gene expression of transforming growth factor beta (TGF-β) was up-regulated in both the early (within 24 hours) and late (2–4 weeks) phases[7]. Because TGF-β is the major cytokine responsible for the fibrotic reaction in lung fibrosis, these two phases were used to investigate the effect of gefitinib on irradiation-induced lung injuries.

Histology and Fibrosis Score

Mice were killed and extensively perfused via the right ventricle with 6 ml of sterile phosphate-buffered saline (PBS) to eliminate blood cells. The left lung was fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for routine histologic examination. The remaining sections of the left lung were processed for immunohistochemical studies. The severity of lung fibrosis was evaluated with the Ashcroft score as described previously[8]. Briefly, the entire field of each 10 sections of the left lung was scanned at a magnification of ×100, and each field was graded visually from 0 (normal lung) to 8 (total fibrotic obliteration of the field). The mean value of the grades obtained for all fields was then used as the visual fibrosis score.

Sircol Assay

The Sircol collagen assay (Biocolor Ltd., Newtownabbey, Northern Ireland, UK) was performed as described previously[9]. For the Sircol collagen assay, the right lung (n=3, in each experimental group) was quickly frozen in liquid nitrogen and saved at −80℃ until use. The collagen content was collected for right-lung protein content.

Immunohistochemical Studies

Lung sections were double-immunostained for proliferating cell nuclear antigen (PCNA) and epithelial marker surfactant-associated protein antigen (SP-C). The sections were irradiated in a microwave oven in a 0.01 M sodium citrate buffer to retrieve epitopes. After the nonspecific binding sites were blocked with 10% normal goat serum, the sections were incubated with a polyclonal antibody against pulmonary surfactant protein C (1 : 200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 minutes at 40℃ followed by a reaction with anti-rabbit IgG conjugated with a horseradish peroxidase-labeled polymer (1 : 500 dilution; Santa Cruz) and 3,3'-diaminobenzidine. After immersion in 0.1 M glycine-HCl buffer (PH 2.2) for 2 hours to remove the prior antibody complex, the slides were incubated with a monoclonal antibody against PCNA (1 : 100 dilution; Santa Cruz) for 30 minutes at 40℃ and reacted with an anti-mouse IgG conjugated with an alkaline phosphatase-labeled polymer (1 : 500 dilution; Santa Cruz) for 15 min at 40℃. Immunoreactants were visualized with a solution containing 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride solution (BCIP/NBT kit IV; Vector Laboratories, Burlingame, CA, USA).

Semiquantitative analysis of epithelial proliferation was performed with the double-stained lung tissue. In brief, each lung sample was quantified in 5 to 8 randomly selected microscopic fields (at × 200 magnification). The average percentages of cells positive for both PCNA and SP-C in the cytokeratin-positive cell population was regarded as epithelial proliferation index.

Double-immunostaining for EGFR and phosphorylated EGFR (p-EGFR) was performed after the retrieval of epitopes and the blocking of nonspecific binding sites using a method similar to that described above. Lung sections were primarily incubated with polyclonal antibodies against EGFR (1 : 20 dilution; Santa Cruz) and against p-EGFR (Tyr 1068; 1 : 20 dilution; Cell Signaling Technology, Beverly, MA, USA) for 30 minutes at 40℃. For secondary staining, tissue sections were incubated with anti-rabbit IgG conjugated with horseradish peroxidase-labeled polymer (1 : 500 dilution; Sant Cruz) for 15 minutes at 40℃. Cells positive for EGFR and p-EGFR were visualized with 3,3'-diaminobenzidine in lung sections, which were counter stained with a hematoxylin solution. Negative control specimens were prepared by immunostaining using serum with no immunity as the primary antibodies. Semiquantitative evaluation of both EGFR- and p-EGFR-positive cells was performed according to the method of Putti et al[10]. The scoring of EGFR or p-EGFR staining was performed as follows: "0 points" for negative staining, "1 point" for <10% positive cells, "2 points" for 10% to 50% positive cells, and "3 points" for >50% positive cells.

Western Blot Analysis

Western blot analysis was performed using a standard procedure[11]. Protein quantification was determined by using a BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Mouse lung lysates, containing 25 μg of protein, were boiled for 5 minutes in sodium dodecylsulfate sample buffer, separated with 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) using an electroblot apparatus (Bio-Rad Laboratories). Endogenous peroxidase activity was blocked by immersing the membrane in 5% not-fat dry milk with Tris-buffered saline with Tween-20 for 1 hour at room temperature on an orbital shaker, and the membranes were incubated overnight at 4℃ with the primary antibody against p-EGFR (1 : 250 dilution, Cell Signaling Technology). The membranes were then incubated in the secondary antibody horseradish peroxidase-conjugated with IgG (1 : 1,000 dilution, Santa Cruz) at room temperature for 1 hour. The protein blot was detected with the ECL Plus system (GE Healthcare Bio-Science Corp, Piscataway, NJ, USA).

Statistical Analysis

The Statistical analysis of differences among the groups was done by one-way ANOVA and post-hoc comparison with Tukey's test or Fisher's PLSD test for multiple comparisons using "Statistica" software

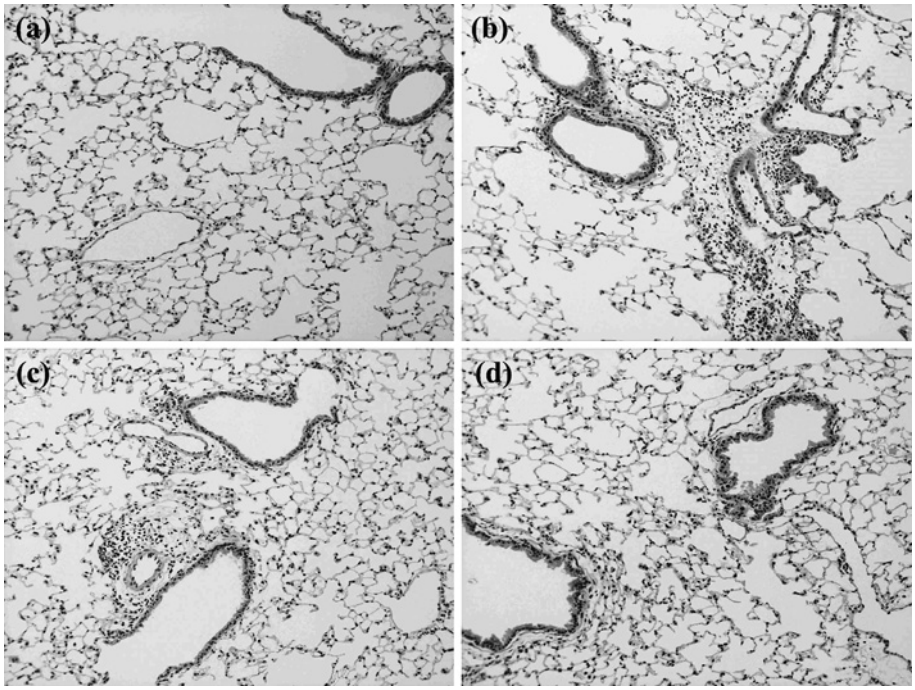

Fig. 1 Pathological changes in radiation-induced lung fibrosis treated with gefitinib
Hematoxylin-eosin stained lung tissues from mice obtained 5 months postirradiation. (a) control (without radiation), (b) radiation only (12 Gy), (c) gefitinib (200 mg/kg) administered in the acute phase after radiation, (d) gefitinib (200 mg/kg) administered in the late phase after radiation. These photographs are representative of 5 mice in each group.

(StatSoft, Tulsa, OK). All data were expressed as the mean ± SE. $p<0.05$ was considered statistically significant.

Results

Irradiation-induced Lung Fibrosis

As shown in **Figure 1**, thickening of the alveolar wall, infiltration of inflammatory cells and fibrosis of alveolar septa were observed 5 months postirradiation, especially in subpleural regions (**Fig. 1b**). There were no differences in survival or body weight between the experimental groups.

Effect of Gefinitib on Lung Fibrosis Induced by Irradiation

Histopathologic examination showed that in both phases high-dose gefitinib (90 or 200 mg/kg) tended to decrease irradiation-induced lung fibrosis (**Fig. 1c and 1d**). As shown in **Figure 2**, compared with irradiation-induced lung fibrosis (Ashcroft score, 2.33 ± 0.47), high dose of gefitinib (200 mg/kg) in the late phase significantly inhibited lung fibrosis (Ashcroft score, 0.80 ± 0.45; $p=0.0038$) 5 months postirradiation. To quantify lung fibrosis, collagen deposition was measured with the Sircol assay. A high dose of gefitinib (200 mg/kg) in the late phase significantly reduced lung collagen levels compared with those in irradiated-lung fibrosis (**Fig. 3**, $p<0.01$). There was no significant difference in either the lung fibrosis score or collagen deposition except, for high dose of gefitinib at late phase.

Effect of Gefitinib on Epithelial Cell Proliferation

Immunostaining with both PCNA and SP-C was used to examine the effect of gefitinib on the proliferation of alveolar epithelial cells. In all mice, alveolar epithelial cells double-immunostained for both PCNA and SP-C were detected (**Fig. 4A**). Thoracic irradiation increased the number of $PCNA^+/SP\text{-}C^+$ alveolar cells. There was no significant difference in the proportion of $PCNA^+/SP\text{-}C^+$ alveolar cells between the mice treated with irradiation alone and those treated with irradiation plus gefitinib in the acute phase. The proportion of

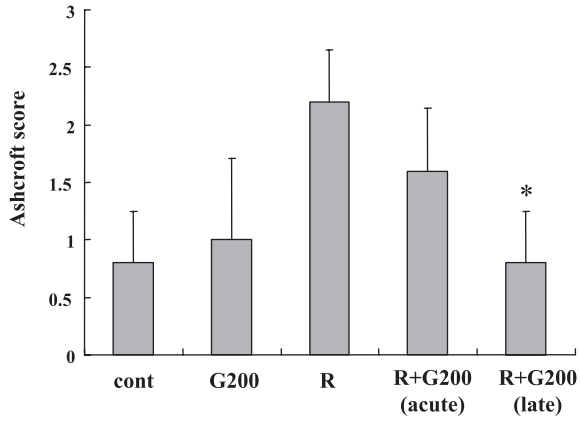

Fig. 2 Effect of gefitinib on lung fibrosis scores (Ashcroft scores)
Ashcroft scores of lung tissues obtained from mice 5 months postirradiation. Data represent the means ± SEM of 5 mice in each group. Control: vehicle (0.1% Tween solution), G200 (gefitinib alone 200 mg/kg/d), R: radiation only (12 Gy), R+G200 (acute): gefitinib (200 mg/kg) administered in the acute phase postirradiation, R+G200 (late): gefitinib (200 mg/kg) administered in the late phase postirradiation. p=0.0038 versus mice treated with gefitinib (200 mg/kg/d) in the late phase postirradiation and radiation only.

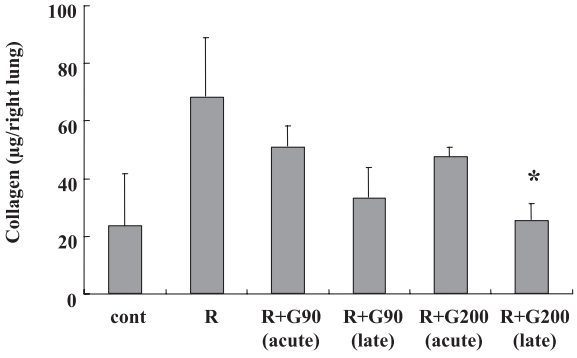

Fig. 3 Effect of gefitinib on collagen content
Collagen content in lung tissues from mice obtained 5 months postirradiation. Data represent the mean ± SEM of 5 mice in each group. Control: vehicle (0.1% Tween solution), R: radiation alone, R+G90 (acute): gefitinib (90 mg/kg) administered in the acute phase postirradiation, R+G200 (acute): gefitinib (200 mg/kg) administered in the acute phase postirradiation, R+G90 (late): gefitinib (90 mg/kg) administered in the late phase postirradiation, R+G200 (late): gefitinib (200 mg/kg) administered in the late phase postirradiation. High-dose (200 mg/kg/d) gefitinib at late phase significantly reduced collagen deposition levels compared with radiation only ($p<0.01$).

$PCNA^+/SP\text{-}C^+$ alveolar cells was significantly decreased when high-dose gefitinib (200 mg/kg/day) was administered in the late phase **(Fig. 4B)**.

Effect of Gefitinib on EGFR and p-EGFR Expression

In mice treated with irradiation, airway epithelial cells and type II pneumocytes were immunostained with EGFR **(Fig. 5Aa)**. Gefitinib did not change the expression of EGFR induced by irradiation **(Fig. 5 Ab)**. On the other hand, gefitinib tended to inhibit the expression of p-EGFR **(Fig. 5Ad)** induced by irradiation. There was a significant difference in the labeling index of p-EGFR between the mice treated with irradiation alone and mice treated with irradiation and high-dose gefitinib in the late phase **(Fig. 5B)**. Gefitinib treatment in both phases inhibited the expression of p-EGFR induced by irradiation **(Fig. 6)**. Four to 5 months postirradiation there was no clear difference in p-EGFR expression between acute-phase treatment and late-phase treatment with gefitinib.

Discussion

To our knowledge this is the first report of the effects of gefitinib on irradiation-induced lung injury and fibrosis in mice. In this study, we demonstrated that gefitinib has no effect on lung-fibrosis or collagen levels induced by irradiation. However, lung fibrosis scores and collagen levels were significantly reduced when high-dose gefitinib (200 mg/kg/day) was administered during the late phase.

Gefitinib, a selective inhibitor of the EGFR tyrosine kinase, is an effective treatment for some patients with advanced NSCLC[1,2]. However, severe lung injury associated with gefitinib treatment has been reported in some Japanese patients[4–6], although the mechanism by which this injury occurs remains unclear. One possible mechanism is the previous alveolar damage caused by the radiotherapy delivered prior to gefitinib treatment[4]. In an animal model with ICR mice, gefitinib has been reported to augment bleomycin-induced pulmonary fibrosis[12]. However, as a recent paper has reported that gefitinib prevents bleomycin-induced fibrosis in C57/BL6 mice[13], the effects of gefiitinib on lung injury

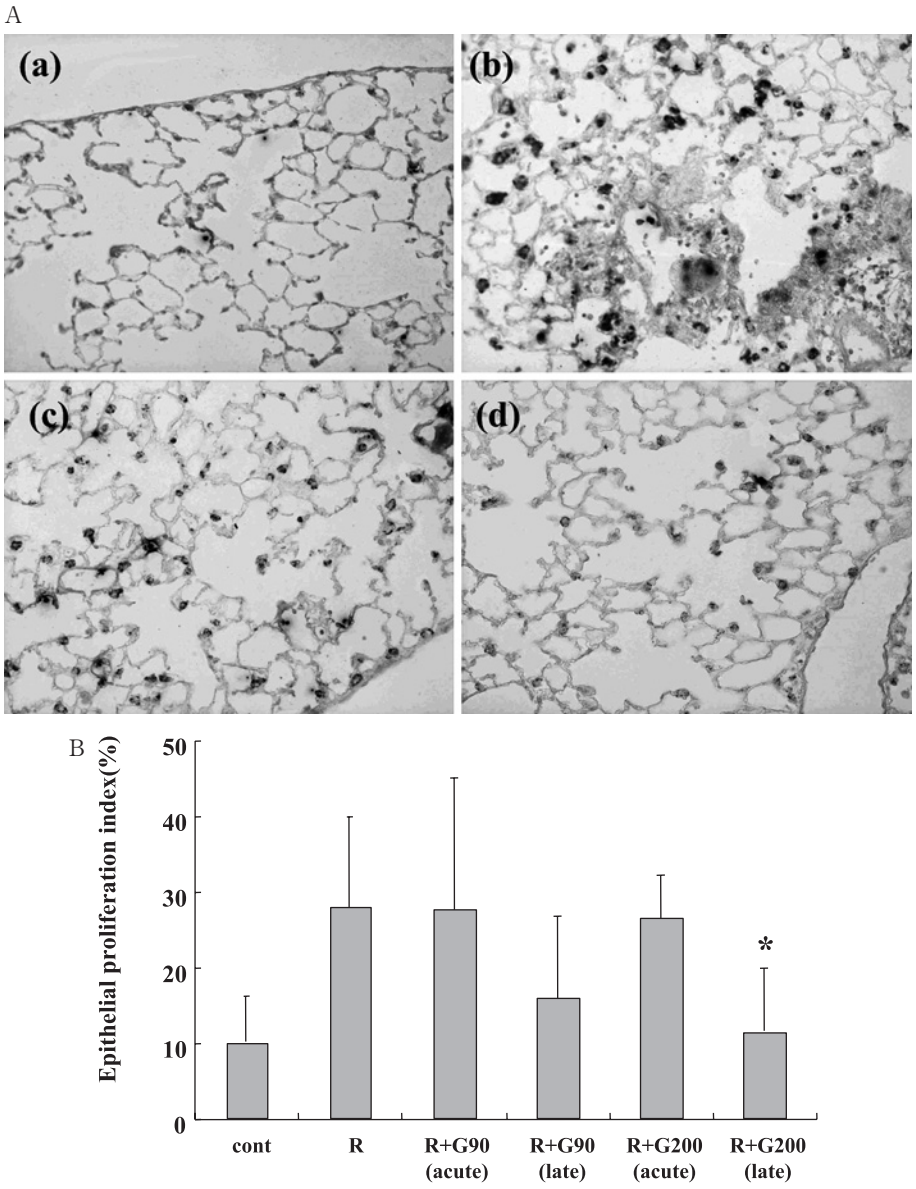

Fig. 4 Effect of gefitinib on epithelial cell proliferation

(A) Immunohistochemical staining of $PCNA^+/SP\text{-}C^+$ in lung tissues from mice obtained 5 months post-radiation. (a) Control (vehicle, 0.1% Tween solution), (b) radiation only (12 Gy), (c) gefitinib (200 mg/kg) administered in the acute phase postirradiation, (d) gefitinib (200 mg/kg) administered in the late phase postirradiation. Original magnification, ×400. These photographs are representative of 5 mice in each group.

(B) Proportion of $PCNA^+/SP\text{-}C^+$ epithelial cells. Control: vehicle (0.1% Tween solution), R: radiation only (12 Gy), R+G90 (acute): gefitinib (90 mg/kg) administered in the acute phase postirradiation, R+G200 (acute): gefitinib (200 mg/kg) administered in the acute phase postirradiation, R+G90 (late): gefitinib (90 mg/kg) administered in the late phase postirradiation, R+G200 (late): gefitinib (200 mg/kg) administered in the late phase postirradiation. High-dose (200 mg/kg/d) gefitinib in the late phase significantly reduced the proportion of $PCNA^+/SP\text{-}C^+$ epithelial cells compared with radiation only ($p=0.018$).

and fibrosis remain controversial. To mimic the clinical situation, a radiation-induced lung injury model was used in this study. To clarify the effects of gefitinib on lung injury and fibrosis, the same doses were used (20, 90, and 200 mg/kg/day) as in previous reports[12,13].

Both EGF and EGFR have been reported to be expressed by type II pneumocytes, suggesting an autocrine regulation of type II pneumocytes[14]. The expression of EGFR has been reported to increase in

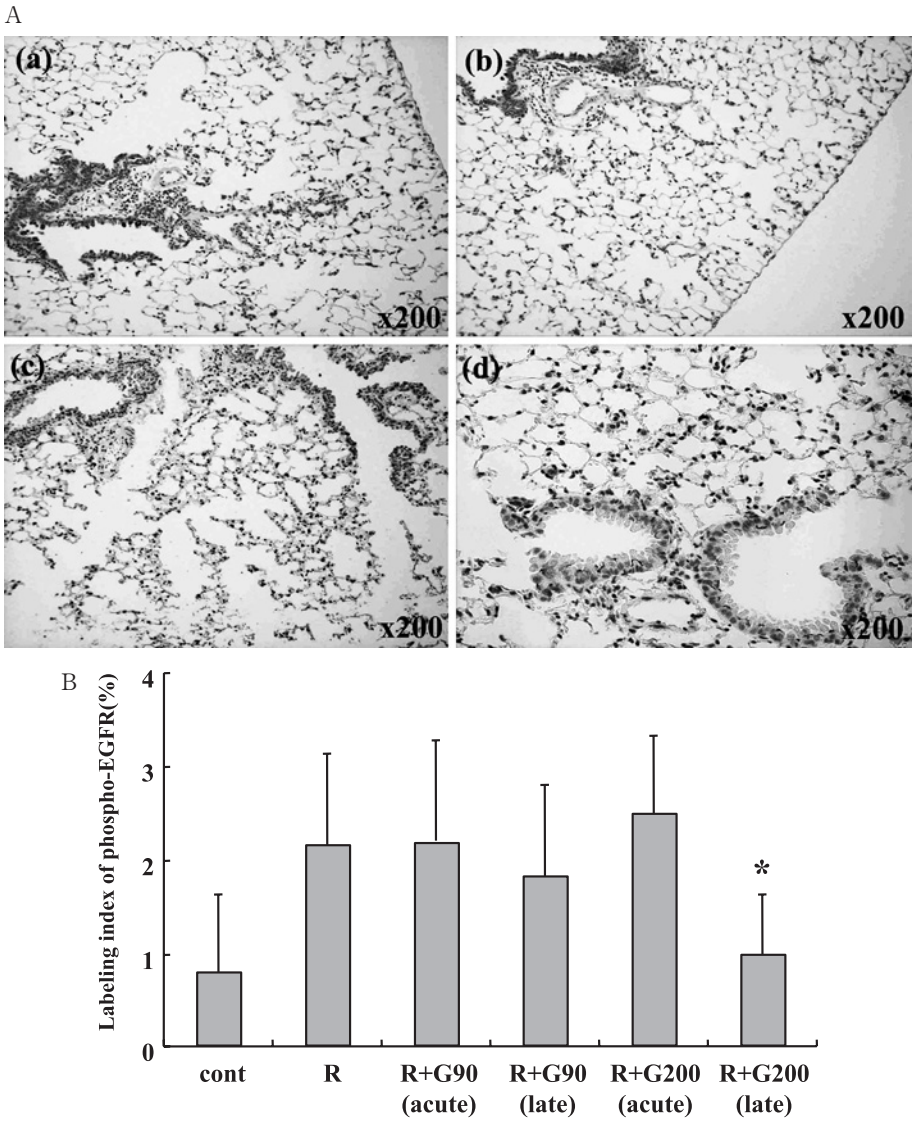

Fig. 5 Immunohistochemical staining for EGFR and p-EGFR

(A) Immunohistochemical staining for EGFR and p-EGFR in lung tissues from mice obtained 5 months postirradiation. (a) Immunostaining for EGFR in the lung postirradiation (12 Gy) only. Airway epithelial cells and type II pneumocytes were immunostained for EGFR. (b) Immunostaining for EGFR in the lung with administration of gefitinib (200 mg/kg) in the late phase postirradiation. Gefitinib did not change the expression of EGFR induced by irradiation. (c) Immunostaining for p-EGFR postirradiation only (12 Gy). (d) Immunostaining for p-EGFR in the lung with administration of gefitinib in the late phase postirradiation. Gefitinib tended to reduce the expression of p-EGFR induced by irradiation. Original magnification, ×200. These photographs are representative of 5 mice in each group.

(B) Proportion of p-EGFR-positive epithelial cells. Control: vehicle (0.1% Tween solution), R: radiation only (12 Gy), R+G90 (acute): gefitinib (90 mg/kg) administered in the acute phase postirradiation, R+G200 (acute): gefitinib (200 mg/kg) administered in the acute phase postirradiation, R+G90 (late): gefitinib (90 mg/kg) administered in the late phase postirradiation, R+G200 (late): gefitinib (200 mg/kg) administered in the late phase postirradiation. High-dose (200 mg/kg/d) gefitinib in the late phase significantly reduced the proportion of p-EGFR-positive epithelial cells compared with radiation only ($p=0.0054$).

bleomycin-induced lung injury[15,16]. In the rat, tyrosine kinase inhibitors specific for EGFR or platelet-derived growth factor (PDGF) have been reported to reduce the proliferation of myofibroblasts and inhibit

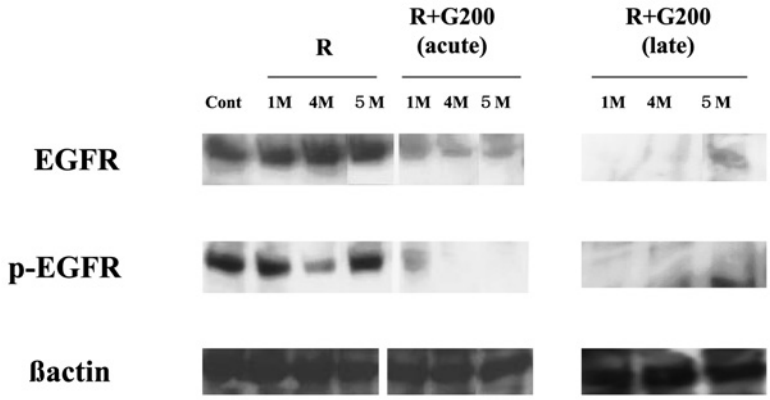

Fig. 6 Expression of EGFR and p-EGFR
Time courses of EGFR and p-EGFR expression in lung tissues were determined with Western blot analysis. Mice treated with or without gefitinib (200 mg/kg) in both the acute and late phases were killed 1, 2, 4, and 5 months postirradiation. Lung-tissue homogenates were analyzed for expression of EGFR and p-EGFR with Western blotting. Gefitinib treatment (200 mg/kg) in both phases inhibited the expression of EGFR and p-EGFR 4 and 5 months postirradiation.

pulmonary fibrosis following injury with vanadium pentoxide (V_2O_5)[17]. Similar results were observed in the present study. High-dose gefitinib (200 mg/kg/day) administered during the late phase significantly decreased the lung fibrosis score and collagen deposition, probably thorough inhibitory effects of gefitinib on irradiation-induced cell proliferation. Suzuki et al. have reported that gefitinib inhibits the proliferation of alveolar epithelial cells[12], and it is, therefore, possible that gefitinib partly inhibits the ability of epithelial cells to respond to lung injury. However, we have demonstrated that gefitinib, except high-dose gefitinib (200 mg/kg/d) administered during the late phase, does not reduce the number of irradiation-induced $PCNA^+/SP\text{-}C^+$ alveolar cells; this finding suggests a minimal effect of gefitinib on alveolar cell proliferation in this irradiation model. Ishi et al. have recently reported that gefitinib at any dose (20, 90, and 200 mg/kg/day) significantly reduces the bleomycin-induced lung collagen accumulation[13]. In the present study, we demonstrated that only the high-dose gefitinib (200 mg/kg/d) during the late phase significantly inhibits lung fibrosis and collagen deposition induced by irradiation. Unlike bleomycin-induced lung injury, protein-rich edema and hyaline membranes were features of radiation-induced injury in C57BL/6 mice[7]. The collagen content (70 μg per right lung) induced by irradiation in the present study was much smaller than in previous reports[12,13]. Lung injury induced by a single dose of irradiation (12 Gy) might be mild for a drug intervention experiment. In addition, in this experimental model, chronic lung injury and fibrosis were longer lasting than in previous reports[12,13]. Gefitinib administration (20 and 90 mg/kg/day), even in the late phase (days 14–19), might not inhibit subsequent collagen accumulation (e.g., 5 months postirradiation). These results suggest that gefitinib does not exacerbate irradiation-induced lung fibrosis. These data are consistent with a recent clinical report that demonstrated that prior thoracic radiotherapy is not a risk factor for interstitial lung disease in patients treated with gefitinib[18]. High-dose gefitinib (200 mg/kg/day) might improve lung injury and fibrosis induced by irradiation. However, 200 mg/kg/day of gefitinib is much higher than the clinical dose (250 mg/body/day), suggesting that it is impossible to apply the results of this study for clinical use. As shown in **Figure 6**, gefitinib (90 and 200 mg/kg) in both phases significantly inhibited the expression of p-EGFR induced by irradiation. The cytodifferentiation of type II alveolar cells is reported to be regulated by EGF and EGFR[14,19]. Inhibition of p-EGFR by high-dose gefitinib could inhibit cell proliferation of type II alveolar cells and, therefore, may contribute to the

antifibrosis effect.

The incidence of lung injury associated with gefitinib treatment in Japanese patients (1.8%–5.4%) is much higher than that in non-Japanese patients[5]. In a recent first-line trial with gefitinib in Japan, the incidence of severe interstitial pneumonia was 10%[20]. Although ethnic differences in the response to gefitinib have been reported, lung injury associated with gefitinib is unlikely to be related to the presence of an EGFR mutation[21]. The reason for the higher incidence of lung injury associated with gefitinib treatment in Japanese patients remains unknown. In mice, genetic backgrounds have been reported to affect the expression of EGFR in epithelial cells, suggesting that mouse strains may affect the response to gefitinib[22]. Two previous reports have demonstrated different effects of gefitinib in bleomycin-induced lung injuries using different mouse strains[12,13]. Furthermore, differences in H2 typing have been reported to affect the response to lung injury in mice[23]. Further studies with other mouse strains will be needed to investigate the effects of gefitinib on lung injury and fibrosis.

In summary, gefitinib did not exacerbate radiation-induced lung injury and fibrosis in this study. These data suggest that thoracic irradiation is unlikely to be a risk factor for lung injury associated with gefitinib treatment. Further clinical studies are needed to confirm the safety of gefitinib in combination with radiotherapy. It is also important to clarify the mechanism of lung injury associated with gefitinib, including the underlying genetic issues.

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(Received, December 17, 2007)
(Accepted, January 10, 2008)