INTRODUCTION

The epidermal growth factor receptor (EGFR) is part of a family of plasma membrane receptor tyrosine kinases that control many important cellular functions, from cell growth and proliferation to cell death (Ranson et al., 2002). Dysregulation of the EGFR signal transduction pathway has been implicated in tumorigenesis and cancer progression, making it a clinically relevant target for novel anticancer treatments. Two tyrosine kinase inhibitors (TKIs) of EGFR, erlotinib (Tarceva®) and gefitinib (Iressa®), were approved for the non-small cell lung cancer treatment. Although these EGFR-TKIs are recognized as relatively safe oral anti-cancer agents, pulmonary toxicity [interstitial lung disease (ILD)] associated with EGFR-TKIs has been reported as a serious adverse effect. Post-marketing surveillance (by AstraZeneca) reported 215 out of 3,222 Japanese patients of gefitinib-induced ILD, 83 of whom died of ILD (Kataoka et al., 2006). On the other hand, the post-marketing surveillance of erlotinib is being performed in medical institutions that meet specific predetermined criteria, with all the patients in the institutions included as study subjects (by Chugai Pharmaceutical Co., Ltd.).

Two studies in mice have investigated the effects of gefitinib on bleomycin (BLM)-induced pulmonary fibrosis. Effects of erlotinib on lung injury induced by intratracheal administration of bleomycin (BLM) in rats

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ABSTRACT — Interstitial lung disease has been reported in cancer patients treated with epidermal growth factor receptor tyrosine kinase inhibitors, erlotinib and gefitinib. Preclinical safety studies with erlotinib did not show any evidence for an induction of injury on intact lungs in rats and dogs. In the present study, we investigated the effects of erlotinib on lung injury induced by intratracheal administration of bleomycin (BLM) in rats. In Experiment 1, we examined the effects of short-term (7- and 21-day) administration of erlotinib (10 mg/kg/day, p.o.; subtoxic dose) on the BLM (0.1 or 0.6 mg/rat)-induced lung injury of slight and moderate severity. In Experiment 2, we examined the effects of long term (up to 63-day) administration of higher-dose (up to 20 mg/kg/day; toxic dose; accompanied with decreased body weight gain and severe skin lesions) erlotinib on the BLM-induced lung injury. In rats receiving erlotinib alone, no lung lesions were noted. In rats receiving BLM alone, diffuse alveolar damage (DAD) and, subsequently, pulmonary fibrosis of slight or moderate severity was observed. The administration of erlotinib to BLM-treated rats showed no exacerbation of lung injuries in indices such as macroscopic findings, lung weights, histopathological scores (lung lesion density and lung fibrosis score), and pulmonary hydroxyproline (HyP) level. These results suggest that erlotinib does not have any exacerbating effects on lung injuries induced by BLM in rats.

Key words: Erlotinib, Epidermal growth factor receptor tyrosine kinase inhibitor, Bleomycin, Lung fibrosis, Rat

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 sis models. Suzuki et al. (2003) reported that inhibition of EGFR phosphorylation augments BLM-induced pulmonary fibrosis by reducing regenerative epithelial proliferation. On the other hand, Ishii et al. (2006) reported that suppression of EGFR prevented BLM-induced pulmonary fibrosis by inhibiting fibroblast migration and proliferation even under conditions related to epithelial regeneration. Thus, the relationship between gefitinib and lung injury has not been clearly explained and the precise mechanisms of ILD induced by EGFR-TKIs remain unknown.

Preclinical safety studies with erlotinib did not show any evidence for an induction of injury on intact lungs in rats and dogs. In the present study, we investigate the effects of erlotinib on lung injuries induced by a single intratracheal administration of BLM in rats in order to clarify whether erlotinib affects the BLM-induced lung injury. Intratracheal administration of BLM in rats has been widely used to produce a model to study the mechanisms of development and repair in lung injury, especially of pulmonary fibrosis (Chandler et al., 1983; Hay et al., 1991; Kawamoto and Fukuda, 1990; Lazenby et al., 1990; Thrall et al., 1979; Usuki and Fukuda, 1995). Furthermore, rat model can induce lung injury with varying severity (Adachi et al., 2003). The development of pulmonary fibrosis induced by BLM-administration can be divided into the following 3 phases: acute injury of lung cells including alveolar and bronchial epithelium, inflammatory response with infiltration of leucocytes, and fibrogenic response (Adachi et al., 2001; Yara et al., 2001). Furthermore, the intensity of chronic lung inflammation and fibrosis after BLM is directly related to the severity of acute injury (Shen et al., 1988). Therefore, in Experiment 1, we examined the effects of short-term (7- and 21-day) administration of erlotinib on BLM-induced lung injury of slight and moderate severity in the inflammatory and fibrogenic phases. In Experiment 2, we examined the effects of long term (up to 63-day) administration of higher-dose erlotinib on the BLM-induced lung injury of moderate severity. In both experiments, histopathological examination was conducted mainly focusing on the effects of the erlotinib on normal or BLM-induced alveolar tissue damage.

MATERIALS AND METHODS

Animals

Seven-week-old male Crl: CD (SD) rats (body weight range: 244-297 g) (SPF, Charles River Laboratories Japan, Inc., Kanagawa, Japan) were housed individually in stainless steel 5-compartment cages (755 mm x 210 mm x 170 mm) in an animal room maintained at 23 ± 2°C and humidity of 55 ± 15% with filtered fresh air changes 12 times per hour, and a 12-hr light and dark cycle (lighting: 6:00 to 18:00). They were given solid feed (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. All procedures associated with this study were reviewed and approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). This study was conducted at Nihon Bioreresearch Inc. (Gifu, Japan) according to guidelines (Nihon Bioreresearch Inc., Animal Experiment Ethics Committee) concerning animal experiments and the protection and management of animals maintained at experimental institutions.

Preparation of BLM-induced lung injury model

The animals were treated intraperitoneally with ketamine hydrochloride (Sankyo Co., Ltd., Tokyo, Japan) at 50 mg/kg and xyladine (Bayer Medical, Ltd., Osaka, Japan) at 2 mg/kg using a disposable polypropylene hypodermic syringe (Terumo Corporation, Tokyo, Japan) with a 23 G hypodermic needle (Terumo Corporation). Rats were incised along the cervical ventral median line under anesthesia, and the trachea was exposed. The nozzle of an intratracheal aerosolizer (1A-1B, Penn Century, PA, USA) was inserted from the mouth into the trachea, and it was ascertained that the nozzle was properly located in the trachea. Then BLM (0.1 or 0.6 mg/100 μl/rat in Experiment 1; 0.6 mg/100 μl/rat in Experiment 2) or saline (100 μl/rat) was injected into the trachea. Finally, the incision site was sutured.

Administration of erlotinib

Erlotinib hydrochloride was used for this study. The required amount of erlotinib hydrochloride was measured out and suspended in the 0.5% methylcellulose (MC, Metolose® SM-400, Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) solution to specified concentrations. The required amount of erlotinib hydrochloride, which was calculated as free base, was measured.

The animals were treated orally by gavage using a disposable polypropylene hypodermic syringe (Terumo Corporation) with a gastric tube for rats (Fuchigami Kikai Ltd., Kyoto, Japan).

Experimental design

Experimental designs are shown in Fig. 1.

In Experiment 1 (Fig. 1A), we investigated the effects of short-term (7 or 21 days) administration of erlotinib on BLM-induced acute lung injury that developed diffuse alveolar damage (DAD) and subsequent pulmonary fibro-
sis of slight and moderate severity. Two treatment groups were administered with 10 mg/kg/day of erlotinib for 7 or 21 days starting from the day of a single intratracheal administration (Day 1) of 0.1 or 0.6 mg/100 μl/rat of BLM (BLM 0.1 mg + ER 10 mg and BLM 0.6 mg + ER 10 mg groups) and sacrificed on Days 8 or 22. The doses of BLM were chosen based on the result of a preliminary study. Briefly, BLM at 0.1 mg/100 μl/rat had no effect on body weight changes but slightly increased the lung weight. BLM at this dose level also caused slight fibrosis thickening of pulmonary alveolar or bronchiolar walls. Furthermore, BLM at 0.6 mg/100 μl/rat caused decreased body weight gains, moderately increased lung weight and pulmonary hydroxyproline (HyP) level, and moderate fibrosis but did not cause death. The doses of erlotinib (10 mg/kg/day) coincided with the maximum dosage in a repeated dose toxicity study. Fig. 1A also shows the corresponding control groups (Saline + Vehicle, Saline + ER 10 mg, BLM 0.1 mg + Vehicle and BLM 0.6 mg + Vehicle groups). Seven animals were assigned to each group.

In Experiment 2 (Fig. 1B), we investigated the effects of long-term (up to 63 days), higher-dose administration of erlotinib on the BLM-induced lung injury. Two groups were administered with 10 or 20 mg/kg/day of erlotinib for 21, 42 or 63 days starting immediately after the administration of 0.6 mg/100 μl/rat of BLM (BLM 0.6 mg + ER 10 mg and BLM 0.6 mg + ER 20 mg groups), and sacrificed at Days 22, 43 or 64. In addition to the 10 mg/kg which was assigned in Experiment 1, 20 mg/kg was employed to assess the effects of the test article at a higher level. Fig. 1B also shows the corresponding control groups (Saline + Vehicle, Saline + ER 10 mg, Saline + ER 20 mg and BLM 0.6 mg + Vehicle groups). Seven animals were assigned to each group.

All animals were weighed twice a week for the duration of the experiment.

In the BLM-treated groups of both experiments, individual animals with no lung lesions or exceptionally different lesions (confirmed histopathologically) from the other animals in their groups, were excluded from the evaluation of the experiments.

Pathological examination

The animals were sacrificed by exsanguination under anesthesia induced by intraperitoneal injection of 4% sodium pentobarbital (40 mg/kg), and necropsied. Abnormalities in external appearance and systemic organs and tissues including lung were macroscopically evaluated. The lung was removed from each animal and weighed. Relative lung weight was calculated by dividing absolute lung

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**Fig. 1.** Study design and experimental protocol of Exp. 1 (A) and Exp. 2 (B). Open arrow, a single intratracheal administration of saline; closed arrow, a single intratracheal administration of BLM; light gray column, oral gavage administration of the vehicle; dark gray column, oral gavage administration of 10 mg/kg of erlotinib; closed column, oral gavage administration of 20 mg/kg of erlotinib; open triangle, times of sacrifice; ER, erlotinib.
weight by body weight and expressed as mg/100 g body weight. The lung was perfused via the trachea with 10% neutral buffered formalin at a water pressure of 20 cm and immersed in the same fixative. Specimens were collected from the right anterior, middle and posterior lobes (total of 3 specimens/rat). According to the standard method, these specimens were paraffin-embedded and cut into 3 μm sections. Thereafter, these sections were stained with hematoxylin and cosin (HE) or Masson’s trichrome.

Histopathological examination was conducted mainly focusing on the effects of the erlotinib on normal or injured bronchiolar epithelium, and re-epithelialization after BLM-induced alveolar tissue damage.

The lung lesions volume density per unit of pulmonary parenchyma [lung lesion density (%)] and the score of lung fibrosis for each animal were histopathologically evaluated using a semiquantitative scoring method as follows. Measurement of the lung lesion density was done according to the method described by Mautz et al. (1988) and Adachi et al. (2003). Briefly, HE-stained sections were viewed under a light microscope at a total magnification of ×100. A 10 × 10 squared grid eyepiece was used to designate the sample field (1 mm²), and 10 randomly chosen microscope fields of each specimen (30 fields/animal) were scored. Within every field, each of the 100 squares was given one of the scores shown in Table 1. The lung lesion density was determined by dividing the number of points scored as slightly to severely by the total number of points that were classified as parenchyma (excluding major vessels and bronchi).

Score of lung fibrosis was assessed semi-quantitatively according to the method of Ashcroft et al. (1988) and Adachi et al. (2003) (“Ashcroft score”). Briefly, lung fibrosis was scored on a scale from 0 to 8 by examining all the parenchyma of each specimen (2 sections/animal) at a magnification of ×100. A 10 × 10 squared grid eyepiece (1 mm²) was used to designate the sample field. The criteria for grading lung fibrosis are shown in Table 2. If there was any difficulty in deciding between two odd-numbered categories, the field would be given the intervening even-numbered grade. The score of lung fibrosis was expressed as a mean score of fibrosis for each animal.

Whether erlotinib exacerbated lung injury was judged from the histopathological score, lung lesion density and the score of lung fibrosis.

**Measurement of Pulmonary HyP level**

Collagen content in the lung was evaluated by determining HyP level using the anterior part of the left lung. The anterior part of the left lung was homogenized in a certain amount (3 to 4 ml) of saline appropriate to the pulmonary tissue weight, and 500 μl of concentrated hydrochloric acid was added to 500 μl of the prepared homogenate. Hydrolytic degradation was performed at 100°C for 20 hr. A mixture of 100 μl of the obtained supernatant and 1.5 ml of lithium hydroxide was used as a sample for determination and analyzed by HPLC.

### Statistical analysis

Group mean values with standard deviations were calculated for the data obtained in the examination and measurement. A statistical software package (SAS statistical processing system version 5.0; SAS Institute, Tokyo, Japan) was used to determine the significance of differences. Data on body weight, lung weight, lung lesion density (%), fibrotic lesion score and pulmonary HyP level were tested by an F test for homogeneity of variance between 2 groups. When the variances were homogeneous, Student’s t-test was performed. When the variances were heterogeneous, Aspin-Welch’s t-test was performed to test the difference in mean values. Significance tests were performed at two-tailed significance levels of 5% and 1%. Intergroup comparison was performed as follows: Saline + Vehicle group and Saline + ER 10 mg, Saline + ER 20 mg groups, BLM 0.1 mg + Vehicle or BLM 0.6 mg + Vehicle groups; BLM 0.1 mg + Vehicle or BLM 0.6 mg + Vehicle groups and BLM 0.1 mg + ER 10 mg, BLM 0.6 mg +

### Table 1. Scoring criteria for lung lesion volume density per unit pulmonary parenchyma

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Nonparenchyma</td>
</tr>
<tr>
<td>1</td>
<td>Alveolar septa and alveolar ducts of normal septal thickness and cellularity</td>
</tr>
<tr>
<td>2</td>
<td>Slightly to moderately injured alveolar tissue with increased cellularity and thickness of the alveolar wall but no disruption of normal alveolar shape</td>
</tr>
<tr>
<td>3</td>
<td>Markedly to severely injured alveolar tissue with clear distortion of the normal alveolar architecture and a more marked increase in septal cellularity and thickness than in 2</td>
</tr>
</tbody>
</table>
mg + ER 10 mg or BLM 0.6 mg + ER 20 mg groups.

RESULTS

Experiment 1: The effects of short-term administration of erlotinib on BLM-induced acute lung injury of varying severity in the inflammatory and fibrogenic phases

General signs and body weight changes

No death or abnormal clinical signs were noted in any of the test groups. In the Saline + ER 10 mg group and BLM 0.1 mg + Vehicle group, body weight was comparable to that in the control group throughout the experiment. In the BLM 0.6 mg + Vehicle group, reduced body weight or body weight gain was noted between Days 4-22. There were no differences in the body weight changes between the BLM 0.1 mg + Vehicle and BLM 0.1 mg + ER 10 mg groups or BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg groups.

Necropsy findings of lung

Necropsy findings of lung in animals necropsied at Days 8 and 22 are shown in Table 3. In the Saline + Vehicle and Saline + ER groups at Days 8 and 22, although very slight inflammatory or foamy cell infiltrations were noted spontaneously in some animals, no abnormalities were observed in almost all animals (Figs. 3A and B).

Histopathological evaluation of lungs

In the Saline + Vehicle and Saline + ER groups at Days 8 and 22, although very slight inflammatory or foamy cell infiltrations were noted spontaneously in some animals, no abnormalities were observed in almost all animals (Figs. 3A and B).

In the BLM + Vehicle groups, DAD and subsequent pulmonary fibrosis were observed. At Day 8, edema, hemorrhage, inflammatory cell infiltration (mainly composed of neutrophils and mononuclear cells), proliferation of myofibroblasts in the alveoli, hyperplasia of bronchial and alveolar epithelial cells, and bronchiolization and disruption of normal alveolar structures were noted. Furthermore, slight fibrosis was also observed. At Day 22, alveoli mainly adjacent to bronchi had been obliterated, the acute inflammation was less obvious, and fibrotic changes were localized (Figs. 3C and E). In the bronchiolization area, the single layer of bronchiolar epithelial cells with uniform cell size and shape was more prominent (Fig. 3G). The severity of lesions was more severe in the BLM 0.6 mg + Vehicle group as compared to BLM 0.1 mg + Vehicle group.

In the BLM 0.1 mg + ER 10 mg group, the histopathological features of the lung were qualitatively and quantitatively similar to those in the BLM 0.1 mg + Vehicle group (Fig. 3D).

In the BLM 0.6 mg + ER groups, high incidence of increased flattened epithelium in the bronchiolization area

Table 2. Criteria for grading lung fibrosis

<table>
<thead>
<tr>
<th>Grade of fibrosis</th>
<th>Histological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal lung</td>
</tr>
<tr>
<td>1</td>
<td>Minimal fibrous thickening of alveolar or bronchiolar walls</td>
</tr>
<tr>
<td>3</td>
<td>Moderate thickening of walls without obvious damage to lung architecture</td>
</tr>
<tr>
<td>5</td>
<td>Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses</td>
</tr>
<tr>
<td>7</td>
<td>Severe distortion of structure and large fibrotic areas; “honeycomb lung” is placed in this category</td>
</tr>
<tr>
<td>8</td>
<td>Total fibrous obliteration of the fields</td>
</tr>
</tbody>
</table>

Lung weights

Lung weights of animals necropsied at Days 8 and 22 are shown in Fig. 2A. In the Saline + ER 10 mg group, no changes of lung weights were noted compared with the Saline + Vehicle group. In the BLM 0.1 mg + Vehicle group, lung weights slightly increased at Days 8 and 22. Furthermore, in the BLM 0.6 mg + Vehicle group, lung weights moderately increased at Days 8 and 22. There were no differences in the lung weights between the BLM 0.1 mg + Vehicle and BLM 0.1 mg + ER 10 mg groups or BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg groups.
was observed at Day 22 (Fig. 3H). Other histopathological features of the lung, including the amount of bronchiolization, were qualitatively and quantitatively similar to those in the BLM 0.6 mg + Vehicle group (Fig. 3F).

The comparisons of the lung lesion density and the lung fibrosis score in each group are shown in Figs. 4A and 5A. In the BLM 0.6 mg + Vehicle group, the lung lesion density and lung fibrosis score were greater than that of the BLM 0.1 mg + Vehicle group. However there were no differences in the lung lesion density or lung fibrosis score between the BLM 0.1 mg + Vehicle and BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg groups.

### Table 3. Necropsy findings of lung in saline-, 0.1 or 0.6 mg of BLM-treated (i.t.) rats with vehicle or 10 mg/kg of erlotinib (p.o.) for 22 days (Experiment 1)

<table>
<thead>
<tr>
<th>Findings</th>
<th>Saline Vehicle (n = 7)</th>
<th>BLM 0.1 mg Vehicle (n = 6)</th>
<th>BLM 0.6 mg Vehicle (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not remarkable changes</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Dark-red/brown focus</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Recessed area</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Emphysema</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 22</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Not remarkable changes</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Dark-red/brown focus</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Recessed area</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Emphysema</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Abbreviations: BLM, bleomycin; Er, erlotinib; i.t., intratracheal administration; p.o., peroral administration.

a Days after the administration of saline or BLM.

b Each value represents the number of animal.

### Pulmonary HyP Level

The comparisons of the pulmonary HyP level among the BLM-untreated and BLM-treated groups are shown in Fig. 6A. There were no differences in the pulmonary HyP level between the Saline + Vehicle and BLM 0.1 mg + Vehicle groups. In the BLM 0.6 mg + Vehicle group, increased pulmonary HyP level was noted at Day 22 compared with the Saline + Vehicle group. On the other hand, statistically significant decreased pulmonary HyP level was noted at Day 8; however this change was considered to be biologically insignificant since it did not correspond with other indices of lung injury including histopathological findings. There were no differences in the pulmonary HyP level between the BLM 0.1 mg + Vehicle and BLM...
Effects of erlotinib on lung injury induced by bleomycin in rats

Experiment 2: The effects of long-term administration of higher-dose erlotinib on the BLM-induced lung injury

General signs and body weight changes

No death was noted in any of the test groups. In the Saline + Vehicle and BLM 0.6 mg + Vehicle groups, no abnormal clinical signs were noted. In the Saline + ER 10 mg group, swelling of the mouth/perinasal/forepaws was observed at Day 47 and thereafter. In the Saline + ER 20 mg and BLM 0.6 mg + ER 20 mg groups, swelling of the mouth/perinasal/forepaws or loss of fur was noted at Day 35 and thereafter. In the Saline + ER 20 mg group, reduced body weight gain was noted at Day 57 and thereafter. In the BLM 0.6 mg + Vehicle group, transient reduced body weight gain was noted between Days 4 and 11. There were no differences in the body weight changes between the BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg or ER 20 mg groups.

Necropsy findings of lung

Necropsy findings of lung in animals necropsied at Days 22, 43 and 64 are shown in Table 4. In the Saline + Vehicle, Saline + ER 10 mg and Saline + ER 20 mg groups, no abnormalities were noted. In the BLM 0.6 mg + ER 10 mg groups or BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg groups.

0.1 mg + ER 10 mg groups or BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg groups.

Fig. 3. Lung sections of rats treated with 10 mg/kg of erlotinib (ER) (B, D, F, H) or vehicle (A, C, E, G) for 22 days after intratracheal administration (i.t.) of Saline (A, B), 0.1 mg (C, D) or 0.6 mg of BLM (E-H). (A) Saline + Vehicle group. No abnormalities are observed. (B) Saline + ER 10 mg group. No abnormalities are noted. (C) BLM 0.1 mg + Vehicle group. DAD and pulmonary fibrosis are observed. Alveoli mainly adjacent to bronchi are obliterated, and slight fibrotic changes were localized. (D) BLM 0.1 mg + ER 10 mg group. The histopathological features of the lung are qualitatively and quantitatively similar to those in the BLM 0.1 mg + Vehicle group. (E) BLM 0.6 mg + Vehicle group. The histopathological features of the lung are qualitatively similar to those in the BLM 0.1 mg + Vehicle group. On the other hand, the severity of lesions is more severe compared to BLM 0.1 mg + Vehicle group. (F) BLM 0.6 mg + ER 10 mg group. The histopathological features of the lung are qualitatively and quantitatively similar to those in the BLM 0.6 mg + Vehicle group. (G) BLM 0.6 mg + Vehicle group. Single layer of bronchiolar epithelial cells with uniform cell size and shape are prominent (open arrow heads). (H) BLM 0.6 mg + ER 10 mg group. Increased flattened epithelium in the bronchiolization area is observed (open arrows). HE staining. Bar = 120 μm (A-F) or 12 μm (G, H).
Fig. 4. Histopathological evaluation of the lung lesion volume density per unit of pulmonary parenchyma (lung lesion density) for saline-, 0.1 or 0.6 mg of BLM-treated (i.t.) rats with vehicle, 10 mg/kg or 20 mg/kg of erlotinib (p.o.) on Days 8, 22, 43 or 64 in Experiment 1 (A) and Experiment 2 (B). Each value represents the mean ± S.D. (n = 6 or 7 in each group). *p < 0.05, **p < 0.01: Significantly different compared with the Saline + Vehicle group.

Fig. 5. Histopathological evaluation of the score of lung fibrosis for saline-, 0.1 or 0.6 mg of BLM-treated (i.t.) rats with vehicle, 10 mg/kg or 20 mg/kg of erlotinib (p.o.) on Days 8, 22, 43 or 64 in Experiment 1 (A) and Experiment 2 (B). Each value represents the mean ± S.D. (n = 6 or 7 in each group). *p < 0.05, **p < 0.01: Significantly different compared with the Saline + Vehicle group. #p < 0.05, ##p < 0.01: Significantly different compared with the BLM 0.1 mg + Vehicle group.

Fig. 6. Pulmonary hydroxyproline (HyP) level for saline-, 0.1 or 0.6 mg of BLM-treated (i.t.) rats with vehicle, 10 mg/kg or 20 mg/kg of erlotinib (p.o.) on Days 8, 22, 43 or 64 in Experiment 1 (A) and Experiment 2 (B). Each value represents the mean ± S.D. (n = 6 or 7 in each group). *p < 0.05, **p < 0.01: Significantly different compared with the Saline + Vehicle group.
Effects of erlotinib on lung injury induced by bleomycin in rats

Table 4. Necropsy findings of lung in saline- or 0.6 mg of BLM-treated (i.t.) rats with vehicle, 10 mg/kg or 20 mg/kg of erlotinib (p.o.) for 64 days (Experiment 2)

<table>
<thead>
<tr>
<th>Findings</th>
<th>Saline Vehicle (n = 7)</th>
<th>Er 10 mg (n = 7)</th>
<th>Er 20 mg (n = 7)</th>
<th>BLM 0.6 mg Vehicle (n = 7)</th>
<th>Er 10 mg (n = 7)</th>
<th>Er 20 mg (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Not remarkable changes</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dark-red/brown focus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Recessed area</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Day 43</td>
<td>Not remarkable changes</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dark-red/brown focus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
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<td>6</td>
<td>7</td>
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<td>Day 64</td>
<td>Not remarkable changes</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>0</td>
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<td></td>
<td>Dark-red/brown focus</td>
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<td>0</td>
<td>6</td>
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<tr>
<td></td>
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</tbody>
</table>
Abbreviations: BLM, bleomycin; Er, erlotinib; i.t., intratracheal administration; p.o., peroral administration.
<sup>a</sup> Days after the administration of saline or BLM.
<sup>b</sup> Each value represents the number of animal.

Vehicle group, dark red/brown focus and recessed area of the lung was noted at Days 22, 43 and 64. There were no differences in the necropsy findings of lung between the BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg or ER 20 mg groups.

Lung weights

Lung weights of animals necropsied at Days 22, 43 and 64 are shown in Fig. 2B. In the Saline + ER 10 mg and Saline + ER 20 mg groups, no changes of lung weights were noted compared with the Saline + Vehicle group. In the BLM 0.6 mg + Vehicle group, lung weights moderately increased at Days 22, 43 and 64. There were no differences in the lung weights between the BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg or ER 20 mg groups.

Histopathological evaluation of lungs

In the Saline + Vehicle or Saline + ER groups at Days 22, 43 and 64, just as in Experiment 1, very slight inflammatory or foamy cell infiltration was noted spontaneously in some animals.

In the BLM + Vehicle groups, pulmonary lesions, qualitatively similar to Experiment 1, were observed. At Day 22, alveoli mainly adjacent to bronchi had been obliterated, the acute inflammation was less obvious, and moderate fibrotic changes were localized. At Day 43, interstitial cells were tightly located in the fibrotic area. Furthermore, the single layer of bronchiolar epithelial cells with uniform cell size and shape was more prominent. Additionally, increased flattened epithelium in the bronchiolization area was noted. At Day 63, the pulmonary changes were almost the same as those on Day 43.

In the BLM 0.6 mg + ER groups, just as in Experiment 1, increased flattened epithelium in the bronchiolization area was observed at Days 22, 43 and 64. Compared to the BLM + Vehicle groups, severity and incidence of this finding was more prominent at Days 22 and 43. However, at Day 64, no marked differences were seen in the BLM 0.6 mg + ER groups. Other histopathological features of the lung, including the amount of the bronchiolization area, were qualitatively and quantitatively similar to those in the BLM + Vehicle groups.

The comparisons of the lung lesion density and the lung fibrosis score in each group are shown in Figs. 4B and 5B. In the BLM 0.6 mg + ER 20 mg groups at Day 22, a statistically significant increased score of lung fibrosis was noted compared with the BLM 0.6 mg + Vehicle group; however this change was considered to be biologically insignificant since it did not correspond with other indices of lung lesions such as macroscopic findings, lung weights, histopathological findings, lung lesion density,
Pulmonary HyP Level

The comparisons of the pulmonary HyP level among the BLM-untreated and BLM-treated groups are shown in Fig. 6B. In the BLM 0.6 mg + Vehicle groups, increased pulmonary HyP level was noted compared with the Saline + Vehicle group at Days 22, 43 and 64. There were no differences in the pulmonary HyP level between the BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 10 mg groups or BLM 0.6 mg + Vehicle and BLM 0.6 mg + ER 20 mg groups.

DISCUSSION

To clarify the potential contribution of erlotinib in the pathogenicity of lung injury, we investigated the effects of erlotinib on lung injury models induced by intratracheal administration of BLM in rats. In Experiment 1, we examined the effects of short-term (7- and 21-day) administration of erlotinib (10 mg/kg/day; subtoxic dose) on the BLM-induced lung injury of slight and moderate severity. As a result, in rats receiving erlotinib alone, no lung lesions were noted. In rats receiving BLM alone, DAD and, subsequently, pulmonary fibrosis of slight or moderate severity was observed. Treatment of rats with erlotinib over 22 days did not exacerbate the BLM-induced lung injury in indices of lung lesions such as macroscopic findings, organ weights, histopathological scores (lung lesion density and lung fibrosis score), and pulmonary HyP level, not only in slight severity of injury but also in moderate injury. In Experiment 2, we examined the effects of long term (up to 63-day) administration of higher-dose erlotinib on the BLM-induced lung injury of moderate severity. As a result, treatment of rats in doses of 10 and 20 mg/kg/day (toxic dose; accompanied by decreased body weight and severe skin lesion) of erlotinib up to 63 days did not exacerbate the BLM-induced lung injury. In BLM-untreated rats receiving erlotinib, no test article-related changes were noted in the lung.

It is known that BLM damages the alveolar capillary endothelium, alveolar epithelium and epithelial basement membrane, and causes inflammation (Kawamoto and Fukuda, 1990; Lazenby et al., 1990; Usuki and Fukuda, 1995). Marked damage in the epithelial basement membrane may cause pulmonary fibrosis. In our experiments, BLM (0.1 or 0.6 mg/100 μl)-treated animals showed lung histopathology such as DAD and subsequent pulmonary fibrosis with slight or moderate severity which were similar to those previously reported (Adachi et al., 2003; Chandler et al., 1983; Thrall et al., 1979).

Two studies have investigated the effects of gefitinib on BLM-induced pulmonary fibrosis models in mice. Suzuki et al. (2003) reported that inhibition of EGFR phosphorylation augments BLM-induced pulmonary fibrosis by reducing regenerative epithelial proliferation. On the other hand, Ishii et al. (2006) reported that suppression of EGFR prevented BLM-induced pulmonary fibrosis by inhibiting fibroblast migration and proliferation even under conditions related to epithelial regeneration. Ishii et al. (2006) mentioned that the difference in strains used, ICR and C57BL/6 mice, in which exposure to gefitinib showed little difference between the two strains. Furthermore, there was a difference in severity of lesion in the BLM-induced lung injury between the two experiments. Scores of lung fibrosis (“Ashcroft score”) for BLM-treated control groups were lower in the ICR mouse treated with 5 mg/kg of BLM (average point: 1.2) and higher in the C57BL/6 mouse treated with 3 mg/kg of BLM (average point: 2.4). It is known that the severity of pulmonary lesions in BLM-treated rats would influence the evaluation of BLM-induced lung injury models (Adachi et al., 2003; Okazaki et al., 2001). However, the effects of EGFR-TKIs on lung injury with varying severity have not been examined under the same experimental conditions. In our experiments, lung fibrosis scores for BLM only-treated control groups were 1.1 or 2.4 (average point in Experiment 1). Treatment of rats with subtoxic or toxic doses of erlotinib did not exacerbate the BLM-induced lung injury, not only in slight severity of injury but also in moderate injury. To our knowledge, this is the first report to date about an investigation on the effects of erlotinib on lung injury models of varying severity.

Histopathologically, the administration of erlotinib had no effects on the normal or injured bronchiolar epithelium, and the amount of bronchiolization, an abnormal, excessive re-epithelialization (Nettesheim and Szakal, 1972; Fukuda et al., 1985; Betsuyaku et al., 2000, 2003). On the other hand, administration of erlotinib to BLM-treated rats increased the number of flattened epithelium in the bronchiolization area at Days 22 and 43. However, no effects of erlotinib on lung lesions induced by BLM were noted at Day 64. Although the erlotinib affected the histopathological changes of re-epithelialization after BLM-induced alveolar damage of a transient nature, it does not have any exacerbating effects on the lung injury induced by BLM in rats. Furthermore, administrations of erlotinib to 0.6 mg of BLM-treated rats tend to reduce the lung injuries (lung weight, lung lesion %, lung fibrosis...
Effects of erlotinib on lung injury induced by bleomycin in rats

score in experiment 1; not statistically significant). EGFR is considered to perform a crucial function in regenerating injured epithelium, since EGFR is expressed by many cell types, especially in epithelial cells, where cell-cycle progression has started (Bierman et al., 2008). It has been reported that EGFR expresses frequently in regenerating epithelium (Madtes et al., 1994), and blockade of EGFR induces G1 growth arrest and reduction in cellular proliferation and apoptosis in the epithelial cells (Gibson et al., 1999; Moyer et al., 1997; Petty et al., 2004). Nakamura et al. (2001) assessed the effect of gefitinib on corneal injury models in rats. They reported that suppression of EGFR delayed wounds from healing in epithelial cells but did not prevent the wound healing itself. From the aspect of biological significance, bronchiolization in the lung injured by BLM has been considered to be a transient, morphological change noted early in the recovery process, which prevents progression of fibrosis of alveoli in the alveolar epithelial cells in the injured lung (Fukuda et al., 1989; Kawamoto and Fukuda, 1990). Accordingly, the high frequency of occurrence of increased flattened bronchiolization in the animals necropsied on Days 22 and 43 was considered to be a reparative change from regenerating alveolar epithelial cells, which occurred transiently as bronchiolization in the recovery process (not anymore seen in Day 64), and the findings were not considered to be an exacerbating effect of erlotinib on lung injury. Taken together, our results rather suggest that the recovery process in the injured lung to normal alveolar epithelium may be promoted by erlotinib through suppression of EGFR.

The mechanism of how clinical ILD is induced could not be clearly explained in our study or previous studies in animal models. Ishii et al. (2006) postulate one hypothesis that blocking EGFR could alter the balance between repair and fibrosis after lung injury in a negative direction due to genetic factors in particular individuals, leading to the induction of fibrosis. Furthermore, our results suggested that EGFR-TKIs affect the process of epithelial repair especially for recession and not significantly for progression. Direct comparison of the (conflicting) results with gefitinib in mice and our results with erlotinib in rats is not possible.

In conclusion, although erlotinib transiently affected the histopathological changes of re-epithelialization after BLM-induced alveolar damage, it does not have any exacerbating effects on the lung injury induced by BLM in rats.

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REFERENCES


