Amphiregulin attenuates bleomycin-induced pneumopathy in mice

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Fukumoto J, Harada C, Kawaguchi T, Suetsugu S, Maeyama T, Inoshima I, Hamada N, Kuwano K, Nakanishi Y. Amphiregulin attenuates bleomycin-induced pneumopathy in mice. Am J Physiol Lung Cell Mol Physiol 298: L131–L138, 2010. First published November 13, 2009; doi:10.1152/ajplung.90576.2008.—Amphiregulin, an EGF receptor (EGFR) ligand, is essential for epithelial development in various organs. A recent report suggested that amphiregulin acts as a protective factor in a liver injury model. Little is known about the roles of amphiregulin in lung injury and pulmonary fibrosis. The purpose of the present study was to investigate the role of amphiregulin in an experimental model of bleomycin-induced pneumopathy in mice. C57BL/6 mice were administered a bleomycin hydrochloride solution intratracheally. Recombinant human amphiregulin was injected intraperitoneally at 6, 8, 10, and 12 days after the bleomycin instillation. The grades of inflammation and fibrosis were assessed histologically and biochemically, and the numbers of apoptotic cells were counted after Tdt-mediated dUTP nick end labeling (TUNEL) staining in the lung tissues. We also examined downstream survival signals of EGFR, namely phosphorylated Akt and phosphorylated Erk, in lung tissues by Western blotting analysis and immunohistochemistry. Expression of intrinsic amphiregulin was increased in murine lung tissues after bleomycin instillation. Administration of recombinant amphiregulin improved the survival rate and suppressed the degrees of inflammation and fibrosis and the number of TUNEL-positive cells in lung tissues. Amphiregulin treatment enhanced the activation of Akt and Erk in lung epithelial cells. Amphiregulin may play a protective role in bleomycin-induced pneumopathy in mice, probably through the activation of survival signals. Administration of amphiregulin may be a novel therapeutic strategy against lung injury and fibrosis.

IDIOPATHIC PULMONARY FIBROSIS (IPF) is defined as a specific form of chronic fibrosing interstitial pneumonia associated with the histological appearance of usual interstitial pneumonia on surgical lung biopsy. The median survival of patients with IPF is reported to be 3–4 yr from the onset of respiratory symptoms (1). Despite this poor prognosis, the etiology of IPF remains uncertain, and no effective therapies have been established. The effects of current anti-inflammatory and immunosuppressive therapies with corticosteroids and cytotoxic agents are limited. Therefore, establishment of additional therapeutic strategies is required.

Alveolar epithelial cells are known to be present at the primary site of lung damage in pulmonary fibrosis. DNA damage and apoptosis in lung epithelial cells also occurs in IPF (2, 15). Although the precise mechanism by which epithelial cell damage and apoptosis lead to pulmonary fibrosis is still unclear, recurrent and persistent epithelial damage and its insufficient repair are thought to break the normal epithelial-fibroblast interactions, which is thought to be followed by fibroblast proliferation and pulmonary fibrosis (9). Therefore, controlling epithelial cell damage and normal repair is important in treatment strategies against pulmonary fibrosis.

Amphiregulin is a member of the EGF family and was initially isolated from conditioned medium of the MCF-7 human breast carcinoma cell line (24). Amphiregulin is expressed in various organs and regulates cell proliferation (25). In lung development, amphiregulin is thought to be produced by mesenchymal cells and serves as a mitogen for the mesenchyme and a growth factor for the epithelium (21). Although little is known about the roles of amphiregulin in lung injury and fibrosis, it was found to be upregulated in lung epithelial cells in a ventilator-associated lung injury model (8). A recent report suggested that amphiregulin acted as a protective factor in a liver injury model. Administration of recombinant human amphiregulin abrogated Fas-mediated liver injury in mice and had direct antiapoptotic effects on primary hepatocytes (3). Since lung epithelial cell damage and apoptosis may play important roles in the pathogenesis of lung injury and fibrosis, we hypothesized that amphiregulin has a protective role against lung injury and fibrosis.

The animal model of bleomycin-induced pneumopathy is extensively used as a model of lung injury followed by pulmonary fibrosis. After intratracheal instillation of bleomycin in rodents, acute alveolitis develops at 2–3 days followed by interstitial inflammation, whereas fibroblast proliferation and collagen synthesis are initiated and progress from 4 to 14 days (5, 22). Using this model, we investigated the roles of amphiregulin in lung injury and fibrosis.

MATERIALS AND METHODS

Animal treatment. The present experiments were conducted in accordance with the guideline of the Animal Care and Use Committee of Kyushu University, approved by the Ethical Committee of Kyushu University Faculty of Medicine, and performed according to the guidelines of the American Physiological Society. Seven-week-old female C57BL/6 mice were purchased from KBT Oriental (Tosu, Japan) and used in all experiments. After measurement of their body weight, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Schering-Plough, Kenilworth, NJ) and administered 50 μl of bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) in sterile saline intratracheally. We used 2.5 U of bleomycin/kg body wt in all experiments except for a survival examination, in which we used 4.0 U of bleomycin/kg body wt. Subsequently, 5 μg/body of recombinant human amphiregulin (Sigma, St. Louis, MO) was injected intraperitoneally at 6, 8, 10, and 12 days after the bleomycin instillation. Since epithelial cell apoptosis of the lung is crucial at late phase (7–14 days after bleomycin treatment) in this model (10), we chose this administration schedule. The dose of amphiregulin was

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determined from previous study (3). Control mice were injected with sterile PBS instead of amphiregulin solution. After the treatments, the mice were returned to their cages and allowed food and water ad libitum. The mice were anesthetized at 14 days after the bleomycin instillation and killed. Samples of the right lung tissues were excised for light microscopy examination. Samples of the left lung tissues were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Histopathology of lung tissues. After a thoracostomy, the pulmonary circulation was flushed with saline, and the lungs were explored. The lung samples were fixed by inflation with 10% formalin at a static pressure of 25 cm H2O overnight, embedded in paraffin, and cut into 5-μm sections. The sections were adhered to glass slides and stained with hematoxylin and eosin. The pathological grades of inflammation and fibrosis in the whole area of midsagittal sections were evaluated under ×40 magnification. The pathological grades were determined according to the following criteria: 0, no lung abnormalities; 1, presence of inflammation and fibrosis involving <25% of the lung parenchyma; 2, lesions involving 25–50% of the lung; 3, lesions involving >50% of the lung.

DNA damage and apoptosis in lung tissues. TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using a DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI) as previously described (11). The number of TUNEL-positive cells was counted in the whole field of each section under a microscope using ×200 magnification.

Collagen content of lung tissues. Frozen lung tissues were homogenized in 0.5 M acetic acid containing 1% pepsin, and the total lung collagen content was measured using a Sircol Collagen Assay Kit (Biocolor, Belfast, Northern Ireland) according to the manufacturer’s instructions.

Western blotting analysis. Frozen lung tissues were homogenized in buffer A (25 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), and the protein concentrations of the supernatants were determined using a BCA Protein Assay Kit (Pierce Chemicals, Rockford, IL). The supernatants were dissolved in sample buffer (133 mM Tris-HCl, pH 6.8, 0.1% SDS, 5% glycerol, 0.67% 2-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and boiled. Aliquots (30 μg of protein) were electrophoresed in the lanes of a SDS-polyacrylamide gel, and the separated proteins were transferred to polyvinylidene fluoride hydrophobic membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at 4°C for 2 h and then incubated with an anti-amphiregulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Erk antibody (Cell Signaling Technology, Beverly, MA), anti-Akt antibody (Cell Signaling Technology), anti-phosphotyrosine Akt antibody (Cell Signaling Technology), anti-PCNA antibody (Santa Cruz Biotechnology), or anti-α-tubulin antibody (Chemicon International, Temecula, CA) in blocking buffer at 4°C overnight. After rinsing, the membranes were incubated with a biotinylated secondary antibody for 30 min at room temperature. The blots were developed using an ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Images of the membranes were scanned, and the relative band intensities were quantified using NIH Image version 1.61 (National Institutes of Health, Bethesda, MD).

Immunohistochemistry. Following deparaffinization of sections, immunohistochemistry was performed by a modified streptavidin-biotinylated peroxidase technique using a Histofine SAB-PO Kit (Nichirei, Tokyo, Japan). After blocking of nonspecific protein staining by incubation with rabbit or goat serum for 30 min at room temperature, the sections were incubated with an antiphosphorylated Erk antibody (Cell Signaling Technology), antiphosphorylated Akt antibody (Cell Signaling Technology), or anti-PCNA antibody (Santa Cruz Biotechnology) at 4°C overnight. For control experiments, the specific antibodies were replaced by nonimmune serum. Next, the sections were incubated with a biotinylated secondary antibody for 30 min, washed, and treated with 0.3% hydrogen peroxide in methanol for 30 min to inhibit the activity of endogenous peroxidase. Finally, the slides were incubated with a streptavidin-biotin-peroxidase complex, mounted on glass slides, and observed under a light microscope.

Statistical analysis. Survival curves (Kaplan-Meier plots) were compared using a log rank test. Pathological grades were compared using the Mann-Whitney U test. Comparisons of the numbers of TUNEL-positive cells and lung collagen contents were carried out using Student’s t-test. Values of P < 0.05 were considered to indicate statistical significance. All statistical analyses were performed with StatView J-5.0 (SAS Institute, Cary, NC).

RESULTS

Expression of intrinsic amphiregulin in lung tissues after bleomycin instillation. The expression of intrinsic amphiregulin in lung tissues was constantly upregulated from day 1 to day

Fig. 1. A: intrinsic amphiregulin expression in murine lung after bleomycin administration. The expression of intrinsic amphiregulin in lung tissues was assessed by Western blotting analysis. Arrows indicate the various forms of amphiregulin. Each lane corresponds to the data for 1 mouse. Bleomycin (2.5 U/kg body wt) was used in this experiment. B: quantitative results of Western blotting analysis for amphiregulin expression. Sum of all optical density (OD) values for each subunit band was normalized by the OD value for the α-tubulin band in the same tissue fragment. Data represent the means ± SE of 3 mice. *P < 0.05, **P < 0.01 compared with untreated group.
14 after bleomycin instillation (Fig. 1A). Various forms of amphiregulin were detected by Western blotting analysis as bands of 50, 43, 28, and 19 kDa, as previously reported (4). Densitometric analysis demonstrated that the percentages of amphiregulin expression at days 1, 3, 5, 7, 10, and 14 after bleomycin instillation were 356, 519, 317, 350, 282, and 267%, respectively, compared with that in untreated mice. Upregulation of amphiregulin was statistically significant at all time points except day 10 after bleomycin instillation (Fig. 1B).

**Effect of amphiregulin on survival.** Mice injected with 4.0 U of bleomycin/kg body wt showed a high mortality rate. The survival of mice treated with amphiregulin after bleomycin administration (14-day cumulative survival rate, 75%) was significantly improved compared with that of control mice (40%) (Fig. 2).

**Effect of amphiregulin on the histological findings in lung tissues.** Figure 3A shows an image of normal lung parenchyma from an untreated mouse. A large number of lymphocytes infiltrating the lung interstitium, thickening of the alveolar septa, collapse of alveolar spaces, and proliferation of fibroblasts were observed at 14 days after bleomycin instillation (Fig. 3B). On the other hand, only minimal changes were observed in mice treated with amphiregulin after bleomycin instillation (Fig. 3C). Furthermore, the pathological grades of inflammation and fibrosis were significantly decreased in amphiregulin-treated group compared with control group (Fig. 3D).

**Effect of amphiregulin on the number of TUNEL-positive cells.** The number of apoptotic cells detected by the TUNEL method is considered to reflect the degree of lung injury (14, 26). Although the types of cells were not clearly identified, some bronchiolar and alveolar epithelial cells or inflammatory cells in inflammatory lesions showed evidence of apoptosis as evaluated by TUNEL staining at 14 days after bleomycin instillation (arrows in Fig. 4B). No TUNEL-positive cells were detected in untreated mice (Fig. 4A). The TUNEL-positive signals were abrogated in the lung tissues of amphiregulin-
treated mice (Fig. 4C). The number of TUNEL-positive cells in amphiregulin-treated mice (means ± SE, 1.82 ± 0.70 cells/×200 field) was significantly decreased compared with that in control mice (3.43 ± 0.64 cells/×200 field).

**Effect of amphiregulin on the total lung collagen content.** The collagen content in lung tissues at 14 days after bleomycin instillation, which represents the extent of fibrosis, is shown in Fig. 3E. The lung collagen content was significantly decreased in amphiregulin-treated mice (50.0 ± 9.4 μg/mg) compared with control mice (83.5 ± 10.8 μg/mg).

Amphiregulin increased phosphorylation of Akt and Erk and decreased expression of PCNA. Since activation of Akt and Erk is the downstream signaling pathway of EGFR receptor (EGFR), and PCNA is correlated with the proliferative state of cells, we assessed the expression of PCNA and phosphorylation of Akt and Erk in murine lung tissues by Western blotting analysis (Fig. 5A). Akt and Erk were constitutively expressed in lung tissues from mice treated with bleomycin and control mice. The bands for phosphorylated Akt and phosphorylated Erk were faint after bleomycin instillation and increased by amphiregulin treatment. Expression of PCNA was upregulated after bleomycin instillation and abrogated by amphiregulin treatment. Densitometric analysis revealed that amphiregulin addition to bleomycin resulted in 6.5-fold significant increase in phosphorylated Akt expression, 37-fold significant increase in phosphorylated Erk expression, and 85% significant decrease in PCNA expression compared with control group (Fig. 5B).

**Immunohistochemical analysis of amphiregulin effect on Akt and Erk phosphorylation and expression of PCNA.** Absent or weak positive signals for phosphorylated Akt, phosphorylated Erk, and PCNA were detected in normal lung tissues from untreated mice (Fig. 6, A, D, and G, respectively). Phosphorylated Akt and phosphorylated Erk were weakly expressed in the cytoplasm of epithelial cells in lung tissues after bleomycin instillation (Fig. 6, B and E, respectively) and upregulated by amphiregulin treatment (Fig. 6, C and F, respectively). The expression of PCNA was upregulated in epithelial cells and interstitial cells after bleomycin instillation (Fig. 6H) and decreased in mice treated with amphiregulin (Fig. 6I).

**DISCUSSION**

We have demonstrated that recombinant human amphiregulin attenuated the development of bleomycin-induced pneumopathy in mice. Administration of amphiregulin reduced the number of apoptotic cells, pathological grade, and collagen content in lung tissues and improved the survival rate of bleomycin-treated mice. We further demonstrated that the expression of various forms of intrinsic amphiregulin were upregulated constantly from day 1 to day 14 after bleomycin administration in murine lung. It is reported that The 50- and 28-kDa bands represent cell surface forms and that the 43- and 19-kDa bands represent soluble forms of amphiregulin (4).
These results may indicate that upregulation of amphiregulin occurs to protect mice from lung injury and fibrosis and that it was insufficient in fibrotic phase.

Great attention has been paid to the roles of EGFR and its ligands in the pathogenesis of lung injury and fibrosis. Expression of EGFR is increased in bleomycin-induced pneumopathy in rats (16). The effect of EGFR inhibitors on bleomycin-induced pneumopathy in mice is controversial. Ishii et al. (12) showed that an EGFR inhibitor, gefitinib, inhibited lung fibroblast proliferation and that it attenuated bleomycin-induced lung fibrosis in mice. Rice and colleagues (20) also reported that EGFR tyrosine kinase inhibitor AG1478 blocked the proliferation of myofibroblasts and that it alleviated pulmonary fibrosis in rat induced by vanadium pentoxide. On the other hand, Suzuki et al. (23) showed that administration of gefitinib suppressed the proliferation of lung epithelial cells and that it augmented bleomycin-induced pulmonary fibrosis in mice. Those inconsistent findings regarding the effect of gefitinib may be explained by the difference in target cells (either fibroblasts or epithelial cells). Regarding gefitinib, it was administered for a longer period by Suzuki et al. (23) (day 19) than Ishii et al. (12) (day 13) after bleomycin administration by Suzuki et al. compared with days 1–13 Ishii et al.).

One possible explanation about the inconsistent findings regarding the effect of gefitinib is that epithelial cell proliferation is very important for tissue repair right after bleomycin treatment, i.e., the beginning of the inflammatory phase and the late fibrotic phase in bleomycin-induced pulmonary fibrosis. In other words, in the Suzuki et al. (23) study, the antiproliferative effect of gefitinib on lung epithelial cells overwhelmed its antiproliferative effect on lung fibroblasts, whereas the latter overwhelmed the former in the Ishii et al. (12) study. Transforming growth factor (TGF)-α, an EGFR ligand, is also upregulated in an animal model of bleomycin-induced pneumopathy and thought to promote pulmonary fibrosis. Conditional expression of TGF-α in the murine lung causes pulmonary fibrosis (12), and TGF-α knockout mice are resistant to pulmonary fibrosis. In the present study, amphiregulin reduced pulmonary fibrosis in mice. Therefore, the issue of whether EGFR signals promote or regulate pulmonary fibrosis may depend on the ligands involved.

Apoptosis of epithelial cells is deeply involved in the pathogenesis of lung injury and fibrosis. In the present study, amphiregulin treatment reduced the number of apoptotic cell.

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**Fig. 5.** A: Western blotting analysis of Akt, Erk, phosphorylated (phospho-) Akt, phosphorylated Erk, and PCNA in lung tissues. Each lane corresponds to the data for 1 mouse. B: quantitative results of Western blotting analysis for phosphorylated Akt, phosphorylated Erk and PCNA expression. The OD value for each band was normalized by the OD value for the α-tubulin band in the same tissue fragment. Data represent the means ± SE of 3 or 4 mice. Bleomycin (2.5 U/kg body wt) was used in this experiment. *p < 0.05, **p < 0.01.
Although the cell type for which apoptosis was decreased by amphiregulin could not be identified under light microscopy, based on our previous study, which demonstrated by using electron microscopy that the cells undergoing apoptosis in bleomycin-treated mice are lung epithelial cells (10), we have concluded that the cell type with decreased apoptosis by amphiregulin is also lung epithelial cell. Phosphorylation of Akt and Erk mediates the downstream survival signals of EGFR. Since administration of amphiregulin was found to increase the expression of phosphorylated Akt and phosphorylated Erk, the protective effects of amphiregulin appear to be at least partly mediated through suppression of epithelial apoptosis.

Several growth factors for epithelial cells have also been reported to exhibit protective effects on pulmonary fibrosis in animal models. Keratinocyte growth factor (KGF) promotes the proliferation of type II alveolar epithelial cells (27). Intratracheal preinjection of KGF reduces lung lesions in bleomycin-induced pneumopathy in mice, whereas postinjection of KGF does not (6, 30). Therefore, the protective effect of KGF seems to be limited to pretreatment. Hepatocyte growth factor (HGF) is recognized as a multipotent growth factor for various cells (17). Intratracheal HGF administration leads to the proliferation of lung epithelial cells and increases the number of PCNA-positive epithelial cells in normal murine lung. Furthermore, it attenuates bleomycin-induced pneumopathy in mice.

Fig. 6. Results of immunohistochemistry for phosphorylated Akt, phosphorylated Erk, and PCNA in lung tissues. Arrows show positive signals for each protein. Absent or weak positive signals for phosphorylated Akt, phosphorylated Erk, and PCNA were detected in normal lung tissues from untreated mice (A, D, and G, respectively). Phosphorylated Akt and phosphorylated Erk were weakly expressed in the cytoplasm of epithelial cells in lung tissues after bleomycin instillation (B and E, respectively) and upregulated by amphiregulin treatment (C and F, respectively). The expression of PCNA was upregulated in epithelial cells and interstitial cells after bleomycin instillation (H) and decreased in mice treated with amphiregulin (I). Bleomycin (2.5 U/kg body wt) was used in this experiment. Original magnifications: ×62.5.
lin may be a novel therapy against lung injury and fibrosis. Administration of amphiregulin proliferation seems to be the key for therapeutic strategies and in vitro conditions. Therefore, the effects of amphiregulin may differ between in vivo and in vitro conditions.

In conclusion, control of epithelial cell damage and fibroblast proliferation seems to be the key for therapeutic strategies against lung injury and fibrosis. Administration of amphiregulin may be a novel therapy against lung injury and fibrosis.

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**DISCLOSURES**

The authors have no conflicts of interest to disclose.

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