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Amphiregulin suppresses epithelial cell apoptosis in lipopolysaccharide-induced lung injury in mice

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ABSTRACT

Background and objective: As a member of the epidermal growth factor family, amphiregulin contributes to the regulation of cell proliferation. Amphiregulin was reported to be upregulated in damaged lung tissues in patients with chronic obstructive pulmonary disease and asthma and in lung epithelial cells in a ventilator-associated lung injury model. In this study, we investigated the effect of amphiregulin on lipopolysaccharide (LPS)-induced acute lung injury in mice.

Methods: Acute lung injury was induced by intranasal instillation of LPS in female C57BL/6 mice, and the mice were given intraperitoneal injections of recombinant amphiregulin or phosphate-buffered saline 6 and 0.5 h before and 3 h after LPS instillation. The effect of amphiregulin on apoptosis and apoptotic pathways in a murine lung alveolar type II epithelial cell line (LA-4 cells) were examined using flow cytometry and western blotting, respectively.

Results: Recombinant amphiregulin suppressed epithelial cell apoptosis in LPS-induced lung injury in mice. Western blotting revealed that amphiregulin suppressed epithelial cell apoptosis by inhibiting caspase-8 activity.

Conclusion: Amphiregulin signaling may be a therapeutic target for LPS-induced lung injury treatment through its prevention of epithelial cell apoptosis.

1. Introduction

Acute respiratory distress syndrome (ARDS) was first proposed by Ashbaugh et al., in 1967 and was defined by the American-European Consensus Conference in 1994 [1]. Acute lung injury is characterized by neutrophil inflammation and epithelial apoptosis [2]. Common causes of clinical disorders associated with the development of ARDS are pneumonia, gastric content aspiration, sepsis, and severe trauma [2]. Although many studies have investigated the mechanisms and risks of ARDS, the mortality rate remains high [3,4].

Lipopolysaccharide (LPS) is strongly associated with lung injury and ARDS. In type II alveolar epithelial cells, LPS induced apoptosis and cytokine production [5]. Intratracheal LPS instillation induced acute lung injury with neutrophil emigration in mice [6,7]. Neutrophil accumulation and cytokine production were reported to be caused by intranasal and aerosol LPS administration [8]. Intravenous LPS induced disseminated endothelial apoptosis [9] as well as non-endothelial tissue damage in the lung, and epithelial cell apoptosis was detected in diffuse alveolar damage [10]. We previously reported that intravenous LPS induced apoptosis of endothelial and alveolar epithelial cells, and that a broad-spectrum caspase inhibitor prevented apoptosis and lung injury in mice [11].

Amphiregulin was first described as a member of the epidermal growth factor family in 1989 [12]. It was reported to be upregulated in a ventilator-associated acute lung injury model [13]. Amphiregulin expression was also increased in damaged lung tissues in patients with chronic obstructive pulmonary disease and asthma [14–16] recently, amphiregulin has been reported to protect against lipopolysaccharide (LPS)–induced acute lung injury in mice [17]. However, the molecular mechanisms have not been well documented. Given that lung epithelial cell damage plays an important role in the pathogenesis of acute lung injury, this study was to determine the effect of amphiregulin on the apoptosis in the

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LPS-induced lung injury.

2. Materials and methods

2.1. Animal treatment

This study was conducted in accordance with the Animal Care and Use Committee of Kyushu University guidelines, approved by the Ethics Committee of Kyushu University Faculty of Medicine (No. A26-130-0), and performed according to the guidelines of the American Physiological Society. Seven-week-old female C57BL/6 mice were purchased from SLC, Inc., Shizuoka, Japan and used in all experiments. The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Schering-Plough, Kenilworth, NJ, USA) and intranasally administered 0.5 mg/kg LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO, USA). Control mice were administered sterile saline instead of the LPS solution. The mice were intraperitoneally injected with 7.5 μg/body weight of recombinant human amphiregulin (Sigma) or phosphate buffered saline (PBS) 6 and 0.5 h before and 3 h after LPS instillation, as previously reported [18]. The treatment schedule and doses of amphiregulin were determined according to previous studies [18,19]. The mice were sacrificed with an overdose of anesthesia 6 or 24 h after LPS instillation.

2.2. Histopathology of lung tissue

Preparation of lung tissue was performed as previously described [20]. Briefly, 10% formalin was infused into the airways at a pressure of 20 cm H_2O, as recommended for lung histology assessment in animal experiments, and fixed lung samples were embedded in paraffin. The lung sections were stained with hematoxylin and eosin. The pathological grades of lung injury in the whole area of the mid-sagittal sections were evaluated under 40 × magnification by two observers in a blinded manner. The pathological grade was scored on a scale of 0–3 as previously described [20,21]. Briefly, the grade criteria were as follows: 0, no lung abnormality; 1, presence of lung injury involving <25% of the lung; 2, lesions involving 25–50% of the lung; or 3, lesions involving >50% of the lung.

2.3. Immunohistochemistry of lung tissue

Paraffin sections (3 μm thick) were adhered to slides pretreated with poly-L-lysine. Following deparaffinization, the sections were treated with 0.3% methanol for 30 min to inhibit any endogenous peroxidase activity. Immunohistochemistry was performed using a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan). Nonspecific protein staining was blocked with rabbit or goat serum for 30 min at room temperature. Sections were immunostained with rabbit polyclonal anti-epidermal growth factor receptor (EGFR) antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal anti-phospho-EGFR antibody (1:50; Cell Signaling, Beverly, MA, USA) at 4 °C overnight. The sections were then incubated with biotinylated secondary antibody for 30 min. The slides were incubated with streptavidin–biotin–peroxidase complexes for 30 min and mounted.

2.4. Bronchoalveolar lavage fluid analysis

Bronchoalveolar lavage (BAL) and analysis were performed as previously described [20]. Briefly, After insertion of a tracheal tube, the trachea was lavaged twice with 1 mL sterile saline at room temperature. The recovered fluids were filtered through a single layer of gauze to remove the mucus. The cells present in the lavage fluid were counted using a hemocytometer. Differential counts of BAL cells were performed on 200 cells stained with Diff-Quick (Baxter Diagnostics, Dearfield, IL, USA). Total protein concentrations in BAL fluid (BALF) were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

2.5. DNA damage and apoptosis in lung tissue

Terminal deoxynucleotidyl transferase -mediated dUTP nick end label (TUNEL) staining was performed using a DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) as previously described [18]. The number of TUNEL-positive cells was counted (the whole field of 10 randomly selected fields) in each section under a microscope using 200 × magnification.

2.6. Effects of LPS on a mouse lung epithelial cell line

The mouse lung epithelial cell line, LA-4, derived from lung adenoma, was purchased from ATCC (Manassas, VA, USA). The cells were cultured in F12K medium with 10% fetal bovine serum and 1% penicillin–streptomycin. The cultures were incubated at 37 °C in a humidified, 95% air/5% CO_2 atmosphere. The cells were treated with 0, 10, or 100 nM amphiregulin for 3 h and then washed with PBS. The cells were then incubated with 0 or 50 μg/mL LPS for 24 h to induce apoptosis and then harvested to prepare for flow cytometry or western blot analysis. The supernatants were used for cytokine/chemokine measurements.

2.7. Apoptotic analysis by flow cytometry

LA-4 cell apoptosis was analyzed using a Annexin V-FLUOS staining kit (Roche Diagnostics, Penzberg, Germany). Cells were washed in PBS and re-suspended in an incubation buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 5 mM $CaCl_2$] with Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide. After incubating for 20 min on ice, fluorescence was measured using a Coulter EPICS XL flow cytometer (Coulter, Miami, FL). Three samples from each group were analyzed.

2.8. Western blot analysis

Cells were lysed in a sample buffer [50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.6% mercaptoethanol], and boiled for 2 min. The samples were subjected to western blot analysis as previously described [13]. The following primary antibodies were used: anti-caspase 11 (Santa Cruz Biotechnology), anti-cleaved caspse-8 (Cell Signaling), anti-cleaved caspase-3 (Cell Signaling), and anti-β-tubulin (Millipore). Signals were measured using ImageJ public-domain software (imagej.nih.gov/ij/) and standardized to β-tubulin.

2.9. Enzyme-linked immunosorbent assay

Interleukin (IL)-1β, IL-6, chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor (TNF)-α, and intercellular adhesion molecule (ICAM)-1 were measured using enzyme-linked immunosorbent assay (ELISA) kits obtained from Thermo Scientific (USA). Chemokine (C-X-C motif) ligand 1 (CXCL1) levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

IL-1β, IL-6, CXCL1, and CCL2 in the supernatant of LPS-stimulated LA-4 cells were measured using the Bio-Plex Pro assay kit on the basis of xMAP suspension array technology (Bio-Rad Laboratories Inc., Hercules, CA, USA).