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Original article:

EFFECT OF ANTIBIOTIC THERAPY ON THE INFLAMMATORY RESPONSES DURING STREPTOCOCCAL PNEUMONIA IN EMPHYSEMATOUS MICE

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ABSTRACT

Background and objective: Bacterial infection is one of the most important causes of acute exacerbation of respiratory failure in patients with chronic obstructive pulmonary disease (COPD). There were few studies evaluating the effects of early intervention by antibiotic on respiratory bacterial infection in COPD subjects. We investigated the effect of early intervention by respiratory quinolone antibiotic on the systemic inflammatory responses induced by streptococcal pneumonia using a mouse model of experimental emphysema.
Methods: Experimental pulmonary emphysema was developed by a single intratracheal instillation of porcine pancreatic elastase in ICR mice. Three weeks later, lethal doses of *Streptococcus pneumoniae* were intratracheally inoculated, followed by oral administration of 50 mg/kg body weight of Grepafloxacin (GPFX) every day from a day after tracheal inoculation.
Results: While all emphysematous mice without GPFX treatment died within 8 days, all emphysematous mice with GPFX treatment survived. Seventy two hrs after infection, serum levels of tumor necrosis factor alpha, chemokine (C-X-C motif) ligand 1, and CXCL2 (Macrophage inflammatory protein-2) in emphysematous mice with antibiotic therapy were significantly lower than those without therapy.
Conclusions: Thus, the early intervention using a respiratory quinolone antibiotic prevents emphysematous mice with pneumonia from severe systemic inflammation, and rescues these mice from death. These results suggest that early intervention using a respiratory quinolone may improve the outcome of the exacerbated COPD patients.

Keywords: COPD, respiratory infection, respiratory quinolone, cytokine

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common disease, physiologically characterized by various levels of airflow obstruction, and pathologically by findings of pulmonary emphysema (Rabe et al., 2007). The prevalence of COPD in the

Japanese male population (40 years or greater) was reported to be at least 8.6 % in the Nippon COPD Epidemiology Study (Fukuchi et al., 2004). COPD is a major, global medical problem. The natural history of COPD is associated with frequent respiratory tract infections, resulting in exacerbation, severe respiratory failure, and death (Rabe et al., 2007; Connors et al., 1996; Hurd, 2000; Schumaker and Epstein, 2004). However, little is known about why patients with COPD are susceptible to bacterial infections.

In our previous study, we have demonstrated abnormal inflammatory process from lung to whole body in emphysematous mice with streptococcal pneumonia (Inoue et al., 2003; Tokairin et al., 2008). All of mice with pulmonary emphysema died after streptococcal infection within several days. Six hours after inoculation, the levels of tumor necrosis factor alpha (TNF-α), interleukin-1β and -6 in bronchial alveolar lavage fluid (BALF) were significantly elevated compared to those of control mice. Twenty-four hours after inoculation, the levels of TNF-α, chemokine (C-X-C motif) ligand 1 (CXCL1), and CXCL2 in bronchial alveolar lavage fluid (BALF) of emphysematous mice are significantly lower than those of control mice. On the other hand, 72 hours after inoculation, the serum levels of TNF-α, CXCL1, and CXCL2 in emphysematous mice are significantly elevated with systemic spread of bacteria compared to those in control mice. These findings suggest that deteriorated inflammatory procedures in respiratory-infected emphysematous mice result in severe systemic cytokine storm by bacteremia. Thus, it is speculated that early intervention by antibiotic may improve the outcome of COPD subjects with respiratory infection by preventing them from the spread of bacteria into whole body.

In the case of respiratory bacterial infection in patients with COPD, we have to consider these deteriorated inflammatory responses, which make the management of acute exacerbation of COPD difficult. Even though some studies reported that antibiotic therapies were effective for the acute exacerbation of COPD (Schumaker and Epstein, 2004;Wilkinson et al., 2004), few studies are available which show the effect of early intervention by antibiotic on the systemic inflammatory responses induced by respiratory infection using a mouse model of experimental emphysema.

Streptococcus (S.) pneumoniae is known to be one of the most virulent gram-positive bacteria in community acquired pneumonia in healthy and diseased individuals (Almirall et al., 2007). *S. pneumoniae* is a frequently encountered pathogen in respiratory infections, and causes acute exacerbation in patients with COPD (Schumaker and Epstein, 2004; Lode et al., 2007; Soler et al., 1998; Miravitlles et al., 1999). Therefore, the prevention of pneumococcal infections in COPD patients is at the core of their management.

Grepafloxacin (GPFX) is a broad-spectrum fluoroquinolone with superior activity against gram-positive bacteria such as *S. pneumoniae* compared with older quinolones, and is classified into the respiratory quinolone (Norrby, 1997). The respiratory quinolones are expected to bring the good outcome for the treatments of respiratory infection including *S. pneumoniae* (Cook et al., 1995; Wakebe et al., 1994). Indeed, many reports have shown their effectiveness in clinic (DeAbate et al., 1999; Reinert et al., 2005; Suzuki et al., 2005). However, the effects of respiratory quinolones on survivals and systemic inflammatory responses in infected COPD animal models have not been demonstrated.

In these backgrounds, we evaluated the usefulness of early intervention by a respiratory quinolone against the streptococcal infection in emphysematous mice. We demonstrated early intervention against streptococcal pneumonia using GPFX greatly improved the survival and inflammatory responses of emphysema mice. This result implicates the clinical usefulness of respiratory quinolone antibiotics against respiratory infection by *S. pneumoniae* in patients with COPD.

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MATERIAL AND METHODS

Animals

Specific pathogen-free male ICR mice, 8 weeks of age, were purchased from Japan Clea Co. (Tokyo, Japan). All experiments using mice in this study were approved by the institutional animal care and use committee.

Preparation of Streptococcus pneumoniae

S. pneumoniae serotype 3 (from Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) is penicillin-sensitive. The minimal inhibitory concentrations (MIC) of penicillin G and GPFX against the organism were ≤0.06 μg/ml and ≤ 0.20 μg/ml, respectively. *S. pneumoniae* were incubated at 37 °C in tryptic soy broth (DIFCO, Detroit, MI) with 10 % fetal bovine serum (Takashima et al., 1996; Tateda et al., 1996).

An emphysematous model

To produce pulmonary emphysema, mice were anesthetized with an intraperitoneal injection of thiopental sodium (150 mg/kg body weight), and the trachea was intubated with a 22 gauge canula. Porcine pancreatic elastase (Calbiochem-Novabiochem Co., USA) in phosphate-buffered salt solution (PBS) was intratracheally administered via a canula in doses of 12 units/ 50 μl (Inoue et al., 2003; Karlinsky and Snider, 1978).

Streptococcal infection

Three weeks after elastase treatment, suspensions containing 10^7 cfu of *S. pneumoniae*/100 μl broth were intratracheally administered to mice under conditions of intraperitoneal anesthesia.

Antibiotic therapy

From 24 hrs after infection, emphysematous mice were treated with antibiotic. Fifty mg/kg body weight of Grepafloxacin (GPFX) (a fluoroquinolone antibiotic, from Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) were dissolved in water, and administered orally every day for 5 days. Same volume of water was administered to emphysematous mice without antibiotic therapy every day.

Preparation of serum

Seventy-two hours after bacterial inoculation, whole blood was obtained by direct puncture of the right ventricular cavity in mice, which had been deeply anesthetized with excess intraperitoneal thiopental sodium (450 mg/kg body weight). Individual sera were separated from the clotted blood by centrifugation, and stored at –80 °C until the assays were performed.

Biochemical analysis of serum

Serum levels of TNF-α, CXCL1, and CXCL2 were measured by an enzyme-linked immunosorbent assay (ELISA), (Quantikine; R&D Systems, Minneapolis, MN), since these cytokines are known to play roles as key molecules in inflammatory responses in bacterial infections (Takashima et al., 1997; Benton et al., 1998; Standiford et al., 1999).

Histological analysis

For morphological examinations, both lungs were inflated under constant positive pressure (25 cm water pressure) of 10 % buffered formaldehyde and were then perfuse-fixed. The fixed lungs were embedded in paraffin, stained with hematoxylin and eosin, and examined using a microscope (BX50F4, Olympus, Tokyo) (Saito et al., 2000).

Statistical analysis

All values are expressed as means ± standard deviation of the mean (S.D.). The differences between the groups were further compared using Mann-Whitney's U test with an adjustment of the p values ($p < 0.05$). A p value of $p < 0.05$ was considered statistically significant.

RESULTS

Survival after infection

Figure 1 shows the survival curves obtained from 2 groups of mice with 10^7 cfu/mouse of *S. pneumoniae* inoculation.

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Mice with emphysema became emaciated and died within several days after infection. In sharp contrast, all emphysematous mice treated with GPFX were survived throughout the observation period.

Histological changes after infection

Representative microscopic findings of the lungs in the emphysematous mice with or without antibiotic therapy 72 hrs after infection are shown in Figure 2. In the emphysematous mice without therapy, polymorphonuclear leukocyte accumulation, alveolar wall thickening with eosinophilic materials and capillary congestion with red blood cells were observed (Figure 2a). There are no inflammatory changes in alveolar wall or capillary in the emphysematous mice with antibiotic therapy (Figure 2b).

Systemic inflammatory changes

Serum TNF-α, CXCL1, and CXCL2 levels are summarized in Figure 3. Seventy-two hours after infection, serum cytokine levels were significantly higher in emphysematous mice without therapy than those with antibiotic therapy.

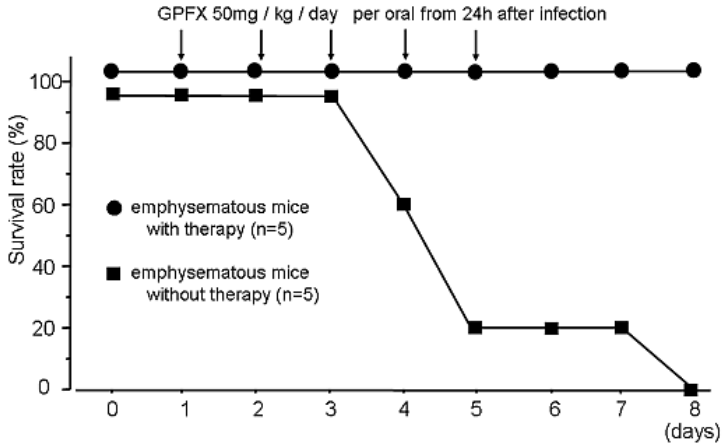

Figure 1: Survival rate in emphysematous mice with or without antibiotic therapy.

Emphysematous mice without antibiotic therapy died after challenged 10^7 cfu/mouse of *S. pneumoniae*. Emphysematous mice with antibiotic therapy did not die after infection.

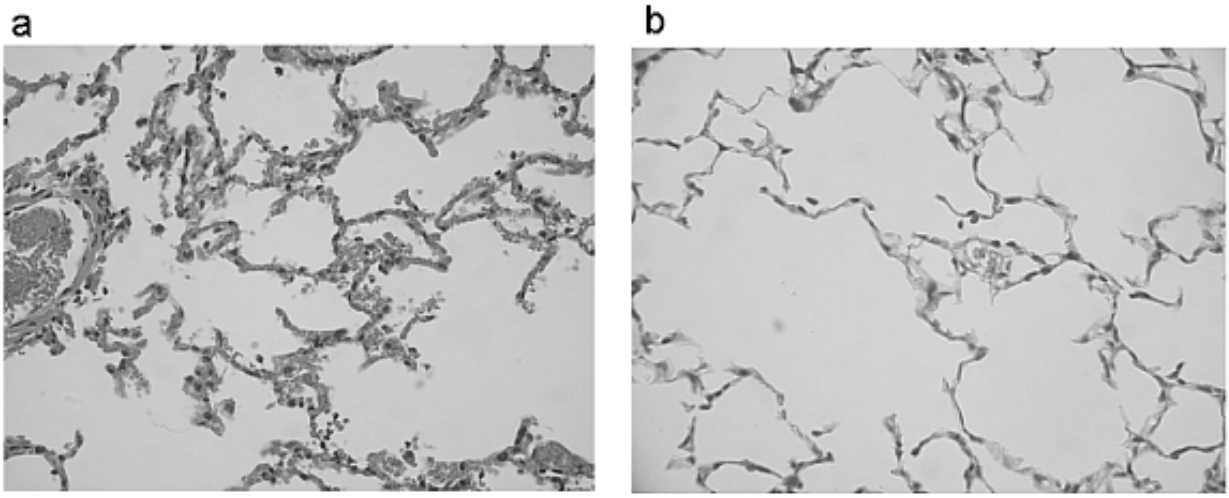

Figure 2: Histological findings of the lung tissue sections.
Representative images of lung tissue sections are shown (Hematoxylin and eosin stain ×100).
a: Emphysematous mice without antibiotic therapy. Polymorphonuclear leukocyte accumulation, alveolar wall thickening with eosinophilic materials and capillary congestion with red blood cells were evident.
b: Emphysematous mice with antibiotic therapy. Significant improvements of these inflammatory changes were observed.

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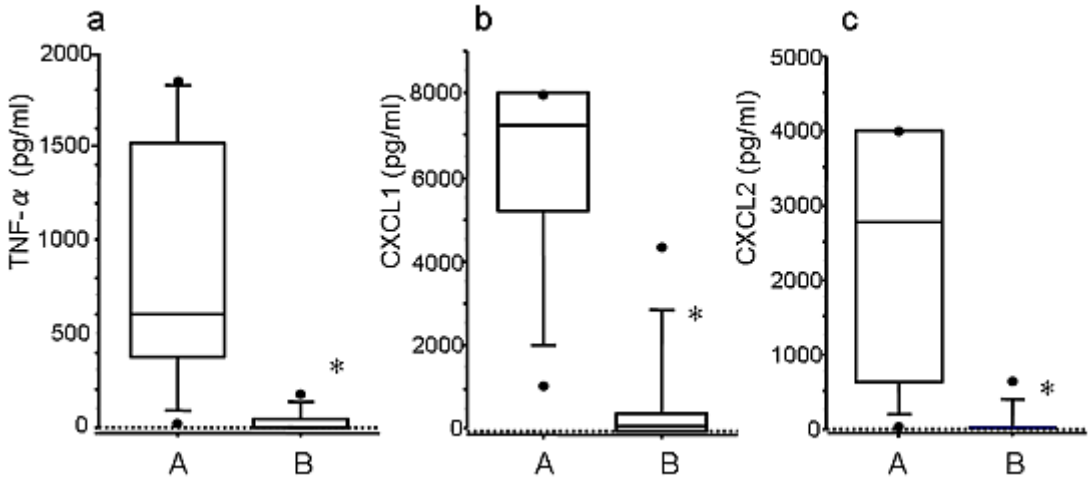

Figure 3: Serum levels of TNF-α (a), CXCL1 (b), and CXCL2 (c) from emphysematous mice with or without antibiotic therapy.
Seventy-two hrs after infection, each cytokine level in emphysematous mice with antibiotic therapy were significantly less than those in emphysematous mice without therapy.
A: Emphysematous mice without antibiotic therapy; B: Emphysematous mice with antibiotic therapy.
* $p < 0.05$ compared with emphysematous mice without therapy.

DISCUSSION

In the present study, we investigated the usefulness of early intervention using fluoroquinolone against streptococcal pneumonia in elastase-induced emphysematous mice. All emphysematous mice were survived by antibiotic therapy after inoculation of lethal dose bacteria, and have less pathological findings regarding to the lung inflammations compared to the mice without antibiotic treatment, suggesting that early treatment by GPFX prevented the mice from development of pneumonia after bacterial infection. Systemic responses such as serum levels of TNF-α, CXCL1, and CXCL2 in emphysematous mice with antibiotic therapy were significantly lower than those without therapy.

In our previous report, we showed that immediate inflammatory responses in the lungs 6 hours after bacterial inoculation were significantly enhanced (Tokairin et al., 2008), and then subsequent inflammatory responses in the lungs 24 hours after inoculation were significantly less in mice with emphysema. In contrast, systemic responses in emphysematous mice were significantly elevated compared to those in control mice 72 hours after inoculation. Mice with pulmonary emphysema died with bacteremia that is caused by the destruction of the lung-blood barrier (Inoue et al., 2003).

In patients with COPD, respiratory infections easily deteriorate their general condition compared with healthy people. It is reported that COPD patients have not only frequent respiratory infections but also subsequent bacteremia (Bouza et al., 2005; Lodise et al., 2007). Thus, the lung-blood barrier in COPD patients is suggested to be also impaired as this experimental model. Our present data demonstrated that antibiotic therapy prevents emphysematous mice with pneumonia from death by severe systemic infections.

Acute exacerbation of patients with COPD is associated with decreased lung function and increased morbidity and mortality. The majority of acute exacerbations of COPD are induced by respiratory pathogens including bacteria, and characterized by increased cough, sputum volume and purulence, dyspnea, and sometimes fever (Schumaker and Epstein, 2004; Soler et al., 1998; DeAbate et al., 1999). Major bacterial pathogens in acute exacerbation of COPD patients include *Haemophils influenzae*, *S. pneumoniae*, and *Moraxella catarrhalis*. In particular, *S. pneumoniae* is one

of the most frequent respiratory pathogens in patients with acute-exacerbated COPD and patients with community-acquired pneumonia (Schumaker and Epstein, 2004; Soler et al., 1998; Miravitlles et al., 1999). Importantly, *S. pneumoniae* rapidly induce very severe pneumonia even in healthy people. Thus, the management against *S. pneumoniae* infection is really important in patients with COPD. Although a vaccination for *S. pneumoniae* is available in clinic, many COPD patient is still dying due to the respiratory infection of this pathogen. Therefore, it is easily supposed that early intervention with antibiotic therapies against respiratory streptococcal infection prevent patient with COPD from lethal illness.

GPFX is a broad-spectrum fluoroquinolone with superior activity against gram-positive bacteria such as *S. pneumoniae* compared with older quinolones. We administered single oral dose of 50 mg/kg of GPFX to mice, which expected to reach as well dose of blood concentration as human which administered ordinary dosage (Cook et al., 1995; Wakebe et al., 1994). Although we performed this study when GPFX has been available, it is no more accessible in the clinic due to the severe adverse effect. However, other fluoroquinolones that are classified into the respiratory quinolone are expected to have similar effects on streptococcal infection in COPD patients.

In conclusion, we demonstrated an animal model of bacterial infection in mouse with pulmonary emphysema. Antibiotic treatment prevented emphysematous mice from severe systemic inflammation and death. We speculate the importance of early antibiotic therapy against streptococcal respiratory infection in COPD patients.

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