Pulmonary Pharmacology & Therapeutics 23 (2010) 292–299

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Experimental pulmonary infection and colonization of *Haemophilus influenzae* in emphysematous hamsters☆

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ARTICLE INFO

Article history:
Received 9 November 2009
Received in revised form
20 February 2010
Accepted 28 February 2010

Keywords:
Haemophilus influenzae
Pulmonary disease, chronic obstructive
Pulmonary emphysema
Disease models, animal
Moxifloxacin

ABSTRACT

Background: Bacterial infection has been considered the main cause of acute exacerbations of chronic obstructive pulmonary disease (AECOPD). However, experimental model of COPD exacerbation induced by *Haemophilus influenzae* infection was not available up to now. Furthermore, only a few studies on evaluation of antibiotics using an *H. influenzae* infection model in mice have been reported. The aim of this work was to evaluate the activity of moxifloxacin on experimental pulmonary infection and colonization of *H. influenzae* in emphysematous hamsters.

Methods: Pulmonary emphysema was developed by intratracheal instillation of porcine pancreatic elastase in golden hamsters, which were infected by agar-beads enclosing *H. influenzae* to establish animal models of AECOPD. Alterations of lung histopathology, inflammatory factor levels in plasma and bronchoalveolar lavage fluids (BALFs), viable cell counting of lung tissue were determined on different days after challenge and moxifloxacin administration.

Results: Lung bacterial counts of BALFs and homogenates were significantly higher in emphysematous hamsters than those in normal non-emphysematous animals from 1 to 3 weeks after intratracheal inoculation of bacterial agar-beads suspensions. Moreover, *H. influenzae* colonized and survived for a longer period of time in emphysematous lungs than in normal non-emphysematous lungs after challenge. Efficacy of 3-day intragastric administration of moxifloxacin was proved by reduction in pulmonary *H. influenzae* burden and alleviation of inflammatory responses on days 4, 8 and 21 post-inoculation. No planktonic bacteria were isolated from BALFs in the first week after moxifloxacin treatment, and bacterial load in lung tissue homogenates declined significantly. Nevertheless, after 3 weeks, bacterial load in BALFs and homogenates of emphysematous lungs recovered to a large quantity. Inflammation in lung tissue, including lung consolidation, hemorrhage, and neutrophils infiltration, was conspicuously improved after administration of moxifloxacin. Levels of inflammatory factors in plasma were significantly decreased on days 8 and 21 after treatment compared with that without drug therapy. Inflammatory factors in BALF were also reduced, among which IL-8 dropped down markedly in early stage.

Conclusion: Our results suggest that chronic bacterial infection and colonization is highly correlated with lung emphysematous lesions, which would be one of the important mechanisms for repeated attacks of acute exacerbations of chronic pulmonary diseases and uncertain efficacies of antibiotics.

1. Background

Acute exacerbation is a primary contributor to the health care costs, quality of life, morbidity, and mortality of patients with chronic obstructive pulmonary disease (COPD) [1–3]. Bacteria have been considered the main infectious cause of exacerbations for a long time. Nevertheless, no exact mechanisms have been proposed to explain how bacterial infection can trigger acute exacerbations of COPD (AECOPD) [4], and precise role of bacteria remains unclear because patients with COPD are often colonized with bacteria even when they are clinically stable [5–7].

Haemophilus influenzae (*H. influenzae*) is the bacterial species most commonly isolated from respiratory tract samples during AECOPD [8,9]. The ability of *H. influenzae* to cause intense airway inflammation and the association of exacerbations with development of humoral immune responses to *H. influenzae* supports the

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doi:10.1016/j.pupt.2010.02.006

role of infection by this organism in causing COPD exacerbations [10]. Although Patients with AECOPD recover more rapidly with antibiotic therapy, exacerbations could relapse and even be precipitated by the increase of number, change in location in the airway of colonized bacteria, or acquisition of a new, more virulent, or more proinflammatory bacterial species or strain [8,11,12].

Therefore, an experimental model of COPD exacerbation would be urgently needed to allow in-depth studies concerning the correlation between bacterial burden and pathophysiology of AECOPD, which regretfully is not available up to now. Furthermore, only a few studies on evaluation of antibiotics using an *H. influenzae* infection model in mice have been reported [13,14].

In this study, we attempted to develop a pilot experimental model of lung bacterial infection and colonization in emphysematous hamsters in order to probe the changes of bacterial burden and inflammatory response of acute exacerbation and stable phase of COPD/emphysema. Meanwhile, the efficacy of moxifloxacin was also evaluated against pulmonary *H. influenzae* infection and colonization in emphysematous hamsters.

2. Materials and methods

2.1. Bacterial isolates, growth, and storage

Haemophilus influenzae 1685, a clinical isolate from the sputum of a patient with AECOPD, was used in this study. The strain was identified according to standard procedures, and propagated on chocolate agar at 37 °C under 5% CO_2 and 95% humidity. Colonies from the agar plates were transferred to 3.7% brain heart infusion medium (BHI broth; Difco Laboratories, Detroit, MI, USA) containing nicotinamide adenine dinucleotide (NAD, 10 mg/l) and hemin (10 mg/l) (both from Sigma Chemical Co., St. Louis, MO, USA). These bacteria were suspended in BHI broth containing 25% glycerol, and the suspension was stored at −80 °C.

2.2. Antimicrobials

Preparation of sterile stock solutions of moxifloxacin (Bayer, Leverkusen, Germany) was performed in accordance with the manufacturer's instructions.

2.3. Susceptibility tests

Minimal inhibitory concentration (MIC) was determined following the broth microdilution method described by Clinical and Laboratory Standard Institute (CLSI, M100-S17) using Haemophilus Test Medium (HTM) as test medium for *H. influenzae* 1685 [15]. Briefly, overnight cultures of bacteria were diluted to a final concentration of 5×10^5 cells/ml. Samples were then added to equivalent volumes of the various concentrations of antibiotics distributed on microplates and prepared from serial two-fold dilutions, ranging from 0.001 to 32 μg/ml. After 20–24 h of incubation at 35 °C, the MIC was recorded as the lowest concentration of drug in which there was no visible growth.

2.4. Animal

Healthy male golden hamsters with body weights of approximately 100 g were purchased from Center of Experimental Animal, PLA general hospital. The hamsters were maintained in barrier-protected animal facilities under specific pathogen free conditions using ventilated microisolator cages. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of PLA general hospital, which followed internationally recognized guidelines.

2.5. Golden hamster model of emphysema

Emphysema was produced in hamsters by the intratracheal injection of 18 units of purified porcine pancreatic elastase (Sigma Chemical Co., St. Louis, MO, USA) [16,17]. Control animals received no injection. On the thirtieth days after injection of elastase, the lungs were fixed by inflation with 3 percent buffered glutaraldehyde through a tracheal cannula at a pressure of 25 cm of fixative for 24 h [18]. Two sagittal sections of each lung, one medial and one lateral, were embedded in paraffin. Sections were cut at 5 μm and were stained with hematoxylin and eosin.

All sections were examined for parenchymal abnormalities, particular for emphysema, which was considered to be present when there was a significant increase in alveolar diameter, usually accompanied by alveolar wall thinning, and diminished prominence of alveolar septa. These subjective judgments were substantiated by morphometric results, including the measurement of mean linear intercept (MLI), mean alveolar area (MAA), maximum alveolar diameter (MAD), and alveolar perimeter (AP), assessed in 5 randomly selected fields on each section [19,20].

2.6. Preparation of bacteria-contained agar-beads for challenge

Inocula of *H. influenzae* in agar-beads were prepared as previously described [21]. *H. influenzae* was cultured in BHI broth at 37 °C for 24 h with shaking (170 rpm). Bacterial cells were harvested by centrifugation (23,000*g*, 30 min, at 4 °C) and resuspended in BHI broth. Equal volume of bacterial suspension and 4% molten sterile agarose cooled to approximately 50 °C were mixed. The mixture was forced with air through a channel into BHI broth. Serial dilutions of the inocula were plated and the colonies counted 18 h later to determine the actual concentration of bacteria. The suspension of agar-beads containing *H. influenzae* was finally adjusted to a concentration of approximately 3×10^8 colony-forming units (CFU)/ml aliquot. The suspension of sterile agar-beads was made from sterile BHI broth and agarose in the same way.

2.7. Lung infection by H. influenzae in golden hamster and moxifloxacin administration

134 Golden hamsters were divided into five groups: one (12 normal lungs with no emphysema, instilled with sterile agar-beads suspension, sacrificed on days 1, 4, 8 after challenge), two (12 emphysematous lungs, instilled with sterile agar-beads suspension, sacrificed on days 1, 4, 8 after challenge), three (40 normal non-emphysematous lungs, instilled with agar-beads suspension containing bacteria, sacrificed on days 1, 4, 8, 21 after challenge), four (40 emphysematous lungs, instilled with agar-beads suspension containing bacteria, treatment with saline, sacrificed on days 1, 4, 8, 21 after challenge) and five (30 emphysematous lungs, instilled with agar-beads suspension containing bacteria, treatment with moxifloxacin, sacrificed on days 4, 8, 21 after challenge).

At the time of challenge, hamsters were anesthetized by intraperitoneal injection of pentobarbital at a dose of 50 mg/kg of body weight and tracheotomized. One-tenth 1 ml of the agar-beads suspension was instilled intratracheally into the lower right lung of each animal. The incision was then sutured with silk thread, which healed without any complications. On the next day after challenge, the hamsters in saline-treated group received intragastric sterile saline once daily for three days, and the hamsters in moxifloxacin-treated group received intragastric administration of moxifloxacin (60 mg/kg/day). Moxifloxacin was administered using a stomach sonde attached to a 1-ml syringe once daily for three days after inoculation.