Effect of clarithromycin on chronic respiratory infection caused by Pseudomonas aeruginosa with biofilm formation in an experimental murine model

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Fourteen-membered macrolides (e.g. clarithromycin and erythromycin), but not 16-membered macrolides (e.g. josamycin), are effective in diffuse panbronchiolitis. However, there are no studies that have compared the effects of 14- and 16-membered macrolide antibiotics on biofilm formation. Treatment with high-dose clarithromycin (100 mg/kg) resulted in a significant decrease in the number of viable bacteria in an experimental murine model. Josamycin at a dose of up to 100 mg/kg had no effect on the number of viable bacteria in the lung. Our results may explain, at least in part, the clinical efficacy of 14-membered macrolide antibiotics in patients with chronic pneumonia caused by Pseudomonas aeruginosa.

Keywords: macrolide antibiotics, chronic respiratory infection, Pseudomonas aeruginosa, biofilm formation, murine model

Introduction

Diffuse panbronchiolitis is a common and representative cause of chronic respiratory tract infections in Japan, treated effectively by a long course of macrolide antibiotics. The latter group of antibiotics is also efficacious against other airway inflammatory disorders such as cystic fibrosis. In this regard, 14-membered macrolides (e.g. clarithromycin and erythromycin), but not 16-membered macrolides (e.g. josamycin) are effective against diffuse panbronchiolitis (DPB). This difference has been reported by other investigations; josamycin failed to influence the proliferation of lymphocytes in a mouse model of chronic infection, and did not inhibit cytokine production from THP-1 cells. However, to our knowledge, there are no in vivo studies that have compared the effects of 14- and 16-membered macrolide antibiotics on biofilm formation.

Recently, we established a new murine model of chronic respiratory Pseudomonas aeruginosa infection produced by placement of a plastic tube precoated with a biofilm-forming organism in the bronchus. In the present study, we compared the effect of a 14- and a 16-membered macrolide on chronic respiratory infection with biofilm formation.

Materials and methods

Laboratory animals

Male, ddY, 7-week-old, 30–35 g body weight, specific pathogen-free mice were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All mice were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at our institution.

Bacterial strain

Animals were infected with mucoid P. aeruginosa NUS10, a clinical isolate obtained from sputum of patients at Nagasaki
University Hospital. The bacteria were stored at –70°C in Brain–Heart Infusion Broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10% (v/v) glycerol and 5% (w/v) skimmed milk (Yukijirushi Co., Tokyo, Japan) until use.

**Intubation tube**
Disposable sterile plastic cut-down intravenous catheters (3 Fr, 1.0 mm diameter; Atom Co., Tokyo, Japan) were used for intubation/infection. The tubes were 3.0 mm long with a few slits made at the proximal end to prevent clogging by oral/airway secretions.

**Preparation of bacterial precoated tube**
A detailed description of the method used was reported previously by Yanagihara et al. In the first step, *P. aeruginosa* was cultured on Trypticase Soy agar (BBL Microbiology Systems) for 24 h. The bacteria were suspended in saline (0.9% NaCl), harvested by centrifugation (3000 g, 4°C for 10 min), resuspended in sterile saline and adjusted to 1 × 10⁶ cfu/mL as estimated by turbidimetry. The intubation tube was then immersed in the bacterial saline suspension for 3 days at 37°C. The number of bacteria on tubes after 3 days of incubation before intubation was 6.24 ± 0.43 log₁₀ cfu/mL (mean ± S.D., n = 10). After immersing the tubes, they were subjected to scanning electron microscopy. These specimens were then fixed for 2 h at 4°C with 1% glutaraldehyde in 0.1 M phosphate-buffered saline, followed by re-fixation for 2 h at 4°C in 1% osmium acid in the same buffer, dehydration in a series of aqueous ethanol solutions (50–100%) and freeze drying. Samples were then coated with platinum/palladium using an ion sputter and observed with a JSC-35C scanning electron microscope (Nihon Dennshi Kogyo, Tokyo, Japan).

**Experimental model of infection**
The methods used were those described previously. The intubation procedure was carried out under anaesthesia. Briefly, the blunted end of the inner needle of an intravenous catheter (Angiocath; Becton Dickinson Vascular Access, Sandy, UT, USA) was inserted through the oral cavity of anaesthetized mice, with the outer sheath and the attached tube at the tip. The tube was advanced through the vocal cords into the trachea. The inner needle was pulled out followed by a gentle push of the outer sheath to place the pre-coated tube into the main bronchus. After intubation, the infected mice were provided with food and water.

**Bacteriological examination**
Both lungs were homogenized and cultured separately. Bacterial enumeration was carried out using serial dilutions on Trypticase Soy agar in NAC agar plates (BBL Microbiology Systems), incubating the plates at 37°C in air overnight and then counting colonies on plates to estimate cfu in lungs of mice.

**Drug administration and MIC determination**
Clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan) and josamycin (Daiichi Pharmaceutical Co., Tokyo, Japan) were dissolved in sterile water immediately before use. The MIC of each agent was determined by the agar dilution technique using Mueller–Hinton agar (Becton Dickinson Microbiology Systems), with an inoculum size of 10⁴ cfu per spot. The MIC was defined as the lowest concentration of agent that inhibited visible growth of *P. aeruginosa* after 16 h of incubation at 37°C. The MICs of clarithromycin and josamycin were 250 and 500 µg/mL, respectively. Treatment commenced 7 days after intubation of the precoated tube with mucoid type *P. aeruginosa*. Thirty-five mice were allocated into seven treatment groups: clarithromycin (10 mg/kg/day), clarithromycin (50 mg/kg/day), clarithromycin (100 mg/kg/day), josamycin (10 mg/kg/day), josamycin (50 mg/kg/day), josamycin (100 mg/kg/day) and saline for the control group. Each drug was administered once a day for 10 days and the animal was watched carefully to ensure that it received the full dose.

**Statistical analysis**
Data were expressed as means ± S.E.M. Differences between groups were examined for statistical significance using the unpaired Student’s t-test. P < 0.05 denoted a statistically significant difference.

**Results**

**Scanning electron microscopy of intubated tube**
A scanning electron micrograph of the surface of the catheter intubated for 7 days in mouse bronchus demonstrated *in vivo* formation of a biofilm containing blood cells, complex fibrous structures and bacteria (Figure 1).

**Therapeutic effect of antibiotics against chronic respiratory *P. aeruginosa* infection**
A significant number of viable bacteria were found in the lungs of control animals (5.50 ± 0.46 log₁₀ cfu/mL, n = 5). Treatment with josamycin had no effect on the number of viable bacteria in the lung (Figure 2). In contrast, high-dose clarithromycin (100 mg/kg/day) resulted in a significant decrease in the number of viable bacteria compared with the control (3.20 ± 0.46 log₁₀ cfu/mL, n = 5, P < 0.01).
Effects of clarithromycin on biofilm

Discussion

Biofilm bacteria are a major concern for clinicians in the treatment of infectious diseases because of their resistance to a wide range of antibiotics. Biofilm has in fact been found on the surface of biomaterials and tissues in chronic bacterial infections characterized by resistance to chemotherapy and resistance to clearance by the humoral or cellular host defence mechanism. Recently, bacterial biofilms have been detected on a number of living and inert surfaces within the human body. Establishment of a biofilm is the prelude to the development of various chronic, refractory infections, such as biomaterial-associated infection and pulmonary infection in intubated patients and patients with cystic fibrosis or DPB. Macrolide antibiotics offer a new strategy for infection with biofilm formation.

In the present study, we demonstrated that high-dose clarithromycin was effective in treating biofilm-associated chronic respiratory P. aeruginosa infection without any antimicrobial agents against P. aeruginosa. It is as yet uncertain whether the activity of clarithromycin to remove glycocalyx is independent of the general modes of antibacterial activity of clarithromycin. With regard to bacterial biofilm, Yasuda et al. showed by using experimentally induced subcutaneous infection in rats that the quantities of alginate and hexose in which bacterial biofilm had been formed clearly decreased in a dose-dependent manner after treatment with clarithromycin. In our model, P. aeruginosa is expected to be eradicated by the mucociliary transportation system and phagocytic cells after the disappearance of biofilm formation.

We have already reported that 14-membered macrolides such as clarithromycin and erythromycin eradicated P. aeruginosa from patients with DPB without any antimicrobial agents against P. aeruginosa after 1–12 months of treatment. The present results add support to these clinical data. Moreover, the need for ‘long-term’ treatment for patients may be related to the ‘high dosage’ in our mouse model. The mechanisms of the differences in the effectiveness of 14- and 16-membered macrolides on biofilm formation are not clear at present. It would be worth looking at other 16-membered macrolides to see whether failure to effect biofilm formation is a ‘group’ effect of 16-membered macrolides. It is possible that such differences are due to differences in the fine structure of these compounds, such as substitutions on the lactone ring and/or sugar composition of these antibiotics.

In conclusion, we reported here that clarithromycin but not josamycin was effective against chronic respiratory infection with biofilm formation. The present data may explain, at least in part, the clinical efficacy of 14-membered macrolide antibiotics in chronic pneumonia caused by P. aeruginosa.

References


