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Anti-inflammatory effects of a novel non-antibiotic macrolide, EM900, on mucus secretion of airway epithelium

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ABSTRACT

Objective: Low-dose, long-term use of 14-membered macrolides is effective for treatment of patients with chronic airway inflammation such as diffuse panbronchiolitis or chronic rhinosinusitis. However, long-term use of macrolides can promote the growth of drug-resistant bacteria, and the development of anti-inflammatory macrolides that lack antibiotic effects is desirable. Previously, we developed EM900, a novel 12-membered erythromycin A derivative, which has potent anti-inflammatory and immunomodulatory activities and lacks any antibacterial activity. We examined the anti-inflammatory effects of EM900 on mucus secretion from airway epithelial cells.
Methods: To examine the *in vivo* effects of EM900 on airway inflammation, we induced hypertrophic and metaplastic changes of goblet cells in rat nasal epithelium *via* intranasal instillation of lipopolysaccharides. *In vitro* effects of EM900 on airway epithelial cells were examined using cultured human airway epithelial (NCI-H292) cells. Mucus secretion was evaluated *via* enzyme-linked immunosorbent assays with an anti-MUC5AC monoclonal antibody.
Results: Oral administration of EM900 or clarithromycin (CAM) significantly inhibited LPS-induced mucus production from rat nasal epithelium. EM900, CAM, or erythromycin significantly inhibited MUC5AC secretion induced by tumor necrosis factor-α from NCI-H292 cells. MUC5AC mRNA expression was also significantly lower in EM900-treated cells.
Conclusion: These results indicated that a novel non-antibiotic macrolide, EM900 exerted direct inhibitory effects on mucus secretion from airway epithelial cells, and that it may have the potential to become a new anti-inflammatory drug for the treatment of chronic rhinosinusitis.

1. Introduction

Low-dose, long-term use of a 14- or 15-membered macrolide such as clarithromycin (CAM), erythromycin (EM), or azithromycin (AZM) is a very effective treatment for patients with a chronic airway disease such as diffuse panbronchiolitis [1], chronic bronchitis [2,3], cystic fibrosis [4], or chronic rhinosinusitis (CRS) [5,6]. These useful clinical effects may depend on the anti-inflammatory activity of macrolides rather than the antibacterial activity. The anti-inflammatory activity includes the immunomodulatory effect on inflammatory cells, the modulation of cytokine production from epithelial cells, and the inhibition of bacterial functions and biofilm formation.

Mucus hypersecretion is an important feature of airway inflammation, and macrolide therapy results in a significant reduction of the amount of secreted mucus; sputum and rhinorrhea. In previous studies, we demonstrated that macrolides inhibit hypersecretion of mucus in airways [7,8]. Intranasal instillation of lipopolysaccharides (LPS) causes inflammation of rat nasal epithelium, and oral administration of CAM, EM, or AZM significantly inhibits the LPS-induced hypertrophic and metaplastic changes of goblet cells in this rat model of airway inflammation. CAM, EM, or AZM also exerts direct inhibitory effects on mucus secretion from cultured airway epithelial (NCI-H292) cells or human nasal epithelial cells. These results indicate that low-dose, long-term macrolide therapy can be useful for the treatment of hypersecretory conditions associated with chronic airway inflammation.

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However, long-term use of macrolides can promote the growth of drug-resistant bacteria, and non-antibiotic macrolide derivatives with anti-inflammatory activities are desirable. Recently, Sunazuka and coworkers developed a novel 12-membered erythromycin A (EMA) derivative, (8R,9S)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (EM900) that has potent anti-inflammatory and immunomodulatory activities, but apparently lacks antibacterial activity [9,10]. In that study, the anti-inflammatory activities of EMA derivatives were evaluated by the THP-1 assay system, which examined the promotion of the differentiation of monocytes to macrophages.

Here, we examined the anti-inflammatory effects of EM900 on mucus secretion from airway epithelial cells; specifically, we evaluated (1) the *in vivo* effects of EM900 on LPS-induced mucus production in rat nasal epithelium and (2) the *in vitro* effects of EM900 on tumor necrosis factor-α (TNF-α)-induced MUC5AC secretion and MUC5AC mRNA expression in cultured human airway epithelial (NCI-H292) cells.

2. Materials and methods

2.1. Mucus hypersecretion in rat nasal epithelium

All experiments were approved by the Committee for the Care and Use of Laboratory Animals of Shiga University of Medical Science. LPS instillation was performed with rats as described previously [11]. Male Fischer 344 rats (6 weeks old) were anesthetized with ether, and 0.1 mL saline containing 0.1 mg LPS from *Escherichia coli* 0111:B4 (Sigma) or 0.1 mL saline control was intranasally instilled once daily for three consecutive days.

EM900 was a gift from T. Sunazuka (Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan). EM900 (1–10 mg/kg) or CAM (10 mg/kg, Taisho Pharmaceutical, Tokyo) in 0.5% carboxymethyl cellulose sodium salt was administered orally exactly 1 h before the intranasal instillation of LPS on each of the three instillation days. Each rat was sacrificed 24 h after its last intranasal instillation; the nasal cavity was then transversely sectioned at the level of the incisive papilla. Paraffin sections were stained with alcian blue-periodic acid-Schiff (AB-PAS) or hematoxylin and eosin (H-E).

2.2. Morphometry

The amount of AB-PAS-stained mucosubstance in the surface epithelium was determined with an image analyzer (Image-Pro Plus, Medical Cybernetics, Maryland) as described previously [11]. The area of nasal epithelium was outlined, and the image analyzer determined the area of AB-PAS-stained mucosubstances within this reference area. The percent area of stored mucosubstance per surface area was calculated over 2 mm (1 mm each side of nasal septum) of the basal lamina at the center of the septal cartilage.

2.3. Cell cultures

A human mucoepidermoid carcinoma cell line, NCI-H292, was grown on plastic dishes in RPMI 1640 medium containing 10% fetal bovine serum, penicillin streptomycin (50 U/mL–50 μg/mL), and HEPES (25 mM). EM900, CAM, or EM was dissolved in *N*,*N*-dimethylformamide at a concentration of 10^{-1} M, and each of these stock solutions was stored at 4 °C until use; each stock solution was diluted with the appropriate medium to result in a final concentration within the range between 10^{-4} and 10^{-6} M in each experiment. When the NCI-H292 cells become confluent, TNF-α, and EM900 or CAM was added to the culture medium for 20 h, then the culture medium was collected and total RNA was extracted from each culture.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Each subsample of culture medium and serial dilution of "standard" purified human nasal mucin [12] were incubated in one well of a 96-well plate at 40 °C until dry. Wells were then incubated with 2% bovine serum albumin for 1 h, and then with 50 μL of mouse monoclonal MUC5AC antibody (1:100, Thermo Scientific, Massachusetts) for 1 h. Wells were then incubated with 100 μL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:10,000) for 1 h. Color reaction was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution. Absorbance was read at 450 nm. Data were expressed as the percent above the control vehicle (RPMI-1640) as described previously [7,8].

2.5. Reverse transcription-polymerase chain reaction (RT-PC)

Total RNA was extracted from cultured cells, reverse transcribed, then the cDNA was amplified by PCR using the Superscript preamplification system kit (Gibco, Grand Island, NY). The MUC5AC cDNA was amplified using the sense primer 5′-CACCAA ATACGCCAACAAGAC-3′ and the antisense primer 5′-CAGGGC-CACGCAGCCAGAGAA-3′. The GAPDH cDNA was amplified using the sense primer 5′-CCACCCATGGCAAATTCCATGGCA-3′ and the antisense primer 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. These steps were described previously [7].

2.6. Statistics

All data are expressed as mean ± standard error of the mean (SEM). Differences between variables were assessed *via* the Mann–Whitney *U* test. Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. In vivo *effects of macrolides on LPS-induced mucus production*

Intranasal instillation of LPS induced hypertrophic and metaplastic changes of goblet cells in rat nasal septal epithelium within 24 h after the last instillation (Fig. 1). Only a few goblet cells were observed in untreated or saline-instilled control rats. Oral administration of EM900 (1–10 mg/kg) or CAM (10 mg/kg) inhibited LPS-induced hypertrophic changes of goblet cells, and quantitative measurement of the area of epithelial mucosubstance revealed a significant inhibition of intraepithelial mucus production in EM900- or CAM-treated rats (Fig. 2).

The number of infiltrating neutrophils in nasal septal mucosa was significantly higher in LPS-treated rats than in saline-treated rats. Oral administration of EM900 or CAM slightly inhibited LPS-induced neutrophil infiltration, although these changes are statistically insignificant (Fig. 3).

3.2. In vitro *effects of macrolides on TNF-α-induced MUC5AC secretion*

TNF-α (20 ng/ml) stimulated the secretion of MUC5AC mucin from cultured NCI-H292 cells. At concentrations from 10^{-4} to 10^{-6} M, EM900 significantly inhibited TNF-α-induced MUC5AC secretion in a dose-dependent manner. CAM and EM showed inhibitory effects on TNF-α-induced MUC5AC secretion similar to those of EM900 (Fig. 4).