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Kenichiro Fujita · Takeshi Shimizu · Yuich Majima
Yasuo Sakakura

Effects of macrolides on interleukin-8 secretion from human nasal epithelial cells

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Abstract Low-dose, long-term macrolide treatment has recently been reported to be very effective in patients with chronic airway diseases. We examined the in vivo and in vitro effects of 14-membered macrolide antibiotics erythromycin (EM) and clarithromycin (CAM) on interleukin (IL)-8 secretion from human nasal epithelial cells. Fifteen patients with chronic sinusitis received macrolide treatment (CAM 400 mg/day) for 1 to 3 months. The number of infiltrated neutrophils and IL-8 concentrations in the nasal discharges of these patients decreased significantly at 1 to 2 months after the treatment. In vitro effects of EM and CAM on IL-8 secretion were examined in nasal epithelial cells cultured at the air-liquid interface. After 14-day culture in the air-liquid interface, macrolide antibiotics were added in medium for 24 h. EM and CAM at concentrations of 10^{-4} M did not affect spontaneous secretions or IL-1β-induced secretions of IL-8 either apically or basolaterally. When cells were preincubated with 10^{-4} M CAM for 7 days, the IL-1β-induced secretion of IL-8 decreased significantly. However, no difference was observed between the effects of 10^{-4} M CAM and 10^{-4} M josamycin, a 16-membered macrolide. These results suggest that macrolide treatment inhibits neutrophil infiltration and IL-8 secretion in nasal epithelium in vivo and that these clinical effects depend on a mechanism other than the direct action of macrolide on nasal epithelial cells.

Key words Chronic sinusitis · Interleukin-8 · Human nasal epithelial cells · Macrolide antibiotics

K. Fujita (✉) · T. Shimizu · Y. Majima · Y. Sakakura
Department of Otorhinolaryngology,
Mie University School of Medicine,
2-174 Edobashi, Tsu, Mie 514-0001, Japan
Tel.: ++81-59-232-1111, ext. 5637, Fax: ++81-59-231-5218

Introduction

Respiratory epithelium plays an important role as a physical barrier between the environment and the underlying tissue and may also contribute to inflammation by producing inflammatory mediators. Human nasal or bronchial epithelial cells have been found capable of releasing various cytokines, including interleukin (IL)-6, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF) on stimulation with IL-1 or tumor necrosis factor [1].

Airway inflammatory diseases are characterized by mucosal neutrophil infiltration. This has been shown by the finding of many neutrophils in the patients with chronic sinusitis (CS) [10, 15] or in bronchoalveolar lavage (BAL) fluid of patients with chronic bronchitis [19]. IL-8 is a strong chemoattractant and activator of neutrophils, with high concentrations in BAL fluid demonstrable with the severity of inflammation [6]. In general, neutrophils and epithelial cells are capable of secreting IL-8, where respiratory epithelial cells are one of the major sources of IL-8 in the airway [1].

In 1984, low-dose, long-term erythromycin (EM) treatment was first reported to be very effective for patients with diffuse panbronchiolitis (DPB) in Japan [9]. The 5-year survival rate of DPB was shown to improve from 57.6% to 93.4% after the use of this treatment. Recently, the usefulness of this treatment has been employed in patients with such chronic airway diseases as chronic bronchitis, bronchiectasis, bronchial asthma and sinobronchial syndrome, as well as CS [8, 11, 12, 14]. Clarithromycin (CAM) and roxithromycin (ROX), 14-membered macrolide antibiotics, are also very effective for treating patients with these inflammatory diseases [14]. In so doing, there is a significant reduction in the amount of secreted mucus, sputum and rhinorrhea [8, 9]. However, the mechanism of these clinical changes is still unclear.

It has been suggested that the effects of macrolide antibiotics depend on its anti-inflammatory action rather than its anti-bacterial one, because low-dose treatment, in which only half of the usual dose is used, has resulted in

good effects when medication is given over long periods of time. This treatment has also been effective for patients with pathogens insensitive to macrolides, such as *Pseudomonas aeruginosa*. In the present study, the clinical effectiveness of this treatment was examined by the measurement of the number of infiltrated neutrophils and IL-8 concentrations in the nasal discharge taken from CS patients. In vitro effects of macrolides on IL-8 secretion were then examined using human nasal epithelial cells cultured at the air-liquid interface.

Materials and methods

Patients with CS were diagnosed by clinical symptoms, rhinoscopic findings and X-ray examination. Fifteen patients with CS (Case range, 7 to 78 years; mean age, 49.1 ± 6.3 years) were given clarithromycin (400 mg/day or 10 mg/kg per day per os) for 1 to 3 months. Nasal discharges were collected by aspiration using the Juhn Tym-tap (Xomed-Tresce, Jacksonville, FLa., USA) at 0, 1, 2 and 3 months after the beginning of the treatment. Nasal discharges from six patients with seasonal allergic rhinitis (AR) to Japanese cedar pollen (case range, 21 to 47 years; mean age, 31.7 ± 3.9 years) were collected in season and those from six healthy subjects (case range, 30 to 72 years; mean age, 51.5 ± 7.3 years) were also obtained. None of these subjects had experienced symptoms of an upper respiratory tract infection within the preceding 4 weeks.

Measurements of neutrophil numbers and IL-8 concentrations

The number of neutrophils was assessed using a cytocentrifuge (Cytospin 2; Shandon Southern Products, Cheshire, UK), according to a technique described previously [10]. Briefly, half of the nasal discharge was diluted 1 : 5 in 10 mM dithiothreitol, incubated for 1 h at 4 °C, mixed, and cytospinned at 1,200 rpm for 10 min. Slides were stained with Wright-Giemsa and the number of neutrophils in the nasal discharge was counted.

A separate half amount of each nasal discharge was immediately diluted 1 : 5 in saline and centrifuged at 10,000 rpm for 10 min. The supernatants were collected and stored at –80 °C. IL-8 concentrations in these samples was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Amersham, UK).

Cell cultures

Nasal polyps were obtained from patients undergoing endoscopic sinus surgery, after which epithelium was dissected from underlying tissue. The nasal epithelium was then minced to fragments that were approximately 1–2 mm^3 in size. These were incubated overnight at 4 °C with 1% protease in Hams F-12 solution (Sigma Chemical, St. Louis, Mo., USA). The enzyme activity was stopped by the addition of fetal calf serum (FCS) (Cansera International, Rexdale, Canada) to a final concentration of 10%. The epithelial cells were filtered through a 20 mm nitex mesh, centrifuged at 1,200 rpm and resuspended in culture medium. Cells were cultured according to a technique described previously [4, 20]. Growth medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Sigma), supplemented with 30 mM HEPES, 0.45 mM L-leucine, 0.50 mM L-lysine, 0.12 mM L-methionine, 6.5 mM L-glutamine, 0.30 mM $MgCl_2$, 0.40 mM $MgSO_4$, 1.05 mM $CaCl_2$, 8.6 mg/ml phenol red, 1.2 g/l $NaHCO_3$, 100 IU-100 μmg/ml penicillin-streptomycin. The medium also included 10 μg/ml insulin, 0.1 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 5 μg/ml transferrin, 25 ng/ml epidermal growth factor, 7.5 μg/ml epithelial cell growth supplement, 60 μg/ml bovine pituitary extract and 3 mg/ml bovine serum albumin. All of these reagents were purchased from Sigma except for penicillin-streptomycin, which was obtained from gibco (Grandisland NY., USA) and bovine pituitary extract perchased from Becton-Dickinson Labware (Bedford, Mass., USA).

Cells were grown on Trans well-Col tissue culture inserts (Corning Costar, Cambridge, Mass., USA), which were prepared permeable membranes (24.5 mm diameter, 0.4 μm pore size). Membranes were coated with 0.4 ml of collagen gel type I-A (Nitta Gelatin, Osaka, Japan) and incubated overnight. The nasal cells were plated onto the membrane in 1 ml of medium at a density of 1×10^5 cells/well, and 1.5 ml of medium with 10% FCS was also added onto the basolateral compartment. FCS was added for the first 24 h only. The medium was then changed every other day until cells had grown to confluence. When the cells become confluent at 7–10 days, an air-liquid interface was created by removing medium from the apical surface and cultures were supplemented with medium containing 5×10^{-8} M retinoic acid every day. All cultures were incubated in a humid environment in 95% air with 5% CO_2 at 37 °C.

Drug and cytokine administration

At the 14-day culture in the air-liquid interface, CAM (Dainabot, Tokyo, Japan), EM (Shionogi Pharmaceutical, Osaka, Japan), or josamycin (JM; Yamanouchi Pharmaceutical, Tokyo, Japan) was added to the culture medium of the lower compartment for 24 h or 8 days. CAM was dissolved in acetone, while EM and JM were dissolved in ethanol. All antibiotics were adjusted in the growth medium to reach final concentrations of 10^{-4} M or 10^{-6} M. The control medium contained the same concentrations of both ethanol and acetone. Human-recombinant IL-1β (20 ng/ml; Genzyme, Cambridge, Mass., USA) was added in the basolateral medium individually or with the macrolide antibiotics for 24 h. The baseline IL-8 secretion was established by harvesting media after a 24-h incubation (period I). Basolateral medium in the lower compartment was collected at 24, 48 and 72 h (period II) after the administration of macrolide antibiotics or IL-1β. The sample from the upper compartment was collected by gentle washing with 0.5 ml phosphate buffered saline (PBS) at the same time. These samples were stored at –80 °C, and IL-8 concentrations were determined by ELISA.

To determine the effects of macrolides on IL-8 secretion, its secretory index was established for each culture dish by comparing the data of period II to period I. To assess whether IL-8 secretion from the cultured nasal cells was the result of cell toxicity by the macrolide antibiotics or IL-1β, lactate dehydrogenase (LDH) determination was performed using an LDH assay kit (Sigma).

Statistical analysis

All data were expressed as the means ± standard deviation (SD) and compared with Student's *t*-test. A probability value of $P < 0.05$ was considered to be significant.

Results

Figure 1 illustrates the IL-8 concentrations and number of infiltrated neutrophils in the nasal discharges from patients with CS and AR. IL-8 concentrations of nasal discharge from CS patients (11.6 ± 5.4 ng/ml) were significantly higher than those of the AR patients (3.5 ± 1.0 ng/ml) and normal controls (3.6 ± 1.1 ng/ml). The number of infiltrated neutrophils in the nasal discharges from the CS patients (10828.8 ± 4981.0 cells/mm^3) was significantly higher than those of the AR patients (214.3 ± 141.3 cells/mm^3) and normal controls (251.0 ± 140.7 cells/mm^3). There was a significant correlation between the IL-8 titers and the number of infiltrated neutrophils ($r = 0.62$, $P < 0.05$).

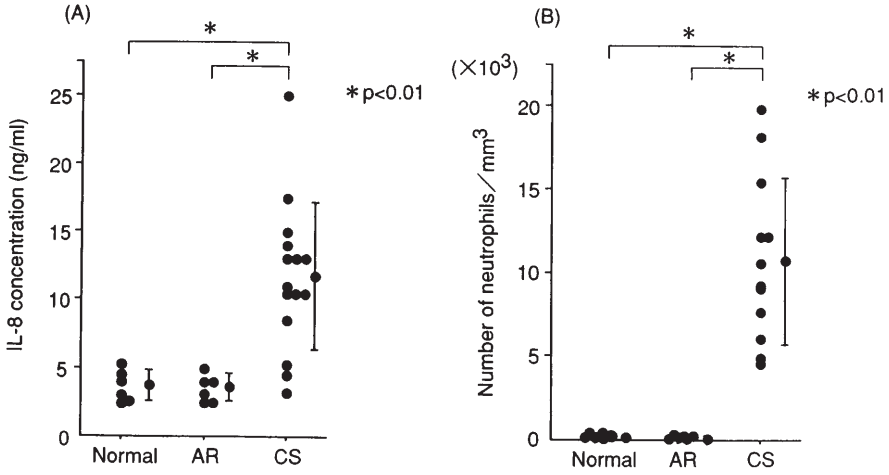

Fig. 1 A IL-8 concentrations and **B** the number of infiltrated neutrophils in the nasal discharges of CS patients (n = 15), AR patients (n = 6) and normal controls (n = 6). IL-8 concentrations and number of infiltrated neutrophils of nasal discharge from CS patients were significantly higher than those of AR patients and normal subjects (*P < 0.01)

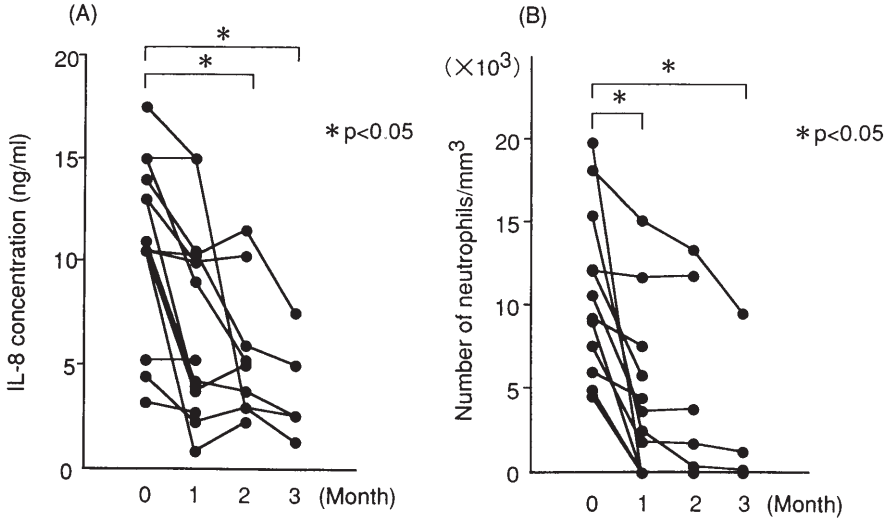

Fig. 2 Effects of low-dose, long-term CAM treatment on **A** IL-8 concentrations and **B** the number of infiltrated neutrophils in the nasal discharges of CS patients (n = 13). The number of infiltrated neutrophils and IL-8 concentrations in the nasal discharges decreased significantly at 1 to 3 months after the beginning of treatment. (*P < 0.01)

Figure 2 illustrates the effects of low-dose, long-term macrolide treatment on the number of infiltrated neutrophils and IL-8 concentrations from the nasal discharges of the CS patients. The number of infiltrated neutrophils decreased significantly at 1 to 3 months after the beginning of CAM treatment. The IL-8 concentrations in the nasal discharge decreased significantly at 2 to 3 months after the beginning of this treatment.

The effects of IL-1β (20 ng/ml) on IL-8 secretion from the cultured nasal cells are shown in Fig. 3. IL-1β stimulated both apical and basolateral secretions of IL-8 with a significant increase in IL-8 secretion observed at 24 h and 48 h after IL-1β administration.

The effects of 10^{-4} M CAM, 10^{-4} M EM and 10^{-4} M JM on spontaneous IL-8 secretion from HNECs cultured at the air-liquid interface are depicted in Fig. 4. The apical secretions of IL-8 concentrations in 0.5 ml PBS were 12.2 ± 3.6 ng/ml and those of the basolateral medium were 17.2 ± 3.2 ng/ml, (baseline IL-8 secretion). CAM, EM and JM did not affect the basolateral secretion of IL-8 from the cultured nasal cells. The apical secretion of IL-8 decreased slightly at 72 h after macrolide treatment, but no significant difference was observed when compared with the control medium.

The effects of 10^{-4} M CAM, 10^{-4} M EM and 10^{-4} M JM on IL-1β (20 ng/ml)-induced secretion of IL-8 from the cultured nasal cells are shown in Fig. 5. Drugs were simultaneously added to the growth medium for 24 h, but CAM, EM and JM did not affect the IL-1β (20 ng/ml)-induced secretion of IL-8 (both apical and basolateral secretion) from the nasal cells.

Figure 6 illustrates the effects of preincubation with 10^{-4} M and 10^{-6} M CAM and 10^{-4} M and 10^{-6} M JM for 7 days on IL-1β (20 ng/ml)-induced secretion of IL-8 from the cultured nasal cells. IL-1β was added to the growth medium for 24 h at day 8. Only the basolateral secretion was examined, because there was almost the same IL-8 concentration between the apical and basolateral compartments. CAM at 10^{-4} M significantly decreased the IL-1β (20 ng/ml)-induced secretion of IL-8 from nasal epithelial cells at 48 h after IL-1β administration when compared with the control medium. However, no significant differences were observed between 10^{-4} M CAM and 10^{-4} M JM. 10^{-6} M CAM and 10^{-6} M JM did not affect the IL-1β (20 ng/ml)-induced secretion of IL-8.

No evidence of macrolides or IL-1β-mediated cytotoxicity in the cultured epithelial cells was detected by LDH determination (data not shown).

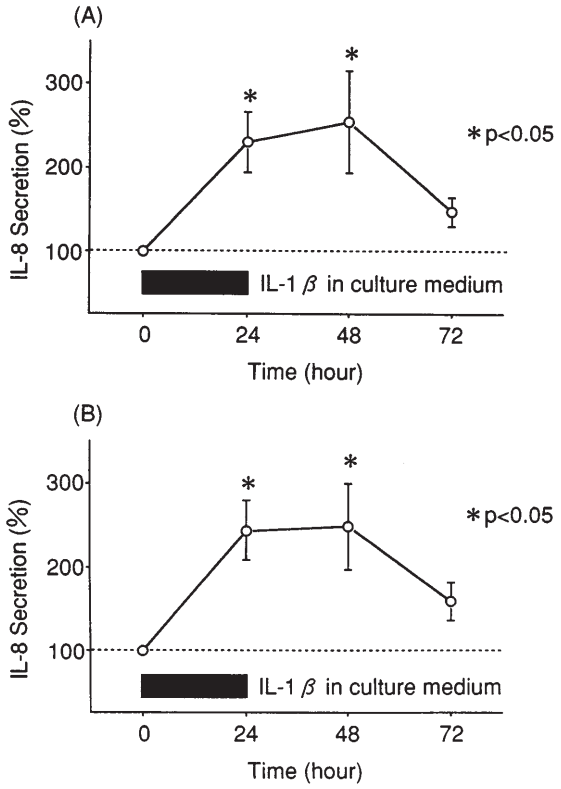

Fig. 3 A, B Effects of 20 ng/ml IL-1β on IL-8 secretion from nasal epithelial cells cultured at the air-liquid interface ($n = 6$). **A** Apical secretions collected with 0.5 ml PBS. **B** Basolateral secretions. IL-1β significantly stimulated IL-8 levels at 24 h and 48 h after treatment ($*P < 0.05$)

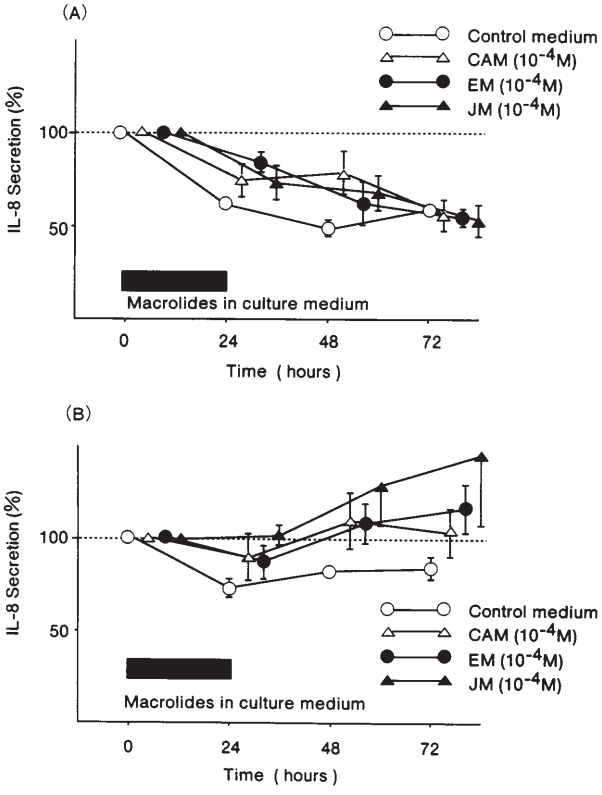

Fig. 4 A, B Effects of 10^{-4} M CAM, 10^{-4} M EM, and 10^{-4} M JM on spontaneous IL-8 secretion from nasal epithelial cells cultured at the air-liquid interface ($n = 6$). **A** Apical secretion collected with 0.5 ml PBS. **B** Basolateral secretions. CAM, EM and JM did not affect IL-8 secretions from the nasal epithelial cells compared with the control medium. Data are expressed as percent changes of baseline IL-8 secretions

Discussion

IL-8 is a potent chemoattractant and activator of neutrophils. High concentrations of this cytokine have been reported in the airway secretions from patients with various respiratory inflammatory diseases [5, 12, 15]. In the present study, we demonstrated that IL-8 concentrations in the nasal discharges of CS patients were significantly higher than those from AR patients and normal controls. The number of neutrophils in the nasal discharges from the CS patients was also higher than those from AR patients or normal controls. The correlation between the IL-8 concentrations and the number of neutrophils in the nasal discharges from the CS patients indicates that IL-8 production in nasal mucosa directly modulates neutrophil infiltration and plays an important role in the activation of inflammation in CS.

IL-8 concentrations and the number of neutrophils in the nasal discharges of patients decreased significantly at 1 to 2 months after oral CAM treatment, suggesting that macrolide antibiotics suppress inflammation through the inhibition of IL-8 secretion. To elucidate the direct effects of macrolide antibiotics on epithelial IL-8 secretion, we examined the in vitro effects of CAM on IL-8 secretion from nasal epithelial cells cultured at an air-liquid interface.

The air-liquid interface is a unique biphasic culture system in which epithelial cells are fed from only their basolateral sides and exposed to air at their apical sides. We have shown previously that cells cultured at an air-liquid interface possess highly differentiated biochemical functions and morphologic structures that are similar to those of in vivo airway epithelium [4, 20]. This cell culture model is thus a better system and well suited to study epithelial function, such as cytokine secretion.

In our present study, 10^{-4} M CAM did not affect spontaneous secretion or IL-1β-induced secretion of IL-8 from the epithelial cells examined. Since long-term macrolide treatment was effective for the treatment of CS patients, we looked closely at the effects of preincubation with both 10^{-4} M and 10^{-6} M CAM for 7 days. Preincubation with 10^{-4} M CAM significantly decreased IL-1β-induced secretion of IL-8 at 48 h after IL-1β stimulation. However, these in vitro results cannot explain the clinical improvement of CS patients, because there was no significant difference between the effects of 10^{-4} M CAM and the 16-membered macrolide JM at a concentration of 10^{-4} M despite findings JM to be ineffective for the treatment of CS patients. CAM at a concentration of 10^{-4} M was also much higher than the therapeutic concentration of 10^{-6} M in tissues. Finally, 10^{-6}

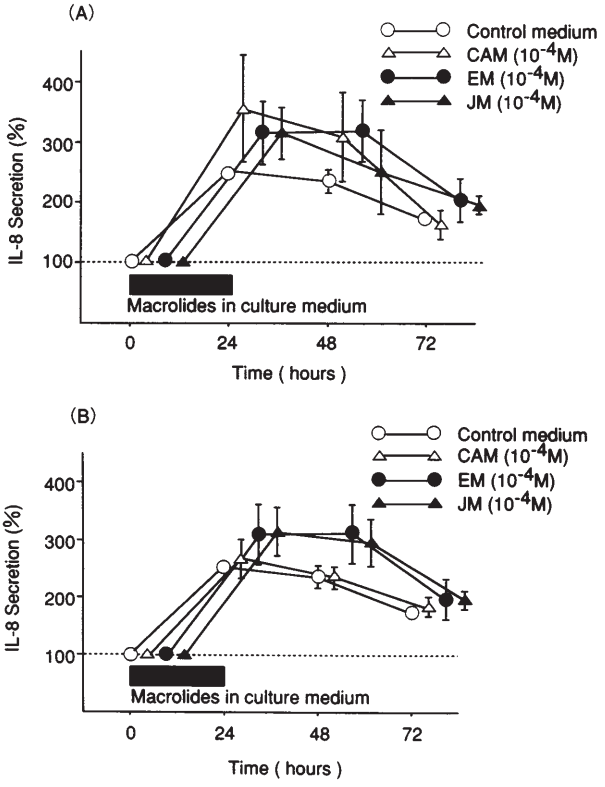

Fig. 5 A, B Effect of 10^{-4} M CAM, 10^{-4} M EM and 10^{-4} M JM on 20 ng/ml IL-1β-induced secretion of IL-8 from nasal epithelial cells cultured at the air-liquid interface (n = 5). **A** Apical secretion collected with 0.5 ml PBS. **B** Basolateral secretion. CAM, EM and JM did not affect IL-8 secretion from the epiyhelial cells. Data are expressed as percent changes of the baseline IL-8 secretion

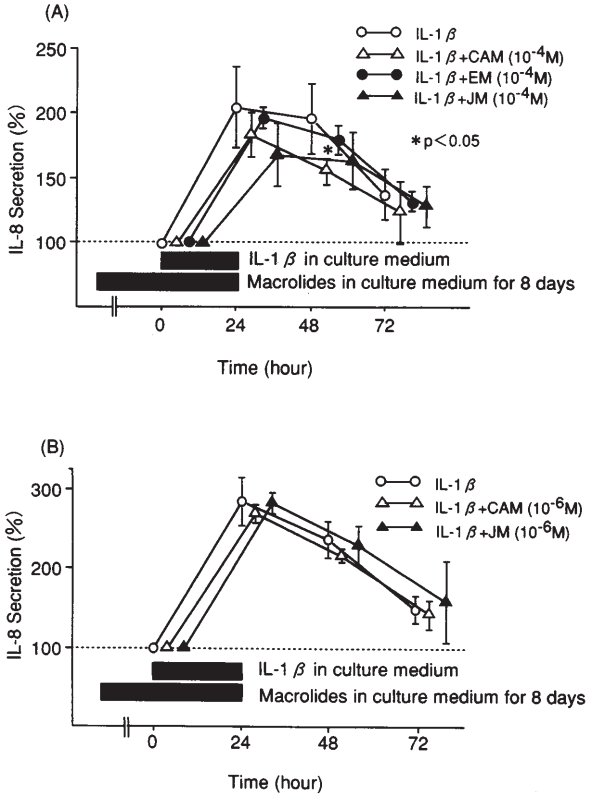

Fig. 6 Effects of preincubation with **A** 10^{-4} M or **B** 10^{-6} M CAM and JM for 7 days on IL-1β-induced secretion of IL-8 (basolateral secretion) from nasal epithelial cells cultured at the air-liquid interface (n = 6). CAM at 10^{-4} M significantly inhibited IL-8 secretion at 48 h after IL-1β administration, but no significant difference was observed between CAM and JM. CAM at 10^{-6} M did not affect IL-1β-induced secretion of IL-8. The data are expressed as percent changes of the baseline IL-8 secretion. *P < 0.05 for CAM at 10^{-4} M vs control medium

M CAM did not affect IL-1β-induced secretion of IL-8 from the nasal epithelial cells.

There is some disagreement as to the effects of macrolides on IL-8 secretion from cultured airway epithelial cells. Takizawa et al. [18] reported that CAM and EM at a therapeutic concentration inhibited IL-1β-induced secretion of IL-8 from cultured bronchial epithelial cells that was dose-dependent, whereas JM showed a minimal inhibitory effect. Khair et al. [7] reported that EM did inhibit *Haemophilus influenza* endotoxin (HIE)-induced secretion of IL-8 from explant bronchial epithelial cells. Suzuki et al. [16] reported that lipopolysaccharide (LPS)-induced IL-8 secretion in nasal epithelial cells was decreased by the addition of CAM and JM at a concentration of 10^{-5} M, but a significant difference in the effects of CAM and JM was only observed at a concentration of 10^{-6} M. All of these experiments were performed by a 24-h incubation of macrolide treatment. In contrast, CAM failed to inhibit IL-8 secretion when compared with JM. The different results may be due to the different responses between the nasal and bronchial epithelial cells and the different culture systems. Previous studies were also performed using an immersion culture system, in which the cultured cells were poorly differentiated and lost the characteristics of matured epithelial cells.

Our findings show that the clinical effectiveness of macrolide treatment for CS patients was reflected by the decrease in the number of neutrophils and the decrease in IL-8 concentrations in nasal discharges. Our in vitro results revealed that the in vivo effects of CAM on neutrophil accumulation and IL-8 secretion were not caused by the direct inhibitory effect on IL-8 secretion from the nasal epithelial cells. Many investigators have reported anti-inflammatory actions of macrolide antibiotics that include an inhibitory effect on neutrophils [5, 12], a modulation of the function of lymphocytes [21], an inhibition of elastase production from *Pseudomonas aeruginosa* [13], an inhibition of cytokine production [3], and an inhibition of mucus secretion from epithelial cells [2, 17]. The in vivo effects of CAM might be caused indirectly through these various anti-inflammatory actions of the macrolide antibiotics.

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