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Anti-Inflammatory Effects of EM900 on Cultured Human Nasal Epithelial Cells

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Objectives: Macrolide therapy is an important conservative therapy for chronic rhinosinusitis, especially in Japan. The mechanism underlying this therapy involves anti-inflammatory and not antimicrobial activity. However, the administration of long-term low-dose macrolides (LTLMs) causes an increase in the number of antibiotic-resistant bacteria. EM900 is a derivative of erythromycin (EM), with anti-inflammatory but not antibacterial effects. It does not induce macrolide-resistant bacteria as shown by LTLM. In the present study, we analyzed the inhibitory effects of EM900 in comparison with those of clarithromycin (CAM) on inflammatory cytokine production in human nasal epithelial cells (HNEpCs).
Methods: After HNEpCs were cultured for 4 days, CAM or EM900 was added into the culture, followed by stimulation with tumor necrosis factor (TNF)-α. Interleukin (IL)-8 and vascular endothelial growth factor (VEGF) levels were measured using real-time polymerase chain reaction (RT-PCR) and an enzyme-linked immunosorbent assay (ELISA).
Results: Both the ELISA and RT-PCR showed that EM900 and CAM significantly inhibited IL-8 production in HNEpCs. In contrast, EM900 and CAM did not suppress the increased VEGF production when HNEpCs were stimulated with TNF-α.
Conclusion: EM900 showed an anti-inflammatory effect, such as that of CAM, due to the inhibitory effect on IL-8 production in HNEpCs. (J Nippon Med Sch 2018; 85: 265–270)

Key words: chronic rhinosinusitis, macrolide, EM900, IL-8, TNF-α

Introduction

Long-term administration of low-dose 14-membered ring macrolides, such as clarithromycin (CAM) and erythromycin (EM), has been shown to markedly improve diffuse panbronchiolitis[1], chronic bronchitis[2,3], and chronic rhinosinusitis (CRS)[2,4]. Therefore, long-term low-dose macrolide (LTLM) therapy is primarily chosen to treat representative chronic inflammatory diseases, such as CRS, in the field of rhinology. Although the mechanism underlying macrolide efficacy has been assumed to involve anti-inflammatory and not antimicrobial activity, the details regarding the exact mechanism remain unclear[5].

LTLM has been shown to contribute to the increase in the number of antibiotic-resistant bacteria. Therefore, EM900 series were developed as the derivatives of EM; EM900 has been characterized by anti-inflammatory but not antimicrobial effects[6,7].

The formation of nasal polyps is a typical mucosal remodeling phenomenon, contributing to the refractory pathology of CRS. However, the details underlying the pathogenesis of polyps remain unclear. Although nasal polyps have been considered to result due to neutrophilic inflammation, the mechanism underlying the occurrence of refractory and recurrent nasal polyps in cases with eosinophilic sinusitis remains unclear. Interleukin (IL)-8 is one of the representative inflammatory cytokines produced by macrophages and neutrophils migrating into the paranasal sinus mucosa under the influence of viral or bacterial infection and microvillus components[8,9]. Fur-

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thermore, vascular endothelial growth factor (VEGF) is produced by inflammatory epithelial cells and fibroblasts. VEGF has two major pathological functions: angiogenesis and hypervascular permeability. The outgrowth of the sinus edematous mucosa due to VEGF is thought to be an important initial step in the formation of nasal polyps[10]. According to studies using fibroblasts from nasal polyps, VEGF production increases under hypoxic conditions in paranasal sinuses[11], particularly in cases with allergic rhinitis; a significant increase in VEGF production from nasal mucosal fibroblasts is regarded as an exacerbating factor for allergic CRS[12]. Hence, VEGF seems to play an important role in the pathogenesis of CRS.

Furthermore, tumor necrosis factor (TNF)-α levels in the remaining fluid increase in the paranasal sinuses of CRS cases[11]. Studies using cultured nasal polyp fibroblasts have shown that macrolides, such as CAM, inhibited the production of IL-8 and VEGF in the presence of TNF-α stimulation[8,13]. Although nasal epithelial cells directly facing nasal and paranasal circumstances exhibit an important initial response in mucosal inflammation, it remains unclear whether macrolides including derivatives inhibit the epithelial inflammatory response monitored by cytokine production. In the present study, to elucidate the anti-inflammatory effect of EM900 compared with that of CAM, cultured human nasal epithelial cells (HNEpCs; Promo Cell, Germany) were stimulated with TNF-α, and the inhibitory effects on inflammatory cytokine production were compared between CAM and EM900.

Materials and Methods

Drugs

We used CAM powder, a conventional 14-membered ring macrolide antibiotic, provided by Taisho Toyama Pharmaceutical Co., Ltd. It was dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 2,000 μg/mL and diluted with sterile water and culture medium to obtain 10^{-6}, 10^{-5}, and 10^{-4} M solutions. EM900 was provided by Kitasato Institute for Life Sciences, Kitasato University. It was dissolved in DMSO and diluted to obtain 10^{-6}, 10^{-5}, and 10^{-4} M solutions. Furthermore, TNF-α (Merck Millipore, Germany) was dissolved in distilled water, and 10 ng was inoculated into each well.

Cell Culture

HNEpCs were subcultured until the fourth passage and then used. The cultured cells were diluted with culture medium using Airway Epithelial Cell Media (Promo Cell, Germany) and seeded onto a 6-well flat-bottomed plate for cell culture to obtain a density of 5×10^5 cells per well. The plate was incubated in a 5% CO_2 incubator at 37°C, and the culture medium (Airway Epithelial Cell Media) was changed after 24 and 76 h to obtain a monolayer culture/outgrowth of epithelial cells on the plate bottom.

Drug Treatment and Stimulation of Cultured Cells

Culture media containing CAM or EM900 at specific concentrations (0, 10^{-6}, 10^{-5}, or 10^{-4} M) were inoculated 4 days after the start of the culture. The intraparanasal level of TNF-α in patients with sinusitis is considered to be 10 ng[14]. Therefore, 10 ng of TNF-α was inoculated in each culture medium on day 7 after the start of the culture. Cells were collected after 16 h for real-time polymerase chain reaction (RT-PCR), and the supernatant and cells were collected after 24 h for an ELISA. The cells and supernatant were stored at −80°C until use.

IL-8 and VEGF

The levels of IL-8 and VEGF in the supernatant were measured using ELISA kits for human IL-8 and VEGF (Life Technologies, MA, USA).

In cultured cells, mRNA expression was determined using RT-PCR. Commercial primers were used. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an intrinsic control.

Statistical Analysis

Wilcoxon's t-test was used for statistical analysis.

Ethical Statement

Because commercially available cultured cells were used in the present study, an application was not submitted to the Ethics Review Board.

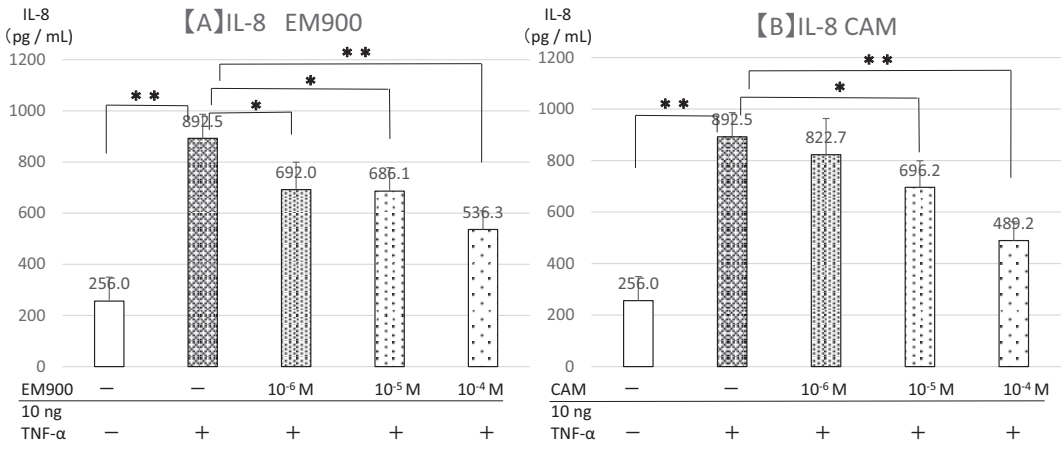
Results

Effects of CAM and EM900 on IL-8 Production

Using both an ELISA and RT-PCR, we confirmed that TNF-α stimulation alone significantly enhanced the production of IL-8 (**Fig. 1 and 2**; n=7). The TNF-α-stimulated cultured HNEpCs were treated with CAM or EM900, and their significant, dose-dependent inhibitory effects on IL-8 production were noted using both the ELISA and RT-PCR.

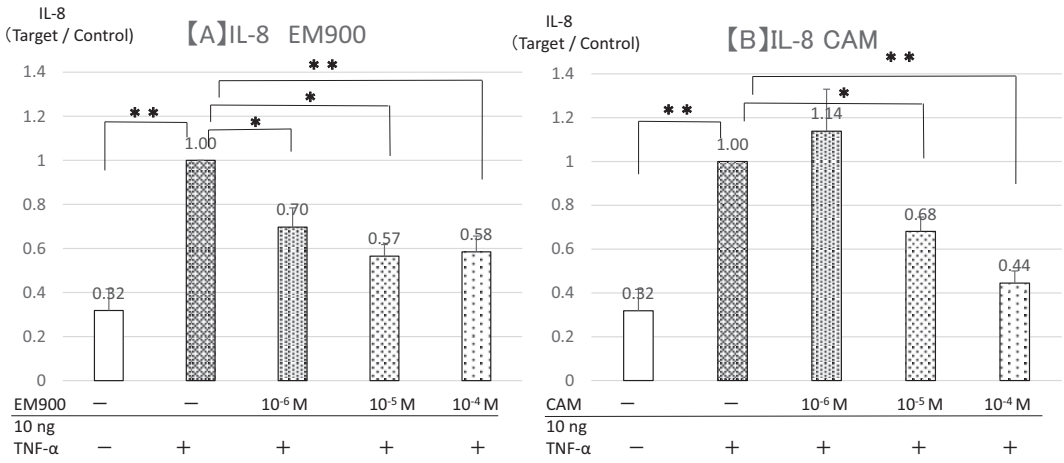
Effects of CAM and EM900 on VEGF production

Using both the ELISA and RT-PCR, we confirmed that TNF-α stimulation alone significantly enhanced the production of VEGF (**Fig. 3 and 4**; n=7). Regarding the inhibitory effects of macrolides, the ELISA demonstrated that the addition of 10^{-6} M EM900 significantly inhibited the production of VEGF. However, there were no significant changes with any other EM900 concentration. In



* : P<0.05 * * : P<0.01 Wilcoxon t-test
A: Administer EM900 and 10 ng/mL TNF-α B: Administer CAM and 10 ng/mL TNF-α

Fig. 1 Result of interleukin (IL) -8 measurements using an enzyme-linked immunosorbent assay (ELISA). Effects of clarithromycin (CAM) and EM900 (n=7). (A) Administration of EM900 and 10 ng/mL of tumor necrosis factor (TNF) -α. (B) Administration of CAM and 10 ng/mL of TNF-α. The ELISA confirmed that TNF-α stimulation alone significantly enhanced the production of IL-8. The TNF-α-stimulated cultured human nasal epithelial cells were treated with CAM or EM900. Their significant, dose-dependent inhibitory effects on IL-8 production were noted using an ELISA. Mann-Whitney U-test; *P<0.05, **P<0.01.



* : P<0.05 * * : P<0.01 Wilcoxon t-test
mRNA of IL-8 expression was standardized by the expression of GAPDH as an internal control.
A: Administer EM900 and 10ng/mL TNF-α B: Administer CAM and 10ng/mL TNF-α

Fig. 2 Result of interleukin (IL) -8 measurement using real-time polymerase chain reaction (RT-PCR). Effects of clarithromycin (CAM) and EM900 (n=7). (A) Administration of EM900 and 10 ng/mL of tumor necrosis factor (TNF) -α. (B) Administration of CAM and 10 ng/mL of TNF-α. IL-8 mRNA expression was standardized with that of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as an internal control. RT-PCR confirmed that TNF-α stimulation alone significantly enhanced the production of IL-8. The TNF-α-stimulated cultured human nasal epithelial cells were treated with CAM or EM900. Their significant, dose-dependent inhibitory effects on IL-8 production were noted using RT-PCR. Mann-Whitney U-test; *P<0.05, **P<0.01.

contrast, RT-PCR confirmed that there were no significant changes due to CAM or EM900 administration at any concentration.

Discussion

IL-8 and VEGF play important roles in the pathogenesis of paranasal inflammation, and their effects can be suppressed by steroids[15]; in the present study, the levels of

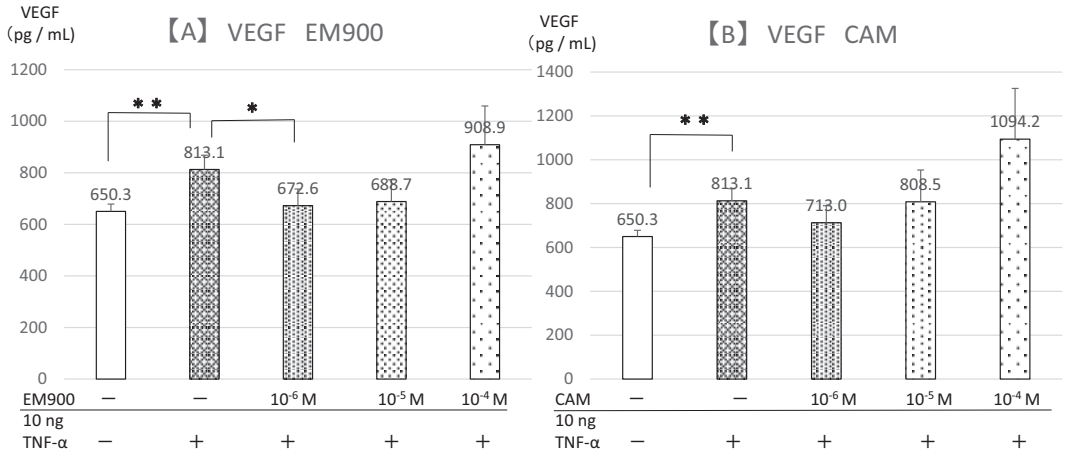


Fig. 3 Result of vascular endothelial growth factor (VEGF) measurement using an enzyme-linked immunosorbent assay (ELISA). Effects of clarithromycin (CAM) and EM900 (n=7). (A) Administration of EM900 and 10 ng/mL of tumor necrosis factor (TNF) -α. (B) Administration of CAM and 10 ng/mL of TNF-α. The ELISA confirmed that TNF-α stimulation alone significantly enhanced the production of VEGF. Regarding the inhibitory effects of macrolides, the ELISA demonstrated that the addition of 10^{-6} M EM900 significantly inhibited the production of VEGF, but there were no significant changes at any other concentration. Mann-Whitney U-test; *P<0.05, **P<0.01.

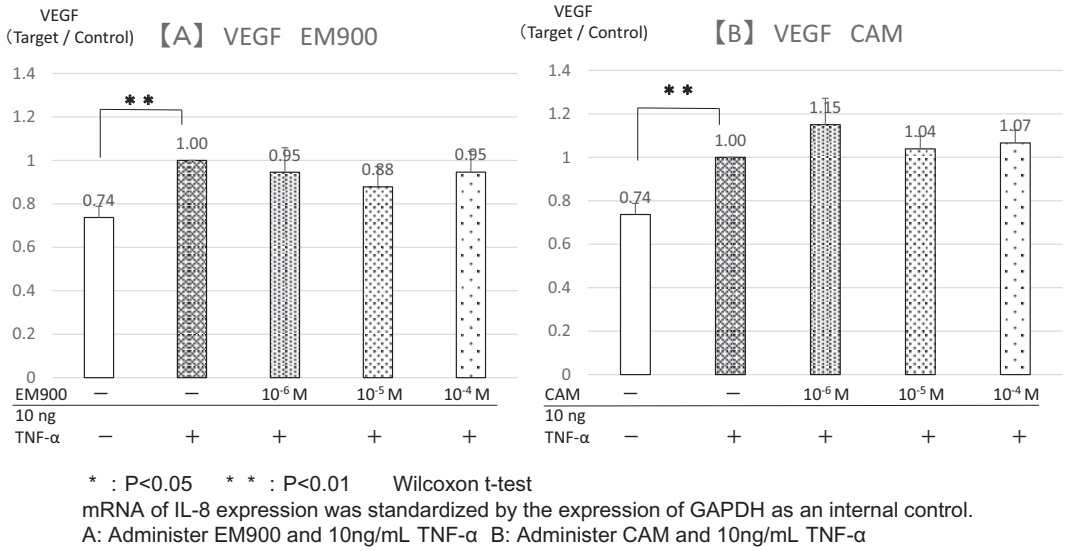


Fig. 4 Result of vascular endothelial growth factor (VEGF) measurement using real-time polymerase chain reaction (RT-PCR). Effects of clarithromycin (CAM) and EM900 (n=7). (A) Administration of EM900 and 10 ng/mL of tumor necrosis factor (TNF) -α. (B) Administration of CAM and 10 ng/mL of TNF-α. VEGF mRNA expression was standardized with that of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as an internal control. RT-PCR confirmed that TNF-α stimulation alone significantly enhanced the production of VEGF. Regarding the inhibitory effects of macrolides, RT-PCR confirmed that there were no significant changes induced by CAM or EM900 at any concentration. Mann-Whitney U-test; *P<0.05, **P<0.01.

both IL-8 and VEGF were significantly increased in cultured cells due to TNF-α stimulation. IL-8 is a representative inflammatory cytokine that particularly activates neutrophils. Previous studies using cultured respiratory epithelial cells have reported that EM and CAM suppress IL-8 production by inhibiting the intranuclear transcription of transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

and activator protein (AP)-1[4,8,9,16–19]. In the present study, we speculated that macrolides may inhibit IL-8 production enhanced by TNF-α stimulation. EM900 inhibited IL-8 production in a dose-dependent manner, as demonstrated by the administration of CAM. We suggested that EM900 as well as CAM, which is a clinically prevalent macrolide, inhibit neutrophil inflammation. According to another study, the concentration of CAM after oral administration of >150 mg was 4.50 μg/mL in the paranasal mucus[20]. In the present study, the maximum concentration of CAM was 10^{-4} M (0.74 μg/mL) and that of EM900 was 10^{-4} M (0.718 μg/mL); similar effects could also be obtained *in vivo*. Therefore, EM900 and CAM are equally effective against neutrophil-mediated CRS with respect to their tissue transferability.

In contrast, VEGF is a cytokine that promotes vascular endothelial proliferation and enhances vascular permeability, suggesting that VEGF-related outgrowth of the paranasal mucosa results in the formation of nasal polyps. Previous studies using cultured fibroblasts indicated that VEGF production is considerably enhanced in the presence of hypoxia or TNF-α stimulation[12,13]. In the present study, TNF-α stimulation enhanced the production of VEGF in cultured HNEpCs. However, the inhibitory effects of CAM and EM900 on VEGF production were not consistently or significantly observed; the results of the inhibition experiment did not show a dose-dependent pattern between the changes in VEGF production and macrolides administered. The intracellular signalling pathway of TNF-α stimulation for VEGF production may be different between epithelial cells and mucosal fibroblasts. Therefore, the inhibitory effects of macrolides may have differed between these two cell types in the previous studies[13]. Further studies are necessary to elucidate the intracellular events related to VEGF production.

The new EM derivative EM900 significantly inhibited IL-8 production, similar to that observed using CAM administration. Therefore, EM900, which does not show antibiotic activity, can demonstrate promising anti-neutrophil inflammatory effects in CRS, similar to those observed using CAM.

Eosinophilic inflammatory CRS is increasing in Japan. However, macrolides have no anti-eosinophil inflammatory effects. Therefore, according to previous reports, macrolides have no effect on eosinophilic inflammatory CRS. There is no experimental data or clinical data at the present time for the effect of EM900 on CRS. Therefore, further investigation is required on suppressive effects of EM900 on cytokines that cause eosinophilic inflammation.

The mucosal epithelium harvested during operations is repeatedly exposed to environmental pathogens and thus may be already stimulated. Therefore, the data may vary because of substances in the sinus fluid. The collection of normal human mucosal epithelium is ethically challenging. On the other hand, commercial nasal epithelial cells can be simply and constantly stimulated using TNF-α alone. Therefore, commercial cells were used in the present study to estimate the anti-inflammatory effects of EM900 compared with those of CAM.

Conclusion

The anti-inflammatory macrolide EM900 significantly inhibited IL-8 production in cultured HNEpCs. However, such an inhibitory effect was not observed on VEGF production, as observed in the experiment using fibroblasts. EM900, as well as CAM, can be a promising drug for the treatment of neutrophil inflammation in CRS.

This study was presented at the 26th Congress of the European Rhinologic Society in Stockholm, Sweden.

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Conflict of Interest: No potential COI to disclose

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