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The Effects of Antibiotics on in vitro Biofilm Model of Periodontal Disease

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Abstract
Objective: Periodontal disease is considered to be a biofilm infectious disease. The effects of macrolide and tetracycline on biofilm were examined in *in vitro* biofilm model made of periodontal disease-associated bacteria.
Methods: Biofilms were made on salivary pellicle by adding *Streptococcus gordonii* for 2 days, followed by *Porphyromonas gingivalis* inoculation for 2, 5, or 12 days. Biofilms were treated with macrolide antibiotics; erythromycin (EM), azithromycin (AZM) and josamycin (JOM) and tetracycline antibiotic, minocycline (MINO). The effects of these antibiotics on biofilms were examined using colorimetric quantification method, scanning electron microscope (SEM) and confocal laser scanning microscopy (CLSM).
Results: When antibiotics were added to the biofilm 2 days after inoculation of *Porphyromonas gingivalis* (biofilm inhibition model), all four antibiotics decreased the number of bacteria by both colorimetric method and SEM observation. When antibiotics were added to biofilms 5 or 12 days after inoculation (biofilm destruction model), those in biofilms were decreased by EM and AZM compared with JOM and MINO. Moreover, CLSM observation demonstrated that EM and AZM killed bacteria in biofilm more deeply than JOM and MINO.
Conclusion: These results suggest the feasibility of EM and AZM for the treatment of periodontal disease as a biofilm infectious disease.

Key words: biofilm, periodontopathic bacteria, azithromycin, erythromycin, macrolide

Abbreviations: AZM, azithromycin; CLSM, confocal laser scanning microscope; EM, erythromycin; JOM, josamycin; MINO, minocycline

Introduction

Caries and periodontal disease are two major oral diseases and are the infectious diseases due to dental plaques which are formed by intraoral bacteria. Recently, Costerton et al. [1] proposed that they are biofilm infectious diseases. The systemic disorders due to biofilm include cystic fibrosis pneumonia and infective endocarditis [1]. Biofilms are bacterial populations which are enclosed by extracellular matrix produced by bacteria *per se* and which adhere to each other and/or surfaces or interfaces such as medical devices [2, 3]. Importantly, bacteria in biofilm are resistant to antibiotics [1, 2].

Periodontal disease as a biofilm infectious disease is considered as below. *Porphyromonas gingivalis* (*P. gingivalis*) is implicated in the initiation and progression or periodontitis [4]. Periodontal disease-associated bacteria formed biofilm in periodontal pockets or on the surface of cementum. Planktonic bacteria from biofilm invade into periodontal tissues and lead to inflammation and destruction of tissues directly and indirectly by elicit host defense mechanism. Supragingival dental plaques (biofilm) are easily removed by professional mechanical tooth cleaning (PMTC), while subgingival dental plaques and bacteria invading into periodontal tissues are difficult to be removed. Therefore, the development of novel methods for periodontal disease is needed to eliminate these biofilms efficiently.

Presently, local administration of minocycline (MINO), one of tetracycline antibiotics, into periodontal pockets has been performed for the treatment of periodontal disease. Moreover, penicillin, nitroimidazole or erythromycin (EM) antibiotics have also been administrated systemically [5-8]. However, these antibiotics are shown to be less effective to biofilm made of *P. gingivalis* [9-11]. In contrast, azithromycin (AZM), 15-membered ring macrolide antibiotics, has a good tissue penetration property [12-15] and inhibits biofilm formation made of *Pseudomonas aeruginosa* (*P. aeruginosa*) [16].

In this study, we examine the effect of macrolide and tetracycline to *in vitro* biofilm model, which is made of *Streptococcus gordonii* (*S. gordonii*) and *P. gingivalis* [17]. Concomitantly, we examined the effects of 14-membered ring macrolide antibiotic EM and 16-membered ring macrolide antibiotic josamycin (JOM) and tetracycline antibiotic MINO.

Materials and Methods

Antibiotics

Erythromycin (EM), azithromycin (AZM), josamycin (JOM) and minocycline (MINO) were obtained from Nihon SiberHegner (Tokyo, Japan), Pfeizer Japan (Tokyo, Japan), Astellas Pharma (Tokyo, Japan) and

Miyuki Yakugyo (Osaka, Japan), respectively. All antibiotics were dissolved in methanol at 0.1 and 1 mg/ml and added to culture media at final concentration of 0.1 and 1 μg/ml.

Saliva

Unstimulated whole saliva was collected from healthy volunteers and centrifuged at 1,000 × g for 10 min at 4 °C. Supernatant was filtrated through 0.45 mm-pore membrane and stored at 4 °C. Saliva was collected at each experiment.

Biofilm Formation

Biofilm formation was carried out as described previously [17] with minor modification. For quantification of bacteria, filtrated saliva was coated on 96-well plate (Asahi Technoglass, Tokyo, Japan) for 30 min at 4 °C, yielding to pellicle. For observation by scanning electron microscope (SEM) and by confocal laser scanning microscope (CLSM), saliva was coated on glass coverslips in 24-well plate (Asahi Technoglass) and 35 mm glass base dish (Asahi Technoglass), respectively. *S. gordonii* (Challis) was inoculated into wells in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) including yeast extract (BBL Microbiology Systems), 5 μg/ml of hemin (Wako Pure Chemical Industries, Osaka, Japan), of 0.5 μg/ml of menadione (Nacalai Tesque, Kyoto, Japan) and 6 μg/ml of Sucrose (Wako) and incubated for 2 days at 37 °C under anaerobic conditions of 85% N_2 and 5% CO_2. Then, *P. gingivalis* (ATCC 33277) was inoculated in the same medium as *S. gordonii* on these biofilms and incubated under anaerobic conditions. On 2 days (biofilm inhibition model) or 5 days (biofilm destruction model) after inoculation of *P. gingivalis*, antibiotics and methanol (as a control) were added to biofilms and incubated for 4 h at 37 °C under anaerobic conditions. For CLSM observation, antibiotics were added on 12 days after inoculation. The durations of biofilm formation were determined by SEM observation in preliminary experiments (data not shown). The effects of antibiotics were examined by a colorimetric method using toluidine blue, SEM and CLSM as described below.

Quantification of Biofilm

For quantification of biofilm, biofilm treated with antibiotics or methanol were stained with 0.1% toluidine blue for 20 min at room temperature and washed three times in distilled water. The stained biofilms were dissolved in 100 μl of ethanol and absorbance was measured 570 nm using microplate reader (Model 550; Bio-Rad, Hercules, CA). The value of background (without bacteria) was subtracted from that of each sample, and the averages were calculated as percentage to control. Data are presented as mean ± standard deviation (SD) of the average of five experiments.

Scanning Electron Microscope (SEM)

After treatment with antibiotics, glass coverslips were washed with 0.1M phosphate buffer (pH7.4) and fixed with 2.5% glutaraldehyde in phosphate buffer for 2 hours at room temperature. Then, specimens were dehydrate with an ethanol series (50 to 100%), freeze-dried and coated with gold-palladium. Observations were carried out on JSM-6300 (JEOL, Tokyo, Japan)

Confocal Laser Scanning Microscope (CLSM)

After treatment with antibiotics, the samples were stained with ViaGram Red+ Bacterial Gram Stain and Viability Kit (Invitrogen, Oregon, OR). Observations were carried out on LSM510 (Carl Zeiss, Jena, Germany). Blue (DAPI) and green (SYTOX Green) stainings represent viable and dead bacteria, respectively.

Statistical Analysis

A one sample t test with Bonferroni correction was used to examine whether the each mean value of experimental groups is different to 100%. All computations were performed with the statistical program R (http://www.r-project.org/). Values with $p < 0.05$ were considered as significantly different.

Results

The Effects of Antibiotics on Biofilm Formation

We first examined the effects of antibiotics on biofilm formation (biofilm inhibition model). Fig. 1 shows that the rate of inhibition was as follows: EM (0.1 μg/ml, 60.4 ± 5.7; 1 μg/ml, 51.0 ± 10.2), AZM (0.1 μg/ml, 66.6 ± 12.3; 1 μg/ml, 55.9 ± 14.6), JOM (0.1 μg/ml, 61.3 ± 3.8; 1 μg/ml, 38.7 ± 5.4), MINO (0.1 μg/ml, 59.6 ± 3.9; 1 μg/ml, 40.2 ± 5.4). Thus, all four antibiotics (EM, AZM, JOM and MINO) dose-dependently decreased the number of bacteria to approximately 40 to 50% of untreated control (methanol alone) at a concentration of 1 μg/ml (Fig. 1). Moreover, we observed the change of biofilm structure. SEM observation showed that bacteria adhered to each other and formed aggregation but that a very small amount of exopolysaccharide (EPS) was present in untreated control (Fig. 2A). In antibiotics-treated biofilm, the numbers of bacteria were decreased to similar extents (Fig. 2B-E). These results indicate that all antibiotics have similar inhibitory effects on the biofilm formation.

The Effects of Antibiotics on Mature Biofilm

Next, we examined the effect of antibiotics on already formed biofilm (biofilm destruction model). Fig. 3 shows that the rate of inhibition was as follows: EM (0.1 μg/ml, 52.4 ± 10.4; 1 μg/ml, 42.5 ± 7.9), AZM (0.1 μg/ml, 65.8 ± 18.2; 1 μg/ml, 50.5 ± 10.5), JOM (0.1 μg/ml, 87.2 ± 6.2; 1 μg/ml, 78.0 ± 9.2), MINO (0.1 μg/ml, 78.0 ± 3.2; 1 μg/ml, 62.8 ± 5.2). Thus, all four antibiotics (EM, AZM, JOM and MINO) dose-dependently decreased the number of bacteria. AZM and EM decreased to approximately 40 to 50% of control (Fig. 3A and B), while JOM and MINO decreased to 80% and 60%, respectively (Fig. 3C and D)

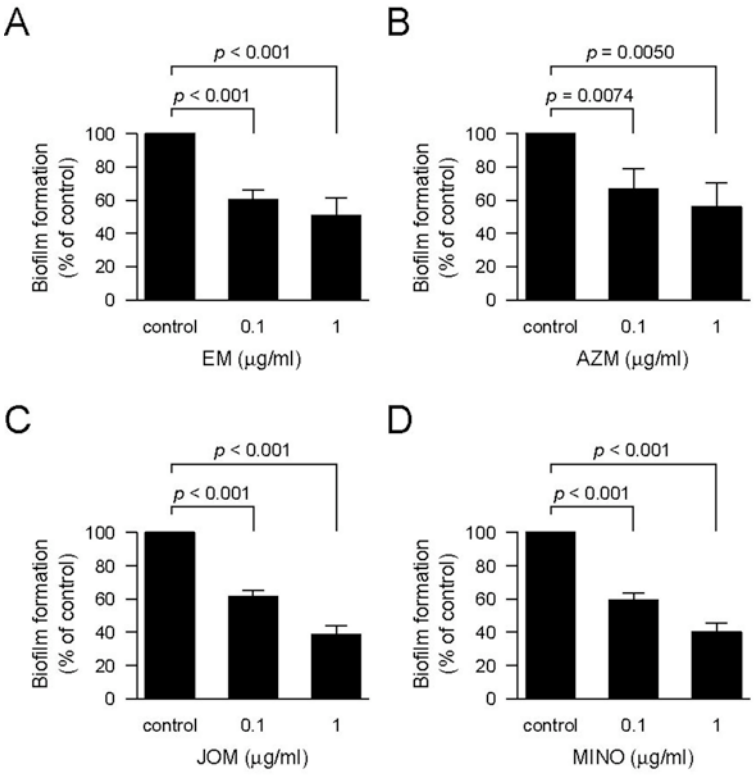

Fig. 1. The effects of antibiotics on biofilm inhibition model. (A) EM, (B) AZM, (C) JOM and (D) MINO. The rate of inhibition (%) = (A_{570} of sample - A_{570} of background)/(A_{570} of control - A_{570} of background) × 100. Data are presented as mean ± SD of the average of five experiments. *p* values using one sample t test (population mean as 100%) with Bonferroni correction were indicated.

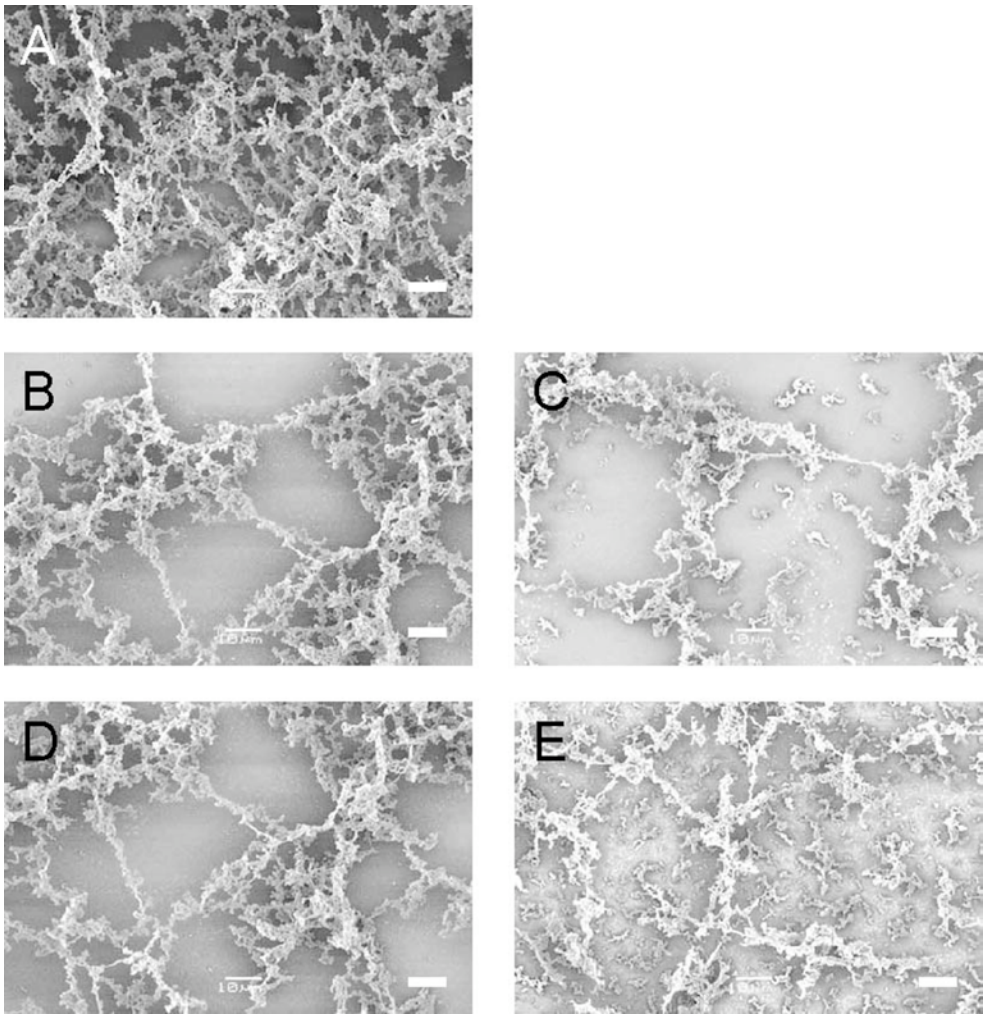

Fig. 2. SEM photographs of biofilm in biofilm inhibition model. (A) control, (B) EM, (C) AZM, (D) JOM and (E) MINO. Bars, 10 μm.

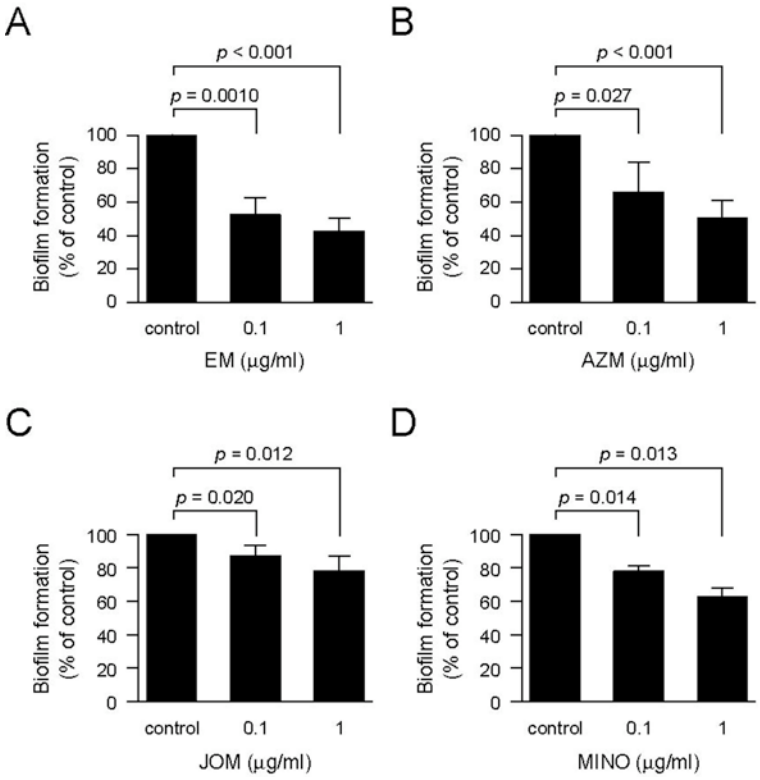

Fig. 3. The effects of antibiotics on biofilm destruction model. (A) EM, (B) AZM, (C) JOM and (D) MINO. The rate of destruction (%) = (A_{570} of sample - A_{570} of background)/(A_{570} of control - A_{570} of background) × 100. Data are presented as mean ± SD of the average of five experiments. *p* values using one sample t test (population mean as 100%) with Bonferroni correction were indicated.

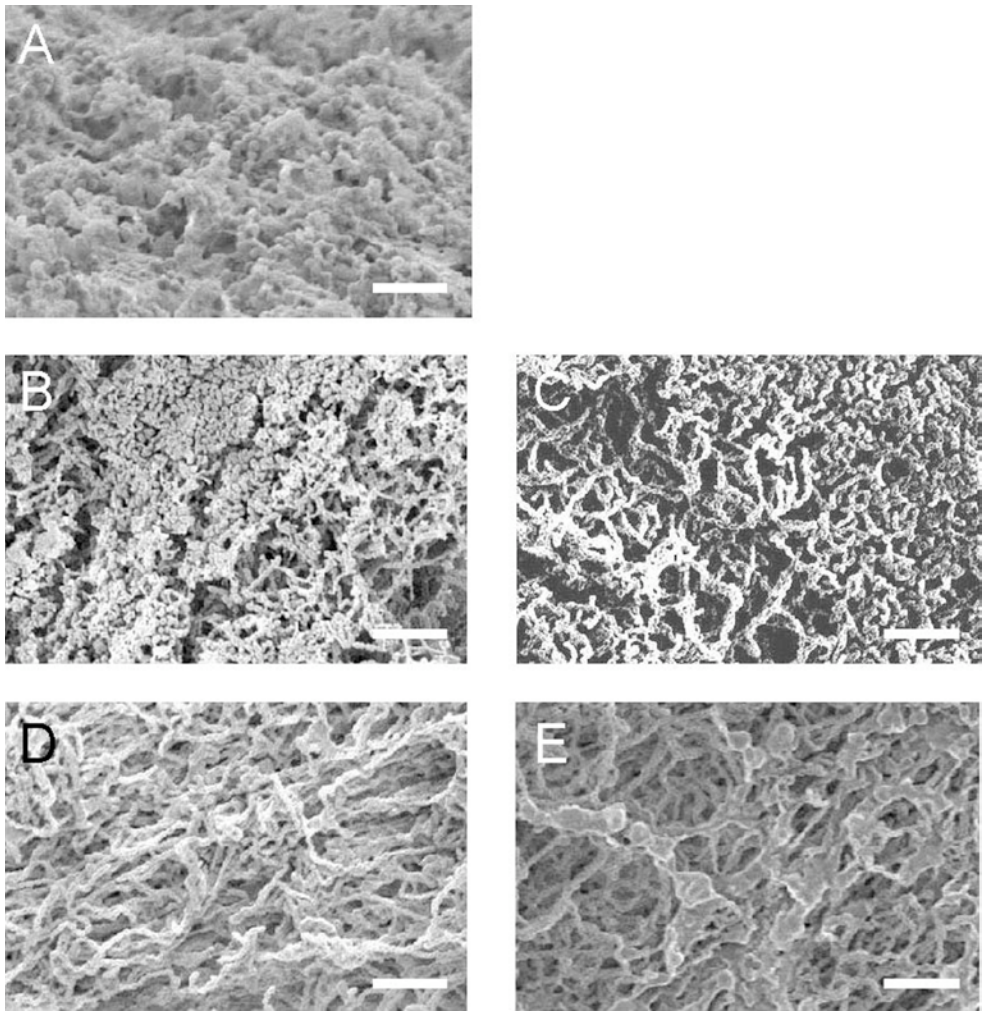

Fig. 4. SEM photographs of biofilm in biofilm destruction model. (A) control, (B) EM, (C) AZM, (D) JOM and (E) MINO. Bars, 20 μm.

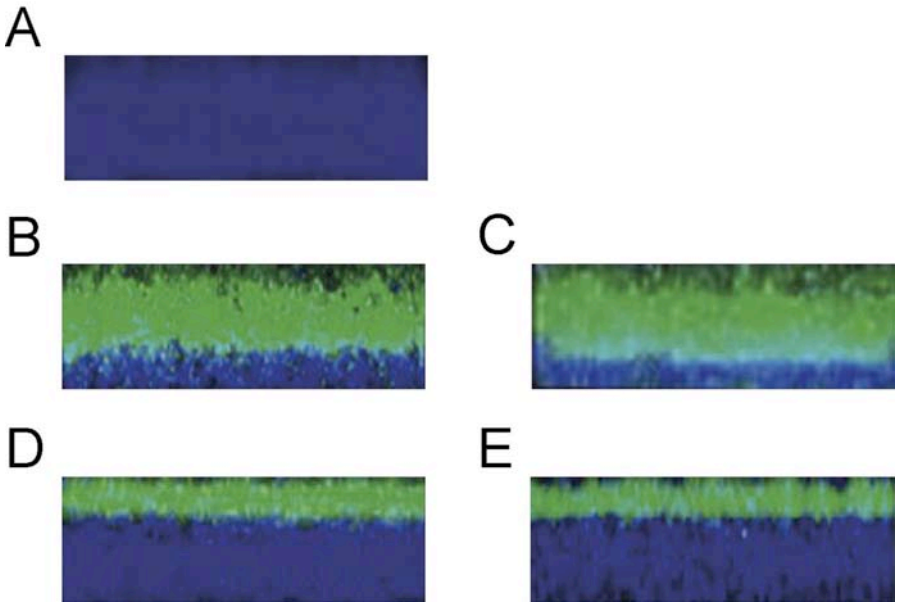

Fig. 5. Confocal images of biofilm in destruction model. Cross sections of biofilm were denoted. Blue and green indicate viable and dead bacteria, respectively. (A) control, (B) EM, (C) AZM, (D) JOM and (E) MINO.

at a concentration of 1 μg/ml. SEM observation showed that numerous number of bacteria aggregated and that large amount of EPS was present in untreated control (Fig. 4A). Surface structures were altered in EM and AZM-treatment groups (Fig. 4B and C) although obvious alterations were not observed in JOM or MINO-treatment groups (Fig. 4D and E). At last, we examined how deeply these antibiotics effect to bacteria in biofilm by CLSM observation. In untreated control, no dead cell was observed (blue area: alive bacteria) (Fig. 5A). In all antibiotics-treated groups, dead bacteria (green area) were observed on the top surface of biofilm. Concretely, EM and AZM killed bacteria in biofilm deeply than JOM and MINO (Fig. 5B-E). These results indicated that EM and AZM have antimicrobial effect to already formed biofilm more than JOM and MINO.

Discussion

In this study, we examined the antimicrobial effects to *in vitro* biofilm model made of *S. gordonii* and *P. gingivalis*. We consider that this model is simple and useful for the biofilm model by periodontopathic bacteria for the following reasons. (i) The biofilm models with streptococci and *P. gingivalis* have been analyzed [18-20]; (ii) *S. gordonii* play a role in initial adhesion to pellicle and periodontopathic *P. gingivalis* adhere to *S. gordonii*; (iii) The molecular mechanisms of adhesion between *S. gordonii* and *P. gingivalis* have been revealed [21, 22]; and (iv) The sequence (*S. gordonii-P. gingivalis*) in this biofilm model is consistent with the situation that prevails in oral cavity [23].

All four antibiotics decreased the number of bacteria to similar extents in biofilm inhibition model, i.e. immature biofilm (Fig. 1). Because only small amount of EPS was produced at this time (Fig. 2), it is suggested that antibiotics freely penetrate into immature biofilm and inhibit biofilm formation. Bacteriostatic antibiotics JOM and MINO decrease the number of bacteria in biofilm as well as bactericidal antibiotics EM and AZM. Macrolide and tetracycline antibiotics suppress protein synthesis by inhibition of bacterial 70S ribosome. Also, this reduction in bacteria suggests bacteria death or inhibition of growth. Moreover, AZM inhibits the production of fimbriae in *P. gingivalis* [24] Because adhesion between *S. gordonii* and *P. gingivalis* is mediated- by cell-surface molecules including the Ssp proteins of *S. gordonii* and the major fimbriae and a 35 kDa protein of *P. gingivalis* [21, 22], it is assumed that these antibiotics suppress aggregation of bacteria in biofilm by inhibiting the synthesis of these adhesion molecules.

In contrast, in biofilm destruction model, EM and AZM decreased the number of bacteria than JOM and MINO (Fig. 3). Because the large amount of EPS was produced at this time, i.e. mature biofilm (Fig. 4), antibiotics are generally hard to penetrate into biofilm. Considering that EM and AZM could destroy the biofilm (Fig. 5), these antibiotics may penetrate into biofilm deeply and kill the bacteria. However, observation by CLSM is unable to distinguish the effects of antibiotics (bactericidal or bacteriostatic) and the permeability of antibiotics into biofilm. Indeed, EM and AZM show bactericidal effects, while JOM and MINO do bacteriostatic ones. Moreover, we could not measure the concentrations of antibiotics in biofilm. Therefore, we did not conclude that the reasons that EM and AZM showed bactericidal effects more deeply in biofilm are due to (1) bactericidal effects, (2) high penetration into biofilm or (3) both. In addition, the precise mechanisms that the amounts of EPS were decreased by the treatment with EM and AZM (Fig. 4B and C) remained unknown. Nevertheless, we consider that our results EM and AZM destroy the biofilm more efficiently than JOM and MINO are very significant because AZM is effective to biofilm infectious disease by *P. aeruginosa* [16]. Further studies are needed to clarify these results. On the other hand, the macrolides for patients with airway biofilm disease depends on the ability of such macrolides to inhibit alginate production by *P. areuginosa*. This suggests that

inhibitory effect observed with 14-, 15- and 16-membered macrolides may depend on the sugar chain connected with the macrolide ring [25].

As a defense mechanism, bacteria mediate signal transduction to each other via biofilm. This regulation at a genetic level is named as quorum sensing [26]. This system is a mechanism which senses a bacterial cell density and, in response, regulates specific sets of genes as shown in part of fungi. Most bacteria produce autoinducer, an important mediator in quorum sensing, and this autoinducer regulates the production of various virulence factors including biofilm forming materials and adherence molecules [27]. Recently, EM and AZM are reported to inhibit quorum sensing system in *P. aeruginosa* [28, 29]. However, the relationship between AZM and EM and the quorum sensing system in *P. gingivalis* is still conversial.

The systemic administration of AZM against patients of periodontal disease is reported to decrease the numbers of periodontal disease-associated bacteria such as *P. gingivalis*, Prevotella intermedia and spirochaetes [30, 31]. Recently, our group demonstrated that systemic administration of AZM is clinically effective to the treatment of early-onset (aggressive) periodontitis [32]. AZM is reported to be incorporated into phagocytic cells such as macrophages *in vitro* and *in vivo* [33]. Therefore, the concentration of AZM in lung or tonsil is more than 20-fold higher than that in blood [34, 35]. AZM concentration is also high in gingival tissue [13, 36]. These findings suggested that AZM concentration is kept to be high in periodontal tissue in the long-term and shows antimicrobial effects to periodontopathic bacteria which have invaded in periodontal tissue. Moreover, AZM may suppress aggregation of bacteria in biofilm by inhibiting the synthesis of these adhesion molecules and make bacteria be sensitive to antibiotics. For these reasons, we consider that AZM may be a very effective antibiotic for the treatment of periodontal disease although the combination with conventional initial preparation is very important.

In conclusion, we demonstrated that EM and AZM have the ability to destroy the biofilm *in vitro*. These results suggest that pharmacotherapy to destroy biofilm using antibiotics such as EM and AZM is effective for the treatment of periodontal disease.

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