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Limitations and Adverse Influences of Antimicrobial Strategy for the Control of Oral Biofilm

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Abstract

Chemical complements such as toothpastes and mouth rinses that contain antimicrobial agents have proven to be effective for the control of oral biofilm. When used with adequate mechanical measures, additional anti-plaque and anti-gingivitis clinical benefits are achieved. However, recent investigations have reported some adverse effects of the antimicrobial strategy for the control of oral biofilm. One adverse effect is that most of the antimicrobial agents failed to remove the biofilm structure. The residual structure may serve as a scaffold for the redevelopment of biofilm. The remaining structure causes adverse effects with regard to host response to pathogens, even if the microorganisms in the biofilm are completely killed. Another effect is that low-dose antibiotics may promote bacterial biofilm formation. The short exposure time of chemical agents will cause gradient of concentration inside the biofilm. It has been demonstrated that a variety of antibiotics or antimicrobial agents at sub-MIC levels can induce biofilm formation *in vitro*, interfering with bacterial biofilm virulence expression. Future strategies that promote the biofilm matrix detachment are therefore expected, without affecting bacterial growth targeting to polymeric substances.

Introduction

The oral cavity has unique properties which differ from most other sites in the human body. A large variety of microorganisms reside in our intraoral environment [1]. The existence of bacteria in the oral environment is natural and essential for the physiology of the oral cavity [1,2]; we all coexist with the oral biofilm community. The resident microflora also contributes to the prevention of exogenous microorganisms becoming established in the mouth [2]. The oral cavity is the only organism that enables removal (without surgical intervention) of biofilms developed in the human body. Therefore, mechanical elimination such as brushing and flossing is fundamental for the control of oral biofilm [3]. The chemical approach is used as an alternative or adjunctive method when elimination using dental instruments proves difficult. Consequently, various antimicrobial agents have been formulated into oral care products in order to enhance their plaque control potential [4-6]. In fact, it has been demonstrated that adjunctive antimicrobials improve clinical parameters including plaque index and gingival inflammation by interfering with metabolic activities [6-10]. It has also been reported that antiplaque biocides do not cause microbial resistance and alterations to microbial flora [4]. However, recent investigations have reported on the limitations of chemical strategies that rely on antimicrobial properties. This article reviews studies demonstrating the adverse effects of antimicrobial strategy against oral biofilm and discusses a possible strategy for the control of oral biofilm.

Retarded Penetration into Biofilm

Recent investigations have demonstrated that antimicrobial compounds do not work as intended [11-14]. Especially in a short time exposure, antimicrobials failed to penetrate the biofilm. This phenomenon can be explained as the result of retarded penetration due to degradation and/or modification by the biofilm matrix. Extracellular polymeric substances (EPSs) produced by microorganisms make up the intercellular space of microbial aggregates and form the architecture of the biofilm matrix which reduces antimicrobial penetration (Figure 1) [15,16]. In direct time-lapse microscopic observation, the penetration of 0.12% chlorhexidine gluconate (CHG) into an oral biofilm model was critically restricted, indicating that the average penetration velocity was only 4.1 μm/min [12]. Wakamatsu et al. [17] have reported the penetration kinetics of mouth rinses into *in vitro Streptococcus mutans* biofilms by direct time-lapse microscopic analysis. The antimicrobial penetration was critically restricted within 30 s of exposure, and the average penetration velocity ranged from 4.2 to 30.1 μm per min. Consequently, the microorganisms inside the biofilm will

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Table 1: A summary of representative experiments demonstrating that chemical approach failed to detach the biofilm structure.

Biocide	Species	Conditions for biofilm formation	Flow speed of biocide	Exposure time	Judgment	Reference
11.6% EtOH 0.12% CHG Biotene	*Streptococcus oralis*, *Streptococcus gordonii*, *Actinomyces naeslundii*	Flow cell chamber	1 ml/ min	20 min	Microscopic observation (transmission image)	[12]
40% EtOH 0.1% SLS 0.03% TRN 0.12% CHG 0.05% CPC 0.005% nisin	*Streptococcus oralis*, *Streptococcus gordonii*, *Actinomyces naeslundii*	Flow cell chamber	1 ml/ min	60 min	Microscopic observation (transmission image)	[14]
0.12% CHG EO CPC IPMP	*Streptococcus mutans*	Glass-based dish	No flow	5 min	Microscopic observation (transmission image)	[17]
0.05 to 0.2% CHG	*Porphyromonas gingivalis*	Glass-based chamber	No flow	5 min	Microscopic observation (transmission image), Quantitative analysis of protein and carbohydrate composition	[19]
0.14mM QAC 0.5mM Glutaraldehyde 14.9 µM nisin	*Staphylococcus epidermidis*	Flow cell chamber	1 ml/ min	60 min	Microscopic observation (transmission image)	[20]

EtOH: Ethanol; CHG: Chlorhexidine Gluconate; SLS: Sodium Lauryl Sulfate; TRN: Triclosan; CPC: Cetylpyridinium Chloride; IPMP: Isopropyl Methyl Phenol; QAC: Quaternary Ammonium Compound

Table 2: A summary of representative experiments demonstrating that sub-MIC of antimicrobial agent's up regulate pathogenic genes.

Biocide	Concentration of biocide	Species	Condition of bacteria	Incbation time	Up regulated genes	Reference
Sodium fluoride Chlorhexidine Tea polyphenol	1/2 MIC	*Streptococcus mutans*	Planktonic	24h	*gtfB, gtfC, luxS, comD, comE*	[38]
Sodium fluoride Chlorhexidine Tea polyphenol	1/2 MIC	*Streptococcus mutans*	Biofilm	24h	*gtfB, gtfC, gtfD, luxS, comD, comE*	[38]
Triclosan	1/2 and 1/4 MIC	*Streptococcus mutans*	Planktonic	2h	*atlA, gtfB, gtfC, comD, luxS*	[39]
MTAD MTADN MTAN	1/4 MIC	*Porphyromonas gingivalis*	Planktonic	1h	*clpC, clpP* (MTAD, MTADN, MTAN). *sprE* (MTAD, MTADN), *ace, clpX, cylB, efaA, gelE* (MTAN).	[40]

MTAD: 3% doxycycline, 4.5% citric acid and 0.5% polysorbate 80 detergent, MTADN: nisin combined with MTAD, MTAN: nisin in place of doxycycline in MTAD.

respond to antimicrobial stress and facilitate a horizontal gene transfer [18].

Residual Structure

Recent investigations have demonstrated that disinfection by antimicrobial agents resulted in leaving the biofilm structure intact. A summary of representative experiments demonstrating that the chemical approach failed to detach the biofilm structure is shown in Table 1. It has been reported that little or no biofilm structure was removed when *in vitro* oral biofilms were treated with ethanol [12,14], chlorhexidine [12,14,17,19], nisin [14,20], glutaral dehyde [20], a quaternary ammonium compound [20], sodium lauryl sulfate [14], triclosan [14], cetylpyridinium chloride [14,17], or essential oil [17]. Davison et al. [20] investigated the dynamic antimicrobial action of four antimicrobial agents within biofilm cell clusters of *Staphylococcus epidermidis* using time-lapse confocal scanning laser microscopy (CSLM). The penetration of antimicrobial activity, as judged by calcein-AM fluorescence loss, was remarkably retarded and chlorine was the only antimicrobial agent that caused any biofilm removal. In the latest investigation by Song et al. [21] it has been demonstrated, using bacterial vibration spectroscopy and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy that oral bacteria adhering to salivary conditioning films became more difficult to remove after exposure to mouth rinses containing CHG, cetylpyridinium chloride, or amine fluoride, due to strengthening of the polysaccharide bond. The residual structure may cause adverse effects in the oral environment even if the microorganisms in the biofilm are completely killed. One possible negative reaction is that the disinfected structure contributes a source of antigen that causes host inflammatory reaction. Since EPS contains carbohydrates, proteins, polysaccharide, lipids, and nucleic acids [22], dead bacteria and their components will work as antigens and induce inflammatory reactions. Also, the structural component of the outer surface membrane of bacteria is known to activate immune cells, such as macrophages and monocytes [23-25]. For example, the lipid A moiety of lipopolysaccharides (LPS) initiates innate immune responses by interacting with Toll-like receptor 4 (TLR4), which results in the production of a wide range of cytokines. Thus, derivatives of LPS have been noted as adjuvants for vaccinations [24]. Augustin et al. [26] noted that the injection of the dead components of *Enterococcus faecalis* into rats, following mechanical aortic damage from a catheter, produced endocarditic vegetation enriched with polymorphonuclear cells. Taken together, the failure to disrupt the structure of the biofilm after the antimicrobial approach may contribute to continuous inflammation. Another possible negative effect is that the residual structure may act as a scaffold for secondary bacterial adhesion. We have demonstrated that the residual structure of *S. mutans* biofilm promoted the secondary bacterial adhesion and biofilm redevelopment, using a rotating disk reactor *in vitro* [27]. At first, *S. mutans* biofilm generated on a resin-composite disk was

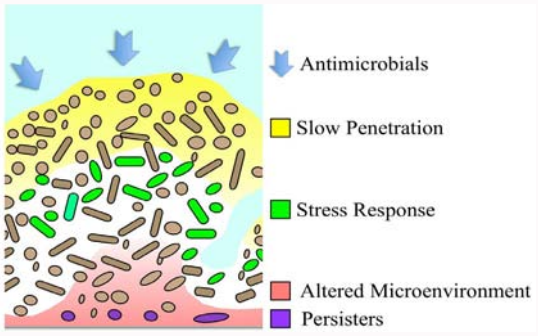

Figure 1: Mechanisms of biofilm tolerance. Antimicrobial penetration is retarded in the presence of EPS (yellow). The some microorganisms in the biofilm change their activity in response to antimicrobial stress (green). The microenvironment in deeper area is altered to resist eradicating (pink). Persister cells are present in deeper area of biofilm (violet). This image was modified from CBE Image Library by the Center for Biofilm Engineering at Montana State University.

disinfected completely with 70% isopropyl alcohol, and then returned to the reactor. The same bacterial strains in the logarithmic phase were then flowed into the reactor for 4 h. The number of secondary cells that adhered to the remaining structure was compared with those on a disk without structure, using CLSM analysis and quantitative analysis. Three-dimensional reconstruction revealed that viable bacteria appear to get caught on the upstream edges of the disinfected biofilm structure. The cryosectioned sample demonstrated stratified patterns of viable cells beside the structure. The mean viable count that adhered to the structure was significantly higher than that on the plane surface. Yamaguchi et al. [19] investigated the volume of *Porphyromonasgingivalis* adherent to the residual biofilm following a CHG treatment for 5 min using CLSM. It was shown that the amount of *P. gingivalis* adhering to the residual structure was greater than that on the non-structural surface. These results indicate that the residual biofilm could serve as a scaffold for secondary biofilm formation.

Antimicrobial-Induced Biofilm Formation

Numerous studies have shown that subminimal inhibitory concentrations (sub-MICs) of various antibiotics and chemicals can inhibit biofilm formation. A representative example is macrolide antibiotics. In the case of *Pseudomonas aeruginosa*, which contributes to the progression of respiratory infection, the bacterium shows a resistance to the macrolide azithromycin. However, in spite of this resistance, low-dose azithromycin has been shown to inhibit protein synthesis [28] and improve clinical symptoms [29,30]. It has also been shown that sub-MICs of azithromycin inhibited quorum sensing and alginate production [31,32]. In contrast to the inhibitory effects of sub-MIC antimicrobials against biofilm formation, recent studies have shown that some antibiotics at sub-MIC can significantly promote biofilm formation by a variety of bacterial species such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Escherichia coli*, and *P. aeruginosa* [33]. Kaplan et al. demonstrated that biofilm formation of some strains of *S. aureus* significantly increased in the presence of four different β-lactam antibiotics at sub-MIC [34]. The amount of biofilm induction was 10-fold at its maximum, and sub-MIC β-lactamantibiotics induced autolysin-dependent extracellular DNA release. This phenomenon is of great clinical significance because bacteria are exposed to sub-MIC of antibiotics at the beginning and end of a dosing regimen [35]. In addition, antimicrobials are retarded in order to diffuse within the biofilm matrix [36,37]. In such cases, the bacteria in deeper areas are exposed to antimicrobials at sub-MICs. As for oral biofilm, some studies reported that sub-MICs of antimicrobial agents up regulate the genes related to pathogenicity [38-40]. A summary of representative experiments is shown in Table 2. Even in limited works with regard to oral biofilms, it is likely that a short exposure time of antimicrobial agents in the oral cavity sometimes causes adverse effects because the microorganisms that survived exposure to the agents will alter gene expression in both positive and negative ways.

Detachment-Promoting Agents

Overall, shifting the focus from the bacteria-targeting to the matrix-targeting approach seems a reasonable strategy for controlling oral biofilms. One possible strategy is to use a type of enzyme to degrade the EPS [41-45]. For example, glycoside hydrolase dispersin B has been shown to degrade poly-N-acetyl-glucosamine polymers, inhibit biofilm formation, and detach established biofilm colonies [41,45]. Another possible strategy is to use a compound that interferes with genes related to EPS production [46-49]. We have recently reported that vizantin, animmuno stimulating compound, caused structural degradation as a result of changing gene regulation related to bacterial adhesion and glucan production of *S. mutans* [49]. Vizantin did not affect either bacterial growth or biofilm formation, whereas the biofilm developed in the presence of 50 μM sulfated vizantin was readily detached from the surface. Furthermore, biofilm development on a hydroxyapatite disk coated with sulfated vizantin was inhibited depending on the concentration, suggesting prevention from bacterial adhesion. Among eight genes related to bacterial adherence in *S. mutans*, the expression of *gtfB* and *gtfC* was significantly up regulated, whereas the expression of *gtfD*, *GbpA*, and *GbpC* was down regulated according to the concentration. This novel immune stimulating compound may be useful in a matrix-targeting approach. However, a control strategy using an enzyme and interfering with exopolysaccharide synthesisis limited in terms of targeting a specific polysaccharide component. Further investigations are needed to explore the possibility of clinical applications of these detachment-promoting agents as the biofilm is composed of EPS produced by a variety of microorganisms.

Conclusion

Numerous and diverse microorganisms reside in the intraoral environment, and we all coexist with oral biofilm. Thus, chemical controls for oral biofilm are entirely different from approaches toward conventional medical pathogens. The aim of the chemical approach is to control oral biofilm rather than eradicate it, thus preserving the benefits of the normal resident oral microflora.

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