Extracellular Dextran and DNA Affect the Formation of *Enterococcus faecalis* Biofilms and Their Susceptibility to 2% Chlorhexidine

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Abstract

Introduction: *Enterococcus faecalis* is frequently recovered from root-filled teeth with refractory apical periodontitis. The ability of *E. faecalis* to form a matrix-encased biofilm contributes to its pathogenicity; however, the role of extracellular dextran and DNA in biofilm formation and its effect on the susceptibility of the biofilm to chlorhexidine remains poorly understood. **Methods:** *E. faecalis* biofilms were incubated on dentin blocks. The effect of a dextran-degrading enzyme (dextranase) and DNase I on the adhesion of *E. faecalis* to dentin was measured using the colony-forming unit (CFU) counting method. CFU assays and confocal laser scanning microscopy were used to investigate the influence of dextranase and DNase I on the antimicrobial activity of 2% chlorhexidine. **Results:** The CFU count assays indicated that the formation of biofilms by *E. faecalis* was reduced in cells treated with dextranase or DNase I compared with that in untreated cells ($P < .05$). In addition, we found that treating *E. faecalis* biofilms with dextranase or DNase I effectively sensitized the biofilms to 2% chlorhexidine ($P < .05$). **Conclusions:** Both dextranase and DNase I decrease the adhesion of *E. faecalis* to dentin and sensitized *E. faecalis* biofilms to 2% chlorhexidine. *(J Endod 2012;38:894–898)*

Key Words

Biofilm, chlorhexidine, dextranase, DNase I, *Enterococcus faecalis*

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Enterococcus faecalis is the dominant species isolated from root canals with persistent endodontic infections (1, 2). It is responsible for many endodontic failures because of its inherent antibiotic resistance, adaptability to harsh environmental changes, and ability to penetrate deeply into dentinal tubules (3). *E. faecalis* can adhere to root canal walls where it can accumulate and form biofilms. Biofilm formation helps this species to rapidly acclimate to changing growth conditions and to survive in the presence of high concentrations of antimicrobial agents (4, 5). The bacteria in biofilms are up to 1,000-fold more resistant to antimicrobial agents than their planktonic form (6). Although some aspects of biofilm resistance are poorly understood, the dominant mechanisms are thought to involve extracellular polymeric substances (EPSs), which act as a barrier to both effectors of the immune system and antimicrobial agents (7).

EPSs provide biofilms with mechanical stability, and it has been suggested that EPSs can interact with antibiotics in a manner that reduces antibacterial activity. In addition, EPSs are thought to be involved in drug tolerance (8, 9). Recently, dextran and extracellular DNA (eDNA) have been found in the matrix of *E. faecalis* biofilms, suggesting that dextran and eDNA are involved in the development of bacterial communities (10–12). Dextran is a class of extracellularly formed glucans produced by bacteria. The eDNA in *E. faecalis* biofilms is presumably derived from cell lysis. Some studies have suggested that the interplay of 2 secreted and coregulated proteases, GelE and SprE, is responsible for regulating the autolysis and release of eDNA (11, 13). However, little is known about the role of EPSs in *E. faecalis* biofilms attached to dentin.

Irrigation is a crucial process for eliminating microorganisms from the root canal system. Because of its substantive antimicrobial activity, chlorhexidine (CHX) is used in irrigation solutions during root canal therapy (14, 15). In various *in vivo* and *in vitro* studies, CHX is effective in reducing or eliminating *E. faecalis* from the root canal space and dentinal tubules (16). However, CHX is less effective against biofilms compared with planktonic cultures (17). This appears to be related to the poor penetration of the irrigation solution through the matrix-encased biofilm (18). Therefore, to remove biofilms, it is necessary to develop strategies that not only counteract microorganisms but also affect the matrix. In the present study, we assessed the effect of 2 EPS-degrading enzymes (dextran and eDNA) on the formation of *E. faecalis* biofilms and the sensitivity of these biofilms to 2% CHX.

Materials and Methods

Bacteria and Culture Conditions

E. faecalis (American Type Culture Collection 29212; Guangdong Provincial Key Laboratory of Microbiol Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou, China) was used in this study. *E. faecalis* was removed from −80°C stocks, plated onto brain-heart infusion (BHI) plates that were supplemented with 1.5% agar, and incubated aerobically at 37°C for 24 hours. The expected colony, cell morphology, and Gram stain were verified.

A single colony from a BHI agar plate was used to inoculate an overnight BHI liquid culture. The cultures were grown aerobically at 37°C for 16 hours. The density of the culture was measured spectrophotometrically and then diluted into fresh BHI medium at a density of 1×10^7 colony-forming units (CFUs) per milliliter (19).

Enzymes

Dextranase (Sigma, St Louis, MO) with an activity of 25 U/mg and bovine pancreatic DNase I (Sigma) with an activity of 2,200 Kunitz units per milligram were used in the present study.

Specimen Preparation

The dentin block models used in this experiment were prepared as previously described (20) with slight modifications. In brief, healthy human third molars were extracted from 18- to 25-year-old adults at the Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, SunYat-Sen University, Guangzhou, China. Informed consent was obtained from each subject, and the protocols were approved by the university's ethics committee. The freshly obtained teeth were split longitudinally, and the coronal pulp chamber surfaces were cut into blocks (4 mm × 4 mm × 0.2 mm) without scraping using a hard-tissue cutting machine (Buehler, Chicago, IL). The dentin blocks were washed with distilled water, ultrasonically cleaned, autoclaved, stored in 10 mmol/L phosphate-buffered saline (PBS) at 4°C, and used within 1 week.

The Effect of Dextranase and DNase I on Biofilm Formation

Dentin blocks were placed in 24-well polystyrene cell culture plates with the pulpal surfaces facing upward. Each well was inoculated with a 1-mL suspension of *E. faecalis* (1×10^7 CFU/mL). For the experimental groups, the dentin blocks were treated with 1 mL BHI containing 40 μg/mL dextranase or 100 μg/mL DNase I. Control samples were treated with 1 mL BHI alone. The plates were left undisturbed in an anaerobic incubator at 37°C for 1, 12, 24, or 48 hours (n = 12). The culture media was replaced every 24 hours. At the end of the incubation, each block was aseptically removed and washed twice with PBS while shaking on a shaker (Unitwist RT; Uniequip Company, Laborgeratebau & Vertriebs GmbH, Martinsried, Germany) for 30 seconds to remove loosely adherent cells. The blocks were then incubated in 1 mL cysteine peptone water for 1 minute (21). Biofilm samples were harvested using sonication (42 kHz) for 5 minutes. The bacteria were serially diluted, and each dilution was plated onto a BHI plate. The plates were then incubated in an anaerobic atmosphere at 37°C for 48 hours, and the number of CFUs per square millimeter was calculated.

The Effect of Dextranase and DNase I on the Susceptibility of 48-hour-old Biofilms to CHX

The formation of *E. faecalis* biofilms on dentin was performed as described previously. To test the effect of dextranase and DNase I, 48-hour-old dentin biofilms were rinsed 3 times with 1 mL PBS buffer and then added to 1 mL BHI containing 40 μg/mL dextranase or 100 μg/mL DNase I. Control samples were treated with 1 mL BHI alone. After incubating for 1, 6, 10, 30, 60, or 180 minutes at 37°C (n = 12), 1 mL 2% CHX was added to each well, and the biofilms were incubated for an additional 5 minutes at room temperature. The control dentin biofilms were treated with 1 mL PBS. The biofilms were washed 3 times in PBS solution, 1 minute for each wash, to remove the CHX. The CFU assay was performed as previously described.

Confocal Scanning Laser Microscopy

Dentin biofilms were grown for 48 hours as described previously. The biofilms were incubated for 10 minutes with EPS-degrading enzymes before adding 2% CHX or BHI medium as a control. All confocal microscopy was performed with an LSM 710 Meta laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) using a 63× oil immersion objective. The images were acquired, and 3-dimensional (3D) renditions of the biofilms were reconstructed using LSM Image Examiner software (Carl Zeiss). For the microscopy, washed dentin blocks were stained for 15 minutes in the dark at room temperature with 50 μl L-7012 LIVE/DEAD BacLight TM bacterial cell stains (Molecular Probes Inc, Eugene, OR). Live and dead cells in the biofilms were differentiated by staining with SYTO9 (green fluorescence) (Invitrogen, Carlsbad, CA) and propidium iodide (red fluorescence), respectively. Excess dye was removed by aspiration, and the biofilms were washed twice with PBS. The stained samples were mounted onto glass coverslips using an antifade solution before image acquisition. Confocal illumination was performed using an argon laser (488-nm laser excitation) fitted with a long-pass 515/30 filter for the green fluorescence signal and a 605/75 filter for the red fluorescence signal. Simultaneous dual-channel imaging was used to display green and red fluorescence. The fluorescence intensity profiles of dead and live bacteria were also analyzed using the LSM Image Examiner software. Confocal laser scanning microscopic images were processed to remove background fluorescence and quantify cells. The percentage of living cells was calculated to determine the spatial distribution of live/dead bacteria in the dentin biofilms. For each group, 12 dentin blocks were used for image analysis. The experiment was repeated at least 3 times.

Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS for Windows; SPSS Inc, Chicago, IL). To examine the effect of dextranase and DNase I on dentin biofilm formation, the CFU counts were analyzed using the analysis of variance method for a 4 × 3 factorial design. To examine the effect of dextranase and DNase I on 48-hour-old biofilms killed by CHX, the reduction in viable cells was calculated using a variance for a 6 × 3 factorial design. The image stacks acquired by confocal laser scanning microscopy were analyzed using 1-way analysis of variance and the Kruskal-Wallis test. $P < .05$ was considered to be statistically significant.

Results

Dextranase and DNase I Decreased the Adhesion of *E. faecalis* to Dentin

Figure 1 shows that both dextranase and DNase I decreased the adhesion of *E. faecalis* to dentin at all the selected time points ($P < .05$, n = 12). The formation of *E. faecalis* biofilms was significantly inhibited by dextranase and DNase I when compared with cells in an unsupplemented medium. The percentages of adherent bacteria after 1 hour of dextranase or DNase I treatment were 15.61% and 18.39%, respectively, which was lower than the control group. There was no significant difference between the dextranase and DNase I

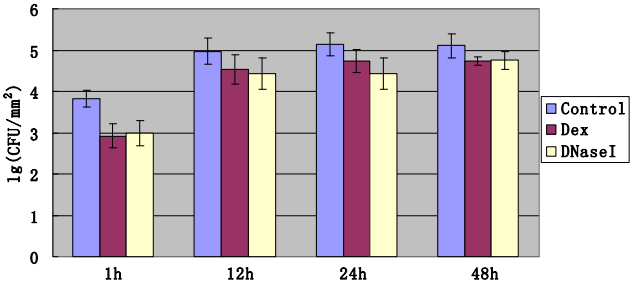

Figure 1. DNase I and dextranase affect the adhesion of *E. faecalis* to dentin (n = 12). The formation of *E. faecalis* biofilms was significantly inhibited by dextranase and DNase I compared with cells in an unsupplemented medium ($P < .05$).