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Plasminogen coating increases initial adhesion of oral bacteria *in vitro*

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ABSTRACT

Plasminogen is a major plasma protein and the zymogen of the broad spectrum protease plasmin. Plasmin activity leads to tissue degradation, direct and through activation of metalloproteinases. Infected tooth root canals, as a consequence of the inflammatory response and eventual necrosis, contain tissue fluid and blood components. These will coat the root canal walls and act as conditioning films that allow bacterial biofilms to grow and be a potential source of hematogenously spreading bacteria. We investigated the effect of *in vitro* surface conditioning with human plasminogen on the initial adhesion of bacteria. Four bacterial species, *L. salivarius, E. faecalis, A. naeslundii, and S. gordonii*, isolated from dental root canals, and three other oral streptococci, *S. oralis, S. anginosus, and S. sanguinis*, were grown in albumin- or plasminogen-coated flow chambers and studied by confocal laser scanning microscopy using the cell viability staining LIVE/DEAD and 16S rRNA fluorescence in situ hybridization (FISH). *A. naeslundii*, *L. salivarius* and in particular *S. gordonii* showed a higher initial adhesion to the plasminogen-coated surfaces. *E. faecalis* did not show any preference for plasminogen. Four-species biofilms cultured for 96 h showed that streptococci increased their proportion with time. Further experiments aimed at studying different streptococcal strains. All these adhered more to plasminogen-coated surfaces than to albumin-coated control surfaces. The specificity of the binding to plasminogen was verified by blocking lysine-binding sites with epsilon-aminocaproic acid. Plasminogen is thus an important plasma component for the initial adhesion of oral bacteria, in particular streptococci. This binding may contribute to their spread locally as well as to distant organs or tissues.

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1. Introduction

Plasminogen is a major plasma protein and the zymogen of the powerful broad spectrum protease plasmin. Plasmin activity leads to fibrinolysis as well as tissue degradation directly and also indirectly through the activation of MMPs. Plasmin is thus involved in tissue degradation and protease activation in inflammation as well as spread of cancer and physiological tissue remodeling and cell migration [10].

Many microorganisms, including oral streptococci, have the ability to bind plasminogen to their surface via lysine-dependent mechanisms that are very specific for plasminogen [11,18]. This binding provides the bacteria with a host-derived mechanism that allows them to migrate through tissues, a property that has been shown to be of importance to their virulence [33].

Oral bacteria have been found to cause bacteremia and infections and other pathological conditions in distant locations [13,14] and plasminogen binding has been found to be an important factor for their capacity to spread [3,15,34,36,37,40].

Bacterial biofilms formed in necrotic tooth root canals are believed to be the main cause of chronic endodontic lesions. The development of bacterial biofilms in root canals seems to follow essentially the same sequence of events as at other environmental sites, i.e. adhesion, microcolony formation, and structural maturation [25,31]. The capacity of a microorganism to adhere to an exposed substratum is the main step leading to biofilm formation and it depends on the bacterial cell surface properties and on the nature of the surface and conditioning [1,17]. When a clean, abiotic surface is exposed to a complex physiological fluid, a conditioning film will form almost instantaneously [38]. This conditioning layer may in turn influence the extent of bacterial adhesion and biofilm formation [1,17].

In infected dental root canals, surfaces are exposed to complex physiological fluids. At a site of inflammation the tissue content of plasma constituents is increased due to transudation. When the tissue in an infected dental pulp chamber becomes necrotic, the

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inner dentin surface becomes exposed to plasma proteins, including the potent zymogen plasminogen, that will form active conditioning films paving the way for subsequent microbial colonization. The presence of plasminogen-binding structures on certain bacteria might increase their ability to adhere to such surfaces. The colonization of internal tooth surfaces may provide a depot of potentially systemically spreading bacteria. The term atopobiosis has been coined for the occurrence of bacteria in places where they are normally not found and this has been recognized as a possible basis for pathology [30].

The aim of this study was to investigate the influence of plasminogen coating on initial bacterial adhesion. Clinical root canal isolates of *Enterococcus faecalis*, *Lactobacillus salivarius*, *Actinomyces naeslundii*, and *Streptococcus gordonii* and three other oral streptococci, *S. oralis, S. anginosus, and S. sanguinis*, were grown anaerobically in a mini-flow chamber system coated with human plasminogen.

2. Materials and methods

2.1. Bacterial strains and culture

The bacterial strains used in the present study, *E. faecalis*, *L. salivarius*, *A. naeslundii*, and *S. gordonii*, were isolated from dental root canals, undergoing endodontic treatment, that presented with persistent infections [6,7]. Three additional strains of oral streptococci were also used; *S. oralis, S. anginosus, and S. sanguinis*. All strains were routinely cultured on blood agar plates and Todd-Hewitt broth (TH) in an anaerobic atmosphere of 10% H_2 and 5% CO_2 in hydrogen at 37 °C. Overnight bacterial cultures were harvested by centrifugation and washed twice with TH broth, and the optical density at 600 nm was adjusted to 0.6, equivalent of about 1×10^8 cells/mL before the start of each experiment.

2.2. Preconditioning of flow chambers

Mini flow chamber system μ-Slide VI for Live Cell Analysis (Ibidi®, Integrated BioDiagnostics) with hydrophilic surfaces were precoated with human plasminogen (25 μg/mL in PBS; Enzyme Research Laboratories) or albumin (40 mg/mL in PBS), respectively, for 1 h at room temperature.

2.3. Biofilm formation

Each flow chamber cell was inoculated with 185 μL of bacterial suspension and incubated in an anaerobic atmosphere at 37 °C for 2 h or 96 h.

2.4. 16S rRNA fluorescent in situ hybridization (FISH)

The root canal four-species consortium was grown as above and analyzed with FISH after 2 h and 96 h incubation. After removal of the supernatants, the flow chambers were washed with PBS, and the bacteria fixed with 4% formalin.

The identity of the different bacterial species was verified through 16S rRNA fluorescent in situ hybridization (FISH). *E. faecalis* was identified using ENF191 probes (GAAAGCGCCTTTCACTCT-TATGC) [41] fluorescently labeled with Pacific Blue (blue) and ATTO-488 (green) respectively. *L. salivarius* was identified using the LAC722 probe (YCACCGCTACACATGRAGTTCCA CT) [32] fluorescently labeled with ATTO-565 (red). *A. naeslundii* was identified using JF201 probes (GCTACCGTCAACCCACCC) [12] fluorescently labeled with Pacific Blue (blue) and ATTO-565 (red) respectively. Streptococci were identified using the STR405 probe (TAGCCGTCCCTTTCTGGT) [28] fluorescently labeled with ATTO-488 (green) or dual-labeled with cyanine3 and ATTO-488 yeilding yellow fluorescence.

The 16S rRNA FISH protocol was performed as previously described [29]. In brief, biofilm cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized using a lysozyme solution 10 mg/mL. The biofilms were then washed with ultra-pure water and dehydrated with 50%, 80% and 99% ethanol for 3 min each, after which 30 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl buffer, pH 7.5, with 0.01% sodium dodecyl sulfate and 25% formamide) containing 20 ng/mL of the oligonucleotide probes was added to the flow cells. Hybridization was performed at 48 °C for 90 min in a humid chamber.

2.5. Study of adhesion to albumin and plasminogen

The effect of the surface-coating proteins on initial adhesion was studied after 2 h biofilm formation. The biofilm bacteria were stained with Live/Dead BacLight Bacterial Viability kit for microscopy (Molecular Probes) or SYTO®62 red fluorescent nucleic acid stain (Life Technologies) and viewed using confocal scanning laser microscopy (see below).

Ten or 15 images (from two or three flow chambers) were randomly obtained and analyzed in each experiment. The experiments were performed in triplicate. The images were analyzed as described below.

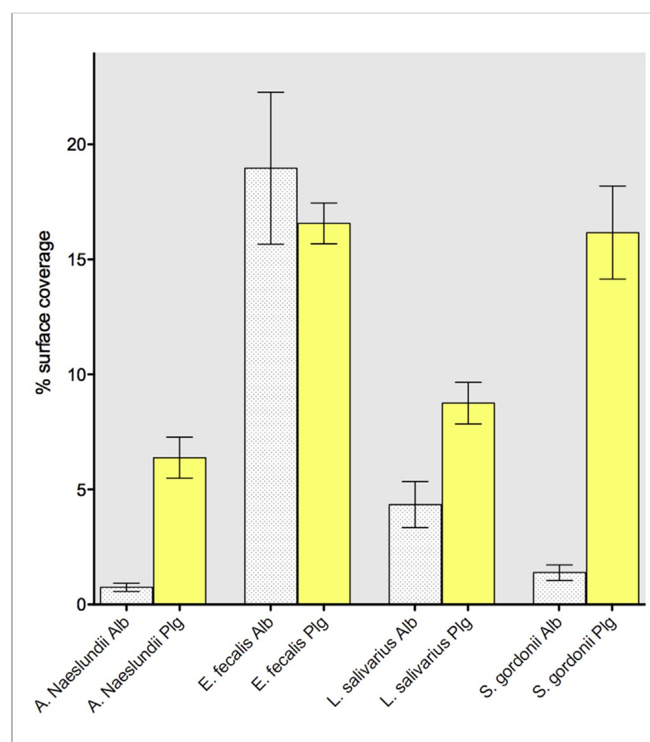

Fig. 1. Adherence of four species of bacteria, *Actinomyces naeslundii* (AN), *Enterococcus fecalis* (EF), *Lactobacillus salivarius* (LS) and *Streptococcus gordonii* (SG) to Ibidi® flow cells coated with albumin (Alb; white bars) and plasminogen (Plg; yellow bars) respectively. The graph shows percent surface area coverage after 2 h incubation (mean ± 95% CI). Data from ten images per culture well and experiment. Mean of three experiments. Mann Whitney test for the comparison between albumin and plasminogen coating gave $p < 0.0001$ for *Actinomyces naeslundii*, $p = 0.74$ for *Enterococcus fecalis*, $p < 0.0001$ for *Lactobacillus salivarius* and $p < 0.0001$ for *Streptococcus gordonii* (SG). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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