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Adhesion of *Porphyromonas gingivalis* and *Tannerella forsythia* to dentin and titanium with sandblasted and acid etched surface coated with serum and serum proteins – An *in vitro* study

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ABSTRACT

Objective: To evaluate the adhesion of selected bacterial strains incl. expression of important virulence factors at dentin and titanium SLA surfaces coated with layers of serum proteins.
Methods: Dentin- and moderately rough SLA titanium-discs were coated overnight with human serum, or IgG, or human serum albumin (HSA). Thereafter, *Porphyromonas gingivalis, Tannerella forsythia,* or a six-species mixture were added for 4 h and 24 h. The number of adhered bacteria (colony forming units; CFU) was determined. Arg-gingipain activity of *P. gingivalis* and mRNA expressions of *P. gingivalis* and *T. forsythia* proteases and *T. forsythia* protease inhibitor were measured.
Results: Coating specimens never resulted in differences exceeding 1.1 log10 CFU, comparing to controls, irrespective the substrate. Counts of *T. forsythia* were statistically significantly higher at titanium than dentin, the difference was up to 3.7 log10 CFU after 24 h ($p = 0.002$). No statistically significant variation regarding adhesion of the mixed culture was detected between surfaces or among coatings. Arg-gingipain activity of *P. gingivalis* was associated with log10 CFU but not with the surface or the coating. Titanium negatively influenced mRNA expression of *T. forsythia* protease inhibitor at 24 h ($p = 0.026$ uncoated, $p = 0.009$ with serum).
Conclusions: The present findings indicate that: a) single bacterial species (*T. forsythia*) can adhere more readily to titanium SLA than to dentin, b) low expression of *T. forsythia* protease inhibitor may influence the virulence of the species on titanium SLA surfaces in comparison with teeth, and c) surface properties (*e.g.* material and/or protein layers) do not appear to significantly influence multi-species adhesion.

1. Introduction

Teeth are continuously exposed inside the surrounding sulcus to a transudate (during homeostasis) or to an exudate during inflammation, called gingival crevicular fluid (GCF), (Griffiths, 2003). Resting volume of GCF is between 0.05 μl and 2 μl depending on the periodontal disease status (Goodson, 2003). Similarly to teeth, oral implants are surrounded by *peri*-implant sulcular fluid (PISF), the volume of which has been determined to be 0.5 μl in health and about 1.3 μl in disease (Arikan, Buduneli, & Lappin, 2011).

The components of GCF derive from many sources, such as serum, connective tissue and epithelium, as well as from inflammatory cells and bacteria which are present in the crevice and the surrounding soft tissues (Griffiths, 2003). Research on GCF protein concentration has suggested a composition similar to that of serum (Tew, Marshall, Burmeister, & Ranney, 1985), and serum proteins, *e.g.* albumin, transferrin and immunoglobulin (Ig)G are incorporated in the pellicles formed on teeth *in vivo* (Carlen, Rudiger, Loggner, & Olsson, 2003). Despite the fact that no exact data about the protein composition in PISF are available, it appears to be similar to GCF (Ozcakir-Tomruk, Chiquet, & Mericske-Stern, 2012).

Although most of the microbiota colonizing the oral cavity is considered being commensal, the presence of specific bacteria, like *Porphyromonas gingivalis*, *Treponema denticola, Tannerella forsythia*

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(*i.e.* "the red complex"), and *Aggregatibacter actinomycetemcomitans*, is strongly associated with an increased risk for periodontitis (Socransky, Haffajee, Cugini, Smith, & Kent, 1998). In particular, *P. gingivalis* is considered a key-stone pathogen due its ability of causing dysbiosis between the host and the oral biofilm (Hajishengallis, Darveau, & Curtis, 2012). Major virulence factors of *P. gingivalis* are its arginine-specific (HRgpA and RgpB; encoded by two genes *rgpA* and *rgpB*) and lysine-specific (Kgp, encoded by *kgp*) cysteine proteases, also called gingipains (Guo, Nguyen, & Potempa, 2010). *T. forsythia* encodes also several proteases (Ksiazek, Mizgalska, Eick et al., 2015), which alike gingipains are secreted *via* a novel Type IX secretion system (T9SS) (Tomek et al., 2014). Moreover it encodes a bacterial serpin (called miropin) that inhibits several host proteases (Ksiazek, Mizgalska, Enghild et al., 2015).

In general, it appears that there are major similarities in the microbioms associated with periodontitis and *peri*-implantitis (Kumar, Mason, Brooker, & O'Brien, 2012). Further, it has been demonstrated, that, as it occurs on natural teeth (Lee, Tanner, Maiden, & Weber, 1999), periodontopathogens colonize implants immediately after placement (Furst, Salvi, Lang, & Persson, 2007; Quirynen et al., 2006). In this context, the initial stages of biofilm formation (Jakubovics & Kolenbrander, 2010) involve bacterial adhesion to the pellicle-coated surfaces and it has been shown *in vitro* that adherence of bacteria associated with gingivitis and periodontitis is mediated by plasma proteins in the pellicle (Carlen et al., 2003). In a recent *in vitro* study, it has been recognized that caries associated bacteria (*i.e.*, *Streptococcus mutans* and *Streptococcus mitis* biovar 2) exhibit a marked upregulation in their proteolytic activity when in contact with salivary proteins coated on a surface, despite very dissimilar patterns of adhesion to the same protein (Kindblom, Davies, Herzberg, Svensater, & Wickstrom, 2012). There is limited information, however, on how different protein coatings on different substrates may influence attachment and/or protease activity of bacteria associated with periodontitis and/or *peri*-implantitis.

Therefore, the aims of this *in vitro* study were: a) to evaluate the adhesion of single strains of *P. gingivalis, T. forsythia,* and of a six-species mixture including *P. gingivalis* and *T. forsythia* and in addition two early colonizers (*Streptococcus gordonii*, *Actinomyces naeslundii*), and two bridging species (*Fusobacterium nucleatum*, *Parvimonas micra*) of oral biofilms on dentin and titanium after having been coated with protein components of gingival crevicular fluid and *peri*-implant sulcular fluid, and b) to analyze the mRNA expression of major virulence factors of *P. gingivalis* and *T. forsythia* in dependence of material and serum-coating.

2. Material & methods

2.1. Bacterial strains

All used bacterial strains were reference strains. As single strains were tested *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037. The six-species mixture consisted of the mentioned *P. gingivalis* and *T. forsythia* strains with the addition of *S. gordonii* ATCC 10558 and *A. naeslundii* ATCC 12104 as early colonizers, and *F. nucleatum* ATCC 25586 and *P. micra* ATCC 33270 as bridging species. Before each experiment, all strains were precultivated on Schaedler agar plates (Oxoid, Basingstoke, UK) with 5% sheep blood and vitamin K addition, in an anaerobic atmosphere or with 5% CO_2 (*S. gordonii* ATCC 10558). Thereafter, suspension was prepared by adding bacteria in 0.9% w/v NaCl equal to $OD_{600nm} = 1.0$. In case of mixed cultures, suspensions of the strains were mixed by adding 1 part of *S. gordonii* ATCC 10558 to each two parts of the other strains. Bacterial suspension was given to Wilkins-Chalgren broth (Oxoid, Basingstoke, UK) supplemented with 5 mg/l β-NAD in a ratio 1:50.

2.2. Test specimens

The test specimens were dentin discs from extracted human teeth and moderately rough titanium discs (SLA, Institute Straumann AG, Basel, Switzerland), with a diameter of 5 mm and a thickness of about 1 mm.

Extracted teeth were collected after the patients had signed an informed consent regarding using their teeth in *in vitro* experiments. According to the Ethical Commission of the Canton Bern, an approval for anonymous use of these by-pass products is not needed. After extraction, teeth were placed in chloramine solution for disinfection for 2 h. Thereafter, they were stored in 0.9% v/w NaCl solution and processed within 2–3 weeks. After crown removal, dentin slices of the appropriate size were cut with a diamond saw. Surface properties were standardized by grinding the dentin specimens with silicon carbide papers of #2400 grit size, corresponding to an abrasive particle size of 6.5 μm (Struers A/S, Ballerup, Denmark).

2.3. Coatings

Human serum (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was heat-inactivated to exclude complement-activity and used in a 25% concentration in 0.9% w/v NaCl. IgG (Sigma-Aldrich Chemie GmbH) was diluted to 5 mg/ml and human serum albumin (Sigma-Aldrich Chemie GmbH) to 10 mg/ml, each with 0.9% w/v NaCl. The control was 0.9% w/v NaCl.

2.4. Adhesion assay

Dentin and titanium discs were placed in a 24 well plate and incubated at room temperature overnight with 25% human serum, 5 mg/ml IgG, 10 mg/ml HSA or 0.9% w/v NaCl (controls). Thereafter, 500 μl bacterial suspension mixed with Wilkins-Chalgren broth was added to each well. After incubation for 4 h or 24 h at 37 °C in anaerobic conditions, the test specimens were transferred to tubes containing 100 μl of 0.9% w/v NaCl. Then, test specimens were subjected to 5 min of ultrasonication. (Preliminary tests have confirmed that by using this procedure viability of bacteria is not influenced and the overwhelming majority of bacteria is detached from the surface.) From these mixtures, in case of *P. gingivalis* one part was immediately frozen (−20 °C) for later evaluation of arginine-specific amidolytic activity. Second, serial dilutions were made and defined volumes were spread on Schaedler agar plates. Agar plates were incubated in the respective atmosphere before the total counts of the colony forming units (CFU) were enumerated. Counting of CFU was performed using an Acolyte colony counter and Acolyte version 3.04 software (Symbiosis, Cambridge, UK). In addition in the multi-species mixtures, the counts of *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037 were determined by using real-time PCR as described recently (Eick, Straube, Guentsch, Pfister, & Jentsch, 2011).

2.5. Determination of the arginine-specific amidolytic activity

Samples taken from the *P. gingivalis* experiments were tested for arginine-specific amidolytic activity by using a chromogenic substance: *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) (Sigma, St. Louis, MO, USA) with a final concentration of 2 mM in the assay buffer (0.2 M Tris-HCl, 0,1 M NaCl, 5 mM $CaCl_2$, pH 7.6, freshly supplemented with cysteine hydrochloride solution (10 mM)) that had 20% DMSO. Ten μl of the samples were mixed with 10 μl of the substrate solution. The absorbance was read at 405 nm (37 °C) at 30 s intervals for 2 h by using a spectrophotometer (BioTek EL808, BioTek, Luzern, Switzerland).

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