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Bacterial profiles and proteolytic activity in peri-implantitis versus healthy sites

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ABSTRACT

Peri-implantitis is a biofilm-induced destructive inflammatory process that, over time, results in loss of supporting bone around an osseointegrated dental implant. Biofilms at peri-implantitis sites have been reported to be dominated by Gram-negative anaerobic rods with a proteolytic metabolism such as, *Fusobacterium*, *Porphyromonas*, *Prevotella* and *Tannerella*, as well as anaerobic Gram-positive cocci. In this study, we hypothesized that protease activity is instrumental in driving bone destruction and we therefore compared the microbial composition and level of protease activity in samples of peri-implant biofluid (PIBF) from 25 healthy subjects (H group) and 25 subjects with peri-implantitis (PI group). Microbial composition was investigated using culture techniques and protease activity was determined using a FITC-labelled casein substrate. The microbial composition was highly variable in subjects both in the H and PI groups but one prominent difference was the prevalence of *Porphyromonas/Prevotella* and anaerobic Gram positive cocci which was significantly higher in the PI than in the H group. A subgroup of subjects with peri-implantitis displayed a high level of protease activity in the PIBF compared to healthy subjects. However, this activity could not be related to the presence of specific bacterial species. We propose that a high level of protease activity may be a predictive factor for disease progression in peri-implantitis. Further longitudinal studies are however required to determine whether assessment of protease activity could serve as a useful method to identify patients at risk for progressive tissue destruction.

1. Introduction

In recent decades, the use of titanium dental implants to treat individuals with lost and missing teeth has increased rapidly. While implant therapy is usually successful, the presence of microbial biofilms on an implant surface can give rise to complications in terms of peri-implant infections and peri-implantitis. Peri-implantitis is a destructive inflammatory process that, over time, results in loss of supporting bone around an osseointegrated implant. Upon placement of a dental implant the abutment surface, which passes through the mucosa, rapidly becomes colonized by bacteria from saliva and the neighbouring oral surfaces. Colonization is initiated by adhesion of pioneer species, including *Streptococcus oralis*, *Streptococcus gordonii*, and *Actinomyces naeslundii*, through interactions with the salivary pellicle. These early colonizers facilitate the adherence of secondary colonizers by co-adhesion, and biofilm formation proceeds through growth and division of surface-associated microorganisms [1]. If the biofilm is allowed to spread on the abutment it may subsequently reach the implant surface. Within implant-associated biofilms, multiple species live in close physical contact and this increases the probability of microbial interactions, both synergistic and antagonistic, between microbial cells [2]. A clear relationship has been established between the formation of microbial biofilms on an implant surface and an inflammatory response in the host tissues [3].

Biofilms from healthy peri-implant sites are characterized by a low ratio of anaerobic to facultative anaerobic species with a high proportion of Gram-positive cocci [4,5]. In contrast, biofilms at peri-implantitis sites have been reported to harbour a microbiota with a large number of Gram-negative anaerobic rods (for a review see [6]) as well as anaerobic Gram-positive cocci for example

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Parvimonas and *Peptostreptococcus* [7] Several studies have indicated that the microbiota associated with peri-implantitis is similar to that found in cases of periodontal disease [8,9] and periodontitis has been identified as a risk factor for peri-implantitis [10]. The microbial communities at periodontitis sites have been shown to vary in terms of species composition and this has led to the concept of "microbial complexes" associated with periodontal disease. While the presence of members of the so-called 'red complex' (*Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola*) and 'orange complex' (*Fusobacterium spp*, *Prevotella spp*, *Parvimonas micra*, *Eubacterium spp* and *Streptococcus constellatus*) have been shown to have an association with periodontal disease [11] the finding that these bacteria can also be present in healthy individuals suggests that other mechanisms are also involved in disease etiology.

It is generally accepted that the composition of biofilms is dependent upon the local environment. In the periodontal pocket, the host inflammatory response to the accumulation of biofilms increases the flow of gingival exudate thus increasing the supply of host proteins and glycoproteins to the bacteria. The resulting nutrient flux gives proteolytic and anaerobic Gram-negative bacteria a competitive advantage, leading to a disruption in the balance of organisms within the biofilm, which has been termed 'dysbiosis'. This concept is encapsulated in the 'ecological plaque hypothesis' [12,13]. As well as an increasing number of proteolytic bacteria, laboratory studies have shown that the protease activity in individual bacterial cells of, for instance *P. gingivalis*, is upregulated in response to proteins such as hemin [14] that are present in the gingival exudate. Thus a high protease activity in the gingival pocket would reflect a shift in both genotype and phenotype within the biofilm. In addition to providing peptides, amino acids and hemin for growth, bacterial proteases are important virulence factors since they can cause tissue damage and are instrumental in subversion and modulation of host defences. The most studied proteases produced by this group of bacteria are the gingipains from *P. gingivalis*, which have been shown to degrade extracellular matrix components and activate host matrix metalloproteinases as well as inhibiting complement [15].

Based on the ecological plaque hypothesis, we propose that the proteolytic phenotype of peri-implant biofilms from subjects with peri-implantitis would differ from that of healthy individuals. Therefore, in this study we have compared subjects with peri-implantitis and healthy individuals with regard to the prevalence of bacterial species and overall levels of protease activity in peri-implant biofluid.

2. Material and methods

2.1. Subjects and sampling procedure

This non-randomised, controlled, clinical exploratory study was approved by the local ethical committee at the University of Gothenburg, Sweden (Dnr: 652-10). The study was limited to a single evaluation time point. Participants were selected from patients previously rehabilitated with dental implants attending scheduled implant maintenance sessions at the Brånemark Clinic, Gothenburg, Sweden. The study included 25 subjects with peri-implantitis and 25 healthy subjects. The inclusion criteria for subjects in the peri-implantitis group (PI group) was at least three implants with (i) crater-shaped bone-loss on radiographs of $\geq$3 mm, (ii) bleeding on superficial probing according to the Mombelli modified Bleeding Index (mBI) [16] and (iii) suppuration upon palpation around the implants. The healthy group (H group) had no radiographic evidence of pathologic bone loss or signs of inflammation around their implants. Subjects who had taken antibiotics within three months prior to the proposed inclusion date were excluded. The investigator performing the clinical examination and sampling of peri-implant biofluid (PIBF) was not blinded to the study parameters but other people involved in sample analyses were blinded to subject identity and group (H and PI).

One implant per subject was selected at random for evaluation and, where necessary, supra-gingival plaque was removed before sampling. PIBF was aspirated from the base of the peri-implant sulcus/pocket using a metal suction tip (diameter 0.7 mm) connected to a 1 ml syringe. After sealing the end with a rubber plug, the suction tip containing the PIBF was transported to the laboratory for analysis within 24 h. On arrival 150 μL of phosphate buffer (PBS) (7 mM K_2HPO_4, 2.5 mM KH_2PO_4, 68 mM NaCl) reduced in 9% H_2 in N_2 for 24 h, (reduced PBS) was introduced into the suction tip containing the PIBF and the sample transferred to a sterile microfuge tube. Each sample was then divided and subjected to both analysis of microbial composition and proteolytic activity.

2.2. Cultivable microflora in PIBF – identification of selected bacterial cultivable taxa

The cultivable microflora in the PIBF sample was investigated by culturing on Brucella agar. Fifty μL of the sample was serially diluted in reduced PBS and 200 μL of each dilution plated on pre-reduced Brucella agar and distributed evenly using glass beads. Plates were incubated at 37 °C under anaerobic conditions (10% H_2 and 5% CO_2 in N_2) for 10 days. The total number of cultivable bacteria in the samples ranged from 10^3–10^7 CFU/sample confirming that the syringe sampling technique resulted in a sufficient amount of PIBF for a reliable assessment of the microbial composition. Bacterial taxa with the highest abundance were isolated and Gram stained. Black-pigmented colonies (*Porphyromonas/Prevotella*) as well as colonies with a morphology consistent with *Tannerella* and *Fusobacterium* were enumerated and isolated irrespective of their number. Gram-positive, catalase-negative, facultative anaerobic streptococci were identified to species level using biochemical tests [17,18]. Anaerobic Gram-positive cocci were identified based on Gram staining and growth under anaerobic conditions but not in 5% CO_2 in air. Members of the *F. nucleatum-periodontium* group were identified using the following criteria: colony morphology [large (5–7 mm), flat, irregular, shimmering colonies ranging in colour from yellow to purple/blue or low convex, white/grey, glistening colonies] and Gram staining (Gram-negative slender rods with sharply pointed ends). *Tannerella* was identified based on colony morphology [small (1–2 mm), grey/purple, colonies with a central ring-like depression], Gram staining (Gram-negative slender rods) and a positive reaction using a chromogenic substrate for trypsin (Na-Benzoyl-DL-Arginine p-Nitroanilide (Sigma Aldrich, St Louis, MO, USA). Black-pigmented bacteria (*Porphyromonas/Prevotella*) were identified based on colony morphology (glossy or matt black colonies) and Gram staining (small, Gram-negative, coccoid rods). Representatives of all the dominating taxa were recovered and stored in skimmed milk at −80 °C.

2.3. Measurement of protease activity

A broad-spectrum protease substrate FITC-casein (QuantiCleave Fluorescent Protease Assay kit, Pierce, Rockford, IL, USA) which, according to the manufacturer, is cleaved by serine-, cysteine- and metallo-proteinases, was used to measure protease activity [19]. To generate a standard curve, trypsin (Thermo Fisher Scientific, Waltham, MA, USA) diluted in PBS at concentrations ranging from 0.06 mg/L to 15 mg/L was mixed with 1 μL FITC-casein (5 mg/mL in 25 mM Tris, 150 mM NaCl, pH 7.2), and incubated for 1 h at 37 °C.