Analysis of early biofilm formation on oral implants in man

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SUMMARY Biofilm formation on oral implants can cause inflammation of peri-implant tissues, which endangers the long-term success of osseointegrated implants. It has been reported previously that implants revealing signs of peri-implantitis contain subgingival microbiota similar to those of natural teeth with periodontitis. The purpose of the first part of this study was an atraumatic, quantitative investigation of biofilm formation on oral implant abutments; the objective of the second part was to investigate whether *Haemophilus actinomycetemcomitans* and *Porphyromonas gingivalis* were present in the crevicular fluid around oral implants. Biofilm formation on 14 healing abutments, inserted for 14 days in 10 patients, was analysed quantitatively by use of secondary-electron and Rutherford-backscattering-detection methods. A 16S rRNA-based polymerase chain reaction detection method was used to detect the presence of *H. actinomycetemcomitans* and *P. gingivalis* in the crevicular fluid. For this investigation, samples of sulcus fluid were collected with sterile paper points at four measurement points per abutment. The difference between biofilm coverage of supragingival surfaces (17.5 ± 18.3%) and subgingival surfaces (0.8 ± 10%) was statistically significant (P < 0.05). By use of universal primers, bacteria were found in all the samples taken, although the two periodontal pathogens were not found in any of the samples. The absence of periodontal pathogens from the sulcus fluid during initial bacterial colonization, despite massive supragingival biofilm formation, substantiates the assumption that cellular adherence of peri-implant tissue by means of hemidesmosoma, actin filaments and microvilli reduces the risk of formation of anaerobic subgingival pockets.

KEYWORDS: biofilm, biomaterials, microbiology, periodontitis, polymerase chain reaction, oral implant, peri-implantitis

Accepted for publication 5 December 2006

Introduction

Use of osseointegrated oral implants has been shown to be an excellent method for replacement of missing teeth. In several studies, favourable long-term results after implant treatment have been published likewise by independent centres (1–6). Different factors, for example excessive occlusal force or bacterial accumulation on implant surfaces, can lead to oral implant failure (7, 8).

After exposure to the oral cavity, an acquired pellicle formed from salivary biopolymers becomes adsorbed on all soft and hard oral tissues (9). This pellicle forms the interface between the implant surface and initial microorganisms, for example *Streptococcus mitis*, *Streptococcus sanguis* and *Streptococcus oralis*. These bacterial microorganisms create the pre-conditions for adhesion of periodontal pathogens such as *Haemophilus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola* or *Tannerella forsythensis*, which can induce peri-implantitis with characteristic inflammation of the peri-implant mucosa and destruction of the peri-implant bone (6). It is assumed that gingiva surrounding natural teeth, and mucosa around implants, have the natural potential to prevent
subgingival biofilm formation and potential to respond to early biofilm accumulation (10–13). Protective mechanisms of this barrier include structural components, for example a well keratinized oral epithelium terminated at the crest of the gingival margin where it is continuous with non-keratinized sulcular epithelium and circular collagen fibres (14).

Research on osseointegrated oral implant surfaces usually entails harvesting the implants (14). Another method for investigation of bacterial colonization of titanium surfaces is to fix titanium discs on acrylic splints (15). However, position of these titanium discs in aerobic surroundings inhibits growth of periodontal pathogens such as *H. actinomyctemcomitans* and *P. gingivalis* in the biofilm. This method is further confounded by tongue and cheek activity. Extrapolation of results generated by use of this method on peri-implant structures is not possible.

As there is only a limited number of reports at the clinical level on the microbiology of the supra- and subgingival plaque on implant abutments (16), the objective of the present study was to investigate peri-implant biofilm accumulation atraumatically by use of implant abutments which were inserted temporarily and removed for biofilm investigation. In the first part of the study, a quantitative analysis in supra- and subgingival areas was performed. The purpose of the second part of the study was to investigate whether periodontal pathogens were present in the crevicular fluid around the implant abutments. This determination was focused on *H. actinomyctemcomitans* and *P. gingivalis*, the predominant microbiota causing periodontitis (17–19).

**Material and methods**

**Patients**

This study was approved by the ethics committee of Hannover Medical School (No. 3791). The examination was performed with the understanding and written consent of each subject.

Quantitative analysis was based on the 14 healing abutments which were inserted for 14 days into 10 patients (seven patients received one implant, two patients received two and one patient received three implants), four women and six men, aged between 18 and 75 years (mean 52 ± 21.2 years). Criteria for exclusion were history of periodontitis and probing depth of the remaining dentition more than 3 mm. In addition, samples of sulcus fluid were taken with sterile paper points at four measurement points per abutment from five randomly selected patients of this group, for the detection of *H. actinomyctemcomitans* and *P. gingivalis*. All subjects were partially edentulous and had at least one oral two-piece implant made of titanium, which had been inserted 3 months before investigation in the lower jaw and 6 months before investigation in the upper jaw. Two weeks after abutment surgery, the previously existing abutments were removed and the analysed healing abutments were inserted. No trimming of surrounding gingiva was performed. All patients were in good systemic health and none had received antibiotic therapy. They were instructed to continue their oral hygiene, but not to use antibacterial mouth rinsing.

**Quantitative analysis of biofilm formation**

Biofilm formation on healing abutments was analysed quantitatively by use of standard scanning-electron microscopy procedures, for example Rutherford-backscattering-detection (RBSD) and secondary-electron (SE) methods (LEO 1455 VP*). The SE pictures afforded a topographical overview, whereas the RBSD pictures were used for detection of biofilm-coated surfaces (Fig. 1a and b). The line of demarcation between supra- and subgingival regions was reproduced through previously taken silicon-impressions. Therefore, control healing-abutments were replaced into the impressions and subgingival located surfaces were marked with high-dispersive colour (Okkluspray†). Supra- and subgingival areas were distinguished in superposing the RBSD-pictures of the probe and the marked control.

The extent of coverage of supra- and subgingival surfaces with biofilm was calculated by using surface-analysis software (IMAGE J 10.2 for Apple‡) to differentiate grey areas.

**Microbial examinations**

A 16S rRNA-based polymerase chain reaction (PCR) detection method was used to determine the preval-

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ence of *H. actinomycetemcomitans* and *P. gingivalis* in the crevicular fluid. Samples of sulcus fluid were taken with sterile paper points at four measurement points per abutment (mesio-buccal, mesio-lingual/palatal, distobuccal, and distolingual/palatal). Sample sites were isolated with cotton rolls. After removal of peri-implant saliva by use of an air spray, sterile paper points were inserted for 10 s in the peri-implant sulcus. By use of forceps, potentially contaminated regions of the paper points were removed before storage of the paper points in Eppendorf tubes§ at −18 °C.

Purification of bacterial DNA was performed by use of the QIAamp® DNA Mini Kit from Qiagen¶, in accordance with a standard procedure for purification of the genomic DNA of bacteria. The pure bacterial DNA was stored at −18 °C. Water, dNTPs, 10× buffer, MgCl₂, and Taq-polymerase were used as Mastermix components for the polymerase chain reaction.

The DNA template and specific upstream and downstream primers were also added before starting the polymerase chain reaction according to a standard PCR program for the thermocycler**. PCR products were analysed by conventional gel electrophoresis.

The nucleotide sequences of the specific primers for *H. actinomycetemcomitans* and *P. gingivalis* are shown in Table 1 (20). Cross-reactions between specific primers and primer bonding at wrong sections were inhibited by using only one primer pair for any process. The presence of any bacterial DNA on the samples was verified by using universal primers targeting conserved regions of the 16S rRNA encoding genes. A positive control, what was the control for universal PCR, was included in all PCR reactions (*H. actinomycetemcomitans*: XY 766; *P. gingivalis*: XY 677).

**Statistical analysis**

Documentation and statistical analysis were performed by use of the data processing software SPSS/PC, version 13.0 for Windows††. Correlation between biofilm coverage and its location was calculated and compared by use of the Wilcoxon test. A *P*-value <0.05 was regarded as statistically significant.

**Results**

Biofilm was detected on all healing abutments after removal from the oral cavity. A total of 52.1% of the abutments surface areas were located supragingival and 47.9% were located subgingival. Figure 2 summarizes results from the quantitative analysis of biofilm formation with respect to location. Biofilm formation occurred on 17.5 ± 18.3% of supragingival surfaces and only on 0.8 ± 1.0% of subgingival surfaces; this difference was statistically significant (*P* < 0.05). This difference resulted in a line of demarcation between supragingival surfaces (Fig. 3). No bleeding of the peri-implant tissues

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was observed after insertion of the sterile paper points in the peri-implant sulcus.

After polymerase chain reaction with universal primers, a distinct band was obtained after gel electrophoresis for all the samples taken, although the two periodontal pathogens were not detected in any of the samples. Results from quantitative analysis of biofilm formation on the healing abutments investigated and from qualitative analysis of the peri-implant crevicular fluid are summarized in Table 2.

Discussion

In this study, quantitative analysis of biofilm formation was performed on healing abutments, because they exhibit a subgingival area with an anaerobic environment. This anaerobic environment enables formation of the same biofilm as during development of periodontitis. A second advantage of the use of healing abutments was the possibility of atraumatic removal. In a recent study, a similar method was used to determine the influence of surface-roughness of abutments on the microbiology of biofilm (16). In this study, long-term biofilm formation on surface modified abutments carrying a fixed dental prosthesis was analysed. To analyse initial biofilm formation of unloaded abutments in supra- and subgingival areas independent from prosthetic treatment, in the present study healing abutments were inserted for 14 days. The investigation was performed over this specific period to assure a similar microbiota in crevicular fluid of implants and natural teeth. In a clinical study, the microbiota around implants remained unchanged for 6 months after a comparable period (21).

These advantages facilitated investigation under realistic conditions, in contrast with previous studies in which bacterial colonization of titanium surfaces was quantified on titanium discs fixed on acrylic splints. The disadvantage of harvesting implants to obtain realistic results (14) is also overcome.

Biofilm-coated surfaces were differentiated from non-coated surfaces by use of the RBSD method, because with this method differentiation could be performed by a computer on the basis of differences between grey values. Results of supragingival biofilm formation demonstrated a high s.d. A possible expla-
ation for this finding may be the influence of interindivid-ual co-factors e.g. oral hygiene, nutrition and tongue activity. A 16S rRNA-based PCR detection method was used to determine whether *H. actinomyce-tetemcomitans* and *P. gingivalis* were present during early biofilm formation on healing abutments. This qualitative analysis was performed, as long-term prognosis of osseointegrated implants not only depends on the quantity of the biofilm, but also on the species in the biofilm (22). In previous studies, *H. actinomyctemcom-itans* and *P. gingivalis* were found in greater amounts in peri-implant lesions (19, 23). Therefore, these two pathogens can be regarded as the pre-dominant microorganisms responsible for a destructive peri-implant infection. Peri-implant infections with periodontal pathogens can be reliably identified by PCR. This method also avoids time-consuming and fault-prone cell culture (20, 24). Detection sensitivity of PCR can be assumed to be 100 colony-forming units.

Planctonic bacteria in the crevicular fluid are a representative spectrum of vital subgingival micro-organisms. Ubiquitous primers detected bacterial contamination in each sample taken and verified results from the quantitative analysis which showed that only a small amount of biofilm was formed in subgingival areas. Universal primers also revealed 16S rRNA amplification as positive control of PCR. The species-specific PCR primers were validated by amplifying DNA of pure cultures of *H. actinomycetemcomitans* and *P. gingivalis*.

Qualitative analysis revealed that none of the tested periodontal pathogens were present in any sample. These results are in agreement with the findings of Botero et al., who found no *P. gingivalis* or *H. actinomyctemcomitans* in stable osseointegrated implant surfaces, in contrast with peri-implant lesions, in which high levels of periodontal pathogens and superinfecting bacteria were present (7). For colonization of abutment surfaces with these micro-organisms, a long-standing biofilm and proliferation in subgingival areas seem to be necessary. In partially edentulous patients, the composition of the subgingival microbiota is similar to teeth and implants (25–27). Transmission of bacteria from residual pockets around neighbouring teeth is also possible (10, 28). In the present study, none of the patients had clinical signs of periodontal disease. Therefore, cross infection between healing abutments and natural teeth was unlikely.

The results from the PCR are in agreement with the quantitative analysis of biofilm formation on healing abutments, in which hardly any biofilm formation was found in subgingival areas. This absence of subgingival biofilm led to a recognizable demarcation line. A possible explanation of the absence of biofilm in subgingival areas and the absence of *H. actinomyctemcomitans* and *P. gingivalis* in the sulcus fluid might be the formation of a tight peri-implant barrier by circular collagen fibres and adherent hemidesmosoma, actin filaments and even microvilli (14, 29). In the present study, a qualitative and quantitative analysis of biofilm formation on healing abutments was performed. In all investigated patients, two key periodontal pathogens could not be detected by PCR, probably due to the healthy state of the remaining dentition. In further studies, the comprehensive composition of long-term supragingival and subgingival biofilm must be determined.

### References


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**Table 2. Results from quantitative and qualitative analysis**

<table>
<thead>
<tr>
<th></th>
<th>Total area</th>
<th>Biofilm covered area</th>
<th>Porphyromonas gingivalis</th>
<th>Haemophilus actinomycetemcomitans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supragingival</td>
<td>52.1%</td>
<td>17.5 ± 18.3%</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Subgingival</td>
<td>47.9%</td>
<td>0.8 ± 1.0%</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>


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