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IN VIVO EARLY PLAQUE COLONIZATION ON SMOOTH TITANIUM SURFACE

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(Received for publication May 11, 1996 and in revised form August 29, 1996)

Abstract

Plaque development on pure titanium in a 24-hour period is described in an in vivo human model. Stents with titanium and root cementum specimens were applied to volunteers, who suspended oral hygiene procedures for 24 hours. The specimens were removed at 2, 4 and 24 hours and studied with a scanning electron microscope. A global area of 240 μm x 300 μm, composed of the sum of twelve 20 μm x 25 μm fields randomly selected, was examined for each specimen. The presence of cocci, short rods, long rods and bacterial aggregation, and bacteria density was recorded for each field. The values quoted for the global area were cumulative of those observed in the fields. At 24-hours, significantly less bacteria was recovered from the titanium than from the corresponding root cementum specimens. The early colonizers of titanium were cocci, frequently located in the roughest part of the specimen. Visible salivary pellicle formation was delayed on the titanium compared to the cementum surface. At 24-hours, fewer rods were recovered from the titanium than from cementum surfaces. These results suggest that early plaque formation is reduced on titanium surface and that morphological irregularities are critical for bacterial adhesion and colonization.

Key Words: Dental plaque, adhesion, dental implant, titanium, microbial colonization, scanning electron microscopy.

Introduction

Bacterial adhesion and colonization are fundamental for the development of dental plaque, the most important, if not the only underlying cause of gingivitis and periodontal diseases (Christersson et al., 1991; Socransky and Haffajee, 1994). Recent data suggest that oral microorganisms colonize a titanium surface with a pattern similar to that described for the tooth surface (Mombelli et al., 1988) and may play a role in the success or failure of oral implants (Mombelli et al., 1987; Mombelli and Lang, 1994; Tonetti and Schmid, 1994). Although several studies have investigated the morphology of early plaque formation on enamel (Lie, 1979; Nyvad and Fejerskov, 1987a,b), dentine (Bjorvatn, 1986), cementum (Nyvad and Fejerskov, 1987a,b; Carrassi et al., 1989), and certain dental materials (Siegrist et al., 1991), information about the interaction between titanium and oral microorganisms during the first hours of contact is scanty.

We describe here, in an in vivo model, the pattern of the early phases of bacterial colonization on a pure titanium surface as examined with the scanning electron microscope (SEM).

Material and Methods

Eight dental student volunteers, aged 20 to 23 years, were involved in the study. They all had excellent general and oral health and no visible plaque or evidence of gingivitis at the beginning of the study. An acrylic stent was prepared and stored for each subject.

The experimental group consisted of 24 3-mm-wide disks of smooth, uncoated, pure, class IV titanium supplied by Straumann (Waldenburg, Switzerland). The surface roughness was verified with a RM 600 Laser Profilometer (Rodenstock, Göttingen, Germany) according to the ISO standard 468:1982 (ISO, 1988). The mean roughness assessment (Ra) value was 0.54 μm.

Specimens of similar dimensions from sound root cementum were used for comparison. The mineralizing front of the root cementum slabs was exposed after removal of periodontal membrane by a fresh 5% sodium hypochlorite solution as previously suggested (Carrassi *Address for correspondence:
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Figure 1. Titanium specimen at 2 hours. Few epithelial cells are present (arrows). No plaque or salivary pellicle are visible. Periodical ridge due to the polishing procedures are evident in this field. Bar = 100 μm.

Figure 2. Root cementum specimen at 2 hours. The morphology of the mineralizing front of inorganic cementum is obscured by a dense and granular pellicle. Pioneer bacteria (coccoids) can be observed in the crack of the pellicle. Bar = 1 μm.

Figures 3 and 4. Titanium specimen at 4 hours. Microbial forms (Fig. 3) and coccabacillary forms and salivary pellicle (Fig. 4) are evident at the base of the ridge. Bars = 1 μm.

Figure 5. Root cementum specimen at 4 hours. The surface of inorganic cementum is completely coated by coccoid bacteria. Bar = 1 μm.

Figures 6 and 7. Titanium specimen at 24 hours. Figure 6. The titanium surface is partially covered by a thin pellicle. The top of the ridge is free of deposits (arrows). Bar = 10 μm. Figure 7. Only a few layers of bacteria colonize the titanium surface. Cocci are predominant and a few short rods are detectable. Bar = 1 μm.

Figure 8. Root cementum specimen at 24 hours. A thick plaque is present. The rods are inserted perpendicularly to the specimen surface. Bar = 1 μm.

Table 1. Bacterial density and presence of cocci, short and long rods, and bacterial aggregations on titanium and root cementum at 2, 4 and 24 hours.

<table>
<thead>
<tr>
<th></th>
<th>bacterial density</th>
<th>cocci</th>
<th>short rods</th>
<th>long rods</th>
<th>bacterial aggregations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>index mean p</td>
<td>mean p</td>
<td>mean p</td>
<td>mean p</td>
<td>mean p</td>
</tr>
<tr>
<td>2-h titanium</td>
<td>5.87 ns</td>
<td>5.75 &lt; 0.5</td>
<td>0.00 ns</td>
<td>0.00 ns</td>
<td>0.50 ns</td>
</tr>
<tr>
<td>2-h root cementum</td>
<td>4.25</td>
<td>3.62</td>
<td>0.50 ns</td>
<td>0.00</td>
<td>4.37 &lt; 0.005</td>
</tr>
<tr>
<td>4-h titanium</td>
<td>16.37 ns</td>
<td>11.37 ns</td>
<td>0.12 ns</td>
<td>0.00 ns</td>
<td>10.62 ns</td>
</tr>
<tr>
<td>4-h root cementum</td>
<td>15.12</td>
<td>11.62</td>
<td>0.37</td>
<td>0.00</td>
<td>0.62</td>
</tr>
<tr>
<td>24-h titanium</td>
<td>34.00 &lt; 0.01</td>
<td>12.00 ns</td>
<td>4.00 &lt; 0.001</td>
<td>12.00 ns</td>
<td></td>
</tr>
<tr>
<td>24-h root cementum</td>
<td>36.00</td>
<td>12.00</td>
<td>9.25</td>
<td>2.37</td>
<td>12.00</td>
</tr>
</tbody>
</table>

Statistical differences were computed by Mann-Whitney's U test using the mean rank; ns = not significant (p > 0.05).

et al., 1989; Gatewood et al., 1993). All specimens were rinsed in distilled water, autoclaved and stored.

At the time of the experiment, the specimens were glued to the stent in the region of the lower premolars and the first molar, close to the gingival margin. Three titanium disks were placed on the right side of the stent, and radicular slabs were fixed on the left side. Then, the volunteers inserted the stent and discontinued oral hygiene procedures for 24 hours. Titanium disks and radicular slabs were removed from each subject at 2, 4 and 24 hours, fixed in 2.5% glutaraldehyde solution buffered with 0.1 M sodium cacodylate for 4 hours, and processed for scanning electron microscopy as follows (Carrassi et al., 1988): (a) rinse in 1 M Na cacodylate for 30 minutes; (b) dehydration in the graded ethanol series (70, 80, 90 and 100% for 15 minutes each); (c) dehydration in 1,1,2-trichloro-1,2,2-t trifluoro ethane overnight; and (d) dehydration at CO₂ critical point in a Top Critical Point 30 unit (W. Pabish, Milano, Italy). The specimens were mounted on aluminum stubs, sputter-coated with 20-nm thick gold-palladium in a E5100 Polaron coating unit (Polaron Equipment Ltd., Watford, Hertfordshire, UK) and blind studied with JSM 840A SEM (JEOL, Tokyo, Japan) operating at an accelerating voltage of 15 keV in the secondary electron detection mode and a 0° tilt angle.

A global area of 240 μm x 300 μm was examined for each specimen. The area was composed of the sum of twelve 20 μm x 25 μm fields randomly selected. The following data was collected for each 20 μm x 25 μm field: presence (score = 1) or absence (score = 0) of cocci, short rods (length ≤ 10 μm), long rods (length > 10 μm) and bacterial aggregations. The values reported for the global area are cumulative of those observed in 20 μm x 25 μm fields.

For bacterial density, the following variables were recorded in each of the 20 μm x 25 μm fields: 0 (score 0); > 0, ≤ 10 (score 1); > 10 ≤ 100 (score 2); > 100 (score 3) bacteria per field. The value computed for each global area was obtained by the cumulative sum.
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of each score multiplied by the frequency of observation in the area:

\[ \text{bacterial density index} = S \text{ (no. fields) (score)}. \]

Mann-Whitney’s U Exact Test with Monte Carlo method (Senchaudhuri et al., 1995) was used for statistical evaluation of the mean ranks. For small data sets, the Exact Tests provide a more accurate estimate of \( p \) value than the common asymptotic Non-parametric Tests.

**Results**

The bacterial density index computed at 2, 4 and 24 hours on titanium and root cementum surfaces are listed in Table 1.

The bacterial density increased consistently from 2 and 4 hours and between 4 and 24 hours in titanium (\( p < 0.0001 \)) and in root cementum groups (\( p < 0.0001 \)).

The 2-hour titanium surfaces were almost free of bacteria or visible salivary pellicle (Fig. 1), whereas the cementum specimen surfaces were uniformly obscured by a thin and homogeneous film of salivary components (salivary pellicle) with a globular aspect in which few coccoid and coccobacillary forms were detectable. These pioneer bacteria were usually observed in the roughest area or in fractures within the pellicle (Fig. 2).

The microorganisms observed on titanium surfaces were located at the base of the circular ridges, whereas the area between two consecutive grooves appeared free of deposits (Fig. 3). At higher magnification (Fig. 4), bacteria able to adhere to the titanium through a salivary pellicle were observed. The root cementum surfaces always appeared obscured by a homogeneous layer of coccoid forms (Fig. 5). The density of bacteria observed on titanium specimens did not differ from that observed on root cementum surfaces after 2 and 4 hours. In contrast, comparison of 24-hour titanium and 24-hour root cementum indicated significant differences (\( p < 0.01 \)).

The presence of cocci increased from 2 to 4 hours on root cementum (\( p < 0.005 \)) and on titanium surface (\( p < 0.0005 \)); however, the presence of short and long rods did not vary. The presence of bacterial aggregation increased only on the titanium specimen after 4 hours.

Between 4 to 24 hours, short rods, long rods and bacterial aggregation increased significantly on root cementum surfaces (\( p < 0.005, p < 0.01, \) and \( p < 0.0005, \) respectively).

On titanium surfaces from 4 to 24 hours, short rods and bacterial aggregation were observed more frequently (\( p < 0.005 \)). In contrast, no differences were noted as regards to the long rods on titanium specimens.

Difference was found between titanium and root cementum surfaces at 2-hour with respect to coccoid mor-

phototypes (\( p < 0.05 \)). After 4 hours, more bacterial aggregation was observed on titanium surface than on root cementum (\( p < 0.005 \)). At 24 hours, the bacterial colonization of titanium differed from root cementum with respect to short and long rod morphotypes (respectively, \( p < 0.001 \) and \( p < 0.05 \)). All root cementum surfaces were observed to recover multilayer plaque. Typically, the rods appeared to be inserted perpendicularly to the specimen surface emerging from the organic pellicle (Fig. 6). In contrast, the 24-hour titanium surfaces were frequently partially covered with a thin plaque (Fig. 7) which, at higher magnification, appeared to be mainly cocci, although a few short-rods were present (Fig. 8). When plaque was multilayered, a few layers of microorganisms, no more then 10 \( \mu \)m thick, made up the plaque as revealed by the occasional fracture of the plaque surface due to dehydration. Typically, the tops of the ridges caused by polishing of titanium disks appeared free of microbial deposits.

**Discussion**

This research shows that colonization of smooth titanium differs from that of inorganic cementum surface for two reasons: (1) visible salivary pellicle formation is reduced; (2) the bacterial plaque colonization is delayed. Compared with cementum surface, only a few bacterial layers cover the titanium surface after 24 hours. The fact that the visible salivary pellicle seemed to be reduced over the titanium surface compared to cementum could be considered the consequence of several phenomena such as different physical and chemical properties of the two substances, different surface free energies and different surface morphology.

Surface morphology and particularly roughness has been claimed to play a pivotal role in bacterial adhesion to hard dental tissues and materials (Quirynen et al., 1990, 1993; Quirynen and Bollen, 1995). The amount of plaque and deposits growing in vivo on enamel, dentine, amalgam, chrome-cobalt and ceramic has been recently proved to be dependent on the surface roughness of the materials (Siegrist et al., 1991). Roughness could influence plaque growth by protection against mechanical shear and salivary flow and an increase in the overall available surface. In the present study, the pioneering bacteria were observed to start the adhesion process at the base of the titanium ridges, probably because in those areas, they are more protected against mechanical shear and salivary flow. Such a hypothesis is also supported by the observation that the tops of the ridges are the last area to be colonized.

The patterns of microbial colonization of titanium and root cementum surfaces observed in the present study are similar to those previously described (Lie,
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1979; Nyvad and Fejerskov, 1987a,b; Carrassi et al., 1989). Any exposed clean surface in the oral cavity is rapidly covered by salivary pellicle. The salivary pellicle was observed to be composed of a thin and granular material on the root cementum surface. On the salivary pellicle, cocci are the first colonizers and usually appear on irregularities of the specimen surface. These cocci have been demonstrated to be facultative Gram positive with a thick cell wall connected to the pellicle by fimбриae (Nyvad and Fejerskov, 1987a,b). From 4 to 24 hours, a 30-35-fold increase in total microbial count of microbiota has been observed (Gatewood et al., 1993). Short and long filamentous rods are encountered after 24 hours over cementum (Carrassi et al., 1989) and titanium (Gatewood et al., 1993). The sequence of appearance of the species does not seem to change among different tissues and materials (Siegrist et al., 1991; Gatewood et al., 1993). Consequently, it was suggested that substrate composition has little or no effect on species selection (Siegrist et al., 1991). However, our results demonstrate that even though the sequence of species appearance did not differ between root cementum and titanium specimens, the presence of short and long rods was lower on titanium surfaces than on root cementum. This phenomenon, particularly evident after 24 hours of exposure in the oral cavity, suggests a lower maturation degree of plaque growing on titanium than on root cementum surfaces.

Different hypotheses have been proposed to explain the delayed plaque colonization on titanium. An attractive but still controversial explanation has related the delayed colonization of titanium to some inherent antibacterial properties of titanium itself (Imoberdorf et al., 1989; Wolonsky et al., 1989; Apse et al., 1989) or to a metal ion release into the periimplant environment (Kean et al., 1988). Other hypotheses may involve the adhesion of bacteria to the titanium substrate. Modification in the number of site-specific-binding between bacteria and the salivary pellicle should not be excluded as a consequence of the reduced pellicle formation observed on titanium. Moreover, micro-morphological observations of titanium and root cementum specimens suggest that the latter is rougher because of the presence of calcified Sharpey’s fibers. Consequently, the roughness of root cementum surface shelters the bacteria and allows them to establish a link with the surface.

The first major interaction between a pathogenic microbiota and its host entails attachment to a surface. To inhibit a pathogenic organism from colonizing a host and establishing an infection, hampering initial attachment is thus a logical place to start. The present findings suggest that a titanium surface is less favorable habitat for bacterial plaque to colonize than natural teeth because surface conditions seem to play a determinant role in the plaque growth rate. Any improvement in the final polishing of titanium oral implants or chemical modification of their surface that allows a reduction of total plaque reduces the occurrence of bacterial infection and guarantees more early and long-term successful results.

Acknowledgments

The study was supported by the Italian Research Council (C.N.R.) grant no. 93.01549.CT11.

References


Discussion with Reviewers

R.J. Doyle: Adhesion patterns frequently appear to contain clusters or aggregates of cells. Do these morphologies represent colonization, co-aggregation or co-operative adhesion as described by Cowan et al. (1987) (Infect. Immun 55: 1552)?

Authors: It is difficult to distinguish bacterial colonization from co-operative adhesion and coaggregation because the SEM is unable to identify to which species the cocccid or the bacillary forms belong. However, it is rational to suppose that the clusters of cocccid forms observed in the less colonized specimens are made of multiplied bacteria, belonging to the same strain which, initially, have adhered in groups. This pattern of adhesion is similar to that described in vitro by Cowan et al. (1987) for Streptococcus sanguis on saliva-coated glass. In contrast, in the highly colonized specimens whose clusters are composed of cocccid and bacillary forms, coaggregations were easily identified. We frequently observed short and long rods surrounded by cocci. Moreover, at 20,000X, we observed the cocci cell walls connected with the filamentous walls. Such observations are suggestive of coaggregation.

R.G. Richards: How do you know bacteria were not removed from the smooth surfaces during processing for electron microscopy? Did you perform any light microscopy with staining of the bacteria to check this?

Authors: The less adherent bacteria are usually removed from the substratum surface by moderate flushing with water spray. The residual bound bacteria are defined as dental plaque. We did not check with light microscopy and staining if any or how many bacteria were removed from the specimen during processing. However, we are convinced that the rinsing procedures could have removed the less bound bacteria. We believe this fact did not influence the clinical relevance of our observations. Actually, even in the oral cavity, bacteria, whose binding with the substratum is not stabilized, are constantly removed by clearance forces. Only adherent bacteria (particularly supragingivally) are able to survive, multiply and be virulent. Anyway, in the future, we will check to what degree bacteria are removed in each stage of SEM processing because the results may be interesting. Probably, the dark-field microscope may be more suitable for such an investigation.

R.G. Richards: For future study, a stated smooth titanium and rough titanium should be compared with the surface roughness as the only variable. Also, to better observe the detail of the surfaces of the bacteria and the pellicle and to aid interpretation, lower accelerating voltages should be used. With your SEM, working at 5 keV, it is possible to get good results which will lessen the charging artefacts observed in the images.

Authors: Thank you, we will consider these suggestions in our future work.