In vitro adherence of periodontopathic bacteria to zirconia and titanium surfaces

Masahiro EGAWA1,2, Tadashi MIURA1, Tetsuo KATO1,3, Atsushi SAITO2 and Masao YOSHINARI1

1 Division of Oral Implants Research, Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
2 Department of Periodontology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
3 Laboratory Chemistry, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Corresponding author, Masao YOSHINARI; E-mail: yosinari@tdc.ac.jp

Tetragonal zirconia polycrystals (TZP) has drawn attention as a potential alternative to titanium (Ti) in dental implant treatment, as it minimizes both allergic reactions and esthetic problems. It is also important for dental implants to maintain plaque-free surfaces to prevent peri-implantitis. The purpose of this study was to investigate in vitro adherence of periodontopathic bacteria to TZP comparing with Ti. Periodontopathic bacteria were cultured on polished discs of two kinds of TZP, and Ti as a control. After incubation, the numbers of adherent bacteria were estimated. No significant differences among specimens were observed in the initial attachment, although a decrease was observed in initial attachment to saliva-coated specimens. In the bacterial colonization, no significant differences were recognized among specimens. The adherence of the periodontopathic bacteria on TZP was similar to that on Ti. These results suggest that a strategy is required for inhibition of the bacterial adherence to TZP.

Keywords: Zirconia, Titanium, Peri-implantitis, Periodontopathic bacteria, Bacterial adherence

INTRODUCTION

Titanium (Ti) and its alloys are used extensively in oral and orthopedic implants due to their mechanical properties and biocompatibility with human tissues. There are, however, esthetic problems associated with the Ti implant abutments due to their gray coloring. Moreover, Ti implants have been reported to trigger allergic reactions1,2. Recently, zirconia has drawn attention as a potential alternative to Ti, as it allows both allergic reactions and esthetic problems to be avoided3-5. Tetragonal zirconia polycrystals (TZP), in particular, yttria-stabilized zirconia (Y-TZP) have been applied to the frameworks for fixed prostheses and dental implants as metal-free restorations8-10. Ce-TZP/Al2O3 nanocomposite (NanoZR) is also used due to its resistance to low-temperature degradation, offering not only higher strength, but also higher fracture toughness than Y-TZP11.

Peri-implantitis is defined as an inflammatory process affecting the tissues around an osseointegrated implant in function, resulting in loss of supporting bone12. Although multiple factors can contribute to implant failure, an increasing number of studies point to the detrimental effect of anaerobic plaque bacteria on peri-implant tissue health13. Studies have shown similarities in the microfauna from implant or tooth sites14-17. High levels of periodontopathic bacteria including Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans confer an increased risk for periodontitis18,19. These pathogens are frequently detected in peri-implantitis sites as well20. In an earlier in vitro study, initial attachment of oral bacteria on Ti surfaces was investigated21. The results showed that comparatively large amounts of P. gingivalis and A. actinomycetemcomitans adhered to Ti even with surface polishing. These findings indicate that there is a considerable risk of adhesion by periodontopathic bacteria at Ti implants.

While much information is available on bacterial adherence to enamel or Ti22-25, information regarding bacterial adherence to TZP is limited26,27. Furthermore, little is known about in vitro adherence activity of periodontopathic bacteria to TZP.

The aim of this study was to compare in vitro adherence (initial attachment and colonization) of selected periodontopathic bacteria to two kinds of TZP with that to Ti under saliva-treated and untreated conditions.

MATERIALS AND METHODS

Specimen preparation

Yttria-stabilized tetragonal zirconia polycrystal (Y-TZP, TZ-3YB-E, Tosoh, Tokyo, Japan), Ce-TZP/Al2O3 nanocomposite (NanoZR, C type, Panasonic Healthcare, Ehime, Japan), and pure Ti (grade 2, Kobe Steel, Kobe, Japan) as a control were used in this study. Density of Y-TZP and NanoZR were approximately 6.1 and 5.5, respectively, and hardness (Hv) of Y-TZP and NanoZR were approximately 1,250 and 1,160, respectively. Disks 13 mm in diameter and 0.5 mm in thickness were ground progressively with silicon carbide paper down to 1200 grit, after which they were finely polished with 3-µm diamond pastes and 0.06-µm colloidal silica using a polishing machine (Ecomet 3, Buehler, Lake Bluff, IL) and then ultrasonically cleaned with acetone and distilled water. The washed samples were sterilized in an autoclave (121°C, 15 min). The sterilized specimens were stored in dry conditions until used. Six specimens...
from each group were used under each experimental condition.

Surface roughness
Arithmetic mean surface roughness (Ra) was measured using a surface profilometer (Surfcom 130A, Tokyo Seimitsu, Tokyo, Japan) with a measuring length of 4 mm and cut-off value of 0.8 mm.

Surface wettability
The surface wettability of the samples was measured against double distilled water (DW) using a contact angle meter (Phoenix α, Meiwa-forces, Tokyo, Japan). Contact angle measurements were made at 3 different locations on each of 3 samples at 15 s after application of droplets. The volume of the drop was maintained at 4 μL.

Bacterial strains and culture conditions
Porphyromonas gingivalis ATCC 33277, Prevotella intermedia ATCC 25611, and Aggregatibacter actinomycetemcomitans 310a (kindly provided by Dr. H. Ohta, Ibaraki University) were used. P. gingivalis and P. intermedia were grown in trypticase soy broth (Becton Dickinson, Sparks, MD, USA) supplemented with hemin (5 µg/mL; Sigma Chemical Co., St. Louis, MO, USA) and menadione (0.5 µg/mL; WakoPure Chemical Industries, Osaka, Japan). A. actinomycetemcomitans 310a was cultured in Todd Hewitt Broth (Becton Dickinson) supplemented with yeast extract (10 mg/mL; Becton Dickinson). These bacterial cultures were grown at 37°C under anaerobic conditions for 24 h. The optical density of each bacterial suspension was adjusted with phosphate-buffered saline (PBS, pH 7.4) to 0.2 at 660 nm (with the exception of P. intermedia, which was adjusted to 0.3 at 660 nm) using a spectrophotometer (Ultraspex 2100pro Amersham Biosciences, New Jersey, USA), which corresponds to a microbial concentration of 3.0×10⁸ microorganisms/mL.

Initial attachment and colonization assay
Initial attachment and colonization were determined in both saliva-unicoated (Saliva−) and saliva-coated (Saliva+) groups. Unstimulated saliva sample was obtained from a healthy male donor aged 28 who had not been on any medication for 3 months prior to the study. The donor had no active periodontal disease or caries. A single donor was used in this study to eliminate alteration in salivary protein composition. The saliva was passed through a 0.45 µm filter and stored at –20°C until use.

An acrylic tube was fitted to cover the experimental disk. An aliquot of 300 µL of each bacterial suspension was inoculated onto each disk and incubated anaerobically at 37°C for 1 h (initial attachment) or 48 h (colonization). In the Saliva+ group, specimen disks were pre-treated with saliva and incubated for 1 h at room temperature, after which the disks were washed twice in buffered KCl (0.05 M KCl, 1 mM potassium phosphate, 1 mM CaCl₂, 0.1 mM MgCl₂, pH 6.0)²⁸. After 1 h or 48 h incubation with bacteria, the disks were washed twice with PBS to remove loosely bound cells. The number of bacteria adhered to the disk surface was estimated by an ATP-bioluminescent assay using a commercial kit (BacTiter-Glo Microbial Cell Viability Assay kit, Promega, Madison, USA). An aliquot of 100 µL BacTiter-Glo reagent was added to 200 µL PBS in each plastic tube and briefly mixed, after which ATP activity in the solution was measured using an auto lumicounter (Model 1422EX, Microtec, Funabashi, Japan), and the relative luminescence was determined. In preliminary experiments a standard curve of ATP activities versus colony-forming units (CFU) obtained by traditional plating methods was established for each bacterium. This relationship was very reproducible within the CFU range of 1×10⁵ to 1×10⁶. All assays were performed using triplicate samples of each material in at least 2 different experiments.

Scanning electron microscopy
The periodontopathic bacteria were grown as described in the adhesion assay. After incubation with each bacterium, the specimens were fixed with 1.25% glutaraldehyde in PBS for 2 h at room temperature. Specimens were then washed 3 times with PBS and dehydrated using a series of graded ethanol (70, 80, 90, 95, and 100%). Specimens were subsequently freeze-dried, sputter-coated with Au-Pd, and observed using a scanning electron microscope (SEM, JSM-6340F, JEOL, Tokyo, Japan).

Statistical analysis
The data were analyzed using an analysis of variance (ANOVA) followed by the Scheffe test for multiple comparisons using a software package (Excel Statistics, 2006, SSRI, Tokyo, Japan).

RESULTS
Surface roughness (Ra) and surface wettability
The Ra values of the experimental specimens were shown in Table 1. All surfaces had mirror-like flat textures with small Ra value of less than 0.1 µm.

Contact angles on the experimental specimens...
against DW were shown in Table 1. Contact angle in the Saliva+ group showed a significant decrease compared with that in the Saliva− group. No clear differences were recognized among specimens in the Saliva− group. The contact angle on Ti was significantly greater than that on Y-TZP or NanoZR (p<0.05) in the Saliva+ group.

Initial bacterial attachment

The CFU values of bacteria (a; P. gingivalis ATCC 33277, b; P. intermedia ATCC 25611, c; A. actinomycetemcomitans 310a) that adhered initially (1 h) to Saliva− and Saliva+ specimens are shown in Fig. 1. No significant difference was observed in initial attachment among specimens in any bacteria under Saliva− or Saliva+ conditions. The initial bacterial attachment to Saliva− specimens was significantly greater than that to the Saliva+ specimens (p<0.05), regardless of the differences in material. This finding was confirmed by SEM (Fig. 2).

Bacterial colonization

In 48 h incubation, there was a general trend towards an increase in bacterial counts (Fig. 3) compared with 1 h incubation (Fig. 1). No significant differences in colonization (48 h) were recognized among specimens under either Saliva− or Saliva+ conditions. In addition, no significant difference was observed in number of bacteria between the Saliva− and Saliva+ groups. Colonization with an aggregate structure was observed by SEM (Fig. 4).

DISCUSSION

In the present study, we compared initial attachment and colonization of the periodontopathic bacteria on two kinds of TZP with that on Ti under saliva-coated and non-saliva-coated conditions. Within our knowledge, this study is first to investigate in vitro adherence activity of periodontopathic bacteria to TZP, in relation to Ti. No significant differences in initial attachment (1 h) of P. gingivalis, P. intermedia or A. actinomycetemcomitans were observed among specimens, although a decrease was noted in initial attachment in Saliva+ specimens. Likewise, no significant differences were observed in colonization (48 h) of the periodontopathic bacteria

Fig. 1 CFU values of bacteria on initial attachment (1 h) a; P. gingivalis ATCC 33277; b; P. intermedia ATCC 25611; c; A. actinomycetemcomitans 310a. Data shown as mean CFU±standard deviation (n=6). Saliva− groups showed significantly higher values than Saliva+ group. *p<0.05.

Fig. 2 Representative SEM images of initial attachment (1 h) for P. gingivalis (a), P. intermedia (b), A. actinomycetemcomitans (c) to Saliva− and Saliva+ Y-TZP specimens.
Fig. 3 CFU values of bacteria on colonization (48 h) 
a: P. gingivalis ATCC 33277; b: P. intermedia ATCC 25611; c: A. actinomycetemcomitans 310a. 
Data shown as mean CFU±standard deviation (n=6). No differences were observed in number of 
bacteria between Saliva− and Saliva+ groups (NS).

Fig. 4 Representative SEM images of colonization 
(48 h) for P. gingivalis (a), P. intermedia (b), A. 
actinomycetemcomitans (c) to Saliva− and Saliva+ 
Y-TZP specimens. 

In an earlier study, we demonstrated that roughened Ti surfaces exhibited significantly higher levels of bacterial adhesion than mirror-polished Ti. Comparatively large quantities of bacteria, however, still adhered to the Ti, even though the surfaces were polished.

In regard to surface wettability, a significant increase was observed in the Saliva+ group compared to the Saliva− group. In general, hydrophobic surfaces enhance bacterial adhesion through hydrophobic interaction between substrates and bacteria. Das et al. reported that the adhesion activity of Streptococcus and Actinomyces strains was positively related to an increase in surface hydrophobicity. Drake et al. showed that hydrophobic Ti surfaces were preferentially colonized by Streptococcus sanguinis. Accordingly, decreasing hydrophobicity, i.e. decreasing the surface contact angle, may lead to a decrease in bacterial adhesion, as observed in the present study.

Bacterial adhesion is also influenced by the electrical charge of the substrate surface, as bacteria have a large specific surface area. In general, bacteria are negatively charged, as are Ti surfaces (pH 7). The isoelectric point of TiO₂ is reported to be around 6.0. Therefore, positively-charged calcium ions are easily adsorbed to negatively-charged Ti surfaces. This results in calcium-mediated bacterial adhesion to Ti via Ti/TiO₂/Ca/(protein)/bacteria. Reports concerning the electrical charge of TZP are scarce. Zirconia is an amphoteric metal.
oxide which exhibits both anion and cation exchange properties depending on the pH and/or composition of the buffer\(^{67}\). It has also been reported that the electrical charge of colloidal TZP powders in aqueous suspension is neutral or positively-charged\(^{68}\). Accordingly, bacterial adherence to TZP appears to be weaker than that to Ti. In the present study, however, no significant differences in the adherence of the periodontopathic bacteria were recognized among specimens. Further study is necessary to clarify the influence of electrical charge of the material surface on bacterial adherence as well as other mechanisms.

Specific bacterial receptors and salivary proteins also influence bacterial adhesion to substrates\(^ {31,39}\). The formation of pellicles by selective adsorption of components from saliva may induce bacterial adhesion\(^ {40}\). In the present study, no differences in initial attachment were recognized among the specimens in the Saliva+ groups. This finding may be supported by an earlier study which found that similar types of salivary protein attached to Ti and zirconia\(^ {41}\). In the present study, no attempt was made to evaluate the content of saliva proteins. Further study is necessary to clarify the adsorption properties of TZP and Ti in saliva and the role of adsorbed proteins on the adhesion of periodontopathic bacteria.

In the present study, an increase in CFU values was observed with 48 h incubation in all specimens compared to those for 1 h incubation, especially in the Saliva+ groups (Figs. 1 and 3). The results may imply the following: (1) Bacterial aggregation was enhanced during the 48 h period (as suggested by SEM observation) (2) Materials used in this study had no intrinsic antimicrobial properties.

Initial attachment and colonization of the periodontopathic bacteria to two kinds of TZP was similar to that to Ti, suggesting a need for strategies to reduce bacterial adherence. Surface modification of TZP is one approach to inhibit biofilm formation. There are at least 2 methods of inhibiting the formation of microbial plaque: the first is to inhibit the initial attachment of bacteria; the second is to inhibit the colonization of bacteria, which involves surface antimicrobial activity. Hauser-Gerspach \textit{et al}. reported that release of F ions from resin composites decreased bacterial adhesion\(^ {29}\). In earlier studies, we demonstrated a number of mechanisms capable of inhibiting biofilm accumulation on a Ti surface: coating with alumina for inhibition of initial attachment\(^ {21}\), application of F-implantation\(^ {53}\) and immobilization of anti-microbial peptides\(^ {69}\). Such surface modification may also prove to be effective on TZP surfaces.

CONCLUSION

The initial attachment and colonization of the periodontopathic bacteria on two kinds of TZP surfaces was similar to that on Ti surfaces, although a reduction in initial attachment by saliva coating was observed. These results suggest that a strategy is required for inhibition of bacterial adherence to TZP.

ACKNOWLEDGMENTS

We would like to thank Associate Professor Jeremy Williams, Tokyo Dental College, for his advice on the English of this manuscript. This research was supported by an Oral Health Science Center Grant HRC7 from Tokyo Dental College, a “High-Tech Research Center” Project for Private Universities: Matching Fund Subsidy from MEXT (Ministry of Education, Culture, Science, and Technology) of Japan, 2006–2010, and a Grant-in-Aid for Scientific Research (B:18390524 and 18659581) from the Japan Society for the Promotion of Science.

REFERENCES

24: 96-105.