Original

Behavior of Human Gingival Epithelial Cells on Titanium Following Abrasion of the Adjunctive Glycine Air Polishing Powder

Reiko Koishi¹⁾, Yoichiro Taguchi¹⁾, Makiko Okuda¹⁾, Akio Tanaka²⁾ and Makoto Umeda¹⁾

¹⁾ Department of Periodontology, Osaka Dental University, Osaka, Japan

²⁾ Department of Oral Pathology, Osaka Dental University, Osaka, Japan

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Abstract: Treatment to replace missing teeth with implants has been available for nearly 50 years. However, many implants are subject to peri-implantitis because of resorption of the bone supporting the implant. Therefore periodontal maintenance to avoid bacterial infection around an implant device is essential. There are several methods of maintenance available to remove bacterial infections. One of these methods involves the direct removal of bacterial infection in the implant sulcus using tooth surface abrasives. Abrasives that contain glycine are recommended because of their biocompatibility with the tooth surface. The purpose of this study was to examine the cellular behavior of human gingival epithelial cells on the surface after abrasion with adjunctive glycine air-polishing powder. We treated the titanium surface with air-polishing abrasives containing glycine or sodium bicarbonate, then examined the structural surface change and wettability, as well as the proliferative potency and gene expression of adherent gingival epithelial cells. The glycine-containing abrasives exhibited higher biocompatibility than the sodium bicarbonate abrasive, and the use of abrasives with small average particle size may be useful in the removal of bacterial infections around implants.

Key words: Implant, Abrasion powder, Epithelial cell

Introduction

Implant procedures have become a common prosthodontic treatment to replace teeth lost as a result of periodontitis. These treatments have been available for about 50 years since Prof. Per-Ingvar Brånemark first advocated them^{1,2)}. Previous studies have indicated a cause-and-effect relationship between microbial plaque colonization and the pathogenesis of peri-implant infections^{3,4)}. The number of cases of peri-implantitis leading to the presence of periodontitis field bacilli in the mouth has increased as well as the number of natural teeth. Therefore, periodontal maintenance after implant placement has become very important.

Maintenance of the peri-implant sulcus is considered to be particularly important, because it is the site where peri-implantitis occurs. There are many methods for maintaining implants⁵⁾. However, few of these procedures are specialized for maintenance of implants, and procedures used for maintenance of chronic periodontitis are often substituted. Because maintenance is often performed not only by the dentist, but also by the dental hygienist, it is more effective to use a procedure designed for periodontal maintenance. Commercially available injection abrasive water jets, also known as air-polishing devices, provide highly efficient and

convenient biofilm removal as part of periodontal maintenance. With the goal of establishing an efficient and safe technique for subgingival biofilm removal, a low-abrasive air-polishing powder was developed for use in commercially available air-polishing devices⁶⁻⁸⁾. This novel approach to biofilm removal substantially facilitates periodontal debridement. Air-polishing devices were first introduced to dentistry in 1945⁹⁾, and have been used for professional mechanical tooth cleaning since the 1980s, primarily using a powder based on sodium bicarbonate^{10,11)}. The air-polishing device technique offers a user-friendly mode of biofilm removal in patients with periodontitis and peri-implantitis. However, the ever-present problem of related hard and soft tissue trauma resulting from the use of sodium bicarbonate air-polishing compromises its routine use. Studies have shown that it is not possible to adjust the working parameters of previous air-polishing devices to allow biofilm removal from root surfaces in a safe, reliable and efficient manner^{12,13)}. However, it may be possible to control and optimize the efficacy and safety of an air-polishing device on dentin and cementum by using air-polishing powders with mechanical properties differing from those of the standard sodium bicarbonate powder commonly used.

To facilitate the removal of biofilm from root surfaces while minimizing trauma to hard and soft tissues, an air-polishing powder consisting of an amino-acid glycine salt, which allows efficient

Correspondence to: Dr. Yoichiro Taguchi, Department of Periodontology, Osaka Dental University, 8-1, Kuzuha-hanazono-cho, Hirakata, Osaka, Japan; Tel: +81-72-864-3084; Fax: +81-72-864-3184; E-mail: taguchi@cc.osaka-dent.ac.jp

Table 1. Powder Characteristics

Powder	Type	Average particle diameter (μm)
A	Sodium bicarbonate	65
B	Amino acid glycine	65
C	Amino acid glycine	25

plaque removal with minimum abrasion of root cementum and dentin, was recently introduced⁶⁻⁸⁾. This glycine powder is intended to be used instead of sodium bicarbonate powder in conventional air-polishing devices of various manufacturers. Moene et al.¹⁴⁾ found that this method is faster and safer than the method of using hand instruments to remove subgingival plaque. Petersilka et al.¹⁵⁾ demonstrated the effect on gums of air polishing using glycine powder *in vivo*, and concluded that glycine powder air-polishing results in less gingival erosion than sodium bicarbonate air-polishing or hand instrumentation. Another study suggested that for maintenance of the soft tissue in the implant sulcus, air-polishing using glycine powder is more effective and less invasive than curettage with a Teflon curette¹⁶⁾.

The purpose of the present study was to examine the movement of human gingival epithelial cells on titanium surfaces after abrasion with adjunctive glycine air-polishing powder.

Materials and Methods

Sample preparation

Titanium discs (15-mm diameter, 1-mm thick) of grade 2 commercially pure titanium were prepared by machining (Daido Steel, Osaka, Japan). The discs were treated with an air-polishing system (Handyjet, Morita Ltd., Kyoto, Japan) using either amino acid glycine in two particle sizes (AIR-FLOW® Soft, AIR-FLOW® Perio, Electro Medical Systems, Nyon, Switzerland) or sodium bicarbonate powder (AIR-FLOW® Classic). Table 1 shows the particle size of the three powders. Each titanium disc received single and repeated treatment procedures by consistently moving the nozzle from the center to the periphery in four circular motions. Treatment time for each procedure was set at 1 minute. The discs were classified into four groups; A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (control). The solution in each flask was replaced and treated with distilled water (200 ml), and this procedure was repeated to remove potential powder deposits. Samples were then dried at room temperature. The surface topography of the discs in each group was qualitatively evaluated using a scanning electron microscope (SEM, S-4000, Hitachi, Tokyo, Japan) and scanning probe microscope (SPM, SPM-9600, Shimadzu, Kyoto, Japan). In SPM analysis, Ra was the average roughness and Rz the maximum height roughness.

Measurement of contact angle

Contact angles were measured with a video contact angle

measurement system (VSA 2500 XE, AST Products, Tokyo, Japan) at room temperature, using 3 μl of ultra-pure water.

Measurement of cell adhesion and proliferation

We used cells from the epi4 human gingival epithelial cell line kindly provided by Professor Shinya Murakami of Osaka University. Cell adhesion was measured using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, human gingival epithelial cells were seeded on the samples at a density of 4×10^4 cells/cm² and allowed to attach for 1, 3, 6, 24 and 72 h. At each prescribed time point, non-adherent cells were removed by rinsing with phosphate buffered saline (PBS). CellTiter-Blue Reagent (50 μl) and PBS (250 μl) were then added to each well. After incubation at 37 °C for 1 h, the solution was removed from the 24-well tissue culture plates (Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and 100 μL was added to a new 96-well tissue culture plate (Falcon). The OD560/590 of the remaining solution was measured. The difference between the two optical densities was defined as the proliferation value.

Real-time PCR analysis

After 3 h of culture, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA (10 μl) from each sample was reverse transcribed into cDNA using a PrimeScript^{RT} Reagent Kit (Takara Shuzo Co, Otsu, Shiga, Japan).

Expression of mRNA was investigated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR), using a StepOne PlusTM Real-Time RT-PCR System (Applied Biosystems, Foster City, CA, USA). First 10 μl of Taqman Fast PCR Master Mix, 1 μl of each primer (Taqman® Gene Expression Assays, Life Technologies Corporation, Carlsbad, USA), 2 μl of sample cDNA, and 7 μl of diethyl pyrocarbonate water (Nippongene, Tokyo, Japan) were added to each well in a Fast 96-well Reaction Plate (0.1 ml well volume; Applied Biosystems). The plate was subjected to 40 reaction cycles of 95 °C for 1s, and 60 °C for 20 s. Gene expression levels were calculated employing the $\Delta\Delta C_t$ method¹⁷⁾, relative to the expression of control genes. Expression of Intercellular Adhesion Molecule-1 (ICAM-1), integrin $\alpha 6$ and integrin $\beta 4$ was quantified.

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The comparison of results was performed by means of the Mann-Whitney U-test and differences were considered to be significant when $P < 0.05$.

Results

Sample analysis

Figure1. Scanning electron microscope images after abrasion with various powders. The upper, middle and lower images show the overall microscale topography.

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (Control)

The surface morphology and the roughness

Figure 2. Scanning probe microscope 3D images showing the surface roughness of the control (CON) and experimental groups.

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (Control)

Table 2. Surface Roughness

	A	B	C	D
Ra (nm)	38.230	32.239	18.431	4.747
Rz (nm)	182.361	304.673	141.912	48.690

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (Control)

SEM of the titanium surfaces after abrasion with the adjunctive glycine air-polishing powder are shown in Fig. 1. The untreated titanium surface appeared to have relatively smooth surface features. The treated titanium surface appeared to be equally abraded on the flat surface, but under high magnification it was evident that the surface irregularities were not uniform. Additionally, the surface irregularities seemed to correlate with particle diameter. The surface morphology and the roughness values of each titanium disk were obtained using a scanning probe microscope, as shown in Fig. 2 and Table 2. As the average particle

diameter increased, the scanning probe microscope image showed that the irregularities also increased in size. It was found that the Ra level correlated with the average particle diameter, but the Rz level did not have this tendency.

Wettability

Cross-sectional views of water droplets on the surface of treated and control discs and their contact angles are depicted in Figs. 3 and 4. A marked difference was found between the contact angles measured for the two test discs (A, B) and the control discs

Wettability

Figure 3. Contact angle measurements of ultra-pure water droplets pipetted on the specimens. The upper panel shows the optical images.

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (Control)

Figure 4. Graph shows the quantitative results of contact angle measurements of ultra-pure water droplets. (*: $p < 0.05$).

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (control)

Figure 5. Graph showing cell proliferation after 1, 3, 6, 24 and 72 h of incubation measured using the CellTiter-Blue Cell Viability Assay. (*: $p < 0.05$, **: $p < 0.01$)

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (Control)

Figure 6. Gene expression of ICAM-1, integrin $\alpha 6$ and integrin $\beta 4$ in human gingival epithelial cells cultured on titanium surfaces after incubation for 3 h. Data were generated by real-time PCR and are shown as mean $\pm$ SD expression relative to GAPDH. (*: $p < 0.05$)

A (AIR-FLOW® Classic), B (AIR-FLOW® Soft), C (AIR-FLOW® Perio), D (Control)

(D).

Cell adhesion and proliferation

Cell adhesion and proliferation on the disks after 1, 3, 6, 24 and 72 h of culture were assessed (Fig. 5). No difference in epi4 dissemination was observed among the groups at 1 h, but the glycine 25 μ m group exhibited significantly more cell adhesion and proliferation than the sodium bicarbonate group at 24 and 72 h.

Gene expression

Cell adhesion-related gene expression of ICAM-1, integrin

$\alpha 6$, and integrin $\beta 4$ in human gingival epithelial cells on the discs was assessed after 3 h of culture (Fig. 6). Expression of ICAM-1 was significantly higher in the control group than in the sodium bicarbonate and glycine 75 μ m groups, but there was no significant difference between the glycine 25 μ m group and the control group. Expression of integrin $\alpha 6$ was significantly higher in the glycine 25 μ m group than in all other groups including the control group. Expression of integrin $\beta 4$ was significantly higher in the glycine 25 μ m group than in the sodium bicarbonate and glycine 75 μ m group, but no significant difference was found with the control group.

Discussion

The present study was designed to evaluate the influence of three different types of air-polishing powders on gingival epithelial cell viability on biologically contaminated titanium dental implant surfaces. In general, the results indicated that glycine air-polishing powder results in higher biocompatibility of the titanium surface than that achieved with sodium bicarbonate abrasives, possibly because it is more difficult for plaque to attach to the treated titanium surface, and the adhesion of epithelial tissue to the surface is good.

The structure and function of the mucosa surrounding the implant abutment have been studied in human and various animal experiment models¹⁸⁾. We generally use sintered alumina ceramics as abutments in our clinic. Abrahamsson *et al.*¹⁹⁾ suggested that the abutment material is an important influence on the adherent properties between the abutment and the surrounding mucosa, and that similar mucoadhesion was obtained for ceramics with titanium as for sintered alumina ceramics. In the present study, we used flat discs of pure titanium as the experimental material. Morphologic observation with SEM to reveal differences in the surface structure after air-polishing with various abrasives did not reveal significant variations. However, the differences became evident after three dimensional observation with the scanning probe microscope, which revealed particularly marked differences in Ra and Rz. Moon *et al.*²⁰⁾ found that there are two layers of connective tissue in the adhesion zone of pure titanium implants. The layer adjacent to the implant surface is 40 μm thick with very few blood vessels, and contains a large number of cells along the implant surface parallel to the major axis of the implant. It is necessary for the surface in this zone to be smoother; that is, with small Ra and Rz values, as near as possible to the control group values. The particle size of the abrasive is a major influence on the surface properties, and it is suggested that more effective adhesion between the tissue and the implant is achieved with a small particle size.

The wettability of the implant surface usually needs to be high to achieve osseointegration. However, a low level of hydrophilia is desirable in terms of biofilm adhesion. Fig. 5 indicates the proliferation of human gingival epithelial cells after abrasion with the three air-polishing powders. The effects of cell proliferation began to appear after 3 h, and at 24 h a significant difference was found between A and B, A and C, and A and D. It is thought that these differences in cell proliferation occurred because of differences in surface properties related to the particle size and the quality of the abrasive. Kreisler *et al.*²¹⁾ reported that cell proliferation decreases in the presence of bacteria when the surface is not treated with abrasives, and significant cell proliferation occurs when the surface is treated with abrasives. The cells used in this study were gingival fibroblasts and the abrasive used was sodium bicarbonate; however, the findings confirm that removal

of the biofilm with abrasives is necessary. Both air powder and Er:YAG laser irradiation demonstrate good potential to remove cytotoxic bacterial components from implant surfaces. Schwarz *et al.*²²⁾ suggested that cell viability at a biologically contaminated titanium surface is mainly influenced by the particle type of the abrasive. This experiment did not investigate the adhesion of the biofilm in isolation, because the properties of the titanium surface influence both cell proliferation and the adhesion of the biofilm. Our findings suggest that the properties of the titanium surface are influenced both by the type of powder and the particle size of the abrasive. Specifically, our study suggests that cell proliferation is more greatly enhanced by the use of glycine as the powder type than with other types of powder, because it is an essential amino acid with a higher level of biocompatibility than sodium bicarbonate and, in addition, the small particle size results in reduced surface coarseness.

Fig. 6 illustrates the expression of the three adhesion molecules (ICAM-1, integrin $\alpha 6$ and integrin $\beta 4$) from human gingival epithelial cells after abrasion with each air-polishing powder. Hormia *et al.*²³⁾ suggested that integrin $\alpha 6$ and integrin $\beta 4$ are significantly involved in epithelial adhesion to the tooth surface and the junctional epithelium. It has been shown that the expression of ICAM-1 is higher in the gingival tissue of patients with chronic periodontitis than in normal gingival tissue²⁴⁾. We suggest that titanium that has been air-polished with tooth surface abrasives with large average particle diameter and high biocompatibility causes inflammation of the gingival epithelial cells and enhances the migration of white blood cells. Integrin $\alpha 6$ and integrin $\beta 4$ are involved in the wound healing of gingival epithelial tissue^{25,26)}, and are also associated with the adhesion and migration of junctional epithelium cells. Miyata *et al.*²⁷⁾ also reported that integrin $\alpha 6$ and integrin $\beta 4$ are involved in the epithelial boundary surface of the implant. Expression of ICAM-1 was substantially higher in the control group than in the sodium bicarbonate and glycine 75 μm groups. Expression of integrin $\alpha 6$ was substantially higher in the glycine 25 μm group than in all other groups, including the control group. Expression of integrin $\beta 4$ was substantially higher in the glycine 25 μm group than in the sodium bicarbonate and glycine 75 μm group. These results suggest that using an abrasive powder with a small average particle diameter and a high level of biocompatibility positively influences the adhesion migration function of epithelial cells. It is also suggested that difference in gene expression 3 h after the culture influences the cell proliferation at 72 and 24 h. The use of tooth surface abrasives with a small average particle size and a high level of biocompatibility exerts few effects on the living body, while providing an effective treatment for periodontal pockets and the implant sulcus to enhance cell adhesion and growth of the epithelial attachment.

The initial homeostasis of implants is almost established, and

implants are useful as a therapy for sites where teeth are missing. However, the current synostosis type implant has been established for 50 years^{1,2)}, and is susceptible to inflammation around the implant. The incidence of peri-implantitis is likely to increase in the future. Inflammation may be reversed by removing the peri-implantitis bacterial infection from the implant sulcus. However, peri-implantitis may extend to the osseointegrated tissue surrounding the implant, causing irreversible disease as the inflammatory process causes the loss of the supporting bone²⁸⁾. It is important that implant treatment in the future focuses on reducing the incidence of peri-implantitis. Kreisler et al.²¹⁾ investigated the efficiency of bacterial infection removal with the Er: YAG laser and different tooth surface abrasives. It is easier to change the quality of bacterial infection removal on the titanium surface by using tooth surface abrasives than by using the Er: YAG laser. However, bacterial infection removal with tooth surface abrasives results in a higher level of cell proliferation and may remove cytotoxic bacteria from the implant surface. Therefore, we consider that mechanical cleaning of the implant sulcus is essential, and tooth surface abrasives can be used to effectively remove bacterial infection from the implant sulcus. This study examined the effects of the use of tooth surface abrasives on epithelial tissue in the implant sulcus. Our findings suggest that different tooth surface abrasives have an influence on the wound healing of the implant joint, and that tooth surface abrasives with a high level of biocompatibility and small average particle size are effective in promoting early wound healing.

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Competing interest

The authors have declared that no COI exists.

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