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Initial attachment of human oral keratinocytes cultured on zirconia or titanium

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INTRODUCTION

Titanium (Ti) and its alloys are used extensively in the dental-implant treatment of patients. There are, however, aesthetic problems associated with the Ti implants due to its gray coloring. Moreover, since Ti originally having the characteristics of biotolerant which cause ion elution by corrosion in some environments, Ti implants have been reported to trigger allergic reactions¹-⁴. Recently zirconia has drawn attention as a potential alternative to Ti, as it allows both allergic reactions and aesthetic problems to be avoided⁵-⁸. Yttrium oxide-stabilized tetragonal zirconia polycrystals (TZP) are used in dentistry and show outstanding mechanical, biocompatible and aesthetic performance. Zirconia enjoys popularity in the dental community due to its bright coloring, and is recommended in cases where the quantity of soft tissue would not be enough to mask the gray color of Ti, especially in areas of esthetic concern⁶. Furthermore, zirconia was found to be as biocompatible as Ti⁷,⁸.

The soft tissue barrier around dental implants serves as a protective seal between the oral environment and the underlying bone. Around natural teeth, the junctional epithelium attaches to the tooth by hemidesmosomes formed by laminin γ₂ and integrin β₄ via the internal basal lamina, acting as a biological seal between the oral environment and the internal structures of the body. On the other hand, it has been reported that the peri-implant epithelium attaches only weakly at the apical portion of peri-implant epithelium-implant surface made from Ti by laminin γ₂ in rat⁹. This means that inflammation around implant can occur more easily than around natural teeth. Therefore, securing a biological seal with the peri-implant epithelium at the abutment is essential for the long-term success of implant treatment.

In terms of the bone-implant interface, the adhesion of osteoblasts to the implant surface is important in achieving osseointegration. A number of studies have described the early stages of osteoblast-zirconia attachment, proliferation, differentiation and activity¹⁰-¹². The initial attachment of fibroblasts, assuming soft tissue adhesion, on zirconia or Ti discs has also been investigated¹³,¹⁴, and these studies have confirmed the similar biological responses of each type of cell on each material. In determining the potential success of epithelium-implant attachment, it is necessary to characterize the response of epithelial cells to zirconia, and initial cell attachment is of particular importance for subsequent cell proliferation, migration and differentiation¹⁵,¹⁶. Raisanen et al. analyzed the extent of epithelial cell coverage on Ti¹⁷. Cochran et al. evaluated the attachment and growth of epidermal keratinocytes on Ti¹⁸, while Shiraiwa et al. examined the initial attachment and subsequent behavior of epithelial cells on Ti¹⁹. Moreover, several studies have reported that Ti modification enhances keratinocyte adhesion²⁰-²². However, to our knowledge, no studies have investigated the initial attachment of keratinocytes on zirconia.

The purpose of this study was to clarify the epithelial cell adhesion to the poor condition surface, especially, the initial attachment of human oral keratinocytes (HOKs) on TZP compared with on pure Ti in vitro.

MATERIALS AND METHODS

Specimen preparation

Commercially pure Ti (grade 2, Tokyo Titanium, Saitama, Japan) and TZP (3 mol% yttria-stabilized, Tosoh, Tokyo, Japan) discs were used in this study. Discs of 13 mm or 30 mm in diameter and 0.5 mm in thickness were ground progressively finer down to 1,200 grit and then finely polished with 3-μm diamond paste and
0.06-µm colloidal silica using a polishing machine (Ecomet 3, Buehler, Lake Bluff, IL, USA). Finally, they were ultrasonically cleaned with acetone and distilled water.

**Surface roughness and surface wettability**
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 a cut-off value of 0.8 mm.

The surface wettability of each sample was characterized by contact angle measurement with distilled water using a contact angle meter (Phoenix®, Meivwa-forces, Tokyo, Japan). Measurements were made at three different locations on each sample at 15 s after application of each droplet. The volume of each drop was maintained at 4 µL.

**Cell culture**
Cell culture experiments were performed using 3-passaged HOKs (ScienCell Research Laboratories, Carlsbad, CA, USA). Cells were cultured at 37°C in a CO₂ incubator (5%) in a keratinocyte medium (ScienCell Research Laboratories) supplemented with 5 mL oral keratinocyte growth supplement (ScienCell Research Laboratories) and 5 mL penicillin/streptomycin solution (ScienCell Research Laboratories). The medium was changed every 2–3 days and the cells were passaged at 80–90% confluence with the use of 0.05% trypsin-EDTA (Gibco BRL, Grand Island, NY, USA).

**Cell attachment assay**
The 13-mm discs were placed in 24-well culture dishes and HOKs seeded into the wells at a density of 5.0×10⁶ cells/mL (in 1 mL). The HOKs were incubated for 1, 3, 12, 24, or 48 h. After washing in PBS, cells were detached using 0.05% trypsin-EDTA at each time period and the number of detached cells determined using a Coulter counter (Beckman Coulter, Fullerton, CA, USA).

The viability of attached cells on the 13-mm discs was evaluated at 1, 3, 12, 24, or 48 h of culture at a density of 2.0×10⁶ cells/mL. Quantification was performed using WST-1 based colorimetry (WST-1, Roche Applied Science, Mannheim, Germany). At each time period, the discs were washed three times with PBS to remove unattached cells and moved to new culture plates. The culture wells were incubated at 37°C for 1 h with 50 µL tetrazolium salt (WST-1) reagent and 500 µL culture medium, after which 110 µL reaction solution was moved to 96 well plates to measure absorbance. Formazan production was measured using a microplate reader (SpectraMax M5, Molecular Device, Tokyo, Japan) at 450 nm.

**Quantitative RT-PCR**
Cells were seeded at a density of 3.0×10⁴ cells/mL on each 30 mm disc for quantitative RT-PCR to measure RNA expression of epithelial cell attachment proteins laminin γ₂, and integrin β₄. Total RNA was extracted from HOKs using the acid guanidium thiocyanate/phenol-chloroform method as follows: after 1, 3, 12, 24 or 48 h incubation, the culture medium of each substrate was removed and the cells rinsed twice using PBS. Cells were homogenized in 500 µL TRIsol Reagent (Invitrogen, Carlsbad, CA, USA) and each solution transferred to a 1.5-mL tube containing chloroform. Each tube was then centrifuged at 14,000 rpm at 4°C for 20 min, after which the supernatant was placed in a 1.5-mL tube containing 250 µL of 100% isopropanol (half the amount of TRIsol® Reagent) at −80°C for 1 h. After centrifugation at 14,000 rpm for 20 min at 4°C, the supernatants were discarded and the remaining total RNA pellets washed with 70% cold ethanol. Total RNA pellets were dissolved in 50 µL RNAase-free (DEPC-treated) water. Total RNAs were reverse transcribed and amplified in 20 µL volumes using a Reverse Transcription Kit (Quanti Tect®, Qiagen, Germantown, MD, USA) containing RNA PCR Buffer, 2.5 µg/mL RNAase inhibitor, 0.25 U/µL reverse transcriptase, 0.125 µM oligo dt-adaptor primer, 5 mM MgCl₂ and RNAase-free water. RT-PCR products were analyzed by quantitative real-time RT-PCR in TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for the target genes, laminin γ₂ (Hs00194345_mL) and integrin β₄ (Hs00236216_mL). The TaqMan® Endogenous Control (Applied Biosystems) for the target gene β-actin (4333762T) was used as an endogenous control. All PCR reactions were performed using the real-time PCR 7500 Fast System. Quantification of gene expression using TaqMan Gene Expression Assays was performed using the real-time PCR 7500 Fast System. Gene expression quantitation using TaqMan® Gene Expression Assays was performed as the second step in two-step RT-PCR. Assays were performed in 20 µL single-plex reactions containing TaqMan® Fast Universal PCR Master Mix, TaqMan® Gene Expression assays, distilled water and cDNA, according to the manufacturer’s instructions (Applied Biosystems). Reaction conditions consisted of a primary denaturation at 95°C for 20 s, then cycling for 40 cycles of 95°C for 3 s and 62°C for 30 s. PCR data were compared with those for Ti at 1 h as the baseline.

**Immunofluorescence observation and morphometry**
The HOKs were seeded at a density of 2.0×10⁶ cells/mL on each 13 mm disc for immunofluorescent observation. To observe the distribution of laminin γ₂ and integrin β₄, the HOKs were washed in PBS after 1, 3, 12, 24 or 48 h culture and then fixed in 10% paraformaldehyde for 30 min at room temperature. The cells were then washed 3 times with PBS. Nonspecific binding was blocked with 3% bovine serum albumin (BSA) for 30 min at room temperature. To observe actin filament, the cells were incubated for 30 min at room temperature with phalloidin. The other cells were incubated overnight at 4°C with a primary rabbit polyclonal antibody against laminin γ₂ (1:100 dilution, Abcam, Cambridge, UK) or an integrin β₄ mouse monoclonal antibody (1:50 dilution, Abcam, Cambridge, UK). After 3 additional washes in PBS, the samples were incubated with a secondary
antibody —either Alexa fluor 543 goat anti-rabbit immunoglobulin G (IgG) (Molecular Probes, Eugene, OR, USA) for laminin γ2, which were detected as a red, or Alexa fluor 488 for integrin β4, which were detected as a green (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. Subsequently, the samples were incubated with 1 µg/mL 4′, 6-diamidino-2-phenylindole (DAPI, Invitrogen), which were detected as a blue for 10 min at room temperature. After 5 washes in PBS, a micro cover glass was mounted over each sample with antifade reagent (ProLong Gold, Invitrogen).

FITC-conjugated phalloidin (1:100 dilution, Invitrogen) was used to observe filamentous actin (F-actin) stress fibers.

These samples were observed using a confocal laser scanning microscope (CLSM, LSM 5 DUO, Carl Zeiss, Jena, Germany) with software (Zen 2008, Carl Zeiss). Perimeter of actin was quantified using an image analyzer (ImageJ, NIH, Bethesda, MD). Over 5 cells were quantified at each 10 randomly selected area per each of 5 discs.

**ELISA assay and comparative analysis of expressed laminin γ2 and integrin β4**

Enzyme-linked immunosorbent assays (ELISA) of HOKs were performed to compare the expression of laminin γ2 and integrin β4. HOKs were seeded at a density of 5.0×10^4 cells/mL on each 30 mm disc. After 1, 3, 12, 24 or 48 h culture, the cells were washed twice with PBS, lysed in RIPA buffer (#R0278, SIGMA ALDRICH, Tokyo, Japan) containing protease inhibitors. The lysates were cleared by centrifugation at 8,000 rpm for 10 min at 4°C to obtain supernatant. Protein concentrations in the supernatant were determined by using BCA protein assay reagent kit (Thermo Fisher Scientific, Illinois, USA). Each sample was diluted to a concentration of 500 ng/mL in PBS. 96-well plastic plates were coated with laminin γ2 antibody at 1.0 µg/mL in 1% BSA or integrin β4 antibody 0.5 µg/mL in 1% BSA at 4°C overnight, and blocked with 3% BSA. 200 µL of samples were added to the microasssay well and incubated at room temperature for 1 h. After washing with PBS containing Tween 20 (PBS-T), samples were incubated with laminin γ2 antibody at 10 µg/mL in 1% BSA or integrin β4 antibody 1.0 µg/mL in 1% BSA for 30 min at room temperature. After washing with PBS-T, either Alexa fluor 488 goat anti-rabbit immunoglobulin G (IgG) (Molecular Probes, Eugene, OR, USA) for laminin γ2 or Alexa fluor 488 goat-anti mouse for integrin β4 (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. 200 µL of PBS was added each well to after washing 5 times with PBS-T. Fluorescence at 485 nm was evaluated with a Microplate reader (Molecular Device).

**Scanning electron microscopy (SEM)**

The HOKs were allowed to adhere to each disk for 1, 3, 12, 24 or 48 h at a density of 2.0×10^4 cells/mL. At each time period, the discs were washed three times with PBS to remove the culture medium and unattached cells were then immersed in 1.25% glutaraldehyde PBS solution for 1 h. The discs were then washed with PBS for 15 min. After dehydration in a graded series of ethanol, all discs were placed in tetrabutylalcohol followed by freeze-drying. The dried specimens were mounted on viewing boards, coated with a gold-palladium alloy and observed using a scanning electron microscope (SEM, JSM6340F, JEOL, Tokyo, Japan).

**Statistical analysis**

Data from each experiment were statistically analyzed with the Student’s t-test to determine differences between cultures on the Ti or TZP discs (p<0.05).

**RESULTS**

**Surface roughness and contact angle assessment**

No significant difference was observed in surface roughness, with a mirror-like surface yielding Ra values of 0.065±0.002 and 0.067±0.003 for Ti and TZP respectively (Table 1). Significant difference was observed in contact angle (Table 1), at 61.5°±2.1° and 68.6°±1.3° on Ti and TZP respectively.

![Fig. 1 Number of HOKs attached to Ti or TZP measured using Coulter counter after 1, 3, 12, 24, 48, 72, 120 or 168 h culture. (n=5).](image)

Table 1  Surface roughness values and contact angle of Ti and TZP. Data represent means (±SD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface roughness (Ra, µm)</th>
<th>Contact angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure titanium (Ti)</td>
<td>0.065±0.002</td>
<td>61.5°±2.1°</td>
</tr>
<tr>
<td>Zirconia (TZP)</td>
<td>0.067±0.003</td>
<td>68.6°±1.3°</td>
</tr>
</tbody>
</table>
Cell attachment assay
The number of attached HOKs on each disc increased until 168 h. No significant difference was observed in number of HOKs between Ti and TZP at any time period examined. Number of attached HOKs reached that of cells initially seeded at between 48 and 72 h (Fig. 1).

Degree of cell attachment as measured by WST-1 assay showed no significant difference between the Ti and TZP groups (Fig. 2). A similar degree of viability was observed between HOKs seeded on each substrate.

mRNA expression (quantitative RT-PCR)
Expression of laminin γ2 and integrin β4 mRNAs in HOKs is shown in Fig. 3. No significant difference in expression of laminin γ2 or integrin β4 mRNA was observed between the two substrates except at 1 h of culture, where it was significantly higher on Ti discs. Laminin γ2 and integrin β4 mRNA was consistently observed on each disc at each time point.

Immunofluorescence observation and morphometry
Laminin γ2 and integrin β4 immunofluorescence images are shown in Fig. 4. After 1 h of culture, immunoreactivity for laminin γ2 and integrin β4 was observed on each substrate. Dotted distribution of laminin γ2 was observed at 1 and 3 h of culture. After 24 and 48 h of culture, laminin γ2 was distributed at the cell periphery more strongly than at 1 or 3 h of culture. Dotted distribution of laminin γ2 at 1 h changed, moving to the periphery of each substrate at 48 h. Distribution of integrin β4, uniform throughout the cytoplasm at 1 and 3 h of culture, became patchy at 48 h of culture. Uniform distribution of integrin β4 within the cytoplasm at 1 h became patchy at 48 h on each substrate. Similar distribution pattern of laminin γ2 or integrin β4 was observed on either substrate between 1 and 48 h.

Immunofluorescence images of actin filaments are shown in Fig. 5. Actin filaments extended with time on both discs. The stretching of cellular processes was similar on both substrates at all time points (Fig. 6).

Protein expression (ELISA assay)
The results of ELISA assay are shown in Fig. 7. No significant difference in expressions of laminin γ2 and integrin β4 proteins was observed between the two substrates except at 1 h of culture, where it was significantly higher on Ti discs. Laminin γ2 and integrin β4 protein was consistently observed on each disc at each time point.

Cell morphology
Changes in cell morphology are shown in Fig. 8, with cells losing their initial roundness to become more spread out with time. The apparent formation of filopodia was observed at 12 h; lamellipodia were observed at 24 and at 48 h. No significant difference was observed in cell morphology between the Ti and TZP discs at any time point.

DISCUSSION
To compare the attachment of epithelial cells on Ti and TZP, mirror-surfaced discs which can’t adhere easily for cells in generally were used in this study. The Ra values of both types of disc were similar in this study, suggesting that its effect on cell attachment would be equivalent.

To determine the initial attachment period, we counted attached cell numbers on each disc using a Coulter counter. At 72 h of culture, the number of attached cells had proliferated. Cell behavior begins with adhesion, followed by spread and, eventually, proliferation. We determined that the initial attachment period was within the first 48 h in this study. The number of attached cells showed a continuous increase, with similar numbers at each time point.

To compare number of viable attached cells on each disc, we used the WST-1 assay. Consistent with the results of the Coulter counter, the results of the WST-1
assay showed that the number of viable cells increased over time and was similar between both groups at every time point. Yamashita et al. reported that for chemically stable bioinert materials with an oxide film such as Ti or TZP, constituents of cell attachment were influenced by surface roughness. Therefore, we conclude that the above results were due to the similarity in surface roughness between the two substrates.

Expression of laminin γ2 and integrin β4 mRNA showed a significant difference only at 1 h, being approximately the same at every other time point. The difference in laminin γ2 and integrin β4 mRNA expression and protein expression at 1 h appears to arise from differences in surface physicochemical properties such as surface wettability (contact angle) and electrical charge (zeta potential). The surface of Ti has been revealed about the functional groups determined wettability, but the surface of TZP has not been clear. The difference in contact.
angle between Ti and TZP was approximately 7°. Wei et al. reported no significant difference in number of attached fibroblasts on discs with a 60° or 80° contact angle at 6, 12 or 24 h, indicating that a small difference in contact angle does not influence expression of laminin γ2 or integrin β4 mRNA. Therefore, it was considered that it was affected by difference in electrical charge. The electrical charge of TiO2 at a neutral pH is known as negative, whereas that of Y-TZP is almost ±0. When exposed to air, Ti reacts immediately with oxygen, forming a surface oxide layer. Accordingly, TiO2 layer adsorbs ionized Ca, Acidity hydroxyl (OH) groups that are charged negatively could be directly adsorbed to ionized Ca. In other words, at first, Ti promotes adsorption of Ca ions, allowing easier attachment of negative keratinocytes. This may explain why expressions of laminin γ2 and integrin β4 mRNAs on Ti were higher than on TZP at 1 h. And the results of protein expressions were considered to reflect differences in mRNA expressions. Distribution of laminin γ2 and integrin β4 proteins were determined by immunofluorescence. Laminin γ2 was observed in the membrane in HOKs cultured on TZP, as well as on other materials, and integrin β4 was also observed as dots in the cytoplasm. No difference was observed in the distribution of laminin γ2 or integrin β4 between HOKs cultured on Ti or on TZP. Distribution of laminin γ2 changed over time, from a punctuate distribution at 1 h to sheet-like at the cell periphery at 48 h in both groups. Laminin γ2 is also localized in epithelial cells, as well as at the track of cell migration. Cell migration is elicited by laminin γ2, which is highly expressed in the direction of migration. In this study, laminin γ2 was deposited more strongly on one side at 48 h, and this was attributed to cell migration. Integrin β4 localizes in the entire cytoplasm of oral epithelial cell in static condition. Various-sized foci are mingled. However, integrin β4 localizes in the opposite direction in
migrating cell. Distribution of integrin β3 changed from throughout the cytoplasm at 1 h to patchy distribution at 48 h on each substrate, as well as on other materials. This may have been indicative of change in cell migration.

Laminin γ2 is structure structural protein of hemidesmosomes. The expression and organization of hemidesmosomes is a reflection and indicator of the efficiency of cell adhesion. Lack of laminin-5 induces epidermal detachment from the hypodermis. Because laminin is localized in the membrane, expression of laminin γ2 was determined to estimate adhesion force. ELISA assay revealed that expression of laminin γ2 on Ti discs was higher than that on TZP discs at 1 h, suggesting that cell attachment on TZP was poorer than that on Ti. Although expression of mRNA was significant difference, the number and viability of attached HOKs were similar. It was thought that the differences of mRNA expression were reflected to expression of laminin γ2 rather than the number of attached HOKs, indicating difference of its adhesion force. Binding of laminin γ2 to integrin αβ6 stimulates the formation of hemidesmosomes. Figure 4 shows that HOKs had attached to the TZP by laminin γ2 and integrin β6, then spread by the development of actin stress fibers. The cell morphology changes from round to flat as the actin fibers develop. Therefore, the perimeter of actin increased continuously, showing no difference between the Ti and TZP substrates at any time point. Taken together with our observations on cell morphology, this suggests that similar changes occur at the same time, regardless of substrate.

Scanning electron microscopy was used to investigate cell morphology during the initial time periods on the TZP or Ti discs. Cell migration began with adhesion and then spread. Cells undergoing chemotaxis extend protrusions such as filopodia and lamellipodia, and then move by forming new adhesions and retracting their tails. In this study, some filopodia formation was observed at 12 h. At 24 and 48 h, lamellipodia formation was observed on both substrates. The time points when filopodia and lamellipodia formation was observed were similar to those of the phalloidin stained images.

The initial attachment of HOKs cultured on TZP was equivalent to or slightly lower than that on Ti. Since TZP was showed similar biological response in comparison to Ti after 48 h, TZP could be considered to be not inferior to Ti in clinical use in terms of epithelial cell attachment. Therefore, it was suggested that TZP has a potential to form epithelial attachment like Ti.

REFERENCES