Effect of surface roughness on initial responses of osteoblast-like cells on two types of zirconia

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The aim of this study was to evaluate the effect of surface roughness on the initial attachment of mouse osteoblast-like cells on ceria-stabilized zirconia/alumina nanocomposite (NANOZR) and yttria-stabilized zirconia (3Y-TZP) in comparison to those on pure titanium (Ti) and alumina oxide (AO). Specimens with smooth and rough surfaces were prepared by grinding with diamond paper or by sandblasting, respectively. For four substrates examined, the number of attached cells on the rough surface specimens was significantly higher than that on the smooth surface specimens (p < 0.05). Integrin α₁ and β₁ expression had a greater increase in rough surface specimens than in smooth surface specimens. Actin cytoskeleton organization was, however, similar for both smooth and rough surface specimens. NANOZR and 3Y-TZP produced good cell attachment, similar to Ti and AO. The overall results demonstrated that NANOZR and 3Y-TZP with rough surface could provide good initial cell responses, adequate for future implant usage.

Keywords: Zirconia, Surface roughness, Osteoblast

INTRODUCTION

Since implant replacement in dentistry is a widely used treatment modality¹,², its application has become more widespread. Dental implants are used in many patients, including those with edentulous situations³,⁴. Commercially pure titanium and titanium alloys are mostly utilized as dental implant materials. These biocompatible materials have been used for 40 years as implant substrates⁵ and have had high success rates⁶,⁷. Although the implant functions optimally, one problem is the possibility of the grayish color of the titanium implant shining through the thin peri-implant mucosa, thus marring the entire aesthetic result⁸. Titanium may be exposed due to recession of the peri-implant mucosa. Furthermore, several studies found that titanium can induce an allergic reaction⁹. With highly sensitive immunologic tests, sensitization to titanium can be observed in some cases³,⁴,¹⁰. Thus, serious efforts are now being made to develop safer implants with high success rates¹¹. To resolve the allergy and aesthetic problems caused by titanium implants, a ceramic implant was developed as a viable alternative. However, a major problem with all-ceramic restorations is their low fracture resistance, which makes it difficult to fabricate an all-ceramic dental implant. Aluminum oxide (alumina) was once used as an implant material¹², but because of its insufficient physical properties, it was withdrawn from the market. Ceramics are generally brittle and subject to premature failure, especially in repeated contact loading and in moist environments.

Recently, research has focused on another ceramic material, zirconium dioxide (zirconia), which has the potential for future use as a dental implant material. Tetragonal zirconia polycrystals, especially 3 mol% yttria-stabilized zirconia (3Y-TZP), serves as a metal substitute in substrates and possesses good physical characteristics¹³,¹⁴; 3Y-TZP has been used as a conventional material for medical and dental restorations¹⁵,¹⁶. Some researchers, however, recently reported that a ceria-stabilized zirconia/alumina nanocomposite (NANOZR) not only had higher strength but might also exhibit higher fracture toughness when compared to 3Y-TZP¹⁷-²². Currently, data regarding the use of zirconia in medical and dental implants mainly come from animal and laboratory trials²³-²⁵.

When biomaterials are introduced into human tissue, the biocompatibility of the materials depends not only on their physical and chemical surface properties but also on the initial response of the cells on the material surface. Furthermore, the interaction of the cells with the material surface is fundamentally relevant and contributes to osseointegration. The initial attachment and adhesion on a material surface²⁶ are followed by cell spreading and migration. The quality of this first phase of cell-material interaction will influence the cell capacity to proliferate and differentiate²⁷. Integrins as transmembrane heterodimeric receptors consisting of an α- and β-subunit play important roles in signal transduction and in the organization of the actin cytoskeleton²⁸-³¹. The...
extracellular domain of the integrins binds to extracellular matrix (ECM) proteins, such as fibronectin (FN), as a prime target of \( \alpha_5\beta_1 \). Actin is a structural component of microfilaments, and actin filaments link integrins through talin, vinculin, or tensin. The bundling of microfilaments produces stress fibers.

The functional activity of cells in contact with the biomaterial is determined by the characteristics of the surface, as observed for titanium. Furthermore, the surface roughness has been established to play an important role in osseointegration. A few studies have reported on the effect of surface roughness on zirconia. Because of the progress made both in material research and cell biology, biocompatibility investigations have increased focus on the initial cellular response that controls the cell physiology on different surface roughness materials. This study was designed to evaluate the effect of surface roughness on the initial attachment of osteoblast-like cells to two types of zirconia and to compare these results with those on titanium and alumina.

**MATERIALS AND METHODS**

**Sample preparation**

As listed in Table 1, 3Y-TZP (15 mm in diameter and 0.5 mm in thickness), NANOZR (15 mm in diameter and 0.5 mm in thickness), commercially pure titanium (15 mm in diameter and 1 mm in thickness, referred to as Ti), and alumina (15 mm in diameter and 0.5 mm in thickness, referred to as AO) disks were used in this study. Smooth surfaces for each substrate were polished by diamond paper (#220, #400, #600, and #1000). The rough surface on Ti was prepared by 70-\( \mu \)m Al\(_2\)O\(_3\) sandblasting and on other specimens by 125-\( \mu \)m Si\(_C\) powder sandblasting at 0.4 MPa, 90° against the surface and 10 mm away from the surface. After sandblasting, NANOZR and 3Y-TZP disks were heated for 5 min at 1000°C in air to recover the crystal phase on the zirconia surface. These specimens were ultrasonically cleaned for 10 min in acetone, ethyl alcohol, and distilled water, and were sterilized in an autoclave for 20 min at 121°C under 2 atm. According to our previous results, low temperature degradation could be assumed to be negligible with this autoclave.

**Surface roughness and morphology**

The surface roughness was measured with a surface profile meter (Surfcom 130A; Tokyo Seimitsu, Tokyo, Japan). The specimens were sputter-coated with gold and characterized by scanning electron microscopy (SEM; JSM-5510LV; JEOL, Tokyo, Japan). An atomic force microscope (AFM; Nanoscope III; Digital Instruments, Santa Barbara, CA, USA) was used to observe the three-dimensional surface topography of the specimens.

**Cell cultures**

Mouse osteoblast-like MC3T3-E1 subclone 4 cells (MC3T3-E1) were purchased from ATCC (Manassas, VA, USA). The cells were grown in \( \alpha \)-modified minimum essential medium (\( \alpha \)-MEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Moregate Biotech, Bulimba, QLD, Australia), 2 mM L-glutamine (ICN Biomedicals, Aurora, OH, USA), 100 units/ml penicillin G (Wako Pure Chemical Industries, Osaka, Japan), and 100 \( \mu \)g/ml streptomycin (Wako Pure Chemical Industries) in a humidified atmosphere of 95% air/5% CO\(_2\) at 37°C. The medium was changed every 2–3 days until the cells reached subconfluence. Next, the cells were detached using 0.25% trypsin (Gibco Laboratories, Paisley, UK) and 0.02% ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Kyoto, Japan) in phosphate-buffered saline (PBS). Cells (5 \( \times \) 10\(^4\) cells/well; \( n = 4 \)) were plated in 24-well tissue culture plates (Iwaki, Tokyo, Japan) on disks composed of Ti, AO, 3Y-TZP, and NANOZR. Ti and AO were employed as the control specimen.

**Cell attachment**

Cell attachment was evaluated using Cell-Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) at 1, 3, 6, and 24 h after incubation. The counting technique employed a tetrazolium salt that produced a highly water-soluble formazan dye. After 1h incubating with the reagent according to the manufacturer's

<table>
<thead>
<tr>
<th>Code</th>
<th>Product Name (Manufacturer)</th>
<th>Composition</th>
<th>Final Sintering</th>
<th>Flexural strength(^{18}) (MPa)</th>
<th>Fracture toughness(^{18}) (MPa m(^{1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti</td>
<td>KS-40 (Kobelco)</td>
<td>100 % Ti</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AO</td>
<td>TM-DAR (Taimei Chemicals)</td>
<td>Al(_2)O(_3)</td>
<td>1400°C, 1 h</td>
<td>500</td>
<td>3.9</td>
</tr>
<tr>
<td>3Y-TZP</td>
<td>TZ-3YB-E (Tosoh)</td>
<td>3 mol Y(_2)O(_3) – ZrO(_2)</td>
<td>1350°C, 6 h</td>
<td>1200</td>
<td>10</td>
</tr>
<tr>
<td>NANOZR</td>
<td>MACZ-100 (Panasonic Electric Works)</td>
<td>10 mol CeO(_2)–ZrO(_2) 30 vol% Al(_2)O(_3)</td>
<td>1450°C, 2 h</td>
<td>1500</td>
<td>18</td>
</tr>
</tbody>
</table>
instructions, the absorbance of the dissolved solute was measured at a wavelength of 450 nm by the formazan dye product in the cultures. These tests were repeated four times for each group.

**Cell morphology**

Cells that attached onto the specimens at 1, 3, 6, and 24 h after incubation were fixed with 2.5% glutaraldehyde containing 0.1 M sodium cacodylate buffer (pH 7.4 and 4°C) for 1 h and post-fixed for 30 min with 1% osmium tetroxide containing 0.1 M cacodylate buffer. After dehydration in graded ethanol, specimens were transferred into t-butyl alcohol and freeze-dried. Specimens were sputter-coated with gold, and the cell morphology was observed by SEM.

**Flow cytometry analysis**

Cells that attached onto the specimens 3 h after incubation were washed with PBS and detached by 0.05% trypsin and 0.02% EDTA in PBS at 37°C. Cells were fixed for 15 min in PBS containing 1% paraformaldehyde. After rinsing with 0.5% bovine serum albumin (BSA; Wako Pure Chemical Industries) in PBS, the cells were incubated with hamster anti-mouse integrin α5 antibody and rat anti-mouse integrin β1 antibody (1:50; BD Biosciences, San Jose, CA, USA) for 30 min at 4°C. Cells were washed with 0.5% BSA again, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-armenian hamster IgG (H+L) antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 30 min at 4°C, and analyzed with a flow cytometer (EPICS XL; Beckman Coulter, Fullerton, CA, USA).

**Actin staining**

Cells that attached onto the specimens at 1, 3, and 6 h after incubation were washed with PBS and fixed for 15 min in PBS containing 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 for 30 min at room temperature and rinsed with PBS. After blocking with 0.5% BSA for 30 min, cells were incubated with Alexa 465-conjugated phalloidin (1:50; Molecular Probes, Leiden, The Netherlands) for 30 min at room temperature. Following a brief wash, specimens with cultured cells were mounted on microscope slide, and the fluorescent images were photographed under a fluorescence microscope (BX-51, Olympus, Tokyo, Japan).

**Statistical analysis**

These data were statistically analyzed by two-way ANOVA.

**RESULTS**

**Characterization of materials**

Table 2 shows the surface roughness Ra (arithmetical mean deviation of the profile) of the smooth and rough groups. The surface roughness Ra of the smooth group had an average of 0.24 µm, and rough specimens had an average of 1.04 µm. Fig. 1 shows an SEM photograph of four kinds of smooth and rough surface.

<table>
<thead>
<tr>
<th>Ra (µm)</th>
<th>Ti</th>
<th>AO</th>
<th>3Y-TZP</th>
<th>NANOZR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>0.33 ± 0.05</td>
<td>0.26 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Rough</td>
<td>1.03 ± 0.08</td>
<td>0.98 ± 0.08</td>
<td>1.01 ± 0.08</td>
<td>1.13 ± 0.10</td>
</tr>
</tbody>
</table>

Fig. 1  SEM photographs of the smooth and rough surfaces of Ti, AO, 3Y-TZP, and NANOZR.
Fig. 2  AFM images of the smooth and rough surfaces of Ti, AO, 3Y-TZP, and NANOZR.

Fig. 3  Attachment of MC3T3-E1 on the smooth and rough surfaces of Ti, AO, 3Y-TZP, and NANOZR at 1, 3, 6, and 24 h after incubation.
Fig. 4  SEM photographs of MC3T3-E1 on the smooth (a) and rough (b) surface of Ti, AO, 3Y-TZP, and NANOZR at 1, 3, 6, and 24 h after incubation.
specimens. Due to the grinding, many scratches were observed on the smooth surface of Ti, 3Y-TZP, and NANOZR; the surface texture of AO was different. After sandblasting, edges and deep depressions were observed on the roughened surfaces for all materials except AO. Fig. 2 shows an AFM picture of the surface of four types of smooth and rough plates. The surface topographies for the smooth surface samples of Ti, 3Y-TZP, and NANOZR were smooth but also had many scratches; AO had an unusual morphology due to the grain boundaries of the sintered Al₂O₃ particles. Shape edges were observed on the rough surface specimens except for AO.

**Cell attachment**

Fig. 3 shows the attachment of MC3T3-E1 on the smooth and rough surfaces of Ti, AO, 3Y-TZP, and NANOZR at 1, 3, 6, and 24 h after incubation. The increase in the number of cells was time-dependent for all specimens and was greater for rough surface specimens than for smooth surface specimens ($p < 0.05$). However, among these surface group specimens, no significant differences were observed in the attachment ($p > 0.05$).

**Cell morphology**

Fig. 4 shows SEM photographs of MC3T3-E1 on the smooth and rough surfaces of Ti, AO, 3Y-TZP, and
NANOZR at 1, 3, 6, and 24 h after incubation. The cell morphology of MC3T3-E1 cells on rough and smooth surfaces was normal. After a 1 h-incubation, a spherical form of cells covered the surface of all the plates. After a 3 h-incubation, the cells spread on all of the plates. After 6 h- and 24 h-incubations, the spread cell was denser with numerous cell–cell contacts on all the plates. These results demonstrated that MC3T3-E1 on all the plates appeared to attach and spread well.

Expression of integrin α5 and β1
Fig. 5 shows the fluorescence intensity of α5 and β1 integrins expressed on smooth and rough surfaces of Ti, AO, 3Y-TZP, and NANOZR at 3 h after incubation. Lines represent a negative control mouse IgG-stained with FITC-conjugated secondary antibody. Gray and black areas indicate the fluorescence profile of cells after indirect fluorescence staining with anti-integrin α5 and β1 monoclonal antibodies. MC3T3-E1 expressed integrin α5 and β1 on the all plates. The increase in

Fig. 6 Analysis of actin cytoskeleton of MC3T3-E1 on the smooth (a) and rough (b) surface of Ti, AO, 3Y-TZP, and NANOZR at 1, 3, and 6 h after incubation.
fluorescence intensities of $\alpha_5$ and $\beta_1$ integrins expression was greater for rough surface specimens than for smooth surface specimens. However, the fluorescence intensities of integrin $\alpha_5$ and $\beta_1$ were almost same among the specimens.

Analysis of actin cytoskeleton

Fig. 6 shows the actin cytoskeleton of MC3T3-E1 after 1, 3, and 6 h of culture on smooth and rough groups of Ti, AO, 3Y-TZP, and NANOZR. After a 1 h-incubation, MC3T3-E1 had a spherical form on the surface of all of the plates. After a 3 h-incubation, the cells spread on all the plates. After a 6 h-incubation, we observed typical long and straight actin stress fibers on all the specimens. The actin filament distribution was similar on both smooth and rough surface specimens.

DISCUSSION

Zirconia has been employed as a conventional material for dental restorations. Zirconia is a bioinert material with excellent biocompatibility. Its mechanical properties, chemical stability, and aesthetics make this material suitable as a dental implant material. It has been shown that in titanium, currently the most popular implant material, its surface roughness improves osseointegration, but the influence of surface roughness on the biomechanical properties of zirconia has not been extensively investigated. In this study, we focused on MC3T3-E1 response on zirconia substrates with different surface roughness.

In the majority of the literature, based on the average surface roughness ($S_a$) of titanium, surfaces with $S_a \leq 1 \mu m$ are considered smooth, and those with $S_a > 1 \mu m$ are described as rough. Wennerberg et al. reported that surfaces with $S_a$ ranging from 1 to 2 $\mu m$ were considered optimal for the surface roughness. In accordance with these reports, we prepared smooth and rough specimens with an average $R_a$ of 0.24 and 1.04 $\mu m$, respectively. Nishimoto et al. investigated cell attachment with three different methods: direct counting, dye binding, and microculture tetrazolium assays. They found that roughened surfaces improved early cell attachment to titanium. This finding is compatible with our results. The surface roughness of materials can profoundly affect cell attachment and spreading, and a roughened surface is generally thought to be preferable for cell attachment. Furthermore, initial cell adhesion on the material surface occurs through mechanical interlocking. Therefore, an appropriate surface roughness can produce beneficial mechanical interlocking at the initial adhesion stage and aid in future cell adhesion.

Recently, Bachle et al. investigated the osteoblastic response to different surface topographies of zirconia for the first time, and showed that in rough surfaces, the cell proliferation was significantly higher on day 3, compared to smooth surface. In the present study, in rough surface zirconia, we detected significant increase in cell attachment as early as after 1h-incubation (Fig. 3). We suggest that the roughness of surface in zirconia specimens is an important factor to facilitate the initial attachment of osteoblasts, similar to titanium.

An integrin $\alpha_5\beta_1$ receptor is selective for FN which is a parameter related to cell adhesion, followed by migration and proliferation. Osteoblasts interact with implant substrates via integrin binding initially to proteins adsorbed on the biomaterial surface and later to proteins in their secreted ECM. Raz et al. reported that the expression of integrin increased when osteoblast-like MG63 cells were grown on Ti surfaces with rough microtopographies. We showed that the expression of integrin $\alpha_5$ and $\beta_1$ was enhanced for rough surface specimens of zirconia. This finding is in excellent agreement with their reported results.

The actin cytoskeleton was important for the control of cell differentiation, movement, and morphogenesis. We also investigated the influence of surface roughness on the actin cytoskeleton in osteoblasts because integrin receptors physically interact with the actin cytoskeleton and the actin stress fibers function as a transmitter of forces. In the present study, the actin filament was expressed on all the specimens at 6 h (Fig. 6). The expression of the actin filament was similar on both smooth and rough surface specimens, although the expression of integrin $\alpha_5\beta_1$ was increased on rough surface specimens than smooth surface specimens. The complexity of signal transduction to the actin cytoskeleton, involving integrin-mediated matrix production and cell–cell binding, might have caused this discrepancy.

All the substrates employed in this study were classified as chemically stable and bioinert materials, because these specimens were covered with stable oxides, such as ZrO$_2$, Al$_2$O$_3$, and TiO$_2$. Our results revealed that the components of cell adhesion were influenced by the surface roughness of the materials but not by the type of substrates. Moreover, the surface roughness is able to influence proliferation, differentiation, matrix production, and calcification of osteoblasts in titanium and has an important role in implant success and prognosis. The quality of the first phase of cell–material interaction will influence the cell capacity to proliferate and differentiate. It has been demonstrated that surface modifications of titanium including apatite coating, etching, or anodization can improve initial cell responses. Thus, it may be necessary to modify the surface of zirconia for the further improvement of initial cell responses, in addition to surface roughness.

Further study is clearly needed to investigate the effects of the surface roughness and surface modification of two zirconia on the proliferation, differentiation, matrix production, and calcification of osteoblasts. Because of its high stability, mechanical properties, and biocompatibility, two types of zirconia could open up new possibilities in implant dentistry as an ideal replacement for titanium. Moreover, the surface structure should be considered when designing...
zirconia implants to improve the success associated with medical devices.

CONCLUSIONS
In conclusion, cell attachment of NANOZR and 3Y-TZP were comparable to those of Ti and AO. The surface roughness of both zirconia significantly influenced the attachment of MC3T3-E1 cells, similar to the effect seen in Ti and AO. The specimens with rough surfaces had better initial cell responses as compared to the smooth surface specimens. Therefore, the surface structure should be taken into consideration in designing zirconia implants to improve the success of medical devices.

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