INTRODUCTION

Biomaterials are widely used in medicine and dentistry and must satisfy many requirements depending upon their intended uses, like material properties, form, and biocompatibility. In orthopedics, titanium and zirconia have been used for hip replacements1). In dentistry, dental implants have a long history, with gold or cobalt-chromium alloys being replaced as the preferred material in the 1950s by titanium. Today, hydroxyapatite and zirconia are also used for dental implants, but titanium is still the predominant material. In the oral cavity, titanium implants made of pure titanium or titanium alloy are widely used, due to their outstanding qualities, such as acid resistance with a stable oxidation film2), biocompatibility, in vivo stability, and excellent osseointegration.

In contrast, several problems with the use of titanium have been pointed out recently. Post-transplantation metal accumulation has been found in organs or lymph nodes3,4) and titanium hypersensitivity has also been reported5-7). Titanium exposed to the oral cavity by gingival recession or peri-implantitis has caused problems with sensitivity. New metal-free biomaterials, like zirconia, are being developed that are hypoallergenic and less likely to cause sensitivity.

Zirconia (zirconium oxide, ZrO_2) is a chemically stable material with a high intensity and toughness compared to other ceramic materials, like aluminum oxide8,9). In dentistry, Y-TZP (a 3 mol% yttria-stabilized tetragonal zirconia polycrystals) is a major material and has been widely used as a post material for endodontically treated teeth10,11). Owing to its white color, zirconia also allows the manufacture of metal-free aesthetic restorations that can match tooth color12). Furthermore, its excellent biocompatibility and mechanical properties overcome many of the shortcomings of metal implants and allow zirconia to be used as the abutment for implants13,14). In Japan, the use of zirconia abutments was authorized by the Pharmaceutical Affairs Law in 2005.

Before Y-TZP can be used clinically as a dental implant fixture, more information is needed to assess the interfacial surface of implants and optimize bone formation with cell adhesion, proliferation, and differentiation to improve osseointegration. Many studies about the surface morphology and cell response have been demonstrated on both titanium and zirconia16-20), but little is known about the initial progression for mineralization on zirconia. In this study, we investigate the cell proliferation, differentiation, and mineral induction of C2C12 cells seeded on Ce-TZP disks.

MATERIALS AND METHODS

Preparation for titanium disk, Ce-TZP disk and PLL glass

The titanium disk (10 mm diameter, 0.7 mm thick) made of commercially pure titanium was purchased...
from Furuuchi Chemical Corporation (Tokyo, Japan). The ceria-stabilized tetragonal zirconia polycrystals/aluminum oxide nanocomposite (Ce-TZP) disk (10 mm diameter, 0.5 mm thick) made of 10 mol% CeO₂ stabilized TZP/30 vol% of Al₂O₃ was kindly provided by Panasonic Healthcare Co., Ltd. (Tokyo, Japan). The poly-L-lysine coated slide glass (PLL glass) (10 mm diameter, 0.12–0.17 mm thick) (culture cover glass, Matsunami Glass IND., LTD., Osaka, Japan) was used as a positive control. The surface of titanium disk was polished with diamond papers (#1200, #2400 and #4000) using a grinding and polishing machine (MODEL 900 Grinder/Polisher, South Bay Technology, Inc., San Clemente, CA, USA). The surface of Ce-TZP disk was first polished with a diamond wheel (#400) using a grinding machine (Surface Grinder PSG52DX, Okamoto Machine Tool Works, Ltd., Gunma, Japan). The polished Ce-TZP was furthermore processed with the slurry paste of diamond particle (6–12 μm, 2–6 μm and 0–1 μm) using a polishing machine (Compact Desktop Lapping System Ed-380IN, ENGIS JAPAN Corporation, Kanagawa, Japan).

Surface roughness, contact angle and microscopic observation
The surface roughness of titanium and Ce-TZP disks were measured using a surface roughness measuring instrument and stylus method (HANDYSURF E-35A, TOKYO SEIMITSU CO., LTD., Tokyo, Japan) with a measuring length of 5 mm and a cut-off value of 0.8 mm. The PLL glass surface area (2×2 μm²) was measured using an Atomic Force Microscope (Nanosurf Easyscan 2 AFM, Nanosurf Inc., Boston, MA, USA) in contact mode with a monolithic silicone probe coated with aluminum (ContAl-G, BudgetSensors, Innovative Solutions Bulgaria Ltd., Bulgaria). The contact angle of each sample was measured by using a contact angle meter (FACE contact angle mater CA-P, Kyowa Kaimenkagaku Co., LTD. Tokyo, Japan). For microscopic observation, each disk was fixed on the specimen mounting stage and gold evaporation was conducted. The disk was observed using a scanning electron microscope (SEM, JSM-5600LV, JEOL, Tokyo, Japan).

Cell culture
The C2C12 mouse myoblast cells were obtained from the RIKEN Cell Bank (Tsukuba Science City, Ibaraki, Japan). C2C12 cells were maintained in alpha MEM (Gibco-BRL, Gaithersburg, MD, USA) containing 10% FBS and 1% antibiotics (50 U/mL of penicillin-G and 0.05 mg/mL of streptomycin) (Gibco-BRL) at 37°C in a humidified 5% CO₂ atmosphere. For mineral induction, the mineralization medium was comprised of growth medium in L-ascorbic acid phosphate magnesium salt n-hydrate (AA; 50 μM, Wako Pure Chemical Industries, Ltd., Osaka, Japan), β-glycerophosphate (β-GP; 10 mM, Sigma-Aldrich, St. Louis, MO, USA), all-trans retinoic acid (ATRA; 1 μM, Sigma-Aldrich) and recombinant bone morphogenetic protein 2 (rBMP2; 10 nM) obtained from a baculovirus/sf-9 insect system was used. Also αMEM media only without mineral-induced factors described above was incubated as a negative control. Media was changed at every 72 h.

Cell proliferation assay
C2C12 cells were spread on disks at a density of 8.0×10⁴ cells/cm² and the number of cells counted at 3, 48 and 96 h. For cell recovery, the C2C12 cells were washed twice with 1 mL PBS (Ca, Mg free) and 0.3 mL trypsin/EDTA solution was added to the dish. After the incubation at 37°C for 5 min, 0.2 mL of αMEM was added to dilute the trypsin/EDTA and the media containing the detached cells was transferred into microtue. An aliquot of cells was counted using a hematemeter (Bright-Line™ Hemacytometer, Sigma-Aldrich) and the number of cells per a disk area was calculated.

Alkaline phosphatase staining
C2C12 cells were spread on disks at a density of 3.16×10⁴ cells/cm². After incubation for 24 h, the medium was changed to mineralization medium. An aliquot of C2C12 cells was incubated with the growth medium only without β-GP, ATRA and rBMP2 as control. After an additional 5 days of incubation, the cells were rinsed twice with PBS, fixed with 10% formaldehyde for 30 min, stained with 0.1 mg/mL of naphthol AS-MX BB salt, and 2 mM MgCl₂ in 0.1M Tris-HCl buffer (pH 8.5) for 30 min at room temperature, and then washed with dH₂O and photographed.

Alkaline phosphatase assay
Determination of ALP activity was carried out by using the modified method of Katagiri et al. C2C12 cells on disks at day 5 following mineral induction were rinsed with PBS twice after the removal of the culture medium and sonicated in 50 mM Tris-HCl containing 0.1% Triton X-100 buffer (pH 7.5) for 10 s (three times) on ice. Cell lysates were collected into a tube and an aliquot (5 μL) was transferred into 96 well plates. ALP activity was determined with 10 mM p-nitrophenylphosphate (95 μL) as the substrate in 0.1 M 2-amino-2-methyl-1-propanol buffer (pH 10.0) containing 2 mM MgCl₂ and 0.1 M disodium p-nitrophenyl phosphate hexahydrate (PNPP, Wako Pure Chemical Industries, Ltd.). The reaction mixture was incubated for 5 min at room temperature and was quenched by the addition of 0.2 M NaOH. The absorbance at 405 nm was read on a plate reader (Bio-Rad Model 450, Hercules, CA, USA).

Protein concentration in an aliquot (10 μL) of cell lysates was measured using a protein assay kit (BCA™ Protein Assay Kit, Thermo Fisher Scientific Inc., Massachusetts, USA). The protein standard was Bovine Serum Albumin (Pre-Diluted Protein Assay Standard BSA Set, Thermo Fisher Scientific Inc., Massachusetts, USA). The enzyme activity was calculated as micromoles of p-nitrophenol produced per min per mg of protein.
**Reverse transcription polymerase chain reaction (RT-PCR)**

RT-PCR was performed to investigate the expression of alkaline phosphatase (ALP), type I collagen, osteocalcin, and osterix. Following mineral induction for 5 days, total RNA from C2C12 cells was extracted with RNA extraction reagent (Isogen, Nippon Gene Co., Ltd., Tokyo, Japan). Purified total RNA (2 μg) was reverse transcribed using cDNA synthesis kit (Ready-To-Go You-Prime First-Strand Beads, GE Healthcare UK Ltd, Buckinghamshire, England) with oligo (dT)12-18 primer and random hexamer (Invitrogen, Carlsbad, CA, USA) for RT-PCR. PCR amplifications used Taq DNA polymerase (50 U/mL) (Thermo Scientific Japan, Tokyo, Japan) containing PCR buffer (Thermo Scientific Japan), dNTP (250 μM) ( Takara Bio Inc., Shiga, Japan) and primer (1 nmol/mL), and ran for 20 and 31 amplification cycles using DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Carlsbad, CA, USA). The specific primer sets and expected amplification product sizes are listed in Table 1.

**Alizarin red staining**

The C2C12 cells were grown on disks at an initial density of 3.16×10⁴ cells/cm². After incubation for 24 h, the medium was changed to the mineralization medium. C2C12 cells were cultured for up to 12 days. Mineralization was visualized by alizarin red S staining. After fixation with 10% formaldehyde for 30 min, the cells were stained with 1% alizarin red S (Sigma-Aldrich) solution for 10 min, and then washed with dH₂O and photographed.

**Quantitative analysis of calcium**

The C2C12 cells were grown on disks at an initial density of 3.16×10⁴ cells/cm². After incubation for 24 h, the medium was changed to the mineralization medium. C2C12 cells were cultured for up to 12 days. Each disk on the plates was rinsed with PBS, and the calcium was dissolved in 1 mL of 0.5 N HCl by gentle rocking for 1 h. The calcium concentration in the eluate was spectrophotometrically determined at 595 nm by following the color development with calcium assay kit (Calcium E-Test Wako, Wako Pure Chemical Industries, Ltd.). All values were normalized against the cultivation area.

**Statistical analysis**

All values were represented as means±standard error (SE). Statistical significance was determined using an unpaired Student’s t-test. In all cases, p<0.05 was regarded as statistically significant.

## RESULTS

### Surface properties of disks

We examined the surface of both polished Ce-TZP...
and titanium disks by SEM and found the surfaces to be very different (Fig. 1). The surface of Ce-TZP disks consisted of densely-distributed black granules of Al₂O₃ on a background of gray-colored Ce-TZP, while the surface of the titanium disks contained homogeneously distributed white granules. The Ce-TZP disk contained longitudinal parallel grooves generated during sintering or polishing.

The results of the surface roughness assessment are listed in Table 2. The \( R_a \) values of titanium and Ce-TZP disks indicated the same measured value \( (R_a=0.054\pm0.003 \ \mu m) \) and no statistically significant difference was observed between the titanium and Ce-TZP disks.

The results of the contact angle assessment are shown in Table 3. The contact angle of PLL glass was significantly higher than that of the Ce-TZP and titanium disks \( (p<0.05) \). Titanium and Ce-TZP disks indicated the similar measured value and no statistically significant difference was observed between both disks.

**Cell proliferation**

The number of C2C12 cells on the titanium and Ce-TZP disks and PLL glass were counted at 3, 48 and 96 h after cell seeding (Fig. 2). The cells proliferated actively on all materials, but the smallest proliferation rate was measured for 3 h cultures. There were no significant differences in cell numbers for the two types of disks and the PLL glass at 3 h. At 48 h after cell seeding, the number of cells on the two types of disks was significantly lower than on PLL glass, but no significant differences were observed between the titanium and Ce-TZP disks. After 96 h, the cell numbers reached \( 2.8\times10^5 \) cells/cm² on both the titanium and Ce-TZP disks and \( 2.9\times10^5 \) cells/cm² on the PLL glass, and the differences were not statistically significant.

**In vitro mineral induction**

After mineral induction for 5 days, the differentiation of C2C12 cells to the osteoblast phenotype on the Ce-TZP and titanium disks and the PLL glass was evaluated by ALP staining. ALP staining for the mineral-induced C2C12 cells on the two types of disks displayed blue-colored staining images comparable with that of control without the mineral induction (Fig. 3a). On the high-magnification images, the cells were extensively and widely distributed on Ce-TZP, while they showed an uneven distribution on titanium (Fig. 3b).

Since differentiation to an osteoblast phenotype was observed, we examined ALP activity for the C2C12 cells on the Ce-TZP and titanium disks, and PLL glass. The ALP activity after the mineral induction of the C2C12 cells in the mineralization medium significantly increased on the Ce-TZP and titanium disks and the PLL glass (Fig. 4). There were no significant differences among all materials following the mineral induction \( (p>0.05) \), although ALP activity on Ce-TZP was slightly higher than that on the titanium disk.

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**Table 2** Surface roughness of PLL glass, titanium (Ti) and Ce-TZP

<table>
<thead>
<tr>
<th>Sample</th>
<th>PLL glass</th>
<th>Ti</th>
<th>Ce-TZP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface roughness ( (R_a, \mu m) )</td>
<td>0.001±0.001</td>
<td>0.054±0.002</td>
<td>0.054±0.003</td>
</tr>
</tbody>
</table>

Data represent means±SE \((n=3)\).

**Table 3** Contact angle of PLL glass, titanium (Ti) and Ce-TZP

<table>
<thead>
<tr>
<th>Sample</th>
<th>PLL glass</th>
<th>Ti</th>
<th>Ce-TZP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact angle (degree)</td>
<td>52.3±1.3</td>
<td>37.6±1.5</td>
<td>32.4±3.0</td>
</tr>
</tbody>
</table>

Data represent means±SE \((n=3)\).
Gene expression of osteoblast differentiation markers
Gene expression of osteoblast differentiation markers (ALP, type I collagen, osteocalcin and osterix) at 5 days following mineral induction were analyzed by RT-PCR (Fig. 5). The expression of ALP and type I collagen dramatically increased in the mineral-induced C2C12 cells on Ce-TZP, titanium and PLL glass. When compared to the control, the expression of osteocalcin and osterix on the two disks and the PLL glass were slightly increased in the mineral-induced C2C12 cells. Expression of ALP on Ce-TZP was slightly higher than on titanium.

Mineralization activity
To further characterize osteoblast differentiation of the C2C12 cells, nodule formation and the mineralization capacity were assessed with Alizarin Red S staining (Fig. 6a). At 12 days following mineral induction, mineral nodules were evident on the Ce-TZP, titanium disk and on the PLL glass compared to cells without mineral induction. High-magnification images showed the mineral deposition was extensively and widely distributed on Ce-TZP, but it was uneven on titanium and PLL glass (Fig. 6b).

The calcium content in C2C12 cells was 0.12 mg/cm² on Ce-TZP, 0.13 mg/cm² on titanium and 0.09 mg/cm² on PLL glass, and was approximately 4–7 fold higher than that of the control (Fig. 7). The amount of calcium on the titanium and Ce-TZP disks was significantly higher than that of the PLL glass, but
Fig. 4 The alkaline phosphatase (ALP) activity for the C2C12 cells on PLL glass, titanium disk, and Ce-TZP disk with or without the mineral induction. An aliquot (5 μL) of cell lysates of C2C12 cells after the mineral induction was incubated in 96 well plates and ALP activity was measured using p-nitrophenylphosphate as substrate. The ALP activity is calculated as micromoles of p-nitrophenol produced per min per mg of protein. Data are means±SE (n=9) of Ce-TZP and titanium and PLL glass (n.s.: not significant).

![Bar graph showing ALP activity]

no significant differences were evident between the titanium and Ce-TZP disks.

**DISCUSSION**

*Surface roughness, contact angle and microscopic observation of titanium disk, Ce-TZP disk and PLL glass materials*

Bone formation is essential for the success of a dental implant, and requires cell adhesion, proliferation and differentiation. Implants therefore must be constructed of materials capable of achieving osseointegration. Pure titanium or titanium alloys have been widely used as implant materials; however, titanium implants have been associated with side-effects, such as allergies and titanium accumulation in body. In dentistry, ceramic implants have been considered as an alternative to titanium to avoid these problems. Ceramic is a sintered compact of metal oxide, which contains inorganic components as do teeth and bones. Owing to its stability in the body, ceramic is a potential alternative to titanium as a dental implant material. Coating the surface of a titanium fixture with hydroxyapatite has also been tried to promote early osseointegration. However, the coating of hydroxyapatite may not be stable and could exfoliate from the titanium surface. Our study focused on zirconia, which has biocompatibility, mechanical properties and osseointegration comparable to titanium. Compared with other ceramics, Y-TZP provides higher toughness by inhibiting the progression of cracks within the crystals by volume expansion due to phase transition. However, the strength of traditional Y-TZP can deteriorate due to the reduction of temperature in the presence of water. Ce-TZP is a complex of cerium-stabilized tetragonal zirconia polycrystals and aluminum oxide nanocomposite (Ce-TZP/Al₂O₃). Its crystalline phase transition improves bending strength and has high toughness relative to Y-TZP. In this study, we compared the cell adhesion, proliferation and differentiation of C2C12 cells on Ce-TZP, titanium and PLL glass.

As well as the surface roughness, the contact angle indicated the same value between titanium and Ce-TZP. This suggests that the wettability of titanium and Ce-TZP have an equal behavior. In general, it is known that the contact angle of glass is hydrophilic. As the glass we used in this study was coated with PLL, it shows hydrophobic.

The surface texture of materials influences cell adhesion, proliferation and differentiation. There are no significant differences in cell adhesion or the expression of adhesion factors between Ce-TZP and titanium disks with equal surface roughness. We adjusted the surface on both the Ce-TZP and titanium disks to equalize surface roughness by polishing (Table 2). In SEM images (Fig. 1), the surface of Ce-TZP disk consisted of densely-distributed black granules of Al₂O₃ on a background of gray-colored Ce-TZP, which is a major feature of ceramic matrix nanocomposite.

*Cell proliferation*

Several studies have demonstrated that cell adhesion and proliferation are similar on Y-TZP and titanium. We first confirmed that no cell proliferation occurred on
a micro cover glass (data not shown), but well proliferated on PLL glass (Fig. 2). To decide appropriate cell numbers, we performed a few pilot studies (data not shown) and decided to use $8.0 \times 10^3$ cells/cm$^2$ of cell numbers for the cell seeding of C2C12 cells on all materials. At 96 h after the cell seeding, all materials showed cell numbers indicating equal proliferation potency (Fig. 2). These results strongly suggest that Ce-TZP disk possesses the equal potency and the suitable environment for cell proliferation as well as titanium disk and PLL glass. It also demonstrates the usefulness of Ce-TZP instead of Y-TZP.

In vitro mineral induction
Recently, it has been shown that there are no significant differences between Ce-TZP, Y-TZP and titanium with respect to the cell morphology, cell adhesion and adhesion factors$^{35}$. It has also been reported that osteoblast cell proliferation and protein synthesis on ZrO$_2$ disks are similar to those on glass coverslips$^{40}$. C2C12 cells are known as express markers for the initial differentiation to osteoblasts (ALP activity or osteocalcin) under the existence of BMP2 and ATRA. The BMP2 is widely known as the differentiative induced factor for osteoblast$^{24}$, while the ATRA is a reaction accelerator for mineral induction$^{43}$. We
selected the mineral induction system with BMP2 and ATRA, and estimated the cell differentiative ability of C2C12 cells into osteoblasts. ALP staining produces a blue color on the cell membrane. C2C12 cells on Ce-TZP at 5 days after the mineral induction were densely stained for ALP activity and the stain was extensively distributed (Fig. 3). In contrast, the cells on titanium and PLL glass were unevenly stained for ALP activity. We have thought that this unevenly ALP staining might be due to the density or distribution of cells, and/or the difference of expression of ALP activity. We also analyzed ALP assay for C2C12 cells on the same day as mineral induction (Fig. 4). There were no statistically significant differences among all materials (p>0.05), but the Ce-TZP disk indicated slightly higher ALP activity than that on titanium disk.

Since the differentiation to osteoblast was expected, we examined the gene expression of tissue-specific differentiation markers for osteoblast by PCR (Fig. 5). Osteocalcin is only distributed in bone and tooth and is well known as the specific marker for mineralized tissue\(^4\). Osterix is a bone-specific transcription factor as is Runx2\(^2\). We analyzed the gene expression of osteoblast differentiation markers. Our data showed the gene expression of ALP and type I collagen was dramatically increased in C2C12 cells on all materials, and ALP expression on Ce-TZP was slightly higher than on titanium or PLL glass, although osteocalcin and osterix were equally expressed on all materials. These results suggest that the initial differentiation to osteoblast and the mineral induction on Ce-TZP disk dominantly occurred than that on titanium disk and PLL glass.

We also demonstrated that nodule formation at 12 days after mineral induction varied for Ce-TZP and titanium (Fig. 6), although no significant differences were observed in calcium contents (Fig. 7). However, the calcium content of both disks showed the significant low amount compared to PLL glass. This suggests that both Ce-TZP and titanium disks may have suitable mineralization environments compared to PLL glass. Based upon the ALP staining, bone formation on Ce-TZP occurs more uniformly than the titanium, suggesting that Ce-TZP has advantages with the initial differentiation of cells associated with mineralization and with mineral formation itself relative to other materials.

In this study, we demonstrated that Ce-TZP has equal ability for cell proliferation and differentiation as titanium disk and PLL glass. This suggests the conclusion that Ce-TZP has a utility as the fixture of dental implants. Future studies are now required to investigate how the surface roughness of Ce-TZP affects the cell differentiation, or to examine the adhesion against the bacteria in terms of peri-implantitis.

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REFERENCES


