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Original

Proliferation and Alkaline Phosphatase Activity of Osteoblast-like Cells on the Sintered Rutile Titanium Dioxide

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Abstract: The purpose of this study is creation of biomaterials from titanium dioxide (TiO_2). This TiO_2 has known for photocatalysis and osteogenesis. For the purpose of applying this function to orthodontic brackets and coating materials for implant, the relationship between surface of sintered and cell proliferation were examined. In addition, crystal structure and the surface property of sintering TiO_2 were investigated. TiO_2 were sintered at 1300°C for use as samples. We examined surface roughness, x-ray diffraction and scanning electron microscopy to make observations of the surface properties and texture. Moreover, mouse osteoblast-like cell line, MC3T3-E1 was cultured on sintered TiO_2 in order to evaluate the cell proliferation and ALP. For the samples sintered at 1300°C, the crystalline phase of rutile-type TiO_2 was confirmed.5000-fold magnified SEM images of the surface of the unsintered samples, needle-like TiO_2 crystals were pressure welded and showed mutual overlap, with pores occurring among the crystals. Sintering at 1300°C produced numerous small pores. Rutile TiO_2 as a starting material was sintered at 1300°C and subjected to a cell culture experiment in which MC3T3-E1 cells were cultured on the sample, followed by viable cell counting and cell morphology observation on days 7, 14, 21, and 28 of culture. In the test of cell proliferation, sintered at 1300°C samples was found to remarkable cell proliferation even after time had passed. ALP activity of cells on 1300°C TiO_2 sample, the values were 110% and 126% on days 14 and 28 of culture, respectively. These changes were calculated using polystyrene dish as the reference condition. Thus, TiO_2 sintered at 1300°C showed good compatibility and increase in the ALP activity in MC3T3-E1 cells.

Key word: Titanium dioxide (TiO_2), Biomaterials, Cell proliferation, Alkaline phosphatase

Introduction

Pure titanium or titanium alloys have been utilized as dental biomaterials in recent years, with many clinical results reported. Good biocompatibility of these implants is considered to rely on a passive film of titanium dioxide (TiO_2) being formed on the surface of the implants[1,2]. However, there are few reports on the biocompatibility of TiO_2 itself in a practical context[3]. When assuming applications of TiO_2 as a dental biomaterial, we believe that it is essential to confirm the basic biosafety of TiO_2, particularly considering the surface properties of sintered TiO_2 as factors that directly influence the living organism. Accordingly, the purpose of this study was to experimentally investigate the impact of TiO_2 in a cell culture system to accumulate basic biological information about TiO_2 in the context of the compound's dental applications.

We prepared the sintered rutile form of TiO_2 and investigated the influences of its surface properties on mouse osteoblast-like cells these same experimental materials were used throughout the study. Here we report some of the results which were obtained.

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Materials and Methods

Experimental materials and sample preparation

Rutile TiO_2 powder in the form of 5.0 μm nanorods with a mean diameter of 0.3 μm (FTL-100, Ishihara, Osaka, Japan) was used as an experimental material. Compacted samples approximately 3 mm thick were prepared by measuring and mixing 0.5 g TiO_2 and 0.3 ml distilled water. The mixture was then transferred to a metal mold with an inner diameter of 12 mm and a depth of 15 mm. A 150 kN load was then applied for 1 min. The compacted samples were dried in an electric furnace (LP-907,

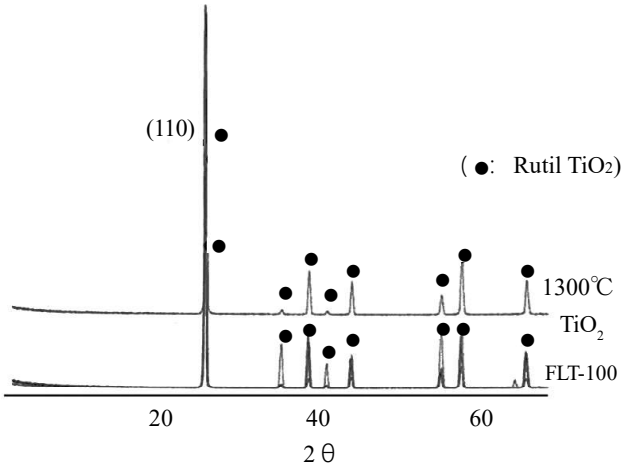

Figure 1. X-ray diffraction patterns of the sintered TiO_2. All peaks were rutile TiO_2.

Koyo Thermo Systems, Nara, Japan) by increasing the temperature from room temperature to 100°C over a period of 30 min. The samples were then dried and sintered by increasing the temperature to the designated sintering temperature over a period of 120 min. Sintering was performed in air at a temperature of 1300°C. After the samples were left at the designated temperature for 30 min, they were cooled in the furnace in preparation for the experiment. The samples were sterilized by autoclaving for 15 min at 120°C. Five samples were prepared on each culture day.

As a control, polystyrene dishes (Cell Desk LF1, Sumitomo Bakelite, Tokyo, Japan) were subjected to molding to produce samples of a size nearly equivalent to that of the experimental samples.

X-ray diffraction analysis of sintered TiO_2

The crystal phase of the starting material and TiO_2 sintered at 1300°C was identified using an X-ray diffraction analyzer (Rad-rC, Rigaku, Tokyo, Japan). Measurement conditions were as follows: 40 kV tube voltage, 60 mA tube current, CuKa tube, 20–60° diffraction range using a Ni filter.

Observation of the surface of sintered TiO_2

The surface properties of the TiO_2 sintered at 1300°C were observed using a scanning electron microscope (SEM; JSM-6000, JEOL Ltd.). The acceleration voltage was 20 kV.

Examination of cell proliferation on sintered TiO_2

A mouse osteoblast-like cell line, MC3T3-E1 (Sumitomo Dainippon Pharma), was used and cell processing was conducted in a culture medium of a-minimum essential medium (GIBCO) in which 10% calf serum (EQUITECH-BIO), 100 U/ml penicillin (GIBCO), and 100 µg/ml streptomycin were added. Samples of sintered TiO_2 were individually placed in each well of a 24-well microplate (Falcon) onto which a 600 µl cell suspension adjusted to 2.0×10^4 cells/ml was plated. The culture was performed for 7, 14, 21, and 28 days under the condition of 37°C and 5% CO_2. To count the cells that had proliferated on each sample, the samples were transferred to a new 24-well microplate containing 600 µl of culture medium per well. Subsequently, 120 µl CellTiter 96® AQueous One Solution Reagent (Promega) was added to each well. After culturing for 2 h, absorbance (492 nm wavelength) of the cultures was measured using a microplate reader (MPR-A4i, Tosoh, Tokyo, Japan) to count the number of viable cells on the samples. As an alkaline phosphatase (ALP) assay, samples after 14- and 28-day culture were subjected to enzymatic treatment for 30 min at 37°C using a TRACP & ALP Assay Kit (TAKARA BIO, Shiga Japan), before measuring its absorbance (at 405 nm wavelength) with the microplate reader. Data were statistically analyzed with a one-way analysis of variance and Bonferroni multiple-comparisons tests ($p < 0.05$).

Observation of cell morphology

The morphology of the cells after 14 days of culture on each sample was observed. Briefly, after fixation for 10 min in 99.8% methanol (Kanto Chemical, Tokyo, Japan) and air drying, each sample was immersed in Giemsa stain (Wako, Osaka, Japan) diluted with distilled water to 20% for 30 min, before washing with running water followed by air drying. The cells on the sample were then observed using an inverted metallurgical microscope (PME3, Olympus, Tokyo, Japan).

Results

X-ray diffraction

Fig. 1 shows X-ray diffraction patterns of the sintered TiO_2. The peaks of X-ray diffraction obtained from the starting material and the materials sintered at 1300°C were identified as peaks of rutile TiO_2 in all cases.

Surface observation

Fig. 2 shows 5000 fold magnified SEM images of the surface of the polystyrene dish (Cell Desk, LF1) and TiO_2 samples. The shape and size of the crystal grains of the TiO_2 samples varied with the sintering temperature. In the unsintered samples, needle-like TiO_2 crystals with a major axis measuring approximately 5 µm overlapped each other in each direction, and many pores were present among the crystals. In the unsintered samples, needle-like TiO_2 crystals were pressure welded and showed mutual overlap, with pores occurring among the crystals. Sintering at 1300°C produced numerous small pores.

Cell proliferation and ALP activity

Fig. 3 shows the relationship between cell proliferation and the TiO_2 sample on which the cells proliferated. A significant

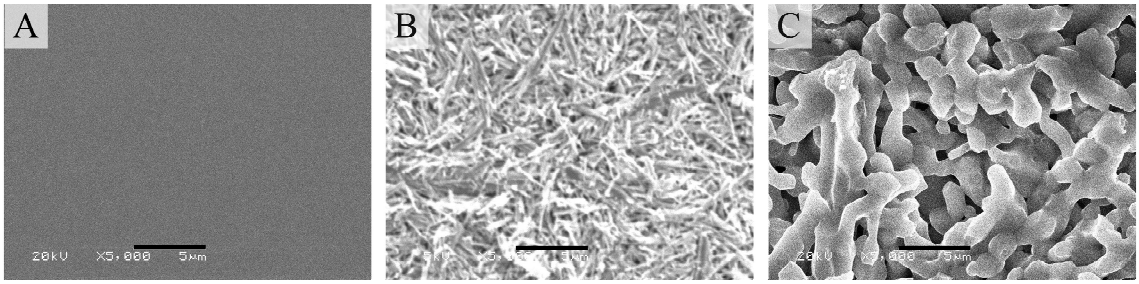

Figure 2. SEM images. Polystyrene dish LF1 (A). Unsintered TiO_2 sampl (B). Sintering at 1300°C (C). Scale bar = 5μm

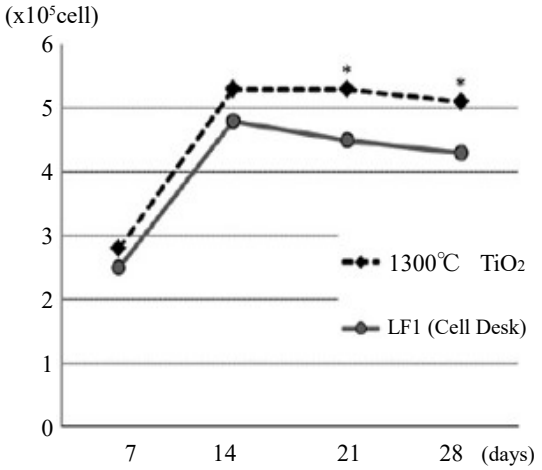

Figure 3. MC3T3-E1 cells proliferation on 1300°C TiO_2 sample and Polystyrene dish LF1. A significant difference in cell proliferation was identified on each day from days 21 to 28 of culture ($P < 0.05$).

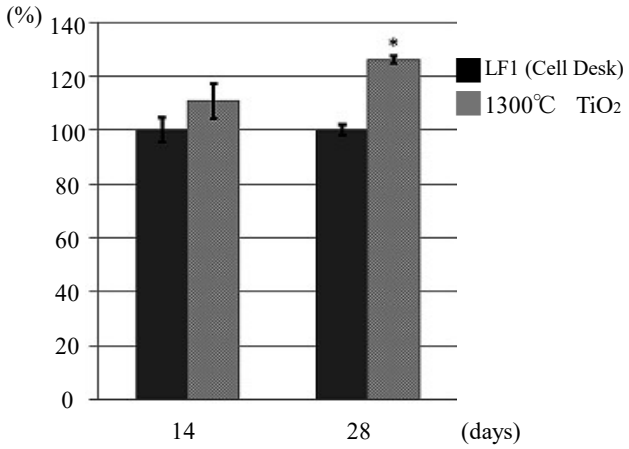

Figure 4. ALP activity of cells on a 1300 °C TiO_2 sample. These changes were calculated using LF1 as the reference condition. The values of 1300 °C TiO_2 samples were both 110% and 126% on days 14 and 28 higher than the reference ($P < 0.05$).

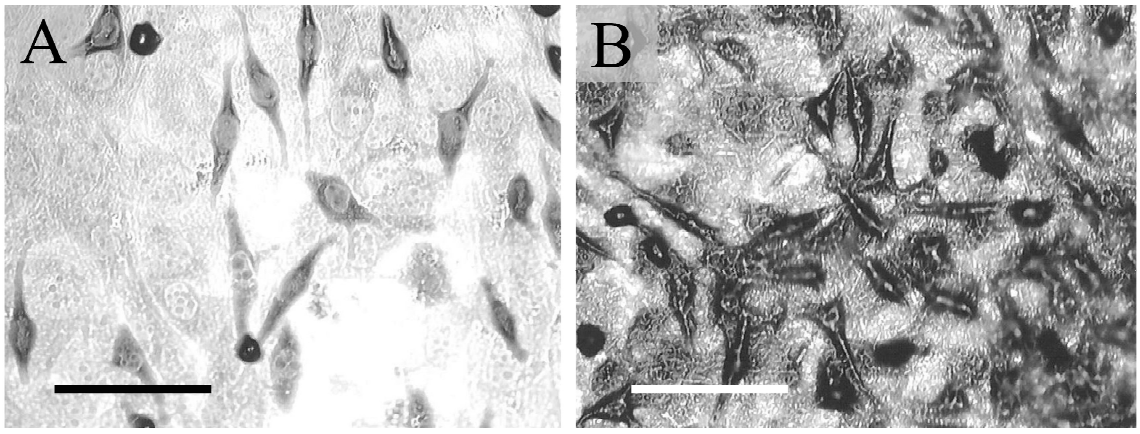

Figure 5. Images of cells on each sample LF1 (A) and 1300 °C TiO_2 (B) after 14 days of culture. Scale bar = 40μm

difference in cell proliferation was identified on each day from days 21 to 28 of culture ($P < 0.05$). Although cell proliferation during days 7 to 14 was noteworthy in both control and experimental groups, the number of cells subsequently decreased from day 21 and reached confluence on day 28.

As shown in Fig. 3, the proliferation rate of cells on sintered TiO_2 showed higher values than that on the polystyrene control (Cell Desk, LF1), with statistically significant increases on days 21 and 28.

Fig. 4 shows changes in the ALP activity of cells on a 1300°C TiO_2 sample. These changes were calculated using LF1 as the reference condition. The values were 110% and 126% on days 14 and 28 of culture, respectively, with both higher than the reference, indicating a higher ALP activity of the cells on the 1300°C TiO_2 sample.

Observation of cell morphology

Fig. 5 shows images of cells on each sample after 14 days of

culture. No cell necrosis was observed on both LF1 and 1300°C TiO_2 samples, with good cell engraftment observed on both samples. On the samples, fusiform and triangular cells were observed to have proliferated to the point of overlapping.

Discussion

Titanium dioxide (TiO_2)

The photocatalytic activity of TiO_2 has garnered attention as a result of the work of Fujishima and Honda[4]. The surface of TiO_2 is known to be highly reactive with light. Such reactivity produces amphophilic properties, i.e., the surface shows both hydrophilic and hydrophobic characteristics[5]. In addition, the photocatalytic activity induced by UV excitation exerts a substantial influence on the environment[6]. Moreover, pure titanium and titanium alloys, which have recently been widely utilized as biomaterials[7], instantly form a nanometer thin oxidized TiO_2 film when the fresh surface generated by processes such as cutting and polishing is exposed to air and/or moisture. TiO_2 is produced by the surface reforming of titanium and it exhibits superior biocompatibility. Surface analyses of the chemical structure of titanium surfaces and composition and thickness of passive films on the surface have been revealed, and many studies have reported on cell kinetics on surface-treated titanium[8] and on TiO_2-coated materials[9-11].

Meanwhile, TiO_2 occurring naturally in ceramic form is used as a starting material in the smelting of titanium metal and is also widely used as raw material for white pigments, constituents of ferroelectric substances, and in cosmetics. This TiO_2 has three types of crystalline structures: rutile, anatase, and brookite. The rutile type examined in this study is used for cosmetics and dentifrices due to the difficulty in the excitation of its catalytic activity. In contrast, anatase shows photocatalytic activity, and many studies are currently being conducted. When anatase TiO_2 with strong catalytic activity is sintered as a starting material, it changes to rutile at approximately 850°C and consequently loses nearly all its catalytic activity[4-6]. In our experiment, we chose rutile TiO_2 and examined the impact of its sintered material on cell proliferation relative to the sintering temperature and surface properties because our focus was on its application as dental biomaterials.

Compact preparation

There are numerous molding methods of ceramics, and the compacting pressure is likely to affect the density and surface properties of the sintered bodies. In this study, we prepared disc-like simple forms of compact material by uniaxial pressing. Compared with the properties of the material prior to sintering, the diameter of the crystal grains increased, number of pores decreased, and disc diameter slightly decreased in 1300°C TiO_2. This trend was similar to that shown in normal ceramic sintering.

Relationships between sintering temperature and cell proliferation/ALP activity

In this experiment, we used mouse osteoblast-like MC3T3-E1 cells to evaluate the biocompatibility of TiO_2. Cell culture test using MC3T3-E1 cells, which are generally used for evaluation of dental biomaterials, is an evaluation method that can be rapidly applied with high reproducibility of cell proliferation[12-16]. ALP activity is an indicator of differentiation of osteoblast-like cells and allows assessment of the presence of cell activation. There are few reports[17] available on evaluations of the biocompatibility of TiO_2 as a ceramic, which we used in our experiment.

In this experiment, the experimental group showed better cell proliferation and ALP activity than the control group. This finding indicated that rutile TiO_2 used in this experiment has stable chemical and physical properties as a biomaterial[18-24]. One of the reasons for the improved cell proliferation in this study is most likely the difference in surface roughness between the experimental material and control material. Materials that are manufactured by coating the surface of titanium metal or ceramic materials, such as hydroxyapatite, have a higher surface roughness than the polystyrene culture dishes we examined, and cell proliferation on our experimental samples was better than that in the control group, the result of which is consistent with that presented in a previous report[8]. In future studies, we intend to observe changes in cell proliferation by varying the surface roughness of samples to examine the relationship between surface roughness and cell proliferation. The ALP activity showed higher values in the 1300°C TiO_2 group than in the control group on all days of culture, indicating that sintered TiO_2 may influence the differentiation of osteoblast-like cells through enhancement of ALP activity.

Sintering temperature and cell morphology

As shown in Fig. 5, the cells observed on the sample were fusiform or triangular and engrafted themselves on the surface by extending their processes radially in a pericellular manner. The cell morphology of the experimental group showed no change and was the same as that of the control group, suggesting that 1300°C TiO_2 had no impact on cell morphology. In toxic samples, dying out, vacuolar degeneration, disruption of the plasma membrane, and/or necrosis of cells in the vicinity of the test piece are normally observed[25,26]. However, in our experiment, none of these cell morphologies were found, and round cells in their mitotic phase, fusiform, and triangular cells were instead observed. This trend is similar to cell morphologies in apatite or calcium triphosphate cultures. This result indicates that the compatibility between TiO_2 and the cultured cells was very high.

In conclusion, Rutile TiO_2 as a starting material was sintered at 1300°C and subjected to a cell culture experiment in which MC3T3-E1 cells were cultured on the sample, followed by viable cell counting and cell morphology observation on days 7, 14, 21, and 28 of culture. The following results were obtained:

1. In 1300°C sintered TiO_2, an increase in crystal grains and a decrease in the number of pores were observed when compared with the material prior to sintering.

2. An increase in the duration of culture resulted in an increase in cell proliferation. The number of viable cells and ALP activity showed higher values in the experimental group with TiO_2 sintered at 1300°C compared with the control group.

Thus, TiO_2 sintered at 1300°C showed good compatibility and increase in the ALP activity in MC3T3-E1 cells.

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Competing Interests

The authors have declared that no COI exists.

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