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Observation and quantitative analysis of rat bone marrow stromal cells cultured *in vitro* on newly formed transparent β-tricalcium phosphate

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To observe living cell morphology on ceramics by light microscopy, we fabricated a new material—transparent β- tricalcium phosphate (*t*-βTCP) ceramic—for the purpose of serving as a tissue culture substrate. Bone marrow stromal cells (BMSCs) were obtained from rat femora and cultured on both *t*-βTCP ceramic disks and culture grade polystyrene (PS) dishes in an osteogenic medium. After 1 day of culture, cell attachment and spreading on both the *t*-βTCP and PS substrata were equally and clearly detected by ordinary light microscopy. After 14 days of culture, extensive cell growth, alkaline phosphatase (ALP) staining, and bone mineral deposition could be detected on both substrata. In addition, quantitative biochemical analyses revealed high DNA content, ALP activity, and osteocalcin content of these cultures. This experiment is significant in that all of the results were similarly observed on both the *t*-βTCP and PS substrata, indicating the excellent properties of βTCP ceramics for BMSCs culture towards osteogenic differentiation.

Introduction

Viable cells can be cultured in suitable scaffolds, and composites of such cells have already been clinically applied to patients. As examples, some polymers such as collagen combined with a variety of cultured cells are currently being used for cartilage and skin regeneration [1, 2], and ceramics combined with mesenchymal cells are being used for bone regeneration [3]. In our clinical cases, culturing a patient's derived mesenchymal cells on any of hydroxyapatite (HA), β-tricalcium phosphate (βTCP), or alumina ceramics has been used in treating patients having skeletal problems. In particular, before cell transplantation to the patients, we forced the mesenchymal cells to become osteoblasts followed by the formation of bone matrix on a ceramic surface [4, 5]. We have therefore defined the tissue-engineered construct fabricated by culturing mesenchymal cells on ceramics as *regenerative cultured bone tissue* [6].

Many researchers, ourselves included, have previously reported that composites of fresh bone marrow (without culture) and porous HA or βTCP [7–9] can show extensive new bone formation after *in vivo* implantation. We considered that the bone formation could be initiated on a ceramic surface by attaching mesenchymal cells from bone marrow. However, due to *in vivo* conditions, observation of the cells/ceramic interaction is difficult. In this regard, *in vitro* culturing of bone marrow stromal cells (BMSCs) on ceramic disks might be another approach for observing cells/ceramic interaction. Usually, cultured cells can be observed with

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an ordinary microscope when the culture is on culture dishes. However, due to the opacity of the ceramics, microscopic observation of the cells on ceramics is very difficult. Scanning electron microscopy (SEM) or other techniques can be used to observe the cells on the ceramics, but these techniques usually require cell fixation by glutaldehyde, paraformaldehyde, or similar fixative solution, and the cells are no longer viable. Moreover, consecutive observation of the same specimen during the culture period is impossible.

If the ceramic were transparent, we could use an ordinary microscope for observation, which would enable us to detect cell attachment, spreading, proliferation, and differentiation cascade. Recently, it has been claimed that transparent ceramics are useful for the observation of cultured cells [10]. Transparent ceramics are mainly made by a spark plasma sintering process (SPS) [11]. SPS systems have many advantages such as rapid sintering, sintering with fewer additives, uniform sintering, low operating expense, and relatively easy operation compared to that of conventional systems using hot press (HP) sintering, hot isostatic pressing (HIP), or atmospheric furnaces.

β-TCP ceramics are known to be highly biodegradable. Porous types of β-TCP have attracted much attention within the field of bone reconstruction [12–15]. The rates of proliferation and differentiation of cultured cells on β-TCP ceramics have been demonstrated by biochemical analyses or gene expression analysis [16, 17] and SEM observation. However the ceramics used were opaque and direct observation was difficult to accomplish. Therefore, whether the surface of the ceramic could support cell attachment, proliferation, and differentiation was not clear. In this study, we made transparent βTCP (t-βTCP) ceramic disks (20 mm in diameter) using the SPS sintering method. We used rat BMSCs as the cell source and observed cell attachment, proliferation, and osteogenic differentiation on the t-βTCP ceramic disks as well as on culture grade polystyrene (PS) dishes as a positive control. PS dishes have been commonly used as cell culture substrates for a long time and their suitability is well known. This report describes not only the morphological but also the biochemical qualitative analyses of the BMSCs culture and includes a comparative study of the culture on t-βTCP and PS.

Materials and methods

Preparation of transparent βTCP (t-βTCP)

A fine powder made of βTCP (Taiheikagaku, Co. Ltd., Japan) was used as the basic material. One gram of this powder was poured into a graphite mold (inner diameter: 15 mm), and then sintered by the spark plasma sintering process (SPS: Dr Sinter-511S, Sumitomo Coal Mining, Tokyo, Japan). The samples were pressed uniaxially under 10 MPa, then heated at 1000 °C for 10 min at a heating rate of 25 °C/min. Each ceramic disk consisted of polycrystalline, transparent materials with a diameter of 20 mm and a thickness of 2 mm. The surface shapes and crystallographic analyses were determined using a scanning electron microscope (SEM, SM-300, Topcon Corporation, Tokyo, Japan) and an X-ray diffractometer (XRD, Geiger flex 2027, Rigaku, Japan), respectively.

Surface characterization

The surface roughness of the t-βTCP ceramic disks and PS dishes was measured by using a profilometer (Surfcorder SE-30D, Kosaka Lab., Ltd.) with a 5-μm tipped diamond stylus. Then, both the average roughness (Ra) and maximum roughness (Rz) were quantified. The sessile contact angles (SCA) of t-βTCP ceramic disks and PS dishes were determined using a Milli-Q water system and a goniometer (Face Contact-Angle Meter, Kyowa Kaimenkagaku Co., Ltd. Tokyo, Japan). A probability (p) of less than 0.05 was considered significant.

Cell culture

The preparation and osteogenic differentiation of bone marrow stromal cells (BMSCs) from 7-week-old male Fischer 344 (F344) rats were performed as described by Maniatopoulos *et al.* [18] with some modifications by us [19]. In brief, rat bone marrow plugs were flushed out and suspended in a culture medium, minimum essential medium (MEM, Nacalai Tesque, Inc., Kyoto, Japan) containing 15% fetal bovine serum (FBS, JRH Biosciences, Inc., KS, USA) and 1% antibiotics. These bone marrow cells were cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C.

The mesenchymal cells, which have osteogenic potentials, were contained in BMSCs. The adherent BMSCs were initially cultured up to 80% confluence in T-75 flasks, harvested, and resuspended to 5×10^5 cells/mL in culture medium using 0.05% trypsin/0.53 mM EDTA. One mL of cell suspension was applied to each sterilized t-βTCP ceramic disk (3.1 cm^2), which were placed into a 12-well PS plate (3.8 cm^2). As a control, the same amount of cell suspension was also applied directly in a PS well having no disks. These cells were cultured with osteogenic medium containing 10 nM dexamethasone (Dex, Sigma-Aldrich Corporation, MO, USA), 10 mM β-glycerophosphate (β-GP, Merck, Darmstadt, Germany), and 0.28 mM ascorbic acid 2-phosphate magnesium salt n-hydrate (AAc, Sigma-Aldrich Corp.). The culture medium was changed two or three times per week. During the culture period, the cell morphologies at different stages of cell attachment, proliferation, and differentiation were observed by light microscopy (Olympus CK41SF, Olympus Optical, Co., Ltd., Tokyo, Japan). Some cultures did not have Dex added but the other conditions were the same as above.

Qualitative biochemical analysis

Alkaline phosphatase (ALP) activity staining

At day 14, the cultured cells on both the *t*-βTCP ceramic disks and the PS dishes were rinsed with phosphate buffer saline (PBS) and fixed with 4% paraformaldehyde for 10 min at 4 °C. The fixed cells were soaked in 0.1% naphthol AS-MX phosphate and 0.1% fast red violet LB salt in 56 mM 2-amino-2-methyl-1, 3-propanediol (pH 9.9) for 10 min at room temperature and then washed with PBS.

Alizarin Red S staining

At day 14, the cultured cells on both the *t*-βTCP ceramic disks and the PS dishes were washed with PBS and fixed with 4% paraformaldehyde for 10 min at 4 °C. The fixed cells were soaked in 0.5% Alizarin red S /PBS for 10 min at room temperature and then washed with PBS. Non-specific reactions were removed by a wash step using 70% ethanol (EtOH). The stain was used to detect calcium in the extracellular matrix. To check a background level of Alizarin Red S, *t*-βTCP ceramic disks without cells were soaked into Alizarin red S in the same condition to cells (Fig. 6(e)).

Calcein uptake

The calcium in the extracellular matrix was also determined by using calcein, which is a calcium-chelating fluorescence chemical [20]. One μg/mL

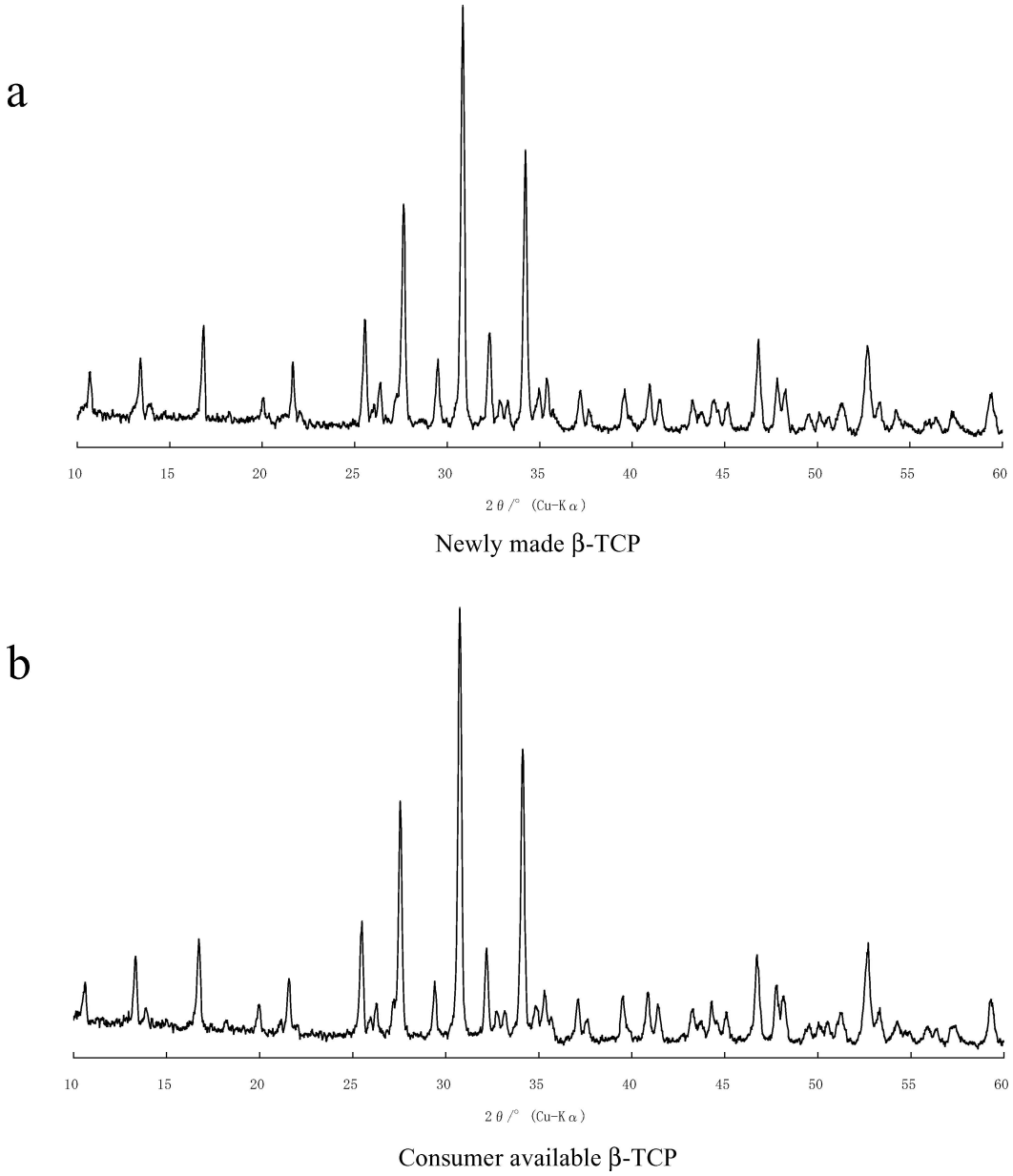

Figure 1 Powder X-ray diffraction (XRD) pattern of transparent βTCP ceramics made by spark plasma sintering (a) and standard βTCP (b). Details are given in the Materials and methods section.

calcein (Dojindo Laboratories, Kumamoto, Japan) was added to the medium during culture. The fluorescence of the incorporated calcein was observed by using a fluorescent microscope (IX70, Olympus). The medium containing calcein was removed and washed with PBS immediately before these analyses. To check a background level of calcein, t-βTCP ceramic disks without cells were soaked into medium containing calcein in the same condition to cells (Fig. 4(e)).

Quantitative biochemical analysis

Measurement of DNA contents

The DNA contents were measured by using Hoechst 33258 and expressed as μg/cm^2. The cells were scraped off the t-βTCP or PS surface into 0.5 mL of buffer (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl (pH 7.4)) and sonicated. 20 μL of suspended cell solution was added to 0.2 mL of buffer containing 5 μg/mL Hoechst 33258 and the fluorescence was measured on a

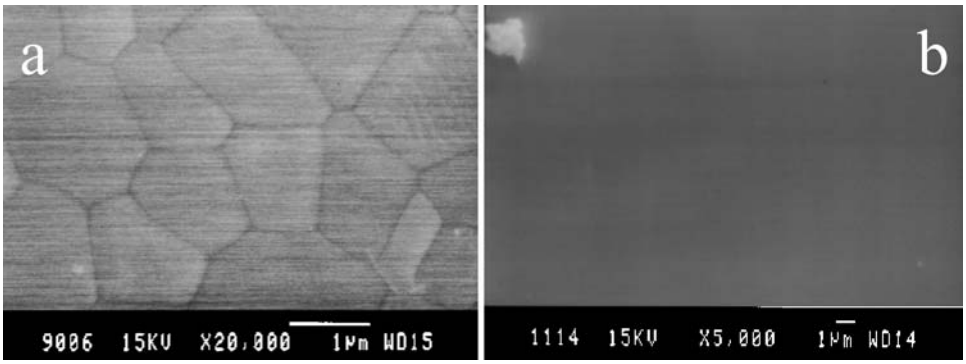

Figure 2 SEM images of transparent βTCP (a) and culture grade polystyrene (PS) (b) Bar: 1 μm. The surface of a culture grade PS dish is very smooth and, therefore, most of the SEM photos were out of focus. To bring the object into focus, we photographed a dish contaminated with a very small dust particle (shown at the upper left) as a pointer.

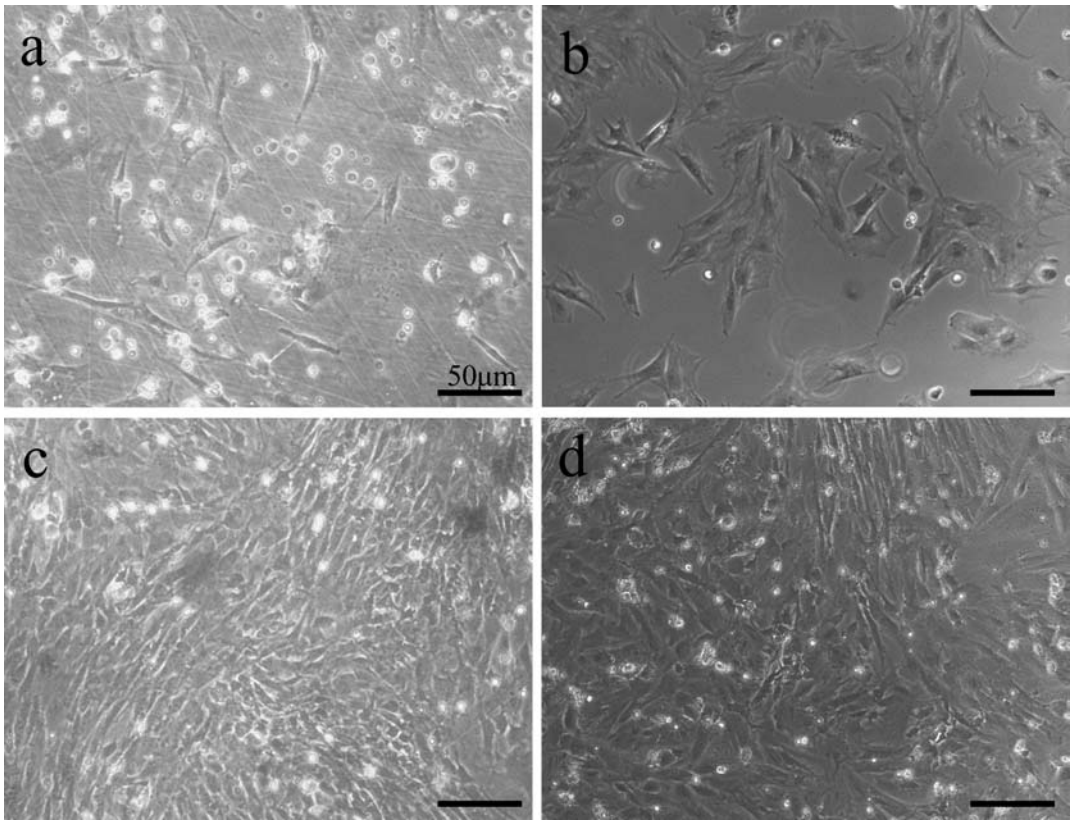

Figure 3 Cell morphology of rat BMSCs seeded on transparent βTCP ceramic disks and culture grade polystyrene (PS) dishes. The cells seeded on βTCP ceramic disks are shown in the left column (a, c). The cells seeded on PS dishes are shown in the right column (b, d). Bar: 50 μm. Cell morphology at day 1 (a, b) and day 4 (c, d) after BMSCs seeding on PS dishes and t-βTCP ceramic disks observed by light microscopy. One day after cell seeding, the cells began to spread on the surface. Cells on both substrata proliferated well and most cells had a fibroblastic shape after 4 days.

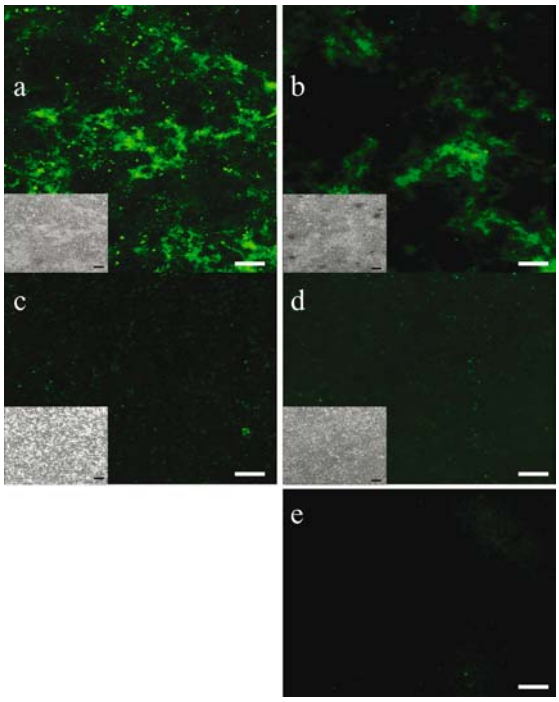

Figure 4 Calcein uptake by rat BMSCs seeded on transparent βTCP ceramic disks and culture grade PS dishes at day 14. Bar: 200 μm. When BMSCs were cultured in the presence of Dex, the culture on both culture grade PS dishes (a) and transparent βTCP (t-βTCP) ceramic disks (b) showed extracellular mineralization indicated by a green fluorescence. In contrast, in the absence of Dex, cells did not differentiate into osteoblasts and the green fluorescence was not detected (c, d). Each inset at lower left shows the phase contrast microscopic view of the corresponding field. Background level of calcein when t-βTCP without cells were soaked into calcein contained medium were shown in e.

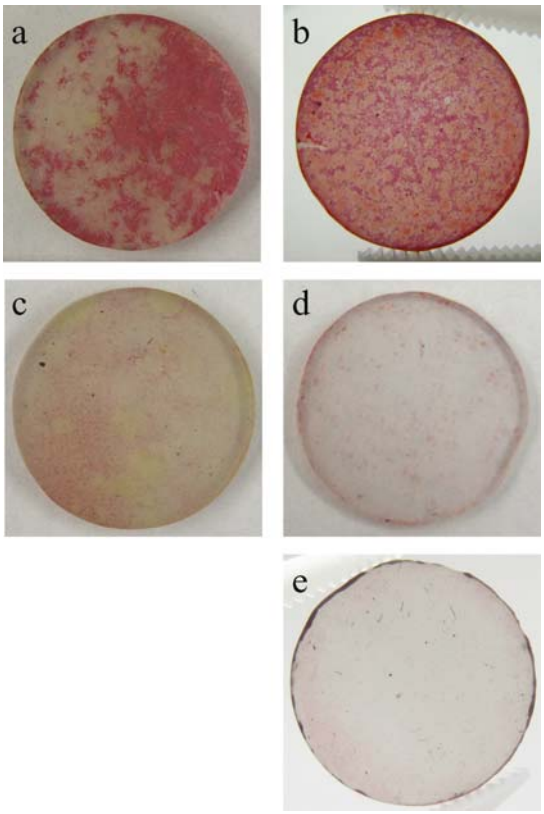

Figure 6 Macroscopic observation of ALP (a, c) and Alizarin Red S staining (b, d). When BMSCs were cultured in the presence of Dex (a, b), the cells on transparent βTCP differentiated into osteoblasts and showed extensive ALP (a) and Alizarin Red S (b) staining over the entire ceramics area compared with the control culture without Dex (c, d). Background level of Alizarin Red S when t-βTCP without cells were soaked into Alizarin Red S were shown in e.

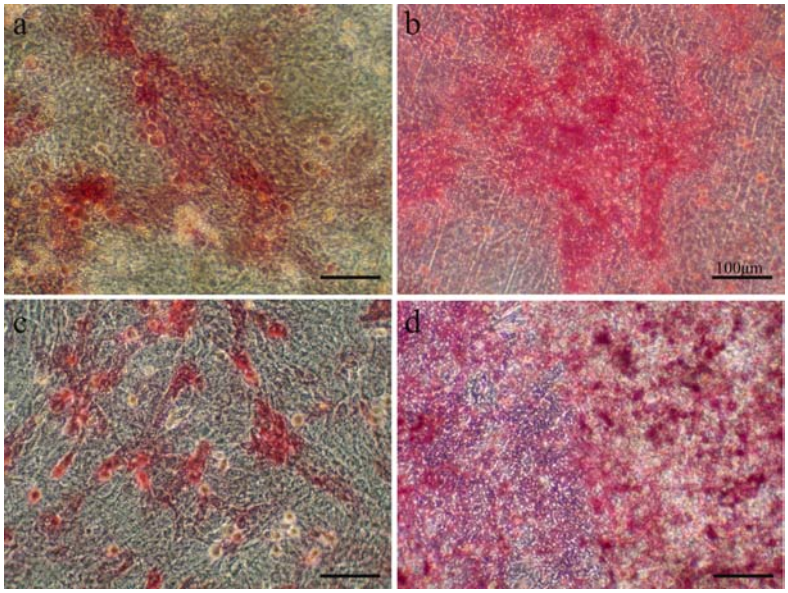

Figure 5 Microscopic observation of ALP and Alizarin Red S staining of the BMSCs culture. Bar: 100 μm. BMSCs were cultured in the presence of Dex for 14 days. Numerous regions of the cultured cells were positive for ALP on both t-βTCP ceramic disks (a) and PS dishes (c). Positive areas are represented in red. Strong Alizarin Red S stain could be detected in many cellular as well as extracellular regions on both the t-βTCP ceramic disks (b) and PS dishes (d).

micro plate reader (Wallac 1420 ARVOsx, PerkinElmer Life & Analytical Sciences, MA, USA). The standard DNA solutions were prepared using salmon sperm DNA (Invitrogen).

Alkaline phosphatase (ALP) activity assay

ALP activity of the cultured cells was examined quantitatively by the specific convention of *p*-nitro phenyl phosphate (pNPP) into *p*-nitro phenol (pNP). The cells were scraped off *t*-βTCP or PS surface into 0.5 mL of 10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl (pH 7.4). The suspended cell solution was sonicated and centrifuged at 10,000 $\times$g for 1 min at 4 °C. An aliquot (20 μL) of the supernatant was assayed for ALP activity using PNPP solution (Zymed Laboratories Inc. CA, USA). The aliquot was incubated at 37 °C for 30 min. After enzymatic reaction was stopped with 0.2 M NaOH, the aliquot was measured for the absorbance of *p*-NP product formed at 405 nm on a micro plate reader (Wallac 1420 ARVOsx). Enzyme activity was expressed as μmol of pNP released/30 min/DNA content.

Measurement of Osteocalcin deposited in extracellular matrix by ELISA

The cells were scraped off and sonicated, then centrifuged at 10000 $\times$g for 10 min. After treatment of the precipitation in 20% formic acid for 2 days, the samples were centrifuged at 800 $\times$g for 10 min and the supernatants were subjected to gel filtration to eliminate inorganic ions. Gel-filtered samples were evaporated for concentration. The concentrated samples were added to an EIA plate and immobilized with anti-rat osteocalcin antibody to measure the concentration of osteocalcin with an intact rat osteocalcin EIA kit (Biomedical Technologies Inc. MA, USA). Osteocalcin deposition was expressed as ng/DNA content.

Results

Fig. 1(a) shows the X-ray diffraction (XRD) pattern of a spark plasma sintering (SPS) specimen of *t*-βTCP. The peaks in both figures clearly show that the specimen we made is similar to that of typical βTCP (Fig. 1(b)). We analyzed the surfaces of the *t*-βTCP ceramic disks by scanning electron microscopy (SEM) before the cell culture. Although the surfaces of the *t*-βTCP ceramic disks were slightly rough compared with the surfaces of the PS (Fig. 2(b)), the grain structure of the surface of the sintered *t*-βTCP ceramic disks was fine (less than 1 μm) (Fig. 2(a)).

It is well known that the surface roughness and wettability of culture substrata influence cell attachment and proliferation. The average roughness and maximum roughness of both the *t*-βTCP ceramic disks and the PS dishes were measured by using a profilometer (Table I). The roughness levels of the specimens were similar. We also checked the wettability of the *t*-βTCP ceramic disks and the PS dishes, which is represented by sessile contact angles (SCA) (Table I). The SCA of the *t*-βTCP ceramic disks and the PS dishes showed no significant difference. The results indicate that surface structure is not an important factor in comparing cellular responses on *t*-βTCP ceramic disks to that on PS dishes.

TABLE I Surface roughness and sessile contact angle of each culture substrate

	Surface roughness (μm)		
Material	Ra	Rz	SCA (°±SD)
Culture dish	0.005	0.040	71.0 ± 3.42
β-TCP	0.008	0.079	74.7 ± 3.92

We observed the cell attachment, spreading, and proliferation of rat BMSCs on both culture substrata during the early culture periods. Then, the capacity of BMSCs for osteogenic differentiation was investigated during the late culture periods. The *t*-βTCP ceramic disks are sufficiently transparent to perform microscopic observations and, thus, the cells cultured on the *t*-βTCP ceramic disks can easily be observed by light microscopy equal to those on PS dishes. At a very early stage of the culture, rat BMSCs attached, followed by spreading on not only the PS dishes but also on the *t*-βTCP ceramic disks. One day after the seeding, most of the cells were able to attach and exhibit the morphological characteristics of mesenchymal types (spindle cell morphology) (Fig. 3(a) and (b)). The cells proliferated well and almost all were spindle-shaped after 4 days on both substrata (Fig. 3(c) and (d)). It is thus suggested that rat BMSCs could easily attach, spread, and proliferate on *t*-βTCP ceramics as well as on PS.

In order to analyze the osteogenic differentiation of BMSCs, the BMSCs were further cultured on both *t*-βTCP ceramic disks and PS dishes in the presence of dexamethasone (Dex) for 14 days, and cell morphology was observed by light microscopy. Dex is used because it is well known as an osteogenic factor. To observe the extracellular mineralized matrix more clearly, we added calcein to the medium and measured its fluorescent intensity. When cultured in the osteogenic condition for 14 days, the same level of mineralized bone matrix on both the *t*-βTCP ceramic disks and the PS dishes was observed (Fig. 4(a) and (b)). In contrast, the BMSCs cultured in the absence of Dex showed no osteoblastic cell shape but did show a fibroblastic shape and no evidence of matrix formation (Fig. 4(c) and (d)). These findings indicate that BMSCs can easily differentiate into osteoblasts on the surface of *t*-βTCP ceramics, resulting in the formation of bone matrix under osteogenic

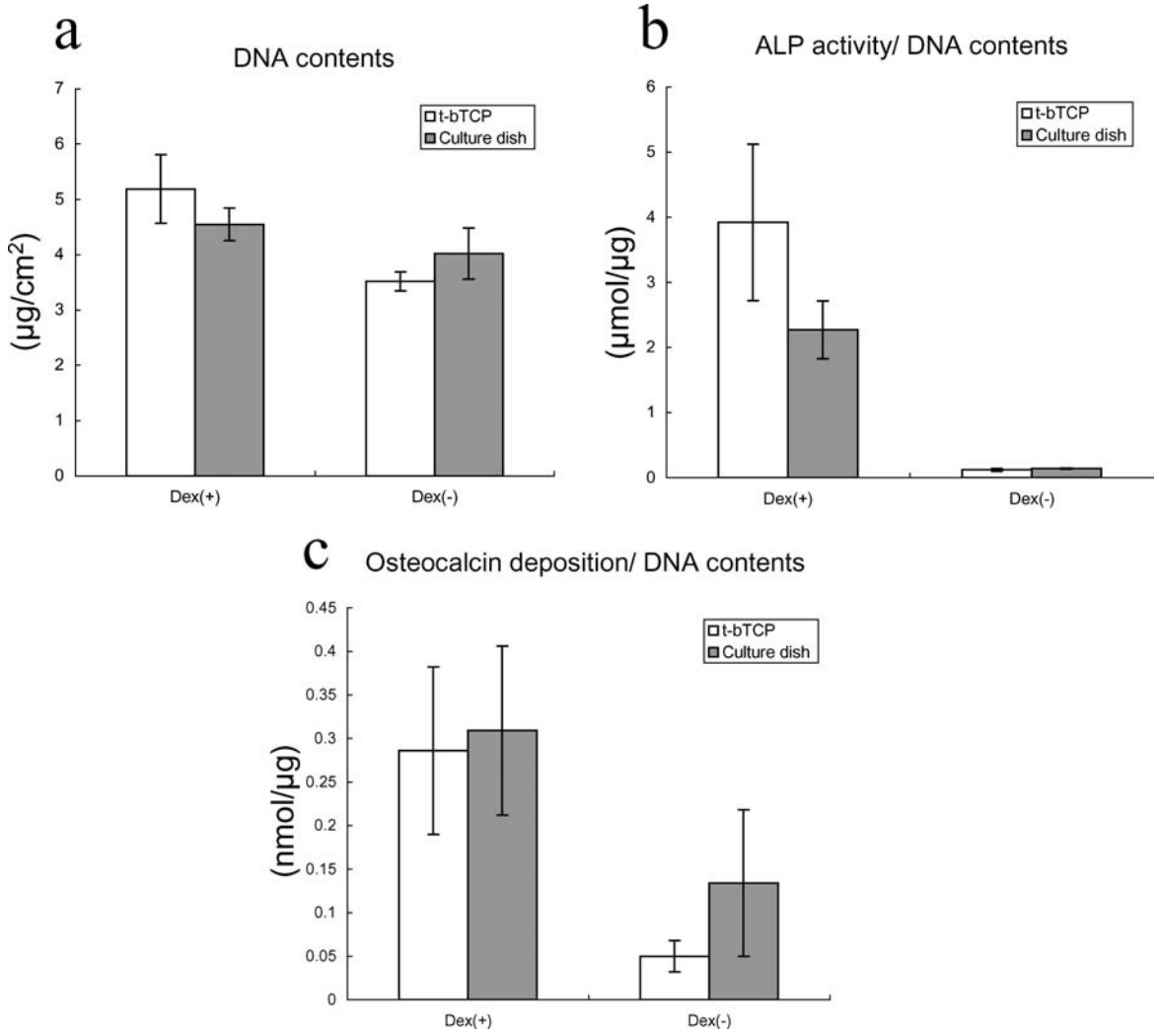

Figure 7 Quantitative and biochemical analyses of BMSCs cultured in the presence (+) or absence (−) of Dex. BMSCs were cultured on both transparent βTCP ceramic disks (t-βTCP) and culture grade polystyrene dishes (culture dish) for 14 days. The DNA contents were represented as micrograms per cm^2 (a). ALP activity (b) and osteocalcin deposition (c) were normalized for DNA content.

conditions. Importantly, the cascade of the differentiation of BMSCs can equally be observed on both t-βTCP ceramics and PS.

To confirm the osteogenic differentiation of BMSCs on the t-βTCP ceramic disks, we performed alkaline phosphatase (ALP) activity and Alizarin Red S staining after two weeks of cultivation. ALP, which is a cell surface protein, is known as an early marker for osteoblastic differentiation and Alizarin Red S can identify calcium in the matrix. Many clusters of cells were well stained with ALP on both the t-βTCP ceramic disks (Fig. 5(a)) and PS dishes (Fig. 5(c)) when cultured with Dex. As seen in the ALP stain, the cultures with Dex were strongly stained with Alizarin Red S (Fig. 5(b) and (d)). In contrast, the cultures without Dex were barely stained with either ALP or Alizarin Red S, as evidenced by macroscopic observation (Fig. 6). These biochemical data showed that the osteogenic differentiation of BMSCs cultured in the presence of Dex could occur on both t-βTCP ceramic and PS substrata.

After 14 days of culture, the DNA contents from each substrate were measured. The DNA contents were similar regardless of the culture substrata (Fig. 7(a)). As seen in ALP staining (Figs. 5 and 6) the cultures on both substrata with Dex showed high ALP activity compared with the culture without Dex (Fig. 7(b)). These data confirmed the morphological data (Figs. 3–6) that βTCP ceramics as well as PS supports the proliferation and osteogenic differentiation of BMSCs cultured in the presence of Dex. To confirm the osteogenic differentiation, we measured the bone-specific protein of osteocalcin. As seen in Fig. 7(C), high levels of osteocalcin were detected in the culture with Dex on both substrata. These quantitative biochemical data were evidence that the surfaces of both the t-βTCP ceramic disks and the PS dishes provide considerable

support for the osteoblastic differentiation cascade of BMSCs.

Discussion

In this study, cell behavior on transparent βTCP ceramic disks was compared with that on culture grade PS dishes. PS dishes are considered the gold standard substratum for cell cultivation and were therefore used in the present experiment as a positive control. It has also been reported that the surface characteristics of the culture substrata affect cell behavior during the culture period [21, 22]. As can be seen in Table I, the surface roughness and wettability of t-βTCP were similar to those of PS. It is considered that the optimum wettability for cell attachment is about 70° (defined by sessile contact angle; SCA), and the SCA of t-βTCP as well as PS is nearly 70°. Additionally, the surface of t-βTCP is very smooth as is that of PS as observed by SEM (Fig. 2). These data mean that the wettability of t-βTCP is suitable for cell attachment and that surface roughness is not a decisive factor for comparing cell behavior on both substrata.

Currently, *in vitro* osteogenic differentiation on the various ceramics can be monitored by ALP staining or calcium staining after fixation as shown in Fig. 6. However, microscopic observation of this staining is difficult due to the opacity of ordinary ceramics. In addition, staining cannot be used for repeated monitoring of the same specimen during the culture period. In this regard, by using t-βTCP ceramic, we can detect the ALP and Alizarin Red S stain at the cellular level (Fig. 5). By adding calcein to the culture medium, we can clearly monitor the differentiation indicated by fluorescence emission (Fig. 4). Fluorescence can be detected repeatedly for the same specimen during the entire culture period. As seen in the results, we confirmed the capability of t-βTCP to enable BMSCs attachment, proliferation (Fig. 3), and osteogenic differentiation as confirmed by ALP stain, Alizarin Red S stain (Fig. 5), and calcein uptake (Fig. 4). We also confirmed that the capability of t-βTCP as a cell culture substrate was similar to that of PS. The qualitative analyses were confirmed by quantitative analyses of DNA, ALP activity, and osteocalcin measurements (Fig. 7). The data verify that the surface of t-βTCP ceramic is equivalent to that of culture-grade PS and has the capability of supporting cellular adhesion and proliferation, which results in the osteogenic differentiation of BMSCs. It is important to demonstrate a functional equivalence of both substrata because PS is suitable to *in vitro* culture but the appropriate materials for tissue engineering is βTCP. It is possible to make various type of βTCP combined with extracellular matrix or growth factors. And we can control a composition of βTCP. Together, we concluded that an observable transparent βTCP is useful materials for *in vitro* research in the field of medicine especially tissue engineering.

Conclusion

We fabricated a new material, a transparent βTCP (t-βTCP) ceramic, and used it as a culture substrate. The ceramic material enables the observation of cultured cells by light microscopy. The shape of the cells on the ceramic disks was as clearly detected as that seen on culture grade polystyrene (PS) dishes. In this report, rat bone marrow stromal cells (BMSCs) were cultured. As a result, cell attachment, proliferation, and differentiation of BMSCs on t-βTCP disks were similar to those on (PS) dishes. The results were confirmed by quantitative biochemical assay. All results confirmed the excellent properties of βTCP for supporting the differentiation capability of BMSCs, which resulted in osteoblastic phenotype expression.

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