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Observation of osteogenic differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite ceramics

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Abstract

The use of bioceramics and cultured cells for tissue engineering is a novel approach, which is available in a wide variety of clinical situations. The approach requires apparent verification of the cellular functions occurring on the ceramic surface, and these functions could be monitored by microscopic observation of the cultured living cells on the ceramic material. However, such observation is difficult due to the opaque nature of ordinary ceramics. To overcome this drawback, we used transparent hydroxyapatite (tHA) ceramics as a culture substrate and a transgenic rat having an enhanced green fluorescent protein (EGFP)-expressing gene as the cell source. Marrow mesenchymal stem cells (MSC) were obtained from the rat and cultured on both tHA ceramics and a tissue culture polystyrene (TCPS) dish. One hour after the cell seeding, many MSC had attached and showed initial cell spreading. The attachment and spreading were more obvious 5 h after the seeding. Following the culture in the osteogenic condition, the cells differentiated into osteoblasts, which fabricated bone matrix on the culture substrate. The phenomena were similarly observed on both the tHA ceramics and TCPS substrata. These results confirm the excellent properties of tHA ceramics, which support cell attachment, proliferation, and differentiation. Transparent materials make us know the biological usefulness of ceramics in tissue-engineering field.

Keywords: Observation; Mesenchymal stem cells (MSC); Transparent hydroxyapatite (tHA) ceramics

1. Introduction

Current technology enables the regeneration of viable tissues or organs by using both cultured cells and suitable scaffolds. Hydroxyapatite (HA) is a well-known scaffold material and plays important roles in the adhesion, proliferation, and differentiation of cultured cells, especially bone-related cells. We have previously reported that mesenchymal stem cells (MSC) derived from bone marrow can be cultured on HA ceramics [1,2]. Significantly, under osteogenic conditions, the cultured MSC further differentiate into osteoblasts, which fabricate bone matrices on the HA ceramic surfaces. The cultured osteoblast/matrix constructs can show in vivo osteogenic capability as evidenced by new bone formation after in vivo implantation [3–5]. Therefore, the tissue-engineered construct fabricated by cultured osteoblasts on a scaffold could be defined as *regenerative cultured bone tissue* [6]. Regenerative cultured bone tissue has already been applied clinically to patients such as in bone tumor cases. Thus, MSC cultured on various ceramics, in particular HA ceramics, has clinical significance. However, due to the non-transparency of the ordinary hydroxyapatite ceramics, analysis of the cellular mechanisms that lead to in vitro bone formation is obscured.

In order to clarify the mechanisms, we tried to use novel materials for cell culture, namely, transparent hydroxyapatite (tHA) ceramics that were made by the

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spark plasma sintering process (SPS). SPS is a new process that enables sintering of materials in short periods by charging the spaces between powder particles with electrical energy and using a high sintering pressure. SPS systems offer many advantages (e.g. rapid sintering, sintering with fewer additives, uniform sintering, low operating expense, easy operation) over conventional systems using hot press (HP) sintering, hot isostatic pressing (HIP), or atmospheric furnaces. The SPS process can be applied to many materials including fine ceramics.

In this report, we have applied cell culture to tHA ceramics. Recently, enhanced green fluorescent protein (EGFP)-expressing transgenic rat has become available; the green fluorescent protein is responsible for the green bioluminescence of the jellyfish, *Aequorea victoria*. The luminescence has caused researchers to use the gene of the protein for real-time bio-imaging monitoring. The cells we used were cultured mesenchymal stem cells derived from the EGFP-expressing transgenic rat bone marrow. As described, because the EGFP-expressing gene affects both fresh as well as cultured cells, the cells fluoresce in green under UV light. This enables clear observation of the shape of cultured cells by using fluorescence microscopy. In this paper, the MSC derived from EGFP transgenic rat were cultured on tHA ceramics as well as on a polystyrene culture dish and were observed under light and fluorescence microscopy. Osteogenic differentiation of the MSC resulting in in vitro bone formation was analyzed both morphologically and biochemically.

2. Materials and methods

2.1. Preparation of ceramics

We previously reported the detailed method of making transparent hydroxyapatite (tHA) ceramics [7]. Briefly, a fine powder of HA (high-purity grade) was utilized as the basic material. One gram of this powder was poured into a graphite mold (inner diameter: 15 mm), 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 800°C, 900°C, and 1000°C for 10 min at a heating rate of 25°C/min. Each ceramic disk was 5 mm in diameter × 2 mm thick. The ceramic samples were finely polished with a paste containing fine Al_2O_3 particles smaller than 0.5 mm and autoclaved for sterilization at 120°C for 20 min. The microstructure was examined using a scanning electron microscope (SEM). A Fourier transform infrared spectrometer (FT-IR) and X-ray diffractometer (XRD) were also applied for characterization.

2.2. Surface characterization

The sessile contact angles (SCA) of the tHA ceramics and TCPS dishes were determined using Milli-Q water and a goniometer (Face Contact-Angle Meter, Kyowa Kaimenkagaku Co. Ltd., Tokyo, Japan). A probability (p)-value of less than 0.05 was considered significant. To evaluate the surface structures of the materials, a scanning electron microscope (SM-300, TOPCON CORPORATION) was used to analyze the surface of each material. Prior to being placed in the test chamber, the specimens were coated with 200 Å-thick platinum to prevent charging.

2.3. Culture methods

Rat bone marrow cell plugs were obtained from 7-week-old female Sprague–Dawley (SD) rats that were EGFP transgenic rats (“green rat CZ-004”, SD TgN (act-EGFP) OsbCZ-004; Japan SCL Inc., Shizuoka, Japan). The EGFP absorbs light in the UV-blue region (400 nm), and emits light in the green (510 nm), facilitating observation of the shape of the transgenic cells using fluorescence microscopy. Preparation and osteogenic differentiation of MSC from the rat were described by Maniatopoulos et al. [8] and modified by Ohgushi et al. [9]. In brief, rat bone marrow cells were flushed out by 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 adherent mesenchymal stem cells (MSC) from rat bone marrow were initially cultured up to 80% confluence in T-75 flasks (Becton, Dickinson and Company (BD), NJ, USA) and resuspended to 5×10^5 cells/ml in culture medium following harvesting using 0.05% trypsin/0.53 mM EDTA. The cell suspension was applied to sterilized, transparent hydroxyapatite (tHA) ceramic disks (5 mm in diameter × 3 mm thick), which were placed into a 24-well plate, and on TCPS dishes (ϕ35 mm, BD) as a control. These cells were cultured with osteogenic medium containing 10 nM dexamethasone (Sigma-Aldrich Corporation, MO, USA), 10 mM β-glycerophosphate (Merck, Darmstadt, Germany) and 0.28 mM ascorbic acid two-phosphate magnesium salt n-hydrate (Sigma-Aldrich Corp.). The culture medium was changed two or three times per week. During the culture period, the cell morphologies were detected by phase-contrast and fluorescence microscopy.

2.4. Alkaline phosphatase (ALP) activity staining

The cultured cells on the tHA ceramics 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 washed with PBS [9].

2.5. Calcium staining by Alizarin Red S

The cultured cells on the ceramics 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 washed with PBS [9].

3. Results

The tissue culture polystyrene (TCPS) dish, which is fabricated from crystal grade polystyrene by a vacuum–gas plasma treatment, shows a slightly hydrophilic nature since the plasma treatment introduces carboxyl and hydroxyl groups onto the surfaces. Under typical culture conditions employing TCPS dishes, a variety of types of cultured cells are able to attach and proliferate on the surfaces, while cells could hardly attach to bare polystyrene because of the high degree of hydrophobicity of the surfaces. Therefore, TCPS is known to be gold standard substratum for cell cultivation. We first analyzed the attachment and spreading of rat mesenchymal stem cells (MSC) on both TCPS and tHA ceramics during early culture periods and then investigated the capacity of MSC for osteogenic differentiation on both substrata during the later stages of culture.

Fig. 1a and b show the X-ray diffraction (XRD) pattern and the Fourier transform infrared spectra (FT-IR) of a spark plasma sintering (SPS) specimen of tHA, respectively. The peaks in both figures clearly show that the specimen we made is typical hydroxyapatite.

It is well known that the roughness and wettability of culture substrata influences cell proliferation and attachment. Therefore, we used scanning electron microscopy (SEM) to analyze the surface of transparent hydroxyapatite (tHA) ceramics before the culture. Although the surfaces of the tHA ceramics were slightly rough compared with the surface of TCPS (Fig. 2b), the grain structure of the surface of the sintered tHA ceramic was fine (less than 1 μm) (Fig. 2a). We also investigated the wettability of tHA ceramics and TCPS, which is represented by sessile contact angles (SCA) (Table 1). The SCA of the tHA ceramics and the TCPS showed no significant difference. The results indicate that the surface configuration is not an important factor in comparing cellular responses on tHA ceramics to that on TCPS.

As tHA ceramic material is highly transparent, the cells cultured on tHA ceramics could be observed by phase-contrast and fluorescence microscopy. The shapes of the cultured cells on the tHA ceramics were clearly detected by phase-contrast microscopy; the resolution of the microscopy was almost the same as on the TCPS.

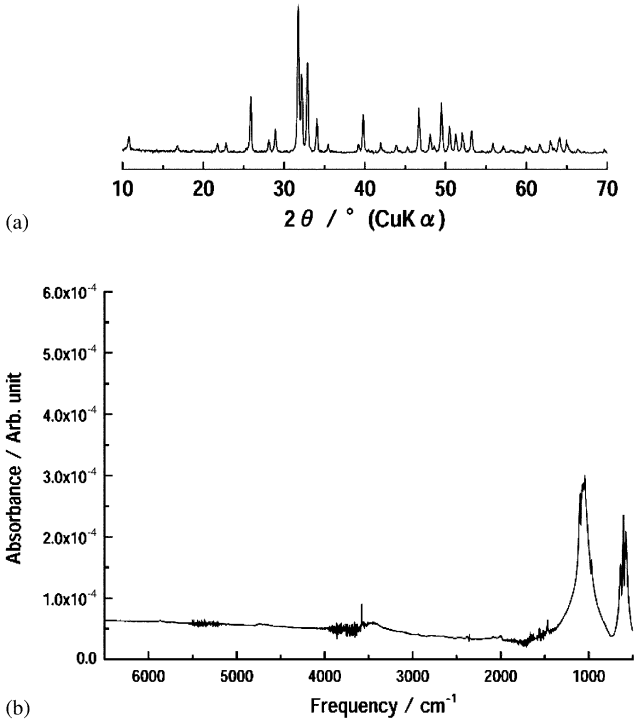

Fig. 1. Powder X-ray diffraction (XRD) pattern (a) and Fourier transform infrared spectra (FT-IR) (b) of transparent hydroxyapatite (tHA) ceramics.

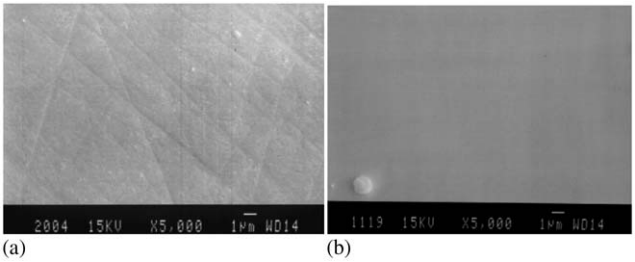

Fig. 2. SEM image of transparent hydroxyapatite (a) and tissue culture polystyrene (TCPS) dish (b). (Original magnification: 5000 ×). Bar: 1 μm. The surface of TCPS dish is very smooth and therefore most of SEM photos were out of focus. To bring the object into focus, we took a photograph of the dish contaminated with very small dust (shown at the lower left) as a pointer.

Table 1
Sessile contact angle (SCA)

	SCA(°) ± SD
tHA	73.13 ± 2.36
TCPS dish	71.00 ± 3.42

Data represent the mean value of eight samples with standard deviation (SD).

These results indicate that ordinary cells without fluorescent characteristics can be monitored even when the cells are cultured on HA ceramics. To our surprise, at a very early stage of the culture (1 h after cell seeding), many MSC had already attached and showed initial spreading, as evidenced by small round cells on the tHA ceramics (Fig. 3a and b). The attachment and spreading were more obvious 5 h after the seeding (Fig. 3e and f). Most of the cells were able to attach and exhibit the morphological characteristics of mesenchymal types (spindle cell morphology) 1 day after the seeding (Fig. 3i and j). The cascade of the cellular response on the tHA ceramics was similar to that of the cells seeded on the TCPS (Fig. 3c, d, g, h, k and l). It is thus suggested that MSC can attach, spread, and proliferate on tHA ceramics as well as on TCPS.

In order to analyze the osteogenic differentiation of MSC on tHA ceramics, the MSC were cultured on both tHA ceramics and TCPS in the presence or absence of dexamethasone (Dex) for 14 days, and cell morphology was observed by fluorescence and phase-contrast microscopy. Dex is well known as an osteogenic factor and cells were cultured in the presence of β-glycerophosphate, ascorbic acid, and Dex (Dex (+)). The cells differentiated into bone-forming osteoblasts, which fabricate an extracellular mineralized matrix around a clump of the cultured cells. As shown in Fig. 4a–d, the MSC differentiated into osteoblasts and fabricated a

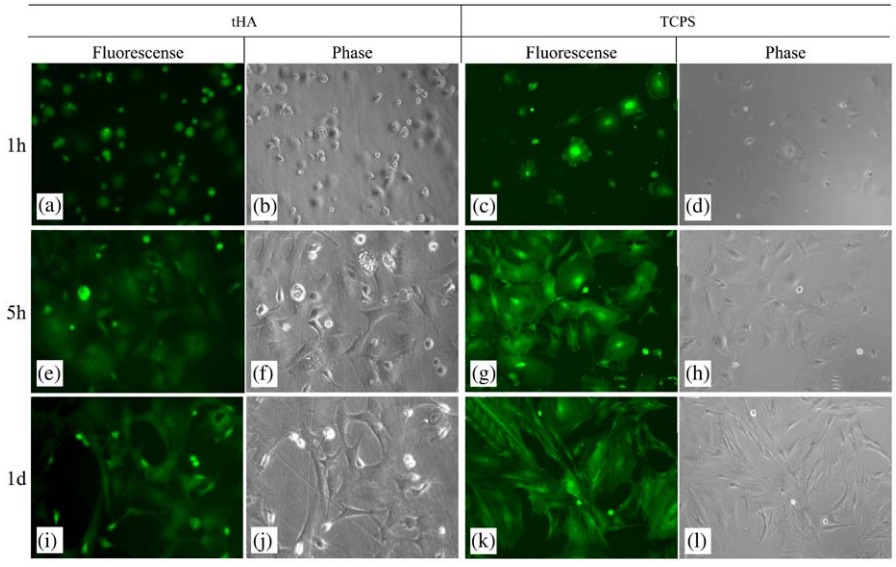

Fig. 3. Cell morphology of rat mesenchymal stem cells (MSC) seeded on transparent hydroxyapatite (tHA) ceramics and tissue culture polystyrene dishes (TCPS). The cells seeded on tHA ceramics are seen in (a), (b), (e), (f), (i) and (j). The cells seeded on TCPS are seen in (c), (d), (g), (h), (k), (l). The cell morphology was observed at 1 h, 5 h and 1 d after MSC were seeded on TCPS dishes and tHA ceramics by fluorescent (a), (c), (e), (g), (i), (k) and phase-contrast (b), (d), (f), (h), (j), (l) microscopy. After 1 h, the cell shape was round (a–d) but after 5 h, the cells began to spread on the surface (e)–(h). Cells on both substrata could spread completely; most cells had a fibroblastic shape after 1 d (i–l). (Original magnification: 200 ×).

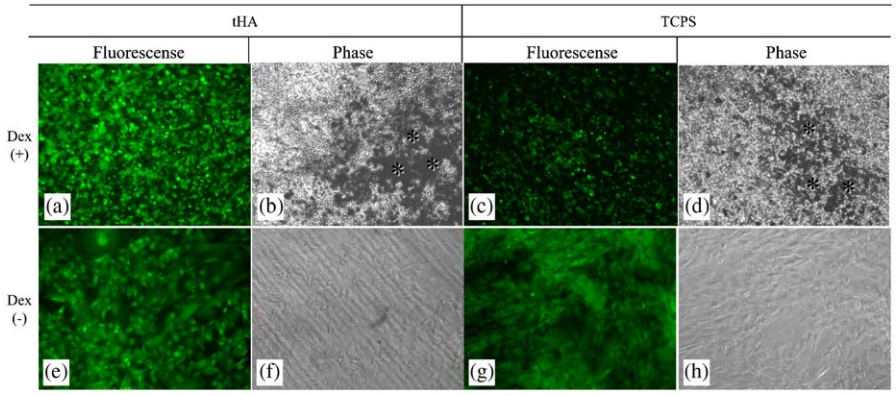

Fig. 4. Cell morphology of rat mesenchymal stem cells (MSC) seeded on transparent hydroxyapatite (tHA) (a, b, e, f) ceramics and tissue culture polystyrene (TCPS) (c, d, g, h) dishes in the presence (+) or absence (–) of dexamethasone (Dex) after day 14 of cultivation. The Dex-treated rMSC differentiated into osteoblasts and formed minerals (asterisk) on those surfaces (a–d) while the MSC not treated with Dex were able to proliferate but not differentiate into osteoblasts (e)–(h). The cell shape can easily be recognized by fluorescent microscopy even after the cells formed minerals (a), (c). (Original magnification: 100 ×).

mineralized bone matrix on both the tHA ceramics and TCPS. Even after an abundant matrix formation, the shape of the cells could easily be recognized under observation by fluorescent microscopy (Fig. 4a and c). In contrast, the MSC cultured in the absence of Dex (Dex(–)) showed no osteoblastic cell shape but did show a fibroblastic shape (Fig. 4e–h) and no evidence of matrix formation. These findings indicate that MSC can easily differentiate into osteoblasts on the surface of tHA ceramics, resulting in the formation of bone matrix under conditions of osteogenic culture. Importantly, the cascade of the differentiation of MSC can be observed equally on both tHA ceramics and TCPS.

To confirm the osteogenic differentiation of MSC on tHA ceramics, we stained the cells with alkaline phosphatase (ALP) and calcium staining (Alizarin Red S) after 2 weeks of cultivation. ALP, which is a cell surface protein, is known as an early marker for osteoblastic differentiation; calcium is the principal inorganic component of bone matrix. Faint ALP stains on both the tHA ceramics (Fig. 5e and f) and TCPS (Fig. 5g and h) were detected in the culture without Dex, but the stains were much more extensive in the culture with Dex (Fig. 5a–d). Alizarin Red S stain is commonly used to demonstrate calcium deposits. As seen in the ALP stain, the Dex-treated cells were strongly stained with Alizarin Red S (Fig. 6a–d), while the cells not treated with Dex were hardly stained (Fig. 6e–h). This biochemical data showed that the osteogenic differentiation of MSC cultured in the presence of Dex could occur on both tHA ceramic and TCPS substrata.

4. Discussion

In the progress of tissue engineering, it is important to develop new biomaterials suitable for cell cultivation. If cultured cells, especially stem cells, are to maintain their

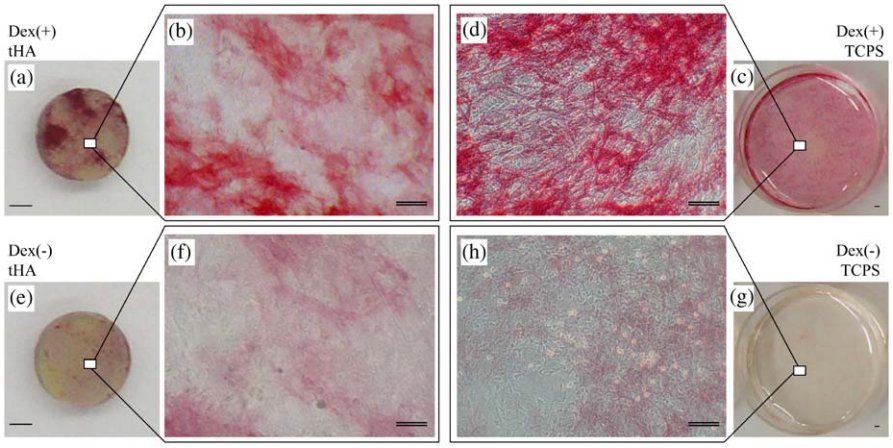

Fig. 5. Alkaline phosphatase (ALP) staining of the cultured cells on day 14. Numerous regions of the cultured cells were positive for ALP in the presence of Dex both on tHA ceramics (a, b) and TCPS (c, d). The positive areas are red. In contrast, in the absence of Dex on tHA ceramics (e, f) and TCPS (g, h), ALP activities were very low and only a few cells showed weak ALP signals. Bar: 1 mm. Double bar: 0.1 mm.

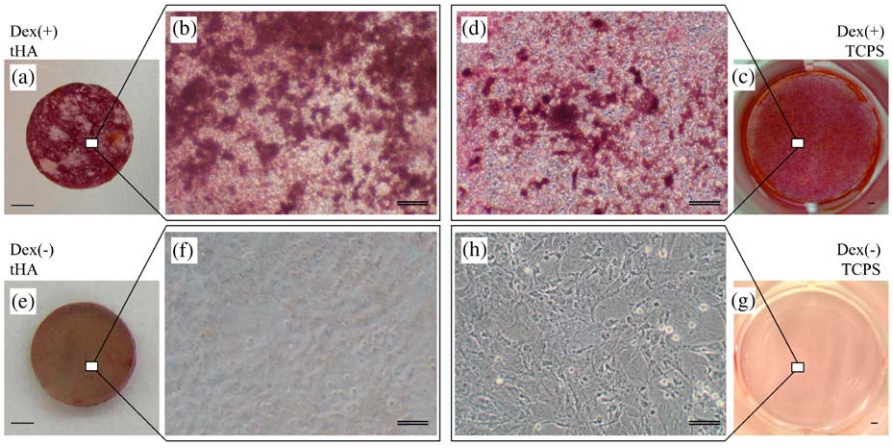

Fig. 6. Calcium staining of the cultured cells with Alizarin Red S on day 14. Many Alizarin Red stainable regions of the cultured cells could be detected in the presence of Dex on tHA ceramics (a, b) and TCPS (c, d); the stain was at almost basal levels for cells cultured in the absence of Dex on tHA ceramics (e, f) and TCPS (g, h). Bar: 1 mm. Double bar: 0.1 mm.

biological activity within the materials, they are expected to have the capability of self-renewal and differentiation into lineage-committed cells after in vivo implantation. Therefore, the combination of cultured cells and biomaterials is a key issue for successful fabrication of regenerative cultured tissues or organs. Furthermore, cell viability and proliferation on these materials is prerequisite for clinical applications. Recently, many materials have been developed for building tissue-engineered scaffolds. We have employed a variety of ceramics such as bioactive beta-tri-calcium phosphate ceramics [1], glass ceramics [10], and bio-inert alumina ceramics [11,12] beside as bioactive hydroxyapatite for hard tissue regeneration. These ceramics can be combined with rat or human mesenchymal stem cells (MSC) derived from bone marrow. The MSC can differentiate into osteoblasts not only under in vitro but also under in vivo conditions.

Cell dynamics are usually detected by observation using an ordinary light microscopy. Unfortunately, cells on many types of ceramics are difficult to observe because of the opaque nature of the ceramics. We previously utilized synthetic transparent alumina ceramics observing cell dynamics. Transparent alumina ceramics also exist naturally, known as sapphire, which consist of single-crystal Al_2O_3. But most other ceramics such as hydroxyapatite are not naturally transparent and it is difficult to create single-crystal or transparent ceramics. Although alumina ceramics are bioinert and do not exhibit bone-bonding properties, hydroxyapatite ceramics are widely known as bioactive ceramics having bone-bonding properties and are thus suitable scaffolds for culturing MSC. However, due to the opaque nature of the ceramics, cell behavior on the ceramics is difficult to observe. To overcome this drawback, the spark plasma sintering (SPS) process has recently been used for making some types of transparent ceramics, previously thought to be impossible.

Using the SPS process, we have already succeeded in fabricating tHA ceramics [7,13] and have used these ceramics for observing the cultured cells [14]. However, the results were preliminary and the cells used did not show fluorescent emission. The present results enabled us to demonstrate the cascade of cell differentiation (osteogenic differentiation) using fluorescent cells. Importantly, we were able to use a microscope to monitor the cascade of the same samples throughout the culture period. In other words, we were able to conduct real-time monitoring of cells cultured on ceramics.

We used fluorescent cells for cultivation on the tHA ceramics, which can be clearly observed with fluorescent microscopy. It is easy to observe the morphology of non-fluorescent cells on tHA ceramics by phase-contrast microscopy during initial culture periods but it is hard to make the same observations during later culture periods, especially when the culture is conducted under osteogenic conditions. The reason for this is that the cultured cells sometimes differentiate into osteoblasts, which make a calcified matrix, and the matrix masks observation of the shape of the cells on both tHA ceramics and TCPS, using a phase-contrast microscope. Consequently, we utilized EGFP-expressed rat mesenchymal stem cells whose cellular morphology can be more clearly detected, as shown in Figs. 1 and 2. These novel approaches using transparent ceramics combined with fluorescent cells emphasized the importance of observing cellular behavior on biomaterials that are to be used as tissue-engineering materials. In fact, the tHA ceramics demonstrated the immediate capability of allowing MSC to attach to the surface. The attachment led to excellent cellular proliferation that resulted in osteogenic differentiation.

Both the TCPS and tHA ceramics discussed in this paper exhibited excellent cellular attachment followed by proliferation. Many factors might be involved in the mechanisms that occur. In this regard, fibronectin is an interesting factor and is known to be essential for promoting cellular attachment and spreading on the substrate [15]. Fibronectin is any of a group of widely distributed glycoproteins that serve as a substrate to promote cellular adhesion and migration through its central-binding domain RGD sequence [16,17]. It can be produced by most mesenchymal and epithelial cells and is also present in serum used in the culture. The interaction of fibronectin with unmodified polystyrene (as is commonly used for bacterial agar plates) is weak and about $\frac{1}{10}$ as effective in cell binding in comparison with polystyrene modified for tissue culture plates (TCPS). This is the reason that cells generally fail to grow on bacterial grade plates. Therefore, TCPS is an excellent substratum for cell culture and was therefore used as a positive control in this study. Cell attachment to tHA ceramic surfaces and the cell shape on the ceramics were quite similar to that on TCPS. This observation, as mentioned above, was easily detected by fluorescence microscopy without the need for a fixation step at any time during the culture period. This suggests that tHA ceramic surfaces are equivalent to culture-grade dishes and have the capability of supporting cellular adhesion and proliferation, which results in the osteogenic differentiation of the MSC. Although further experiments are required to clarify the mechanisms of the excellent properties of tHA, cell-binding factors such as fibronectin and laminin might easily be adsorbed on the surface of tHA ceramics, and promote the adhesion and/or proliferation of the cells on the surface.

The composite of biomaterials and cells can be applied for the treatments in a variety of clinical situations. In particular, we have succeeded in differentiating patients' derived mesenchymal stem cells (MSC) into osteoblasts together with forming bone matrix on various ceramics. Importantly, the constructs

having the cells/matrix (*regenerative cultured bone*) can be made from not only fresh cells but also cryopreserved cells from a geriatric patient's marrow cells [6]. Evidence of adequate cellular functions before their implantation into a patient is strongly needed in order to go through cell-based therapy and tissue engineering. Here, we demonstrated for the first time that transparent hydroxyapatite ceramics enable us to observe living cells directly and the ceramic surface clearly support the attachment/proliferation/differentiation of the MSC. Therefore, these results confirm the excellent properties of the hydroxyapatite ceramics to be used in hard tissue regeneration.

5. Conclusion

We used transparent hydroxyapatite (tHA) ceramics as a cell culture substrate. The ceramics make us possible to observe cell behavior on ceramics by light microscopy with ease. In this report, rat mesenchymal stem cells (rMSC) were applied and as a result, cell attachment, proliferation, and osteogenic differentiation of rMSC on tHA observed by light microscopy were significantly similar to those on tissue culture polystyrene (TCPS) dish, which is the gold standard material for cell culture. The observation of living cells on tHA is a new significant and revealed reliable nature of the hydroxyapatite ceramics for the purpose of tissue engineering in hard tissue repair.

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