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In vitro Mineralization by Mesenchymal Stem Cells Cultured on Titanium Scaffolds

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Titanium has been utilized in the field of orthopaedic and dental reconstructive surgery, but mineralization through osteogenic differentiation of osteogenic cells on titanium surfaces has not been fully investigated. Here we cultured rat mesenchymal stem cells (MSCs) on the surfaces of titanium dishes in osteogenic media containing calcein which is a calcium-binding fluorescence dye. On titanium dishes, MSCs showed high viability to adhere to the surfaces and excellent proliferation. At day 14 of culture, MSCs differentiated into osteoblasts to form mineralized matrices on titanium dishes as well as tissue culture polystyrene (TCPS) dishes which are widely recognized as optimal culture substrates. Calcein was incorporated into the bone minerals fabricated by MSCs cultured on both substrates to show green emission under fluorescence microscopy. The fluorescence intensity was quantified with an image analyser during culture periods. These results indicate that the surfaces of titanium showed a high adhesion/proliferation potential to MSCs and that the titanium effectively supported the osteogenic differentiation of MSCs comparable to TCPS dishes. Therefore, the titanium is an effective scaffold that is applicable in bone reconstruction surgery.

Key words: calcein, cell adhesion, mesenchymal stem cell, osteogenic differentiation, titanium.

Abbreviations: calcein-AM, calcein-bis [(acetyloxy) methyl] ester; Calcein F. I., calcein fluorescence intensity; Dex, dexamethasone; EDX, energy dispersive X-ray spectroscopy; EthD-1, ethidium homodimer-1; FBS, fetal bovine serum; hFbs, human dermal fibroblasts; MEM, minimum essential medium; MSCs, mesenchymal stem cells; PBS(−), calcium/magnesium-free phosphate-buffered saline; P-1, passage-1; P-3, passage-3; Ra, surface roughness; SEM, scanning electron microscopy; TCPS, tissue culture polystyrene.

Mesenchymal stem cells (MSCs) can differentiate into osteoblasts and undergo mineralization when they are cultured in the presence of ascorbic acid, β-glycerophosphate and dexamethasone (Dex) (*1*, *2*). From physicochemical and biochemical analyses, such mineralization is not a simple precipitation of calcium and phosphorous but is a biological accumulation of apatite, which exists in natural bone (*3*). Cultured MSCs can differentiate into osteoblasts not only on tissue culture polystyrene (TCPS) dishes but also on the surfaces of biomaterials such as bioactive calcium phosphate ceramics (*4*, *5*) and bioinert alumina ceramics (*6*) or titanium alloys (*7–9*). The cultured osteoblast/biomaterial constructs show *in vivo* osteogenic capability as evidenced by new bone formation after implantation (*10–13*). Based on these results, it has been proposed that autologous MSCs can be used for the treatment of bone/joint diseases; they have already been implanted in patients resulting in excellent therapeutic effects (*14*, *15*). In clinical applications, we must evaluate the osteogenic capability of cultured cells non-invasively and accurately because we have found individual differences in the osteogenic capabilities of patient's MSCs (*16*). Therefore, in order to set suitable implant conditions, the evaluation of the regenerative cultured bone on the biomaterial seems to be very important (*17*). In this regard, we reported a novel method of real-time monitoring of a mineralized matrix by MSCs cultured on TCPS dishes with a calcium-binding fluorescent dye, calcein (*18*). Using the novel method, we stained the calcium deposited on the bone matrices with calcein, and quantified the intensity of the fluorescence emission of calcein with an image analyser.

Titanium alloys including pure titanium are widely utilized as substitutes for dental implants (*19*) and the fixing of fractures (as fixation plates and screws) (*20*). Titanium is bioinert and biocompatible, and shows osteoconductive property when implanted in bone defects. In previous study, Ohgushi and Caplan (*10*) were reported that osteoinductivity was given by making the marrow cell/titanium composites (*10*). However, mineralization through the osteogenic differentiation of osteogenic cells on titanium has not been fully examined. In contrast, TCPS dishes are recognized as the gold standard of substrates for cultured cells because their surfaces are chemically modified to be suitable for cell adhesion.

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In this article, we confirmed the *in vitro* osteogenic differentiation of rat MSCs cultured on titanium surfaces as well as on TCPS dishes and investigated real-time monitoring of mineralized matrices produced by rat MSCs on the titanium surfaces throughout the cultivation period.

MATERIALS AND METHODS

Pure Titanium as Cell Culture Dishes—We prepared titanium dishes (Hi-Lex Corporation, Takaradsuka, Japan) for cell culture. Each titanium dish was 17 mm in diameter including the rim (3 mm height). The cell culture surface was 15 mm in diameter × 2 mm deep; the culture surface of the dish bottom was finely polished with a No. 800 diamond paste (Fig. 1a). The surface roughness (Ra) of the titanium dish was 0.25 (μm) ± 0.02 (mean value ± SD; Handysurf E-35A, Tokyo Seimitsu Co., Ltd, Tokyo, Japan). Scanning electron microscopy (SEM) revealed that the culture surface of the titanium dishes had a smooth surface with a few abrasive marks (Fig. 1b). In addition, an elementary analysis of the culture surface was conducted with energy dispersive X-ray spectroscopy (EDX). The EDX analysis confirmed that the culture surface of the titanium dishes were exclusively pure titanium (Fig. 1c). The SEM and EDX analyses were performed with a scanning electron microscope (Model S-3500N, Hitachi High-Technologies Corp., Tokyo, Japan).

Cell Preparation and Culture Method—MSCs derived from rat marrow or human dermal fibroblasts (hFbs; Cat. No. 2F0-C25, Cell systems, CA, USA) were used in this study. Animal experiments were carried out in compliance with the Japanese (No. 105) law on animal protection and administration as well as according to the regulations on the Implementation of Animal Experimentation of the AIST (Independent Administrative Organization, National Institute of Advanced Industrial Science and Technology). The rat bone marrow cells were obtained from the bone shafts of the femora of male, 6-week-old Fisher 344 rats. Preparation and osteogenic differentiation of the rat MSCs were as described by Maniatopoulos *et al*. (*1*) and modified by Ohgushi *et al*. (*3*). In brief, rat bone marrow was 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 (100 units/ml penicillin, 0.1 mg/mL streptomycin and 0.25 μg/ml amphotericin B; Sigma-Aldrich Co., CA, USA). The bone marrow cells were cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. Adherent cells from rat bone marrow cells were initially cultured up to 80% confluence in T-75 flasks (Becton, Dickinson and Company (BD), NJ, USA). The adherent cells were released from the substrates using 0.05% Trypsin/0.53 mM EDTA (P-1; passage-1) and were seeded into 12-well TCPS dishes at a cell density of 1×10^4 cells/cm^2 (for the hFbs: 5×10^3 cells/cm^2), or into titanium dishes placed in 12-well plates. The culture was in osteogenic media containing 10 nM Dex (Sigma-Aldrich Corporation, MO, USA), 10 mM β-glycerophosphate (Merck, Darmstadt, Germany) and 0.28 mM ascorbic acid 2-phosphate magnesium salt *n*-hydrate (Sigma-Aldrich Corp.). The medium was changed three times per week during the culture period. The cells were counted by a NucleoCounter® (ChemoMetec, Allerød, Denmark). The P-1 cells were also cultured in MEM with FBS until 80% confluency in T-75 flasks and the passages were repeated three times (P-3; passage-3). The P-3 cells were cultured in the osteogenic media on both substrates.

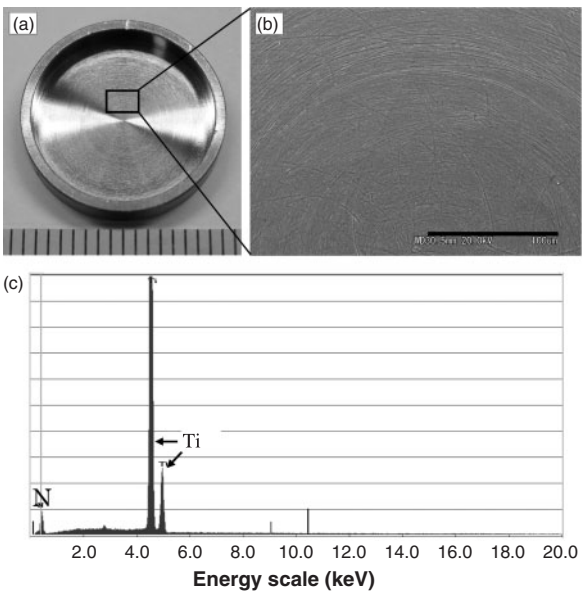

Fig. 1. **Surface characterization of titanium dishes.** (a) Macroscopic view of titanium dish (b) SEM image and (c) an EDX pattern on culture surface of a titanium dish. Bar: 100 μm.

Observation of Cell Culture—The cell proliferation and osteogenic differentiation on the TCPS dishes were observed by a phase-contrast microscope (Model IX70; Olympus Optical, Tokyo, Japan) for 14 days. Extracellular mineralization was observed by a fluorescence microscope (Model IX70), as described later. Observation of the cell culture on the titanium dishes was assessed with a combination dye (LIVE/DEAD Viability Assay Kit; Molecular Probes) based on a simultaneous determination of living and dead cells with two probes, calcein-bis [(acetyloxy) methyl] ester (calcein-AM) for intracellular esterase activity and ethidium homodimer-1 (EthD-1) for plasma membrane integrity (*21*). The cells cultured on titanium dishes for 3, 7 and 14 days were washed with calcium/magnesium-free phosphate-buffered saline (PBS(−); Invitrogen), and the working solution (2 mM calcein-AM and 5 mM EthD-1 in PBS(−)) was added to the cultures. After 10 min incubation at 37°C, the stained cells were observed under objective fluorescence microscopy (Model SZX12; Olympus).

Fluorescence Image Analyses of Extracellular Mineralization—Calcein (1 μg/ml) was added to the osteogenic media for the entire culture periods. Immediately before the analyses, the medium containing calcein was removed and the sample was washed with PBS(−). The fluorescence of calcein incorporated into the

extracellular mineral ingredients was visualized with an image analyser (Typhoon 8600; 526 nm short-pass filter; Amersham Biosciences, NJ, USA). The scanned fluorescence images were quantified with ImageQuant TL software Ver. 5.1 (Amersham Biosciences) and represented as fluorescence intensity per culture well. The results were transferred to Excel (Microsoft, WA, USA) and calculated as the pixel intensities per selected area. The calculated fluorescence intensity values were referred as to calcein fluorescence intensity (calcein F. I.) in this article. Extracellular mineralization was also observed with an objective fluorescence microscope (Model SZX12, Olympus). Measurement of calcein F. I. of the MSCs cultured on the titanium dishes was performed as shown in Fig. 2 after turning the dish upside down.

Calcium Staining by Alizarin Red S—The MSCs on both substrates (TCPS dishes and titanium 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(−) solution for 10 min at room temperature and washed with water to remove the remaining stains. After staining, the specimens were observed by an objective microscope (Model SZX12).

Measurement of Calcium Concentration—After quantification of the calcein uptake by using an image analyser, the cells were washed with PBS(−) and then 0.5 ml of 20% formic acid was added. The samples were stored at room temperature for 3 h. The acid-hydrolyzed samples were diluted to 1.0 ml with water. The calcium contents of the diluted samples were measured using an inductively coupled plasma atomic emission spectrometer (SPS7800 plasma spectrometer; Seiko Instruments Inc., Chiba, Japan).

Quantification of Osteocalcin Deposited on Cell Layers—After measurement of the calcium contents, remnants of the samples in 10% formic acid were centrifuged at 20 000 × *g* for 10 min. An aliquot (0.5 ml) of the formic acid extract was applied to a column of NAP-5 (Sephadex G-25 DNA grade, Amersham Bioscience, Uppsala, Sweden) and was eluted with 1.0 ml of 10% formic acid. Gel-filtered samples were evaporated to concentrate and prepared for the assay of intact rat osteocalcin as previously described (*22*). We utilized two antibodies that recognize the N- and C-terminal amino acid regions of rat osteocalcin for an enzymatic immunoassay (Rat Osteocalcin EIA Kit, No. BT-490; Biomedical Technologies Inc., MA, USA). Purified rat osteocalcin was used for a standard and a positive control.

Statistical Evaluation—The correlation between the calcein F. I. and the amount of bone matrices (calcium and osteocalcin) was tested by Pearson's correlation coefficient. The significance of the correlation coefficient was followed by Fisher's *r* to *z*-test (*P*-value). These data were analysed using the StatView J Version 4.5 (SAS Institute, Cary, NC).

RESULTS

Proliferation and Differentiation of Rat MSCs—Under osteogenic conditions, Dex induces undifferentiated MSCs into osteoblasts that produce mineralized matrices as we previously reported (*3*). During 14 days of culture, the MSCs cultured on TCPS dishes formed a calcium deposit stained with calcein (Fig. 3c and d). In contrast, calcein deposition was never observed for hFbs (Fig. 3g and h). Regardless of the differentiation, both the MSCs and hFBs showed good adhesion and proliferation on the TCPS dishes (Fig. 3a–c and e–g). As shown in Fig. 4, cell adhesion, proliferation (viability) and differentiation of MSCs on the titanium dishes were observed by objective fluorescence microscopy. Because titanium dishes are not transparent, cultured cells are usually never observed under light microscopy. To visualize the cells under microscopy, LIVE/DEAD staining was used. In this method, cell-permeable calcein AM enters the cell plasma to be cleaved into calcein and AM by intracellular

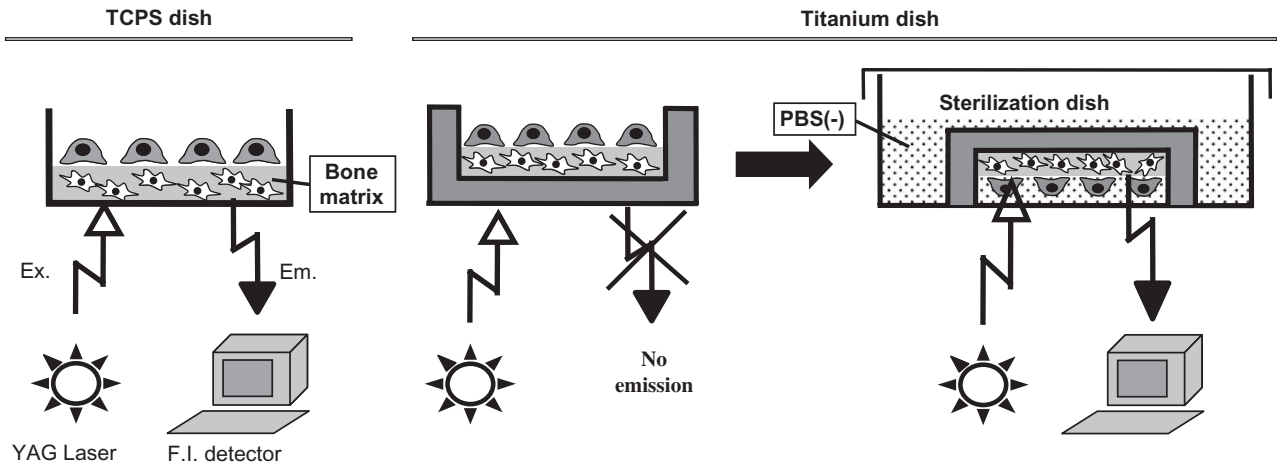

Fig. 2. **Schematic representation of procedures for measurement of fluorescence emission.** During the culture of rat MSCs and hFbs, calcein calcium-binding dye was added to the culture medium. The emission from the calcein deposited on the mineralized matrices in the TCPS dish can be detected due to the transparency of the TCPS dish (left figure). However, the titanium dish is opaque, and fluorescence emission cannot be generated under ordinary condition (middle figure). Therefore, the titanium dish was put upside down in another dish (right figure).

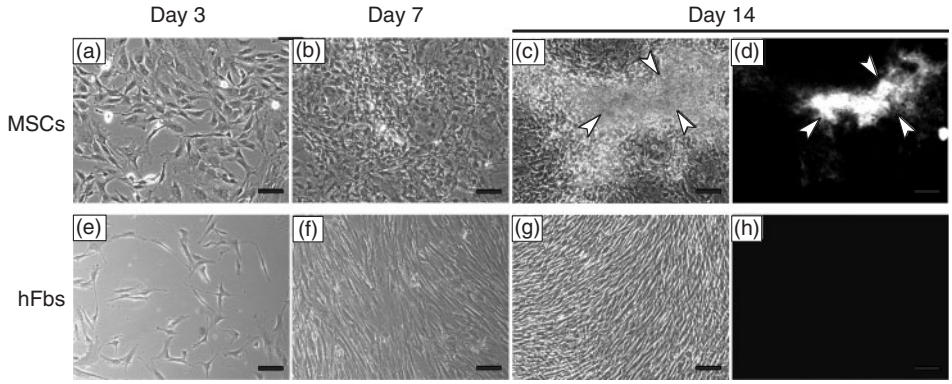

Fig. 3. **Observation of rat MSCs and hFbs with calcein seeded on TCPS dishes.** Both rat MSCs and hFbs were seeded on TCPS dishes. The cells were cultured for 14 days in osteogenic media containing calcein. The cultures were observed with a phase-contrast microscope (a–c and e–g), or a fluorescence microscope (d and h). The rat MSCs and hFbs showed good adhesion and proliferation from day 3 to day 14 of culture (a–c and e–g). Mineralization with calcein deposition (green emission) was confirmed by fluorescent microscopy on 14 days of culture (d; arrowhead). In contrast, hFbs showed no mineralization (h). Bar: 100 μm. (Original magnification: ×100).

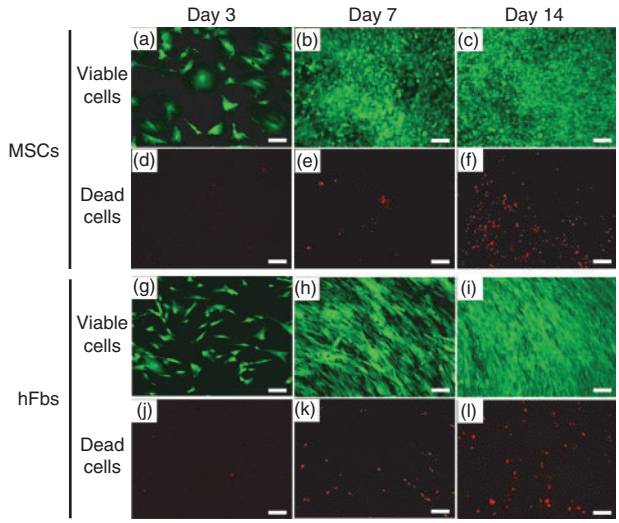

Fig. 4. **Visualization of rat MSC and hFb cultured cells on titanium dishes by LIVE/DEAD staining.** MSCs (a–f) and hFbs (g–l) were seeded on titanium dishes. The cells were cultured in osteogenic media without calcein for 14 days. The cells were observed with an objective fluorescence microscope after treatment with LIVE/DEAD staining at the culture days indicated. Cytoplasm of the viable cells (green emission, a–c and g–i) and the nuclei of the dead cells (red emission, d–f and j–l) are shown. MSCs as well as hFbs adhered/proliferated on the titanium surface and reached confluency at day 7 of culture (a, b, g and h). At day 14 of culture, MSCs formed mineralized nodular aggregates (c). The high cell viability of both rat MSCs and hFbs were shown throughout the culture periods. Bar: 100 μm. (Original magnification: ×100).

esterase of living cells. The free calcein shows a green emission with a fluorescence microscope. On the titanium dishes, both types of cells showed good cell adhesion accompanied by cell spreading (Fig. 4a and g). Subsequently, MSCs cultured on TCPS dishes as well as titanium dishes showed a similar pattern of proliferation, and reached confluency at day 7 (Figs. 3b and 4b). The hFbs also showed good cell adhesion and proliferated to reach confluency at day 7 (Figs. 3f and 4h). At day 14, the MSCs cultured on both substrates formed nodular aggregates (Figs. 3c, d and 4c) to show mineralization. In contrast, the hFbs did not exhibit these aggregates (Figs 3g, h and 4l). As seen in Fig. 4e, f, k, and l, most cells on both culture substrates appeared viable and only a few cells appeared dead.

Visualization of Extracellular Mineralized Matrix of Rat MSCs by Fluorescence Staining—We obtained the calcein fluorescence images of rat MSCs cultured on TCPS dishes and titanium dishes by using an image analyser. The visualized fluorescence images were compared with Alizarin Red S staining. On both substrates, calcein deposited on the mineralized regions appeared to be a green fluorescent emission (Fig. 5d and h), and objective microscopy showed the mineralized nodules surrounded by the MSCs to be stained with Alizarin Red S (Fig. 5b and f). The results confirmed that calcein was specifically incorporated and deposited into the extracellular bone matrices as evidenced by the costaining with Alizarin Red S (Fig. 5).

Correlation between Calcein Uptake and Contents of Extracellular Matrix Components of Calcium and Osteocalcin—The relationship between calcein F. I. calculated from a scanning image and the bone matrices fabricated by MSCs was evaluated by measuring the calcium content and the osteocalcin content. The MSCs cultured on TCPS dishes in osteogenic media were cultured for 7–35 days in the presence of calcein. The calcein F. I. values increased with the calcium and osteocalcin contents, and the data showed a strong correlation between calcein F. I. and extracellular bone matrices (Fig. 6). MSCs cultured on titanium dishes in osteogenic media were cultured for 5–28 days in the presence of calcein. As shown in Fig. 7, there were also strong correlations between calcein F. I. and extracellular bone matrices for MSCs cultured on titanium dishes.

Real-time Monitoring of Extracellular Mineralized Matrix Formation—Real-time monitoring of the amount of mineral deposition was performed by using an image analyser with visualization of the degree of fluorescence

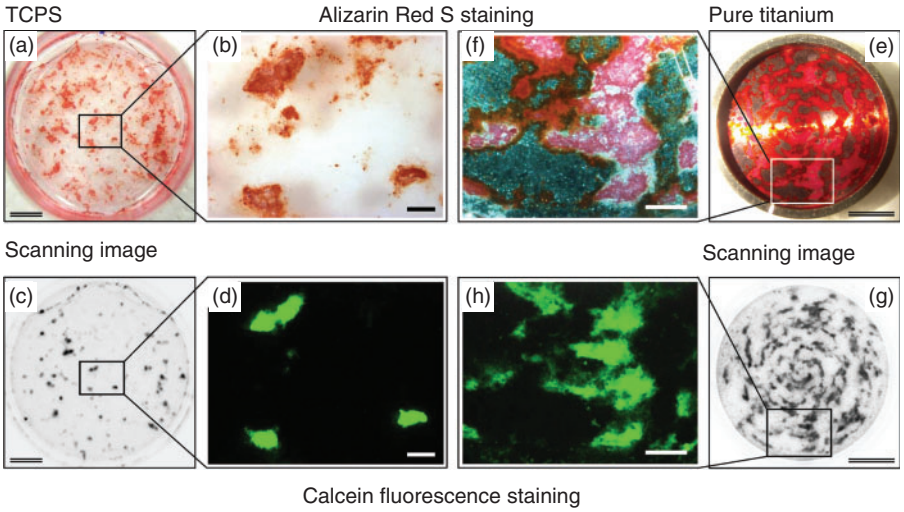

Fig. 5. **Alizarin Red S staining and fluorescence emission of mineralized matrices of rat MSCs cultured on TCPS dishes and titanium dishes.** The rat MSCs were cultured on TCPS dishes (a–d) for 13 days or on titanium dishes (e–h) for 16 days in osteogenic media with calcein. The cells were washed with PBS(−) and observed with an image analyser (c and g) or with an objective fluorescence microscope (d and h). After observation with the image analyser, the cells were stained with Alizarin Red S and observed with an objective microscope (a, b, e and f). The magnification of panels b, d, f and h are higher than those of a, c, e and g indicated in the rectangles, respectively. The objective micrographs of panels b and f show the same objective fluorescence microscopic regions of panels d and h, respectively. Bar: 1 mm. Double bar: 5 mm.

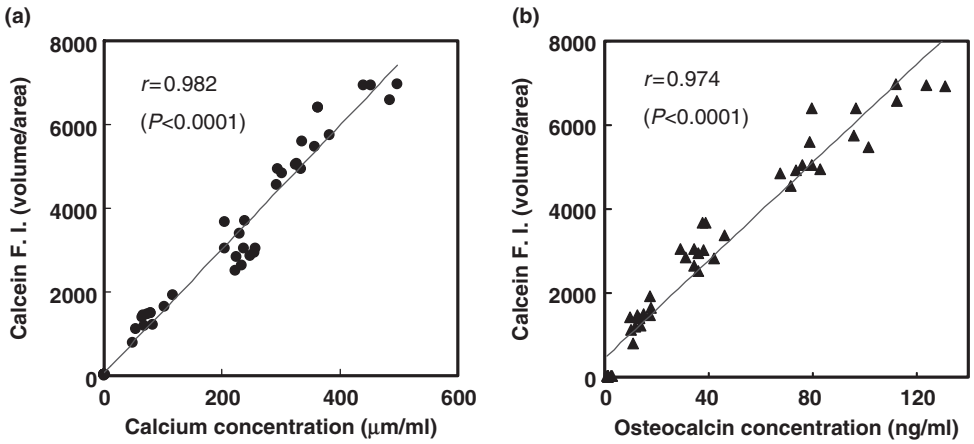

Fig. 6. **Correlation between calcein uptake and contents of extracellular matrix components of calcium and osteocalcin of rat MSCs cultured on TCPS dishes.** The rat MSCs were cultured on TCPS dishes for 35 days in osteogenic media containing calcein. At each culture period of day 7, 14, 18, 21 and 35, calcein F. I. of rat MSCs was calculated. After calculation of calcein F. I. at each culture period, the calcium contents and osteocalcin contents were measured using the same samples. Calcein F. I. was plotted against the calcium contents (a) or the osteocalcin contents (b) of the corresponding cells. The total sample number is 48.

intensity of the calcein. Time-course images of MSCs cultured on titanium dishes are shown in Fig. 8. Areas of mineralized matrices on the titanium dishes are seen as black spots. In the case of P-1 MSCs (Fig. 8a–f), black spots appeared around 11 days of culture, and these areas increased in a time-dependent manner. In the case of P-3 MSCs (Fig. 8g–l), mineralization started around 14 days of culture, but the level of mineralization was very low compared with that of P-1 MSCs. In contrast, no black spots appeared during the entire culture period for hFbs (Fig. 8m–r). The calcein F. I. value increased from 11 days of culture in P-1 MSCs, while the value for P-3 MSCs increased only slightly (Fig. 9). In contrast, calcein F. I. of hFbs stayed at the base line throughout the culture period (Fig. 9).

DISCUSSION

Various materials have been used as cell culture scaffolds for supporting *in vitro* osteogenic properties of cells (*23–26*). For bone regeneration, we confirmed that rat and human MSCs derived from bone marrow could differentiate into active osteoblasts, which produced bone matrices on bioactive ceramics such as β-tricalcium phosphate and hydroxyapatite under culture conditions with Dex (*27–29*). Furthermore, we demonstrated that

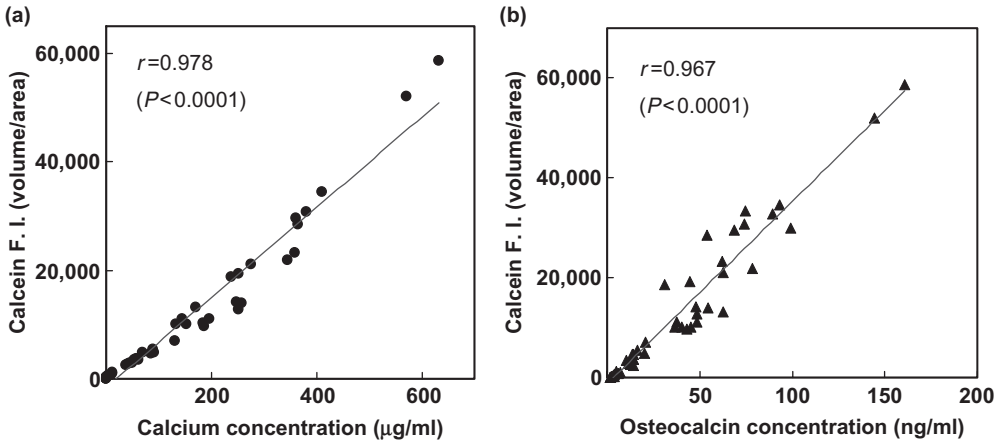

Fig. 7. **Correlation between calcein uptake and contents of extracellular matrix components of calcium and osteocalcin of rat MSCs cultured on titanium dishes.** The MSCs were cultured on titanium dishes for 28 days in osteogenic media containing calcein. At each culture period of day 5, 8, 11, 14, 18, 21 and 28, calcein F. I. of rat MSCs was calculated. After calculation of calcein F. I. at each culture period, the calcium contents and osteocalcin contents were measured using the same samples. Calcein F. I. was plotted against the calcium contents (a) or the osteocalcin contents (b) of the corresponding cells. The total sample number is 44.

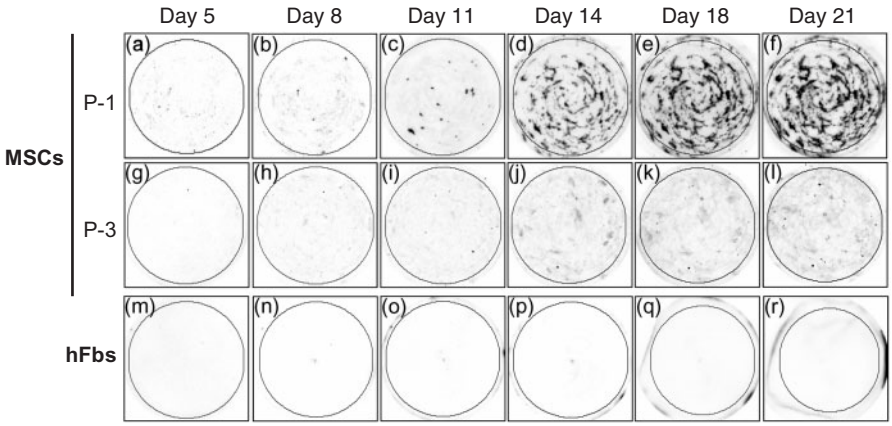

Fig. 8. **Real-time fluorescence monitoring of mineralized matrices formed by rat MSCs.** The rat MSCs (passage-1: P-1 and passage-3: P-3) and hFbs were cultured on titanium dishes for 21 days in osteogenic media containing calcein. After the cultures were washed with PBS(−), the entire titanium surfaces were visualized with an image analyser at each culture day indicated. The rat MSCs of P-1 fabricated mineralized matrices shown as black spots from day 11 of culture; the black spots increased as the culture days progressed (a–f). The rat MSCs of P-3 showed weak mineralization levels throughout the culture periods (g–l). In contrast, hFbs showed no mineralization (m–r).

human MSCs could show *in vitro* osteogenic differentiation and bone formation on bioinert alumina ceramics. Similar to alumina ceramics, titanium is well known as a bioinert material (*10*, *30*). However, quantitative analyses of bone formation by cultured cells throughout the duration of osteogenic differentiation have not been performed on titanium surfaces. In the present study, we tried to monitor the cell behaviour of rat MSCs cultured on titanium surfaces and confirm whether rat MSCs could show osteogenic differentiation on titanium surfaces by quantitative analyses.

In this study, cell behaviours including cell adhesion, cell proliferation and osteogenic differentiation on titanium surfaces were compared with those on TCPS dishes. TCPS dishes are fabricated from crystal-grade polystyrene by using a vacuum-gas plasma treatment. Since the treatment introduces carboxyl and hydroxyl groups onto their surfaces, the TCPS surfaces show slightly hydrophilic characteristics. Under appropriate culture conditions, various types of cultured cells can adhere and proliferate on the surfaces. For this reason, TCPS dishes are commonly used as cell culture substrates. Consequently, TCPS dishes were used in the present study as positive controls. Observation of cell behaviours might be important for clarifying cell/material interactions. Cell behaviours including cell adhesion and cell proliferation are usually observed by light microscopy. However, observation of cells cultured on titanium surfaces is difficult by light microscopy since the titanium is opaque. In order to visualize cells cultured on titanium surfaces, we stained the cells with fluorescent materials, LIVE/DEAD reagents, which are

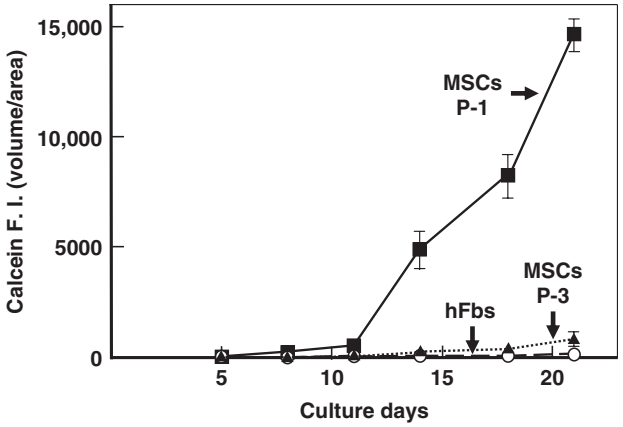

Fig. 9. **Change in fluorescent quantity of rat MSCs with time**. The rat MSCs of P-1 (filled square), P-3 (filled triangle) and hFbs (open circle) were cultured on titanium dishes for 21 days in osteogenic media containing calcein. Calcein F. I. was measured with an image analyser at the culture days indicated. Each symbol represents the mean value ± SD of calcein F. I. taken from 5 cultures.

used for assessing cellular viability. After staining the cells, we could clearly observe the cells on titanium surfaces by fluorescence microscopy during the culture periods (Fig. 4). As shown in Figs. 3 and 4, titanium surfaces exhibited cellular adhesive and proliferative characteristics comparable with those of TCPS dishes. As observed by SEM, the surface of titanium is very smooth, similar to TCPS surfaces (Fig. 1). Cell adhesion molecules could be considered the reason that titanium surfaces showed high cellular affinity. Although further experiments are required to clarify the mechanisms of the excellent properties of titanium surfaces, extracellular matrix proteins such as collagen and fibronectin in serum or produced by the cells are thought to be the principal factors promoting cellular adhesion and spreading on the substrates. The degree of cell adhesion and cell proliferation on titanium dishes was similar to that on TCPS dishes (Figs. 3 and 4). The results suggest that the surface properties of titanium are almost equivalent to those of TCPS dishes, which results in the capability of supporting cellular adhesion and proliferation.

For evaluating the *in vitro* osteogenic differentiation of cultured cells, quantitative measurement (protein synthesis or gene expression) (*18*, *31*) and qualitative staining (immunochemical staining or chemical staining) have usually been used. The measurement of calcium content is also important, since calcium is the main component of extracellular bone matrix (*6*, *32*). As shown in Figs. 3, 4 and 5, we demonstrated that the MSCs differentiated into osteoblasts, which formed mineralized bone matrices on both the TCPS and titanium surfaces. MSCs maintained high viability on the titanium dishes throughout culture periods by assessment of LIVE/DEAD staining (Fig. 4). As shown in Figs. 3 and 5, a green fluorescent emission was seen when the MSC culture was done in the presence of calcein. The fluorescent areas were also stained with Alizarin Red S (Fig. 5), confirming that calcein was incorporated into the regions corresponding to the calcium of the extracellular matrices (Fig. 5F and H). When the MSCs were seeded on the titanium dishes, mineralization as evidenced by calcein uptake occurred on the titanium dishes as well as on the TCPS dishes (Figs. 3D, 5D and H). In addition, calcein F. I. values accurately reflected the amount of mineralized matrices formed by the MSCs because there were strongly positive correlations among the calcein F. I., calcium content and osteocalcin content (Fig. 7). The results also showed the capabilities of titanium in supporting the *in vitro* osteogenic differentiation of rat MSCs.

We also performed real-time monitoring of mineralization by cultured cells for the duration of the culture periods (Fig. 8). In addition, quantification of calcein F. I., which reflects osteogenic capability, could be done among different cells, as shown in Fig. 9. Since rat MSCs have the characteristic of showing decreased osteogenic capability after repeated passages (*33*), we monitored the mineralization by the cells at P-1, P-3 and the control cells of fibroblasts (hFbs). As shown in Fig. 8, we could easily compare the mineralization levels among the cells having different levels of osteogenic capability in a time-dependent manner and demonstrated the decreased capability of mineralization after passage.

In conclusion, we evaluated the efficiency of titanium surfaces used as bone regeneration scaffolds using rat MSCs. Cell adhesion, proliferation and osteogenic differentiation of rat MSCs on titanium dishes were significantly similar to those on TCPS dishes. Therefore, titanium is an excellent material for hard tissue repair for use in the field of bone tissue engineering.

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REFERENCES

1. Maniatopoulos, C., Sodek, J., and Melcher, A.H. (1988) Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res.* **254**, 317–330
2. Leboy, P.S., Beresford, J.N., Devlin, C., and Owen, M.E. (1991) Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. *J. Cell Physiol.* **146**, 370–378
3. Ohgushi, H., Dohi, Y., Yoshikawa, T., Tamai, S., Tabata, S., and Suwa, Y. (1996) *In vitro* bone formation by rat marrow cell culture. *J. Biomed. Mater. Res.* **32**, 333–340
4. Kotobuki, N., Ioku, K., Kawagoe, D., Fujimori, H., Goto, S., and Ohgushi, H. (2005) Observation of osteogenic differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite. *Biomaterials* **26**, 779–785
5. Kotobuki, N., Kawagoe, D., Nomura, D., Katou, Y., Muraki, K., Fujimori, H., Goto, S., Ioku, K., and Ohgushi, H. (2006) Observation and quantitative analysis of rat bone marrow stromal cells cultured *in vitro* on newly formed transparent beta-tricalcium phosphate. *J. Mater. Sci. Mater. Med.* **17**, 33–41
6. Kitamura, S., Ohgushi, H., Hirose, M., Funaoka, H., Takakura, Y., and Ito, H. (2004) Osteogenic differentiation of human bone marrow-derived mesenchymal cells cultured on alumina ceramics. *Artif. Organs* **28**, 72–82
7. Datta, N., Pham, Q. P., Sharma, U., Sikavitsas, V.I., Jansen, J.A., and Mikos, A.G. (2006) *In vitro* generated extracellular matrix and fluid shear stress synergistically

enhance 3D osteoblastic differentiation. *Proc. Natl. Acad. Sci. USA* **103**, 2488–2493
8. Khadra, M., Lyngstadaas, S.P., Haanaes, H.R., and Mustafa, K. (2005) Effect of laser therapy on attachment, proliferation and differentiation of human osteoblast-like cells cultured on titanium implant material. *Biomaterials* **26**, 3503–3509
9. Beloti, M.M., Bellesini, L.S., and Rosa, A.L. (2005) The effect of purmorphamine on osteoblast phenotype expression of human bone marrow mesenchymal cells cultured on titanium. *Biomaterials* **26**, 4245–4248
10. Ohgushi, H. and Caplan, A.I. (1999) Stem cell technology and bioceramics: from cell to gene engineering. *J. Biomed. Mater. Res.* **48**, 913–927
11. Mendes, S.C., Tibbe, J.M., Veenhof, M., Bakker, K., Both, S., Platenburg, P.P., Oner, F.C., De Bruijn, J.D., and Van Blitterswijk, C.A. (2002) Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture condition and donor age. *Tissue Eng.* **8**, 911–920
12. de Bruijn, J.D., van den Brink, I., Mendes, S., Dekker, R., Bovell, Y.P., and van Blitterswijk, C.A. (1999) Bone induction by implants coated with cultured osteogenic bone marrow cells. *Adv. Dent. Res.* **13**, 74–81
13. Nishikawa, M., Myoui, A., Ohgushi, H., Ikeuchi, M., Tamai, N., and Yoshikawa, H. (2004) Bone tissue engineering using novel interconnected porous hydroxyapatite ceramics combined with marrow mesenchymal cells: quantitative and three-dimensional image analysis. *Cell Transplant.* **13**, 367–376
14. Ohgushi, H., Kotobuki, N., Funaoka, H., Machida, H., Hirose, M., Tanaka, Y., and Takakura, Y. (2005) Tissue engineered ceramic artificial joint—*ex vivo* osteogenic differentiation of patient mesenchymal cells on total ankle joints for treatment of osteoarthritis. *Biomaterials* **26**, 4654–4661
15. Morishita, T., Honoki, K., Ohgushi, H., Kotobuki, N., Matsushima, A., and Takakura, Y. (2006) Tissue engineering approach to the treatment of bone tumors: three cases of cultured bone grafts derived from patients' mesenchymal stem cells. *Artif. Organs.* **30**, 115–118
16. Ohgushi, H., Kitamura, S., Kotobuki, N., Hirose, M., Machida, H., Muraki, K., and Takakura, Y. (2004) Clinical application of marrow mesenchymal stem cells for hard tissue repair. *Yonsei Med. J.* **45**, 61–67
17. Matsushima, A., Kotobuki, N., Machida, H., Morishita, T., Takakura, Y., and Ohgushi, H. (2007) In vivo osteogenic potential of mesenchymal stromal cells derived from patient's bone marrow on hydroxyapatite ceramics. *Key Eng. Mat.* **330**, 1157–1160
18. Uchimura, E., Machida, H., Kotobuki, N., Kihara, T., Kitamura, S., Ikeuchi, M., Hirose, M., Miyake, J., and Ohgushi, H. (2003) *In-situ* visualization and quantification of mineralization of cultured osteogenetic cells. *Calcif. Tissue Int.* **73**, 575–583
19. Albrektsson, T., Branemark, P.I., Hansson, H.A., and Lindstrom, J. (1981) Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone-to-implant anchorage in man. *Acta. Orthop. Scand.* **52**, 155–170
20. Wittwer, G., Adeyemo, W.L., Yerit, K., Voracek, M., Turhani, D., Watzinger, F., and Enislidis, G. (2006) Complications after zygoma fracture fixation: is there a difference between biodegradable materials and how do they compare with titanium osteosynthesis? *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **101**, 419–425
21. Papadopoulos, N.G., Dedoussis, G.V., Spanakos, G., Gritzapis, A.D., Baxevanis, C.N., and Papamichail, M. (1994) An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. *J. Immunol. Methods* **177**, 101–111
22. Dohi, Y., Ohgushi, H., Tabata, S., Yoshikawa, T., Dohi, K., and Moriyama, T. (1992) Osteogenesis associated with bone gla protein gene expression in diffusion chambers by bone marrow cells with demineralized bone matrix. *J. Bone Miner. Res.* **7**, 1173–1180
23. Ohgushi, H. and Okumura, M. (1990) Osteogenic capacity of rat and human marrow cells in porous ceramics. *Acta. Orthop. Scand.* **61**, 431–434
24. Okumura, M., Ohgushi, H., and Tamai, S. (1991) Bonding osteogenesis in coralline hydroxyapatite combined with bone marrow cells. *Biomaterials* **12**, 411–416
25. Goshima, J., Goldberg, V.M., and Caplan, A.I. (1991) Osteogenic potential of culture-expanded rat marrow cells as assayed in vivo with porous calcium phosphate ceramic. *Biomaterials* **12**, 253–258
26. Yoshikawa, T., Ohgushi, H., Uemura, T., Nakajima, H., Ichijima, K., Tamai, S., and Tateisi, T. (1998) Human marrow cells-derived cultured bone in porous ceramics. *Biomed. Mater. Eng.* **8**, 311–320
27. Ohgushi, H., Dohi, Y., Yoshikawa, T., Tamai, S., Tabata, S., Okunaga, K., and Shibuya, T. (1996) Osteogenic differentiation of cultured marrow stromal stem cells on the surface of bioactive glass ceramics. *J. Biomed. Mater. Res.* **32**, 341–348
28. Ohgushi, H., Okumura, M., Tamai, S., Shors, E.C., and Caplan, A.I. (1990) Marrow cell induced osteogenesis in porous hydroxyapatite and tricalcium phosphate. *J. Biomed. Mater. Res.* **24**, 1563–1570
29. Ohgushi, H., Yoshikawa, T., Nakajima, H., Tamai, S., Dohi, Y., and Okunaga, K. (1999) Al2O3 doped apatite-wollastonite containing glass ceramic provokes osteogenic differentiation of marrow stromal stem cells. *J. Biomed. Mater. Res.* **44**, 381–388
30. Goto, T., Kajiwara, H., Yoshinari, M., Fukuhara, E., Kobayashi, S., and Tanaka, T. (2003) In vitro assay of mineralized-tissue formation on titanium using fluorescent staining with calcein blue. *Biomaterials* **24**, 3885–3892
31. Holtorf, H.L., Jansen, J.A., and Mikos, A.G. (2005) Ectopic bone formation in rat marrow stromal cell/titanium fiber mesh scaffold constructs: effect of initial cell phenotype. *Biomaterials* **26**, 6208–6216
32. van den Dolder, J., Vehof, J.W., Spauwen, P.H., and Jansen, J.A. (2002) Bone formation by rat bone marrow cells cultured on titanium fiber mesh: effect of in vitro culture time. *J. Biomed. Mater. Res.* **62**, 350–358
33. Sugiura, F., Kitoh, H., and Ishiguro, N. (2004) Osteogenic potential of rat mesenchymal stem cells after several passages. *Biochem. Biophys. Res. Commun.* **316**, 233–239