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Research Article

β-Tricalcium Phosphate Micron Particles Enhance Calcification of Human Mesenchymal Stem Cells *In Vitro*

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β-Tricalcium phosphate (β-TCP) micron particles whose diameters range from 1 μm to 10 μm have been recently developed, however, their biological effects remain unknown. We investigated the biological effects of β-TCP micron particles on proliferation, cytotoxicity, and calcification of human synovial mesenchymal stem cells (MSCs). MSCs were cultured without dexamethasone, β-glycerophosphate, or ascorbic acid. 1.0 mg/mL β-TCP micron particles inhibited proliferation of MSCs significantly and increased dead cells. In the contact condition, 0.1 mg/mL β-TCP micron particles promoted calcification of MSCs evaluated by alizarin red staining and enhanced mRNA expressions of runx2, osteopontin, and type I collagen. In the noncontact condition, these effects were not observed. 0.1 mg/mL β-TCP micron particles increased calcium concentration in the medium in the contact condition, while 1.0 mg/mL β-TCP micron particles decreased calcium and phosphorus concentrations in the medium in the noncontact condition. By transmission electron microscopy, β-TCP micron particles were localized in the phagosome of MSCs and were dissolved. In conclusion, β-TCP micron particles promoted calcification of MSCs and enhanced osteogenesis-related gene expressions *in vitro*.

1. Introduction

β-Tricalcium phosphate (β-TCP) blocks with suitable porous structure are an osteoconductive bone graft substitute [1–4]. β-TCP micron particles whose diameters range from 1 μm to 10 μm have been recently developed and have attracted attention because these particles are smaller than cells, and their biological effects seem to be different from those of larger particles [5]. However, the precise interaction between β-TCP micron particles and cells remains unknown.

Mesenchymal stem cells (MSCs) play important physiological roles in maintaining homeostasis and repairing damaged tissues including bone [6, 7]. When a bone is fractured, MSCs are mobilized to the site differentiate into osteoblasts, then the bone tissues heal completely [8, 9]. Modifications of MSCs have a possibility to improve the function of MSCs for bone formation [10, 11].

In this study, we investigated the biological effect of β-TCP micron particles on proliferation, toxicity, and calcification of MSCs when these particles were treated with MSCs.

We also performed morphological analysis on MSCs treated with β-TCP micron particles. Our results revealed for the first time the *in vitro* effect of β-TCP micron particles on MSCs.

2. Experimental Procedures

2.1. Preparation of β-TCP Micron Particles. To produce the micron particles, porous-type β-TCP block (Osferion, Olympus Terumo Biomaterials Corp, Tokyo, Japan), whose porosity was 75%, was crushed into granules by an alumina rod. The granules were filtered by stainless sieves for sorting into sizes less than 500 μm in diameter, and they were further filtered by air classifiers. The range of the particle sizes was measured by laser diffraction method using a laser particle sizer (Microtrac HRA, Nikkiso, Japan). The particles were dispersed in an ultrasonic bath for 1 minute. Triton X-100 and distilled water were used as dispersing agents. Morphological characteristics and chemical elements of the particles were observed and analyzed by scanning electron microscopy (S-4500, Hitachi, Hitachinaka, Japan), and energy-dispersive X-ray spectroscopy (EMAX-7000, Horiba Ltd., Kyoto, Japan). The crystal structure of the particles was examined by X-ray diffraction instrument (MiniFlex, Rigaku, Tokyo, Japan) using CuK_β radiation. The data were collected from 10° to 60° with a step size of 0.02°.

2.2. Isolation and Culture of Human Synovial MSCs. The study was approved by an institutional review board, and informed consents were obtained from all donors. Human synovium was harvested during knee operations. The synovium was minced into small pieces, digested with 3 mg/mL Collagenase D (Roche Diagnostics, Mannheim, Germany) for 3 hours, and cultured at a clonal density in complete culture medium (CCM: αMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 250 ng/mL amphotericin B (Invitrogen) for 14 days. Passage 2 MSCs were mainly used for the following analyses. Three days after replating, CCM was replaced with CCM supplemented with β-TCP micron particles. Then, the medium was changed with CCM without any β-TCP micron particles two times per week.

2.3. Colony-Forming Assays. One thousand cells at passage 2 were plated and cultured for 14 days in 60 cm^2 dishes. The cells were subsequently fixed with 4% paraformaldehyde, stained with 0.5% crystal violet (Wako, Osaka, Japan) for 5 minutes.

2.4. In Vitro Differentiation Assay. For chondrogenesis, 250,000 cells were placed in a 15 mL polypropylene tube (BD Falcon, Bedford, MA) and pelleted by centrifugation at 450 ×g for 10 minutes. The pellets were cultured for 21 days in chondrogenic medium, which contained 1000 ng/mL bone morphogenetic protein 7 (BMP-7) (Stryker Biotech, Boston, MA), 10 ng/mL transforming growth factor-β3 (TGF-β3) (R&D Systems Inc., Minneapolis, MN), and 10^{-7} M dexamethasone (Sigma-Aldrich, St. Louis, MO). For histological analysis, the pellets were embedded in paraffin, cut into 5 μm sections, and stained with 1% toluidine blue [12]. The sections were immunostained for type II collagen using a primary anti-rat monoclonal antibody against human type II collagen (1 : 200 dilution with PBS containing 1% BSA; Daiichi Fine Chemical, Toyama, Japan) as described previously [13].

For adipogenesis, the cells were cultured in the adipogenic medium that consisted of a CCM supplemented with 0.5 μM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), and 50 μM indomethacin (Wako). After 21 days, the adipogenic cultures were stained with 0.3% oil red-o solution.

For calcification, the cells were cultured in the calcification medium in the presence of 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid. After 3 weeks, the dishes were washed with PBS twice and fixed in 10% formalin for 5 minutes and stained with 2% alizarin red solution (pH 4.1; Sigma-Aldrich) for 2 minutes. The staining was used to detect calcium deposition in the extracellular matrix. For alkaline phosphatase (ALP) activity, MSCs were plated at 5000 cells/cm^2 in 6-well culture dishes and incubated in CCM and calcification medium. ALP activity was measured after 21 days of differentiation. The cells were harvested with lysis buffer (0.1 M Tris-HCl, 5 mM $MgCl_2$, 2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) and sonicated. Total protein concentrations of the supernatant were determined by the Bradford method (Bio-Rad, Hercules, CA). An aliquot (10 μL) of supernatant was added to 100 μL 50 mM p-nitrophenylphosphatase hexahydrate containing 1 mM $MgCl_2$, and the mixture was incubated at 37°C for 30 minutes. Absorption at 405 nm was measured with a spectrophotometer. The ALP activity per total protein represented millimoles of p-nitrophenol release after 30 minutes of incubation at 37°C.

2.5. Epitope Profile. Synovial MSCs at passage 2 were harvested 7 days after plating. One hundred thousand cells were suspended in 1000 μL FACS buffer (0.2% BSA and 0.09% sodium azide with PBS) containing 400 ng/mL phycoerythrin- (PE-) coupled antibodies against CD34 and CD90 (BD Bioscience, CA), APC-H7 coupled antibodies against CD44 (BD), PE-Cy7 coupled antibodies against CD45 (BD), and PerCP-Cy 5.5 against CD73 and CD105 (BD). As an isotype control, APC-H7, PE-Cy7, PerCP-Cy5.5, or PE-coupled nonspecific mouse IgG (BD) was substituted for the primary antibody. After incubation for one hour at 4°C, the cells were washed with PBS and resuspended in 1 mL FACS buffer for analysis. Cell fluorescence was evaluated by flow cytometry in a FACSVerse instrument (BD); data were analyzed by using FACSite software (BD).

2.6. Cell Proliferation. The proliferation of cells was evaluated by an MTT (3-(4,5-dimethylthiazol-2yl)-2,5-disphenyl-2H-tetrazolium-bromide) assay kit (Roche Diagnostics). MSCs were plated at 5000 cells/cm^2 in 96-well plates (Nalgene Nunc International, NY) with CCM supplemented with β-TCP micron particles at a concentration of 0, 0.01, 0.1, and 1.0 mg/mL. A 10 μL MTT solution was added into each well and incubated at 37°C in a 5% CO_2 humidified incubator.

After 4 hours, 100 μL dimethylsulfoxide was added to dissolve purple crystals. The next day, absorbance was determined at 560 nm by a microplate spectrophotometer (LS-PLATE Manager 2004, Wako).

2.7. Cell Viability. In the contact condition, MSCs were plated at 5000 cells/cm^2 in 6-well plates (BD) with CCM supplemented with β-TCP micron particles at a concentration of 0, 0.01, 0.1, and 1.0 mg/mL. In the noncontact condition, transwell inserts (Corning Incorporated Life Sciences, MA) consisting of 0.4 μm pores were used, and CCM supplemented with β-TCP micron particles was added in the inserts so that the MSCs and β-TCP micron particles became separated in the same culture medium. Two days after incubation, a working solution containing 2 μM Calcein AM and 4 μM ethidium homodimer-1 (EthD-1) (Live/Dead Viability/Cytotoxicity Kit for mammalian cells, Invitrogen) was added to each well. After incubation for 20 minutes at 37°C with 5% humidified CO_2, the labeled MSCs were observed by confocal microscopy (Olympus IX71, Olympus Corporation, Tokyo, Japan). Calcein AM-positive cells which refer to living cells and EthD-1-positive cells which refer to dead cells were counted in five high-power fields (at 200 times magnification), whose area was 500 × 700 μm.

2.8. Calcification. MSCs were plated at 5000 cells/cm^2 in 12-well plates with CCM supplemented with β-TCP micron particles at a concentration of 0, 0.01, and 0.1 mg/mL in both the contact and the noncontact condition. MSCs were also plated at 5000 cells/cm^2 in 12-well plates with CCM and calcification medium. Twenty-one days after incubation, cell cultures were stained with 2% alizarin red solution (pH 4.1; Sigma-Aldrich) for 2 minutes. Incubation with calcification medium was performed as a positive control. Pictures of these plates stained by alizarin red were taken by stereoscopic microscope (Olympus MVX10, Olympus Corporation) and light microscopy (Olympus IX71). The area of alizarin red positive staining was measured by Image J software (National Institutes of Health, Bethesda, MD). In the contact condition, the area of alizarin red positive staining was calculated by subtracting the area of alizarin red positive staining at 3 days from those of at 21 days in order to exclude staining of the β-TCP micron particles themselves.

2.9. Real-Time PCR Analysis. MSCs were plated at 5000 cells/cm^2 in 12-well plates (BD) with CCM supplemented with β-TCP micron particles at a concentration of 0, 0.01, and 0.1 mg/mL in both contact and noncontact condition. MSCs were plated at 5000 cells/cm^2 in 12-well plates with CCM and calcification medium. Twenty-one days after incubation, total RNA was isolated from cells with the RNeasy Total RNA Mini Kit (Qiagen, CA). cDNA was synthesized with oligo-dT primers from total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol. Reverse transcription (RT) was performed by 30 minutes of incubation at 55°C, followed by 5 minutes of incubation at 85°C. Real-time PCR was performed in a LightCycler 480 instrument (Roche Diagnostics) using FastStart TaqMan Probe Master and TaqMan probes for runx2 (no. 87), osteopontin (no. 61), type I collagen (no. 60), osteocalcin (no. 81), and β-Actin (no. 11) (Roche Diagnostics). After an initial denaturation step (95°C for 10 minutes), amplification was performed for 45 cycles (95°C for 15 seconds, 60°C for 60 seconds). Relative amounts of mRNA were calculated as previously described [14].

The primers were as follows:

runx2 (forward): 5′-gtgcctaggcgcatttca-3′

runx2 (backward): 3′-cacctgcctggctcttctta-5′

osteopontin (forward): 5′-cgcagacctgacatccagt-3′

osteopontin (backward): 3′-ggctgtcccaatcagaagg-5′

type I collagen (forward): 5′-aggtccccctggaaagaa-3′

type I collagen (backward): 3′-aatcctcgagcaccctga-5′

osteocalcin (forward): 5′-tgtgaaggcgtctcaaacag-3′

osteocalcin (backward): 3′-ctcacacacctccctcctg-5′

β-actin (forward): 5′-attggcaatgagcggttc-3′

β-actin (backward): 3′-tgaaggtagtttcgtggatgc-5′

2.10. Measurement of Calcium and Phosphorus Concentrations. MSCs were plated at 5000 cells/cm^2 in 12-well plates with CCM supplemented with β-TCP micron particles at a concentration of 0, 0.01, 0.1, and 1.0 mg/mL in both contact and noncontact condition. Culture medium was taken at 2, 7, 14, and 21 days, centrifuged to remove the particles, and the supernatant was measured for calcium and phosphorus concentration by using an automatically analytical instrument of LABOSPECT008K (Hitachi High-Technologies, Tokyo, Japan). For measurement of calcium concentration, a Clinimate CA test kit (Sekisui Medical, Tokyo, Japan) was used, and for phosphorus concentration, a Clinimate IP-2 (Sekisui Medical) was used.

2.11. Light Microscopy. MSCs were plated at 5000 cells/cm^2 in 6-well plates (BD Bioscience, CA) with CCM supplemented with β-TCP micron particles at a concentration of 0.1 mg/mL. During the culture period, the cell morphologies and β-TCP micron particles were observed by light microscopy (Olympus IX71).

2.12. Transmission Electron Microscopy. Ultrathin sections at 90 nm were collected on copper grids, double-stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy (H-7100, Hitachi). The specimens 7 days after incubation with CCM supplemented with β-TCP micron particles at a concentration of 0.1 mg/mL were analyzed.

2.13. Statistical Analysis. The StatView 5.0 program (SAS Institute, Cary, NC) was used for statistical analyses, and P values less than 0.05 were considered to be statistically significant. Comparisons between two groups were analyzed using the Paired t-test. Comparisons between multigroups

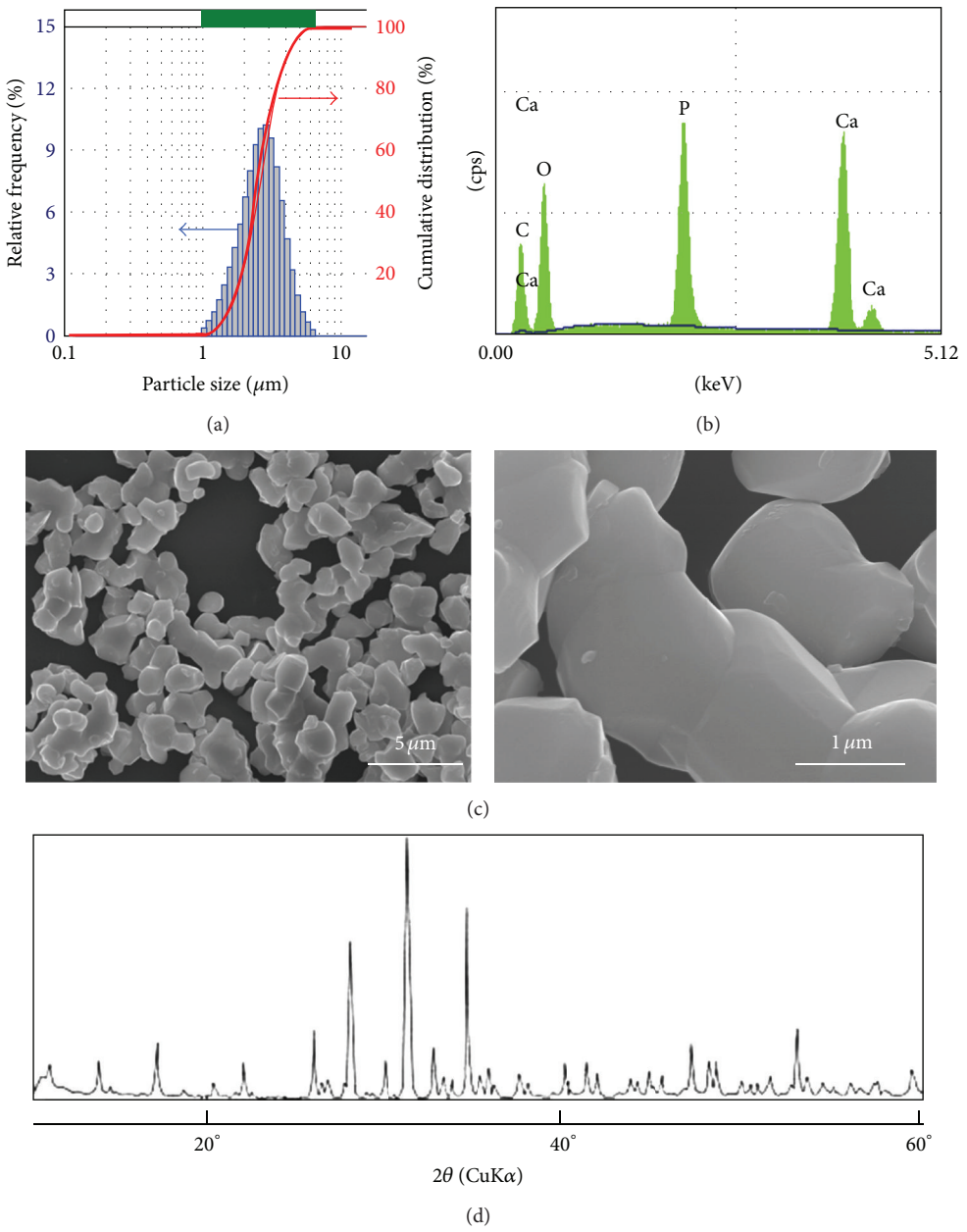

Figure 1: Structural properties of β-TCP micron particles. (a) Distribution of particle size (bar) and cumulative particle size (red line) by laser diffraction method. (b) Energy dispersive X-ray analysis. (c) Images of scanning electron microscopy. (d) X-ray diffraction analysis.

were analyzed using the Kruskal-Wallis test and the Scheffe test.

3. Results

3.1. Structural Properties of β-TCP Micron Particles. Particle sizes of β-TCP micron particles ranged from 1.06 μm to 6.54 μm in diameter, and the most frequent size was 3.00 μm with a percentage of 10% (Figure 1(a)). Fifty percent of the particles were smaller than 2.67 μm, which indicates D50 = 2.67 μm. Standard deviation (SD) was 0.93 μm, and D50/SD was 0.35. EDX analysis confirmed that phosphorus and calcium were present in the particles (Figure 1(b)). Scanning electron microscope showed connected particles which had smooth surfaces and whose diameter per unit was approximately 1 μm (Figure 1(c)). X-ray diffraction analysis of β-TCP micron particles showed that distraction peaks matched with those of the standard β-TCP (JCPDS09-169) (Figure 1(d)).

3.2. Characteristics of Synovial Cells as MSCs. Cells derived from human synovium formed colonies (Figure 2(a)). These cells differentiated into chondrocytes (Figure 2(b)), adipocytes (Figure 2(c)) and were calcified (Figure 2(d)).

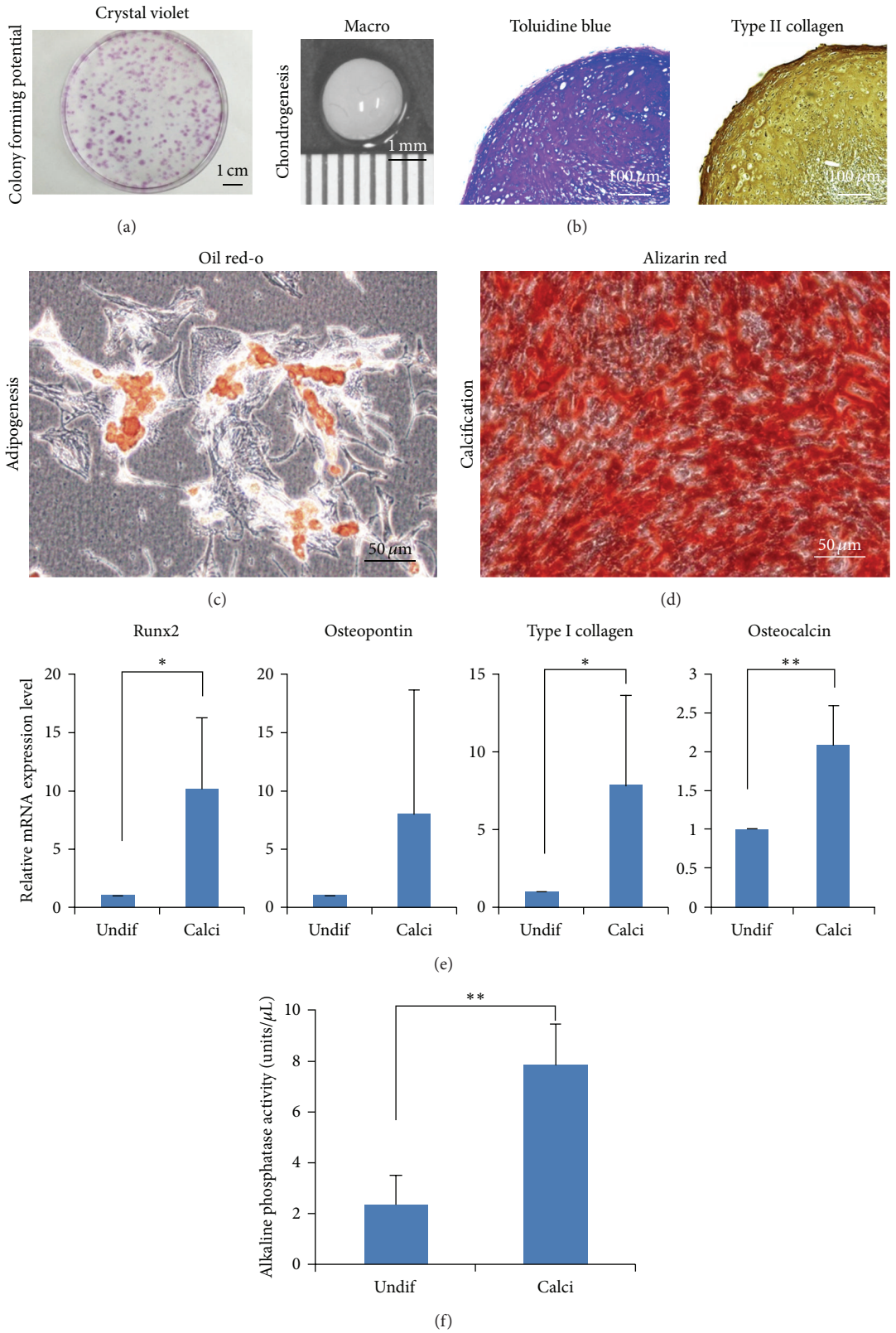

FIGURE 2: Continued.

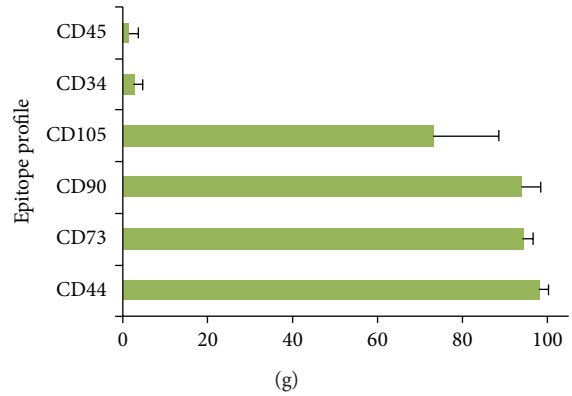

Figure 2: Characteristics of synovial MSCs. (a) Colony forming potential. (b) Chondrogenesis. (c) Adipogenesis. (d) Calcification. (e) Osteogenesis-related gene expressions by real-time PCR analysis. Averaged relative mRNA expression level with standard deviation is shown ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ by the paired t-test). Synovial MSCs cultured in calcification medium were compared with undifferentiated MSCs. (f) Averaged alkaline phosphatase activity with standard deviation ($n = 7$, $^{**}P < 0.01$ by the paired t-test). (g) Epitope profile of synovial MSCs. Positive expression rates (%) with standard deviation are shown ($n = 4$).

Expressions of osteogenesis-related genes, runx2, type I collagen, osteocalcin, and activity of alkaline phosphatase increased significantly when cultured in calcification medium (Figures 2(e) and 2(f)). Flow cytometric analysis demonstrated that the cells expressed CD44, CD73, CD90, and CD105 at a high level and CD34 and CD45 at a low level (Figure 2(g)). These results indicate that the cells derived from human synovium had characteristics of MSCs [15] and a potential for osteogenesis.

3.3. β-TCP Micron Particles Inhibit Proliferation of MSCs. MSCs were cultured with β-TCP micron particles in the contact or noncontact condition (Figure 3(a)). β-TCP micron particles at 1.0 mg/mL significantly inhibited proliferation of MSCs at 2, 7, and 14 days (Figure 3(b)). 1.0 mg/mL β-TCP micron particles increased the ratio of dead cells at 2 days in the contact condition (Figures 3(c) and 3(d)). A similar result was obtained in the noncontact condition (Figures 3(c) and 3(d)).

3.4. β-TCP Micron Particles Promote Calcification of MSCs. In the noncontact culture condition, β-TCP micron particles did not affect stainability with alizarin red (Figure 4(a)). In the contact condition, 0.1 mg/mL β-TCP micron particles already increased stainability with alizarin red at 3 days, and β-TCP micron particles stained red were observed both inside and outside of MSCs (Figure 4(b)). In the contact condition, 0.1 mg/mL β-TCP micron particles further increased the positively stained area with alizarin red at 21 days (Figures 4(a)–4(c)). 0.1 mg/mL β-TCP micron particles increased expression levels of runx2, osteopontin, and type I collagen significantly in the contact condition but not in the noncontact condition (Figure 4(d)).

3.5. β-TCP Micron Particles Affect Calcium and Phosphorus Concentrations in Culture Medium. In the contact condition, 0.1 mg/mL β-TCP micron particles increased calcium concentration in medium at 7, 14, and 21 days (Figure 5). In the noncontact condition, 1.0 mg/mL β-TCP micron particles decreased calcium and phosphorus concentrations in culture medium at 2, 7, 14, and 21 days.

3.6. β-TCP Micron Particles Are [Phagocyted] by MSCs. Microscopic analyses showed that β-TCP micron particles were still located in extracellular spaces at 30 minutes (Figure 6(a)). Many particles were contained in intracellular spaces, but some particles were still observed in extracellular spaces at 3 days. Most particles were contained in MSCs at 7 days, and MSCs containing particles formed multilayered at 21 days.

Transmission electron microscopic analyses revealed that β-TCP micron particles were located in the cytoplasm of MSCs at 7 days (Figure 6(b)). A magnified image showed no cytotoxic findings such as disruptions of the cell membrane, nuclear envelope, or cell organelle. A further magnified image demonstrated that the particles were contained in membrane which was called phagosome (open arrow). The phagosome was adjacent to lysosome (black arrow head) and lamellar body (white arrow head). The border of the particles was blurred (arrow).

4. Discussion

The effects of dosages and particle sizes of calcium phosphate have been reported in the past studies. Huang et al. reported macrophage released lactate dehydrogenase (LDH) when in contact with high concentrations of nanosized hydroxyapatite [16]. Contrary, Lange et al. reported that the release of LDH or TNF-α from macrophages was not altered among four different amounts of particles. They also reported that smaller particles, whose sizes were between 1 and 3 μm, did not increase release of LDH and TNF-α compared to those of larger ones [5]. All of these studies mentioned the effect of calcium phosphate particles from the standpoint of immunogenic responses and cytotoxicity through macrophages.

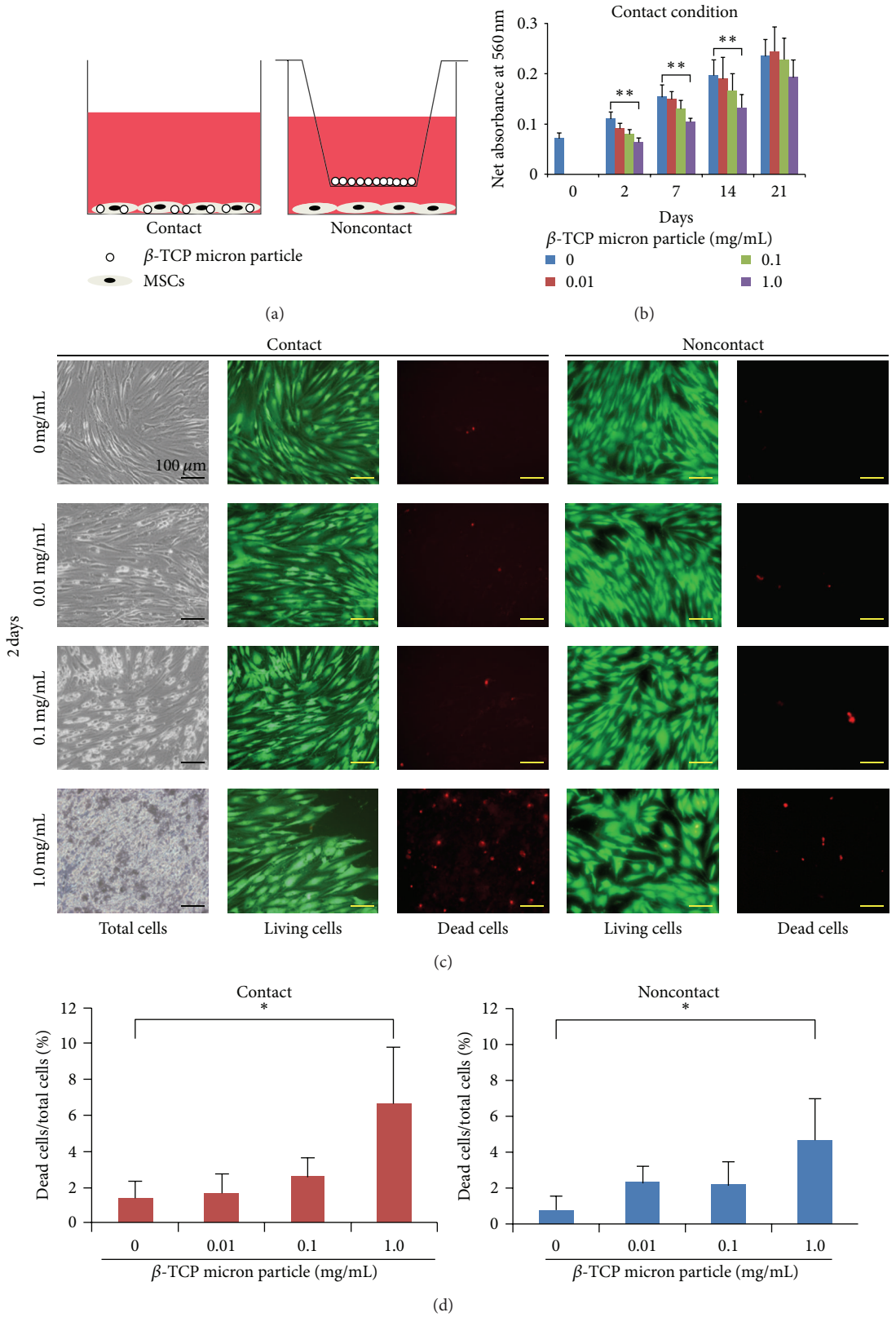

Figure 3: Effect of β-TCP micron particles on proliferation and cytotoxicity of MSCs. (a) Scheme for assays in the contact condition and the noncontact condition. (b) Proliferation. MSCs were cultured with complete culture medium (CCM) supplemented with various concentrations of β-TCP micron particles in the contact condition. Total cell number was measured by MTT assay. Averaged net absorbance with standard deviation is shown ($n = 7$, $^{**}P < 0.01$ by the Kruskal-Wallis test). (c) Morphology of total, living, and dead cells in the contact and noncontact condition. (d) The ratio of dead cells to total cells (%) with standard deviation ($^{*}P < 0.05$ by the Kruskal-Wallis test).

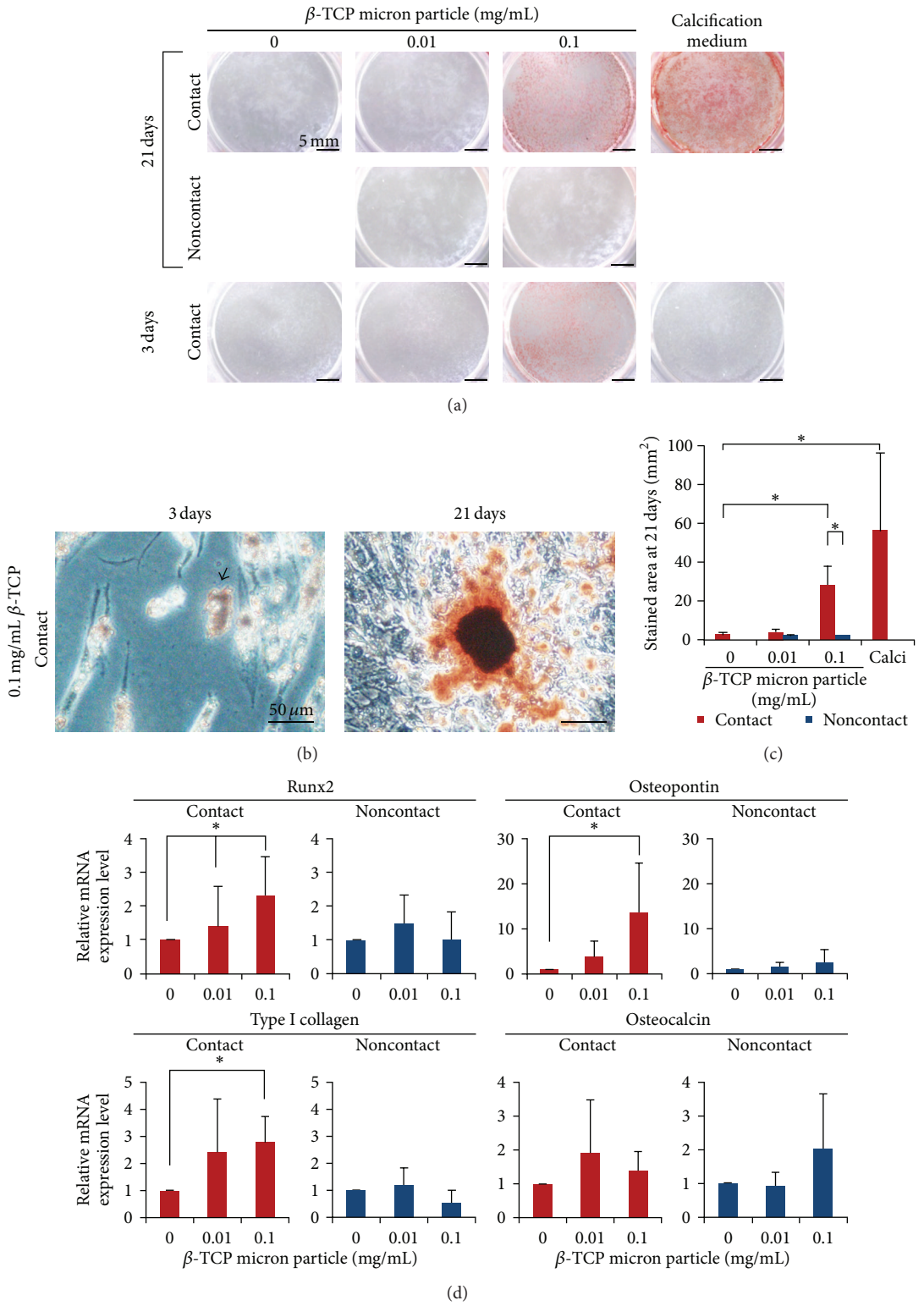

Figure 4: Effect of β-TCP micron particles on calcification of MSCs. (a) Alizarin red staining. MSCs were cultured without or with β-TCP micron particles in the contact or the noncontact condition for 21 days and in the contact condition for 3 days. As a positive control, MSCs were cultured in calcification medium. (b) High magnified pictures for MSCs stained with alizarin red. Black arrow indicates β-TCP micron particles stained with alizarin red located outside of MSCs. (c) Stained area at 21 days with alizarin red, which was calculated by subtracting stained area at 3 days in the contact condition. Averaged area with standard deviation is shown ($n = 6$, $^*P < 0.05$ by the Kruskal-Wallis test). (d) Osteogenesis-related gene expressions by real-time PCR analysis. Averaged relative mRNA expression level with standard deviation is shown ($n = 6$, $^*P < 0.05$ by the Kruskal-Wallis test).

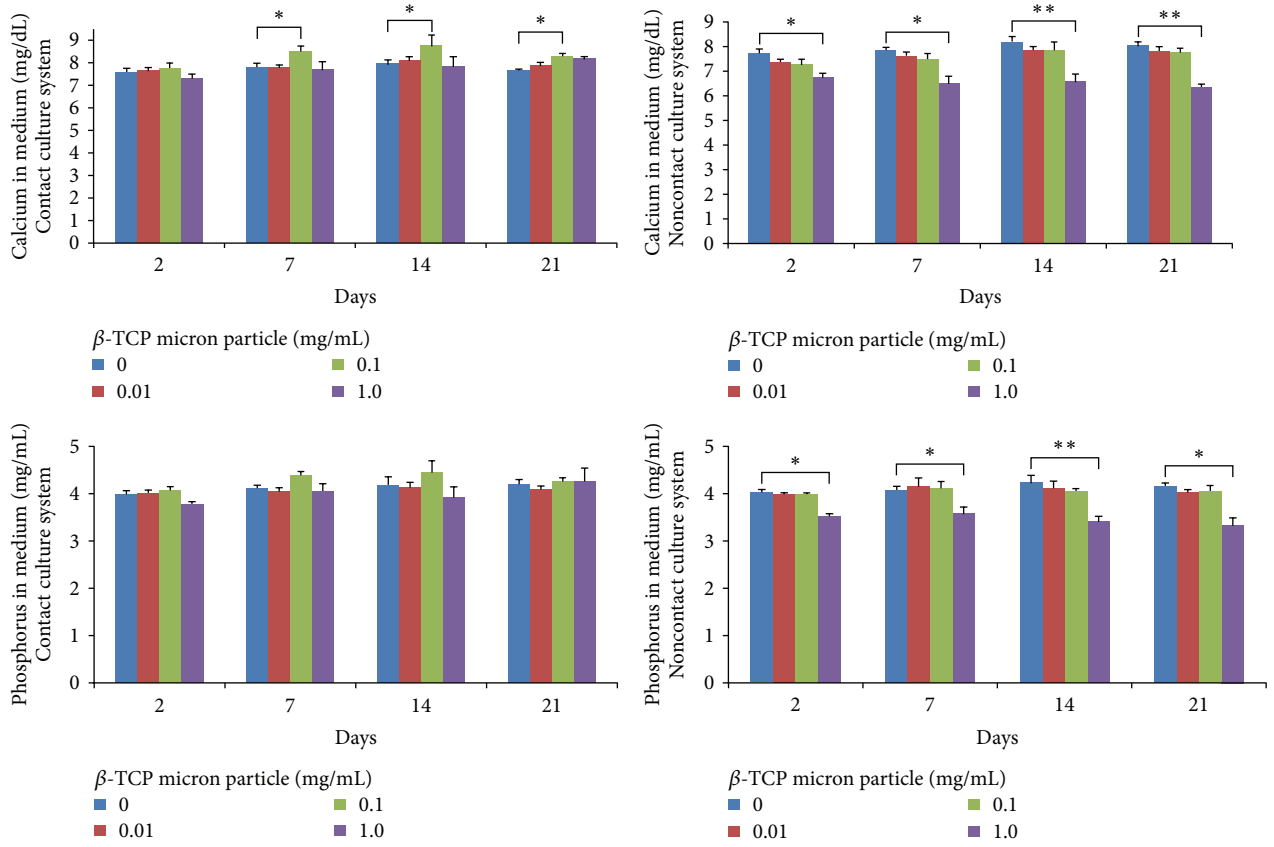

FIGURE 5: Calcium and phosphorus concentrations in medium treated with β-TCP micron particles. Averaged concentrations with standard deviation are shown ($n = 7$, $^{*}P < 0.05$, $^{**}P < 0.01$ by the Kruskal-Wallis test).

In this study, we used MSCs in order to examine the effect of β-TCP micron particles. MSCs have much greater expansive potential than osteoblasts [17] and multilineage differentiation potential including osteogenesis. Moreover, the risk of tumorigenesis is extremely low [7]. MSCs are one of the promising cell sources for bone regeneration [18]. A combination of cytokines and scaffolds improves the function of MSCs for bone formation [10, 11]. Several papers have reported that the use of β-TCP scaffold enhances bone formation of MSCs [19]. Therefore, we focused on MSCs in this study.

MSCs could be isolated from various tissues such as synovium [20], adipose tissue [21], and skeletal muscle [22], in addition to bone marrow [6]. In this study, we used MSCs derived from synovium, and we confirmed that synovial MSCs had an osteogenic potential by alizarin red staining, RT-PCR analysis, and measuring ALP activity. According to our previous studies, synovial MSCs had a similar osteogenic potential to bone marrow MSCs [23, 24], and synovial MSCs had a higher proliferative capacity than that of bone marrow MSCs when cultured with autologous human serum [25]. To examine whether the effect of β-TCP micron particles on MSCs depends on their original tissues would be an interesting future study.

1.0 mg/mL β-TCP micron particles inhibited proliferation and increased dead cells of MSCs in the contact condition. To account for these results, three possible reasons could be proposed. Firstly, low concentrations of calcium and phosphorus in medium might have affected proliferation and viability of MSCs. 1.0 mg/mL β-TCP micron particles significantly decreased calcium and phosphorus concentrations and increased the ratio of dead cells at 2 days in the noncontact condition. These results correspond to those of a previous study in which nanoparticles of hydroxyapatite were examined by Liu et al. A high concentration of nanosized hydroxyapatite particles reduced calcium and phosphorus concentrations in culture medium, resulting in inhibited proliferation of bone marrow MSCs [26]. Secondly, an excessive amount of β-TCP taken up by MSCs might have been toxic because the inhibitory effect of β-TCP micron particles on proliferation of MSCs appeared to be dose-dependent at 2, 7, and 14 days without significant changes of calcium and phosphorus concentrations in medium in the contact condition. Thirdly, the transition of the MSCs from the proliferative phase into the differential phase might have resulted in inhibition of proliferation. MSCs are a heterogeneous population and only MSCs in the osteogenic lineage survive and others die in calcification medium.

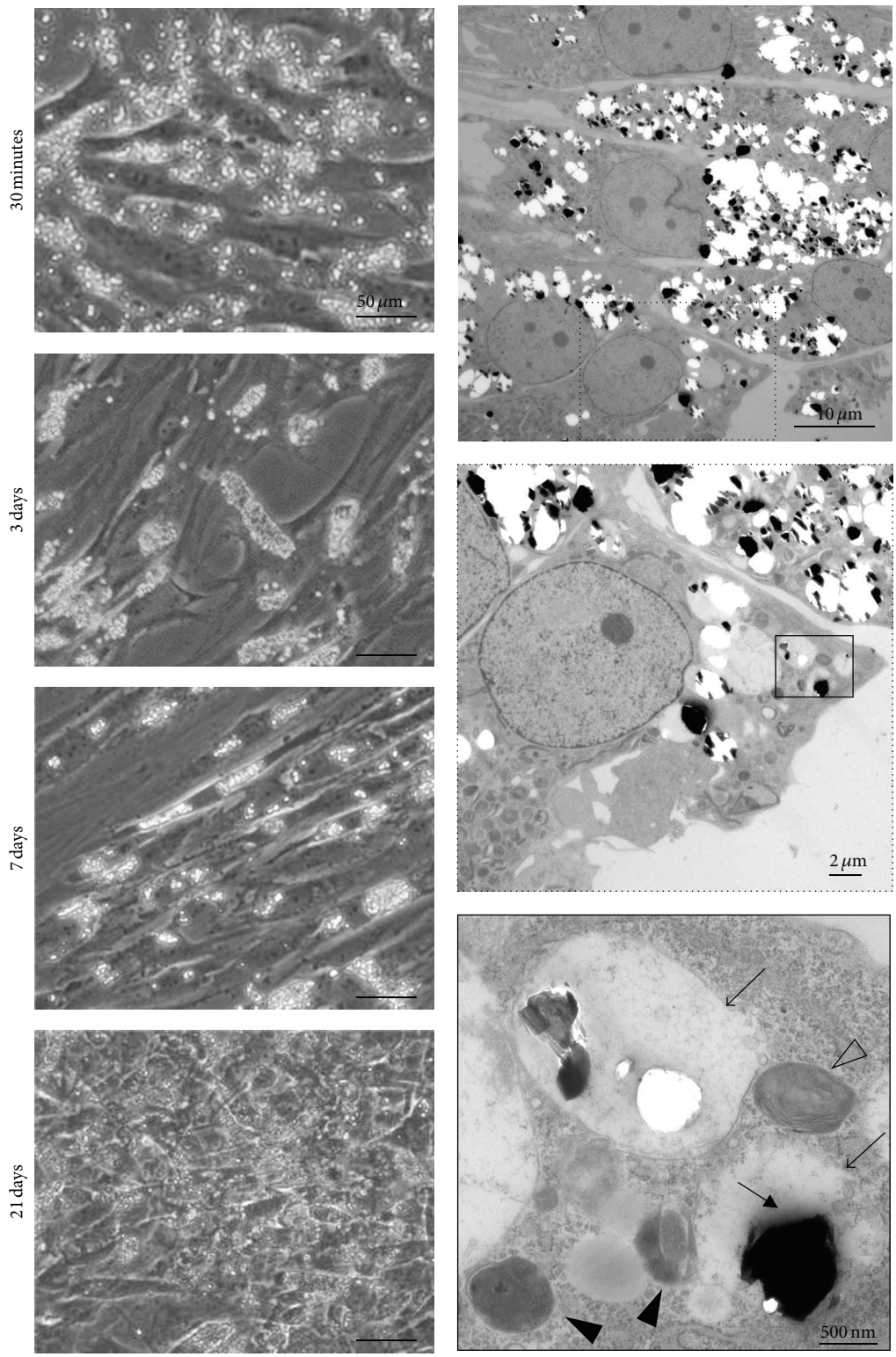

Figure 6: Morphology of MSCs cultured with β-TCP micron particles. (a) Light microscopic images. MSCs were cultured with 0.1 mg/mL in the contact condition. (b) Transmission electron microscopic images for MSCs at 7 days. Each area indicated by a square is magnified. β-TCP micron particles are indicated with arrow, phagosome with open arrow, lysosome with black arrow head, and lamellar body with white arrow head.

We evaluated the pH value in medium by litmus paper, and it was maintained between 7.4 and 7.6 after treatment of β-TCP micron particles in both contact and noncontact condition (data not shown). This indicates that pH was not affected by β-TCP micron particles.

As mentioned above, β-TCP micron particles affected calcium and phosphorus concentrations in medium. Interestingly, 1.0 mg/mL β-TCP micron particles decreased calcium and phosphorus concentrations only in the noncontact condition. In addition, we evaluated calcium and phosphorus concentrations in medium without any cells and found that their concentrations decreased dose dependently (data not shown). One possible reason for this could be that the β-TCP micron particles absorbed calcium and phosphorus in medium. This finding corresponds to the one observed in the previous study in which nanosized hydroxyapatite particles were used [26]. Contrarily, in the contact condition, 0.1 mg/mL β-TCP micron particles significantly increased calcium concentration at 7 days and thereafter, instead of decreasing it. β-TCP micron particles were already taken up by MSCs at 3 days and they appeared to be dissolved in phagosomes at 7 days. These findings suggest that MSCs dissolved β-TCP micron particles and supplied calcium in medium in the contact condition.

Treatment of 0.1 mg/mL β-TCP micron particles for MSCs in the contact condition increased stainability with alizarin red at 21 days and also enhanced expressions of runx2, osteopontin, and type I collagen, markers for osteogenic differentiation [27, 28]. However, expression of osteocalcin, a late marker for osteogenesis, was not significantly increased. These findings indicate that MSCs did not fully differentiate into mature osteoblasts.

For evaluation of calcification by alizarin red staining, the influence of β-TCP micron particles themselves should be taken into account. In the contact condition, apparently, 0.1 mg/mL β-TCP micron particles already increased stainability with alizarin red at 3 days, but β-TCP micron particles stained red were observed outside of MSCs in addition to inside. Osteogenesis-related gene expressions were not increased at 3 days (data not shown). The difference calculated by subtracting the positively stained area with alizarin red at 3 days from that at 21 days would be the actual positively stained area produced by MSCs, as we demonstrated in Figure 4(c).

0.1 mg/mL β-TCP micron particles in the contact condition promoted calcification of MSCs. On the other hand, in the noncontact condition, β-TCP did not alter alizarin red staining and osteogenesis-related gene expressions, showing that the indirect effect of β-TCP micron particles did not affect osteogenic differentiation of MSCs. Two possible mechanisms could be proposed to account how β-TCP micron particles promoted calcification of MSCs. The first mechanism is due to increase of calcium concentration inside and outside of MSCs. Previous studies reported that an elevated concentration of calcium was crucial for *in vitro* mineralization of osteoblast-like cells [29, 30]. The second mechanism is due to increase of bone matrix adhering to MSCs induced by β-TCP micron particles. Weißenböck et al. reported that bone substitute easily bound to osteogenic proteins due to its high affinity, and it supplied bone matrix proteins to MSCs and promoted osteogenic differentiation [31]. Huang et al. reported that MSCs adhering to bone substitute led to change of cytoskeletal organization of osteoblast-like cells [16]. It would be an interesting future study to distinguish the mechanisms of enhancing mineralization of MSCs between β-TCP micron particles used in this study and larger ones which cannot be taken up by MSCs.

β-TCP micron particles were localized in phagosome and not in lysosome. This may have been due to the diameter of the β-TCP micron particles. The diameter of the β-TCP micron particles we used was 1 to 10 μm, larger than that of lysosome whose size ranged from 0.5 to 1.0 μm in diameter. Some lysosomes made contact with the phagosome which included the particles (Figure 6(b)). Thus, we supposed that the phagosome might have become phagolysosome by connecting with the lysosome and that the particles were dissolved in it. van Buul et al. demonstrated that MSCs had a potential to phagocyte inorganic substances [32]; however, there have been no reports showing an ability of MSCs to phagocyte bone graft substitute.

In conclusion, 0.1 mg/mL β-TCP promoted calcification of MSCs evaluated by alizarin red staining and enhanced osteogenesis-related gene expressions. 0.1 mg/mL β-TCP also increased calcium concentration in medium. Transmission electron microscopy demonstrated that β-TCP micron particles were localized in the phagosome of MSCs, thus dissolving the particles.

Conflict of Interests

Y. Hakamatsuka is an employee of the manufacturers of β-TCP micron particles. He did not interfere with the conduct of the study, and the authors take full responsibility for the study. None of the authors has any direct financial relation with the commercial identities mentioned in this paper that might lead to a conflict of interests.

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