# Osteoclastic resorption and osteoinduction in the highly purified β-tricalcium phosphate implanted in the rat subcutaneous tissue are promoted by autologous bone marrow cells.

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***Abstract*—** *Sequential cellular events after highly purified β-tricalcium phosphate (β-TCP) implantation with or without rat bone marrow (BM) cells were investigated. β-TCP disks were soaked with BM cells harvested from femora of syngeneic rats, and were implanted into subcutis of the eight-week-old rats. Specimens were harvested in the intended stage and histological examinations were performed.*

*In BM-treated group, new bone formation was detected and intense signals of α1 chain of type Ⅰ procollagen mRNA were expressed in osteoblasts after day 14. On day 56, new bone never degenerated and normal bone marrow tissue was observed. TRAP positive-multinucleated cells appeared after day 7 and cathepsinK-positive osteoclasts appeared after day 14 with directly attached to β-TCP. Conversely, in BM non-treated group, new bone formation was never observed even on day 56 and neither TRAP nor cathepsinK positive-osteoclast was detected in the whole stage. Quantitative analysis showed that β-TCP area decreased time-dependently in BM-treated group, but not in BM non-treated group. In conclusion, these findings indicate that β-TCP loaded with BM cells has osteoinductive ability. β-TCP is resorbed mainly by osteoclasts and this event promotes ectopic bone formation. BM cells play an important role in osteoinduction and osteoclast differentiation and function.*

***Keywords*— Bone marrow cells; Osteoinduction; Osteoclasts; β-tricalcium phosphate.**

## I. INTRODUCTION

For bone defects such as after malignant tumor resection, revision arthroplasty, trauma, and infection, autologous cancellous bone graft is the first choice because it provides scaffolding for osteoconduction, growth factors for osteoinduction, and progenitor stem cells for osteogenesis. However, due to its limited amounts of supply, this is not the perfect treatment method. A further disadvantage of allografts could be that allografts could cause host immune response or transfer disease. Instead, synthetic bone substitutes such as hydroxyapatite (HA), tricalcium phosphates (TCP), and combination of HA/TCP (biphasic calcium phosphate; BCP) have also been used [1-3] . Highly purified β-tricalcium phosphate (β-TCP) (OSferion®) is widely used in Japan for bone substitute for the treatment of bone defects [4,5]. This material shows good osteoconductive property and biocompatibility in human bone and rat bone [6-8]. The ideal character of biomaterial is to promote bone induction as well as bone conduction. It has been reported that calcium phosphate ceramics has osteoinductivity in the optimal condition [9-14], and various trials have been performed to achieve good bone induction in extra-skeletal sites in various animal species. Bone morphogenic protein-2 (BMP-2) and osteogenic protein-1 (OP-1) protein, and cultured bone marrow mesenchymal stem cell implantation are thought to be prominent candidates for further powerful bone induction [15-19]. However, these tools have disadvantages because they require a lot of processes or cost too much to maintain safely within the animal body. For example, it has been reported that long-term culture of mesenchymal stem cells leads to carcinogenesis [20,21]. Autologous bone marrow (BM) cells graft with β-TCP is an alternative way that is readily available and relatively lower cost than other osteogenic proteins or ex vivo grafts that require mesenchymal stem cell cultures. We hypothesized that autologous BM cells promoted osteoinductive ability well. The purpose of the following study is to analyze the effects of BM cells after implantation with β-TCP into rat subcutaneous tissues using detailed histological examinations.

## II. MATERIALS AND METHODS

### 2.1 Preparation of β-TCP

β-TCP (OSferion®) was obtained from Olympus Biomaterial Corp. (Tokyo, Japan) [4]. Fine β-TCP powder was synthesized mechanochemically by wet milling: $CaHPO_4 \cdot 2H_2O$ and $CaCO_3$ at a molar ratio of 2:1 were mixed into a slurry with pure water and beads of zirconia in a pot mill for 24 hrs, and then dried at 80°C, leading to formation of calcium-deficient hydroxyapatite. This crystalline solid was converted to β-TCP by calcination at 750°C for 1 hr. Upon sintering of β-TCP powder at 1050°C for 1 h, a porous β-TCP block was obtained, which was then characterized through assessment of the surface area and pore-size distribution. The porosity of the block was 75% and the surface area, as measured by the Brunauer-Emmett-Teller method, was 1.4 $m^2/g$ [8,22]. The β-TCP possessed macropores of 100-400 μm and micropores of less than 5 μm. Nearly all macropores were interconnected via 100-200 μm pores [14]. Five-millimeter in diameter and 2.5 mm in height of β-TCP columns were used in this study.

### 2.2 Animal model and tissue preparation

Thirty female, 8-week-old, F344/Fisher rats were used and randomly divided into the two groups. Under general anesthesia, subcutaneous tissue of back in each rat was longitudinally exposed. At the same time, after euthanized, both ends of bilateral femora of a syngeneic 8-week-old female rat were cut and bone marrow plugs were flushed out using 250 μl of phosphate buffer saline expelled from a syringe through a 23-gauge needle and collected in a sterile 10-cm dish. Each 6 β-TCP disks per bone marrow cells obtained from a femur were soaked into extracted BM cells for 30 min in the Petri dish. Six β-TCP disks with BM cells per rat were implanted into subcutaneous tissue in back of a rat and defined as BM-treated group. As a control, six β-TCP disks without BM cells per rat were implanted as well and defined as BM non-treated group. After irrigation with normal saline, the wound was closed. All animal experiments were conducted according to the "Guideline for Animal Experimentation" of OLYMPUS CORPORATION.

On days 4, 7, 14, 28, 56 after the operation, the three rats in each group were euthanized and the implanted β-TCP disks were extracted and immersed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 5-7 days, and then, decalcified with 0.5 M EDTA·2Na solution for 2 days at room temperature and dehydrated with a graded series of ethanol treatments prior to being embedded in paraffin. Paraffin sections of 4-μm thickness were cut using a microtome (Leica, Tokyo, Japan) and stored at 4°C for the following histological evaluations.

### 2.3 TRAP staining

To detect osteoclasts, TRAP staining was carried out according to Burstone's Azo dye method [23], with some modifications [24]. Briefly, a mixture of 3 mg of naphthol AS-BI phosphate (Sigma, St. Louis, MO), 18 mg of red violet LB salt (Sigma, St. Louis, MO) and 2.4 mM L(+)-tartaric acid (Wako, Osaka, Japan) diluted in 0.1 M sodium acetate buffer (pH 5.0) were dropped onto the deparaffinized sections. These sections were incubated for 20-30 min at 60°C and then counterstained with hematoxylin.

### 2.4 Immunohistochemistry of ED1 and cathepsin K

The anti-ED1 monoclonal antibody recognizes a single chain glycoprotein of MW 90,000- 110,000 that is expressed predominantly on the lysosomal membrane and at low levels on the cell surface [25]. For ED1 immunohistochemistry, antigen retrieval with 0.2% trypsin at 37°C for 20 min was required. The tissue sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase, and then incubated with 10% goat serum for ED1 and with 1% bovine serum albumin for cathepsin K for 20 min to reduce nonspecific reactions. The sections were then incubated with anti-ED1 monoclonal antibody diluted 1:500 (Beringer Mannheim, August, Switzerland) for 16h at 4°C or with mouse anti-human cathepsin K antibody (Daiichi Finechemical, Takaoka, Japan) diluted 1:100 for 2h at room temperature, and reacted for 1h with rat MAX-PO (MULTI) secondary antibody (Nichirei, Tokyo, Japan) without diluted for ED1 or with horseradish peroxidase-conjugated goat anti-mouse IgG+IgA+IgM antibody (Zymed Laboratories, Inc., South San Francisco, CA, U.S.A) diluted 1:100 for cathepsin K at room temperature. The peroxidase reaction products were visualized with 3'-diaminobenzidine tetrahydrochloride (Nichirei, Tokyo, Japan). Sections were counterstained with hematoxylin.

### 2.5 In situ hybridization

To examine the differentiation stages of bone-forming cells, deparaffinized serial sections were subjected to mRNA in situ hybridization, as previously described [26,27]. Plasmid containing 0.37-kb fragments of mouse α1 chain of type Ⅰ procollagen (COL1A1) cDNAs was obtained as a gift from the Life Science Research Institute (Asahi-Chemical Industry Co., Shizuoka, Japan). After dewaxing in xylene and rehydrating through a series of graded ethanol treatments, tissue sections were treated with 10 μg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) for 20 min at 37°C, refixed with 4% PFA solution, immersed in 0.1 M triethanolamine containing 0.25% acetic acid for 10 min, and washed in 0.1 M phosphate buffer (pH 7.4). The samples were then incubated in a hybridization solution [10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1 × Denhart's medium, 50% (v/v) deionized formamide / 0.5 μg/ml probe RNA, and 10% dextran sulfate] at 50°C in a moist chamber for 16 hrs. Negative controls were incubated with DIG-labeled sense RNA probes. After hybridization, the slides were washed at 55°C with 50% deionized formamide in 2 × saline-sodium citrate (SSC) (1 × SSC; 0.15 mol/l NaCl, 0.015 mol/l sodium citrate) for 20 min to remove excess riboprobes. Non-specifically hybridized riboprobes were digested with 10 μg/ml of RNase A (Roche Diagnostic) solution at 37°C for 30 min. The specimens were then washed with 2 × SSC for 15 min and with 0.2 × SSC for 15 min twice. To visualize the hybridized probe, the slides were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) at room temperature for 60 min after blocking with 1.5% blocking reagent (Roche Diagnostics) in 100 mM Tris-HCl (pH 7.5) for 55 min. The specimens were then washed twice with 100 mM Tris-HCl (pH 7.5) for 15 min, and briefly immersed in 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$ for 5 min. The colorimetric reaction was performed with nitro blue tetrazolium salt and bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics) in the dark for 20-120 min, and then the reaction was stopped with 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA. Slides were mounted with micro cover glass (Matsunami, Tokyo, Japan) and analyzed under a light microscope with 0.5% methyl green counterstaining.

### 2.6 Semi-quantitative evaluation of the area of β-TCP and the ratio of newly formed bone per unit area of β-TCP

Specimens were stained with hematoxylin and eosin (HE). The areas of β-TCP and newly formed bone were measured using NIH Image Ver. 1.63 (developed at the U.S. National Institutes of Health, and available at http://rsb.info.nih.gov/nih-image/download.html). About the area of β-TCP, the specimens on day 4 were inappropriate for the measurements due to hyperdecalcification and their data were excluded. These data were then used to calculate the ratio of newly formed bone per unit area of β-TCP. Statistical analyses were performed using StatView software for Windows (Version 5.0) with a Bonferroni-Dunn test (either one way ANOVA or a post hoc test) used. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

## III. RESULTS

No newly formed bone was observed on days 4 and 7 in both bone marrow (BM)-treated group and BM non-treated group. On day 4, clotting was observed in the interconnected macropores of β-TCP (Fig. 1A). On day 7, a large number of fibroblast-like cells and blood vessels were observed in the interconnected macropores, and lots of attached cells appeared (Fig. 1B). After day 14, newly formed bone was detected with attaching to the surface of β-TCP in BM-treated group. On day 28, the thickness of newly formed bone was larger than that on day 14 (Figs. 1C,E). On day 56, most β-TCP surface was covered with newly formed bone, and β-TCP area seemed to decrease compared to those on days 14 and 28. Furthermore, normal bone marrow tissue was detected in interconnected macropores (Fig.1G).

However, in the BM non-treated group, no new bone formation occurred even in the peripheral region of implanted area on days 14, 28, and 56 although blood vessels were observed in β-TCP macropores (Figs.1D, F, and H).

To examine the cellular events of monocyte-macrophage lineage cells, we performed TRAP staining, immunohistochemistry of cathepsin K protein, which is a specific matrix degradation enzyme synthesized by osteoclasts [28], and ED1 protein, which is detected in the cells of the mononuclear phagocyte system in rats [25].

On day 4 after implantation, no TRAP positive cells were detected in both groups (data not shown). On day 7, a few TRAP positive multinucleated cells were attached to β-TCP surface in BM-treated group in the peripheral region (Fig. 2A). On days 14 and 28, abundant TRAP positive-multinucleated cells were detected on the surface of β-TCP even in the central region in BM-treated group. HE staining in BM-treated group on day 14 showed that some multinucleated giant cells were directly attached to β-TCP (Fig. 2G). However, they were sparsely observed around newly formed bone (Figs. 2C,E).

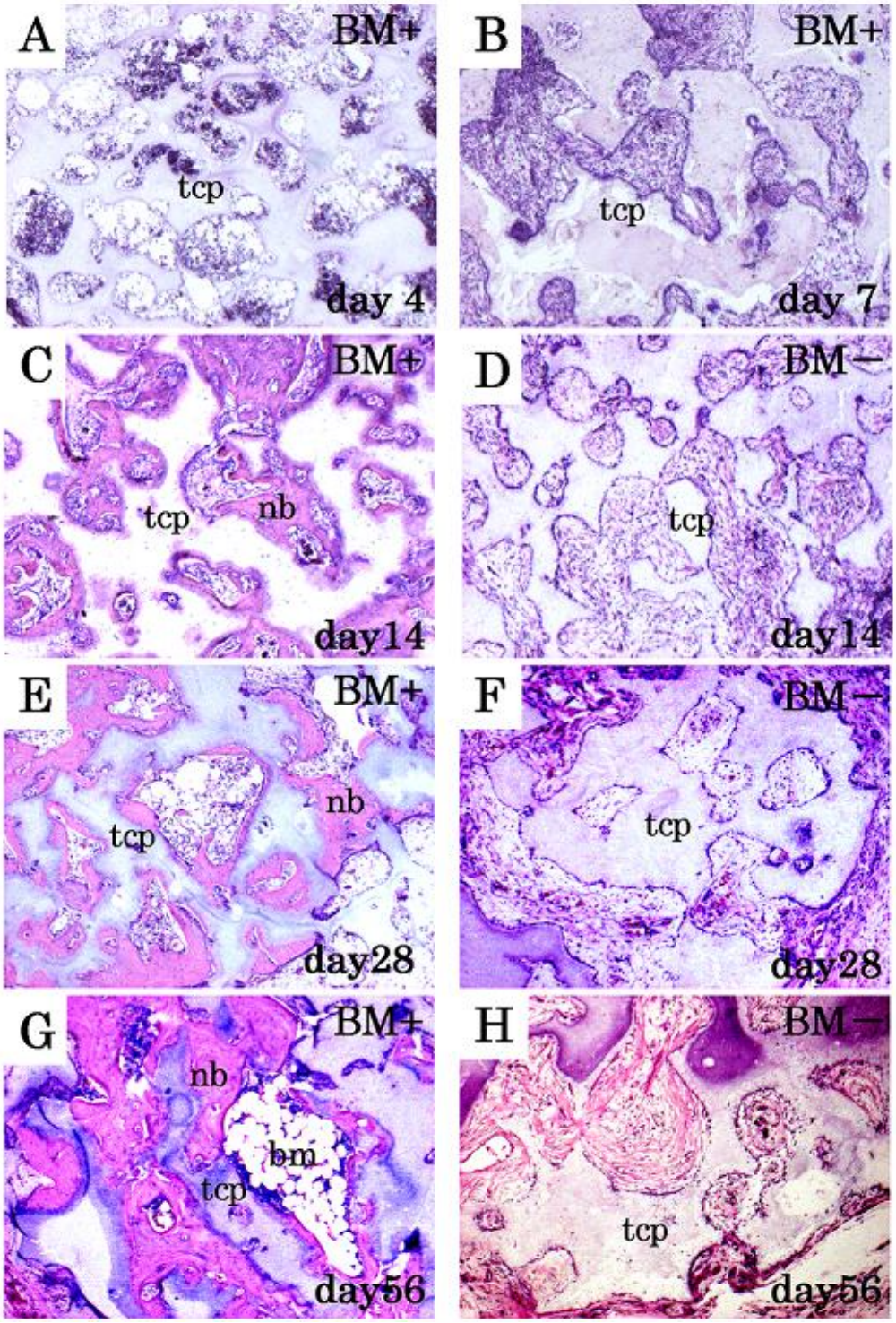


**FIGURE 1. TIME COURSE AFTER IMPLANTATION.**

**[On days 4 and 7, the new bone could not be detected any implanted area in BM-treated group (A and B). No newly formed bone was detected either in BM non-treated group.**

**In the BM-treated group, ectopic bone formation was detected in the macropores of β-TCP with attaching to the surface of β-TCP on day 14 (C).**

**On day 28, newly formed bone became thicker than that of day 14, and almost all surface of β-TCP was covered with new bone (E). On day 56, in addition to abundant newly formed bone, bone marrow tissue was observed in the macropores of β-TCP (G). In contrast, in the BM non-treated group, newly formed bone could not be detected on days 14 (D), 28 (F), and 56 (H) though abundant blood vessels were observed. BM+, BM-treated group, BM–, BM non-treated group, tcp, β-tricalcium phosphate; nb, newly formed bone; bm, bone marrow; original magnification, A-H: x 40. ]**

In contrast, in BM non-treated group, no TRAP positive-multinucleated cells were detected even in the peripheral region on days 7, 14, 28 (Figs. 2B,D,F), and 56.

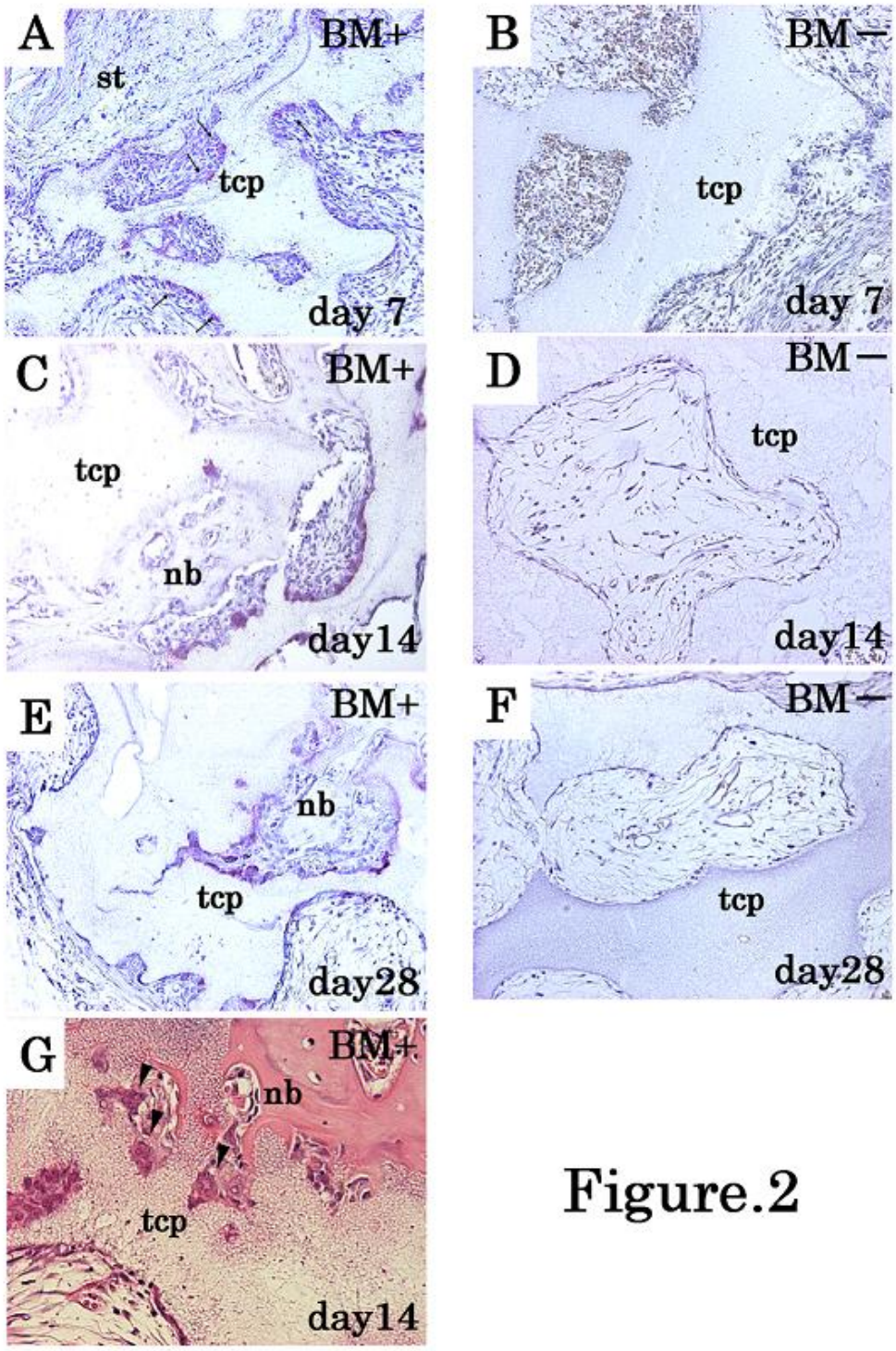


Figure.2

**FIGURE 2. TRAP STAINING AND FINDING OF MULTINUCLEATED GIANT CELLS ON HE STAINING.**
**[On day 7, in BM-treated group, a few TRAP-positive multinucleated cells (black arrows) were attached to β-TCP in the periphery of the implanted area (A). On days 14 band 28 in BM-treated group, a lot of TRAP-positive multinucleated cells were directly attached to β-TCP (C,E). These multinucleated giant cells (black arrow heads) were confirmed also in HE staining on day 14 in BM-treated group (G). In contrast, in BM non-treated group, no TRAP positive cells were detected on days 7 (B), 14 (D), and 28 (F). tcp, β-tricalcium phosphate; st, surrounded soft tissue; nb, newly formed bone, original magnification, A-F: x 100, G:x 200.]**

A lot of cathepsin K positive-osteoclasts were detected after day 14 in BM-treated group and most of them were directly attached to β-TCP or some of them attached to newly formed bone (Figs. 3A,C). Conversely, no cathepsin K positive-osteoclasts were observed on days 14 (Fig.3B), 28 (Fig.3D), and 56 in BM non-treated group.

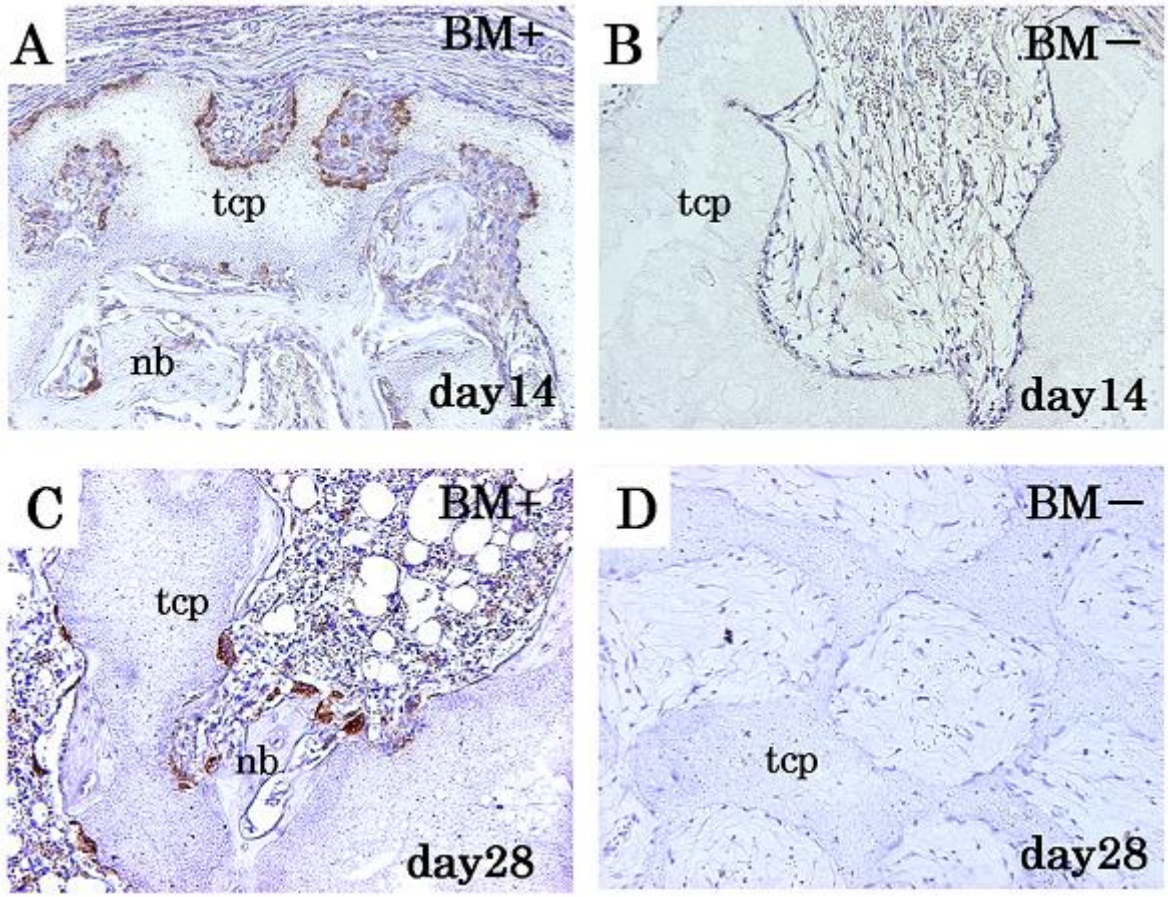


**FIGURE 3. CATHEPSIN K IMMUNOHISTOCHEMISTY**

**[In BM-treated group, cathepsin K positive osteoclasts were attached to β-TCP or newly formed bone on both days 14 and 28 (A,C). However, in BM non-treated group, no cathepsin K positive osteoclasts were observed on days 14 and 28 (B,D). tcp, β-tricalcium phosphate; nb, newly formed bone, original magnification, A-D: x 100.]**

On days 4 and 7, abundant ED1 positive-monocytes gathered in β-TCP macropores and were attached to β-TCP in both BM treated and non-treated groups with no difference (Figs. 4A,B).

However, ED1 protein immunohistochemical findings were quite different between the two groups after day 14. On day 14, abundant ED1 positive- multinucleated cells were attached to β-TCP in BM-treated group (Fig. 4C). Furthermore, on days 28 and 56 in BM-treated group, ED1 positive-multinucleated cells were still attached to β-TCP though their number decreased compared to that on day 14 (Figs. 4E,G). On the contrary, in BM non-treated group, no ED1 positive-multinucleated cells were attached to β-TCP on days 14, 28, and 56 although ED1 positive-monocytes were sparsely observed in macropores (Figs. 4D,F,H).

Next, to detect bone-forming cells, in situ hybridization of COL1A1 mRNA was performed. On days 4 and 7, no difference was detected between both BM-treated and non-treated groups. On day 7, COL1A1 mRNA was expressed in mononuclear cells which were attached β-TCP and was expressed in fibroblast-like spindle shaped cells in β-TCP macropores in both BM-treated (Fig. 5A) and non-treated (Fig. 5B) group. On day 14, in BM-treated group, osteoblasts lining the newly formed bone were strongly positive for COL1A1 mRNA, and their signals were much stronger than those of the fibroblast-like spindle-shaped cells on day 7 (Fig. 5C). However, in BM non-treated group, COL1A1 mRNA-positive cells were observed at surface of β-TCP similar to the finding on day 7, and their intensities were much weaker compared to those on day 14 of BM-treated group (Fig. 5D). Same tendency was preserved on days 28 (Figs. 5E,F) and 56.

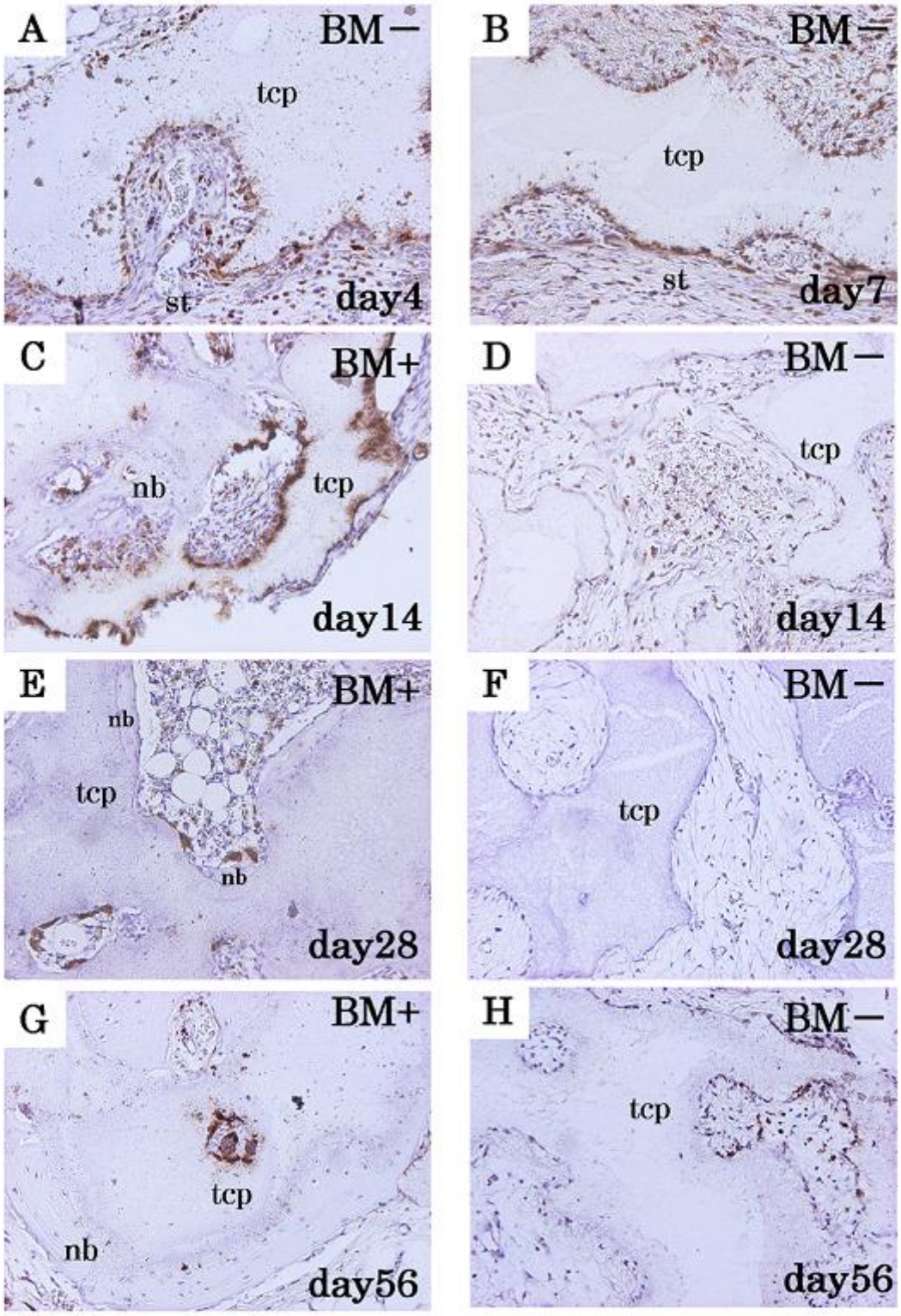


**FIGURE 4. ED1 IMMUNOHISTOCHEMISTRY**

**[On days 4 (A) and 7 (B) in BM non-treated group, abundant ED1 positive mononuclear cells were attached to β-TCP surface or adjacent to β-TCP in the peripheral region. On day 14 in BM-treated group, lots of ED1 positive multinucleated cells were attached to β-TCP (C). Also on day 28 (E) and 56 (G) in BM-treated group, ED1 positive multinucleated cells were observed. However, in BM non-treated group, on days 14 (D), 28 (F), and 56 (H), no ED1 positive-multinucleated cells were attached to β-TCP, although ED1 positive-mononuclear cells were detected in the surface of β-TCP or β-TCP macropores. tcp, β-tricalcium phosphate; nb, newly formed bone, original magnification, A-H: x 100]**

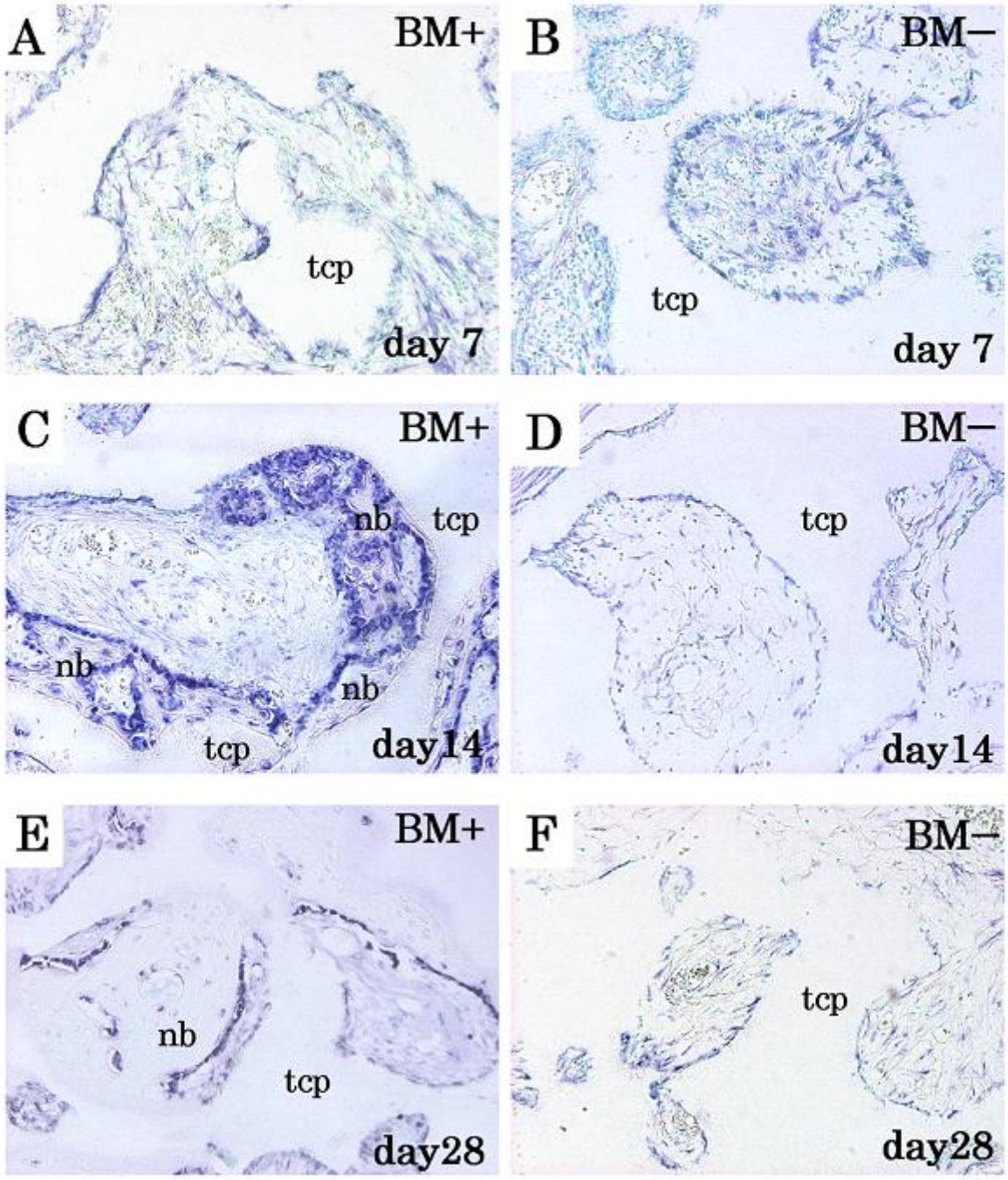


**FIGURE 5. IN SITU HYBRIDIZATION OF COL1A1 MRNA**

**[A. On day 7 in BM-treated group. COL1A1 mRNA positive mononuclear cells were directly attached β-TCP and fibroblast like spindle shaped cells were expressed in COL1A1 mRNA in β-TCP macropores. B. On day 7 in BM non-treated group showed similar finding as A. C. On day 14 in BM-treated group, cuboidal shaped COL1A1 mRNA positive cells lined to newly formed bone, and their intensities were much stronger than fibroblast like COL1A1 mRNA positive cells in β-TCP macropores. D. On day 14 in BM non-treated group, no cuboidal shaped COL1A1 mRNA positive cells were detected. COL1A1 mRNA positive mononuclear cells were still expressed in the surface of β-TCP, but their intensities were much weaker than those shown as C. E. On day 28 in BM-treated group, COL1A1 mRNA positive cells lined to the newly formed bone, and their shapes were slightly flatter than those shown as C. F. On day 28 in BM non-treated group, the finding was similar to that shown as D. tcp; β-tricalcium phosphate, nb; newly formed bone, original magnification; A-F:x 100]**

Quantitative analysis demonstrated that the area of β-TCP in BM-treated group decreased in a chronological manner and the area on day 56 was significantly smaller than that on day 7 ($p<0.05$) (Fig. 6A). However, in BM non-treated group, the tendency was not detected (Fig. 6B). The ratio of newly formed bone per unit area of β-TCP clearly increased from day 14 to day 56 in a time-dependent manner. The ratio of newly formed bone per unit area of on days 28 and 56 were significantly larger than that on day 14 ($p<0.05$) (Fig. 6C).

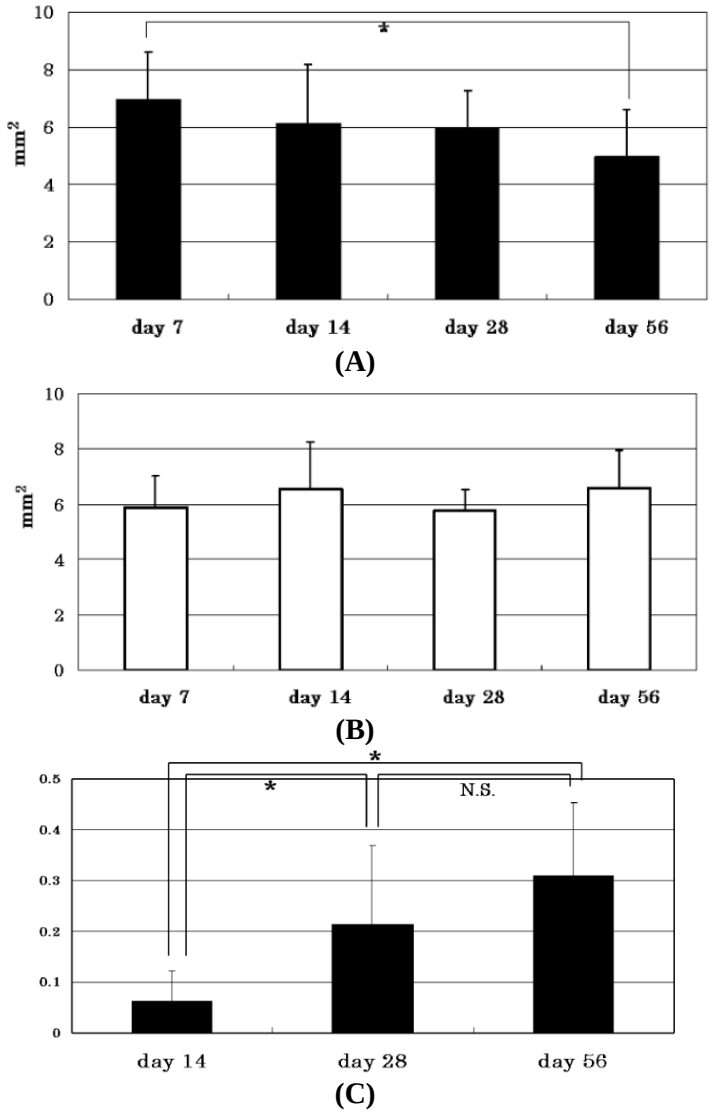


**FIGURE 6. QUANTITATIVE ANALYSIS**

**[A. The area of β-TCP in the BM-treated group. The area of β-TCP decreased in a time-dependent manner and the area on day 56 (4.97±1.63 $mm^2$) was significantly smaller than that on day 7 (6.97±1.61 $mm^2$) ($p<0.05$). B. The area of β-TCP in the BM non-treated group. It had no tendency that was shown in A. C. The ratio of newly formed bone per unit area of β-TCP, shown as the mean ± standard deviation. The amount of newly formed bone increased with time; the amounts of newly formed bone per unit area of β-TCP on days 28 (0.21±0.15) and 56 (0.31±0.14) were significantly higher than that on day 14 (0.06±0.06) ($p<0.05$). *, $p<0.05$, N.S., not significance; Bar, standard deviation.]**

## IV. DISCUSSION

In this study, newly formed bone was detected after day 14 in BM-treated group after β-TCP implantation in rat subcutaneous tissue. This bone was not formed by endochondral ossification but through intramembranous ossification since no cartilaginous tissue was observed. New bone formation appeared from the peripheral region of implanted site and gradually extended to the central region. This pattern is considered as reasonable since peripheral region is closer from host

tissue and is vascularized earlier and more efficiently than the central region. Some reports have also supported this interpretation [29-31]. However, no bone formation was induced in BM non-treated group. These findings indicate that BM cells are very important as a source of osteoinduction in this model.

Moreover, even on day 56, newly formed bone was preserved without being degenerated and normal bone marrow tissue was observed, suggesting that β-TCP loaded with fresh BM cells has a good biocompatibility and osteoinductive ability.

The findings of TRAP staining and cathepsin K immunohistochemistry show that BM cells induce the early appearance of osteoclasts in this model and that osteoclasts resorb β-TCP with directly attaching after day 14. Especially, TRAP positive-multinuclear cells were detected on day 7 in BM-treated group though their number was small.

This phenomenon is in agreement with those after implantation in rat bone and dog dorsal muscles as we previously reported [8,14]. As reported by Jarcho [32], bioresorption of calcium phosphate ceramics such as hydroxyapatite (HA) and TCP consists of solution-mediated processes (the implant dissolves in physiologic solutions) and cell-mediated processes (phagocytosis). Given that osteoclasts which appeared in the early stage were directly attached to β-TCP in BM-treated group and β-TCP area decreased in a time-dependent manner, these data strongly support that cell-mediated resorption plays an important role for bioresorption of β-TCP although the involvement of solution-mediated bioresorption should not be ignored since microporosity possibly could aid in bioresorption by causing microscopic "break-up" secondary to solution-mediated resorption [32] and this β-TCP has a microporous structure.

ED1 immunoreactivities on days 4 and 7 showed that ED1 positive-monocytes gathered adjacent to β-TCP or were directly attached to β-TCP, and no difference was detected between the two groups. These findings suggest that monocytes infiltrate adjacent to β-TCP even in the absence of BM cells. After day 14, ED1 positive-multinucleated cells were detected only in BM-treated group. Nagayama et al. reported that the intensity of ED1 immunostaining decreased with time after implantation of β-TCP and carbonate apatite into the subcutaneous tissue in back of rats, and that it was persisted longer time in β-TCP group [33]. This report is very suggestive in that β-TCP itself induces macrophages in extra-osseous sites. We indicate that additional BM cells promote much more inductive ability of macrophage around β-TCP in this model. In addition, osteoclasts belong to monocyte-macrophage lineage cell and monocytes are supposed to fuse and differentiate into osteoclasts, suggesting that BM cells induce differentiation and proper function of osteoclasts. In vitro, osteoclasts or closely related to osteoclasts were induced after 3-week-culture of bone marrow cells in the presence of 1,25-dihydroxyvitamin D3 [34].

About in situ hybridization of COL1A1 mRNA, no difference was detected on day 7 between BM-treated and BM non-treated group. However, mononuclear cells (osteoblasts) lined to newly formed bone were strongly COL1A1 mRNA positive on day 14, 28, and 56 in BM-treated group, which was quite different from those in BM non-treated group. These results indicate that BM cells also induce bone forming ability.

Quantitative analyses in this study showed the area of β-TCP decreased in a time-dependent manner in BM-treated group, but not in BM non-treated group, suggesting that β-TCP is continuously resorbed in BM-treated group due to the constant osteoclast appearance. In addition, new bone formation consistently increases in BM-treated group, suggesting that bone forming ability is preserved after day 14.

Several previous studies reported that fresh bone marrow cells (without cultured) promoted bone induction in extra-osseous sites after calcium phosphate ceramics implantation such as HA, β-TCP, and biphasic calcium phosphate (HA/TCP). [30,31,35]. The earliest ectopic bone formation was 3 weeks after implantation of biphasic calcium phosphate (HA:TCP was 60:40) into rat muscle [30]. Ectopic bone formation was shown on 1 month and 2 months after implantation of HA and β-TCP with syngeneic rat bone marrow cells into rat subcutis [35]. It is noteworthy that ectopic bone formation was detected 2 weeks, earlier than these previous reports [30,31,35], after implantation in our study.

Cultured bone marrow stromal cells implantation with calcium phosphate ceramics was also reported, and bone formation was detected day 7 days [19,29], or 2 weeks [36] after implantation. These methods are based on culture for good differentiation to osteogenic cells using by dexamethasone or β-glycerophosphate. Given that mesenchymal stem cells implantation may lead to carcinogenesis [20,21], it is indispensable to investigate the safety of mixed implantation combined calcium phosphate ceramics with mesenchymal stem cells. On the other hand, there is no report that had adverse effect such as infection to bone marrow implantation into bone defects or non-union [37].

Moreover, osteoinductive cytokines such as BMP-2, OP-1, and fibroblast growth factor (FGF) have been investigated as potent promoters of osteoinduction in extra-osseous site [15-17,38]. In general, these cytokines induce ectopic bone formation in the early stage after implantation with a scaffold. However, some problems such as immune response, infection, and higher cost, have been concerned. Recently, it has been reported that recombinant human BMP-2, which is widely used for spine surgery, has adverse effects such as infection, hematoma, and excessive edema [38,39].

Bone marrow cells loading to β-TCP is a very simple and lower cost method to induce ectopic bone formation. We consider that it is reasonable_than the calcium phosphate ceramics implantation with the other concomitant use of osteoinductive cytokines and cultured mesenchymal stromal cells in that it does not cost very much and safer and bone formation was confirmed as early as on day 14. Erbe et al. have shown that bone marrow aspiration and β-TCP composite graft is as good outcome as autograft with bone marrow cells [1]. They used "Vitoss", which is a porous, low-density construct prepared by lightly fusing particles of β-TCP, is average approximately 1-2 μm in diameter, and has 90 % porosity and interconnected void space [1].

It remains not elucidated why oseteoclasts proliferated and are differentiated after implantation of β-TCP. Some possibilities about the origin of osteoclasts are considered as follows; (1) stromal cells in implanted bone marrow induce osteoblasts and such osteoblasts induce osteoclasts via receptor activator of NF-kB (RANK)-RANK-ligand (RANKL) system and (2) hematopoietic cells in implanted bone marrow directly induce osteoclasts.

Little report was focused on cellular events about the appearance or the function of osteoclast after implantation of β-TCP. In our study, it is noteworthy that the early (day 7) appearance of osteoclasts prior to new bone formation was confirmed after implantation of BM cells-loaded β-TCP into rat subcutaneous tissue and that osteoclasts play an important role in cell-mediated bioresorption of β-TCP. After implantation of β-TCP into rabbit bone, the peak of osteoclasts was 2 week and it was earlier than that of osteoblasts. Chazono et al. called this event as "coupling-like phenomenon" [22]. In our previous study of β-TCP implantation in dog dorsal muscles, osteoclasts were induced in muscles where these cells never exist and then new bone formation occurred [14]. We indicate that the cellular event shown in our study resembles "coupling-like phenomenon" and is also very important for osteoinduction. Further studies are required for elucidating the mechanism of macrophage or osteoclast responses to biomaterials.

## V. CONCLUSION

β-TCP (OSferion®) is resorbed by osteoclasts and leads to new bone formation when implanted with syngeneic rat bone marrow cells into subcutaneous tissue. These cellular events follow the appearance of a large number of osteoclasts and their attachment to β-TCP. The data in this study suggest that β-TCP implantation with autogenous bone marrow cells is safe and not time-consuming method and promotes osteoinduction method compared to that in non BM cells condition.

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