ORIGINAL ARTICLE

Effects of Vitamins Added to Culture Medium for Calcified Nodule Formation by Rat Bone Marrow Cells

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Synopsis

The present study attempted to obtain factors that induce the calcified nodule formation in a mesenchymal stem cell (MSC) culture through the aggregation of micro- or nanoparticles, such as calcium carbonate. There are few suitable cell sources in the oral cavity other than dental pulp tissue, from which only a small number of MSCs may be obtained. However, a large number of MSCs are needed for tooth regeneration. Therefore, factors that promote MSC proliferation and differentiation are required. Vitamin B_1 (VB_1), vitamin B_3 (VB_3) and vitamin B_5 (VB_5), which are present in physiologically active substances, were selected and investigated their effects to bone formation *in vitro*. VB_1 and VB_3 significantly increased the ability of dexamethasone (Dex) to form calcified nodule aggregates *in vitro*, whereas VB_5 exerted the opposite effects. Therefore, it was confirmed from the results of this *in vitro* study that VB_1 and VB_3 may affect to Dex to promote MSCs in rat bone marrow cells for proliferation and differentiation into hard tissue-forming cells and the formation of calcified nodule aggregates.

Key words: *Vitamin B, Calcified nodules, Bone marrow cells, Dexamethasone*

Introduction

The regeneration of a missing tooth or the restoration of a partially defective one using tissue engineering methods is desired in dentistry. Dentine is one of the hard tissues of a tooth and its structure is similar to that of bone [1]. Unlike bone fractures, tooth defects cannot repair themselves. Clinical applications for the replacement of missing teeth through the transplantation of tooth germs [2] and regeneration of dental pulp tissue [3] have been considered. However, their practical use in clinics has not yet been realized. Bones are present inside the body and, thus, are afforded a level of protection against infection, whereas teeth are exposed to a large number of microorganisms in the oral cavity. Infection is a significant inhibitor of tooth regeneration. There is no doubt that infection is an important factor in inhibiting tooth regeneration in the oral cavity. Additionally, obtaining a large number of mesenchymal stem cells (MSCs) would be need for tooth regeneration. Many MSCs would be essential for the rapid formation of large amounts of hard tissue in basic research *in vitro* on tooth and bone regeneration.

Tooth or bone regeneration requires a large number of MSCs. Few tissues in the oral cavity are suitable as a cell source for MSCs. Although the tooth germ [2] or dental pulp tissue [3] is the preferred tissue as a cell source for MSCs, the number of MSCs obtained from dental pulp tissue is small [4]. Dental pulp tissue is a rare tissue in the oral cavity that has potential as a cell source; however, the number of cells in this tissue is small and, thus, a very limited number of

MSCs may be present [5]. Therefore, these MSCs need to be proliferated *in vitro* using factors that increase their number to that required for the regeneration of tooth hard tissue. The percentage of MSCs present in nucleated cells obtained from human iliac bone marrow is approximately 0.001-0.01 %, which is small [6]. Because of the small number of MSCs, in experiments on *in vitro* osteogenesis using MSCs derived from rat bone marrow cells (rBMCs), dexamethasone (Dex) [7, 8], β-glycerophosphate (β-GP) [7, 9], and ascorbic acid (VC) [7, 10] are typically added to culture media as substances that promote calcified nodule formation.

Vitamins are bioactive substances that may induce MSC proliferation and differentiation. B vitamins (VBs) have been suggested to enhance the effects of Dex on rBMCs *in vitro* [5, 11], thereby promoting the formation of calcified nodule aggregates composed of micro- or nanoparticles, such as calcium carbonate [5, 11-15]. A previous study reported that the ability of Dex to induce calcified nodule formation by rat dental pulp cells *in vitro* was enhanced by the addition of vitamin B_{12} (VB_{12}) to the culture medium [5]. However, there is no evidence to show that vitamin B_1 (VB_1) [16] enhances the effects of Dex and osteogenesis by MSCs *in vitro*. In another study, the amount of bone regenerated by osteoblasts was shown to be increased in a hydrogel scaffold with vitamin B_2 (VB_2) [11].

VB_1 [16], vitamin B_3 (VB_3) [12, 13], and vitamin B_5 (VB_5) [14, 15] were selected for investigation. The purpose of the present study was to establish whether these VBs enhanced the effects of Dex on calcified nodule formation by MSCs in culture media. The effects of these VBs on calcified nodule formation *in vitro* by BMCs from rat femurs were assessed using Ca^{2+} levels measured in decalcified aliquots of aggregated calcified nodules in a rBMC subculture supplemented with each VB.

Materials and Methods

1. Animals

Animal experiments to obtain rBMCs were performed in the Laboratory Animal Facilities at the Research Laboratory Center in the Translational Research Institute for Medical Innovation of Osaka Dental University. Approval was obtained from the Osaka Dental University Institutional Animal Care and Use Committee for this experiment and the use and care of animals (21-07001, 23-07003). The present study was performed in accordance with the Osaka Dental University Regulations on Animal Care and Use established by the Committee and with the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources (8^{th} edition, 2010).

Four 6-week-old male Fischer 344/N Slc rats (Japan SLC, Inc., Shizuoka, Japan) were purchased. They were housed in standard rat cages, given free access to dry pellets and water, and allowed unrestricted movement until used in experiments.

2. Harvest of rBMCs from rat femurs

Animals were euthanized by the excessive inhalation of isoflurane and femurs were removed to harvest rBMCs. Both ends of the femur were cut at the epiphysis. Bone marrow in the bone shaft was flushed out by expelling 10 mL of minimum essential medium (MEM; E-MEM containing L-glutamine and phenol red: FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) through a 21-gauge needle from a syringe. The rBMCs removed from each femur were collected in a 15-mL centrifuge tube.

3. Adjustments of vitamin solutions

Solutions of VB_1, VB_3, and VB_5 were prepared to a concentration of 100 mM in the following volumes of ultrapure water in 15-mL conical tubes: 230 mg of cocarboxylase (VB_1: FUJIFILM Wako Pure Chemical Corp.) in 5 mL of ultrapure water, 12.2 mg of nicotinamide (VB_3: Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in 10 mL of ultrapure water, and 219 mg of calcium (+)-pantothenate (VB_5: FUJIFILM Wako Pure Chemical Corp.) in 10 mL of ultrapure water. The agent in each tube was stirred using a vortex mixer. Concentrations of 10 μM, 100 μM, 1 mM, and 10 mM of every VB solution were obtained by repeating a 10-fold dilution of each VB solution prepared to a concentration at 100 mM. Using a representative concentration of 10 μM, the final contents in 20 μL of each vitamin solution at a concentration of 10 μM added to MEM were 92.15 ng for VB_1, 24.42 ng for VB_3, and 95.31 ng for VB_5.

4. Culture of rBMCs

MEM used to rBMC culture was supplemented with 15 % fetal bovine serum (FBS; Biocera Inc.,

MO, USA) and antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B, Sigma-Aldrich Co. LLC., MO, USA).

A primary culture of rBMCs obtained from the bone marrow of the rat femur was incubated in cell culture flasks (T-75; BD Biosciences, MA, USA) at 37˚C for 1 week at 5 % CO_2 and 95 % relative humidity. The culture medium was replaced 3 times during this period. After the primary culture, rBMCs in T-75 culture flasks were washed 3 times using phosphate-buffered solution without Ca^{2+} and Mg^{2+} (PBS (-); FUJIFILM Wako Pure Chemical Corp.). Cells were isolated from the bottom of T-75 culture flasks with 2 mL of trypsin-EDTA solution (0.05 w/v% Trypsin-0.53 mM/L EDTA-4 Na solution without phenol red: FUJIFILM Wako Pure Chemical Corp.) and incubated at 37˚C for 3 hours. Flasks were shaken to disperse attached cells. To stop the effects of trypsin, 8 mL of MEM containing FBS was poured into flasks. rBMCs were transferred to centrifuge tubes, washed 3 times by centrifugation at 120×*g* using 30 mL of MEM, and then re-suspended in MEM containing 15 % FBS.

For calcified nodule formation, rBMCs were passaged in multi-well culture plates (6-well culture plates; CORNING Inc., NY, USA) for 11 days. The rBMC suspension of 2 mL at 5×10^4 cells/mL was poured into each well of a 6-well culture plate. Culture medium was renewed three times in 1 week. To promote rBMC differentiation and calcified nodule formation in each well, 10 nM of Dex (Sigma-Aldrich Co., LLC.), 1 mM of β-GP (EMD Biosciences, Inc., CA, USA), and 82 μg/mL of VC (Sigma-Aldrich Co., LLC.) were each added at a volume of 20 μL to MEM with medium renewal during the subculture. Calcified nodule formation in the subculture of rBMCs in MEM with Dex, β-GP, and VC (MEM including Dex, β-GP, and VC is abbreviated as MEM-Dex), which was the positive control, was compared to that in MEM with β-GP only (MEM with β-GP is abbreviated as MEM-GP) as the negative control. Medium was exchanged three times during the 11-day subculture of rBMCs for calcified nodule formation, and 20 μL solution at every concentration of VB_1, VB_3, or VB_5 was added to each renewed MEM-Dex and MEM-GP respectively.

5. Observations of calcified nodule aggregates formed by rBMCs in the subculture

1) Phase-contrast inverted microscopic observations

After the subculture of rBMCs for 11 days, calcified nodule aggregates deposited on the bottom of wells in 6-well culture plate were observed macroscopically as well as under a phase-contrast inverted microscope (ECLIPSE Ts2; Nikon Corporation, Tokyo, Japan).

2) Von Kossa staining for aggregated calcified nodules

Only calcium phosphate salts and calcium carbonate salts present in calcified substances are stained dark blue or black by von Kossa staining. Therefore, staining was performed after the subculture to confirm that the aggregates generated by rBMCs on the bottom of wells were calcified components, such as calcium phosphate or calcium carbonate.

Aggregated deposits in cultures of rBMCs with the addition of 10 μM of each VB in MEM-Dex were stained. Each well was washed with tap water to remove as much water as possible. A 5 % aqueous silver nitrate (FUJIFILM Wako Pure Chemical Corp.) solution was then poured into each well. The plate was placed in sunlight for 20 minutes to allow silver to react with lime phosphate in the aggregates formed by rBMCs in the wells. Reactants in the wells were washed three times with distilled water, a 5 % sodium thiosulfate (FUJIFILM Wako Pure Chemical Corp.) solution was poured into the wells for fixation, and plates were allowed to stand for 1 minute. After the reaction, cells were washed with tap water for 10 minutes and reacted with Kernechtrot stain solution (MUTO PURE CHEMICALS CO., LTD. Tokyo, Japan) for 5 minutes for nuclear staining. Cells were dehydrated using 70, 95, and 100 % ethanol sequentially, and an aqueous-based mounting medium (Crystal/Mount: Biomeda Corp., Foster, CA, USA) was then poured into wells to mount and clear cells.

6. Measurement of alkaline phosphatase activity

All procedures for measurement of alkaline phosphatase (ALP) activity were performed at 3-5°C. In the biochemical analysis of ALP activity, the supernatant in each well of all culture plates was collected after the subculture for 11

days. Each cell layer in the wells containing aggregated calcified nodules was washed three times with PBS (-). For the quantitative analysis of ALP, 1 mL of TNE buffer solution (pH 7.4), which consisted of 1 mM of 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (FUJIFILM Wako Pure Chemical Corp.), 0.1 mM of ethylene-diamine-N, N, N', N'-tetra-acetic acid tetrasodium salt tetrahydrate (FUJIFILM Wako Pure Chemical Corp.), and 10 mM of sodium chloride (FUJIFILM Wako Pure Chemical Corp.), was poured into each well and the cell layer on the plate was scraped off and transferred into a 1.5-mL microtube.

rBMCs in 1 mL of buffer solution containing 100 μL of polyoxyethylene sorbitan monolaurate (Tween®20: Tokyo Chemical Industry Co., Ltd.) were crushed using a mixer mill (MM301: Verder Scientific Co., Ltd., Tokyo, Japan) with agitation at 30 rps for 20 seconds and then a 1-minute rest interval followed by an additional agitation for 20 seconds. In the quantitative analysis of DNA, 20-μL aliquots of sonicated cell suspensions were mixed with 100 μL of Hoechst 33258 at a concentration of 2.5 μg/mL (FUJIFILM Wako Pure Chemical Corp.). Salmon sperm DNA (Life Technologies Inc., CA, USA) was used as the standard. The amount of DNA was calculated by measuring fluorescence absorbance with a multimode microplate reader (SpectraMax® Series M5; Molecular Devices, Inc., CA, USA) at an excitation wavelength of 355 nm and fluorescence emission of 460 nm.

The crushed cell suspension remaining in the microtube was used to measure ALP levels. The suspension was spun down by centrifugation at 16,000 ×*g* (Tabletop Micro Refrigerated Centrifuge Model 3520; KUBOTA Corp., Tokyo, Japan) at 4 ˚C for 1 minute. To measure ALP levels, 100 μL of *p*-nitrophenyl phosphate solution (PNPP (1×)-Substrate; Invitrogen Corporation, CA, USA) as the substrate was added to 20 μL of the supernatant and incubated at 37˚C for 30 minutes. To stop the reaction, 100 μL of NaOH at a concentration of 0.2 M was added. The amount of *p*-nitrophenol produced by reacting the supernatant with *p*-nitrophenol phosphate was evaluated by absorbance at a wavelength of 405 nm using the multimode microplate reader employed to measure the amount of DNA. The level of ALP activity in cultured rBMCs was represented as μM of *p*-nitrophenol released after a 30-minute incubation at 37 ˚C. The ALP/DNA ratio was calculated as ALP activity. Results are presented as the mean ± standard error.

7. Quantitative analysis of Ca^{2+} from calcified nodules in the rBMC subculture

The quantity of calcified aggregated nodules produced by rBMCs in the subculture was expressed as the level of Ca^{2+}, which measured using the commercially available Calcium-E test WAKO® (FUJIFILM Wako Pure Chemical Corp.). After the ALP analysis, samples in 1.5-mL microtubes were centrifuged at 15,000 ×*g* for 10 minutes. After removing the supernatant from the microtube, 1 mL of 20 % formic acid solution was added to each precipitate containing calcified nodules in the microtube. The tubes were then shaken using a laboratory shaker (Double shaker NR-3, TAITEK, Koshigaya, Japan) for 4 days at room temperature to demineralize the calcified nodules in the precipitate. After decalcification, the tubes were centrifuged at 1600 ×*g* for 10 minutes. The supernatant of 50 μL was added to 2 mL of mono-ethanolamine buffer included in the kit. To the mixture, 1 mL of methyl xylenol blue in the kit was added as the coloring reagent. After mixing well, Ca^{2+} produced in the rBMC culture was measured by absorbance at 610 nm using a fluorescence-spectrum photometer with a multimode microplate reader (SpectraMax® Series M5). Results are presented as the mean ± standard error.

8. Statistical analysis

All experiments were performed in triplicate (n = 3). Quantitative data are presented as the mean ± standard error. Statistical comparisons between the levels of ALP activity and Ca^{2+} in each well of rBMC cultures were conducted using a two-way unrepeated ANOVA followed by a post hoc analysis using the Tukey-Kramer test. Differences with P <0.001, 0.01, or 0.05 were considered to be significant. For the statistical analysis, Easy R (EZR: Ver. 1.61) was used.

Results

1. Observations of calcified nodule aggregates formed by rBMCs in the subculture

1)Phase-contrast inverted microscopic observations

A representative illustration of the densely arranged calcified nodules formed by rBMCs in

MEM-Dex as the positive control is shown in Fig. 1-a. Nodules appeared to be composed of aggregated fine particles. In the subculture of rBMCs in MEM-Dex supplemented with VB_1 (Fig. 1-b), VB_3 (Fig. 1-c), or VB_5 (Fig. 1-d), densely arranged calcified nodules were observed at the bottom of wells macroscopically and under the phase-contrast inverted microscope. As shown in Fig. 1-b, aggregates appeared to be more densely arranged in the subculture than in the positive control. The density of calcified nodules in MEM-Dex supplemented with VB_3 was similar to those of the positive control and MEM-Dex supplemented with VB_1 (Fig. 1-c). The density of calcified nodules in MEM-Dex supplemented with VB_5 was similar to those of the culture of the positive control (Fig. 1-d)**.**

Calcified nodules were not observed in the subculture of rBMCs in MEM-GP as the negative control (Fig. 2-a). Furthermore, calcified nodules were not detected in the subculture of rBMCs in MEM-GP supplemented with VB_1 (Fig. 2-b), VB_3 (Fig. 2-c), or VB_5 (Fig. 2-d).

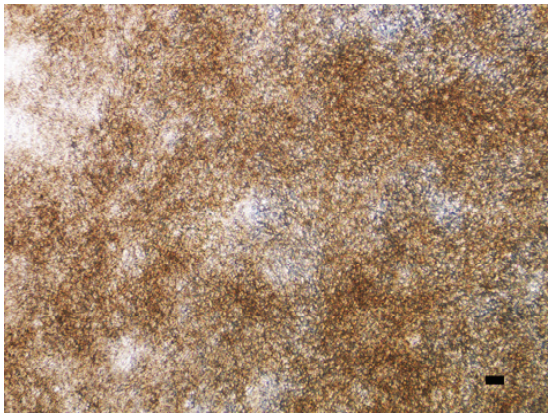

Fig. 1-a
A representative illustration of aggregated calcified nodules under a phase-contrast inverted microscope

Densely arranged calcified nodules formed by rat bone marrow cells (rBMCs) are observed. Dexamethasone (Dex), β-glycerophosphate (β-GP), and vitamin C (VC) were added to MEM (MEM-Dex) as the positive control. rBMCs are present between the area of aggregated nodules. (Bar: 100 μm)

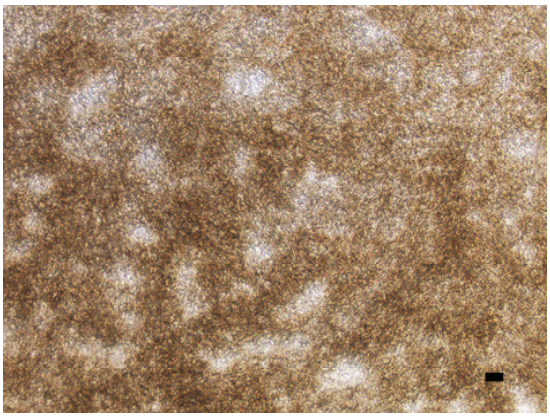

Fig. 1-c
A representative illustration of aggregated calcified nodules under a phase-contrast inverted microscope

rBMCs were cultured with the addition of vitamin B_3 (VB_3) to MEM-Dex. rBMCs are present between the arranged calcified nodule aggregates. (Bar: 100 μm)

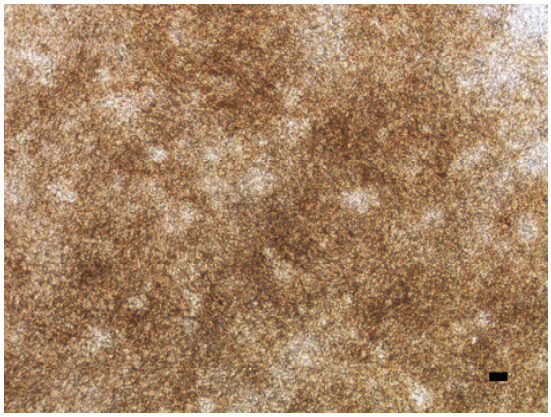

Fig. 1-b
A representative illustration of aggregated calcified nodules under a phase-contrast inverted microscope

Calcified nodules by rBMCs are observed in MEM-Dex supplemented with vitamin B_1 (VB_1). Aggregates appear to be densely arranged. (Bar: 100 μm)

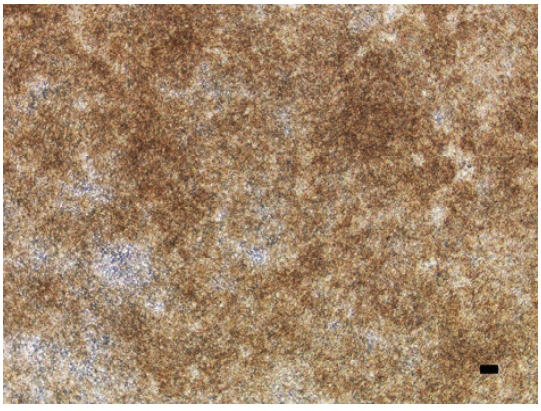

Fig. 1-d
A representative illustration of calcified nodules under a phase-contrast inverted microscope by cultured rBMCs in MEM-Dex

Nodules were formed by cultured rBMCs in MEM-Dex with the addition of vitamin B_5 (VB_5). rBMCs are present between the aggregated nodules. (Bar: 100 μm)

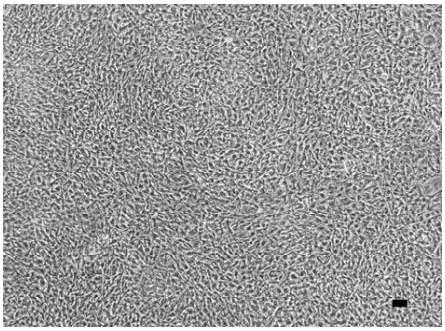

Fig. 2-a
A representative illustration of subcultured rBMCs under a phase-contrast inverted microscope

rBMCs are observed in MEM containing β-GP (MEM-GP) as the negative control. No calcified nodules are present. (Bar: 100 μm)

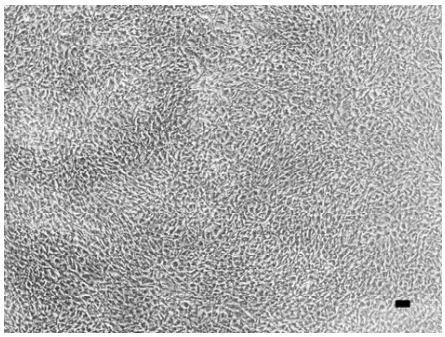

Fig. 2-b
A representative illustration of subcultured rBMCs under a phase-contrast inverted microscope

VB_1 was added to MEM-GP. Only rBMCs are observed without aggregated calcified nodules. (Bar: 100 μm)

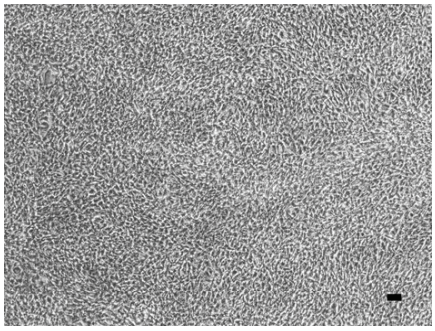

Fig. 2-c
A representative illustration of subcultured rBMCs under a phase-contrast inverted microscope

VB_3 was added to MEM-GP. No calcified nodules are present between rBMCs cultured in MEM-GP. (Bar: 100 μm)

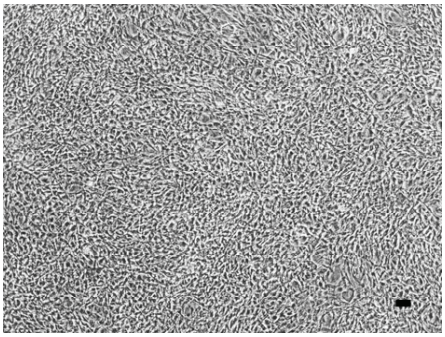

Fig. 2-d
A representative illustration of subcultured rBMCs under a phase-contrast inverted microscope

VB_5 was added to MEM-GP. No calcified nodules are present in the rBMC subculture. (Bar: 100 μm)

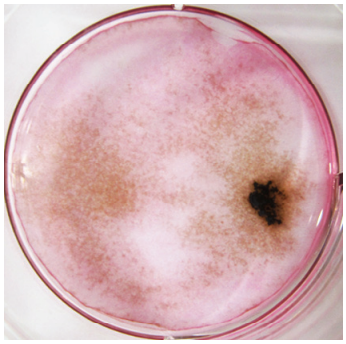

Fig. 3-a
Illustration of aggregated calcified nodules stained by the Von Kossa method on the bottom of a well: a positive control

After the subculture of rBMCs in MEM-Dex, aggregated nodules are macroscopically observed. Nodules are partially stained as darkish brown and widely as light brown.

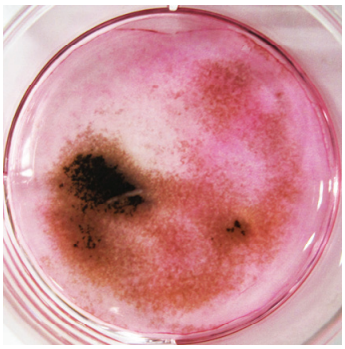

Fig. 3-b
Illustration of aggregated calcified nodules stained by the Von Kossa method on the bottom of a well: after a subculture of rBMCs in MEM-Dex containing VB_1

Calcified nodules are partially stained darkish brown in a small area and widely stained light brown in a wide area.

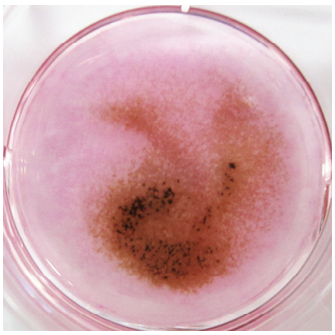

Fig. 3-c
Illustration of aggregated calcified nodules stained by the Von Kossa method on the bottom of a well: after a subculture of rBMCs in MEM-Dex containing VB_3

Macroscopically, calcified nodules are stained darkish brown in a small area and light brown in a wide area.

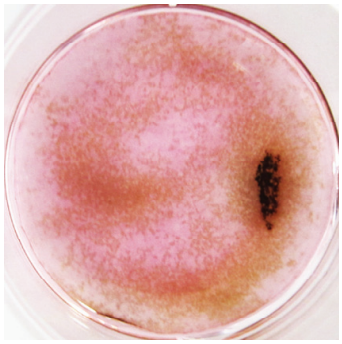

Fig. 3-d
Illustration of aggregated calcified nodules stained by the Von Kossa method on the bottom of a well: after a subculture of rBMCs in MEM-Dex containing VB_5

Aggregates are stained darkish brown in a limited area and light brown in a wide area.

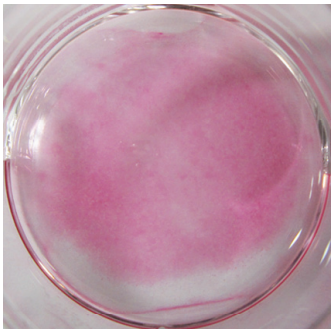

Fig. 3-e
Illustration of macroscopically observed rBMC layer on the bottom of a well: after a subculture of rBMCs in MEM-Dex as the negative control

No stained calcified nodules by the Von Kossa method are observed.

2) Von Kossa staining for aggregated calcified nodules on the bottom of wells

In MEM-Dex as the positive control, aggregated nodules stained dark brown in a narrow area and light brown in a wide area (Figure 3-a). Aggregated calcified nodules stained darkish brown or light brown in MEM-Dex supplemented with each VB (Figs. 3-b, c, d). As shown in Figs. 3-b and -c, calcified nodules in the rBMC culture stained dark or light brown in a wide area of the well with MEM-Dex supplemented with VB_1 and VB_3. Calcified nodules stained dark brown or light brown in a narrow area of wells with MEM-Dex supplemented with VB_5 (Fig. 3-d). Stained aggregates were not observed in wells with MEM-GP as the negative control (Fig. 3-e) or in any culture with MEM-GP supplemented with each VB.

2. ALP activity of rBMCs after the subculture

The ALP activity levels of rBMCs cultured in medium supplemented with 20 μL of 10 μM, 100 μM, 1 mM, 10 mM, or 100 mM of each VB solution with or without Dex and VC are shown in Figs. 4-a, b, c. The subculture of rBMCs in MEM-GP showed low ALP activity levels with no significant difference between cultures with MEM-GP supplemented with each concentration of VB.

As shown in Fig. 4-a, cells exhibited ALP activity of 0.535 ± 0.050 μM/μg DNA in MEM-Dex supplemented with 20 μL of 100 mM VB_1. This value was significantly higher than that of Dex (+) as the positive control (P <0.05). In cultures of rBMCs supplemented with different concentrations of VB_1, the mean level of ALP activity increased at higher concentrations of VB_1. No significant differences were observed between activity levels in medium supplemented with each concentration of VB_1, except for that with 20 μL of 100 mM VB_1 (P <0.05).

ALP activity of 0.568 ± 0.002 μM/μg DNA was the highest value detected among rBMCs cultured in MEM-Dex supplemented with each concentration of VB_3 (Fig. 4-b). No significant differences were noted in ALP activity levels in media supplemented with each concentration of VB_1, except for 100 mM.

ALP activity levels were reduced by culturing rBMCs in MEM-Dex supplemented with a lower concentration of VB_5. As shown in Fig. 4-c, the ALP activity level of rBMCs cultured in MEM-Dex as the positive control significantly differed from that of rBMCs in MEM-Dex supplemented with 20 μL of 10 or 100 μM VB_5 (P <0.01).

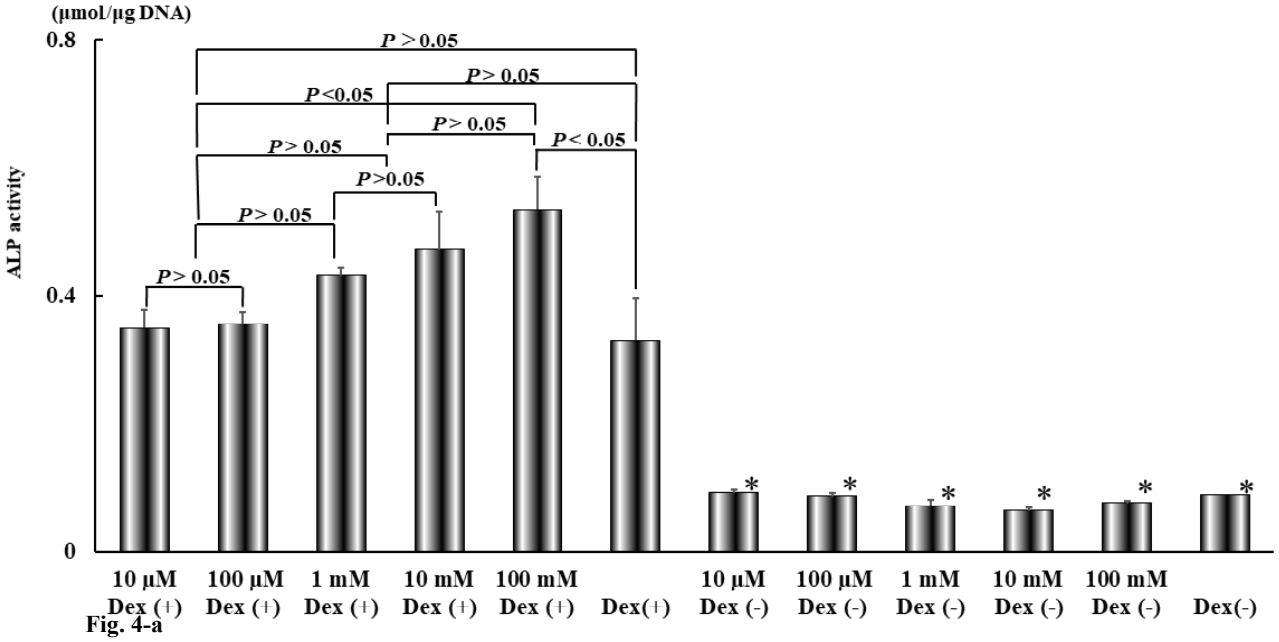

Fig. 4-a

Alkaline phosphatase (ALP) activity of rBMCs after a subculture with and without the addition of VB_1 to MEM-Dex or MEM-GP

No significant difference was observed between the ALP activities of cultured cells in MEM-GP supplemented with each concentration of VB_1. The addition of Dex to MEM significantly induced different levels of ALP activity by cultured rBMCs regardless the presence of VB_1 (P <0.001).

Dex (+): MEM-Dex,

Dex (−): MEM-GP

Concentrations of VB_1 added to MEM-Dex and MEM-GP: 10 μM, 100 μM, 1 mM,10 mM, and 100 mM

*: significantly different at each level from rBMCs cultured in MEM-Dex with VB_1 (P <0.001)

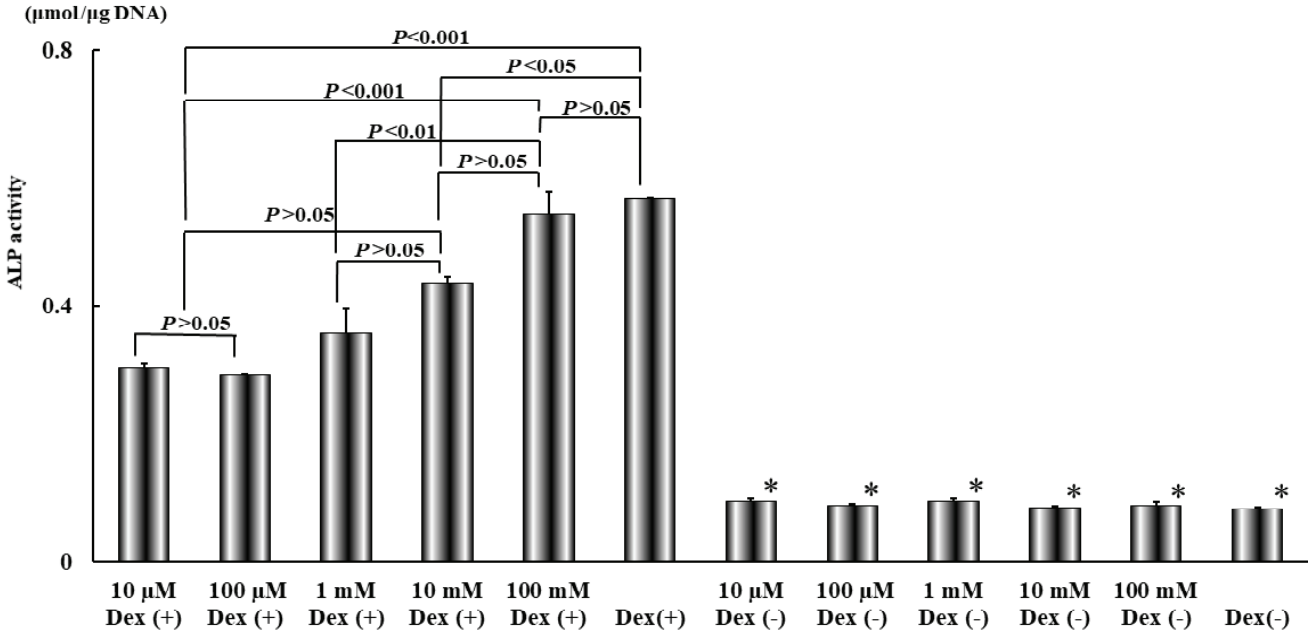

Fig. 4-b

ALP activity of rBMCs after a subculture with and without the addition of VB_3 to MEM-Dex or MEM-GP

The ALP activity of rBMCs in a subculture in MEM-GP was generally low. No significant differences were observed in ALP activity between each concentration of VB_3 added to MEM-GP. ALP activities significantly differed from that in the positive control and in the presence of 100 mM VB_3 (P <0.001).

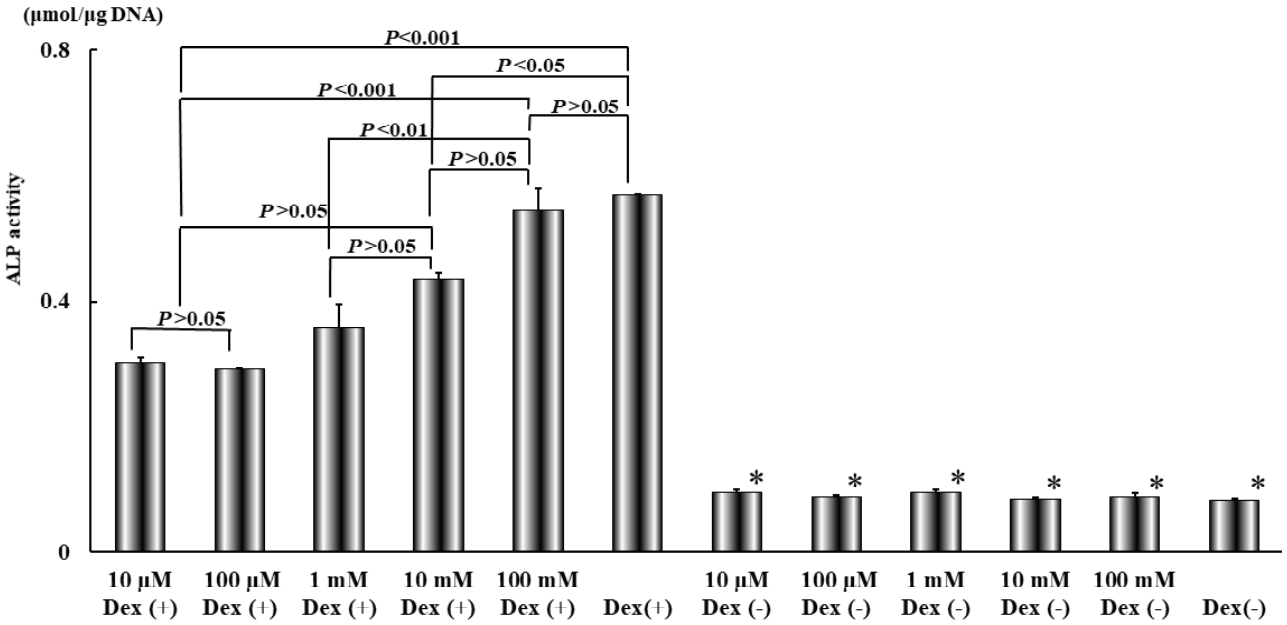

Fig. 4-c

ALP activity of rBMCs after a subculture with and without the addition of VB_5 to MEM-Dex or MEM-GP

No significant differences were observed in the ALP activity of rBMCs after a subculture in MEM-GP supplemented with each concentration of VB_5. The level of ALP activity in MEM-Dex supplemented with 10 or 100 mM VB_5 did not significantly differ from that in the positive control. The level of ALP activity in MEM-Dex supplemented with 10 or 100 mM VB_5 and that of the positive control are higher than those of MEM-Dex supplemented with the other concentrations of VB_5.

3. Quantitative analysis of Ca^{2+} from calcified nodules in the rBMC subculture

1) Effects of VB_1 on rBMCs in the subculture

In the subculture of rBMCs, the level of Ca^{2+} in calcified nodules that formed in MEM-Dex as the positive control was 6.452 ± 0.407 mg/dL. In the culture supplemented with 20 μL of 10 μM VB_1, the level of Ca^{2+} was 8.921 ± 0.141 mg/dL. The level of $Ca^{2+}$was high in the positive control and MEM-Dex supplemented with 20 μL of each concentration of VB_1. Among the Ca^{2+} levels measured in subcultures of rBMCs in MEM-GP supplemented with several different concentrations of VB_1, the highest Ca^{2+} level of 2.294 ± 0.068 mg/dL was observed with 10 mM VB_1. This value was significantly different from that of MEM-Dex (P <0.001), but not from that of MEM-GP (1.119 ± 0.124 mg/dL, P >0.05). No significant differences were noted in Ca^{2+} levels among subcultures of rBMCs in MEM-GP supplemented with different concentrations of VB_1.

As shown in Fig. 5-a, MEM-Dex or MEM-GP markedly affected the level of Ca^{2+} measured. The level of Ca^{2+} in the subculture of rBMCs in MEM-Dex as the positive control was significantly higher than that in MEM-GP (P <0.001). The increase observed in Ca^{2+} levels following the addition of VB_1 to MEM-Dex indicated that VB_1 effectively promoted nodule formation. In comparisons with calcified nodule formation in the subculture of rBMCs in MEM-Dex, significant differences were observed between Ca^{2+} levels in cultures supplemented with VB_1 at concentrations of 10 mM (P <0.05), 1 mM (P <0.01), 100 μM (P <0.001), and 10 μM (P <0.001). The level of Ca^{2+} measured in MEM-Dex as the positive control was 6.452 ± 0.407 mg/dL. The level of Ca^{2+} in rBMCs in MEM-Dex supplemented with 20 μL of 10 μM VB_1 was 8.921 ± 0.141 mg/dL, which was approximately 138.3 % that in MEM-Dex. Following the addition of 20 μL of 100 μM VB_1 to MEM-Dex, the level of Ca^{2+} measured (8.559 ± 0.220 mg/dL) significantly differed from that in the positive control (P <0.001). The level of Ca^{2+} in MEM-Dex (6.452 ± 0.407 mg/dL) increased to 8.073 ± 0.446 mg/dL following the addition of 1 mM VB_1. In the subculture treated with 20 μL of 1 mM VB_1, a significant difference was observed in the level of Ca^{2+} at 8.073 ± 0.446 mg/dL (P <0.01). The level of Ca^{2+} in MEM-Dex supplemented with 20 μL of 10 mM VB_1 was 111.3 % that in the positive control; however, a significant difference was not observed.

The level of Ca^{2+} measured was approximately 99.6 % of Ca^{2+} by rBMCs in MEM-Dex with 20 μL of 100 mM VB_1. No significant difference was observed between its value and that from the culture in MEM-Dex without VB_1.

2) Effects of VB_3 on rBMCs in the subculture

In the subculture of rBMCs, the level of Ca^{2+} in MEM-Dex as the positive control was 8.333 ± 0.220 mg/dL. In cultures with MEM-Dex supplemented with 20 μL of each concentration of VB_3, the level of Ca^{2+} in MEM-Dex supplemented with 10 μM VB_3 was the highest in an average at 10.277 ± 0.288 mg/dL.

As shown in Fig. 5-b, no significant differences were observed in Ca^{2+} levels in the calcified nodules that formed in MEM-GP supplemented with the different concentrations of VB_3. The level of Ca^{2+} was 8.333 ± 0.220 mg/dL in MEM-Dex and 10.277 ± 0.288 mg/dL in MEM-Dex supplemented with 20 μL of 10 μM VB_3 (P >0.05). Additionally, the level of Ca^{2+} in MEM-Dex supplemented with 20 μL of 100 μM or 1 mM VB_3 was 101.2 % that in MEM-Dex, with no significant differences. The level of Ca^{2+} in MEM-Dex supplemented with 20 μL of 10 mM VB_3 was 7.158 ± 0.186 mg/dL, which was 85.9 % that in the positive control. Following the addition of 20 μL of 100 mM VB_3 to MEM-Dex, the level of Ca^{2+} measured was 4.107 ± 0.186 mg/dL, which was 49.3 % that in MEM-Dex as the positive control. A shown in Fig. 5-b, the addition of high concentrations of VB_3 to MEM-Dex inhibited calcium nodule formation by rBMCs during the subculture. The levels of Ca^{2+} in cultures with MEM-GP supplemented with VB_3 were significantly lower than that in the positive control (P <0.001).

3) Effects of VB_5 on rBMCs in the subculture

The levels of Ca^{2+} detected in calcified aggregates formed by rBMCs cultured in MEM-Dex or MEM-GP supplemented with each VB are shown in Fig. 5-c. The level of Ca^{2+} obtained in the subculture of rBMCs with MEM-Dex as the positive control was significantly higher than that in MEM-GP as the negative control (P <0.001). The level of Ca^{2+} in MEM-Dex as the positive control was 11.542 ± 0.153 mg/dL. The level of Ca^{2+} produced by rBMCs cultured in MEM-Dex supplemented with 20 μL of 100 mM VB_5 was 8.134 ± 0.003 mg/dL, which was 70.5 % that in MEM-Dex as the positive control.

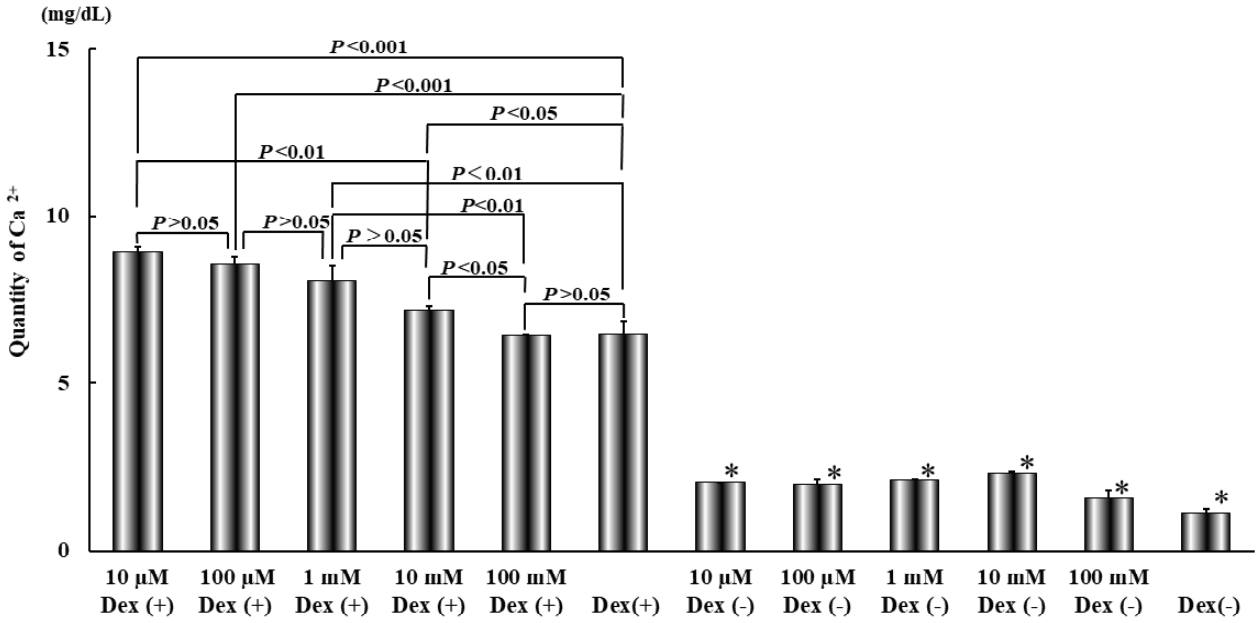

Fig. 5-a

Quantitative analysis of Ca^{2+} from calcium nodules after a subculture with and without the addition of VB_1 to MEM-Dex or MEM-GP

Significant differences were observed in Ca^{2+} levels in rBMCs cultured in MEM-GP and in MEM-Dex (P <0.001). rBMCs cultured in MEM-Dex supplemented with 10 and 100 µM VB_1 showed significantly higher Ca^{2+} levels than that in the positive control (P <0.001). No significant differences were observed between Ca^{2+} levels in rBMCs cultured in MEM-GP supplemented with each concentration of VB_1.

Dex (+): MEM-Dex

Dex (−): MEM-GP

Concentrations of VB_1 added to MEM-Dex or MEM-GP: 10 µM, 100 µM, 1 mM, 10 mM, and 100 mM

*: significantly different at each level from rBMCs cultured in MEM-Dex with VB_1 (P <0.001)

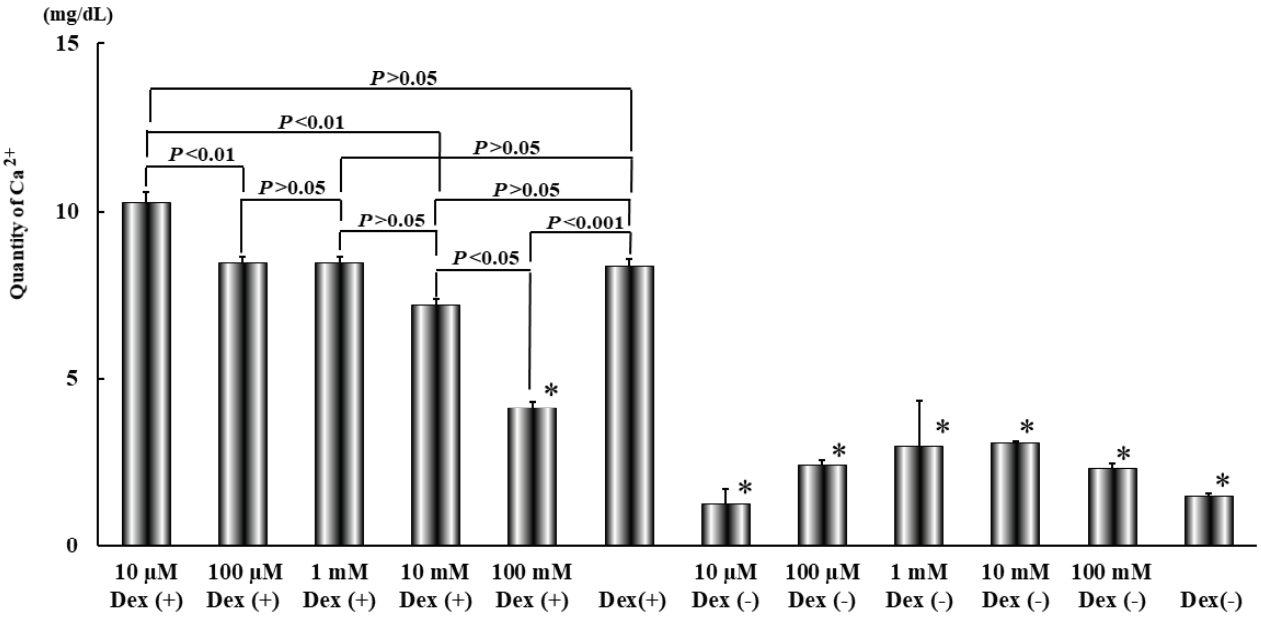

Fig. 5-b

Quantitative analysis of Ca^{2+} from calcium nodules after a subculture with and without the addition of VB_3 to MEM-Dex or MEM-GP

Ca^{2+} levels in rBMCs cultured in MEM-GP were significantly lower than those in rBMCs cultured in MEM-Dex (P <0.001). No significant differences were observed in Ca^{2+} levels between rBMCs cultured in MEM-GP with every concentration of VB_3. The level of Ca^{2+} in the positive control did not significantly differ from those in MEM-Dex supplemented with each concentration of VB_3, except for 100 mM.

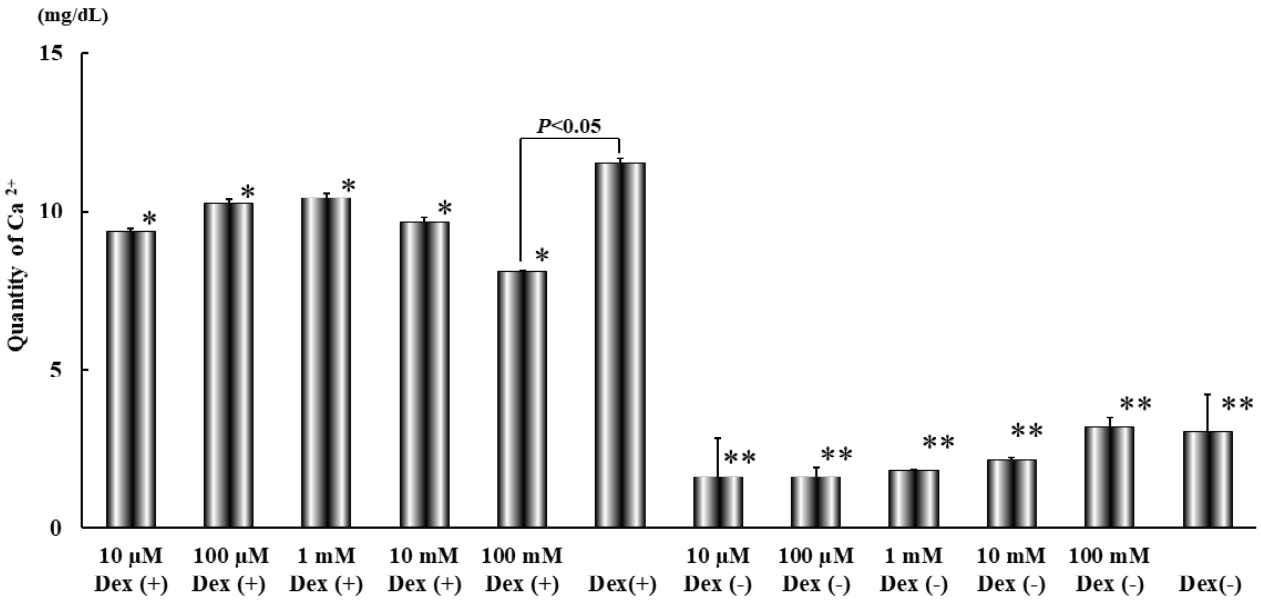

Fig. 5-c

Quantitative analysis of Ca^{2+} from calcium nodules after a subculture with and without the addition of VB_5 to MEM-Dex or MEM-GP

A significant difference was observed in Ca^{2+} levels between rBMCs cultured in MEM-Dex and MEM-GP ($P < 0.001$). A significant difference was noted in Ca^{2+} levels between rBMCs cultured in MEM-Dex supplemented with 100 mM VB_5 and those in MEM-Dex as a control ($P < 0.05$).

The level was significantly lower than the one measured in the culture using MEM-Dex as the positive culture ($P < 0.05$). Although Ca^{2+} levels were higher than 10 mg/dL in cultures using MEM-Dex supplemented with 20 μL of 100 μM or 1 mM VB_5, no significant differences were observed from that in the positive control. The level of Ca^{2+} in the positive control was higher than those in MEM-Dex supplemented with 20 μL of each concentration of VB_5. A significant difference in the level of Ca^{2+} was observed between MEM-Dex as the positive control and MEM-Dex supplemented with 100 mM VB_5, but not with other concentrations of VB_5. No significant differences were detected in the level of Ca^{2+} produced by rBMCs cultured in MEM supplemented with different concentrations of VB_5 in both MEM-Dex in positive and MEM-GP in a negative control. These results suggest that VB_5 added to MEM-Dex for the culture of rBMCs inhibited the ability of Dex to induce calcified nodule formation. In MEM-Dex supplemented with each concentration of VB_5, except for 100 mM, the average level of Ca^{2+} decreased and did not significantly differ among the concentrations examined. The addition of each concentration of VB_5 to MEM-Dex reduced the level of Ca^{2+}. No significant differences were noted, except for 100 mM.

Discussion

The present study examined the effects of VB_1 [16], VB_3 [12, 13], and VB_5 [14, 15] on the formation of osteogenic aggregates by rBMCs *in vitro* with calcified micro- or nanoparticles. MSCs in rBMCs produce ultrafine granules, such as calcium carbonate, and nodules may be formed by the aggregation of these ultrafine particles, which are considered to be lime phosphate. The results obtained herein showed that 20 μL of 10 μM VB_1 added to MEM induced the formation of significantly more calcium nodules by rBMCs. Similar results were obtained with the addition of a low concentration of VB_3 to MEM. On the other hand, the formation of calcium nodules by rBMCs in MEM supplemented with VB_5 was significantly reduced. VB_5 added to MEM attenuated the activity of rBMCs or the effects of Dex on bone formation. This *in vitro* study demonstrated that the addition of VB_1 and VB_3 besides Dex, β-GP, and VC to MEM may be more beneficial for normal bone maintenance and growth. Vitamins and bone health are closely related [18, 19]. However, the mechanisms underlying the potential link between VBs and bone health remain unclear. Based on these findings, VBs were selected for investigation in the present study on bone formation. The level

of calcification of aggregated nodules was assessed from the darkish or light brown staining of aggregates cultured in MEM-Dex as the positive control and those in MEM-Dex supplemented with each VB. Nodules that stained darkish brown were considered to be highly calcified, while those that stained light brown were calcified to a lower level.

Even MSCs derived from bone marrow require factors to induce their rapid proliferation and differentiation. A long time is needed for rBMCs to reach confluence in primary cultures [20, 21], with a previous study reporting a period of approximately 1-2 weeks [22]. Dex has long been used as an effective factor to induce *in vitro* bone regeneration by MSCs [23]. β-GP has been identified as an important phosphate source for bone mineralization by blast cells differentiated from MSCs [7]. Dex, β-GP, and VC are added to MEM in order to induce the formation of calcified nodule aggregates by rBMCs [23]. VC is one of the essential factors for bone formation because of its involvement in the production of collagen, which connects cells [24]. In the present study, Dex, β-GP, and VC were added to MEM for the induction of calcified nodule deposition by rBMCs in the subculture. Similar to VC, other vitamins may enhance the function of MSCs. A physiologically active substance that effectively promotes the proliferation and differentiation of MSCs is desired. The results of this study demonstrated that VB_1 and VB_3 sufficiently compensate for these promoting factors. Effective uses of these vitamins should be considered.

Vitamins act catalytically or directly on metabolism and physiological functions within the body. Nine types of water-soluble vitamins [25] and four types of fat-soluble vitamins [26] have been discovered based on their solubilities in water and organic solvents [27]. Water-soluble vitamins are VB_1, VB_2, VB_6, and VB_{12}, niacin, pantothenic acid, folic acid, biotin, and VC. Vitamin A (VA), D (VD), E (VE), and K (VK) are fat-soluble vitamins. Regarding VB_1, the amount needed to maintain bones under physiological conditions remains unclear [16]. VBs, particularly, VB_2, VB_6, folate, and VB_{12}, have been suggested to play a protective role in bone health [28]. A previous study indicated the involvement of VA in the early stages of osteogenesis through its enhancement of osteoblastic differentiation and, thus, it has potential as a bone-protecting agent that maintains healthy bones [29]. In bone, VC contributes to collagen synthesis [30] and VD to mineralization [31]. VE has been suggested to play an essential role in the regulation of osteoblastic differentiation and production of the bone matrix [32]. VK promotes bone organic matrix synthesis [33].

VB_1, VB_3 and VB_5 were examined as potential factors that induce MSCs to proliferate and differentiate into osteoblasts. A relationship has been suggested between VB_1 deficiency and poor bone health. VB_1 deficiency ultimately leads to cell death [16, 34]. VB_3, known as niacin, is an essential part of the coenzymes involved in metabolism [13, 35]. A previous study reported that a decrease in nicotinamide was associated with reductions in both bone mass and osteoblast differentiation [36]. The potential beneficial mechanisms of action of niacin on bone appear to involve the attenuation of inflammation [37]. In MEM-GP, which was used as the negative control in the present study, calcium deposition was not induced in any well by rBMCs in the presence of absence of VBs. These results indicate that VBs added to MEM did not directly affect the formation of calcified nodules by rBMCs. Previous studies on the effects of VB_5 reported that a deficiency of this vitamin affected osteogenesis [38]. The results of our study contradicted those in the report. No other reports have been described the effect of VB_5 on bone formation in living organisms. It is considered that VB_5 did not act as a catalyst in this *in vitro* study and inhibited the effects of Dex or β-GP. On the other hand, the amount of aggregated calcified nodules formed by rBMCs increased in MEM containing VB_1. The addition of VB_3 to MEM appeared to enhance the hard tissue-forming ability of rMSCs. VB_1 and VB_3 may act on rBMCs to enhance the ability of Dex to activate rMSCs and have the potential to promote stem cell proliferation and differentiation to osteoblasts. However, VBs did not exert direct effects on rBMCs in the subculture.

The present study investigated the effects of VB_1, VB_3 and VB_5 as factors that promote bone formation *in vitro*. Pantothenic acid, folic acid, niacinamide, pyridoxal, and thiamine, which were classified as the VB group, were present at concentrations of 1 to 2 mg/L in the commercially available culture medium used herein. There is no evidence to suggest that these additives do not affect the formation of calcified nodules by rBMCs. However, the addition of VB_1, VB_3, and VB_5 to MEM clearly affected

calcified nodule formation by rBMCs. This result indicated that nodule formation was not affected by additives in the commercially available medium. Moreover, in this *in vitro* study, FBS was added to MEM for the rBMC culture to form calcified nodules according to conventional methods. In the clinical use of regenerated bones and teeth, MSCs need to be cultured in serum-free medium or in medium containing autologous or artificial serum. However, in examinations of the regeneration of teeth and bones *in vitro*, FBS has generally been added to the culture medium of MSCs. Since the aim of this *in vitro* study was to identify the factors promoting the formation of calcified nodules, the inclusion of FBS in the culture medium of rBMCs for this purpose was acceptable.

Conclusions

The present results indicate that VB_1 and VB_3 effectively enhanced the effects of Dex and, thus, have potential in *in vitro* tooth or bone regeneration by rBMCs. The addition of VB_1 to medium increased aggregates of calcified nodules. VB_3 added to MEM functioned as an effective osteoinductive factor in the subculture of rBMCs by enhancing the effects of Dex, β-GP, and VC. The addition of VB_1 or VB_3 to MEM simultaneously with Dex, β-GP, and VC may increase the formation of calcium nodules at a higher density. The addition of VB_5 to MEM suppressed the production of aggregated calcium nodules by rBMCs. These VBs indirectly exerted their effects on rBMCs. The present results confirmed the importance of adding Dex to MEM for the proliferation and differentiation of rBMCs in subcultures.

Data Availability

Data on calcified nodule formation by rat dental pulp cells in the present study were merged to support the results obtained and included within this published article. The data analyzed in this study are available from the corresponding author upon reasonable request.

Conflict of Interest

All authors declare no conflicts of interest regarding the publication of this paper.

Acknowledgments

This study was performed in the Morphological Research Facilities, Biomaterials Research Facilities, Low-Temperature Facilities, Tissue Culture Facilities, Laboratory Animal Facilities and Photograph-Processing Facilities at the Research Laboratory Center in the Translational Research Institute for Medical Innovation of Osaka Dental University.

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(Received: October 16, 2023/
Accepted: November 17, 2023)

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