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Biochemical and Biophysical Research Communications 311 (2003) 32–38
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Physical stress by magnetic force accelerates differentiation of human osteoblasts☆

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Received 14 August 2003

Abstract

We examined the effect of magnetic force on differentiation of cultured human osteoblasts. Magnetic microparticles (MPs) were introduced into the cytoplasm of a human osteoblast cell line and the cells were cultured in a magnetic field (MF) in group MP–MF. Three groups of controls were used: cells without MPs were cultured out of MF (group C), cells without MPs were cultured in MF (group MF), and cells with MPs were cultured out of MF (group MP). The cells in group MP–MF became larger and were elongated along the axis of the magnetic poles. Appearance of alkaline phosphatase (AlPase) activity, formation of bone nodules, and calcium deposition were accelerated depending on the intensity of the magnetic field. It takes longer culture in the other three groups to exhibit these changes. Core-binding factor A1 (Cbfa1: transcription factor for osteoblast differentiation) and osteocalcin (a bone-matrix protein involved in controlling osteogenesis) were expressed earlier or stronger in group MP-MF than the other groups. Then we compared phosphorylation of mitogen-activated protein kinase (MAPK) between group MP–MF and group C. Phosphorylation of $p38^{MAPK}$ (p38) was increased in group MP–MF, while total p38 as well as total and phosphorylated forms of MAPK/ERK 1/2 and SAPK/JNK were not changed between the two groups. When a p38 inhibitor, SB 203580, was added to the culture medium in group C, AlPase activity, formation of bone nodules, and calcium deposits were completely inhibited. On the other hand, they were inhibited only partially by a MAPK/ERK 1/2 inhibitor, U-0126. Based on these results, it is concluded that (1) osteoblast differentiation is accelerated by a magnetic force, (2) this acceleration is mainly attributed to the activation of p38 phosphorylation, and (3) the stimulus induced by a magnetic field offers a new approach to osteoblast differentiation.
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Keywords: Osteoblast; Differentiation; Magnetic microparticles; Magnetic field; Mitogen-activated protein kinase

Physical force plays a crucial role in development and maintenance of many tissues including bone. Bone tissue responds to mechanical loading with bone formation and inhibition of bone resorption [1–4]. However, much less is known about cellular response to physical stimuli and this in part reflects lack of understanding of mechanotransduction mechanisms [1]. We have

☆ *Abbreviations:* MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; SAPK/JNK, stress-activated protein kinase/jun N-terminal kinase; MAPKK, mitogen-activated protein kinase kinase; AlPase, alkaline phosphatase; Cbfa 1, core-binding factor A1.

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.156

developed a novel physical stimulation method in which magnetic microparticles (MPs) were introduced into cultured myoblasts and then the cells were placed in a magnetic field [5], which allows the induction of controllable stimulus in culture for 3 weeks or more. In the present study, we applied this method to human osteoblast culture and examined morphology including AlPase activity and calcium deposit. In addition, core-binding factor A1 (Cbfa1, an essential transcription factor for osteoblast differentiation) and osteocalcin (a bone-matrix protein involved in controlling osteogenesis) were examined for essential molecules of bone development [6,7].

Mitogen-activated protein kinase (MAPK) pathways transmit environmental signals from the cell membrane to the nucleus through phosphorylation cascades, resulting in regulation of gene expression [8]. In mammalian systems, three subgroups of MAPKs have been known: extracellular signal-regulated kinases (ERK1/2), stress-activated protein kinase/jun N-terminal kinase (SAPK/JNK), and $p38^{MAPK}$ (p38). Stress kinases, SAPK/JNK and p38, have been known to respond to various stimuli, including ultraviolet radiation (UV), oxidative stress, heat shock, and so on [8]. However, cellular response is little known to the magnetic force.

In the present study, we found that magnetic force accelerated osteoblast differentiation and this effect was largely attributed to the increased phosphorylation of p38.

Materials and methods

Cell culture. A normal human osteoblast (NHOst) cell system was purchased from BioWhittaker (Walkersville, MD). The cells were seeded in 60-mm culture dishes (Nalge Nunc International, Roskilde, Denmark). The cell concentration was 2×10^6 cells/ml and maintained in an osteoblast basal medium (OBM, CC-3208: BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS: CC-4102), 200 nM ascorbic acid (CC-4398), and 200 nM gentamicin/amphotericin-B (CC-4381).

An osteoblast differentiation medium (ODM), which consisted of OBM supplemented with SingleQuots containing 200 nM hydrocortisone, 10 mM β-glycerophosphate, and 10 μM of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3] (Calbiochem, La Jolla, CA), was used for NHOst cell differentiation. The cells were transferred to the ODM and the cell concentration was adjusted to 1.2×10^4 cells/mm^3 with the aid of a hemocytometer (Erma, Tokyo, Japan). The cells were then seeded in the 60-mm culture dish (day 0 of culture). The cells were cultured up to day 21 at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The medium was renewed every 3 days.

In this study, we used total number of 252 culture dishes. We used three dishes for each analysis.

Introducing magnetic microparticles. When NHOst cells were cultured in the supplemented OBM to point 2×10^6 cells/ml, they were treated in 0.25% trypsin/EDTA (OBM, CC-5012: Clonetics, San Diego, CA, USA). Then the cell concentration was adjusted to 4×10^6 cells/ml with the aid of a hemocytometer. Fluorescein isothiocyanate-labeled MPs, 0.05 μm in diameter and suspended in a medium at a concentration of 20,000 particles/ml, were purchased from Polysciences (Warrington, PA, USA). One milliliter of this MP suspension was added to 250 ml of cultured cell suspension. Using an Electroporator II (Invitrogen, San Diego, CA, USA) at 330 V, 25 mA, and infinite resistance, the MPs were driven into the cells.

Magnetic field generator. A prototype magnetic field generator was constructed with a rare earth magnet having a stable 0.5 T surface magnetic flux density [5]. Using a whole probe (Toyo Jiki Ind., Tokyo, Japan), the magnetic field was adjusted to 0.01, 0.03, or 0.05 T at the center of the 60-mm culture dish. The magnetic field generator was put into an incubator in such a way that the magnetic poles horizontally faced each other on either side of the culture dish. When we measured the magnetic flux, the generator by the magnetic field tesla meter (Toyo Jiki Ind., Tokyo, Japan), the magnetic flux was not affected by placing a culture dish containing with MPs in the generator.

Experimental groups. The cells were grouped into 10 groups as shown in Table 1. Cells without MPs that were cultured without a magnetic field (group C); cells with MPs that were cultured without a magnetic field (group MP); and cells without MPs that were exposed to a magnetic field (group MF); and cells with MPs that were exposed to a magnetic field (group MP–MF, 0.01, 0.03 or 0.05 T); group C was added with 0.5 μM U-0126 (group C + U); group C was added with 10 μM SB 203580 (group C + SB); group MP–MF 0.05 T was added with 0.5 μM U-0126 (group MP–MF 0.05 T + U); and group MP–MF 0.05 T was added with 10 μM SB 203580 (group MP–MF 0.05 T + SB).

The culture media containing SB 203580 (a p38 inhibitor) or U-0126 (an inhibitor against mitogen-activated protein kinase kinase, MAPKK) were prepared as follows: 10 μM SB 203580 (Promega, Madison, WI) or 0.5 μM U-0126 (Promega, Madison, WI) was added to the ODM. The concentrations of the inhibitors used were based on the studies by Tokuda et al. [9] and Bhat et al. [10]. The inhibitors were dissolved in dimethyl sulfoxide (Me_2SO) to give concentrations of 50 mM and 1 μl of this solution per 1 ml of the culture medium was added. The equivalent volume of Me_2SO was added to control cultures in each experiment. The medium was replaced with fresh medium (with or without inhibitors) every 3 days. We cultured the cells up to day 21.

Analysis of cell permeability and DNA synthesis. Twenty-one dishes of each group C, group MP, group MF, group MP–MF 0.01 T, group MP–MF 0.03 T, and group MP-MF 0.05 T were used for a trypan blue-uptake test for cell viability. On days 0, 1, 3, 5, 7, 14, and 21 of the

Table 1
Experimental groups

Groups	
Group C	Cells without MPs that were cultured in 0 T
Group MP	Cells with MPs that were cultured in 0 T
Group MF	Cells without MPs that were cultured in 0.01, 0.03 or 0.05 T
Group MP–MF	Cells with MPs that were cultured in 0.01, 0.03 or 0.05 T
Group C + U	Cells were cultured in a medium containing 0.5 μM U-0126
Group C + SB	Cells were cultured in a medium containing 10 μM SB 203580
Group MP–MF 0.05 T + U	Cells containing MPs were cultured in a medium containing 0.5 μM U-0126 and cultured in 0.05 T
Group MP–MF 0.05 T + SB	Cells containing MPs were cultured in a medium containing 10 μM SB 203580 and cultured in 0.05 T

culture (three dishes for each day), the cells were dispersed in Hanks' balanced salt solution (HBSS: pH 7.5) containing 0.05% trypsin and 0.02% EDTA and suspended in 2% trypan blue in HBSS. The stained and unstained cells were counted under a microscope (40× magnification) using a hemocytometer.

Fifteen dishes of each group C, group MP, group MF, group MP–MF 0.01 T, group MP–MF 0.03 T, and group MP–MF 0.05 T were used for monitoring DNA synthesis. Bromodeoxyuridine (BrdU) was added to ODM at the final concentration of 0.01% on day 0.5 (12 h) of culture and the cells were incubated in this medium for 1 h, 1, 2, 3, and 5 days. Cells in three dishes were fixed with absolute ethanol on each culture day. Cells labeled with BrdU were visualized using a cell proliferation kit (Amersham International Plc., Amersham, UK) and counted under a microscope with the aid of hemocytometer. In our preliminary experiments, almost 100% of cells were labeled by BrdU on culture day 3 and thereafter (data not shown).

Data of trypan blue and BrdU positive cells were statistically analyzed for differences on a day-by-day basis. A probability value of <0.01 was considered to be statistically significant.

Morphology of cultured cells, alkaline phosphatase activity, and calcium deposits. Three dishes of each group C, group MP, group MF, group MP–MF 0.01 T, group MP–MF 0.03 T, and group MP–MF 0.05 T were used on every day 0 to 21 of the culture. The numbers of bone nodules were counted in randomly selected 10 areas (the area inside the photomask, 0.5 cm × 0.75 cm) for one dish, using an inverted phase contrast microscope (TE300 Eclipse, HB-10103AF, Nikon, Tokyo, Japan). These data were statistically analyzed for differences on a day-by-day basis. A probability value of <0.01 was considered to be statistically significant.

The same dishes were used for detecting alkaline phosphatase (AlPase) activity and calcium deposits on days 1, 7, 15, and 20 of the culture. The AlPase activity was detected histochemically by the naphthol AS-phosphate method [11]. In brief, cultures were washed in HBSS and distilled water and then incubated in an incubation medium containing 3% naphthol AS-BI phosphate sodium salt, 5% *N*,*N*′-dimethylformamide, and 5% fast red violet LB salt in 0.1 M Tris buffer (containing 10% $MgCl_2$, pH 8.5) for 30 min at 37 °C. Then the same cultures were fixed with 4% formaldehyde and calcium deposits were stained by the von Kossa method [12]. Namely, the cultured cells were stained with 3% silver nitrate for 1 h and then washed for 3 min in 5% sodium thiosulfate. The cells were examined under a light microscope.

Reverse transcription polymerase chain reaction (RT-PCR). We examined core-binding factor A1 (Cbfa1) mRNA on days 0, 1, 3, 5, and 7 of culture, osteocalcine mRNA on days 1, 3, 6, 9, 12, 15, 18, and 21, and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), on days 1, 3, 5, 6, 7, 9, 12, 15, 18, and 21. Three dishes were used for each day for each group C and group MP–MF 0.05 T.

Total RNA from the cultured cells was extracted using Isogen (Nippon Gene, Tokyo, Japan) based on the method of Chomczynski [13]. One microgram of total RNA was used for reverse transcription with SuperScript II (Gibco Laboratories, Rockville, MD). The primers used to amplify Cbfa1 cDNA fragments were: sense, 5′-TTT CTC GAG TGG TTA ATC TCT GCA GGT-3′; and antisense, 5′-TTT GAA TTC GCT CAC GTC GCT CAT CT-3′. The primers used to amplify osteocalcine cDNA fragments were: sense, 5′-AGC CCT CAC ACT CCT CGC CCT ATT G -3′; and antisense, 5′-GGA GAG GAG CAG AAC TGG GGT TGC C-3′. G3PDH cDNA was amplified using the primer supplied by TOYOBO (Osaka, Japan). The PCR was performed after denaturation for 3 min at 94 °C. The PCR conditions were 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 50 s at 72 °C followed by extension at 72 °C for 10 min for Cbfa1; 30 cycles of 45 s at 94 °C and 45 s at 55 °C followed by extension for 72 °C for 1 min for osteocalcin; and 24 cycles of 45 s at 94 °C and 45 s at 60 °C followed by extension for 2 min at 72 °C for G3PDH.

Protein extraction and Western blot analysis. Whole cell proteins of the cultured cells were extracted with Tricine SDS–PAGE sample buffer from culture days on 1, 3, 6, 9, 12, 15, 18, and 21 (three dishes for each day for each group C and group MP–MF 0.05 T). The proteins were subjected to Tricine SDS–PAGE and then electrophoretically transblotted onto a nitrocellulose membrane (Hybond-C super, Amersham International Plc., Amersham, UK). A monoclonal anti-bovine osteocalcin antibody, clone OCG2 (Takara Shuzo, Shiga, Japan), and monoclonal anti-β-actin antibody, clone AC-74, IgG2a (Sigma Chemical, St. Louis, MO), were used as primary antibodies at a dilution of 1:1000. After the membranes had been blocked for 1 h at room temperature in PBS containing 0.1% Tween 20 and 5% non-fat dried milk, they were incubated overnight at 4 °C with either primary antibody. Detection was carried out using an ECL Western blotting analysis system (RPN 2108: Amersham International Plc., Amersham, UK). The membranes were washed and incubated with LumiGLO, and detected by exposure to ECL X-ray film. The cytosolic protein content was determined using a Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, Ontario, Canada) using bovine serum albumin as a standard.

Analysis of phosphorylation status of MAPK. The phosphorylation status of MAPK was analyzed from 0, 10, 30 min, 1, 3, 5 h, and 7, 14, 21 days of culture by using three dishes for each time period for each group C and group MP–MF 0.05 T. To obtain a protein sample, the cultured cells were washed with HBSS, lysed by adding an SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, at 25 °C, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue), and sonicated. After heating to 95 °C for 5 min, the protein samples were separated by SDS–PAGE and blotted onto polyvinylidene difluoride membranes (Hybond-P, Amersham International Plc., Amersham, UK). A biotinylated protein maker was also loaded to determine molecular weight.

Western blots were performed for analysis of the activation of p38 (Thr180/Tyr182), ERK1 (p44)/2 (p42) (Thr202/Tyr204), and JNK/SAPK (Thr183/Tyr185) using primary antibodies (rabbit polyclonal IgG) specific to the phosphorylated and total forms of the three MAPKs (PhosphoPlus MAP Kinase Antibody Kit, New England Biolabs, Beverly, MA). The membranes were blocked with blocking buffer (Tris buffered saline, pH 7.6, containing 0.1% Tween 20, and 5% non-fat dried milk) for 3 h at room temperature and incubated with the primary antibody with gentle agitation overnight at 4 °C. After extensive washing, the membranes were incubated for 1 h at room temperature with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) and an anti-biotin antibody conjugated to HRP (PhosphoPlus MAP Kinase Antibody Kit, New England Biolabs, Beverly, MA) for detection of MAPKs and the biotinylated protein marker, respectively. The membranes were washed, incubated with LumiGLO, and exposed to ECL X-ray film (Amersham International Plc., Amersham, UK). The blotting images were scanned with a computer-associated scanner and the densities of the bands were analyzed with NIH image (National Institutes of Health, USA).

Results

Uptake of trypan blue and BrdU

There was no difference in the percentages of viable cells (trypan blue-negative cells) or in the percentages of BrdU-uptake cells among group C, group MP, group MF, and group MP–MF 0.01, 0.03, 0.05 T (Fig. 1).

Morphology of cultured osteoblasts in the magnetic force

Differentiation of human osteoblasts in group C was compared with that in group MP, group MF, and group MP–MF (0.01, 0.03, and 0.05 T). In the group C the osteoblasts were thin spindle-shaped. The cells are

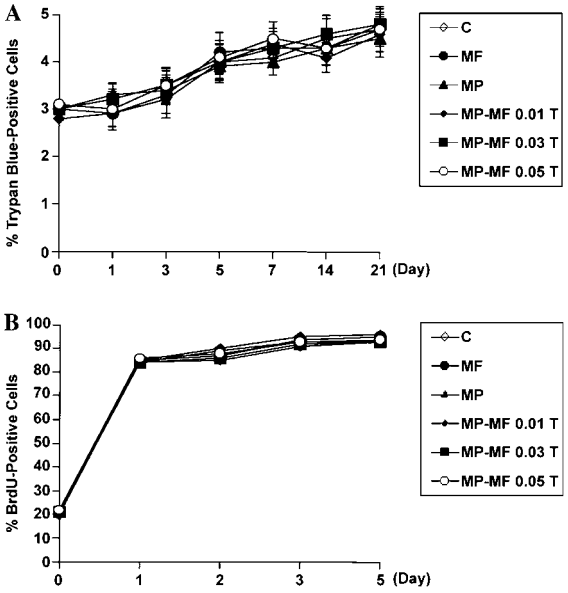

Fig. 1. Percentages of cells showing uptake of trypan blue (A) and bromodeoxyuridine (BrdU: B). There were no differences between group C, group MF, group MP, and group MP–MF 0.01, 0.03, 0.05 T in cell viability (trypan blue-negative) or BrdU uptake. Values are shown as means ± standard deviation (SD).

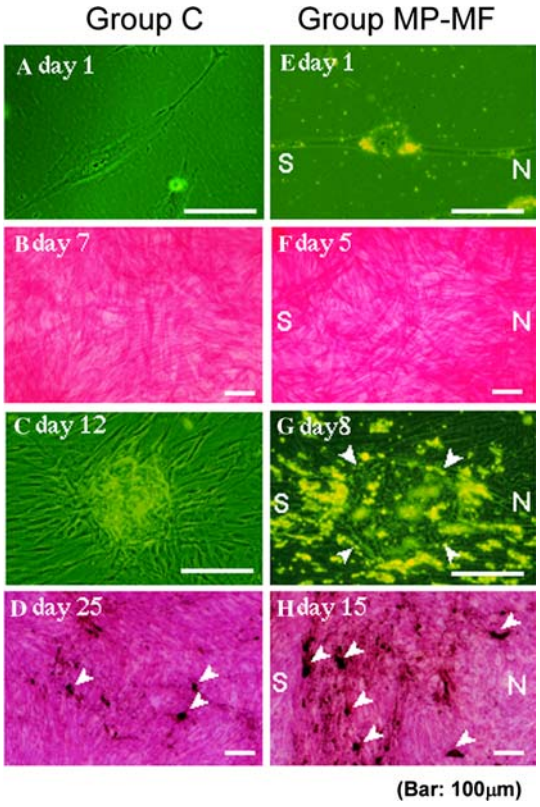

Fig. 2. Phase-contrast microscopic examination of cultured osteoblasts (A, C, E, and G) and conventional microscopy after staining with AlPase and von Kossa's silver impregnation (B, D, F, and H). Group C: cells were spindle-shaped on day 1 (A) and bone nodules were formed on day 12 (C). AlPase activity (red) was detected, but calcium deposits (brown) were not seen on day 7 (B). Both Alpase activity and calcium deposits were detected on day 25 (D). Group MP–MF 0.05 T: cells were elongated and oriented in the north–south direction of the inducing magnet and MPs gathered in the cytoplasm near the both ends of the nucleus on day 1 (E). Bone nodules were formed on day 8 (G). AlPase activity was found on day 5 (F). Calcium deposits were seen on day 15 (H). N, S: directions of north and south poles of the magnetic field. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

oriented randomly on day 1 (Fig. 2A). They began to form colonies by day 3. The number and size of colonies increased, though each cell was oriented randomly in a colony on day 7. AlPase activity was detected on day 7 (Fig. 2B). The cells in a colony tended to arrange parallely on day 10. The accumulation of cells to form a three-dimensional nodule has been called a bone nodule [14–16]. A few bone nodules appeared on day 12 (Fig. 2C) and the number of bone nodules increases with elapse of culture time (Fig. 3). Calcification detected by von Kossa staining was first observed on day 25 (Fig. 2D).

Groups MF, MP, and MP–MF 0.01 T showed the same tendency as group C, though first bone nodules appeared on day 11 in these groups.

Figs. 2E–H show group MP–MF cultured at 0.05 T magnetic field (group MP–MF 0.05 T). On day 1, the cells became oriented in the north–south direction of the inducing magnet along the axis of the magnetic poles and MPs gathered in the cytoplasm near both ends of the elongated nucleus (Fig. 2E). Colonies were formed on day 3. The tendency that the cells oriented in the direction parallel to the magnetic field continued up to day 3 but decreased thereafter. Nevertheless the parallel arrangement of the cells maintained better as compared to the group C. AlPase activity was detected on day 5 (Fig. 2F). The first bone nodules began to form on day 8 at 0.05 T (Figs. 2G and 3). The number and size of bone nodules increased by longer culture. On day 15, the cells showed strongly positive staining by the von Kossa method (Fig. 2H). In group MP–MF 0.03 T, first bone nodules appeared on day 10 and von Kossa staining became slightly positive on day 18.

Fig. 3 shows the number of bone nodules seen in different experimental groups over the course of the experiment. Statistical analysis revealed that more numerous bone nodules were formed in group MP–MF 0.05 T ($p < 0.001$) and group MP–MF 0.03 T ($p < 0.01$) than in groups MP–MF 0.01 T, MF, MP, and C. The differences were also found between group MP–MF 0.05 T and Group MP–MF 0.03 T ($p < 0.01$). However, no significant differences were seen among groups MP–MF 0.01 T, MF, MP, and C.

Expression of Cbfa1 and osteocalcin

Expression of Cbfa1 was examined from day 0 to day 7 of the culture period by RT-PCR (Fig. 4) in group C

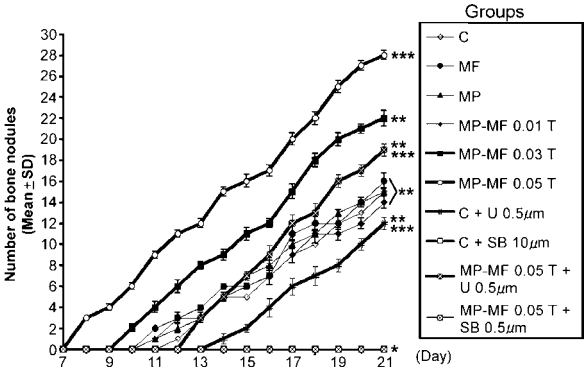

Fig. 3. Number of bone nodules in $0.5 \times 0.75\,cm^2$. Bone nodules were formed on day 12 of culture and increased in number in the control group (C). Three experiments in group MP–MF were shown by the intensity of the magnetic field, 0.01, 0.03, and 0.05 T. Bone nodule differentiation was accelerated in order in 0.05 and 0.03 T magnetic field while no differences were seen among groups C, MF, MP, and MP–MF 0.01 T. Cultures in the media containing SB 203580 (C + SB 10 μM; C + SB 20 μM) had not formed bone nodules. U-0126 inhibited formation of bone nodules depending on its concentration (C + U 0.5 μM; C + U 1 μM). Significant differences were found between two groups shown by asterisks: $*p < 0.001$, $**p < 0.01$, and $***p < 0.01$, respectively. Values are shown as means ± SD.

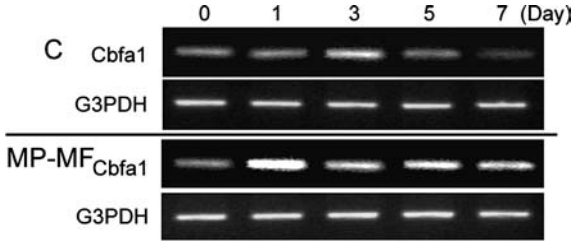

Fig. 4. Expression of core-binding factor A1 (Cbfa1) was examined from day 1 to day 7 of the culture period by reverse transcription polymerase chain reaction (RT-PCR). The expression level of Cbfa1 increased up to day 3 and decreased from day 5 in group C. On the other hand, Cbfa1 mRNA is strongly increased from day 1–7 during culture in the 0.05 T magnetic field. House-keeping gene, G3PDH, expression was not changed during time point.

and MP–MF 0.05 T. The expression level of Cbfa1 was similar in both the groups on day 0. In group C, it slowly increased to day 3 and then decreased on days 5 and 7. On the other hand, in group MP–MF 0.05 T, it reached a maximum on day 1 and then decreased (Fig. 4).

Osteocalcin was examined on day 1 through day 21 by Western blot analysis (Fig. 5). The expression level of osteocalcin appeared on day 9 and increased during culture in group C. On the other hand, osteocalcin appeared on day 6 and increased in group MP–MF 0.05 T. The expression profile of osteocalcin obtained by RT-PCR analysis of osteocalcin mRNA expression was the same as that obtained by Western blot analysis (data not shown).

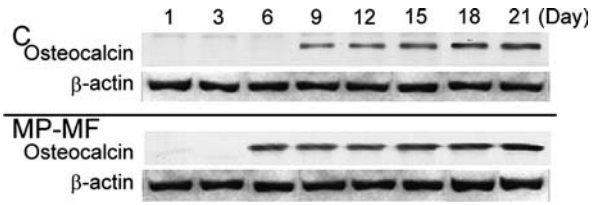

Fig. 5. Western blot analysis of osteocalcin expression. Whole cell proteins from group C and group MP–MF osteoblasts cultured up to 1 day were blotted onto nitrocellulose membrane. Lane C: osteocalcin is increased during culture in normal environment. Lane MP–MF: osteocalcin is strongly increased from day 6–21 during culture in the 0.05 T magnetic field. Lane β-actin: expression level of a house-keeping protein, β-actin, did not change during culture.

Effect of MAPK inhibitors

Fig. 3 also shows the effect of the inhibitors on the number of bone nodules. In group C, bone nodules were first formed on day 12 and increased in number thereafter. The cells did not form bone nodules in the medium containing a p38 inhibitor (group C + SB and group MP–MF 0.05 T + SB). A MAPK/ERK 1/2 inhibitor, U-0126, partially inhibited formation of bone nodules (group C + U and group MP–MF 0.05 T + U). The first appearance of bone nodules was delayed in group MP–MF 0.05 T + U. However, this group formed more nodules than group C after day 19.

Phosphorylation of MAPK cascade

To determine the effects of a magnetic force environment on MAPK cascades, the phosphorylation statuses of various MAPK cascades, i.e., p38, ERK1/2, and SAPK/JNK, were examined (Fig. 6). In group C, phosphorylated and total forms of p38 culture showed

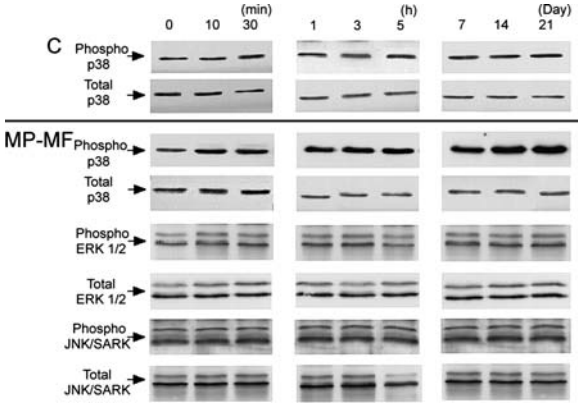

Fig. 6. MAPK phosphorylation was examined by Western blot analysis. Phosphorylation status of MAPK in control culture (C) and at 10 and 30 min, 1, 3, and 5 h, and 7, 14, and 21 days after transfer to culture in the 0.05 T magnetic field (MP–MF). Phosphorylated p38 was increased in the 0.05 T magnetic field, whereas ERK1/2 MAPK and SAPK/JNK were not changed. Total forms of p38, ERK1/2 MAPK, and JNK/SAPK were not changed.

no change throughout the culture period. When the cells were placed in the magnetic field (group MP–MF 0.05 T), the intensity of the bands was gradually increased at 10 min through day 21, while total forms of p38 were not changed. Phosphorylated and total forms of ERK1/2 and SAPK/JNK were not changed in both group C and group MP–MF 0.05 T throughout the culture period.

Discussion

We originated a physical stimulation method, in which magnetic microparticles were introduced into cultured cells and the culture was placed in a magnetic field [5]. This physical stimulation accelerated differentiation of myoblasts [5], as well as osteoblasts shown in the present study in which the appearance of AlPase activity, formation of bone nodules, and calcification were seen earlier. In the present study we also found that this osteoblast differentiation acceleration is mainly attributed to the activation of p38 phosphorylation.

A few papers have been published on the mechanism of physical force affecting osteoblast differentiation via so-called mechanoreceptor. Kotani et al. [17] investigated the effects of a strong static magnetic field (8 T) on bone formation in both vivo and vitro systems recently. They suggested that the magnetic parallel alignment of MC3T3-E1 osteoblasts accelerated the differentiation to express AlPase activity and calcification. In the present study, NHOst cells were also oriented parallely until day 3, which might bring in early differentiation. However, we still do not know what kind of signal transduction system is engaged in this.

Some studies have shown that the osteoblast differentiation is accelerated by the MAPK-related intracellular signals initiated by ECM (extracellular matrix)-integrin interaction [18,19]. Matsuda et al. [20], Peverali et al. [21], and Ziros et al. [22] showed increased MAPK phosphorylation in osteoblasts on silicon membrane that was continuously stretched. Following the idea that integrin receptors act as mechanotransducers, Pommerenke et al. [23] placed osteoblastic cells on integrin-coated paramagnetic microbeads, cultured in a magnetic field, and found activation of focal adhesion kinase (FAK) and MAPK.

The stress kinase (p38 and SAPK/JNK) pathways can be rapidly activated by a number of common stress agents, including heat shock, osmotic shock, UV radiation, X- and γ-rays, and oxidative stress [8]. It has been known that p38 plays an important role in differentiation of various cells, such as adipocyte differentiation of 3T3 Ll fibroblasts [24], myogenic differentiation of C2C12 myoblasts [25], and L8 myoblasts [26], and neuronal differentiation [27,28]. Recently, Suzuki et al. [29] and Yuge et al. [30] reported the role of p38 on osteoblast differentiation. The present study proved that a physical stimulation applied through magnetic force environment increased p38 phosphorylation to accelerate osteoblast differentiation, and that p38 inhibitor, SB 203580, inhibited the osteoblast differentiation. These results suggest that the stress environment activated p38-related cell differentiation mechanism, which is to be elucidated.

Cbfa1, a transcription factor that belongs to the runt domain gene family, is an essential factor for osteoblast differentiation [31–33], and binds the promoter region of the genes related to osteoblast differentiation such as osteopontin and osteocalcin [34–37]. Cbfa1 also regulates the transcriptional activity of the AlPase gene [38]. It was proved in the present study that osteoblasts stimulated by magnetic force expressed Cbfa1, osteocalcin, and AlPase activity earlier than osteoblasts in normal culture condition.

Here we proposed that a physical stress applied through magnetic force accelerated differentiation of cultured osteoblasts by increasing phosphorylation of p38, at least in part. The method using MP-containing cells cultured in a magnetic field enables to culture cells for sufficiently long period for studying cell differentiation.

This method will provide an effective stimulation technique in cell biology and other fields for cell differentiation. Furthermore, the use of osteoblasts and myoblasts [5] differentiated by this method enables us to open the possible new approach in regeneration medicine.

Acknowledgments

We are grateful to Mr. Toshiaki Mouri of Himeji Denshi Co. for providing technical information. We also thank Mrs. N.J. Johnson for comments on the manuscript. This study was supported by the "Ground Research Announcement for Space Utilization" promoted by the National Space Development Agency of Japan and the Japan Space Forum (to L.Y.), "Space Utilization Research" from ISAS (to L.Y.), and by Grants-in-Aid for scientific research (C), No. 10838021 and No. 12832032 (to L.Y.), from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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