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ABSTRACT

Although the promotional effects on osteoblasts of pulsed electromagnetic fields have been well-demonstrated, the effects of static magnetic fields (SMF) remain unclear; nevertheless, magnets have been clinically used as a 'force source' in various orthodontic treatments. We undertook the present investigation to study the effects of SMF on osteoblastic differentiation, proliferation, and bone nodule formation using a rat calvaria cell culture. During a 20-day culture, the values of the total area and the number and average size of bone nodules showed high levels in the presence of SMF. In the matrix development and mineralization stages, the calcium content in the matrix and two markers of osteoblastic phenotype (alkaline phosphatase and osteocalcin) also showed a significant increase. Accordingly, these findings suggest that SMF stimulates bone formation by promoting osteoblastic differentiation and/or activation.

KEY WORDS: static magnetic fields, osteoblast, bone nodule, alkaline phosphatase, osteocalcin.

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INTRODUCTION

The proliferation and/or differentiation of osteoblasts, which are responsible for the growth, remodeling, and repair of bone, is modulated by several extracellular factors, such as cytokines and hormones. Bone formation is also affected by pulsed electromagnetic fields. At present, pulsed electromagnetic fields are extensively applied in clinical treatments involving the non-union of bone fractures, bone grafts, osteotomies, fresh fractures, osteonecrosis, and osteoporosis (Bassett, 1993). As for the effects of pulsed electromagnetic fields on bone, much evidence has suggested that they enhance the activities of osteoblasts, i.e., proliferation (De Mattei *et al.,* 1999) and differentiation (Takano-Yamamoto *et al.,* 1992; Landry *et al.,* 1997), the expression of bone morphogenic protein-2 and -4 (Nagai and Ota, 1994; Bodamyali *et al.,* 1998), extracellular matrices (Heermeier *et al.,* 1998), alkaline phosphatase (Vander Molen *et al.,* 2000), and net flux and the uptake of calcium (Ozawa *et al.,* 1989; Fitzsimmons *et al.,* 1994). The inhibition of osteoblastic differentiation (McLeod and Collazo, 2000) was also suggested. Although pulsed electromagnetic fields yield both a magnetic field and an electric current, no definite conclusion can be drawn as to which factor is more responsible for bone formation.

Rare-earth magnets, which generate SMF, have also been used advantageously as a 'force source' in orthodontic treatments, such as molar distalization, palatal expansion, and impacted tooth movement (Vardimon *et al.,* 1989, 1991; Bondemark and Kurol, 1992; Noar and Evans, 1999). Nevertheless, there is little evidence for the advantage of SMF application, except as a source of force. Recently, some studies have suggested that SMF may increase the rate of bone repair (Darendeliler *et al.,* 1997) and new bone deposition (Darendeliler *et al.,* 1995), and prevent decreases in bone mineral density caused by surgical invasion or implantation (Yan *et al.,* 1998).

To date, there have been no studies examining whether SMF directly influences the proliferation and differentiation of osteoblasts. The purpose of the present study, therefore, was to investigate the effects of SMF on bone formation, as well as rat osteosarcoma osteoblast-like cells (ROS 17/2.8 and UMR 106), in a fetal rat calvaria cell culture system.

MATERIALS & METHODS

Static Magnetic Fields—Exposure System

In the present study, neodymium-iron-boron magnet disks (9.5 mm in diameter and 1.4 mm thick; NEOMAX, Sumitomo Co., Osaka, Japan) and 48-well plastic culture plates (0.75 mm thick) were used. Fig. 1 is a diagrammatic representation of the placement of the magnet and the distribution of magnetic flux densities. The magnet was placed below the well to expose the cultures to north fields. The magnetic flux density was monitored with a Gauss meter (SERIES 9900, F.W. BELL, Orlando, FL, USA) at the bottom of each well,

where rat calvaria cells attached themselves to the culture plates. The magnetic field showed an average flux of 160 mT. Two adjacent wells used for culture were set apart by more than 10 mm as the edge-to-edge distance, so that the influence of the fields of the adjacent magnets would be excluded. In the SMF group, the cells were subjected to continuous SMF exposure. In the control group, the non-magnetic disks of neodymium-iron-boron were placed below the wells. The culture plate of the control group was placed next to that of the experimental group in the same incubator. The flux density values of the wells of the control culture plates were no greater than 0.05 mT, the level of the natural magnetic field of the earth. In our preliminary study, bone nodule formation was examined under SMF strengths of 280 or 340 mT, with 2 or 3 magnets, respectively, in a pile. No apparent differences, however, were noted in comparison with 160 mT (data not shown). Accordingly, 160 mT was used in this study.

Animal Treatment

Our animal use protocol was reviewed and approved by the committee for the care and use of laboratory animals at Kyushu University.

Cell Culture

Rat calvaria cells were isolated by the method of Bellows *et al.* (1986). Briefly, pieces of calvariae from 21-day-old fetal Sprague-Dawley rats were digested in a solution containing 0.1% collagenase and 0.05% trypsin at 37°C. After 10-minute digestion, the solution was discarded, and a new enzyme solution was added to the sediment and re-digested for 10-20 min. Isolated cells were maintained in α-Minimum Essential Medium containing 10% fetal bovine serum and antibiotics (100 μg/mL penicillin, 50 μg/mL gentamicin, and 0.25 μg/mL fungizone) at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. After 48 hrs, the cells were plated at a density of 6000 cells/well in 48-well plates. At the beginning of the culture, 10 mM of β-glycerophosphate, 50 μg/mL of ascorbic acid, and 10^{-8} M dexamethasone were added to the medium. ROS 17/2.8 and UMR 106 cells were also cultured in Dulbecco's Modified Eagle's Medium. Each medium was changed every other day. After days 2-20 of the culture, cells were washed with phosphate-buffered saline (PBS) three times. They underwent lysis in lysate buffer (0.1% Triton X-100, 25 mM Tris-HCl buffer) for the determination of alkaline phosphatase (ALP) activity and protein, and were extracted with 1% trichloroacetic acid for calcium determination. Thereafter, cells were scraped off and transferred to microtubes. They were treated with ultrasonics for 30 sec and centrifuged for 30 min at 10,000 x *g*, and the supernatants were collected. Aliquots were used for each determination.

Bone Nodule Formation Assay

For the bone nodule formation assay, mineralized extracellular matrices were stained by the von Kossa technique. Cells were washed with PBS three times, followed by fixation with 3.7% paraformaldehyde in 3.5% sucrose for 10 min. They were washed three times with PBS and distilled water, respectively, and stained with 5% $AgNO_3$ solution for 1 hr. After being stained, they were washed with distilled water three times and fixed in 3% $Na_2S_2O_3$ solution for 3 min. The area and the number of bone nodules stained by von Kossa were measured with the use of computerized image analyzer software (MCID-TFM ver.3.0, Canada).

Proliferation Assay

To determine cell proliferation, we detached cells from the culture wells with 0.05% trypsin/EDTA on days 2, 4, 6, 8, and 10, and counted them using a hemocytometer.

Alkaline Phosphatase Activity and Protein Assay

ALP activity was determined in the cell lysate with the use of an alkaline phospha B-test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples of the supernatants were added to *p*-nitrophenyl phosphate as the substrate and reacted for 15 min. The reaction was stopped with 0.02 N NaOH, and the products were determined by means of a spectrophotometer at 405 nm. Cell lysates were also analyzed for protein content by means of a BCA protein assay kit (Pierce, Rockford, IL, USA), and activity was normalized for total protein concentration.

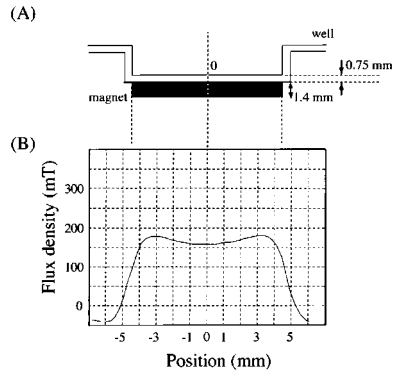

Figure 1. Diagrammatic representation of magnet placement **(A)** and the distribution of magnetic flux density **(B).**

Calcium Assay

Calcium content *per* well was determined with the use of a calcium C-test Wako kit (Wako), which is based on the o-cresolphthalein complexon color development method. Samples of the extractions were added to the reaction buffer and the color development solution. After 5 min, we determined the calcium content in the samples by measuring the absorbance at 570 nm.

Osteocalcin Assay

Osteocalcin concentrations in the conditioned media were measured *via* an osteocalcin rat enzyme-linked immunosorbent assay (ELISA) system (Amersham Pharmacia Biotech K.K., Tokyo, Japan). The conditioned media were reacted with anti-osteocalcin antibody, horseradish peroxidase-conjugated antibody, and tetramethylbenzidine as the substrate. The reaction was stopped with 1 N sulfuric acid, and the absorbance was measured at 450 nm.

Statistical Analysis

Numerical values are expressed as the mean ± SD, n = 4 *per* group. In all studies, three similar experiments were performed for each type of experiment. Statistical differences among the experimental groups were evaluated by analysis of variance followed by Student's *t* test; p values < 0.05 were considered statistically significant.

RESULTS

To investigate the effect of SMF on bone nodule formation, we cultured rat calvaria cells. They reached confluence on days 5-6, and bone nodules could be seen on days 8-9. The total area of the nodules clearly increased with time. At the end of the experimental period (day 20), culture plates were stained by the von Kossa technique. As shown in Fig. 2A, the area of mineralized extracellular matrices had increased significantly in the experimental groups exposed to SMF, compared with the unexposed controls. That is, the area of nodules in the

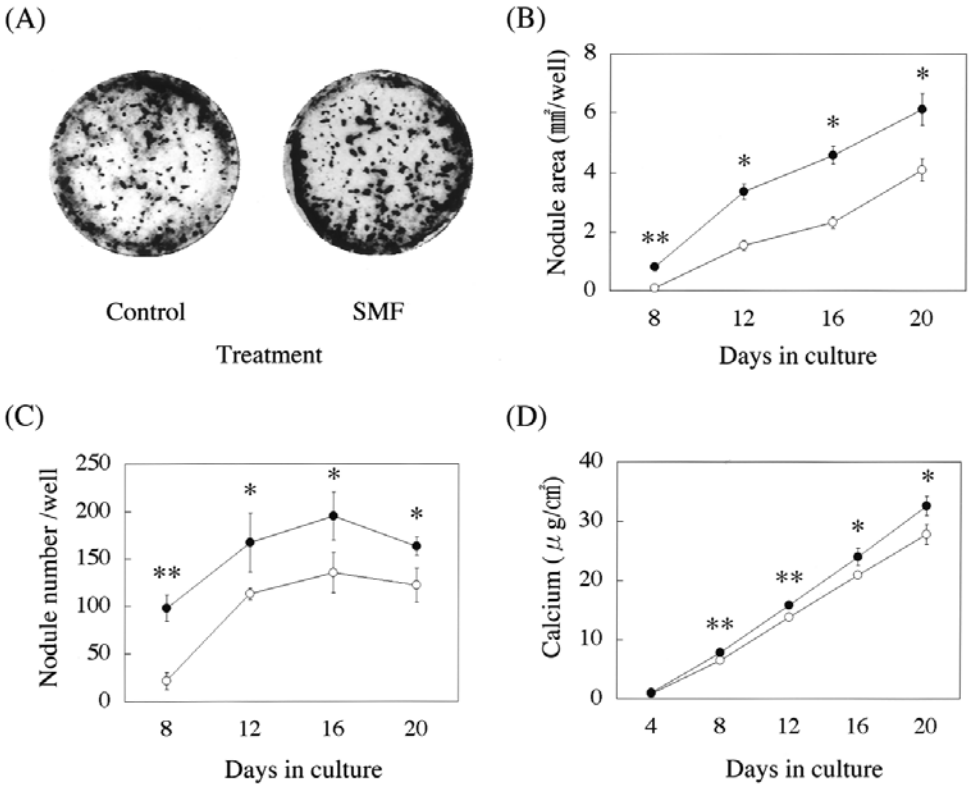

Figure 2. Effect of SMF on mineralized bone nodule formation in rat calvaria cell cultures. Photograph of wells after von Kossa staining **(A)**, time-course of total area **(B)**, number of bone-like nodules **(C)**, and calcium content in the cell matrices **(D)**. Filled circles, exposed to SMF; open circles, non-exposed controls. Data are presented as mean ± SD (n = 4). Asterisks show a significant difference from control values at *P < 0.05 and **P < 0.01, by Student's *t* test.

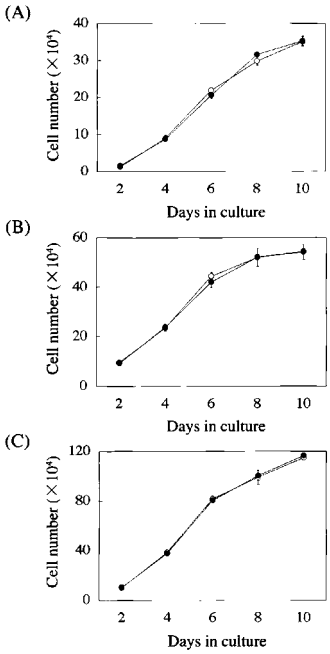

Figure 3. Effect of SMF on growth of rat calvaria cells **(A)**, ROS 17/2.8 cells **(B)**, and UMR 106 cells **(C)**. Filled circles, exposed to SMF; open circles, non-exposed controls. Data are presented as mean ± SD (n = 4).

experimental groups revealed 1.5-fold increases on day 20.

Both the area and the number of mineralized bone nodules *per* well showed a significant increase after day 8 of the culture in the presence of SMF (Figs. 2B, 2C). The total area of nodules showed a continuous increase throughout the experimental period, and the number of nodules continued to increase until day 16; the increase in the experimental groups was more rapid than that in the controls. Furthermore, a significant increase in the average size of the nodules was observed in the cultures exposed to SMF for 12 days when compared with unexposed controls (control, 0.014 ± 0.0010 mm^2/nodule; SMF, 0.021 ± 0.0031 mm^2/nodule; $P < 0.05$). To examine the nodule development, we determined the calcium content in the cell layer every 4 days (Fig. 2D). The calcium content in both the experimental and the control cultures increased with time. At day 4, the calcium content was not significantly enhanced by the application of SMF. In the wells exposed to SMF, however, the calcium content increased significantly after day 8, indicating advanced calcification.

To analyze the effect of SMF on cell proliferation, we counted rat calvaria cells, ROS 17/2.8, and UMR 106 on days 2-10 (Fig. 3). The cell numbers in each culture increased with time, but there was no significant difference between the wells exposed to SMF and the controls.

We analyzed ALP activity and the content of osteocalcin (bone-characteristic protein) on days 4-20 to examine whether SMF influences osteoblastic differentiation. ALP activity increased rapidly from day 4 and reached higher levels in the experimental groups compared with the controls (Fig. 4A). In particular, ALP activity in rat calvaria cells was stimulated by exposure of SMF from days 8 to 12. ALP activity in both ROS 17/2.8 and UMR 106 cells was also stimulated by SMF (Figs. 4C, 4D). The ELISA analysis for osteocalcin production revealed that SMF significantly affected the increase in osteocalcin concentration in the culture medium in a time-dependent manner (Fig. 4B). The amount of osteocalcin in the experimental media was higher than that of the controls at days 8 through 20.

DISCUSSION

The promotional effect of pulsed electromagnetic fields on osteoblasts has been proposed from several points of view (Takano-Yamamoto *et al.,* 1992; Bodamyali *et al.,* 1998; Heermeier *et al.,* 1998), although there have been some contradictory findings (Gonzalez-Riola *et al.,* 1997; McLeod and Collazo, 2000). With respect to the comparison between pulsed electromagnetic fields and SMF, Darendeliler *et al.* (1995, 1997) reported that SMF may promote new bone deposition in the osteotomized mandibles of guinea pigs (flux density, 4-8 mT) and also in the alveolar bone of guinea pigs (flux density, 0.5 mT), when tension was loaded to move a

tooth. On the other hand, Camilleri and McDonald (1993), who evaluated the effects of SMF (flux density, 100 mT) on bone remodeling and mitotic activity of osteoblasts in rat calvaria, suggested that SMF did not affect bone growth, but that thymidine uptake was significantly inhibited. The effect of SMF on bone formation is thus a controversial subject. In the present study, we showed that SMF (flux density, 160 mT) stimulated bone formation by promoting differentiation and/or the activity of osteoblasts, using the rat calvaria cell culture system and two osteoblast-like cells, ROS 17/2.8 and UMR 106.

According to Owen *et al.* (1990), three stages can be distinguished in osteoblastic differentiation: proliferation, matrix development/maturation, and mineralization. The transition from proliferation stage to matrix maturation stage was suggested by the up-regulation of genes associated with matrix development and maturation, such as collagen synthesis and ALP activity. Osteocalcin, a marker of late-stage osteoblasts, is expressed by highly differentiated osteoblasts during the mineralization stage in rat calvaria cell cultures. Calcium accumulation starts at the matrix development stage and reaches its maximum during the mineralization stage.

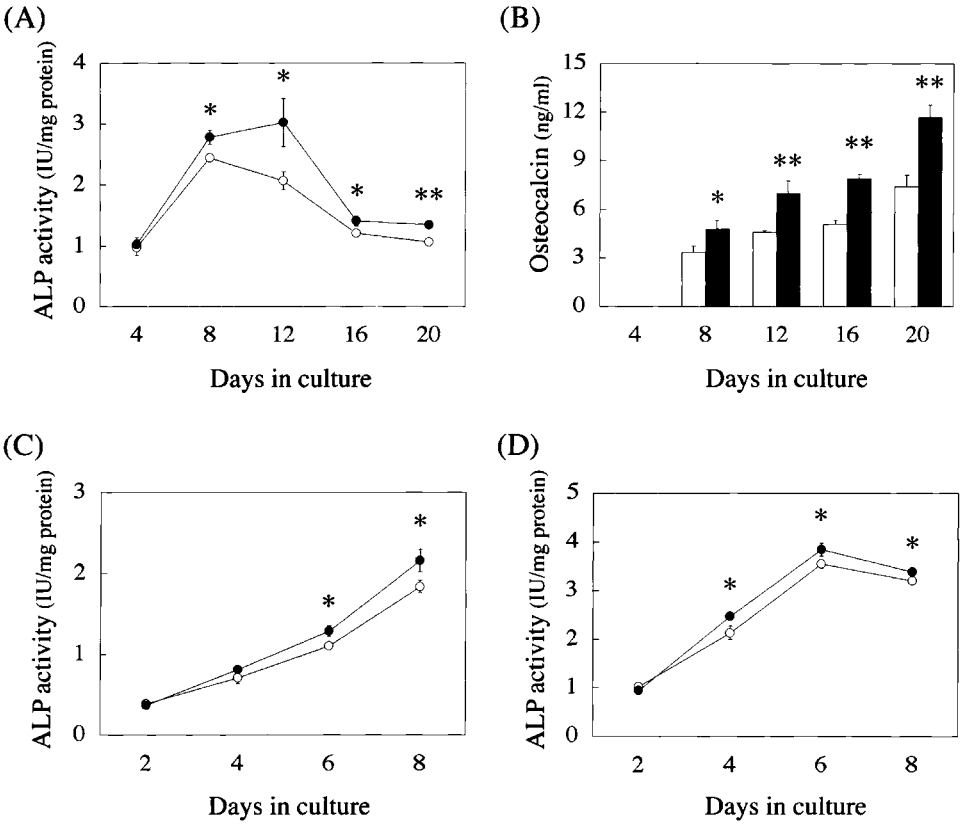

Figure 4. Effect of SMF on two markers of osteoblastic phenotype: ALP activity in rat calvaria cells **(A),** ROS 17/2.8 cells **(C),** and UMR 106 cells **(D);** and osteocalcin content in the conditioned media of rat calvaria cell cultures **(B).** Filled circles and bars, exposed to SMF; open circles and bars, non-exposed controls. Data are presented as mean ± SD (n = 4). Asterisks show a significant difference from control values at *P < 0.05 and **P < 0.01, by Student's *t* test.

We undertook the present investigation to study the effects of SMF on osteoblast activity. Using the cell culture system exposed to SMF, we analyzed osteogenesis in terms of cell proliferation, the expression of markers of osteoblastic phenotype, and bone nodule formation. During the 20-day culture period, the values of the total area, the number, and the average size of bone nodules showed high levels in the presence of SMF. Since the average size of a bone nodule corresponds to the proliferation of osteoprogenitor cells (Bellows and Aubin, 1989), our findings suggest that SMF stimulated the differentiation and proliferation of osteoprogenitor cells. The fractional exposure study also revealed that SMF at the early stage of culture is rather more effective than that at the late stage for bone nodule formation (unpublished data).

In the matrix development and mineralization stages (after 8 days of culture), the calcium content in the matrix showed a significant increase following the application of SMF. The promotional effects of SMF were also evident on two markers of osteoblastic phenotype, ALP activity and osteocalcin content in the culture medium. It seems that the increase in calcium and osteocalcin content of the rat calvaria cell cultures up to day 16 resulted from an increase in nodule number, whereas the increase from days 16-20 resulted from an increase in nodule size. Furthermore, both ROS 17/2.8 and UMR 106 cell cultures showed a significant increase in ALP activity following exposure to SMF. It can thus be suggested that SMF may promote differentiation from osteoprogenitor cells and pre-osteoblasts into mature osteoblasts and/or the activation of osteoblasts.

Using *in vitro* assays for the effect of SMF (flux density, 450 mT) on rat calvaria cells, McDonald (1993) reported that the proliferation of osteoblasts was not affected, whereas that of fibroblast-like cells was accelerated. In the present study, the analysis of cell numbers in 10-day culture also revealed no significant difference between the wells exposed to SMF and the controls in rat calvaria, ROS 17/2.8, and UMR 106 cells. Therefore, it is likely that SMF does not affect the proliferation of osteoblasts.

In conclusion, the present findings suggest that SMF stimulates bone formation by promoting osteoblastic differentiation and/or activation.

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