ORIGINAL ARTICLE

Effects of a Static Magnetic Field on Mineralization of MC3T3-E1 Cells

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Clinical significance

Static magnetic field (SMF) has been utilized for the retention of removable partial dentures. This study indicated that an SMF influences not only the mechanical attachment but also promotes osteoblast-like cells calcification. Although these effects of an SMF remain to be biologically understood, increased in vitro bone-like formation suggests its future applicability.

Abstract

Purpose: The stage at which bone formation was influenced by a static magnetic field (SMF) remains to be elucidated. The purpose of this study was to investigate the initial and long-term effects of moderate-intensity SMF on mouse osteoblastic cells (MC3T3-E1).

Methods: MC3T3-E1 osteoblastic cell cultures were exposed to SMF (250 mT, Nd-Fe-B disc magnet). The long-term effect of this exposure was evaluated by measuring mineralized nodule formation. Osteoblastic cell proliferation was assessed using a colorimetric proliferation assay (WST-8); differentiation was evaluated by measuring alkaline phosphatase (ALP) activity; and noncollagenous gene expression was evaluated using real-time PCR to determine the initial and early responses.

Results: After a month of continuous SMF exposure, mineralized nodule formation increased significantly. The initial proliferative activity decreased and was not related to apoptotic changes. ALP activity and the gene expression levels of secreted phosphoprotein 1 (SPP1), integrin binding sialoprotein, and bone gla protein were not influenced by the SMF exposure.

Conclusion: Moderate-intensity (250 mT) SMF exposure increased mineralized nodule formation in mouse osteoblastic MC3T3-E1 cells. However, gene expression related to decreased cell proliferation and unaltered cell differentiation and bone matrix did not

correlate with the long-term effects observed following SMF exposure. The mechanism by which SMF exposure influences osteoblast mineralization remains unknown.

Key words: static magnetic fields, osteoblast, mineralization, apoptosis

Introduction

Magnetic attachments for removable partial dentures (RPDs) were introduced 3 decades ago.^{1,2} Since the introduction of rare earth element magnets, the use of magnetic attachments has increased and has become one of the treatment modalities for designing an RPD for cases with multiple missing teeth.³ It has been clinically proven that a benefit of magnetic attachment involves an increase in retention and stability of an RPD; further, the decrease in harmful lateral forces facilitates the long-term survival of the abutment teeth.⁴ On the other hand, shortcomings of the magnetic attachments include a mandatory root canal treatment and interference for diagnostic tools that use magnetic resolution. Although the recently designed attachments employ closed magnetic circuits, magnetic flux leakage cannot be eliminated.

The biological effects of the static magnetic field (SMF) remain unclear and depend on various factors such as the intensity of the magnetic field, exposure conditions, and the types of organs, tissues, and cells being treated. With regard to bone tissue effects, several studies have reported the stimulation of bone formation and osteoblast proliferation and differentiation.^{5,6} However, other studies have reported regarding the suppressive effects of an SMF on osteoblasts.⁷ Therefore, there is still lack of understanding regarding the cell based mechanism of SMF exposure.

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Fig. 1 Static magnetic field exposure system.

Bone formation is a sequential event involving osteoblasts and proceeds via 3 stages, namely, proliferation, differentiation, and matrix mineralization. Each stage involves genetic and transcriptional factors that strongly promote or inhibit bone formation. For instance, core-binding factor A1 (Cbfa1) is an essential factor in osteoblast differentiation that genetically regulates the osteoblast phenotype,^{8,9} and osteocalcin is a bone matrix protein that is released simultaneously along with the onset of mineralization.^{10,11} The stage at which bone formation is influenced by an SMF remains to be elucidated. The purpose of this study was to investigate the initial and long-term effects of a moderate-intensity SMF on mouse osteoblast-like cells (MC3T3-E1).

Materials and methods

Static Magnetic Field Exposure System

In the present study, neodymium-iron-boron magnet disks (20.0 mm in diameter and 5 mm in thickness; NE086, 26 SEISAKUSYO Co., Ltd. Kyoto, Japan) and 6-well plastic culture plates were used. The cultures were exposed to the magnet by placing the magnet below the wells. The N-pole was directed toward the culture but did not come in contact with it. The magnetic flux density was monitored using a Tesla meter (TM-601, KANETEC Co., Ltd., Nagano, Japan) present at the bottom of each well, where the mouse osteoblastic cells (MC3T3-E1 cells) attached themselves to the culture plates. The

magnetic field demonstrated a maximum flux strength of 250 mT. The cells in the SMF group were subjected to continuous SMF exposure. The culture plate of the control group and that of the experimental group were placed next to each other in the same incubator. The flux density values of the control culture plates were observed to be no greater than 0.05 mT, that is, it was the same level as that of the natural magnetic field on the earth (Fig. 1).

Cell Culture

The MC3T3-E1 cells, derived from newborn mouse calvaria, are a clonal osteogenic cell line whose cells can differentiate into osteoblasts. The cells were plated in the 6-well plates at a concentration of 1×10^4 cells/well. These cells were cultured in plastic plates containing α -minimal essential medium (α -MEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 10 mM β -glycerophosphate, and 50 μ g/mL of ascorbic acid at 37°C in a humidified atmosphere containing 5% CO₂ in air. SMF exposure was initiated when the culture reached 70% confluence. The medium in each plate was changed every 3 days.

Bone Nodule Formation Assay

With regard to the bone nodule formation assay, mineralized extracellular matrices were stained using the von Kossa technique. The cells were then washed 3 times with PBS, followed by fixation in ethanol for 10 min. Subsequently, they were washed 3 times each with PBS and distilled water, and then stained with 5% AgNO₃ solution for 10 min. After staining, the cells were washed 3 times with distilled water and fixed in a 3% Na₂S₂O₃ solution for 10 min. The area and number of calcified nodules stained by the von Kossa technique were digitally photographed and measured using the NIH image software.

Proliferation Assay

Cell proliferation was assessed using a colorimetric proliferation assay (WST-8 Cell Counting Kit, Dojindo, Kumamoto, Japan). We determined the hormazan dye content in the samples by measuring the absorbance of the 1-, 4-, and 7-day cultures at 450 nm. The measurements were performed after changing the medium by applying the hormazan solution for 30 min to 1 h, depending on the change in color.

DNA Fragmentation Assay

With regard to the cellular DNA fragmentation assay, solid-phase immobilized anti-DNA mAb and anti-BrdU mAb labeled with peroxidase were used to detect BrdU-labeled DNA fragments in the cytoplasm of the cell lysates (Cellular DNA Fragmentation ELISA, Roche Molecular Biochemicals, Mannheim, Germany). In brief, the MC3T3-E1 cells grown in culture dishes (1×10^5 cells/ml) were labeled with BrdU for 24 h, the cell concentration was then adjusted to 5×10^4 cell/ml, and 100 μ l of the suspension was transferred to each well of a microtiter plate (96-well, flat bottom). TNF- α -treated cells were used as the positive control. The concentration of BrdUrd-labeled DNA released into the cytoplasm of the apoptotic cells was quantified by ELISA.

Alkaline Phosphatase Activity and Protein Assays

Alkaline phosphatase (ALP) activity was determined using the BluePhos Microwell Phosphatase Substrate System (Kirkegaard & Perry Laboratory, Gaithersburg, USA). Briefly, the samples of the supernatants were added to the substrate solution and reacted for 30 min. The products were detected at 595 nm by using a spectrophotometer. Protein concentration of the cell lysate was estimated using the Bradford method (Bio-Rad Laboratories). The ALP activity was normalized to that of the total protein content.

Real-Time Transcript Analysis

Total RNA was extracted using Qiazol (QIAGEN, California, USA)¹² and genomic DNA was eliminated by DNase I treatment. The quality and quantity of RNA was measured by spectrophotometry. Subsequently, an Oligo-dT primer and the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL; Grand island NY, USA) were used in a reverse transcriptase reaction to obtain cDNA. The expression level of secreted phosphoprotein 1 (SPP1, osteopontin), integrin-binding sialoprotein (Ibsp), and bone gla protein (BGP, osteocalcin) mRNAs was determined by performing quantitative real-time PCR using a sequence detector (ABI 7700; Applied Biosystems, Foster City, USA). The primers and Taq Man probes were designed according to the UniGene IDs (Applied Biosystems, Foster City, USA); the primers for SPP1(OPN) and Ibsp were NM_009263, and NM_008318, respectively.

The reaction mixture containing the cDNA

sample was prepared according to the manufacturer's protocols to yield a final concentration of $1 \times$ TaqMan Universal PCR MasterMix and 200 nM of each primer. The reaction mixture also contained target hybridization probes (250 nM each). These probes were labeled at the 5' end with the reporter fluorescent dye 6-carboxyfluorescein (FAM). The thermal cycling conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and denaturing and annealing-extension at 60°C for 1 min. All data were calculated by a standard curve, and the results were standardized by comparison with the endogenous control (TaqMan Rodent GAPDH Control Reagents kit, Applied Biosystems, Foster City, USA).

Statistical Analysis

Values are presented as means $\pm$ S.E. with 4-6 cultures per group. Each experiment was repeated 3 times. Differences between the control and experimental groups were assessed by paired Student *t* test.

Results

Analysis of Mineralization

After maintaining the culture for 3 weeks, mineralized nodules appeared on the cell layers. The culture was stopped on Day 31 and the visualization of mineralized nodules became clearer by Von Kossa staining (Fig. 2). The experimental culture displayed scattered black stained nodules that were equally distributed in the culture dishes. On the other hand, the control culture displayed sparse nodule formation. The results of image analysis revealed that the total mineralized area had increased significantly ($P < 0.05$). Moreover, the average number of nodules had also increased significantly ($P < 0.01$).

Proliferation and Apoptosis Assay

The effect of SMF contentious exposure in the early phase was determined by the proliferation assay. The results were normalized using the corresponding control culture, and these results indicated the relative ratio against the control. With regard to proliferative activity, the 1-day culture did not reveal any difference, but the 4- and 7-day cultures demonstrated a significant decrease ($P < 0.05$) of approximately 10% (Fig. 3). The DNA fragmentation assay was performed in

Fig. 2 Mineralized nodule formation (a) von Kossa staining (day 31), (b) Number of nodules, (c) Area occupied by the nodules.

Fig. 3 Cell proliferation activity.

Fig. 4 DNA fragmentation assay (apoptosis).

order to assess the relationship between partial inhibition of proliferation and apoptosis due to SMF exposure. Consequently, identical levels of fragmentation were observed in 1- and 3-day cultures and the control. Challenge with TNF- α represented the positive control for apoptosis (Fig. 4).

Differentiation Assay

Osteoblast differentiation is characterized by an increase in in vitro ALP activity. Indeed, MC3T3-E1 osteoblast-like cells gradually increase ALP activity until Day 4. However, SMF exposure had little effect on ALP activity (Fig. 5). Another marker of osteoblast differentiation is the expression of noncollagenous proteins, including SPP1, Ibsp, and BGP. After 24 h of SMF exposure, the mRNA expression levels were quantified using real-time PCR. These results are illustrated in Figure 6. However, SMF exposure did not alter any of the expression levels.

Discussion

Although the effects of SMF have been studied in various tissue cultures, the results of previous studies are conflicting. The growth of HeLa cells displayed no difference after 120 mT SMF exposure.¹³ On the other hand, Feinendegen and Muhlenstiepen reported that a 1.4-T exposure for 30 min promoted the tyrosine kinase activity in mouse bone marrow cells. Contrary to the above observation, Butler and Dean demonstrated that 0.4 T SMF exposure had inhibitory effects on KB cells.¹⁵ Therefore, it is suggested that the effect of SMF depends on the duration of SMF exposure, tissue penetration, and heat generation.¹⁴⁻¹⁶ Using mouse osteoblastic cells, this study has focused on the long-term effect of continuous SMF exposure and the early responses to SMF.

In this study, we used rare earth magnets, including Neodym (Nd), Iron (Fe) and Boron (B) that are similar to those used in dental magnetic attachments. The magnetic fluxes of the culture wells were unevenly distributed. SMF strength

Fig. 5 Measurement of ALP activity.

was the highest at the center of the magnet (approximately 250 mT) and the lowest at rim of the well (approximately 15 mT). Clinically, the maximum strength of the attachment surface is 100 mT. The SMF exposure simulates periodontal tissue reaction, particularly with regard to bone formation, when the magnet is placed close to the bone.

Regarding the effect of an SMF on the osteoblasts, cell growth and differentiation were the focus because the therapeutic benefits of SMF exposure have been confirmed by clinical results in orthopedics. Hence, the mechanism of the magnetic effect was studied *in vitro* to elucidate its effect. Indeed, osteoblasts, which are responsible for bone formation, are sensitive to SMF exposure. However, the effects of SMF exposure were inconsistent. In well-differentiated osteoblastic cells (ROS17/2.8, UMR106, rat calvaria cells), 160 mT SMF exposure did not increase cell growth; instead, it promoted ALP activity and mineralization.⁶ Camillreri and Macdonald reported that 100 mT SMF exposure decreased the proliferation of the rat calvaria osteoblastic cells.⁷ In this study, we observed that 250 mT SMF exposure decreased cell proliferation and did not increase ALP activity in the mouse osteoblastic MC3T3-E1 cells. Since this cell line possesses pre-osteoblastic cell characteristics, the effect of SMF exposure is dependent on the stage of osteoblastic differentiation.

It has been suggested that SMF causes morphological alterations in cell shape¹⁷ and bioelectrical properties, including the influence of transmembrane calcium flux.¹⁸ An increase in $[Ca^{2+}]$ influx might trigger the development of apoptosis. In this study, we observed that SMF partially inhibited cell growth in cell cultures in which apoptotic cells were not detected. Teodori

Fig. 6 mRNA expression after 24 h of SMF exposure.

reported that 6 mT SMF exposure modulated Ca^{2+} fluxes in human glioblastoma cells and interfered with the stress-induced apoptotic pathway.¹⁹ Therefore, our results were consistent with those of a previous report that demonstrated that SMF alone did not exert apoptogenic activity.

The final outcome observed in the osteoblastic culture was mineralization, which involves the proliferation and differentiation stages, and is essential for mineralization of the extracellular matrix and inorganic phosphate. During the stages in bone formation, the expression of noncollagenous proteins such as SPP-1, Ibsp, and bone gla-protein plays an important role in calcified matrix formation. We also observed that SMF increased mineralized nodule formation; however, the expression of the genes of noncollagenous proteins involved in bone formation were not elevated, and their expression levels did not have an influence on alkaline phosphatase activity, which is believed to be involved in the extracellular breakdown of pyrophosphate, a potent inhibitor of calcium and phosphate deposition.²⁰ Therefore, it is suggested that the SMF-induced mineralization did not mediate cellular gene alteration. Since these results are not in agreement with those of a previous study of a similar nature,⁶ it is possible that factors other than cell proliferation and differentiation, such as apoptosis and matrix-related gene expression, did not induce this long-term effect following SMF exposure. Further study is required to clarify the biological and physiological mechanism of SMF exposure.

Clinical application of SMF exposure is limited to diagnostic appliances, such as MRI, and its physiological effects include relief from pain or controlling of blood pressure.²¹ Since 1992, magnetic attachments used for oral treatments have found greater application. However, their design

did not permit the tissue around the attachment to be exposed to SMF; our results suggest that magnetic leakage might not be a risk. Further, with regard to the alveolar bone, it would be favorable to stimulate bone formation by SMF exposure using a prosthetic device. This study suggests that an SMF could be used for tissue engineering for regenerating the maxillary bone.

Conclusion

Moderate-intensity (250 mT) SMF exposure increased mineralization in mouse osteoblastic MC3T3-E1 cells. However, gene expression related to decreased cell proliferation and unaltered cell differentiation and bone matrix did not correlate with the long-term effects observed following SMF exposure. The mechanism by which SMF exposure influences osteoblast mineralization remains unknown.

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