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Differentiation of Human Osteoprogenitor Cells Increases after Treatment with Pulsed Electromagnetic Fields

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Abstract. *Human mesenchymal stem cells (hMSC) have become an important resource in developing strategies for regenerative medicine and tissue engineering, owing to their ability to renew and their potential for differentiation into cells of various types of tissues. Pulsed electromagnetic field (PEMF) stimulation has been used for several years in the treatment of fracture healing, with clinical beneficial effects, and several studies have demonstrated its capacity to increase bone tissue regeneration. In the present study, stromal cells of human bone marrow (BMSC), obtained from healthy donors, were appropriately expanded and underwent PEMF stimulation eight hours a day for fourteen days. Parameters such as proliferation and differentiation ability were evaluated on stimulated cultures. The evaluation of the marker expression was performed by RT-PCR for osteocalcin, by alkaline phosphatase quantitation and by histochemical stains. The results we obtained showed that BMSC treated with PEMF begin differentiation earlier than untreated BMSC, as shown by the markers used. The data show that PEMF is able to increase the osteogenic differentiation potential in adult mesenchymal cells isolated from young patients.*

Bone marrow is the flexible tissue found in the interior of bones. In humans, bone marrow in large bones produces new blood cells. The stroma of the bone marrow is tissue not directly involved in the primary function of hematopoiesis. It provides the hematopoietic microenvironment that facilitates hematopoiesis by the parenchymal cells. Cells that constitute the bone marrow stroma are: fibroblasts (reticular connective tissue); macrophages; adipocytes; osteoblasts; osteoclasts; endothelial cells (1-4).

From bone marrow it is possible to isolate different kinds of stem cells, among which hematopoietic stem cells (HSC), endothelial progenitor cells (EPC) and mesenchymal/stromal stem cells (MSC). In particular Human MSCs have become an important resource in developing strategies for regenerative therapies, owing to their ease of use and potential for differentiation into cells of various types of tissues, such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma (5-11).

Great attention has recently been generated regarding characterization and control of bone marrow stromal cells (BMSCs). They can be cultivated *in vitro* and have the ability to differentiate. In particular, the ease with which they can be obtained and expanded in number, makes then a simple source to be used to obtain cells with an osteogenic potential useful for the reconstruction of bone tissue; therefore they are an interesting target for use in cell and gene therapy (12-16). Interest in BMSC is due to the fact that they can be isolated from the donor marrow, amplified *in vitro* and reinserted into the same individual, leading to important applications.

Electromagnetic stimulation in humans has been studied in order to increase the spontaneous regenerative capacity of bone tissue (17-20). *In vitro* experiments in humans have shown that exposure to pulsed electromagnetic field (PEMF) promotes proliferation of cells of the immune system and osteoblasts, and promote new angiogenesis in endothelial cell cultures (21, 22). The effects of electromagnetic fields are mainly attributable to the interaction of PEMFs with the cell membrane and in particular to the transduction processes, inside the cell, of the signals present on the surface of the cell itself. Although PEMF stimulation may be clinically beneficial during fracture healing and for a wide range of bone disorders, there is still debate on the mechanisms involved (23, 24). MSCs are likely mediators facilitating the observed clinical effects of PEMF.

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Drawing from this background, we decided to investigate the effects of PEMF on human BMSCs from small volumes of bone marrow aspirated from the iliac crest of healthy donors in order to evaluate whether PEMF can stimulate their osteogenic differentiation and the advantage to use pulsed electromagnetic fields in the clinical field.

Materials and Methods

BMSC isolation and osteogenic differentiation. Stromal cells were obtained from iliac crest marrow aspirates from four young donors in Pausilipon Hospital, Naples. All procedures were approved by the Hospital Ethical Committee and informed consent was obtained from the donors.

BMSC cultures were performed essentially as previously described by Banfi *et al*. (13). Briefly, BMSC cells were cultured in expansion medium consisting of Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) supplemented with 10 ng/ml basic fibroblast growth factor (FGF-2; R&D Systems, Minneapolis, MN, USA), 100 units penicillin, 1000 units streptomycin, and 2 mM L-glutamine. Once the cells reached 50-60% confluence, they were washed twice with phosphate-buffered saline (PBS), detached by 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) and then replated at a ratio of 1:4 under the same culture conditions. BMSC cells before passage 5 were used for the experiments in this study.

BMSC cell cultures were stimulated with an osteogenic medium consisting of high-glucose DMEM (Gibco, Grand Island, NY, USA) and 10% FBS supplemented with 0.1 mM dexamethasone, 10 mM β-glycerol phosphate, 0.2 mM ascorbic acid 100 U penicillin, 1000 U streptomycin, and 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO, USA). The medium was changed 2-3 times a week to maintain the capability for cell proliferation during the process of differentiation.

Cell Growth by Trypan blue exclusion methods. Cell growth was determinate by trypan blue assay exclusion method. Cell concentration of BMSC was adjusted to 4×10^5 cells/ml and 1 ml of the cell was added into 6 well culture plate. Cells were incubated for 24 and 48 h at 37˚C, 5% CO_2 in the presence or absence of PEMF stimulation. After the incubation period, the cultures were detached by trypsin-EDTA, collected and centrifuged at 2000 × g at 4˚C, washed with phosphate buffered saline (PBS) and stained with trypan blue assay. Only viable cells were counted.

PEMF treatment. Plates were placed inside an incubator at equal distance from two solenoids arranged in parallel with each other and perpendicular to the support base, thus respecting the principle of focus signal in order to maintain constant physical characteristics of the stimulation signal for eight hours per day continuously. The external device produced an electromagnetic signal of the square wave type with a frequency of 75Hz and an intensity of 18-30 Gauss. The control experiments were performed on the BMSCs isolated from the same donors but incubated in a separate incubator under the same conditions but without PEMF treatment.

Flow cytometry for cell cycle analysis. After PEMF treatment for 24 and 48 h the BMSCs were collected and centrifuged at 2000 × g at 4˚C. The pellets were fixed in 1 ml with 70% ethanol for 4h at –20˚C. Then cells were centrifuged at 2000 × g at 4˚C, washed with PBS and incubated in 500 ml of a hypotonic buffer (0.1%TritonX-100, 0.1% sodium citrate, 50 mg/ml propidium iodide, and 100 mg/ml RNase). The cells were then analyzed using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Diego, CA) and the percentages of G_1, S and G_2/M populations were calculated by ModFit version III software (Verity Software House, Topsham, USA) respectively.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) measurements. Semiquantitative RT-PCR measurements were performed as previously described by Banfi *et al*. (13). Briefly, total RNA was extracted from BMSC as described by Chomczynski and Sacchi (25). Semiquantitative RT-PCR was performed using the GeneAmp RNA PCR kit from Perkin Elmer (Perkin Elmer, CA, USA). For each RNA sample, a master RT reaction was performed with 2 μg total RNA in a 40 μL mixture. The reaction mixture was made up and the reaction performed as described by Banfi *et al*. (13). Each master cDNA product was divided into two equal parts that were used for PCR amplification either of the housekeeping gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) or osteocalcin (OC).

The number of cycles used was in the linear range of amplification for the specific gene product as described by Banfi *et al*. (13). RT-PCR products were analyzed by electrophoresis of 20 μl aliquots in 1% agarose gels and visualized by ethidium bromide. The amount of PCR product for each single gene was normalized according to the corresponding *GADPH* PCR product.

Alkaline phosphatase assay (ALP). The activity of ALP was measured in cell lysates and used as a cell differentiation marker to evaluate osteogenesis. Established cell cultures in duplicate were washed with cold PBS (pH 7.4) (Sigma Chemical Co., St. Louis, MO, USA) collected in 2 mM Tris HCl/50 mM mannitol (pH 7.2) (final volume 1 ml), sonicated in ice and used for measurements (5 and 10 mL). The phosphatase activity was measured by determining the kinetics of *p*-nitrophenylphosphate hydrolysis using a commercial kit (Boerhinger Ingelheim, Germany). The results are expressed in milliunits per milligram of protein. The determination of total proteins was performed by the Bradford method.

Alizarin red staining. Osteogenesis was quantified by alizarin S staining performed according to the following protocol. After aspirating the culture medium from each plate, plates were washed with 1× PBS and fixed with 4% formalin for 10 minutes. The plates thus prepared were then washed with deionized water and stained with alizarin red for 10 minutes. After aspirating the excess dye, the plates were washed firstly with absolute ethanol and then with deionised water. Images of the colored plates were acquired with a Leitz Orthoplan microscope (on 100 asadin-21 film).

Statistical analysis. Each experiment was performed in triplicate. The results are expressed as means±standard deviations (SD). Student's *t*-test was used to determine statistical differences between the means, and $p<0.05$ was considered a significant difference.

Results

BMSCs were isolated from iliac crest marrow aspirates of four young donors. For the BMSCs isolated, the growth kinetics of the different donors showed the same trend. Cells obtained from the donors were split on the second day after

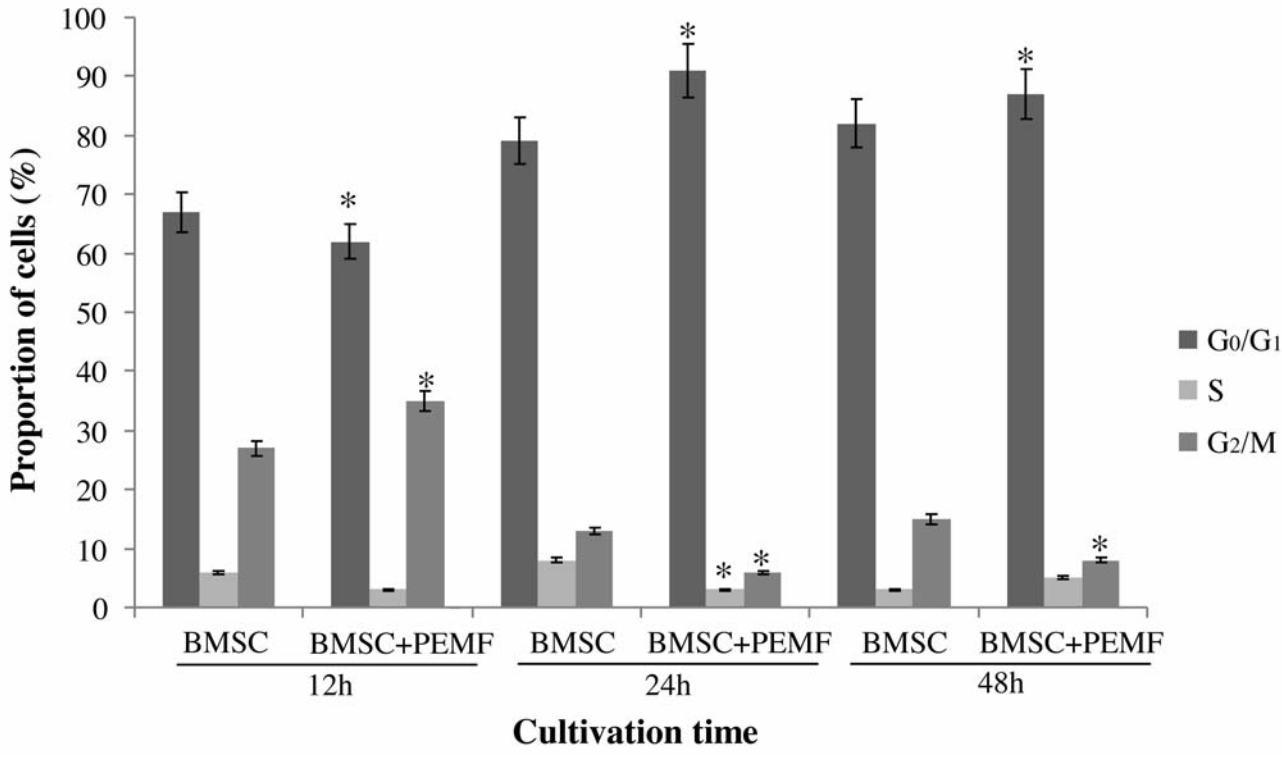

Figure 1. *The cell cycle distribution of Bone Marrow Stem Cells (BMSC) with and without Pulsed Electromagnetic Field (PEMF) exposure. The figure show the percentages of cells in each phase of the cell cycle. Each bar represents the average of three independent experiments±SD. *Significant difference compared with the control (p<0.05).*

the isolation and the ratios of cell density were examined comparing the PEMF-exposed cells vs non-exposed control cells. Both groups exhibited a spindle-shape morphology, however, an increasing of cell number a little more than twice was observed in trypan blue direct cell counting at 24 h in PEMF-exposed cells compared to control cells. At 48h it was not observed any important differences in cell number between the two groups (data not shown).

In order to investigate the enhancement of cell proliferation by PEMF, flow cytometry was carried out of treated and untreated cells. PEMF treatment led to a higher percentage of cells in the G_2/M phase compared with the untreated cells within the first 12 h; at 24 h and 48 h the percentage of cells in the G_2/M phase was lower, while the proportion of cells in the G_0/G_1 phase increased (Figure 1) compared with the untreated BMSCs. These observations demonstrated that PEMF treatment influences the cell cycle distribution.

To investigate if PEMF was also able to affect osteogenesis, BMSCs were grown in osteogenesis stimulation medium and treated with and without PEMF for 8h/day for two weeks. BMSCs were collected and total RNA was isolated at days 3, 7, 10 and 14. The mRNA expression of osteocalcin was examined with a semi-quantitative RT-PCR analysis in order to evaluate the changes in expression of osteocalcin during the two weeks with and without treatment with PEMF.

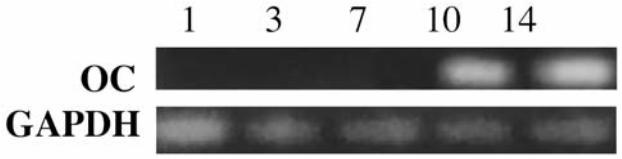

Figure 2. *Reverse Transcription Polymerase Chain Reaction analysis for the expression of osteocalcin (OC) gene in cells treated with Pulsed Electromagnetic Field (PEMF). Total RNA was extracted at day 3, 7, 10 and 14 of treatment with PEMF and used to evaluated the expression of OC and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as control.*

As shown in Figure 2 the electrophoresis of the semiquantitative RT-PCR demonstrated that expression of osteocalcin from the beginning of the treatment was barely at 1-7 days, while it is well expressed at day 10. The expression level was similar at days 10 and 14.

The control with untreated cells exhibited almost undetectable expression of osteocalcin for 10 days and only at day 14 it was detectable (data not shown).

ALPs are a group of enzymes found primarily in the liver (isoenzyme ALP-1) and in the bone (isoenzyme ALP-2).

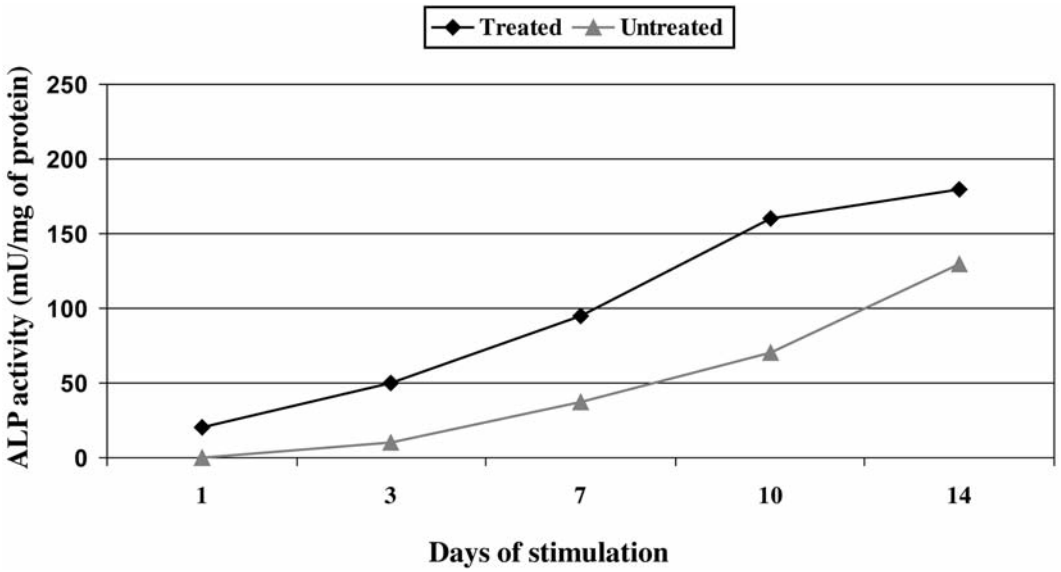

Figure 3. *Assay for alkaline phosphatase (ALP) activity in Pulsed Electromagnetic Field treated and untreated cells.*

ALP activity plays an important role in the initial steps towards formation of new bone; indeed the ALP level is considered an indicator of osteoblastic activity.

The ALP activity of cells was evaluated in untreated cells and cells treated with PEMF. As shown in Figure 3, already after the first 48 h ALP activity was observed in the treated cells. At 72 h ALP activity was found in treated and untreated cells. The ALP activity of treated cells was higher than that of untreated cells.

Finally, on BMSCs treated with PEMF, alizarin red staining was used to evaluate calcium-rich deposits by cells in culture. Figure 4 shows the rapid increase of mineralization starting from the midpoint of the cell treatment.

Discussion

The development of new strategies to improve osteogenesis is an important goal in the clinical field. It is well known that the ability of bone for self-repair is the basis for most reconstruction orthopedic procedures. Although the results of orthopedic surgery in this field are usually good, complications frequently occur in the process of skeletal repair that lead to new interventions which necessarily require the use of bone graft or bone substitute material (26, 27).

Several studies to improve osteogenesis have been performed using human BMSCs. These cells have the advantage that they can be easily isolated from bone marrow aspirates and can be conveniently expanded, and later on, transplanted into the lesion.

During bone regeneration, proliferation and differentiation of new osteoblasts are required and towards this goal, electromagnetic stimulation in humans has been used in order to increase the spontaneous regenerative capacity of bone tissue. In particular, PMEF therapy is used successfully to treat a wide range of bone fractures and diseases (28, 29).

Our study has experimented with the possibility of a combined use of cell therapy and PEMF. PEMF treatment was able to accelerate cell proliferation, enhance cellular differentiation, and increase the formation of bone tissue.

In particular in our experimental model, we observed that a higher percentage of cells were present in the G_2/M phase was present compared with the untreated cells within the first 12 h. The subsequent 24 h showed an increase of G_0/G_1 phase cells compared with the untreated control. This observation demonstrates that the first effect of PEMF treatment is to increase division of cells with a rapid increase in cell density. During these 48 h we did not observe synthesis of extracellular matrix as demonstrated before (30), however other groups, in a different model, showed that PEMF is able to promote the synthesis of extracellular matrix (31).

The histological data obtained by alizarin red staining showed early expression of calcium deposits in the extracellular matrix in the first days of PMEF stimulation. This finding was also confirmed by assay of enzymatic activity for ALP which showed a moderate activity of the enzyme from the first three days of stimulation.

Even the expression of the gene for osteocalcin, one of the specific markers for osteoblastic differentiation detected by semiquantitative RT-PCR, was positive in three days of stimulation, as well as by comparison with gene expression of *GAPDH*. It must be said, however, that quantitatively, the most significant expression of this gene occurs only from

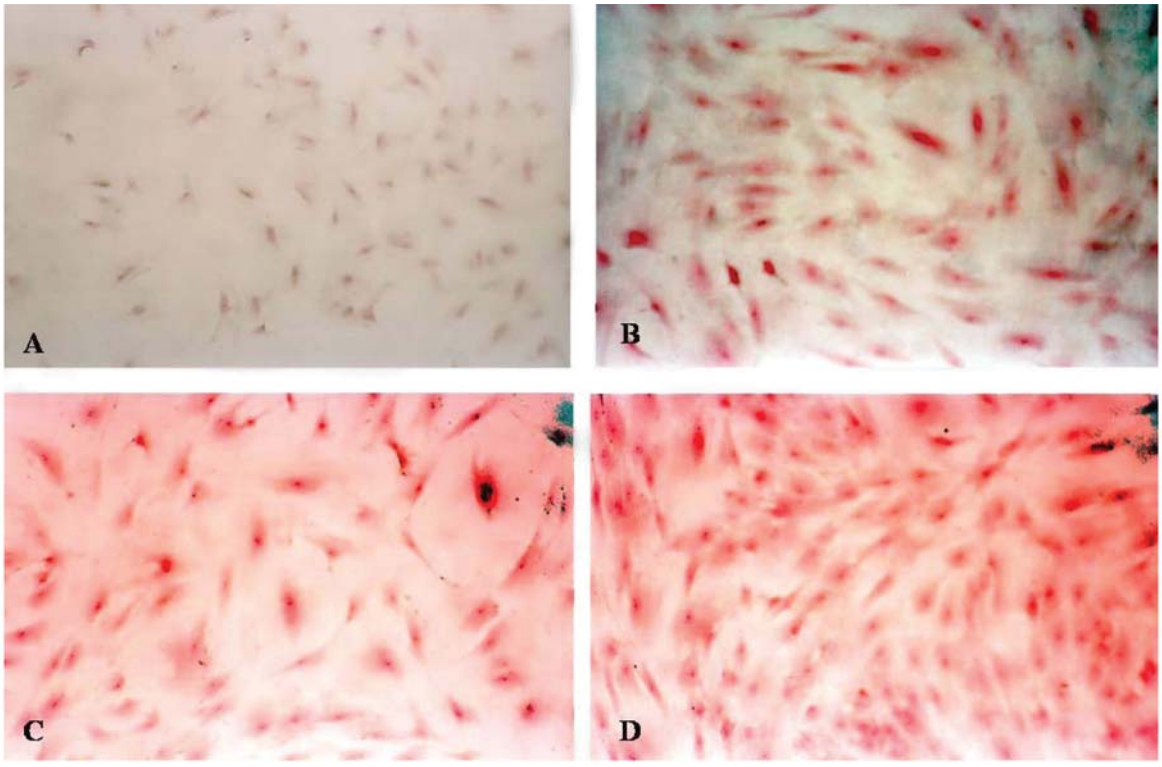

Figure 4. *Alizarin red staining at day 3 (A), 7 (B), 10 (C) and 14 (D) of stimulation with Pulsed Electromagnetic Field (A: original magnification ×10, B, C, D: original magnification ×20).*

day 10 of stimulation. This expression, however, is quite comparable to that obtained at 14 day with specific means for osteoblastic differentiation. This figure could indicate a higher capacity of PEMF to induce early differentiation of adult mesenchymal cells compared to the the standard laboratory methods.

In conclusion, we can state that the data from this study seem to show that PEMF, in addition to the properties well documented in the literature inhibiting osteoclastogenesis (30, 33) and modulating osteoblastic activity (34-37), is also able to induce osteoblastic differentiation of adult stem cells in a relatively short time.

Therefore the use of non-invasive methods for cell differentiation could be a new therapeutic approach to the repair of bone injuries. This method also determines a reduction in the time of regeneration with a consequent reduction of costs per patient.

It is easy to appreciate the enormous possibilities of treatment that a combined approach of cell therapy and physical therapy with electromagnetic fields can open. However, further studies are required to better understand how PEMF influences the kinetics of growth and the differentiation potential of adult MSCs and to evaluate whether this differentiation is stable or not *in vivo*.

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