Effects of electromagnetic field activated-ERK signaling pathway on proliferation and osteogenic differentiation of rat bone marrow mesenchymal stem cells*★

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Abstract

BACKGROUND: It has been demonstrated that electromagnetic field (EMF) can adjust proliferation and differentiation of bone marrow mesenchymal stem cells, but the specific mechanism is not clear.

OBJECTIVE: To investigate the effects of EMF-activated ERK1/2 pathway on proliferation and osteogenic differentiation of rat bone marrow mesenchymal stem cells.

METHODS: The 3rd passage of rat bone marrow mesenchymal stem cells were received EMF treatment (15 Hz, 1 mT, sine wave), 20 μmol/L PD98059 + EMF treatment, or only PD98059 treatment. Simultaneously, a normal control group was established. Western blotting was applied to detect the activation of ERK signal pathway after EMF exposure. MTT assay was used to determine the activation of proliferation of cells. And alkaline phosphatase (ALP) activity in cells was detected by an ALP kit.

RESULTS AND CONCLUSION: The ERK1/2 phosphorylation, proliferation and ALP activity of rat bone marrow mesenchymal stem cells were remarkably increased after exposure to EMF ($P < 0.01$). PD98059 could effectively block the increasing of ERK1/2 phosphorylation and cell proliferation ($P < 0.01$), but elevate ALP activity in a certain level ($P < 0.01$). EMF stimulation can fast activate ERK1/2 signal pathway and then promote the proliferation of rat bone marrow mesenchymal stem cells, however, ERK1/2 signal pathway activation has a less effect on osteogenic differentiation of bone marrow mesenchymal stem cells.

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INTRODUCTION

Bone marrow mesenchymal stem cells (BMSCs) are a class cells derived from bone marrow, which have multi-directional differentiation potential and strong proliferation and differentiation ability[1-4]. Studies have shown that electromagnetic field (EMF) can promote BMSCs proliferation and osteogenesis[5-6], but the specific mechanism of which is not clear. Extracellular signal-regulated kinase (ERK) is an important member of mitogen-activated protein kinase (MAPK) family[7-9], which involved in regulating many biological behaviors of cells. A variety of stimulating factors, such as growth factors, cytokines, viruses, *etc*. can activate this signaling pathway, thereby inducing a variety of cell fate, such as proliferation, differentiation, cell cycle changes and apoptosis[10-13]. Some studies have shown that 60 Hz EMF can activate ERK signaling pathway and cause ERK1/2 phosphorylation increasing in human breast cancer cells[14]. However, the effect of EMF on ERK1/2 signaling pathway in BMSCs, and the relationship between ERK1/2 signal pathway and the cell proliferation and differentiation which effected by EMF have not been reported. Here, under a condition of 15 Hz, 1 mT EMF stimulation, whether the level of phosphorylation ERK1/2 has changed was observed. In addition, ERK1/2 inhibitor PD98059 was used to observe the relationship between ERK1/2 signal pathway and proliferation and differentiation of BMSCs affected by EMF.

MATERIALS AND METHODS

Design

An *in vitro* cytological observation.

Time and setting

This study was performed in Laboratory of Orthopedic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, between May and December in 2007.

Materials

Six Sprague-Dawley rats with 4–5-week old, weighed 100–120 g, clean grade, irrespective of genders, were provided by Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Certificate No. scxk(e)2008-0004.

Reagents and instruments are as follows:

Reagent and instrument	Source
DMEM/F12 (1:1) medium, high-quality fetal bovine serum	Hyclone, USA
Trypsin	Amersco, USA
PD98059	Biosource, USA
Tetrazolium salt (MTT), dimethyl sulfoxide (DMSO)	Sigma, USA
Alkaline phosphatase detection kit, Coomassie brilliant blue protein determination kit	Nanjing Jiancheng Bio-engineering Institute, China
Mouse anti-pERK1/2, rabbit anti-ERK1/2 antibody	Santa Cruz, USA
HRP labeled goat anti-rabbit/mouse IgG	Jackson ImmunoResearch Laboratories, USA
Electromagnetic field generator	Department of Electrical Engineering Design and Manufacture, Naval University of Engineering, China
Inverted phase contrast microscope	OLYMPUS, Japan
Enzyme-linked immunosorbent assay instrument	Huadong Electron Tube Factory, China

Methods

Isolation and culture of rat BMSCs

The rats were sacrificed by the dislocation of the cervical vertebra and were immerged into 75% alcohol for 5 minutes. The bilateral femur and tibia were cut and the attached soft tissues were removed on the super clean bench. The medullary cavity of the bone was washed by DMEM-F12 medium containing 10% fetal bovine serum (containing penicillin, streptomycin each 100 IU/mL). The cells were suspended by adding medium and washing, repeatedly. The cells were counted and adjusted to a concentration of about 5×10^6 cells/mL, and then inoculated to a 50 mL culture bottle, cultured in saturation humidity incubator at 37 ℃ in 5 % CO_2. The culture medium was first changed at 24 hours, and replaced every 3 days. After about 7–10 days, the cells were digested with 0.25% trypsin and transferred when they grew to 90% confluency[15].

Western blot detection of ERK1/2 phosphorylation level after exposure to EMF

The 3rd passage of BMSCs were adjusted to 1×10^5/mL concentration and seeded into 7 9-cm culture dishes, with 8 mL cells in each dish. Cells were stimulated with EMF exposure (15 Hz, 1 mT) or sham-exposure for 5, 10, 15, 30 minutes and 1 hour in the EMF or control groups, those in the PD98059+EMF group were received 20 μmol/L PD98059 combined EMF stimulation for 5 minutes and 1 hour. Then the cells were lysed (50 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 1% Triton-100, 1 mmol/L EDTA, pH 8.0, 100 μg/mL PMSF, 1:1 000 Cocktail) and ultrasonic fragmentation for 10 seconds for 3 times. The lysate was centrifuged at 12 000 g and 4 ℃ for 15 minutes before some of the supernatant was sequestered for a protein assay. Subsequently, the appropriate volume of the supernatant was removed, based upon the concentration values provided by the protein standard assay, and resuspended in the appropriate volume of Laemmli sample buffer. Proteins were denatured at 100 ℃ for 5 minutes, and the supernatant was dissolved in Laemmli sample buffer (2 mol/L Tris-HCl pH 6.8, 20% SDS, 0.25% glycerol, 10% 2-mercaptoethanol, 0.5% bromophenol blue). Aliquots of the protein were resolved on a SDS-polyacrylamide gel (12%) and transferred to nitrocellulose membranes. The membranes were incubated with blocking solution [5% nonfat dried milk in phosphate-buffered saline (PBS), 0.2% Tween-20 (PBST)] for 1 hour, then probed with various primary antibodies (1:500) overnight at 4 ℃. After three washes with PBST for 10 minutes, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary IgG (1:10 000) for 2 hours, followed by another washes with 0.01% PBS for 10 minutes for 3 times. Immunoreactive bands were detected using the Super Signal Chemiluminescent reagent and analyzed quantitatively by normalizing band intensities to the controls on scanned films or using a ChemiImager 4400 Gel imaging system (Alpha Innotech, San Leandro, CA, USA) by Adobe Photoshop software.

MTT assay for detecting cell proliferation activity

The 3rd passage of BMSCs were cultured in DMEM-F12 medium containing 10% fetal bovine serum with density of 2×10^3 cells/well in 96-well plates in the control and EMF groups, additional 20 μmol/L PD98059 was added in the PD98095 + EMF group. After 3 days normal culture, cells were received EMF exposure twice a day, 30 minutes per time, for 3 days. The survival and proliferation activity of BMSCs were detected by MTT method. The absorbance values were read by microplate Reader at λ = 490 nm.

Assay of alkaline phosphatase (ALP) activity

The 3rd passage of BMSCs was plated at a density of 1×10^5 cells/well in 6-well plates for EMF exposure or EMF exposure+PD98059. The cells were exposed for 8 hours per day for 5 days in electromagnetic field. And then the cells were collected by adding Triton X-100 into each well for 1 mL and overnight at 4 ℃. The concentration of protein was detected by using coomassie brilliant blue and the ALP activity were assayed according to ALP kit instructions. The absorbance values were read by microplate Reader at λ = 520 nm. The ALP activity was represented as nkat/g.

Main outcome measures

Effect of EMF on ERK1/2 activity; proliferation, differentiation and ALP of BMSCs; MTT value.

Statistical analysis

All values were expressed as mean±SD. The analyses were conducted with the SPSS 10.0 software. Statistical significance was assessed by Student's *t* test. For all statistical tests, a *P* value < 0.05 was considered significant.

RESULTS

Level of ERK1/2 phosphorylation in cells

Western blot showed that, compared with the control group, the level of ERK1/2 phosphorylation was increased at 5 minutes after EMF stimulation, and continued at high level for 10 minutes to 1 hour ($P < 0.01$, Figure 1). After PD98059 intervention, the ERK1/2 phosphorylation level returned to a lower level ($P < 0.01$, Figure 2). The results suggested that PD98059 could effectively block the activation of ERK1/2 pathway.

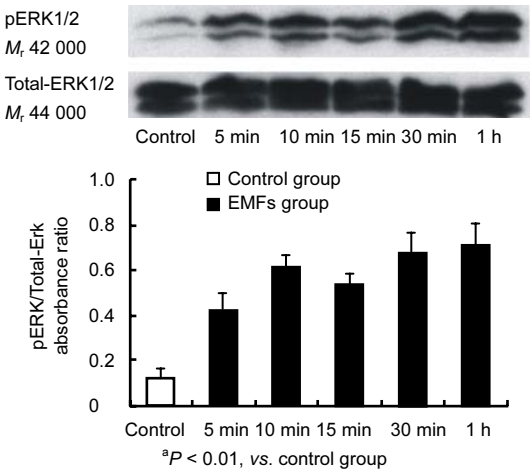
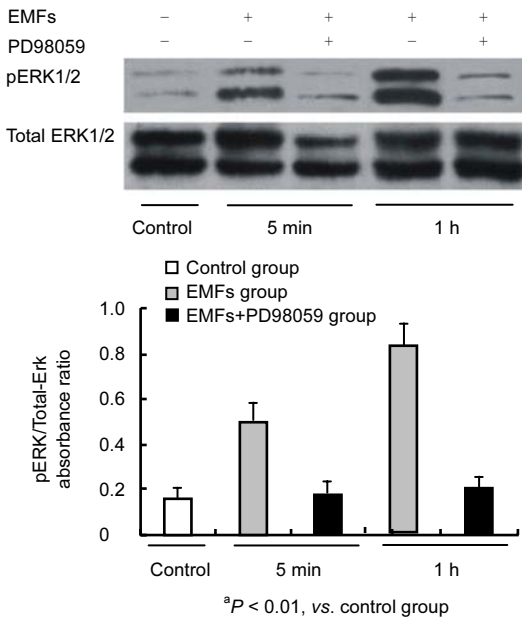


Figure 1 Phosphorylation ERK1/2 stimulated by the electromagnetic field



[a]$P < 0.01$, *vs.* control group

Figure 2 Effect of PD98059 on level of ERK1/2 phosphorylation activated by electromagnetic field

MTT assay results of cell proliferation activity

Compared with the control group, the proliferation activity of BMSCs were dramatically increased at 3 days after EMF stimulation ($P < 0.01$), but concentration of 20 μmol/L PD98059 could significantly block this effect. The proliferation activity of BMSCs in the of PD98059 + EMF group were obviously lower than that of the EMF and control groups ($P < 0.01$, Table 1).

Table 1 MTT absorbance value (*A*) and ALP activity of the cells in each group ($\bar{x}\pm s$)

Item	Control group	EMF group	PD98059 group	PD98059+ EMF group
MTT value (*A*)	0.465±0.023	0.512±0.025[a]	0.387±0.023[a]	0.394±0.022[ab]
ALP activity (nkat/g)	353.90±52.34	447.76±53.51[a]	372.57±67.18[a]	478.60±64.51[ab]

EMF: electromagnetic field; ALP: alkaline phosphatase; [a]$P < 0.01$, *vs.* control group; [b]$P < 0.01$, *vs.* EMF group

Results of ALP activity

The ALP activity of EMF group was significantly higher than that of the control group at 5 days after EMF stimulation ($P < 0.01$). Compared with the EMF group, the ALP activity of the PD98059+ EMF exposed group was also increased ($P < 0.01$, Table 1).

DISCUSSION

Many studies have confirmed that EMF can promote BMSCs proliferation and osteogenesis[16], while the specific mechanism is unclear. In this study, we detected the ERK1/2 signal pathway affected by EMF, and also discussed the relationship between proliferation and osteogenesis of BMSCs and ERK1/2 stimulated by EMF. Our results show that, EMF activate ERK1/2 signal pathway, and the proliferation and osteogenesis of BMSCs are related with the ERK1/2 signal pathways regulated by EMF. ERK1/2 signal pathway is an important intracellular signal pathway, regulates cell proliferation and differentiation[17-19]. The activation of ERK1/2 is implanted by phosphorylation of ERK1/2[20-22]. PD98059 is a selectively blocking of ERK1/2[23-25]. The results of this study show that, 5 minutes of EMF exposure (15 Hz, 1 mT sine wave) actives rat BMSCs ERK1/2 signal pathway, and the level of phosphorylation ERK1/2 remained in a high level after 1 hour. This suggests that EMF can be quickly activated ERK signaling pathway of rat BMSCs, and then causing a series of biological effects. To further study the relationship between ERK1/2 pathway and the biological effects of BMSCs promoted by EMF, the inhibitor of ERK1/2 PD98059 was added to cells and the proliferation and ALP activity were detected. As shown, EMF could significant promote proliferation of BMSCs; the proliferation activity was significant inhibited, and the proliferation stimulated by EMF was also inhibited after blocking ERK1/2 pathway; but the proliferation activity was still slightly higher in the PD98059 + EMF group than that of the PD98059 group, these suggested that: (1) EMF promoted proliferation of MSCs through activating the ERK1/2 signal pathway. (2) EMF may promote proliferation of MSCs through other pathways. According to the results of ALP assay, EMF could significant increase the activity of alkaline phosphatase, after blocking the ERK1/2 pathway using PD98059, the ALP activity of PD98059 group had mild elevated compared with the control group and the ALP activity of PD98059 + EMF group also increased slightly compared with the EMF group, suggesting that the role of ERK1/2 pathway in the alkaline phosphatase activity promoted by EMF was not very significant. Why the activity of alkaline phosphatase is slightly increased when blocking the ERK1/2 need to be further studied.

Suzuki *et al*[26] demonstrated that the cell proliferation period were obviously inhibited when blocking ERK signaling pathway of osteoblast-like cells MC3T3-E1, while there was no changes of ALP activity. Here, we obtain a consistent result. The relationship between ERK1/2 signal pathway and osteogenesis affected by EMF still need further study.

In summary, 15 Hz, 1 mT, sine wave EMF can activate ERK1/2 signal pathway and then promote cell proliferation, however, ERK1/2 signal pathway activation has a less effect on osteogenic differentiation of BMSCs.

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骨髓间充质干细胞增殖与成骨分化中电磁场激活 ERK 通路的作用*★

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摘要

背景：研究表明电磁场可调节骨髓间充质干细胞的增殖和分化，但其具体机制尚不清楚。

目的：从 ERK 信号途径探讨电磁场诱导大鼠骨髓间充质干细胞增殖与分化成骨的作用。

方法：取第 3 代生长良好的大鼠骨髓间充质干细胞，暴磁组给予 15 Hz、1 mT 的正弦波电磁场刺激，PD98059+暴磁组在电磁场刺激前给予 20 μmol/L ERK 阻断剂 PD98059，PD98059 组仅给 PD98059 不进行电磁场刺激，对照组正常培养。电磁场刺激后，收集各组细胞，Western blot 法检测 ERK 通路的活性，MTT 法检测细胞增殖活性，碱性磷酸酶试剂盒检测细胞碱性磷酸酶活性。

结果与结论：电磁场刺激后，细胞 ERK1/2 磷酸化水平、细胞的增殖活性、及碱性磷酸酶活性均明显升高($P < 0.01$)；PD98059 可明显抑制 ERK1/2 磷酸化水平及细胞增殖活性的升高($P < 0.01$)，而在一定程度上提高细胞的碱性磷酸酶活性($P < 0.01$)。说明电磁场刺激可通过激活骨髓间充质干细胞 ERK 信号通路，并且主要通过该途径促进骨髓间充质干细胞的增殖；而在脉冲电磁场促进骨髓间充质干细胞分化成骨的过程中，激活 ERK 信号通路所起的作用较小。

关键词：电磁场；ERK 信号通路；骨髓间充质干细胞；细胞增殖；分化
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提供证据：在 Pubmed 中检索“bone marrow mesenchymal stem cells and ERK and electromagnetic”，检索年限不作限定，只检索出 1 篇相关文献。

创新点说明：阐明了电磁场激活的 ERK1/2 信号通路在电磁场促增殖和成骨分化中的作用。