Effects of Weak, Low-Frequency Pulsed Electromagnetic Fields (BEMER Type) on Gene Expression of Human Mesenchymal Stem Cells and Chondrocytes: An In Vitro Study

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In vitro effects of electromagnetic fields appear to be related to the type of electromagnetic field applied. Previously, we showed that human osteoblasts display effects of BEMER type electromagnetic field (BTEMF) on gene regulation. Here, we analyze effects of BTEMF on gene expression in human mesenchymal stem cells and chondrocytes. Primary mesenchymal stem cells from bone marrow and the chondrocyte cell line C28I2 were stimulated 5 times at 12-h intervals for 8 min each with BTEMF. RNA from treated and control cells was analyzed for gene expression using the affymetrix chip HG-U133A. A limited number of regulated gene products from both cell types mainly affect cell metabolism and cell matrix structure. There was no increased expression of cancer-related genes. RT-PCR analysis of selected transcripts partly confirmed array data. Results indicate that BTEMF in human mesenchymal stem cells and chondrocytes provide the first indications to understanding therapeutic effects achieved with BTEMF stimulation.

Keywords BEMER; Chondrocytes; Gene chip analysis; Mesenchymal stem cells; Pulsed electromagnetic fields.

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Introduction

Numerous studies in the literature indicate that electromagnetic fields are able to modulate the behavior of cells (Aaron et al., 2004). Despite these findings, the physical interaction between pulsed electromagnetic fields and the exposed cells is not understood until today. It is assumed that an extracellular signal is generated, physical or chemical in nature, which in turn somehow affects intracellular signal transduction pathways resulting in altered cell behavior.

Current applications of pulsed electromagnetic fields to patients are based on early findings that bone tissue represents a connection between biomechanical stimulation and the generation of an electric signal (Foster and Schwan, 1989; Fukada and Yasuda, 1957; Lakes and Saha, 1978). Furthermore, it was shown that electromagnetic fields result in enhanced osteogenesis at fracture sites (Bassett et al., 1982).

Various case reports and studies published in literature (Brighton et al., 2001; Cohly et al., 2003; Diniz et al., 2002; Guerkov et al., 2001; Lohmann et al., 2000; Torricelli et al., 2003) provide assumptions that a variety of cellular mechanisms may be affected and supported by electromagnetic fields. Especially in bone (Ciombor and Aaron, 2005; Heckman et al., 1981) and cartilage (De et al., 2003; Hulme et al., 2002; Schmidt-Rohlfing et al., 2002) differentiation, there is scientific evidence of those effects. Based on the fact that these molecular interactions are initiated by energetic activations and that these activations depend on the electromagnetic energy levels of the respective electron configuration, a well-designed electromagnetic stimulation (regarding the temporal applied intensity) might drastically enhance its width of the therapeutic application.

With regard to cells of the musculoskeletal system, the majority of the studies have focused on osteoblasts and/or induced osteogenesis as a consequence of electromagnetic field exposure. Quite a variety of systems, cell lines and—particularly—numerous different electromagnetic fields have been described in the literature and thus are difficult to compare. Nevertheless, these studies from osteogenic and cartilage cells identified several targets of electromagnetic fields such as the expression of extracellular matrix proteins (Sakai et al., 2006), stimulation of kinase signal transduction pathways, e.g., the TOR pathway (Patterson et al., 2006), growth factor synthesis, and others (Chang et al., 2004).

Although these studies on bone and cartilage significantly expanded our understanding how electromagnetic field exposure interacts with cells and tissues, other important cells of the musculoskeletal system, such as mesenchymal stem cells, have not been sufficiently analyzed. The analysis of global gene expression patterns in all musculoskeletal cells and tissues has been largely omitted. A better understanding of molecular consequences of electromagnetic field exposure to cells of the musculoskeletal system and, even more important, the knowledge which genes are not affected, are ultimately needed.

The study was done to investigate the effect of BEMER type electromagnetic fields on in vitro gene-expression patterns in human mesenchymal stem cells and chondrocytes. According to our knowledge, this is the first study to use affymetrix gene chip analysis to study pulsed electromagnetic field effects on mesenchymal stem cells and chondrocytes.
Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich (Munich, Germany) unless specified otherwise. Cell culture media were purchased from PAA (Coelbe, Germany), and fetal bovine serum (FBS) from Gibco (Karlsruhe, Germany).

Cell Culture

Primary human mesenchymal stem cells (MSCs) from bone marrow were isolated as described below and cultured in DMEM/F12 (1:1) medium with 10% heat inactivated FCS at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The chondrocyte cell line C28 I2 (Finger et al., 2003) was cultured in appropriate culture flasks similar to MSCs.

Isolation and Culture Expansion of MSCs

MSCs were isolated from trabecular bone obtained from patients undergoing total hip arthroplasty as described previously using a modified protocol (Noth et al., 2002; Schutze et al., 2005b) originally published by Haynesworth et al. (1992). Experiments were performed upon approval by the Local Ethics Committee of the University of Würzburg and informed consent. Cells were propagated in expansion medium consisting of DMEM/Ham’s F-12 (1:1) with L-Glutamine supplemented with 10% heat inactivated FBS, 1 U/ml penicillin, 100 μg/ml streptomycin (PAA, Coelbe, Germany), and 50 μg/ml L-ascorbic acid 2-phosphate. At confluence, cells were subcultured after exposure to trypsin/EDTA (PAA, Coelbe, Germany) and plated for electromagnetic field treatment in passage 1 in 25 cm² flasks.

Application of BEMER Type Electromagnetic Fields

MSCs and chondrocytes were subcultured into 25 cm² flasks until 50% confluence. For electromagnetic stimulation the flasks were placed on top of the field generating flat coil system (diameter: 48/42 cm) (BEMER3000™, INNOMED, International AG, FL-Triesen) outside the incubator. During subsequent treatments of cells, the magnetic flux density ($B$) ascended from 0 to a maximum mean of 35 mikroTesla according to an intensity time course with $B(t) = t^3 \cdot e^{t \cdot sin(t/3)}$, $[1:4]$, for each pulse, pulse-duration 30 ms; pulse-frequency 30/s. The value of 35 mikroTesla is below the local, geomagnetic flux density of approximately 50 microTesla.

To potentially alter mRNA levels, treatment was performed within two consecutive days for a period of 8 min at 12-h time intervals. In order to additionally monitor putative short-term effects on gene expression levels on day 3, another 8 min treatment was performed 12 h past the last treatment the day before and RNA isolation was done 1 h after the last treatment (see below). Control cell flasks were taken from the incubator in parallel to electromagnetic field treated flasks and placed on a lab bench in order to mimic a similar general handling procedure of treated and control cells.
RNA Isolation

Total RNA was isolated by lysing the electromagnetic field treated cells and control cells with Trizol Reagent (Invitrogen, Karlsruhe, Germany), according to the manufacturer’s instruction, prior to RNA purification by the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. RNA yield and quality was assessed photometrically (260/280 nm). Prior to microarray analysis the quality of the RNA was further controlled by agarose gel electrophoresis.

Microarray Analysis

The microarray assay was performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (www.affymetrix.com). For all samples, 10 μg of biotinylated cRNA was hybridized onto an Affymetrix Gene Chip HG-U 133A containing more than 22,000 25-meroligonucleotides corresponding to 18,400 transcripts and 14,500 genes, respectively. Arrays were scanned with the Affymetrix GeneArray 2500 scanner. Gene expression data were obtained using the Affymetrix softwares Microarray Suite 5.0, GeneChip Operating Software 1.2, and Data Mining Tool 3.0. The gene expression of control cells was compared to the gene expression of treated cells. Differentially expressed genes were obtained as follows: only genes with an increase or decrease call in the comparison were selected which have at least one present call in one of the two compared samples. Further analysis was only performed for genes that fulfilled following criteria: signal log 2 ratio < −1.0 or > 1.0 (representing a fold change of more than 2.0), change p-value < 0.001 or > 0.999, and present call in at least one of the two compared samples.

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNA was synthesized from identical amounts of total RNA (1 μg) using the M-MLV RT, Rnase H(-) Point Mutant with the provided buffer system (Promega, Mannheim, Germany), according to the manufacturer’s instructions. PCR was run using a PTC-200 Peltier thermal cycler (Biozym, Hessisch Oldendorf, Germany) in a volume of 30 μl containing 1 μl cDNA for the housekeeping gene eukaryotic translation elongation factor 1 alpha 1 (EF1α) and for selected gene products (see Table 3 for primer sequences). Primer sequences were obtained by using the online software at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, developed by the Whitehead Institute for Biomedical Research (Rozen and Skaletsky, 2000). Primer oligo-nucleotides were purchased by Operon (Cologne, Germany). The PCR reaction mix for each sample consisted of 1.5 units Taq-polymerase and 1 x reaction buffer (Amersham Biosciences, Freiburg, Germany). Final standard assay concentrations were 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, and 50 mM KCl, 0.3 mM dNTPs plus 5 pmol forward and 5 pmol reverse primer. The PCR reaction steps were as follows: 3 min at 94°C, 23–38 cycles of 94°C for 45s, 54–57°C for 45s, and 72°C for 1 min, with a final 72°C step of 3 min.

To verify the specificity of PCR products, sequence analyses were performed using the Big Dye Terminator v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instructions.
Gel electrophoresis of differential PCR product intensities was performed as described previously (Schutze et al., 2005a) using the LTF Bio ID software (LTF, Wasserburg, Germany) and normalizing on house keeping mRNA amounts.

Results
The pulsed weak electromagnetic field strength (BEMER type) was applied to human mesenchymal stem cells and a human chondrocyte cell line (TC28I2). Since data in the literature are quite variable due to the differences in model systems and, particularly, due to the different field strengths applied to these models, we chose a more general approach to monitor potential effects on cells of the musculoskeletal system, i.e., we studied the alterations in global gene expression patterns due to magnetic field application using affymetrix gene chip analyses.

Changes in Gene Expression Pattern in Human Mesenchymal Stem Cells from Human Bone Marrow
Total RNA from BEMER type electromagnetic field treated MSCs was compared to similarly handled but untreated control cell by hybridization of labeled RNA to the affymetrix HG-U 133A gene chip covering 14,500 genes. A rather short number of gene products were observed with transcript levels changing significantly according to the bioinformatic comparison analysis due to the magnetic field exposition (Table 1).

Figure 1. BEMER type electromagnetic field.
Table 1
Regulated transcripts according to the array analysis of BEMER type electromagnetic field application to MSCs

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin 1</td>
<td>CALB1</td>
<td>−3.3 a*</td>
</tr>
<tr>
<td>Uncoupling protein 3</td>
<td>UCP3</td>
<td>−9.4 a*</td>
</tr>
<tr>
<td>DKFZP586A0522 protein (unknown gene product)</td>
<td>DKFZP0522</td>
<td>−2.1</td>
</tr>
<tr>
<td>KIAA0701 protein (unknown gene product)</td>
<td>KIAA0701</td>
<td>−6.8 a*</td>
</tr>
<tr>
<td>Cytochrome P450 family 3 subfamily member a,</td>
<td>CYP3A5</td>
<td>−6.7 a*</td>
</tr>
<tr>
<td>polypeptide 5</td>
<td>PLEC1</td>
<td>3.3 b*</td>
</tr>
</tbody>
</table>

Results of gene chip analysis according to the affymetrix analysis of raw data. Gene name, symbol, and fold change of transcript levels are presented. (a*) change from present (control cells) to absent (treated cells); (b*) change from absent (control cells) to present (treated cells).

Changes in Gene Expression Pattern in Human Chondrocytes
Similar to MSCs human chondrocytes (cell line TC28I2) was analyzed. Again, a small number of gene products were observed which changed significantly according to the bioinformatic comparison analysis due to the magnetic field exposition (Table 2).

Re-Evaluation of Array Data by RT-PCR
In order to verify the results obtained by gene chip analyses, a selected number of genes were analyzed by RT-PCR was performed using the identical RNA which

Table 2
Regulated transcripts according to the array analysis of BEMER type electromagnetic field application to chondrocytes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golgi autoantigen, golgin subfamily a, 3</td>
<td>GOLGA3</td>
<td>−8.5 a*</td>
</tr>
<tr>
<td>Lysosomal-associated membrane protein 3</td>
<td>LAMP3</td>
<td>−2.9 a*</td>
</tr>
<tr>
<td>Calbindin 1, 28 kDa</td>
<td>CALB1</td>
<td>−2.4 a*</td>
</tr>
<tr>
<td>DKFZP586A0522 protein (unknown gene product)</td>
<td>DKFZPA0522</td>
<td>−1.8 a*</td>
</tr>
<tr>
<td>N-terminal asparagines amidase</td>
<td>ATAN1</td>
<td>−2.7 a*</td>
</tr>
<tr>
<td>Amyloid beta (A4) precursor protein</td>
<td>APP</td>
<td>−3.3</td>
</tr>
<tr>
<td>Signal sequence receptor gamma</td>
<td>SSR3</td>
<td>−2.8</td>
</tr>
<tr>
<td>PI-3 kinase related kinase SMG-1-like</td>
<td>KIAA0220</td>
<td>−3.9 a*</td>
</tr>
</tbody>
</table>

(hypothetical gene product)

Results of gene chip analysis according to the affymetrix analysis of raw data. Gene name, symbol, and fold change of transcript levels are presented. (a*) change from present (control cells) to absent (treated cells).
has been used in the gene chip analyses. The housekeeping gene elongation factor alpha 1 was used to control the similarity of the PCR procedure for the cDNAs under study. As is shown in Fig. 2, the transcript levels of PLEC1 increase due to the magnetic field application whereas the unknown gene product DKFZ0522 apparently does not respond.

MSCs were cultured and treated with the BEMER weak, low-frequent pulsed electromagnetic field as is indicated in materials and methods. Control cells were handled similarly without treatment. One hour past the last field exposition total RNA was isolated and subjected to RT-PCR-analysis as is indicated in materials and methods. For specificity reasons a negative control lacking cDNA was run in parallel. Subsequently to gene specific PCR (PLEC1 and DKFZ0522 38 cycles; E1α 23 cycles) using primers of Table 3, PCR products were separated by gel electrophoresis. Position of a size marker is indicated on the left. Abbreviations used: PLEC1 = plectin 1; DKFZ0522 = unknown gene corresponding to clone DKFZP586A0522; E1α = elongation factor alpha.

As can be depicted from Fig. 3 in a similar approach to electromagnetic field target genes in chondrocytes two transcripts were reevaluated by PCR which indicated a regulation due to BEMER type magnetic field application in the case of the gene product for signal sequence receptor gamma whereas the golgi autoantigen did not display alterations in mRNA levels.

Chondrocytes were cultured and treated with the BEMER weak, low-frequent pulsed electromagnetic field as is indicated in materials and methods. Control cells were handled similarly without treatment. One hour past the last field exposition total RNA was isolated and subjected to RT-PCR-analysis as is indicated

Figure 2. RT-PCR analysis of two examples of transcripts regulated in the array analysis of MSCs.
Table 3
Primer used in RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’ sequence</th>
<th>Reverse primer 5’-3’ sequence</th>
<th>Annealing temperature [°C]</th>
<th>Length of PCR product [bp]</th>
<th>Annotation ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping gene (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1 alpha 1 (EF1a)</td>
<td>AGGTGATTATCCTGAACCATCC</td>
<td>AAAGGTGGATAGTCTGAGAAGC</td>
<td>54</td>
<td>235</td>
<td>NM_001402</td>
</tr>
<tr>
<td>Transcript from array analysis of MSCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plectin 1 (PLEC1)</td>
<td>CTTCACAAAGTTGGTGCAACA</td>
<td>CAGCAGGGGAGATGAGGTTGT</td>
<td>56</td>
<td>99</td>
<td>NM_201378</td>
</tr>
<tr>
<td>Unknown gene (DKFZ586A0522)</td>
<td>GGTGCTGTGCTCTGTGAAGA</td>
<td>GCAACCATAAAACAGAAGGT</td>
<td>56</td>
<td>171</td>
<td>AK02409</td>
</tr>
<tr>
<td>Transcript from array analysis of chondrocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golgi autoantigen (GOLGA3)</td>
<td>TCCGGGAGCTGAGTGAGGT</td>
<td>CTGTTGCTGAGCTGCTGCTGT</td>
<td>57</td>
<td>200</td>
<td>NM_005895</td>
</tr>
<tr>
<td>Signal sequence receptor gamma (SSR3)</td>
<td>GCACATATTGGTAGCTTGT</td>
<td>CATCTTCTCCTCCGAGACA</td>
<td>55</td>
<td>141</td>
<td>NM_007107</td>
</tr>
</tbody>
</table>

Primers were designed using software tools available via the internet (see materials and methods). Genes were selected from Tables 1 and 2. Primers were checked for specificity against sequence data via the blast search program blast available at http://www.ncbi.nlm.nih.gov/BLAST/.
Figure 3. RT-PCR analysis of two examples of transcripts regulated in the array analysis of chondrocytes.

in materials and methods. For specificity reasons a negative control lacking cDNA was run in parallel. Subsequently to gene specific PCR using primers of Table 3 (GOLGA3 33 cycles; SSR3 35 cycles; E1α23 cycles), PCR products were separated by gel electrophoresis. Position of a size marker is indicated on the left. Abbreviations used: GOLGA3 = golgi autoantigen, golgin subfamily a, 3; SSR3 = signal sequence receptor gamma; E1α = elongation factor alpha.

Discussion

Data in the literature remain controversial on the potency of magnetic fields on cellular systems. Discrepancies in in vitro data arise due to different cellular model systems but also largely reflect the variability in the magnetic field strengths and frequencies that have been applied to these models. Thus, results on specific gene products or signal transduction systems are difficult to compare between studies. Therefore, we applied a general approach to our cellular models, i.e., we aimed to analyze changes in global gene-expression patterns due to magnetic field application to the cells. The rational to use the BEMER type magnetic field exposure was to treat cells with field strengths similar to those which are frequently applied to human subjects.

The results of the present study indicate that the exposure to weak, low-frequent pulsed electromagnetic fields is able to alter the gene expression of a limited number of gene products in human mesenchymal stem cells and human chondrocytes.
Regulated genes identified in this study via gene chip analyses mainly affect cell metabolism and the cellular matrix.

From the known genes identified in MSCs, calbindin 1 buffers cytosolic calcium and represents a vitamin D target protein (Christakos et al., 2003). The uncoupling protein 3 (UCP3) represents a mitochondrial transporter protein that participates in thermogenesis and energy balance. It is remarkable that in our study UCP3 is downregulated since the upregulation of UCP3 has been associated with an accumulation of fatty acid/acyetylCoA ratio (Russell et al., 2003) and since the protein also is activated in fasting (Tunstall et al., 2002). The cytochrome P450 family 3 subfamily a polypeptide 5 (CYP3A5) belongs to a group of heme-thiolate monoxygenases which are involved in an NADPH-dependent electron transport pathway and oxidize a variety of compounds, including steroids, fatty acids, and xenobiotics. The downregulation of CYP3A5 observed in our study is interesting since polymorphic variants have been described that are associated with elevated blood pressure (Fromm et al., 2005). Plectin 1 (PLEC1) represents an intermediate filament binding protein and thus behaves as a structural component of the cytoskeleton. Its function is crosslinking and stabilization of the cytoskeleton and its dynamics (Andersen et al., 2005). The observed upregulation of PLEC1 in our study with MSCs appears to be protective since the deletion of PLEC1 results in muscular dystrophy (Smith et al., 1996).

Among gene products identified in chondrocytes, the golgi autoantigen golgin subfamily a 3 (GOLGA3) is involved in transport processes in the Golgi apparatus (Hicks and Machamer, 2002). The function of the lysosomal-associated membrane protein 3 (LAMP3) which was downregulated in our analysis is not well understood. However, it is remarkable that the reverse situation, compared to our study, namely an overexpression of LAMP3 has been associated with uterine cervical cancer (Kanao et al., 2005) and others (Ozaki et al., 1998). Further identified gene products in chondrocytes include the signal sequence receptor gamma 3 (SSR3) which is localized in the membrane of the endoplasmic reticulum and part of a complex whose function is to bind calcium to the ER membrane and thereby regulate the retention of ER resident proteins. The amyloid beta (A4) precursor protein (APP) which represents a cell surface receptor involved in cell mobility.

A limitation of this study is the single array analysis which has been performed. Therefore, the results should be regarded as a first hint on BTEMF effects on these cellular systems. Nevertheless our findings indicate that matrix dynamics and cell metabolism/energy balance are processes which are affected by the electromagnetic field application. Except for calbindin 1 which was identified from both MSCs and chondrocytes, rather the effects appear to be cell specific, this also includes our previous findings in human osteoblasts (Kafka et al., 2005). The advantage of these global screens on gene expression also relies in the gain of knowledge on which genes are not regulated in the individual experimental system investigated. There was no increased expression of genes known to be linked to cancer development nor did we observe inductions of mRNA levels related to other diseases in this study on MSCs and chondrocytes as well as in our previous study on human osteoblasts. (Kafka et al., 2005). This study confirmed the data retrieved from several other trials where, so far, there is no evidence that low-energy pulsed electromagnetic fields may induce the development of malign tumors (Feychting and Forssen, 2006; Johansen and Olsen, 1998; Loberg et al., 2000; Tynes and Haldorsen, 2003).
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