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Effects of a low-intensity electromagnetic field on fibroblast migration and proliferation

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The aim of this study was to test if an extremely weak 1 GHz electromagnetic field (EMF), known to be in resonance with clusters of water molecules, has biological effects on human fibroblasts. We demonstrated that in an *in vitro* model of wound healing, this EMF can activate fibroblast migration. [^{3}H]thymidine incorporation experiments demonstrated that the EMF could also activate fibroblast proliferation. Activation of the expression of human fibroblast growth factor 1 (HFGF1) after EMF exposure showed that molecular wound healing pathways are activated in response to this water-resonant EMF.

Keywords Electromagnetic field, Water-resonant, Wound, Fibroblast, Gene activation

INTRODUCTION

A fibroblast is a type of cell that synthesizes extracellular matrix and collagen, the structural framework for animal and human tissues, and plays a critical role in wound healing. Fibroblasts are the most common cells of the connective tissue in animals. The main function of fibroblasts is to maintain the structural integrity of connective tissues by continuously secreting precursors of the extracellular matrix, and as a result this cell type is critically involved in the process of wound healing. Fibroblasts begin to enter a wound site two to five days after wounding, as the inflammatory phase ends, and their numbers peak at one to two weeks post-wounding. By the end of the first week, fibroblasts are the principal cell lineage in the wound, and they are the main cell type that lays down the collagen matrix in the wound site (Stadelmann et al., 1998). Collagen deposition is important because it increases the strength of the repairing wound; in addition, cells involved in the regulation of inflammation, angiogenesis, and further connective tissue construction attach to, proliferate, and differentiate on, the collagen matrix laid down by fibroblasts (Ruszczak, 2003).

It is known that EMFs play an important role in the cascade of processes determining cell migration, adhesion, and differentiation. The electrical currents and the related fields are generated by passive Na^+ uptake from the environment,

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leading to an internally positive transepithelial potential difference (TEP) (Funk and Monsees, 2006). Endogenous EMFs also exist in the immediate vicinity of wounds, where they are created due to a disruption of the TEP in the epithelial layer. This electric potential collapses at the wound site, but rises to the potential of healthy cells with increasing distance from the wound (Song et al., 2002). For this reason, the application of EMFs may have therapeutic relevance for wound healing and other pathologies. Currently, studies of the biological effects of EMFs are performed with high-frequency EMFs (1–100 Hz) or with those of low physiological frequencies (8–30 Hz) (Funk and Monsees, 2006).

In a number of studies, it has been demonstrated that EMFs of different frequencies can accelerate wound healing (Huo et al., 2009; Strauch et al., 2007). The first study employed an industrially produced therapy device, the action of which is based on an EMF-induced direct current. The authors were able to observe the stimulation of keratinocyte cells participating in wound re-epithelialization, but did not detect significant effects on fibroblast migration or proliferation.

It has been previously demonstrated that EMF frequencies of 50, 65, and 100 GHz (millimeter wavelength) and 1 GHz (decimeter wavelength) correspond to the resonant (natural) frequencies of clusters of water molecules, and also to animal tissues which have similar water resonant spectra. This resonance interaction with aqueous media is observed at very low powers (below 1 $\mu W/cm^2$), and it has been hypothesized that such EMF energy can be transferred between clusters of water through hydrogen bonds; however, at powers of above 1 $\mu W/cm^2$, transfer of EMF energy between clusters of water molecules is reduced by heating effects which disrupt hydrogen bonds (Sinitsyn et al., 2000). The absence of such heating effects at EMF energies below 1 $\mu W/cm^2$ may be associated with uninterrupted resonance interactions within aqueous media which could reach internal tissue regions and cause biological effects due to an enhancement of the natural oscillations of water molecules, and probably also by generation of super-weak electrical currents.

Electromagnetic radiation at frequencies of 1 GHz is easily transmitted in water. The absorption coefficient in water of millimeter wavelength EMF is at least two orders of magnitude larger than the absorption of 1 GHz EMF. We have therefore chosen to study the biological effects of weak water-resonant 1 GHz EMF on fibroblast maintained in culture.

MATERIALS AND METHODS

Cell culture and EMF treatment

Human Dermal Fibroblasts (HDFs) (PromoCell, Heidelberg, Germany) were cultured in DMEM supplemented with 2 m ML-glutamine, 100 IU/ml penicillin and streptomycin, and 10% heat-inactivated FBS in twelve-well plates that were pre-coated with collagen (50 μg/ml) and blocked with BSA (3% BSA in PBS). A conical antenna connected to Aquaton-2 generator of 1 GHz EMF (Telemak, Saratov, Russia) was placed at a distance of 10 cm over cell culture plates. When placed at a distance of 10 cm over cell culture plates, the Aquaton-2 conical antenna (10 cm in diameter and height) creates an circular area of exposure (20 cm in diameter) in which power density (5 nW/cm^2) is evenly distributed within a circular region of 10 cm diameter below the antenna. The remainder of the exposed corona is subject to a power density which decreases from 5 to approximately 3,5 nW/cm^2 at its inner and outer outer perimeters, respectively. In all experiments, cells exposed to EMF treatment were located within the internal circular area of exposure of 10 cm diameter. At a frequency of 1 GHz, the absorption coefficient in water is 0.1 cm^{-1}, and so we consider that absorption losses within a 2, 5 mm layer of cell culture media in the

present experiments are negligible. Two plates were used in every experiment. One of the plates was treated with the EMF for 20 min, and the other served as the control. The control and experimental plates were removed and replaced in the CO_2 incubator simultaneously to avoid the effects of possible differences in environment.

^{3}H-thymidine incorporation assay

Cells were seeded in 35 mm plates and cultured until they reached 70% confluence. Cells were treated for 20 min with the Aquaton-2 device in 5 independent experiments. Five microliters ^{3}H-thymidine (1mCi/ml) was then added directly to the incubation media; the cells were incubated for 3 h and then treated. The cells were then washed twice with ice-cold PBS, followed by two washes with 5% TCA and one wash with PBS. The cells were then solubilized by adding 800 μl 0,5N NaOH /0,5% SDS followed by thorough aspiration. The solubilized cell solution was then collected into 5 ml scintillation vials, 4 ml Ultima gold scintillation cocktail was added, and the vials were counted.

Fibroblasts migration assay

Human Dermal Fibroblasts (HDFs) (PromoCell, Heidelberg, Germany) were cultured in DMEM supplemented with 10% FBS in twelve-well plates that were pre-coated with collagen (50 μg/ml) and blocked with BSA (3% BSA in PBS). A scratch was made with a micropipette tip on the confluent cell culture. The relative migration of the cells was calculated using ImageJ software on images taken immediately and 8 h after scratching.

A scratch was performed with a micropipette tip on the confluent cell culture. The relative migration of the cells was calculated using ImageJ software on images taken immediately and 9 h after scratching.

Quantitative RT-PCR

cDNA was prepared by reverse transcription of total RNA with SuperScript III (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. cDNA was amplified with primers selected for specificity from the Harvard University primer bank (http://pga.mgh.harvard.edu/primerbank/). The human fibroblast growth factor 1 promoter (hFGF1) cDNA specific primers were as follows: sense 5′-CTCCCGAAGGATTAAACGACG-3', and antisense 5′-GTCAGTGCTGCCTGAA-TGCT-3′. The human vascular endothelial growth factor-A (hVEGFA) specific sense and antisense primers: 5′-AGGAGGAGGGCAGAATCATCACGA-3′ and 5′-CTCCTGGAAGATGTCCACCAGGGTC-3′. cDNAs of two reference genes, ribosomal protein (RPL32) and GNBL2, were amplified with the following primers sense: 5′-CATCTCCTTCTCGGCATCA-3′ and antisense: 5′-AACCCTGTTGTCAATGCCTC-3′ (RPL32); sense: 5′-GAGTGTGGCCTTCTCCTCTG-3′ and antisense: 5′-GCTTGCAG-TTAGCCAGGT-3′TC. Real-time PCR was performed on an Applied Biosystems 7300 instrument using Platinum SYBR Green qPCR supermix (Invitrogen). Amplification products were verified by sequencing.

RESULTS

Fibroblast migration

Firstly, we studied the effects of the 1 GHz EMF on fibroblasts in a migration assay which has been widely used as an *in vitro* test of wound healing. It is possible to quantify the speed of fibroblast migration by measuring the area of the artificial *in vitro* wound (Fig. 1a).

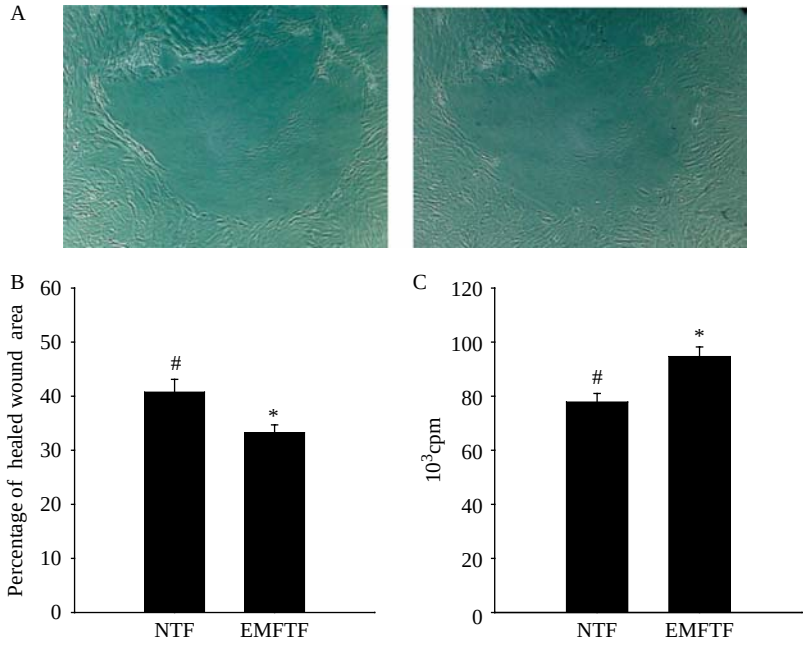

FIGURE 1 Effect of a 1 GHz EMF on fibroblast migration and proliferation. **A.** Representative images of fibroblasts in artificial wound healing experiments taken immediately (left) and 8 h later after scratching (right). (A microscope with a digital camera was used to capture images.) **B.** Quantification of fibroblast migration. The "wound" area was calculated in pixels with ImageJ 1.32 Software (National Institute of Health) immediately and 9 h later after scratching and expressed as percentage of healed wound of the original area (mean ± SEM). *P = 0,017. **C.** Fibroblast proliferation efficiency based on ^{3}H-thymidine incorporation assays measured as counts per minute (cpm) (mean ± SEM). *P = 0,005. NTF, Non-treated fibroblasts; EMFTF, Fibroblasts treated by EMF.

To evaluate weather EMF had effects on fibroblast migration, we performed 5 independent experiments in which scratches were generated in 5 wells of two 12-well plates. The measurement of wound gaps was performed before treatment and after 9 h of incubation, and demonstrated an acceleration of fibroblast migration of approximately 18% (P = 0,017) (Fig. 1b).

Fibroblast proliferation

We further measured the efficiency of DNA replication which is directly related to cell proliferation. To address the effect of the 1 GHz EMF on the proliferation of fibroblasts, we performed *in vitro* ^{3}H-thymidine incorporation assays. Comparisons between [^{3}H]thymidine incorporation into control and EMF-treated cells demonstrated that HDFs responded to Aquaton-2 treatment with an approximate 22% (P = 0,005) stimulation of ^{3}H-thymidine incorporation (Fig. 1c).

Measurement of gene expression

To examine whether molecular biological effects related to artificial wound healing occur in response to EMF treatment, expression of human fibroblast growth factor 1 (hFGF1) and human vascular endothelial growth factor (hVEGF) were measured before and 1 h after treatment. Both FGF1 and VEGF mRNA levels were found to be associated with the EMF treatment. While FGF mRNA levels were significantly increased in fibroblasts treated by the 1 GHz EMF (Fig. 2a), an association between VEGF mRNA and EMF treatment was not significant, although treated cells tended to have higher VEGF mRNA levels than untreated cells (Fig. 2b).

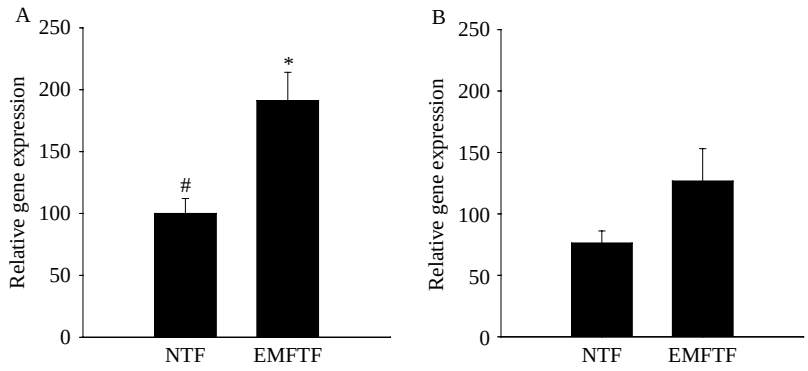

FIGURE 2 Effect of a 1 GHz EMF on hFGF1 (A) and hVEGFA (B) mRNA expression. mRNA expression level were calculated by normalizing average Ct-value of the two reference genes with the experimental gene values (mean ± SEM). **A.** hFGF1 mRNA expression in skeletal muscle. *P = 0,012; **B.** hVEGFA mRNA expression in skeletal muscle. P = 0,118. NTF, Non-treated fibrobasts; EMFTF, Fibroblasts treated by EMF.

DISCUSSION

Here, we demonstrated the biological effects of a super-weak water-resonant 1 GHz EMF. This suggests that a very low-intensity EMF can cause significant biological effects. The biological action of EMFs of much higher energies which are used at present in biological studies may mainly be related to effects involving heating and induced direct currents. The idea of using a weak pulsed EMF which can resonate with certain molecular oscillations and can produce biological effects is not new. Rosenspire et al. (2001, 2005)) employed a weak EMF to the amplitude of NAD(P)H oscillations. We used a similar approach using EMF resonant to water clusters to evaluate the effects of the EMF on fibroblasts in an *in vitro* model of wound healing. Recently, it was demonstrated that a much stronger EMF could accelerate normal and diabetic wound healing by increasing vascular density. The authors demonstrated that the EMF could activate the proliferation of endothelial cells (Callaghan et al., 2008). Another study demonstrated that human skin keratinocyte (a key cell type in wound re-epithelisation) migration and proliferation can also be enhanced by a non invasive EMF (Huo et al., 2009). The data concerning fibroblasts is more controversial. While Huo et al. did not find any influence of an EMF on fibroblast migration and proliferation, another study reported the stimulation of human fibroblast migration in response to an EMF (Sun et al., 2004). In the study of Hou et al. (1999), it is hypothesized that the main mechanism of non invasive EMF influence is the induction of electrical currents in cells surrounding the target cells. It is possible that similar effects are induced by the super-week (5 nW/cm^2) high-frequency EMF employed in our fibroblast experiments, where enhancement of natural oscillations in water molecules might enhance super-weak currents in cellular structures. FGF1 is involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, cell migration and proliferation. The activation of hFGF1 expression, and the tendency for increased VEGFA expression following EMF treatment demonstrated in the current study show that molecular wound healing pathways are activated in response to water resonant EMFs.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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