

Electromagnetic fields increase in vitro and in vivo angiogenesis through endothelial release of FGF-2

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SPECIFIC AIMS

Pulsed electromagnetic fields (PEMF) have been shown to be clinically beneficial in repairing bones and other tissues, but the mechanism of action is unclear. The present study examined the effect of PEMF on angiogenesis in order to elucidate a potential mechanism for its therapeutic effect.

PRINCIPAL FINDINGS

1. PEMF induces endothelial tubule formation

Endothelial cell tubulization is critical to the process of angiogenesis. The effects of PEMF on endothelial tubule formation were assessed using a gelatin microcarrier assay that allowed endothelial cells to tubulize from a single focus, which could subsequently be quantified. In PEMF, a several fold increase in tubulization of one and two diameters was seen (41/50 vs. 24/50, 21/50 vs. 3/50; $P < 0.01$), and only those human umbilical vein endothelial cells (HUVECs) exposed to PEMF developed tubules greater than three diameters (6/50 vs. 0/50; $P < 0.05$). Exposure to PEMF also led to a significant increase in the total number of tubules per microcarrier (2.25 ± 0.45 vs. 1.00 ± 0.25 ; $P < 0.05$) (Fig. 1A–D).

2. PEMF stimulates endothelial proliferation

Endothelial proliferation is another component essential to the formation of new blood vessels. Thymidine incorporation established that HUVECs exposed to PEMF demonstrated enhanced proliferation compared with controls (9.2×10^4 vs. 3.5×10^4 cpm; $P < 0.01$). This increase in proliferation correlated with an increase in absolute cell number ($220 \pm 14 \times 10^3$ cells/well in PEMF vs. $117 \pm 9 \times 10^3$ cells/well in controls) (Fig. 1E, F). However, PEMF did not increase osteoblast or fibroblast proliferation (Fig. 1G).

3. PEMF releases the soluble proangiogenic protein FGF-2

To clarify the precise cellular mechanism by which PEMF exerts its proangiogenic effects, media harvested from HUVECs cultured in PEMF (PEMF-conditioned media) was assessed for angiogenic factors. Proteomic screening demonstrated FGF-2 production to be increased by exposure to PEMF. Northern blot analysis revealed a 150% increase in FGF-2 mRNA and ELISA confirmed 5-fold elevations in FGF-2 protein by PEMF (147.9 ± 011.8 pg/mL vs. 29.5 ± 5.1 pg/mL; $P < 0.05$). Addition of FGF-2-neutralizing antibody decreased the stimulatory effects of PEMF on HUVEC proliferation ($147.28\% \pm 9.73\%$ vs. $94.85\% \pm 3.70\%$, $P < 0.05$). Other proteins elevated to a lesser extent by PEMF included angiopoietin-2 (2320.3 ± 1128.4 vs. 3323.8 ± 1168.7 pg/mL; $P < 0.05$), thrombopoietin (46.7 ± 4.3 vs. 133.1 ± 51.4 pg/mL; $P < 0.05$), and epidermal growth factor (4.8 ± 1.3 vs. 7.1 ± 0.4 pg/mL; $P < 0.05$) (data not shown).

4. Conditioned media stimulates HUVEC and fibroblast proliferation and migration, but not osteoblast proliferation

To confirm the release of functional FGF-2 from PEMF-stimulated HUVECs, we studied HUVEC, fibroblasts, and osteoblasts under the influence of media collected from HUVEC cultures after 24 h PEMF exposure. The average HUVEC and fibroblast proliferative response to PEMF-conditioned media was at least double that of cells given media from control cultures not exposed to PEMF (data not shown). In osteoblasts, proliferation in conditioned media was not significantly different from controls. Addition of a cyclooxygenase inhibitor (indomethacin) was unable to block PEMF-induced stimulation

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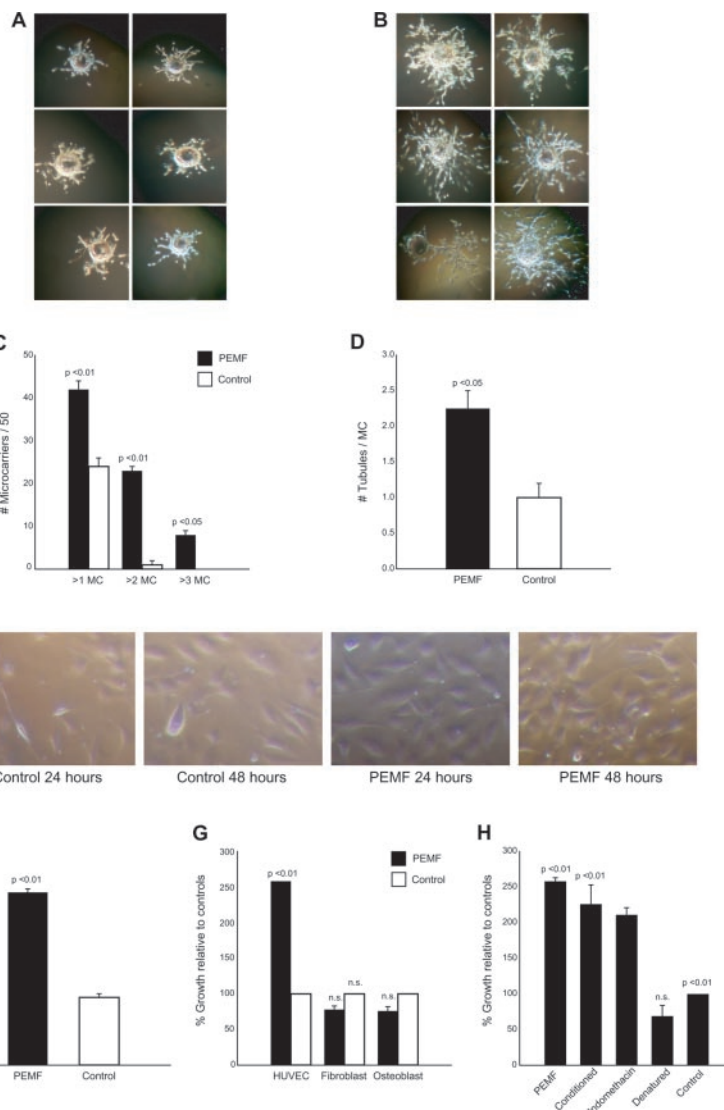


Figure 1. PEMF stimulates 3-dimensional angiogenesis in vitro. A 3-dimensional angiogenesis assay was performed on HUVECs grown on gelatin microcarriers and embedded in a fibrin gel. Representative pictures 7 days after HUVEC-seeded microcarriers were cultured in normal conditions (A) or PEMF (B) demonstrating increased tubulization of PEMF. C) 50 microcarriers were chosen at random and the number of microcarriers exhibiting tubulization of > 1 (>1MC), 2 (>2MC), or 3 diameters (>3MC) was quantified. D) Microcarriers were assessed for the number of tubules present on each microcarrier. The extent of proliferation of HUVECs over 48 h was examined by light microscopy (E) and quantified by thymidine incorporation (F), revealing that PEMF significantly augmented the proliferation of HUVECs ($P<0.01$) but had no effect on osteoblasts or fibroblasts (G). H) Media cultured in PEMF was able to enhance the proliferation of HUVECs, but denaturing the media ablated this effect. HUVEC proliferation in PEMF was not inhibited by the addition of indomethacin, a prostaglandin (PGE₂) synthesis inhibitor.

in HUVECs, suggesting that arachidonic acid metabolites were not involved. However, heat denaturing eliminated the stimulatory effects of PEMF-conditioned media on HUVECs ($77.8\pm 10.2\%$ vs. $222.0\pm 2.3\%$; $P<0.01$), demonstrating that a soluble protein was responsible for the proliferative activity (Fig. 1H). Migratory populations of fibroblasts and HUVECs more than doubled under the influence of conditioned media (data not shown).

5. PEMF stimulates in vivo angiogenesis

Having demonstrated that PEMF has a potent effect on endothelial cells in vitro, we examined whether PEMF was able to stimulate angiogenesis in vivo. Using a Matrigel model, PEMF increased vascular in-growth >2-fold by day 3 (13.3 ± 0.41 vs. 5.8 ± 0.28 cells/hpf; $P<0.01$). This increase in vascular in-growth persisted through days 10 and 14 (16.6 ± 0.49 vs. 12.6 ± 0.43 cells/hpf; $P<0.01$, and 19.4 ± 0.55 vs. 14.8 ± 0.40 cells/hpf; $P<0.01$, respectively) (Fig. 2).

CONCLUSIONS

In this study, we demonstrate that PEMF stimulates processes critical for angiogenesis. The delivery of PEMF at low doses, identical to that currently in clinical use, significantly increased endothelial cell proliferation and tubulization, processes important for vessel formation. The ability of PEMF to increase cellular proliferation was unique to endothelial cells. This suggests that endothelial cells are a primary target of PEMF stimulation, releasing protein in a paracrine fashion to induce changes in neighboring cells of different lineages and up-regulating angiogenesis. However, direct stimulation and conditioned media assays did not produce significant changes in osteoblast proliferation. Thus, the ability of PEMF to enhance the healing of complicated fractures is likely the result of increased vascularity rather than a direct effect on osteogenesis as previously believed.

Although VEGF is the most ubiquitous mediator of angiogenesis, it was not responsible for the angiogenic effect of PEMF in these experiments. Angiogenic pro-

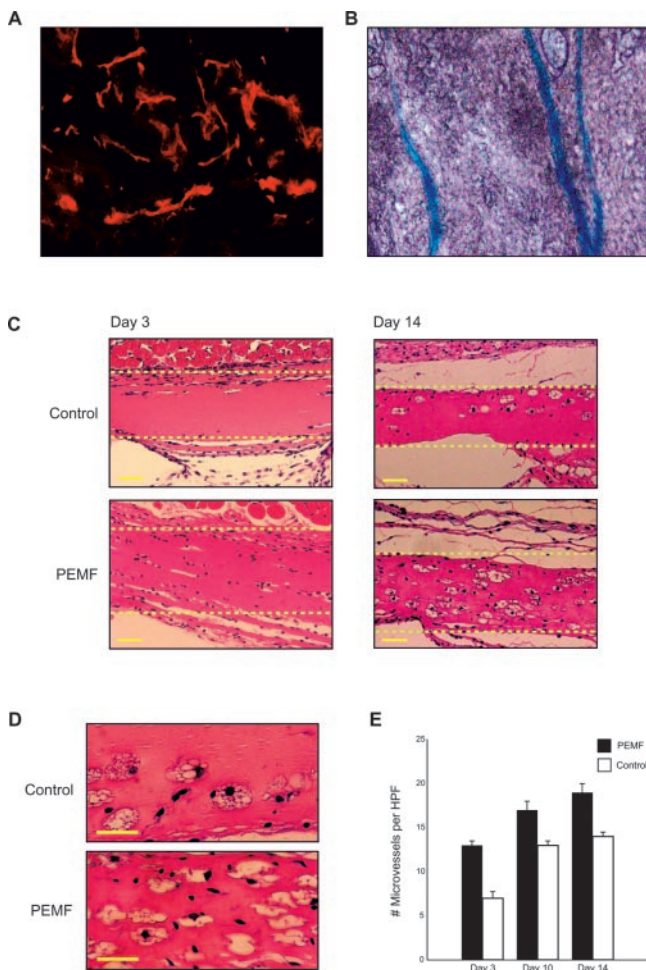


Figure 2. PEMF promotes angiogenesis in an in vivo Matrigel plug assay. Vascular in-growth within Matrigel 2 wk after implantation was confirmed by staining for CD31 and Tie2 (A, B, respectively). C) Representative pictures of Matrigel (dotted box) from mice in control and PEMF cages show that exposure to PEMF led to an increase in the degree of vascular in-growth relative to controls. Scale bars = 25 μ m. D) High-power views of a representative section of Matrigel from control mice (top) and PEMF-exposed mice (bottom) are depicted. Scale bars = 25 μ m. E) Quantification of cells within the Matrigel demonstrated a significant PEMF stimulation of vascular in-growth at days 3, 10, and 14.

tein screening demonstrated a 5-fold increase in FGF-2, a well-described mediator of angiogenesis. Addition of an FGF-2-neutralizing antibody reduced PEMF-stimulation of endothelial cells, but proliferation did not return completely to baseline. It is therefore possible that PEMF does not simply act through the up-regulation of a single agent (i.e., FGF-2). However, it seems likely that FGF-2 signaling is the predominant mechanism and other cytokine changes are secondary. The in vitro potency of PEMF to increase endothelial cell proliferation was comparable to that of high doses of VEGF or FGF, suggesting that this phenomenon is of true biologic relevance in vivo.

To support this, we examined the effect of PEMF on in vivo angiogenesis. Using the well-established Matrigel assay, we demonstrated that PEMF was sig-

nificantly increased angiogenesis in vivo. This suggests that the effects of PEMF are directed toward preexisting endothelial cells rather than bone marrow-derived endothelial precursor.

If PEMF is able to augment angiogenesis, its clinical utility may extend well beyond its current role in bone healing. One obvious application is in the field of *therapeutic angiogenesis*, defined as the artificial manipulation of blood vessel growth to treat ischemic conditions. The majority of existing techniques for therapeutic angiogenesis are based on the delivery of single proangiogenic cytokines or the supplementation of vascular stem cells. Agents such as VEGF or FGF have shown promise in animal models, but clinical trials have been disappointing. PEMF may offer distinct advantages as a noninvasive and targeted modality that is able to release several growth factors to achieve therapeutic angiogenesis. Since PEMF uses commonly available, clinically approved technology, it may have rapid applicability in the treatment of ischemic conditions. Data from this study provide a rational basis for use in these conditions.

The finding that PEMF was able to stimulate endothelial cell kinetics raises important questions regarding the relationship between PEMF and carcinogenesis. Epidemiological studies have suggested a link between electromagnetic fields and malignancies including breast cancer, brain cancer, and leukemia, but the precise mechanism, if any, remains unknown. Although multiple papers confirm that electromagnetic fields are not directly mutagenic or carcinogenic, none have examined the possibility that electromagnetic fields may promote tumor progression once malignant transformation has occurred. Since angiogenesis is believed to be essential for tumor growth, spread, and eventual clinical disease, the present study suggests that the link between electromagnetic fields and cancer may be via increased angiogenesis. Epidemiological studies suggest that exposure to PEMF (i.e., high-tension power lines) at a wide range of frequencies can be correlated with an increased risk of cancer. However, a direct comparison to the field strength used in this study is difficult given the wide-amplitude window produced by pulsed delivery. Although clinical data suggest that PEMF is safe, the possibility that electromagnetic fields are not themselves carcinogenic, but promote tumor progression via increased angiogenesis, warrants further investigation.

In conclusion, although PEMF has been used for years by clinicians to supplement bone healing, its precise mechanism of action has not been determined. Our data provide evidence to support the concept that PEMF acts by promoting angiogenesis primarily through the coordinated release of FGF-2. This suggests that PEMF may facilitate healing by augmenting the interaction between osteogenesis and blood vessel growth. This finding not only elucidates a novel mechanism for PEMF action, but suggests extended applications for PEMF in the treatment of ischemic disease and a potential linkage between electromagnetic fields and tumor biology. EJ