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EMF Induces Differentiation in HL-60 Cells

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Abstract This investigation provides evidence that a 60-Hz electromagnetic field (EMF) at 1 gauss (G) can drive differentiation of cultured hematopoietic progenitor cells. HL-60 cells are known to differentiate from a nonphagocytic suspension culture to an attached fibroblast-like culture with high phagocytic activity in the presence of the tumor-promoting phorbol ester 12–0-tetradecanoylphorbal-13-acetate (TPA). The effect of 60-Hz EMF at 1 G on differentiation is approximately equivalent to treatment of the cells with 250–500 pg/ml TPA. Furthermore, the effect of both EMF and TPA treatment on differentiation is additive at low TPA concentrations. The results strongly suggest similarities between the effects of TPA treatment and EMF exposure and thus provide an approach for tracing the origins of the molecular effects of EMF exposure, as many transduction pathways in the differentiative process are defined. J. Cell. Biochem. 73:212–217, 1999. © 1999 Wiley-Liss, Inc.

Key words: electro-magnetic fields; differentiation; signal transduction

The possibility that relatively low electromagnetic fields can cause changes in cellular activity is a contentious, but persistent, postulate that remains to be resolved. Initially, research on electromagnetic fields (EMF) was driven by the possibility of negative health effects resulting from exposures to electric power lines or other environmental sources of EMF. Some epidemiological studies linked EMF exposure to increased cancer incidence. A pivotal study by Wertheimer and Leeper, 1979] found that the effects of repeated exposures to weak magnetic fields resulted in twice the normal incidence of leukemia in children. Subsequent studies replicated critical aspects of these findings [cf. Savitz, 1988; London et al., 1991]. The presence of confounding environmental factors, however, can make interpretation of epidemiological studies controversial. On the other hand, laboratory studies show that biological effects can be caused by exposure to EMF, but it is still not clear how EMF brings about the reported effects, nor have the effects always been well understood in terms of the cell function or malfunction.

The purpose of the present study was to devise an experimental design that could provide a means of simply confirming or negating a connection between EMF exposure and outcome to the cell. Further, it was desirable that an inherent quality of the endpoint be one in which there was some understanding of the cell signaling involved, as this could be ultimately used to test pathways involved in the proposed health effects. This approach is critical to biologically testing the results reported in environmental studies, as it is highly unlikely that EMF exposure per se could initiate tumor development and no direct genotoxic effects have been confirmed [McCann et al., 1993]. If EMF exposure plays a role in tumorigenesis, or any health effect, it either promotes a process that is already initiated and/or modifies cell regulatory processes.

The model for the present experiments was based on a report by Dertinger et al. [1995]. In this study phagocytic activity was used as a measurement of differentiation. Treatment with phorbol or phorbol esters of different efficacies

Abbreviations: elf, extremely low frequency; EMF, electromagnetic fields; G, gauss; Hz, Hertz; TPA, 12–0-tetradecanoylphorbol-13-acetate.

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was measured in HL-60 cells as a function of in vivo tumor promoting activity. The relative induction of differentiation in HL-60 cells was shown to be proportional to the known in vivo promoting activity of phorbol esters. Importantly, this study demonstrated that the sensitivity of the assay relative to TPA induction of differentiation was <100 pg/ml TPA. Thus, based on a very sensitive assay, if EMF also induces differentiation, it is possible that exposure could have tumor promoting effects.

Induced cellular differentiation in hematopoietic progenitor cells by TPA turned out to be a perfect paradigm for investigating the effects of EMF; our results show that exposure to EMF can also induce differentiation. Within 24-h treatment with EMF or TPA, a portion of the cells undergo transition from nonphagocytic cells in suspension culture to attached fibroblast-like cells with phagocytic activity.

Significantly, signaling pathways have been identified in induced differentiation. Mitogens, including TPA, cause cell proliferation, or initiate early steps consistent with proliferative induction before cells proceed to protein kinase C (PKC) depletion and differentiation [Kharbanda et al., 1994]. PKC is the primary receptor for TPA with concomitant phosphorylation of endogenous proteins. This is consistent with previous findings of the effects of EMF on cells [Luben, 1991; Wallaczek, 1992; Uckun et al., 1995; Dibirdik et al., 1998; Kristupaitis et al., 1998]. The present experiments provide proof that EMF exposure can interact with cells to produce a definitive morphologic endpoint and are complementary to previous findings.

MATERIALS AND METHODS

Cell Lines

HL-60 cells, derived from a promyelocytic leukemia patient, are an in vitro model for AML and a standard for studies of induction and differentiation [Rovera et al., 1979]. The subline we use was obtained from Dr. I. Weinstein, Columbia University Health Sciences. The karyotype of this line is consistent with known characteristics of HL-60 cells. Stored cells are thawed at regular intervals. They are kept in culture for about 2 weeks before they are used for experimental procedures. The cells are maintained in RPMI 1640 (Gibco BRL Life Technologies, Inc., Grand Island, NY) with 10% fetal calf serum (FCS) and 1% Pen-Strep. The medium is changed on a regular schedule (normally 3 times weekly MWF). Calf serum is purchased from Sigma Chemical Co. (St. Louis, MO) on an annual basis to ensure that the serum lot is uniform; new batches are tested relative to cell growth. The viability of cells is determined by trypan blue dye exclusion. Cells are maintained in exponential growth; density is determined with a hemocytometer

Exposure Conditions

Cells are exposed to EMF (60 Hz; 1 G) for periods up to 48 h in a Helmholtz Coil Exposure System designed by Electric Research and Management, Inc. (ERM State College, PA). The exposure conditions were selected as those typical of other experiments which have tested the effects of EMF on signaling pathways. The ERM exposure system provides a graded series of field settings, which are then maximized using the function generator. A sine-wave generator (Wavetek 11-MHZ function generator, model 21) with variable frequency control is used. The ERM system has the capacity to conduct simultaneous sham exposure under blind conditions. The exposure coils consist of two double-wound coils in an approximate Helmholtz configuration (164-turn rectangular windings of gauge 19 magnet wire measuring ~13 × 14 cm with 8-cm spacing; the coils are 1 cm in diameter wound around an approximate square form). The coils are supported by an acrylic frame in which the test samples are placed. The coils are placed in 2 mu metal cans each inside a separated compartment of a double-door incubator. Flasks or dishes are placed on a plexiglass stand in the horizontal plane in an area shown to have a uniform magnetic field and maximum field strength. Field characteristics were measured using a Tektronix 2245A oscilloscope and a Metex Digital Multimeter.

Cells are taken from a single "parental" flask and placed into six wells of a 12-well (2 ml/well) plate. The plate is obtained by dividing a 24-well plate [this approximates the exposure geometry using a T-25 flask]. Cells are changed to new media ~24 h before experiments and the concentration adjusted to 0.5 × 10^6 cells/ ml medium. The concentration of the cells is adjusted to about 1 × 10^6 cells/ ml before exposures. The coil is turned on at least 30 min before experimental procedures are initiated, and the cells are removed from the coil while the signal is still on. The signal generator is

placed outside the incubator. Media temperatures are monitored under conditions equivalent to exposures (Physotemp thermocouple probe sensitive to 0.1°C). The magnetic fields in the current incubator have been measured over a period of 5 years at about 2 mG (rms).

For the present experiments, the cells were subjected to: no treatment, EMF exposure, growth in increasing doses (pg to ng) of TPA and TPA plus EMF exposure, all under the same environmental conditions.

TPA

Comparison with TPA ensures that conditions are correct to obtaining differentiation, and allows the development of points for comparison with TPA; experiments included cells incubated in TPA at concentrations between 0 and 5,000 pg/ml.

Assaying Phagocytic Activity

Fluorescent microspheres (3 µm, Polyscience Fluoresbrite) were sterilized in methanol for 10 min. The spheres were collected by centrifugation, sonicated for one minute in RPMI medium and added to a T-25 flask containing the cells (density of 10 spheres/cell). The spheres and cells were then placed in wells for exposure. After exposure, the medium was removed and replaced with RPMI containing 0.25% trypsin for 5 min to remove adherent spheres and disaggregate cells. The cells were then pipetted vigorously to further reduce aggregates and remove spheres attached to the cell membrane. The medium was changed several times and to reduce free-floating fluorescent particles. This was monitored by microscopy.

Flow Cytometry

Flow cytometry is used as the primary method for determination of the phagocytic uptake of fluorescent particles. The analysis used conditions essentially as described by Dertinger et al. [1995]. Free microspheres and fluorescent cells were distinguished with the forward scatter parameter (particle size). Cells are sorted at various gates to confirm the mean number of fluorescent beads/cell. Essentially, gating (in these experiments, 2 or more particles per cells) is used to provide the most precise measurement of increases in fluorescent particle number over the pattern exhibited by unexposed cells [Dertinger et al., 1995]. Excitation of fluorescence used the Innova Enterprise (TM) laser (488 nm beam at 135 mW). Imaging microscopy was used to set conditions and to confirm the differentiation state based on morphology. Microscopy was also used to assess possible anomalous binding of the fluorescent particles to cells. Internalized Fluoresbrite particles are large and bright enough to be recognized under 10× phase; image magnification is used to confirm internalization.

RESULTS

Initially, three methods of measuring phagocytized fluorescent particles were compared, imaging microscopy, fluorometric analysis, and flow cytometry. A significant increase in phagocytic activity was observed after 24-h exposure to 60-Hz EMF at 1 G, irrespective of method. The most consistent results were obtained using flow cytometry. This was coordinated with microscopic examination for determination of nonspecific sticking of the particles to the cell surface. Results that used microscopic analysis of phagocytic activity in cells exposed to a 60-Hz, 60-mG field have been reported previously [Tao et al., 1998].

Flow cytometry proved to be an excellent and efficient way to measure fluorescent particles. Data from a series of exposures at 60 Hz, 1 G are summarized in Figure 1. A significant difference in the number of phagocytic cells was

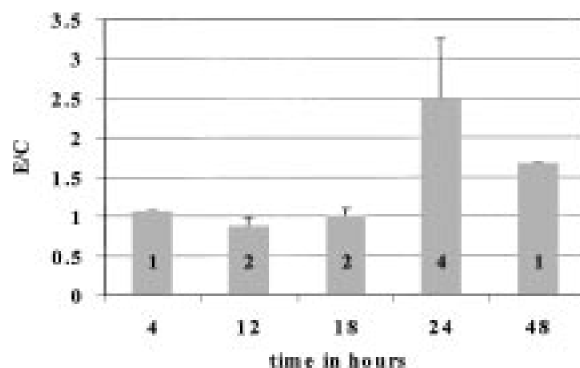

Fig. 1. Effect of time of exposure on EMF induction of differentiation. The figure summarizes a series of exposures of HL-60 cells to 60-Hz, 1 G EMF at timed intervals. Measurements used flow cytometric analysis for determination of phagocytized fluorescent particles in cells isolated from (C) or exposed to (E) EMF (10,000–20,000 cells/determination). The ratio, E/C, is used here to summarize data after gating. It is expressed as mean ±SE. Essentially, gating (here, selection of cells that contain two or more particles) allows the most precise measurement of increases in fluorescent particle number over the pattern exhibited by unexposed cells. Numbers in bars are number of exposures.

observed after exposure to 60 Hz, 1 G for 24 h. The results of exposure for periods less than 24 h confirmed that this is the earliest point at which adequate numbers of phagocytic cells are present for flow cytometry measurements. The relative effects of TPA and EMF were then compared in an experimental series. As can be seen in Figure 2, the effect of EMF relative to induced differentiation in HL-60 cells is approximately equivalent to 250–500 pg/ml TPA. This was tested in further experiments. EMF exposure and TPA treatment at 500 pg/ml were compared over a timed series (Fig. 3). The results show a similar endpoint at 24 h (16% gated), but some cells treated with TPA showed differentiation at as early as 12 h.

One possibility is that TPA and EMF used in concert could have an additive effect. If this is the case, additivity would be most noticeable at lower TPA concentrations since at higher concentrations, the effects would be swamped out by TPA. An example of experimental analysis of a series in which cells were exposed to an EMF of 60 Hz (1 G), TPA, or [EMF + TPA] is illustrated in Figure 4.

These data provided the initial observation that the effects of TPA and EMF could be additive at lower concentrations of TPA. This is an important finding, which was confirmed in further experiments (Fig. 5). Obviously, differences in phagocytic activity between cells that were not treated with TPA and those treated with TPA are highly significant. This finding suggests that TPA and EMF could share a common pathway(s), as previously proposed, and/or work together in an additive manner.

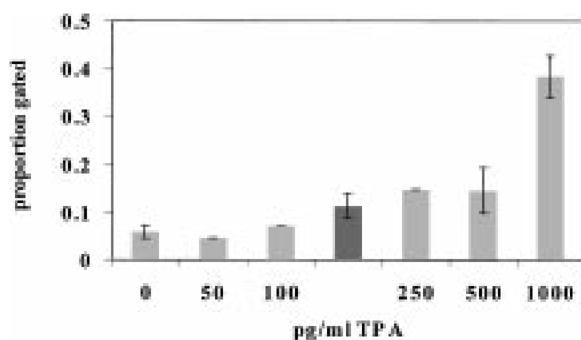

Fig. 2. Effect of EMF exposure on differentiation compared with treatment with TPA. Cells were treated with TPA at the concentrations shown or exposed to 60-Hz, 1 G EMF for 24 h (dark bar). Bars with SE markers represent three or more exposures. Other experimental points were done in duplicate. Experimental conditions were as given in Fig. 1.

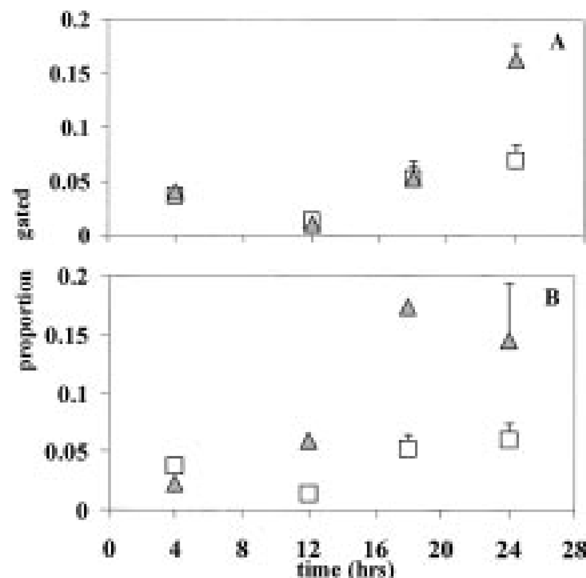

Fig. 3. Comparison of the effect of time on the differentiative response after EMF exposure **(A)** or TPA treatment **(B)**.Cells were either exposed to EMF (60-Hz, 1 G) or treated with TPA (500 pg/ml). For comparison, the proportion gated at 250 pg/ml TPA was 0.14; at 1,000 pg/ml, the proportion gated was 0.38. Data from sham controls is represented by light figures; dark figures are samples exposed to EMF (A) or TPA (B).

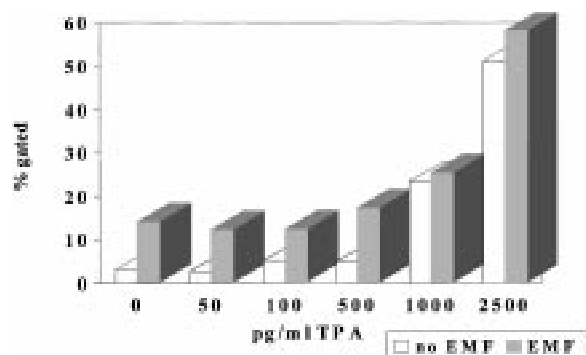

Fig. 4. EMF exposure and TPA treatment together are additive. Cells were exposed to an EMF of 60-Hz, 1 G or [EMF + TPA]. Data from a single experimental series, but other experiments gave comparable data (see Fig. 5). Accordingly, the graph also serves as an example of analysis of flow cytometry data using HL-60 cells. Dotted bars, data from sham-exposed cells; solid bars, measurements from cells exposed to 60 Hz, 1 G for 24 h in the presence (50–2,500 pg/ml) or absence (0 point) of TPA. For the experiment illustrated, Kolmogorav-Smirnov statistics (PC-Lysis 1.1) gave a value of D = 0.03 when two sham control populations were compared; D = 0.45 for EMF-exposed vs control, where the D statistic is the maximum vertical distance between two histograms.

The reasoning is as follows. Assume an effect, X, which can occur in cells exposed to EMF or TPA [EMF +0 = X , or TPA + 0 = X]. If this is the case, some appropriate quantity of each

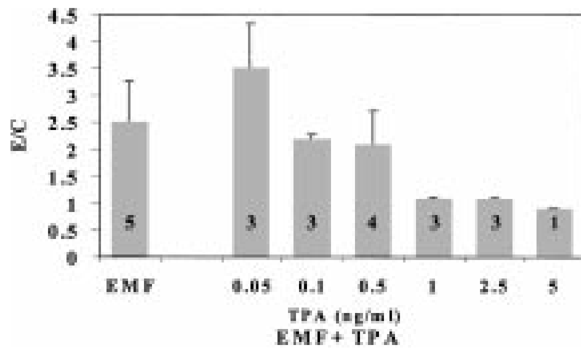

Fig. 5. EMF exposure and TPA treatment together are additive (experimental series). Graphic representation of the results of exposure of HL-60 cells to 60 Hz, 1 G EMF or TPA for 24 h. The profile for 48-h exposure is similar, but the effect of EMF exposure is less dramatic. Measurements used flow cytometry for determination of phagocytized fluorescent particles in cells isolated from (C) or exposed to (E) EMF. E/C is expressed as mean ±SE. Numbers in bars are number of experiments.

could also give X [aEMF + bTPA = X]. The expectation is synergistic activity between the two treatments, but only up to a saturation point (the point at which X is initiated). The data show that there is TPA "saturation" between 500 pg/ml and 1 ng/ml TPA under exposure conditions that use 1 G. Increasing the concentration of TPA to concentrations of >1 ng/ml eliminates the additive effect.

DISCUSSION

This research provides evidence that 60-Hz EMF at 1G influences differentiation of cultured hematopoietic progenitor cells. The use of a sensitive assay circumvented some of the problems inherent in measurements of the influence of EMF on cells. A common outcome of many experimental attempts to measure the effect of EMF is a low, and sometimes controversial, level of change in a cell parameter that could be related to signaling phenomena. It is noted that one explanation for experimental variability is that not all cells in a population are sensitive to EMF exposure, but rather a minor, and presently undefined, population. These experiments address these potential problems directly by studying large cell numbers.

The experimental strategy was initially based on observations that TPA can induce differentiation in HL-60 cells. The present results strongly indicate similarities between the effect of low concentrations of TPA treatment and EMF exposure. This is in keeping with results from other laboratories that show changes in signaling pathways resulting from EMF exposure.

There are persuasive experiments suggesting that EMF influences at least some of the same signal transduction pathways affected by phorbol esters. An initiation point that related EMF and cell signaling were reports of an increase in calcium flux following EMF exposures [cf. Wallaczek, 1992]. Ca^{2+} influx, an early event in signaling cascades, serves as a second messenger in cell regulatory processes. As one example of experiments that support the role of calcium, lymphocytes exposed to a sinusoidal magnetic field (60 Hz, 220 G) and the mitogen, Concavalin A, show increases in calcium influx [Liburdy, 1992]. There are also correlative experiments that support a role of calcium in EMF-cell interactions. In EMF-exposed (60 Hz, 60 mG) HL-60 cells an increase in expression of the steady state levels of c-*fos* mRNA is observed; this effect is abolished when the cells were treated with EGTA to deplete calcium [Karabakhtsian et al., 1994].

The present findings support previous proposals that TPA treatment provides a model for EMF-cell interaction. For example, increased PKC activity was observed in HL-60 cells after 10-, 15-, or 20-min exposures to a pulsed field [Monti et al., 1991]. In B-lineage lymphoid cells exposed to a 60-Hz (1-G) EMF, LYN as well as a downstream substrate SYK was stimulated, resulting in subsequent PKC activation [Uckum et al., 1995]. Further studies showed tyrosine kinase-dependent activation of phospholipase Cγ2 (PLC-γ2) with a resulting increase in inositol phospholipid turnover after EMF exposure [Dibirdik et al., 1998]. LYN plays a essential role in the EMF-induced events, in that PLC-γ2 activation is mediated as the result of LYN stimulation of SYK.

The present results are consistent with the hypothesis that a cell surface phenomenon initiated by EMF exposure could activate second messengers either directly or indirectly. Theoretically, this could include modification (loss or gain of function) in signal transduction systems in which amplification and transmission of signals normally act coordinately in regulation of cell activity. Many previously proposed means of EMF-cell interaction have included interacting mechanisms that normally control cellular differentiation, proliferation, and the maintenance of the differentiated state. There is a dichotomy in EMF studies, however, in that both beneficial and possibly hazardous effects have been proposed following EMF exposures.

It is of importance to develop models to explain the effects of EMF that have been observed, whether beneficial or unsafe. The possibility of synergistic activity of EMF with other cellular activity, and importantly, the possibility of health risk, provides a strong impetus to continue experiments on EMF.

In addition, the approach described in this paper can pave the way for testing a broad range and types of EMF and provide a means for determining what cellular factors are required for EMF induced expression. Primary dose-response curves can be developed as a guide for further delineation of dose-related effects, and a comparison of EMF exposures to nongenotoxic carcinogens can be made.

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