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Letter

Electromagnetic fields (EMF) facilitate cell migration and BrdU incorporation during an EMF-sensitive phase in a rat neurosphere assay *in vitro*

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ABSTRACT — There are both advantages and disadvantages in the application of electromagnetic fields (EMF) for health: the former is deep brain stimulation for neurodegenerative disease in medicine and the latter is the possible association with a tumor. In this study, we examined the effect of EMF (50 Hz; 100 μT) on rat neural stem cells *in vitro*. During handling with culture cells, there are two phases of the state of neural stem cells isolated from rat brains; one phase is a cellular suspension in the medium (no anchorage), and another phase is anchorage to the bottom of the culture dishes. The effect of EMF on neural stem cells *in vitro* was dependent on these cellular phases. Upon anchorages, the cultured neural stem cells migrated along the radial axis, and exposure of these migratory neural stem cells to EMF (50 Hz; 100 μT) facilitates the migration and incorporation of BrdU 1.3~1.4 folds. However, these effects of EMF were not seen once the cellular suspension (no anchorage) was exposed. Even when the neural stem cells fully migrated, there were no effects of EMF on the retinoic acid-induced differentiation. Thus, there is a cell phase sensitive to EMF in the cultured neural stem cells.

Key words: Neural stem cells, 50 Hz, Electromagnetic fields, Migration, BrdU incorporation

INTRODUCTION

There are advantage and disadvantage in utilization of electromagnetic fields (EMF). The former is the use of magnetics of it in medical therapies such as MRI (magnetic resonance imaging) and DBS (deep brain stimulation) for Parkinson's disease (Hartmann *et al*., 2019).

On the other hand, epidemiological data reported by Wertheimer and Leeper stimulated the research area of the biological effects of magnetic fields (Wertheimer and Leeper, 1982). They first identified the association between childhood leukemia and brain tumour and residential magnetic fields of extremely low frequency. Subsequent epidemiological studies showed the association among them (Ahlbom *et al*., 2000; Greenland *et al*., 2000; Schuz *et al*., 2001; Draper *et al*., 2005; Kabuto *et al*., 2006). The International Agency for Research on Cancer classified power-frequency magnetic fields as a possible human carcinogen (IARC, 2002).

In this study, we evaluated the effect of electromagnetic fields of rat neural stem cells *in vitro*, since the sensitivity of the stem cells to EMF would be expected to be higher than other subcultured cells.

MATERIALS AND METHODS

Magnetic fields exposure

In order to expose the cells to magnetic fields, a 50 Hz sinusoidal magnetic field was generated in a chamber with four Merritt-coil devices. Special features of exposure

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coil are: (1) a perforated Plexiglas platform table, within (2) a double-wound, four-coil Merritt exposure system (plastic frame wound with double-wrap, bifilar cable, in the Merritt's turn ratio of 26/11/11/26). The exposure coil was placed in the chamber. The chamber had four ventilation holes (2.54 cm in diameter) on the top and bottom. A temperature probe was placed inside the chamber to monitor temperature continuously. The coil system was driven by signal generators obtained from NF Electronic Instruments Corp. (Kanagawa, Japan).

Preparation of rat neurosphere

Pregnant Wistar rats at embryonic day 14 (E14) were obtained from Clea (Tokyo, Japan). The animals were maintained in home cages at 22°C with a 12-hr light-dark cycle. They received the MF diet (Oriental Yeast Corp., Tokyo, Japan) and distilled water *ad libitum*. All animal care procedures were in accordance with the National Institute for Environmental Studies guidelines. The embryos were removed and transferred to minimal essential medium (MEM; Sigma-Aldrich, Tokyo, Japan). Subsequently, the mesencephalons were dissected from the embryos and were enzymatically digested with 50 U deoxyribonuclease I (Takara Corp., Kyoto, Japan) and 0.8 U papain (Sigma-Aldrich) at 32°C for 12 min. After stirring, the digestion mixture was passed through a 70-μm cell strainer (BD Biosciences, Bedford, MA, USA). The run-through containing the neural stem cells was centrifuged at 800 x g for 10 min. It was then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) and F12 medium (1:1; Invitrogen, Tokyo, Japan) supplemented with B27 (Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, MN, USA) and 10 ng/mL epidermal growth factor (EGF; Roche Applied Science, Tokyo, Japan), and cultured in uncoated dishes without serum. Fresh culture medium containing EGF and bFGF was added after 3-4 days.

A neurosphere assay

The neurospheres were seeded in an amine-coated dish (Coaster Co., Cambridge, MA, USA) in the presence of bFGF and EGF for 16 hr, allow cells to adhere. The migrating distance of the cells was measured from the edge of the sphere, using the National Institute of Health ImageJ 1.38x softwar (public domain software).

To examine the effects of EMF on the differentiation of the neural stem cells, spheres were inoculated onto amine-coated plates and allowed the cells to fully migrate in culture medium containing EGF and bFGF, after which the medium was exchanged for a new one containing 100 ng/mL *all-trans* retinoic acid and 1% FBS, but not EGF and bFGF.

Bromodeoxyuridine (BrdU) incorporation

DNA synthesis was determined via BrdU incorporation as previously described (Ishido *et al*., 2001). Neural stem cells were grown in an amine-coated dish (Coaster Co.). The cells were exposed to no EMF (active sham) or EMF (100 μT) for 24 hr at 37°C. Then, 10 μM BrdU was added for a further 3 hr at 37°C. The cells were fixed with 70% ethanol in 0.5 M HCl for 30 min at -20°C. Following treatment with nuclease, the cells were incubated with anti-BrdU antibody conjugated to peroxidase (Boehringer-Mannheim, Mannheim, Germany). Bound enzymes were detected with the substrates ABTS® (2,2'-azino-di-[3-ethylbenzthiazolinesulfonate]) (Boehringer-Mannheim) and quantified by measuring absorbance at 405 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (DS Pharma BioMedical, Osaka, Japan).

Immunocytochemistry

The spheres and migratory cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5 % Triton X-100, and labeled using primary antibodies diluted 1:100. Secondary antibodies were diluted 1:200. After washing twice with PBS containing 0.01 % Tween 20, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) for 5 min, then analyzed with a Leica TSC SP5 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistics

Statistical analyses were carried out with Student's t-test using JMP version 6.03 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Previously, a neurosphere assay *in vitro* used in this study was validated, using several kinds of chemicals (Ishido, 2009, 2018; Ishido *et al*., 2015; Ishido and Suzuki, 2014, 2010; Suzuki and Ishido, 2011). To examine the effects of EMF on the anchorage state of neural stem cells, isolated neural stem cells were seeded in amine-coated dishes, cultured for 16 hr in the presence of EGF and FGF, and then exposed to 100 μT EMF for 24 hr (Fig. 1A). Control cells received no EMF exposure. The migratory distance was measured (Fig. 1B). EMF significantly facilitated cell migration

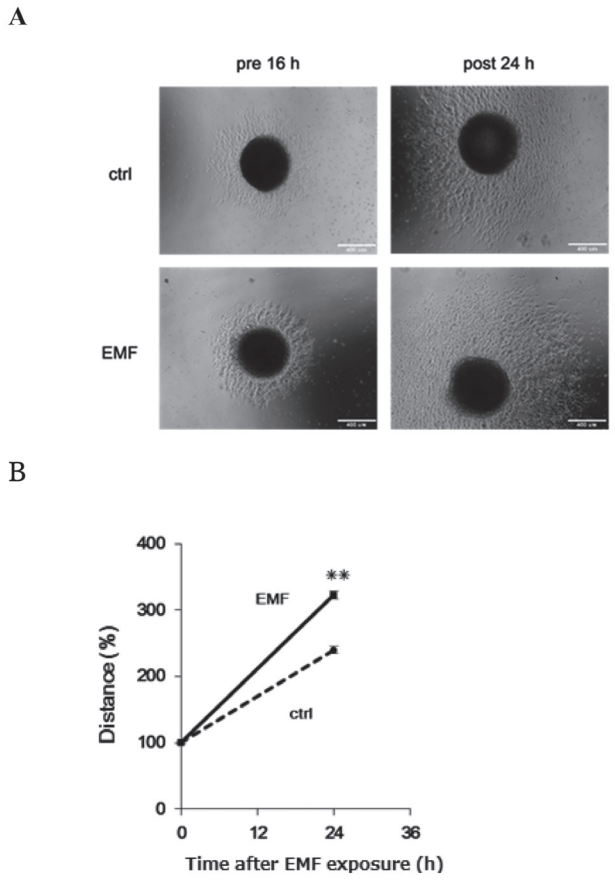

Fig. 1. Effects of EMF on fully migrated neural stem cells *in vitro*. Prior to exposure of the neurosphere to EMF, the cells were incubated for 16 hr to fully migrate (A; *left*). The migratory cells were exposed to 100 μT EMF for 24 hr (B; *right*). Then, migration distance was measured with ImageJ. ctrl: control. **; statistical significance ($p < 0.01$). Bar = 400 μm.

1.3~1.4 fold ($p < 0.01$).

Since cell migration might be coupled with cell growth, we examined BrdU incorporation. The cells were incubated for 16 hr and then, they were exposed to none or 100 μT EMF for 24 hr. Treated cells were then incorporated with BrdU. Incorporated BrdU was quantified with an immunoassay. Figure 2 shows that EMF significantly facilitated BrdU incorporation into anchorage cells ($p< 0.02$).

To examine the effects of EMF on no anchorage state of neural stem cells, at the time when seeding with cell suspension, EMF started to expose them for 24 hr. The migratory distance was then measured. Figure 3 shows

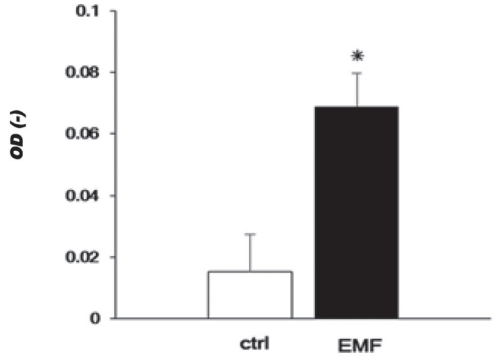

Fig. 2. Effects of EMF on BrdU incorporation in fully migrated neural stem cells *in vitro*. Prior to exposure of the neurosphere to EMF, the cells were incubated for 16 hr to fully migrate, followed by incorporated with BrdU under EMF(100 μT; 24 hr). Then BrdU incorporated was immunoassayed (n = 3~4). ctrl: control. *; statistical significance ($p < 0.02$).

there are no statistical differences between them.

To examine the effects of EMF on the differentiation of neural stem cells, we cultured the cells in the medium containing 100 ng/mL *all-trans*-retinoic acids and 1% FCS, but not EGF and FGF, and then stained them with antibodies for Tuj1, an immature neuron marker, GFAP, an astrocyte marker, and Gal-C, an oligodendrocyte marker. Figure 4 shows that the migratory cells from the neurospheres had differentiated into several types of neural cells, neurons, astrocytes, and oligodendrocytes as indicated. Therefore, neural stem cells in the cultured assay system could retain to differentiate. Then, we examined the effects of EMF under the same condition. There were no differences in each immunocytostaining. Thus, exposure of neural stem cells to EMF (100 μT) did not affect the action of retinoic acid, indicating no change in cellular differentiation.

The sensitivity of cells to EMF was first to be shown by Dr. Liburdy. They originally demonstrated that 60 Hz MF at 1.2 μT inhibits the antiproliferative effect of melatonin on the MCF-7 cells (Liburdy *et al*., 1993). In addition, other laboratories independently reported the results that are consistent with this finding concerning the effect of MF on melatonin (Blackman *et al*., 2001). We extended their findings to show the molecular mechanism of the biological effects of magnetic fields; exposure of magnetic fields causes the uncoupling of the melatonin signal transduction pathway (Ishido *et al*., 2001). Thus, it is

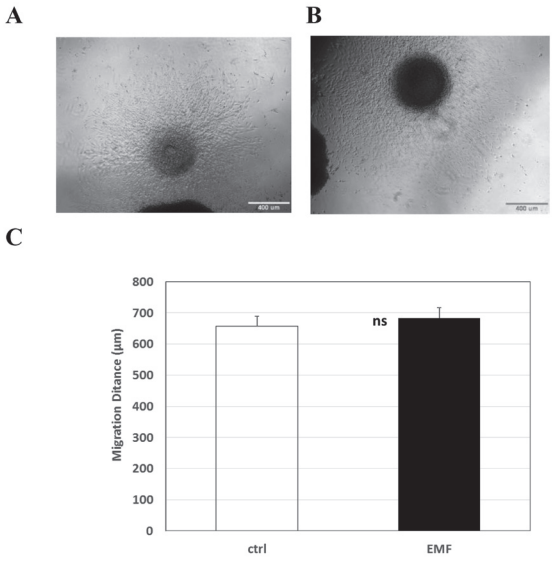

Fig. 3. Effects of EMF on cell suspension of neural stem cells before migration *in vitro*. At the time when the neural stem cells were seeded, it started to be exposed to none (*A*) or EMF (*B*) at 100 μT for 24 hr. Then, migration distance was measured with ImageJ. ctrl: control. ns: no statistical significance. Bar = 400 μm.

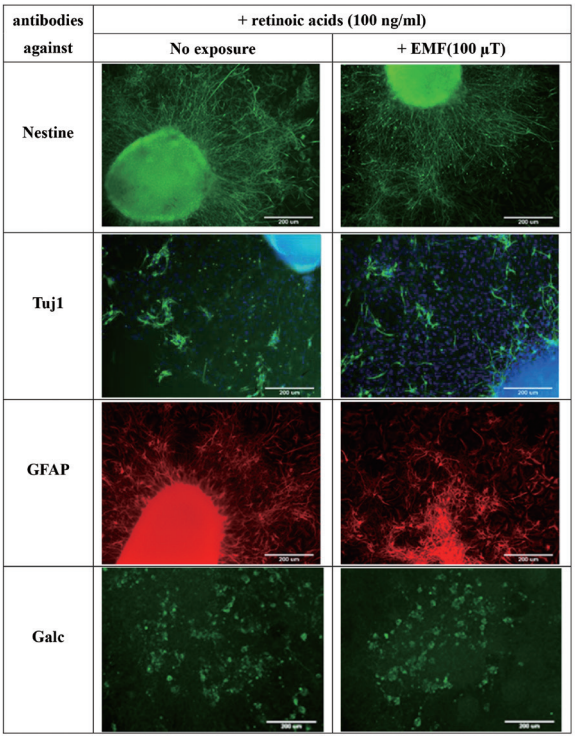

Fig. 4. Effects of EMF on *all-trans* retinoic acid-induced differentiation of neural stem cells *in vitro*. The fully migrating neural stem cells were exposed to none or EMF (100 μT; 16 hr) as indicated, then further incubated with *all-trans* retinoic acids (100 ng/mL). The treated cells were immunostained with antibodies as indicated. Bar = 200 μm.

certain that there are EMF-sensitive cells, MCF-7 cells, at least on the protein level.

Epidemiological studies show that EMF exerts the primary or secondary effects on the nervous system, resulting in a brain tumor. Therefore, it is necessary to study EMF-sensitive neural cells. In this study, we could demonstrate that EMF could affect on cultured neural stem cells on the protein level.

We employed rat DNA arrays for transcriptional analyses of bioeffects of magnetic fields. Go analyses suggested the terms such as nervous system development, neurogenesis, organ morphogenesis, and anatomical structure development (data not shown). Since alteration in transcription programs are a fundamental feature of cancer, it is further needed to examine this sense.

The neural stem cells have self-renewal and multipotency for neural lineage. During neural development, neural stem cells migrate and result in the correct neurocircuit. In this study, we demonstrated the stimulatory effects on the going migratory neural stem cells, certainly implying the disruption of the correct neural circuit *in vivo*.

The availability of cultured neural stem cells would be useful to reveal the molecular mechanisms underlying EMF sensitivity.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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