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Developmental Effects of Low Frequency Magnetic Fields on P19-Derived Neuronal Cells

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Abstract— **Modulation of pluripotent stem cell differentiation by several environmental factors, such as physical stimulation, is important theme in tissue engineering. In this study, we report the effects of extremely low frequency magnetic fields (ELF-MFs) exposure (1 mT or 10 mT, 50 Hz, sinusoidal) on the neuronal differentiation process of P19 embryonal carcinoma cells (P19 cells). Here, during induction of differentiation, the ELF-MFs exposed to embryoid bodies (EBs). After neuronal differentiation, the effects of ELF-MFs were evaluated by morphological analysis, immunochemical analysis (MAP2, GFAP), and the developmental neuronal network activities recorded by the micro-electrode arrays (MEAs). As a result, the percentage of MAP2 positive cells and the spike frequencies were increased by 10 mT ELF-MF, and then the percentage of GFAP positive cells were reduced. However, these effects were not seen in 1 mT exposed cells. Therefore, these results suggested that the intensity of a magnetic field was important for affecting a characteristic of neuronal differentiation and a functional neuronal network property.**

I. Introduction

MANY biological functions are modulated by extremely low frequency magnetic fields (ELF-MFs) [1-3]. Biological effects of the ELF-MFs are thought to be mediated by changes in intracellular Ca^{2+} signaling [4-6], therefore, gene expression, cell fate, and cell differentiation may be modified. Actually, previous studies revealed that the neural or cardiac lineage-promoting genes were regulated by electric or magnetic stimulation on mouse embryonic stem (ES) cells and neural stem cells [7-9]. On the other hand, the electric or magnetic effect to the cell differentiation also needs to investigate the functional properties of differentiated cells. In particular, the neuronal cells metamorphose the functional network properties in the developmental process. The spontaneous electrical activities are generated in the differentiation and the formation process of neuronal networks, and these activities show clearly different behavior according to differentiation period [10, 11]. However, the evaluation to the neuronal differentiation of electric or magnetic stimulation in consideration of a cell function was not performed. P19 embryonal carcinoma cells (P19 cells) are used for the good model of pluripotent stem cells, and specialized to neuronal and glial cells with retinoic acid (RA). The aim of this study is to investigate the effect of ELF-MFs on the differentiation and developmental activities. Therefore, we attempted exposure to ELF-MFs on embryoid bodies (EBs) formed at induction of neuronal differentiation process of P19 cells. Here, the percentages of neuronal differentiation and spontaneous electrical activities of maturing neuronal networks were evaluated by immunochemical analysis and micro-electrode arrays (MEAs) analysis after induction of differentiation. Based on these results, we discussed the possibility of the magnetic effects on the characteristic of neuronal differentiation and neuronal network property.

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II. Materials and Methods

A. Cell Culture

Undifferentiated P19 cells were cultured in alpha-modified minimum essential medium (α-MEM) (Gibco) containing 10 % (v/v) fatal bovine serum (FBS) (Gibco) and 1 % (v/v) penicillin-streptomycin (Pe-St) (Sigma-Aldrich) at 37 ° C in humidified atmosphere with 5 % CO_2 in air. To induce neuronal and glial cells, P19 cells were seeded into 85 mm diameter bacterial grade Petri dish (Fisherbrand) at a density of 2×10^6 cells/cm^2 in α-MEM containing 1 μM RA (Sigma-Aldrich) and were incubated in this medium for 96 hours. In this condition, the floating cells to form EBs. After induction of differentiation, these EBs were dissociated into single cells by trypsin-EDTA, and were replated in a polyethylenimine (PEI) coated tissue culture dish at a density of 3×10^6 cells/cm^2 in Neurobasal medium (Gibco) containing 2.0 % (v/v) B-27 supplement (Gibco), 1.25 ml L-glutamine (Gibco) and 1 % (v/v) Pe-St. Subsequently, differentiated neurons and glial cells were cultured about 4 weeks with replacement of the medium 3 days intervals.

B. Experimental Protocol

ELF-MFs exposure (1 mT or 10 mT, 50 Hz, sinusoidal) were applied to EBs in the induction of differentiation process. Magnetic fields were produced by the acrylic supported Helmholtz coil which was installed in the inside of a CO_2 incubator (010-0211, Ikemoto). The ELF-MF exposure system was constructed by a bipolar AC power supply (BWS40-7.5, Takasago) and a function generator (SG-4111, Iwatsu). And stabilizing magnetic flux densities were set up by measuring with a gauss meter (GM-5005, EMIC). During ELF-MF exposure, the temperature in the exposure space was controlled by circulating water and confirmed stable at 37 ± 0.2 ° C by continuously monitored of temperature using the digital thermometric probe (IT-2000, As One).

C. Immunochemical analysis

For immunochemical analysis, cultured P19-derived neuronal cells were fixed with 4 % paraformaldehyde for 20 min at room temperature. After washing in phosphate buffered saline (PBS) (1 min × 3), fixed cells were permeabilized with 0.25 % TritonX-100 in PBS for 10 min and blocked with 4 % bovine serum albumin (BSA). Primary antibodies used in this study were mouse anti-MAP2 (1:400, Chemicon) and rabbit anti-GFAP (1:1000, Chemicon). These antibodies were applied over night at 4 ° C. Next day, cells are washed in PBS (5 min × 3) and incubated for 2 hours at room temperature with Alexa Fluor 488-mouse IgG (1: 500, Molecular Probes) and Alexa Fluor 546-rabbit IgG (1:500, Molecular Probes) which are secondary antibodies for anti-MAP2 and anti-GFAP, respectively. Immunochemical images were obtained with a phase-contrast microscope (IX-71, Hamamatsu Photonics) and cooled charge-coupled device (CCD) camera (C8800-21C, Hamamatsu Photonics). And obtained images were analyzed with HiPic (Hamamatsu Photonics) which was adjunctive software attached to the C8800-21C cooled CCD camera.

D. MEAs Electrical Recording System

The MEAs consists of 64 indium-tin-oxide micro-electrodes, arranged two square grid with a distance of 500 μm. The size of electrode was 30 μm × 30 μm, and the distance of between the adjacent terminals was 150 μm (Fig.1A). The recoding method for extracellular voltage was described previously [12]. Briefly, extracellular voltages at 64 electrodes were amplified by using a 64 channel amplifier (NF Corporation), and stored on hard disk (Fig.1B). The recoding bandwidth was 100 Hz-20 kHz. Sampling rate per channel was 25 kHz. Recorded data were analyzed by using LabView software (National Instruments).

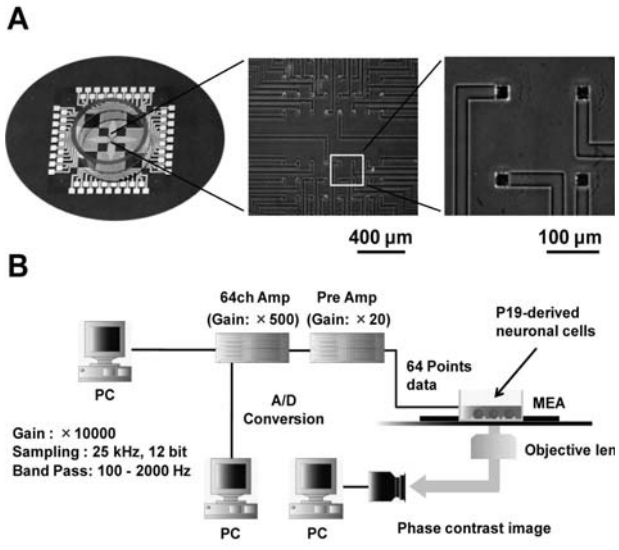

Fig.1 The MEAs recording system. (A) Whole image of the MEAs, (B) Schematic diagram of the system.

E. Data Analysis

All data are analyzed by Student's t-test and are shown as mean ± SD. The stored electrical data analyzed by using PV-wave software (Visual Numerics). To quantify the neuronal network activities, the number of spikes and the distribution of active electrodes were estimated.

III. Results and Discussion

A. Effects of ELF-MF on Morphological Changes of EBs

To confirm the effect of the ELF-MFs exposure on the morphogenesis of EBs, we analyzed the cross-section area of EBs in the 4 days in culture (Fig.2). As a result, we confirmed the tendency for the size of EBs to become small depending on magnetic field intensity. In addition, the percentage of cell death measured with Trypan blue staining, the significant difference between each samples were not seen (data not shown).

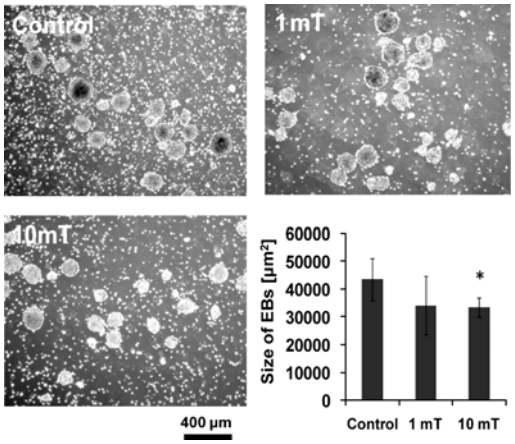

Fig. 2 Morphologies of EBs and the comparison result of the size of EBs at 4 days in culture (N=4, ±SD). *$P < 0.05$

B. Effects of ELF-MF on Time Course of P19-derived Neuronal Differentiation

The EBs formed in P19 cells has the ability to differentiate into neurons, astrocytes and oligodendrocytes in various stimuli. When these EBs enzymatically dissociated into single cells by trypsin-EDTA and plated on the PEI coated polystyrene dish, each cells formed small aggregates and extended neurite within 72 hours. The prolonged incubation for 1-4 weeks, terminally differentiated neurons and glial cells appeared from small aggregates and formed the complicated neuronal networks. In this condition, the P19-derive neurons generate the spontaneous electrical activity via connecting other neurons which is called synapse connection. In contrast, glial cells do not generate the electrical activity but regulate the neuronal function with to act the synapse formation and the cell-cell junction. When neurotransmitters are released from presynaptic neurons, astrocytes release proteins which modulate the activity of postsynaptic neurons. It is suggested that the difference in the neuronal networks constructed by neurons and astrocytes affect the spontaneous electrical activity. To examine the effect of the ELF-MFs on the development of neuronal networks, the percentage of neurons and astrocytes were

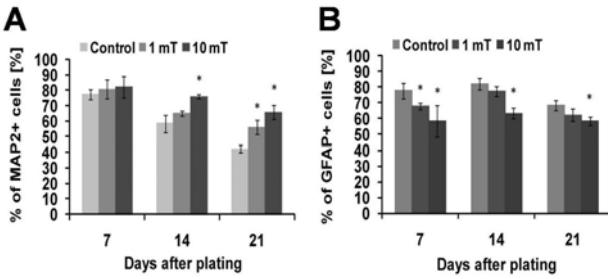

Fig.3 Effects of neuronal and glial differentiation of P19 cells. Quantitative changes in (A) MAP2 positive and (B) GFAP positive cells at 7, 14, and 21 days in culture after ELF-MFs (1 mT or 10 mT) exposure (N=4, ±SD). *$P < 0.05$.

examined by using immunochemical neuronal marker MAP2 and glial marker GFAP. Expression of MAP2 and GFAP were evaluated for whole the cells, and the area consist of each immunoreactive cells were referred to as "MAP2 positive" and "GFAP positive", respectively. About the depending on cultivation period, the percentage of MAP2 positive cells decreased with long-term culture, whereas the percentage of GFAP positive cells did not change. Moreover, the percentage of MAP2 positive cells increased significantly in 10 mT ELF-MF exposed cells (Fig. 3A). Correlated with MAP2 positive cells, GFAP positive cells decreased in same condition (Fig. 3B). Therefore, these results suggest that exposure to 10 mT ELF-MF would change the differential ability of P19 cells.

C. Effects of ELF-MF on Developmental Spontaneous Activities in P19-derived Neuronal Networks

To determine whether exposure to ELF-MF also affects the functional neuronal network properties, time-dependent changes of spontaneous electrical activities of P19-derived neuronal networks were recorded by using the MEAs. And then, we observed that attachments of P19-derived neuronal cells on MEAs were similar in comparison to the attachment on the cell culture dishes. When the morphologies and the spontaneous electrical activities in P19-derived neuronal networks were measured simultaneously, some electrodes detected the extracellular membrane action potentials (Fig. 4A-C). In these figures suggested that the detected spontaneous electrical activities synchronized between adjacent electrodes and the detected electrodes exist near the edge of cell aggregates. In this study, these electrodes that detected action potentials were defined as "active electrodes". The shape and the amplitude of action potential are depended on the distance between the electrodes and the signal sources. For these reasons, the "spike" was defined by signal processing as a value above 5 times of the standard deviation of a noise level. In addition, we confirmed that P19-derived neuronal networks had not mostly covered the electrodes in the early stage of differentiation (0-4 days). Therefore, the recording of spikes was performed on 5-26 days after initiating of differentiation at 3 days intervals (Fig. 4D). The spike was not observed before 5 days after initiating differentiation, but spikes were detected after 8 days. In particular, at 11-17 days after plating, correlated or un-correlated high-frequency spikes were detected significantly at about 10-20 electrodes. In these periods, the number of detected spikes increased approximately 2-fold with time. At 20 days, however, the firing rate of spikes subsided suddenly. And firing frequencies were mostly stable in 23-26 days. These findings suggested that it is the 2-3 weeks that the functional neuronal network is most activated. And generating of these spontaneous electrical activities differed significantly from the neuronal network activity

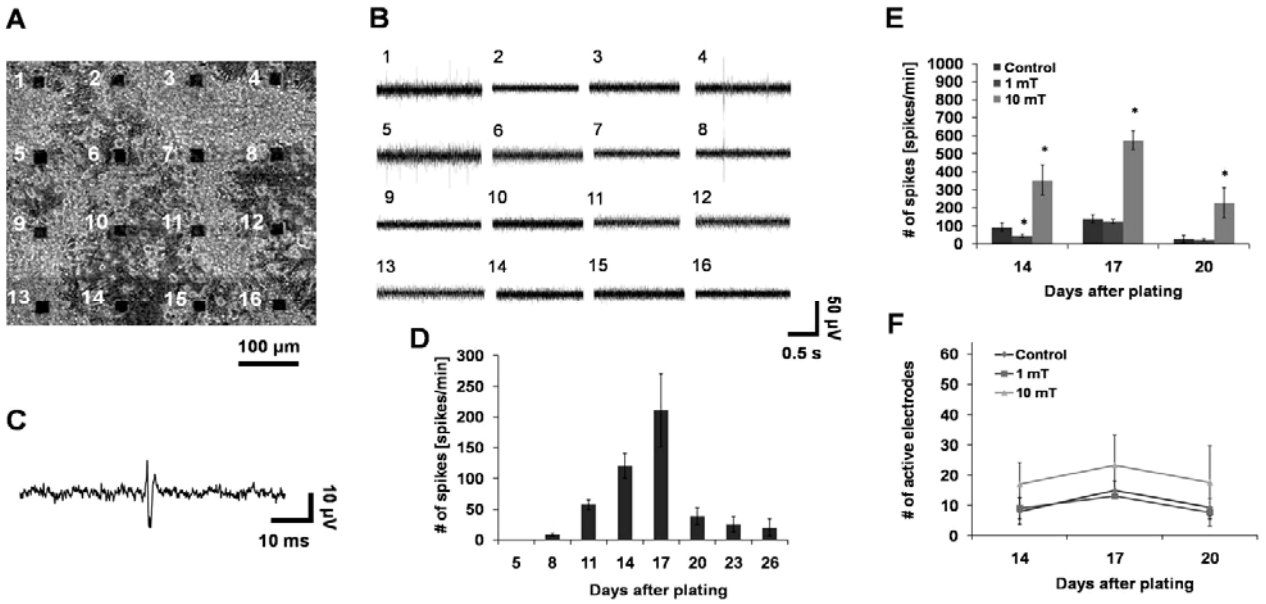

Fig.4 Developmental changes of P19 cell-derived neuronal networks activities. (A) Phase-contrast image of P19 cell-derived neuronal network on MEA. (B) Snap shot images of the distribution of recording signals and (C) the typical shape of action potential. (D) Time-dependent changes of spontaneous activities in P19-derived neuronal networks (N=4, ±SD). (E) Influence to the spontaneous activities after ELF-MF (1 mT or 10 mT) exposure (N=3, ±SD). (F) Comparison of the number of electrodes which detected the spikes (N=3, ±SD). *$P < 0.05$

formed by ES cell derived neuronal neurons [13]. Therefore, we investigated the effect of exposure to ELF-MF on functional neuronal network ability during 14-20 days after plating (Fig. 4E). We measured the spike frequency by asking for the average for 1 min of the number of spikes detected from all the electrodes. Consequently, the spike frequency of ELF-MFs exposed P19-derived neuronal network became a tendency which increases or decreases depending on the magnetic flux density. At 14 days, spike frequencies of 1 mT ELF-MF exposed cells were reduced by 2-fold, but 10mT ELF-MF exposed cells were increased by 3-fold. Similar effects were observed in 10mT ELF-MF exposed cells, but the significant difference was not seen in the 1mT ELF-MF exposed cells at 17 and 20 days in culture. Next, we attempted to determine whether the increases or decreases observed in spontaneous electrical activities were exerted by the distribution of spikes (Fig. 4F). Here, we confirmed that P19-derived neuronal networks were in the state which has covered all the 64 electrodes and counted the number of electrodes which detected one or more spikes for 1 min. In this result, the number of active electrodes in 10 mT ELF-MF exposed cells became approximately 2-fold increase as compared with control cells during 14-20 days. However, similar increases were not observed in 1 mT ELF-MF exposed cells. In fact, immunochemical results showed that the percentages of the MAP2 positive cells were significantly high at the 2-3 weeks on 10 mT ELF-MF exposed cells. The increases in the number of active electrodes of the spontaneous electrical activities in 10mT exposed cells corresponded with the increase in the percentages of neuronal differentiation. These results suggest that the increases in spike frequencies are considered to have been influenced by the increases in this number of active electrodes. Moreover, in the increase in spike frequencies, the percentages of MAP2 positive cells became significantly high as compared with the percentages of GFAP positive cells. At the same time, the modulation was seen by the differentiation rate and spike frequency by exposure to 1mT ELF-MF, the equivalence relation of each result was not found.

IV. Conclusion

In this study, we applied the exposure to ELF-MF (1 mT or 10 mT, 50 Hz, sinusoidal) on P19-derived EBs at induction of differentiation period. Here, we evaluated time-dependent changes in the percentage of the neuronal and glial differentiation and the spike frequency in spontaneous electrical activities. In addition, the effects of ELF-MFs on the morphological changes in forming EBs and the neuronal outgrowth of early stage of differentiation were analyzed. As a result, the size of EBs which applied the exposure to 10 mT ELF-MF decreased significantly as compared with non-exposed EBs. Next, we investigated the properties of the ELF-MFs exposed neuronal networks with long-term culture by immunochemical and electrophysiological analysis. Also by these results, the differentiation rate of neuronal cells and functional neuronal network activities were significantly modulated by exposure to 10 mT ELF-MF. About the immunochemical result of 10mT ELF-MF exposure, the percentage of MAP2 positive cells was increased and GFAP positive cells was decreased at 14 and 21 days after plating. Additionally, the spike frequencies and the active electrodes of the spontaneous electrical activity in this period were also increasing. Moreover, in the comparison result of the differentiation rate and the spike frequencies of 1mT exposed cells, the clear influence which was seen by exposure of 10mT was not able to be observed.

In conclusion, exposure to 10 mT ELF-MF on EBs at the induction of differentiation period had functional influence in the neuronal differentiation process, and it was suggested that the forming the neuronal network and generating the spontaneous electrical activity were affected by these developmental effects.

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