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Effects of Pulsed Magnetic Stimulation on Tumor Development and Immune Functions in Mice

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We investigated the effects of pulsed magnetic stimulation on tumor development processes and immune functions in mice. A circular coil (inner diameter = 15 mm, outer diameter = 75 mm) was used in the experiments. Stimulus conditions were pulse width = 238 μs, peak magnetic field = 0.25 T (at the center of the coil), frequency = 25 pulses/s, 1000 pulses/sample/day and magnetically induced eddy currents in mice = 0.79–1.54 A/m^2. In an animal study, B16-BL6 melanoma model mice were exposed to the pulsed magnetic stimulation for 16 days from the day of injection of cancer cells. A tumor growth study revealed a significant tumor weight decrease in the stimulated group (54% of the sham group). In a cellular study, B16-BL6 cells were also exposed to the magnetic field (1000 pulses/sample, and eddy currents at the bottom of the dish = 2.36–2.90 A/m^2); however, the magnetically induced eddy currents had no effect on cell viabilities. Cytokine production in mouse spleens was measured to analyze the immunomodulatory effect after the pulsed magnetic stimulation. tumor necrosis factor (TNF-α) production in mouse spleens was significantly activated after the exposure of the stimulus condition described above. These results showed the first evidence of the anti-tumor effect and immunomodulatory effects brought about by the application of repetitive magnetic stimulation and also suggested the possible relationship between anti-tumor effects and the increase of TNF-α levels caused by pulsed magnetic stimulation. Bioelectromagnetics 27:64–72, 2006.

Key words: tumor weight; cytokine production; TNF-α production; B16-BL6 melanoma

INTRODUCTION

Biomagnetic stimulation is a method for stimulating biomedical tissues non-invasively. The basis of magnetic stimulation is to induce eddy currents in a target by using a time-varying pulsed magnetic field [Barkar et al., 1985; Ueno et al., 1988]. It has been widely applied to neurological research, such as mapping studies of the cerebral cortex [Ueno et al., 1990; Cracco et al., 1999], and cognitive sciences [Grafman and Wassermann, 1998; Bailey et al., 2001]. Recently, many studies have provided clinically effective evidence of transcranial magnetic stimulation (TMS) on neurological disorders such as depression and Parkinson's disease [Fleischmann et al., 1995; George et al., 1995; Keck et al., 2001; Khedr et al., 2003].

Electrical stimulation such as direct current (DC) is known to induce various biological responses such as anti-tumor effects [Humphrey and Seal, 1959; David et al., 1985; Nordenstrom, 1989] and/or immunomodulatory effects, and a relationship between the anti-tumor effects was suggested [Sersa et al., 1992; Chou et al., 1997; Miklavcic et al., 1997; Cabrales et al., 2001]. Magnetic stimulation induces eddy currents in the body, and the biological responses to magnetic stimulation have been well reported in regard to neurological tissues such as hippocampus [Fujiki and Steward, 1997; Ogiue-Ikeda et al., 2003a,b]. However, the effects of magnetically induced eddy currents on

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tumors and immune systems have not been well clarified [Jovanova-Nesic and Skokljev, 1990; Okada et al., 2002; Roman et al., 2002]. In our previous study, we reported on effects of pulsed magnetic stimulations on tumor growth in vivo and in vitro [Yamaguchi et al., 2004]. A decrease tendency was seen in the tumor weight of the magnetically treated group, suggesting that magnetically induced eddy currents potentially have tumor-suppression effects. However, the anti-tumor effect due to this stimulus condition was insufficient and we were unable to propose its mechanisms.

In this study, we carried out in vivo and in vitro experiments to widen the basic understanding of the effects of magnetic stimulation, especially on tumor growth processes and immune functions. The possible mechanisms of the anti-tumor effect caused by the pulsed magnetic stimulation were also examined.

MATERIALS AND METHODS

Cell Line

Murine melanoma-derived B16-BL6 cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University. B16-BL6 cells were cultured and maintained in RPMI-1640 (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) and 1% antibiotic–antimycotic solution (Gibco).

Tumor Induction

All experimental procedures performed in this study were approved by Animal Ethics Committee of the University of Tokyo. Female C57BL/6J mice (6–8 weeks old) (Saitama Exp. Animal Supply Co., Ltd., Saitama, Japan) were housed under controlled temperature and 12 h light/dark cycle conditions, with food and water freely available. On the first day (day 0), 3.0×10^5 B16-BL6 cells were suspended in 200 μl of a phosphate-buffered saline (PBS, Gibco), and subcutaneously injected into the flanks of the mice.

Exposure Systems and Stimulus Conditions

Magnetic stimulations were performed with a magnetic stimulator (Nihon Kohden Co., Tokyo, Japan), which delivered biphasic cosine current pulses with a period of 238 μs (Fig. 1A,B). A circular coil (inner diameter = 15 mm, outer diameter = 75 mm) was used in the experiments. Stimulus parameters were determined based on former studies about high frequency magnetic stimulation [Fujiki and Steward, 1997; Ogiue-Ikeda et al., 2003a,b] and our previous examination [Yamaguchi et al., 2004]. These high frequency stimulus conditions were reported in regard to the effect on biological responses; e.g., ''upregulating the astroglial gene expression'' or ''LTP-elongation'' in the rat hippocampus. Stimulation conditions were peak magnetic field = 0.25 T (at the center of the coil, 50%

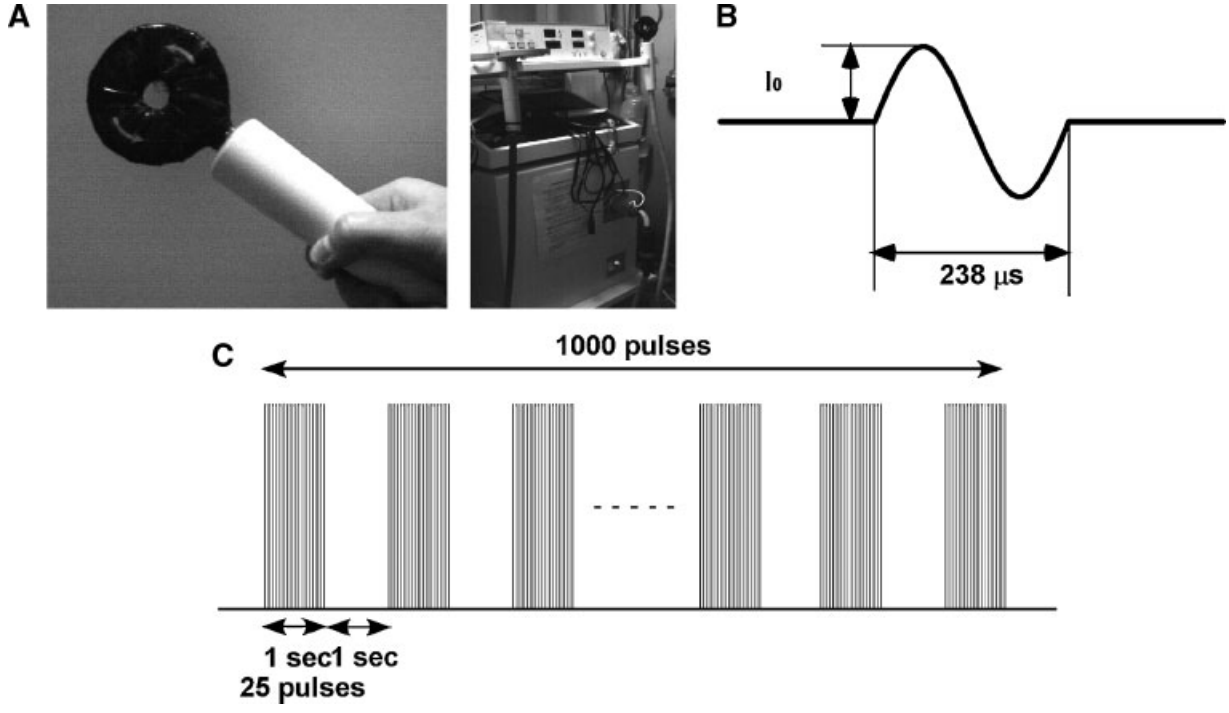

Fig. 1. **A**: Magnetic stimulator (**right**) and stimulus coil (**left**). **B**: Waveform of coil during stimulation. I_0 = peak intensity. **C**: Stimulation pattern for 1 day.

of the motor threshold), frequency = 25 pulses/s, and 1000 pulses/sample/day. Figure 1C shows the stimulus pattern in 1 day. The motor threshold was used as the index of animal stress and was determined by the stimulus intensity when the motor-evoked potential (MEP) peak was greater than 5% of the maximum peak of the MEP. The average motor threshold was approximately 0.5 T in the present study.

Tumor Growth Study

Based on our previous study [Yamaguchi et al., 2004], pulsed magnetic stimulations were performed on the mice from day 1 (the day following cancer cell injection) to day 17. Figure 2A shows an example of a B16-BL6 cell-induced tumor model mouse. The mice of the stimulated group were placed in plastic holders (100 mm in length, 30 mm in diameter) during the stimulation (Fig. 2B). The stimulation coil was positioned 5 mm from the surface of the skin under the spot where the cancer cells were injected (Fig. 2B). The stimulation time was about 80 s for each mouse. The sham group mice were placed in the same holder and exposed to the same noise produced during the stimulation. On day 17, all mice were sacrificed and each tumor was weighed. The tumor samples were fixed in 10% buffered formalin and embedded in paraffin. Three- to four-micrometers-thick sections were then stained with hematoxylin and eosin using standard histological techniques (Mitsubishi Chemical BCL Co., Tokyo, Japan).

Animal viability after the exposure was also examined. The mice treated by the pulsed magnetic stimulation for 16 days, using the same procedure as described above, were observed afterward for 9 days (until day 25).

Eddy Current Calculation

The eddy currents induced in the animals were estimated based on our previous work [Yamaguchi et al., 2004]. Briefly, magnetically induced eddy currents were calculated using a computer program based on the finite-element method [Ogiue-Ikeda et al., 2003a; Sekino and Ueno, 2004]. A mouse calculation model was constructed from a set of magnetic resonance images. We assumed the body to be a homogeneous conductor with a conductivity of 0.07 S/m, according to previous research [Xi et al., 1994]. The element size of the model was $0.7 \times 0.7 \times 0.7$ mm. The stimulation coil was positioned 5 mm from the mouse flank. The waveform of the coil current $I(t)$ in the stimulation is shown in Figure 1B. In this study, we used a pulse width of 240 μs and the peak intensity (I_0) was set at 9.47 kA-turns, which induced a magnetic flux density of 0.25 T at the center of the coil.

Figure 2C shows the calculated result of the eddy current map. The magnetically induced eddy currents were high at the upper and lower body parts of the model with a maximum of 2.46 A/m^2. The eddy current density in the tumor area was 0.79–1.54 A/m^2. Table 1 shows the values of magnetic flux (T) and eddy current density (A/m^2) at the tumor, spleen, and minimum–maximum range in the whole body.

Cell Viability Assay of B16-BL6 Cells

B16-BL6 cells were seeded on a dish at a density of 3.0×10^5 cells in 3 ml of medium and incubated for 17 h. We used a doughnut dish (inner diameter = 30 mm, outer diameter = 54 mm, thickness = 10 mm: Falcon #353653, Franklin Lakes, NJ) in this experiment. The dish was then placed 10 mm above the stimulation coil and pulsed magnetic stimulation (25 pulses/s, 1000 pulses/sample) was performed. Figure 3 shows the pulsed magnetic stimulator of the cells. Since the conductivity is different between the mice (0.07 S/m) and the RPMI medium (0.79 S/m), the peak magnetic field was adjusted to induce the same degree of eddy currents in the dish. Magnetically induced eddy currents were 2.36–2.90 A/m^2 ($\approx$ induced in a stimulated mouse of the 0.25 T group).

The effect of magnetic stimulation on cell viability was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazorium bromide) assay [Mosmann, 1983]. The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Twenty-four hours after the stimulation, 300 μl of a tetrazolium salt solution (5 mg/ml MTT: Sigma) was added to the dish and incubated for 2 h. The blue dye was dissolved in 3 ml of DMSO and then diluted five times. The absorbance of a 550 nm wavelength was detected from 1 ml of the blue dye solution by a spectrometer. Data measurement was repeated three times for one sample and averaged.

Cytokine Measurement

To discriminate the effects caused by pulsed magnetic stimulation on tumor immunity, normal C57BL/6J mice were used. Normal C57BL/6J mice were placed in plastic holders, then exposed to the magnetic stimulation for 3 and 7 days under the conditions described in the method of tumor growth study. The stimulation coil was positioned on the right flank of the mouse. The mice were sacrificed 24 h after the final stimulation, and the spleen from each mouse was

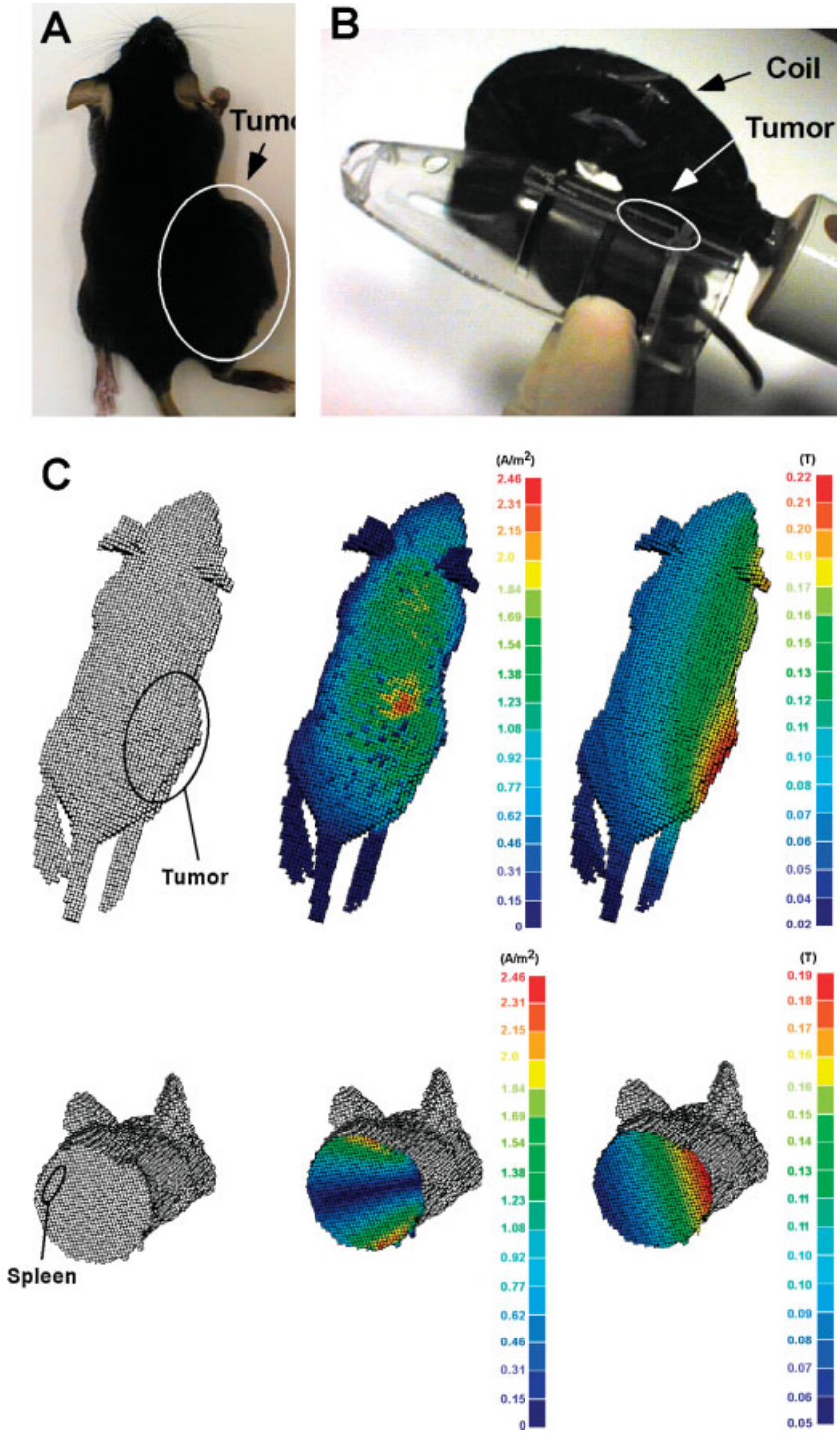

Fig. 2. **A**: Example of tumor in a mouse on day 17, induced by injection of B16-BL6 cells. **B**: Magnetic stimulation of a mouse. A mouse was placed in a plastic holder (100 mm in length, 30 mm in diameter), and the flank of the right side was exposed to pulsed magnetic fields. **C**: Magnetic flux map and eddy current density map of a mouse.

removed aseptically. The spleens were then homogenized. The homogenate was centrifuged and the supernatant was collected. Samples from the exposed sham mice were prepared following the same procedure. IL-2 and tumor necrosis factor (TNF-α) were measured using the Biosource Immunoassay Kit (Biosource Co., Camarillo, CA). Measurements were performed following the technical protocol of the kit and the

TABLE 1. Values of Magnetic Flux (T) and Eddy Current Density (A/m^2) at the Tumor, Spleen, and Minimum–Maximum Range in the Whole Body

Stimulus intensity	At the center of the coil	Tumor area	Spleen area	Min–max
Magnetic flux (T)	0.25	0.13–0.22	0.06–0.1	0.023–0.22
Eddy current (A/m^2)	0	0.79–1.54	0.15–0.92	0–2.46

optical density (OD) at 450 nm was measured in a 96-well plate reader (Bio-Rad Model 450, Bio-Rad Co., Hercules, CA). The standards and samples were assayed in duplicate.

Statistical Analysis

For data analysis of the tumor growth study and cytokine measurements, the Mann–Whitney *U*-test was performed. The Kaplan–Meier procedure was used in the animal survival analysis. The Student's *t*-test was used in the cell proliferation assay. The probability (P) values $<.05$ were considered to be statistically significant.

RESULTS

Tumor Growth Study

On day 17 following the injection of cancer cells, obvious tumors were formed in all mice. Animal body weights were measured to examine the physiological responses to pulsed magnetic stimulation. There were no significant changes in body weights on day 17 (mean $\pm$ SE) between the stimulated group (19.91 ± 0.33) and sham group (19.27 ± 0.36 g).

Figure 4A shows the mean tumor weight of each mouse group. The average tumor weights (mean $\pm$ SE) of the stimulated and sham group were 0.72 ± 0.08 g ($n = 14$) and 1.33 ± 0.16 g ($n = 14$), respectively. The tumor weight of the stimulated group showed a significant decrease (54% vs. sham group, $P < .001$), suggesting that pulsed magnetic stimulation inhibited tumor growth. Figure 4B shows the histological appearance of tumors in the stimulated and sham group. Obvious necrosis tissues were observed in the tumor tissue specimen in the stimulated group. Figure 4C shows the animal viability after the exposure. The mice in both groups were gradually dying from day 17, but the magnetically treated mice survived longer than those of the sham group ($P < .05$).

Cell Viability

Figure 5 shows the in vitro effect of pulsed magnetic stimulation on cell viability. Relative viabilities of the stimulated groups at 0 and 24 h after stimulation were 101.0% and 99.0%, respectively. No in vitro effects were observed in cell viabilities by the pulsed magnetic stimulation.

Cytokine Measurement

Figure 6 shows the TNF-α and IL-2 production of each mouse group. The results are expressed as mean $\pm$ SE. The TNF-α production of the stimulated and sham groups of the 3 day group was 36.06 ± 4.80 and 24.63 ± 1.38 pg/ml, respectively. The results of the 7 day group are 46.77 ± 2.64 pg/ml (stimulated group) and 28.49 ± 4.22 pg/ml (sham group). TNF-α production was significantly increased in both stimulated groups compared with the shams (3 day group: $P < .01$,

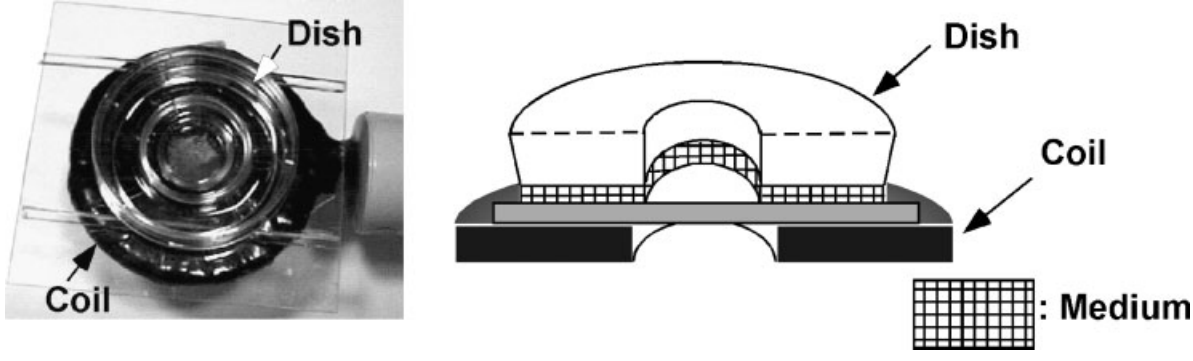

Fig. 3. **A**: Magnetic stimulation of the cells. **B**: The culture dish was placed 10 mm above the stimulation coil and exposed to magnetic stimulation.

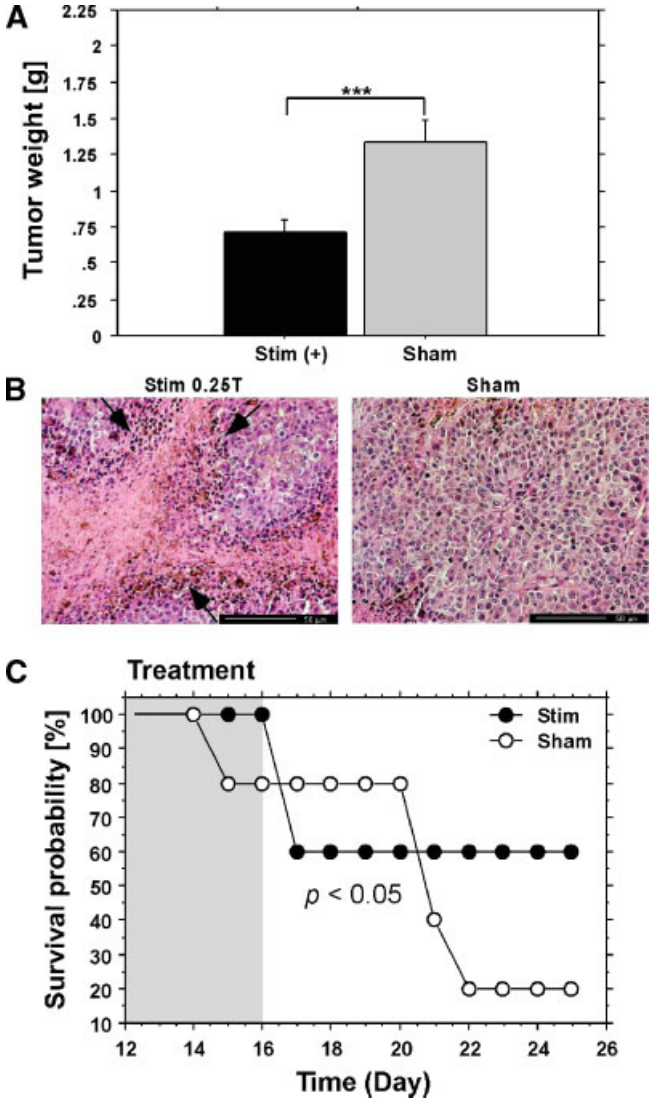

Fig. 4. Result from tumor growth study and histological appearance of tumors treated by magnetic stimulation. **A**: The tumor weights of each group on day 17 were expressed as mean ± SE. $^{***}P < .001$. $n = 14$. **B**: Specimen treated by magnetic stimulation (**left figure**) and that of sham group (**right figure**). The arrow shows where tissue necrosis occurred. Magnification 400×. **C**: Animal survival rates after exposure to pulsed magnetic stimulation. Mice in the stimulated group were exposed to pulsed magnetic stimulation from day 0 to day 17. $n = 5$. $P < .05$.

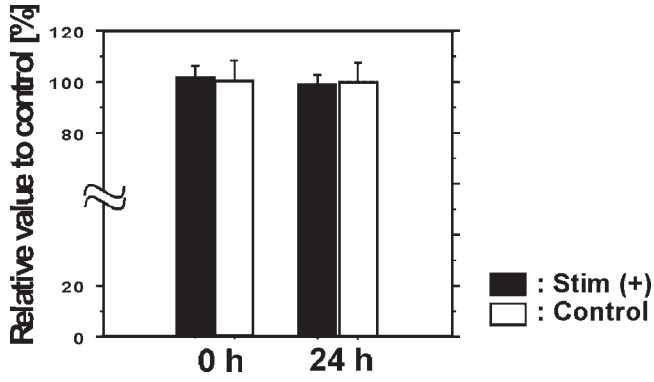

Fig. 5. In vitro effect of magnetic stimulation on cell viability. Magnetically induced eddy currents were 2.36–2.90 A/m^2 (≈ induced in a stimulated mouse of 0.25 T group). Results are expressed as mean ± SE, $n = 5$.

and 7 day group: $P < .05$). The TNF-α production of the stimulated group of the 7 day group significantly increased ($P < .05$) compared with that of the 3 day group.

IL-2 production in both of the stimulated groups showed up-regulation compared with the shams; however, statistical significance was not detected. IL-2 production of the stimulated and sham groups in the 3 day group was 154.71 ± 20.50 and 107.44 ± 9.70 pg/ml, respectively. The results of the 7 day group are 124.50 ± 15.67 pg/ml (stimulated group) and 94.50 ± 12.11 pg/ml (sham group).

The animal spleen and body weights of the 7 day group were measured to examine the inflammatory effects to pulsed magnetic stimulation. Table 2 shows the relative spleen weight (spleen weight/body weight) of the two groups. There were no significant changes in the relative spleen weights (mean ± SE) of the stimulated group (3.25 ± 0.29 mg/g) and sham group (3.64 ± 0.64 g mg/g).

DISCUSSION

In this study, we have presented the anti-tumor effects caused by the application of pulsed magnetic stimulation and we also showed that magnetic stimulation has immunomodulatory effects. These results suggest the possible relationship between anti-tumor effects and the increase of TNF-α levels caused by pulsed magnetic stimulation.

High frequency pulsed magnetic stimulation has been reported to affect biological responses [Fujiki and Steward, 1997; Ogiue-Ikeda et al., 2003a,b] and recent studies indicate that the stimulus intensity has an influence on the effectiveness of these responses [Ogiue-Ikeda et al., 2003a,b]. In our previous research, we focused on the effects of pulsed magnetic stimulation (0.75 T, 150% of the motor threshold) on tumor development, however, the tumor weight decrease was insufficient [Yamaguchi et al., 2004]. The average tumor weights (mean ± SD) of the 0.75 T experiment were 1.16 ± 0.48 g (stimulated group: $n = 18$) and 1.35 ± 0.16 g (sham group: $n = 18$) [Yamaguchi et al., 2004]. Since the stimulus conditions in the previous report induced convulsive seizure in mice due to eddy current effects, which potentially affect biological responses, the present study focused on a gentle stimulus

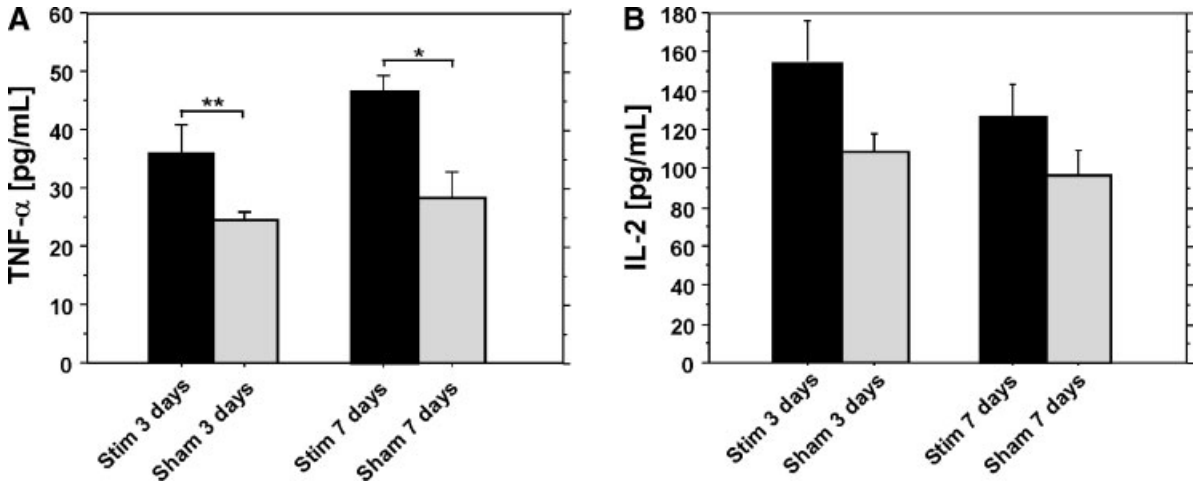

Fig. 6. Effect of magnetic stimulation on tumor necrosis factor (TNF-α) and IL-2 production. $^{*}P < .05$, $^{**}P < .01$; $n = 7$. Results are expressed as mean ± SE. **A**: TNF-α production of stimulated and sham group in 3 day and 7 day groups. **B**: IL-2 production of the stimulated and sham group in 3 day and 7 day groups. IL-2 production in both of the stimulated groups showed the up-regulation compared with the shams; however, statistical significance was not detected.

condition (0.25 T: 50% of the motor threshold) to reduce the non-magnetic effects and for the safety of the animals. As shown in Figure 4A, the tumor weight of the 0.25 T-stimulated group significantly decreased on day 17 and elongated the animal survive (Fig. 4C). Compared with the stimulated mice in our previous study, the stimulated group in the 0.25 T group showed a 62% decrease in tumor weight. Therefore, we suggest that this stimulus condition is effective for tumor model mice. However, further research is needed to clarify the dose dependency of the stimulus intensity.

One hypothesis of the anti-tumor mechanism in the present study is the pH change or heat effects caused by magnetically induced eddy currents. Although the anti-tumor effect was observed in an in vivo experiment, pulsed magnetic stimulation had no effect on the viability of cancer cells in vitro (Fig. 5). Several studies have reported that electrical stimulation such as DC induces cell damage in vitro by causing local pH alteration and electrochemical effects, which play crucial roles in cell death [Nordenstrom, 1994; Li et al., 1997; Yen et al., 1999]. In contrast, the magnetically induced eddy currents in our study induced neither a pH change (data not shown) nor heat (2.66×10^{-10} °C/pulse), so they did not play a role in the anti-tumor effect observed in our present study.

TABLE 2. Relative Spleen Weights of 7 Day Groups*

	Stim	Sham
Relative spleen weight (spleen weight/body weight: mg/g)	3.25 ± 0.29	3.64 ± 0.14

*Results are expressed as mean ± SE.

Several reports showed the functional activation of the immune system brought about by the application of electrical stimulation [Sersa et al., 1992; Chou et al., 1997; Miklavcic et al., 1997; Cabrales et al., 2001]. However, the effects of magnetic stimulation on immune systems were poorly understood. The present study indicates evidence for immunomodulatory effects being caused by pulsed magnetic stimulation for 3 or 7 days (Fig. 6). The TNF-α and IL-2 are associated with many pathological processes, including tumor immunity. TNF-α plays a tumor-suppression role in tumor immunity mainly by TNFR1–TRADD–FADD–Caspase-8–Caspase-3 apoptosis pathways [Ashkenazi, 2002; Aggarwal, 2003] and IL-2, which are produced by T cells, activate the proliferation and functional development of T cells (helper T cells and cytolytic T cells) and B cells [Smith, 1988; Nelson, 2004]. In recent decades, a number of immunotherapies for the treatment of cancer by IL-2 treatment were reported [Thomas and Hersey, 1998; Hersey and Zhang, 2001; Zhang et al., 2001; Andersen et al., 2003]. These reports support the idea that the possible relationship between anti-tumor effects and the increase of TNF-α levels caused by pulsed magnetic stimulation.

As shown in Figure 6, high TNF-α production was induced by magnetic stimulation for 7 days. Since TNF-α is closely related to inflammatory processes [Ashkenazi, 2002; Aggarwal, 2003], the inflammatory effects were examined, but no such obvious effect was detected in the present study (Table 2).

In our experiments, pulsed magnetic stimulation started from 1 day after injection of cancer cells to day 16, including different tumor development stages such as tumor colonization, formation and growth. Tumor immunity also develops followed by these stages [Houghton, 1994; Vonderheide et al., 1999; Rosenberg, 2001]. It is difficult to determine the sensitive period of anti-tumor effects observed in the present study. Results from the cytokine measurement indicate that magnetic stimulation has an influence on at least the early phase of tumor immunity (day 3–7), which is a non-specific immune response.

Moreover, since mice spleens received lower eddy currents (0.15–0.92 A/m^2) compared with tumor areas (0.79–1.54 A/m^2) during magnetic stimulation, it would appear that a neuronal pathway exists rather than a direct effect on the lymphocytes under the immunomodulatory effect in our study. Autonomic nerves (vagus nerves) have been reported as playing a role in signaling immunomodulatory responses to peripheral organs [Elenkov et al., 2000; Tracey, 2002]. Recent studies indicate that the efferent vagus nerve also has an immunomodulatory effect [Borovikova et al., 2000; Tracey, 2002]. In addition, pulsed magnetic stimulation is known as an effective neural-stimulating method and it has been used in neurological studies [Ueno et al., 1990; Cracco et al., 1999]. Thus, we propose that magnetically stimulated nerve systems, such as autonomic nerves, mediate the immunomodulatory effects shown in Figure 6. Further research is required to maximize the functional activation of immune system by pulsed magnetic stimulation and to clarify its mechanisms.

In conclusion, we have demonstrated the anti-tumor and immunomodulatory effects brought about by the application of repetitive magnetic stimulation. These findings show the first evidence that pulsed magnetic stimulation may have effects on tumor-developing processes and immune functions, and also suggest the possible relationship between anti-tumor effects and the increase of TNF-α levels caused by pulsed magnetic stimulation.

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