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Effects of Static Magnetic Fields on Phagocytic Activity of Murine Peritoneal Macrophages

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Abstract – Electro-magnetic fields and static magnetic fields generated from diverse home/environmental sources have been reported that these could make harmful effects on the human health such as suppression of immunity and tumorigenesis. However, the mechanisms for the biologic effects of electro-magnetic fields or static magnetic fields are still remained unclear. In this study, we examined the *in vitro* effects of static magnetic fields (SMF) on murine peritoneal macrophages. The cells were exposed *in vitro* to SMF of 150~250 or 350~450 G in 5 % CO_2-incubator. The phagocytic activity of murine peritoneal macrophages was inhibited under exposure to SMF. In order to provide a more complete picture of molecular mechanism for the biological effect of SMF, we compared the levels of total proteins from macrophages with or without exposure to SMF using quantitative proteomic analysis. Proteins which were differentially expressed in macrophages exposed to SMF compared with non-exposed macrophages, were identified. Among them, the levels of trypsinogen 16, lactose-binding lectin Mac-2, galactoside-binding lectin, actin-like (Put. β-actin, vimentin) and electron transferring flavoprotein beta polypeptide were enhanced under exposure to SMF. These results suggest that SMF can affect the phagocytic activity of macrophages *via* diverse mechanisms.

Key words □ Static magnetic fields, Macrophages, Phagocytic activity

INTRODUCTION

The effects of magnetic fields on physiological processes in living organisms were demonstrated and investigated as early as 1888. Recently nonthermal interactions of extremely low frequency (ELF) electromagnetic fields (EMF) with cellular systems such as immune cells have been demonstrated (Cadossi *et al.*, 1992; Walleczek, 1992). Furthermore, environmental exposure of human body to weak EMF increased the risk for developing certain type of leukemia and cancer (Tynes *et al.*, 1992). For example, the exposure to power-frequency electric and magnetic fields may increase the risk of cancer, specifically breast cancer (Pollan *et al.*, 1999; Coogan *et al.*, 1996). From all of these, ELF-EMF generated from several home/environmental sources such as 50~60 Hz high-voltage transmission lines, video display terminals, electric blankets and clinical nuclear magnetic resonance (NMR) imaging procedures, may affect the human body function. In spite of many studies, the effects of static magnetic fields (SMF) on macrophages are not well understood. In the present study, we examined the effects of SMF on peritoneal macrophages of C57BL/6 mice. Additionally, the levels of total proteins from macrophages with or without exposure to SMF were compared using quantitative proteomic analysis.

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MATERIALS AND METHODS

Animals

The male C57BL/6 mice weighing about 20±2 g, were purchased from Dae-Han Experimental Animal Center (Eumsung, Chungbuk, Korea) and were maintained at 22±3°C with a day/night cycle of 12/12 h. before use, and were fed with lab Chow (Cheil Jedang Co.) and tap water *ad lib.*

Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin-

streptomycin, γ-interferon (Hu, γ-IFN), lipopolysaccharide (026:B6, LPS), N,N'-methylene-bis-acrylamide, zymosan, lucigenin, acrylamide, TEMED, iodoacetamide, silver nitrate, FITC-conjugated *E. coli* K12 particles and EDTA-2Na·2H_2O were purchased from Sigma (St. Louis, MO, U.S.A.). RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco BRL. (Grand Island, U.S.A.). Urea, CHAPS, dithiothreitol (DTT), acrylamide-Isoelectro Foccusing (IEF), and immobilized pH gradients (IPG)-buffer (pH 3-10) were purchased from Amersham Pharmacia Biotech AB (Amersham, UK).

Cell preparation and culture

Murine macrophages were isolated from peritoneal exudate cells of mice pretreated with 2.0 ml of 3% thioglycollate intraperitoneally 3 days before cell isolation. The cells were purified by adherence to plastic dish for 2 h at 37°C in 5% CO_2 incubator. The adherent cells were scraped and more than 95% of the adherent cells were macrophages when confirmed by Wright and nonspecific esterase staining (John *et al.*, 1991). The culture medium was supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured for 24 h in 5% CO_2-incubator at 37°C under static magnetic fields ranged of 150~250 G or 350~450 G monitored by a Tesla meter (Kanetec TM-401).

Assay of phagocytic activity

The obtained macrophages were washed twice in DPBS solution before being resuspended in DMEM without phenol rèd. The cells were diluted to 1×10^6 cells/ml. Zymosan was prepared by the method of Lachmann and Hobert (Lachmann *et al.*, 1978) and diluted to 1×10^7 particles/ml in DPBS-A solution before use. Zymosan and serum were pre-incubated at 37°C for 30 min to opsonize the zymosan. The cells were pre-incubated with fresh lucigenin at 37°C for 15 min. in a white multi-well plate (Costar), and then zymosan was added to individual wells. The chemiluminescence (CL) assays were performed at 5 min intervals for 30 min using a luminometer (Blair *et al.*, 1988). Another method we used for phagocytic activity was developed using FITC-conjugated *E. coli* K12 particles (Chok *et al.*, 1993). The particles were suspended in HBSS and stored at -20°C in the dark. The suspension was then thawed and briefly sonicated just before use. Trypan blue was dissolved in citrate buffer (pH 4.4) at a concentration of 250 μg/ml (Sahlin *et al.*, 1983). The incubation was performed in a humidified 37°C incubator with 5% CO_2. The obtained macrophages were harvested and resuspended in RPMI1640 supplemented with 10% FBS. The cell concentration was adjusted to 5×10^5 cells/ml and 100 μl of cell suspension was then pipetted into each well. The cells were cultured for 1 h and then the culture medium was aspirated. The *E. coli* suspension, pre-warmed to room temperature, was then briefly sonicated to disperse any aggregates and 25 μl was added to each well. The plate was covered and incubated for 1 h, and then the buffer in the plate was removed by aspiration. Extracellular fluorescence was quenched by adding 100 μl of trypan blue (250 μg/ml, pH 4.4). After 1 min, the dye was removed, and the morphology of macrophages was observed with an inverted fluoromicroscope.

Two-dimensional SDS-PAGE

The obtained macrophages were stored in liquid nitrogen. After lysis buffer (8 M Urea, 4% CHAPS, 40 mM Tris base, 1 % DTT, 0.5% IPG buffer, protease inhibitor) was added, the cell suspension was sonicated in short bursts on ice. The lysate was centrifuged at 30,000×*g* for 15 min at 18°C. The supernatant was saved at -80°C until used. The first dimension of 2-D electrophoresis was performed on an IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech.). Linear pH 3~10 Immobiline DryStrips (24 cm) were rehydrated overnight at room temperature in rehydrating buffer (8 M Urea, 1% DTT, 2% CHAPS, 0.5% IPG buffer, bromphenol blue). The supernatant (total protein; 300 μg) was applied during rehydration. The first dimension was run for 53,500 Vhr using the following conditions at 20°C (500 V for 1 h, 1,000 V for 1 h and 8,000 V for 6.5 h). Next, gels were equilibrated for 30 min in equilibration buffer I {50 mM Tris-Cl (pH 8.8), 6 M Urea, 30% Glycerol, 2% SDS, 0.1% DTT} and equilibration buffer II {50 mM Tris-Cl (pH 8.8), 6 M Urea, 30% Glycerol, 2% SDS, 0.25% IAA}. The second dimension was run according to Ettan DALT II system operating and maintenance manual. A 12.5% SDS-polyacrylamide slab gel was used for the second dimension gel electrophoresis. The IPG strips were placed on the surface of the second dimension gel, then the IPG strips were sealed with 0.5% agarose in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). The gels were placed into Ettan DALT II system chamber containing 1×SDS electrophoresis buffer. The gels were run overnight at 110 V until the dye front reached the bottom of the gel.

Silver staining

Silver staining of the gels was performed as a modified silver staining protocol using Silver Stain Plus One kit. The use of glutaraldehyde in the sensitization step and formaldehyde in the

silver impregnation step was omitted. After electrophoresis, the gels were fixed with 40% methanol/10% acetic acid for 30 min. The gels were sensitized by incubating in sensitizing solution (0.2% sodium thiosulphate, 30% methanol, sodium acetate 68 g/L), and it was then rinsed with three changes of distilled water for 5 min. each. After rinsing, the gels were incubated in 0.25% silver nitrate for 20 min. After incubation, the silver nitrate was discarded, and the gels were rinsed twice with distilled water for 1 min. and then developed in 0.15% formaldehyde in 2.5% sodium carbonate with intensive shaking. After the desired intensity of staining was achieved, the development was terminated with 1.46% EDTA.

Image analysis

We digitized the silver-stained gel image using a scanner (UMAX PowerLookIII). ImageMaster 2D (Amersham Pharmacia Biotech) was used to analyze the gel images.

Destaining

Silver-stained proteins were destained with chemical reducers to remove the silver as described previously with following critical modifications (Scheler *et al.*,1998). Potassium ferricyanide and sodium thiosulfate were prepared as two stock solutions of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, both dissolved in water. A working solution was prepared by mixing a 1 : 1 ratio prior to use. After interesting protein spots were excised from the gel, 30~50 μl of working solution were added to cover the gels and occasionally vortexed. The stain intensity was monitored until the brownish color disappeared, then the gel was rinsed a few times with water to stop the reaction. Next, 200 mM ammonium bicarbonate was added to cover the gel for 20 min and was then discarded. Subsequently, the gel was cut into small pieces, washed with water, and was dehydrated repeatedly with changes of acetonitrile until the gel pieces turned opaque white. The gel pieces were dried in a vacuum centrifuge for 30 min.

Trypsin digestion of proteins in-gel

Enzymatic digestion was performed as previously described (Hellman *et al.*, 1995; Shevchenko *et al.*, 1996). Briefly, digestion was performed with 5~10 ng/μl of trypsin and 50 mM ammonium bicarbonate and incubated overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted three times with 10-20 μl of 5% trifluoroacetic acid in 50% acetonitrile and dried using a vacuum centrifuge for 30 min.

MALDI-TOF and ESI Q-TOF mass spectrometric analysis

MALD-TOF and ESI Q-TOF mass spectrometry analysis was performed at Korea Basic Science Institute (Taejon, Korea).

Statistical analysis

The data were shown as the mean±S.E.M. and the evaluation was done using Student's *t*-test.

RESULTS

Effect of SMF on lucigenin chemiluminescence of murine peritoneal macrophages

Chemiluminescence provides a simple method of assessing phagocyte function *in vitro*. The maximum peak of the chemiluminescence response of murine peritoneal macrophages to opsonized zymosan was seen at 20 min. The chemiluminescence from macrophages cultured under SMF was inhibited compared with those in control group (Fig. 1). This result indicates that SMF inhibit the phagocytic activity of murine peritoneal macrophages. We observed the phagocytic activity using FITC-conjugated *Escherichia coli* K-12 bio-particles. Engulfment of the particles in macrophages cultured under SMF was decreased (Fig. 2), and thus it is apparent that SMF inhibit the phagocytic activity of macrophages.

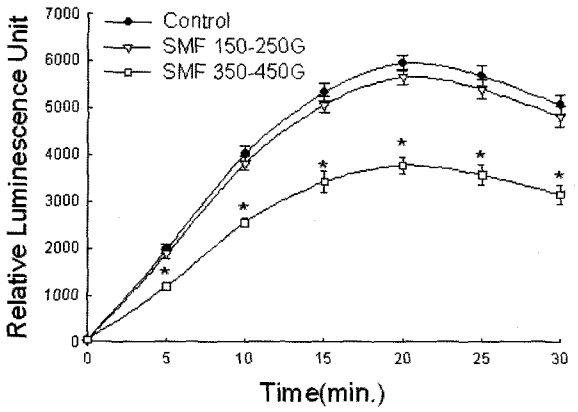
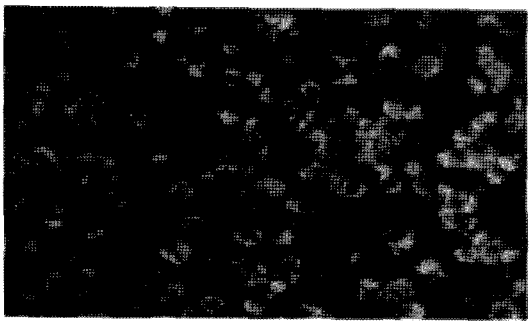
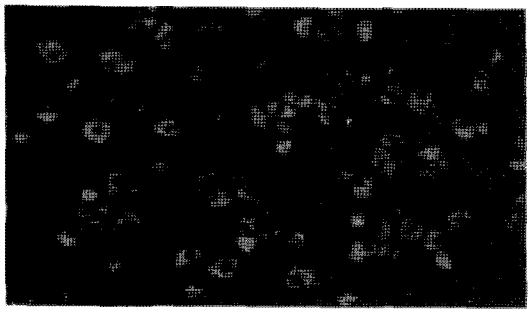


Fig. 1. Effects of static magnetic fields on lucigenin chemiluminescence of murine peritoneal macrophages. The macrophages were cultured for 24 hrs under SMF of 150~250 or 350 ~ 450 G in RPMI1640 media. The cells (5×10^5 cells/ml) were cultured in DME media without phenol red mixed with opsonized zymosan. The chemiluminescence was measured for 30 min with luminometer. Each bar represents the mean± S.E.M. of 5 times. *; Significantly different from control group ($p<0.001$).

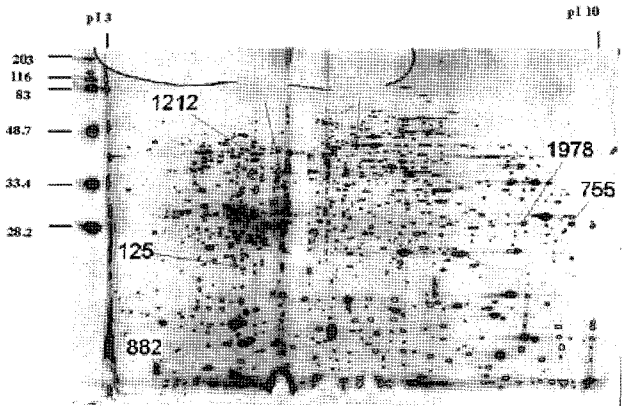


Control



SMF

Fig. 2. Photomicrographs of the engulfment of FITC-conjugated *E. coli* particles in peritoneal macrophages under SMF. Photographs (taken at 200×magnification) showing the uptake of FITC-conjugated *E. coli* particles.



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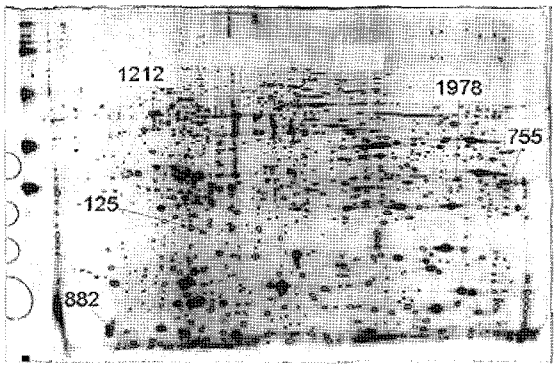

SMF

Fig. 3. Representative 2-DE patterns of control and SMF showing significant and consistant change between two groups.

Effect of SMF on protein profiles of murine peritoneal macrophages

Protein profiles from the macrophages cultured under SMF were analyzed by 2-D PAGE and stained with silver. A representation of one set among them is shown in Fig. 3. We routinely observed 850~1,000 spots from protein of 300 mg which stained with silver staining procedures (Fig. 3). Five proteins which were expressed in macrophages exposed to SMF were enhanced compared with non-exposed macrophages (Fig. 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8). The pI/mass (kDa) of five proteins were about 5.1/23.6, 8.0/26.7, 9.7/28.6, 5.2/43.1 and 9.0/28.8. The spots were excised and destained. Then it was digested by trypsin overnight and was analyzed by MALDI-TOF MS for peptide finger printing as well as by ESI Q-TOF MS for peptide sequencing. Five proteins were identified by MALDI-TOF MS and ESI Q-TOF MS. The five spots were trypsinogen 16, lactose-binding lectin Mac-2, galactoside-binding lectin, actin-like (Put. β-actin, vimentin) and electron transferring flavoprotein beta polypeptide (Table I).

Table I. Proteins significantly increased in murine peritoneal macrophages under SMF

Spot no.	Protein	Accesstion no.	Measured pI/mass	pI/Mw (in database)
125	Trypsinogen 16	16716569	5.1/23.6	/26118
755	Lactose-binding lectin Mac-2	539907	8.0/26.7	/27398
882	Galactoside-binding lectin	387112	9.7/28.6	/15904
1212	Actin-like	8850209	5.2/43.1	/43572
	Put. Beta-actin	49868		/39161
	Vimentin	2078001		/51533
1978	Electron transferring flavoprotein, beta polypeptide	38142460	9.0/28.8	/27293

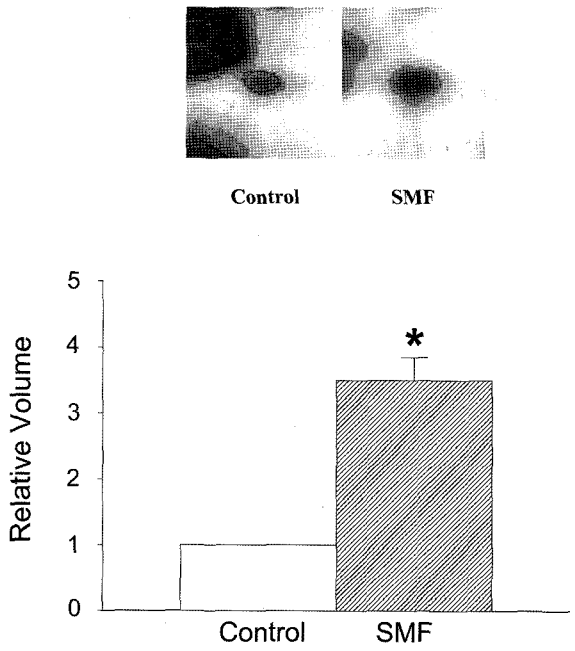

Fig. 4. The protein of spot 125 consistantly up-regulated in SMF compared with control.

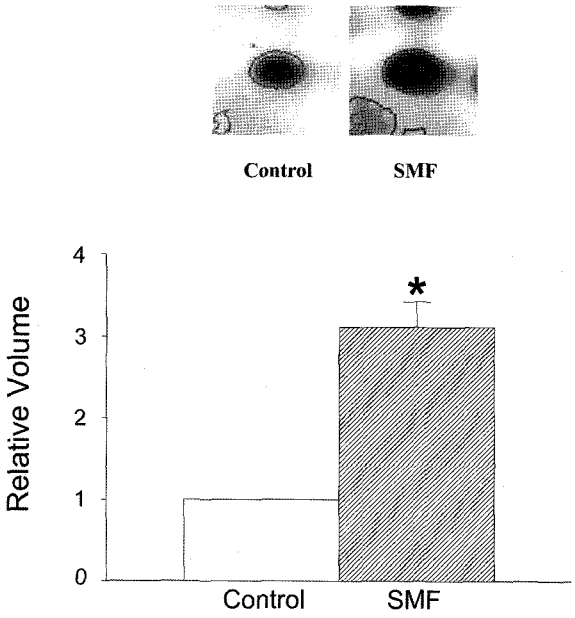

Fig. 5. The protein of spot 755 consistantly up-regulated in SMF compared with control.

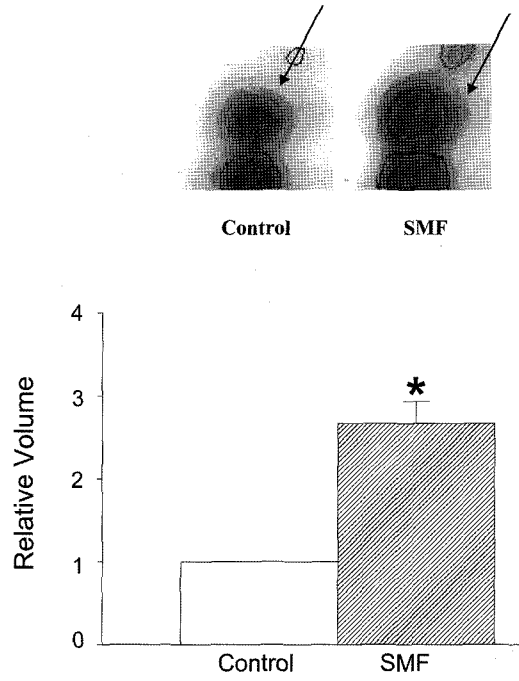

Fig. 6. The protein of spot 882 consistantly up-regulated in SMF compared with control.

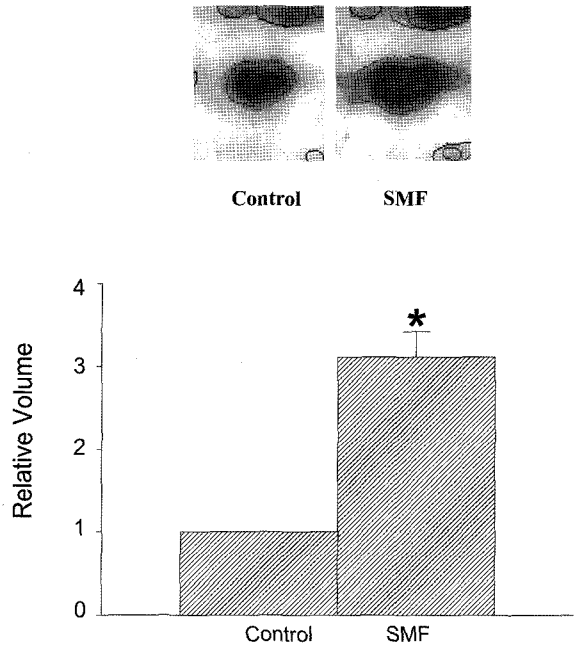

Fig. 7. The protein of spot 1212 consistantly up-regulated in SMF compared with control.

DISCUSSION

At a very low magnitude of SMF of 0.35 G equivalent to the background geomagnetic fields in laboratories, lymphocyte functions were not affected (Yost *et al.*, 1992). The fact that weak magnetic fields, such as those from power lines could never induce physiologically relevant transmembrane potentials has been generally accepted (Weaver *et al.*, 1990). Several *in vitro* studies have shown biological effects of SMF in a vari-

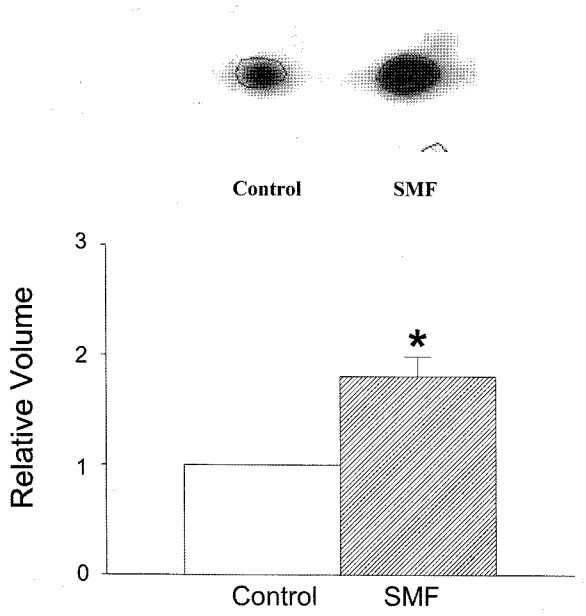

Fig. 8. The protein of spot 1978 consistantly up-regulated in SMF compared with control.

ety of cells, such as in fibroblasts (McDonald *et al.*, 1993), HeLa cells (Hiraoka *et al.*, 1992), melanoma cells (Imajo *et al.*, 1989), and immune cells (Flipo *et al.*, 1998). As of today, the exact mechanisms of SMF-related changes in immune cells are not clear. However, our data support that SMF of 350~450 G exposure could be sufficient to produce biological effects. Previous assays of phagocyte chemiluminescence have required large numbers of cells and have not been able to follow responses from a large number of samples in a single experiment. Recently, sensitive luminometers which use a 96 well microplate format have become available (Boudard *et al.*, 1994). We applied this equipment to the measurement of phagocyte chemiluminescence using lucigenin to enhance the response for estimation of opsonic activity. The basis of the technique is the detection of oxygen radicals produced by phagocytes during the respiratory burst following phagocytic activity of particles or by stimulation with various humoral factors (Holt *et al.*, 1984). We found that SMF of 150~250 G did not affect the phagocytic activity of murine peritoneal macrophages, but SMF of 350~450 G inhibited the phagocytic activity. Simko *et al.* (2001) reported that short-time exposure (45 min) to electro-magnetic fields of 50~150 G stimulated the phagocytic activity of murine bone marrow-derived macrophages. In this study, the cells were exposed to static magnetic fields and much higher (350~450 G) magnetic fields, as well as for prolonged periods of time (24 h). Santoro *et al.* (1997) reported that after long-term exposure to EMF (2 mT, for 72 h), components of the cytoskeleton were reorganized and the membrane fluidity was modified in human Raji cells. Differences among these results probably are due to power of magnetic field and culture-time of cells. To understand the mechanism for the biologic effect of SMF, we used a proteomics analysis method. In the present study, five proteins which were significantly different between control group and SMF-exposed group were identified by MALDI-TOF MS and ESI Q-TOF MS. We suggested that the five proteins were trypsinogen 16, lactose-binding lectin Mac-2, galactoside-binding lectin, actin-like (Put. ß-actin, vimentin) and electron transferring flavoprotein beta polypeptide. Rowen *et al* reported trypsinogen 16 (NP_444473) from *Mus musculus*. The murine Mac-2 gene is a member of an expanding family of soluble lactose-binding lectins (Cherayil *et al.*, 1990; Leffler *et al.*, 1989). Mac-2 has been shown to be secreted from macrophages and to bind to laminin as well as to two recently described glycoproteins, M2BP-1 and M2BP-2, in a sugar-specific manner (Woo *et al.*, 1990; Rosenberg *et al.*, 1991). Mac-2 is expressed at high levels by inflammatory macrophages (Cherayil *et al.*, 1989) and in advanced stage of colorectal cancer (Irimura *et al.*, 1991). Endogenous carbohydrate-binding proteins have been purified from a wide variety of normal and malignant tissues and cells. Although lectins with different sugar-binding specificities have been described, the most prevalent are those that bind ß-galactosides (gal-lectins). Many of these lectins have an apparent molecular weight subunit within the range of 13,000 to 70,000, their most common forms being in the molecular weight range of ~14,000 and 29,000 to 35,000. Carbohydrate recognition mediated by lectins has been implicated in *in vivo* and *in vitro* growth, morphogenesis, differentiation, intercellular interaction, adhesion and metastasis (Raz *et al.*, 1987; Meromsky *et al.*, 1986). The γ-actin-like protein may be an activator of amino acid transporter or may play some role in recruiting transporters to the plasma membranes. It is noteworthy that the a, β, and γ-actins of the mouse and E51 contain a sequence closely related to a type II SH3 binding domain, namely protein XX proline, where X is any amino acid, at amino acid residues 115-118 followed by a lysine residue (Lin *et al.*, 1996). Alonso *et al.* (1986) reported put. beta-actin (CAA27396) from *Mus musculus*. The vimentin can be demonstrated in normal (Fedoroff *et al.*, 1983), reactive (Schiffer *et al.*, 1986) and neoplastic (Paulus *et al.*, 1988) astrocytes, where the high concentrations of vimentin may reflect dedifferentiation. Vimentin, however, is also found in macrophages (Phaire-Washington *et al.*, 1987)

so it cannot be used to distinguish astrocytes from microglia. The astrocytes may actively participate in the phagocytosis and degradation of myelin, a function generally attributed to reactive macrophages (Vinores *et al.*, 1993). The vimentin is an acidic protein which is related to the cytoskeleton (Siffert *et al.*, 1993). Electron transfer flavoprotein and electron transfer flavoprotein dehydrogenase are two mitochondrial emzymes that are successively required for the transfer of electrons between several mitochondrial matrix dehydrogenases and the main electron transport chain (White *et al.*, 1996). Glutaric acidemia type II is a human inborn error of metabolism characterized by hypoketotic hypoglycemia, metabolic acidosis, and excretion of organic acids derived from the substrates of flavin-containing enzymes whose electrons enter the respiratory chain *via* electron transfer flavoprotein and electron transfer flavoprotein dehydrogenase. In some patients, glutaric acidemia II is due to electron transfer flavoprotein deficiency, while in others it is due to electron transfer flavoprotein dehydrogenase. In the future, we are going to study the validation of these proteins.

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