Self-regulating hyperthermia induced using thermosensitive ferromagnetic material with a low Curie temperature

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Hyperthermia has been used for many years to treat a variety of malignant tumors. The Curie temperature (Tc) is a transition point at which magnetic materials lose their magnetic properties, causing a cessation of current and thus heat production. The Tc enables automatic temperature control throughout a tumor as a result of the self-regulating nature of the thermosensitive material. We have developed a method of magnetically-induced hyperthermia using thermosensitive ferromagnetic particles (FMPs) with low Tc (43°C), enough to mediate automatic temperature control. B16 melanoma cells were subcutaneously injected into the backs of C57BL/6 mice, after which tumors were allowed to grow to 5 mm in diameter. FMPs were then injected into the tumors, and the mice were divided into three groups: group I (no hyperthermia, control); group II (one hyperthermia treatment); and group III (hyperthermia twice a week for 4 weeks). When exposed to a magnetic field, the FMPs showed a sharp rise in heat production, reaching the Tc in tissue within 7 min, after which the tissue temperature stabilized at approximately the Tc. In groups I and II, all mice died within 30–45 days. In group III, however, 6 of 10 mice remained alive 120 days after beginning treatment. Our findings suggest that repeated treatment with magnetically-induced self-regulating hyperthermia, mediated by FMPs with a low Tc, is an effective means of suppressing melanoma growth. A key advantage of this hyperthermia system is that it is minimally invasive, requiring only a single injection for repeated treatments with automatic temperature control. (*Cancer Sci* 2008; 99: 805–809)

Hyperthermia has been used for many years to treat a wide variety of malignant tumors. Its use is based on the fact that tumor cells are more sensitive to temperature in the range of 42–45°C than normal tissue cells.[1–4] Higher temperatures up to 56°C, which yield widespread necrosis, coagulation, or carbonization (depending on temperature), is called 'thermo-ablation'. Hyperthermia is advantageous in that it has fewer side-effects than thermo-ablation, chemotherapy, or radiation, and it has been investigated for many years in the treatment of a wide variety of malignant tumors in both experimental animals and patients.[5] The most commonly used method of heating in clinical settings is capacitive heating using a radiofrequency electrical field.[4,6,7] The great advantage of capacitive heating is that it is non-invasive. However, this method can cause excessive heating of the fat layer and is not suitable for site-specific hyperthermia because it is difficult to selectively heat only the local tumor region to the intended temperature without also damaging normal tissue. As the electrical field energy is conducted through the normal tissue, it is imperfectly transduced to heat in a manner reflecting the specific rates of absorption by the tissues, which are dependent on the specific electrical properties (e.g. permittivity and resistance) of each tissue.

To overcome the disadvantages of capacitative heating using an electrical field, attempts have been made to use inductive heating with magnetic nanoparticles.[8–17] When placed within a magnetic field, ferromagnetic materials develop an electric current and generate heat due to hysteresis loss.[18] Magnetic induction heating using thermosensitive material is able to produce highly localized hyperthermia in deep-seated tumors. However, the correct control of temperature has been a difficult and complicated challenge. To address that challenge, we have developed 'thermosensitive ferromagnetic particles' (FMPs) that produce a sufficient amount of heat through production of eddy currents and also have a Curie temperature (Tc) sufficient to provide automatic temperature control. The Tc is a transition point at which a material loses its magnetic properties, which causes current flow, and thus heat production, to cease.[19] A low Tc (43°C) can enable automatic temperature control throughout a tumor because the self-regulating nature of the thermosensitive materials will correct for local variations in heat loss due to blood perfusion. Furthermore, because the average diameter of FMPs is approximately 100 μm, it is possible to inject the material directly into any tumor site, either percutaneously or using a fiberscope (e.g. for tracheal or gastrointestinal tumors), and the FMPs will remain at the injected site because they are larger than blood capillaries and lymphatic vessels.[20] This feature makes this method minimally invasive, as it is possible to give repeated hyperthermia treatments after a single injection with automatic temperature control.

In the present study, we used a mouse melanoma model to examine the antitumor effect of FMP-mediated hyperthermia. We found that our repeated hyperthermia protocol induces a significant antitumor response.

Materials and Methods

***In vitro* experiment using 1% agar phantoms in polypropylene tubes.** FMPs (average diameter 100 μm), developed and supplied by TDK (Tokyo, Japan) and composed of Fe_2O_3, CuO, ZnO, and MgO (49:7:30:14 mol%, respectively), were placed into 1% agar (dissolved in distilled water) in a 50-mL polypropylene tube. A magnetic field (600 A, 188 kHz) was then created using a horizontal coil (diameter 10 cm, two turns) with an induction

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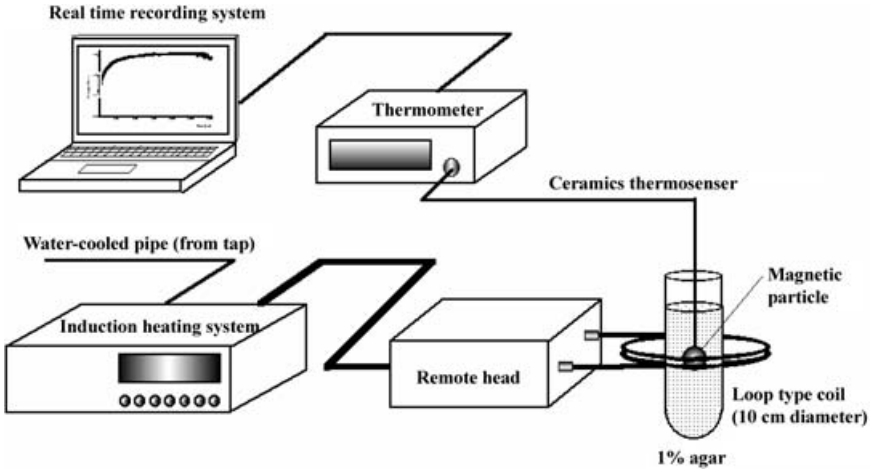

Fig. 1. System for *in vitro* experimentation. Ferromagnetic particles were placed into 1% agar in a 50-mL polypropylene tube. A magnetic field (600 A, 188 kHz) was created using a horizontal coil with an induction heating system. During exposure to the magnetic field, the temperatures were measured continuously using a ceramic thermocouple and recorded on a computer.

heating system (Hot Shot 5; Ameritherm, Scottsville, NY, USA). In some experiments, the temperatures were measured continuously during exposure to the magnetic field using a ceramic thermocouple (Amoth FL-2000; Anritsu Meter, Tokyo, Japan) and recorded simultaneously on a computer (Fig. 1). Each polypropylene tube, incubated in a water bath at 37°C before the hyperthermia experiment, was placed inside the coil such that the FMPs were positioned at the center of the coil. The area within which the temperature was stable in the vicinity of the Tc was also examined to adjust the distance of the ceramic thermosensor tip from the edge of the FMPs deposit.

Tumor cell line and animal model. Mouse B16 melanoma cells (Stratagene, La Jolla, CA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) at 37°C under an atmosphere containing 5% CO_2. Female 6-week-old C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) or Charles River Japan (Yokohama, Japan). To prepare tumor-bearing animals, cell suspensions consisting of approximately 1×10^6 melanoma cells in 100 μL phosphate-buffered saline (pH 7.4) were injected subcutaneously into the backs of C57BL/6 mice. After approximately 10 days, the melanoma nodules had grown to approximately 5 mm in diameter and used for experimentation. Tumor diameters were measured every 2 days, and their sizes were determined by applying the following formula: Tumor size = 0.5 × (length + width, in mm).

Animal experiments were carried out according to the principles laid down in the 'Guide for the Care and Use of Laboratory Animals' prepared under the direction of the Office of the Prime Minister of Japan.

Injection of FMPs. After the melanoma nodules had grown to approximately 5 mm in diameter, mice were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg body weight), and approximately 500 mg of FMPs in 500 μL phosphate-buffered saline was injected into the center of each nodule using a syringe with a 21-gauge needle. The mice were then divided into three groups of 10 mice each. Group I mice were not subjected to the alternating magnetic field (control group). Group II mice were subjected to hyperthermia for 30 min once, and group III mice received hyperthermia treatments twice a week for 4 weeks. Group IV mice were injected with the vehicle and received the alternating magnetic field twice a week for 4 weeks as a control for group III.

In every experiment, each mouse was anesthetized by intraperitoneal injection of pentobarbital and placed inside the coil such that the nodule was positioned at the center of the coil. In some experiments, a ceramic thermocouple was used to measure temperature at the tumor edge and rectum while the alternating magnetic field was on.

Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) analysis. To determine whether hyperthermia induces apoptosis within tumors, on day 20 after injecting the FMPs, five mice from groups I and III were killed and the tumors were removed, fixed in 10% neutral buffered formalin for 18 h at 4°C, and embedded in paraffin. After preparing 3-μm sections, the TUNEL method was used to identify apoptotic cells using an ApopTag Peroxidase In Situ Apoptosis Detection kit (S7100; Intergen, NY, USA) in which 3-3′-diaminobenzidine tetrahydrochloride served as the colorant. After staining, 10 random fields of the slide (five mice in each group, total 50 fields in each group) under constant magnification (×400) were observed, and the percentage of apoptotic cells was evaluated.

Statistics. Group data are expressed as mean ± SD. The statistical significant was assessed by one-way ANOVA with Scheffe's multiple comparison tests, and differences in survival rates were analyzed using the log–rank test in StatView 5.0 (Macintosh).

Results

Fig. 2 summarizes the results of our *in vitro* experiment using 1% agar in a polypropylene tube containing 500 mg FMPs. When exposed to the magnetic field, the temperature at the edge of the FMPs increased sharply, reaching the Tc of 43°C within 5 min, after which it stabilized at the Tc. The area within which the temperature was stable in the vicinity of the Tc extended to a distance of almost 10 mm from the edge of the FMPs (Table 1).

When FMPs were injected into tumors and exposed to the magnetic field, the temperature within the tumor increased to the Tc within approximately 7 min, and was then maintained in the vicinity of the Tc with little deviation during the entire period of exposure to the magnetic field (Fig. 3). Rectal temperature remained at approximately 37°C throughout the period, although it increased slightly. Thus our hyperthermia system using FMPs appeared able to mediate localized heating of the tumor without damaging healthy tissue.

Fig. 4 shows the time courses of tumor growth. After melanoma nodules had grown to 5 mm in diameter, hyperthermia was induced in groups II, III, and IV. In group I (control), which did not receive hyperthermia treatment, and group IV, which received the alternating magnetic field twice a week for 4 weeks

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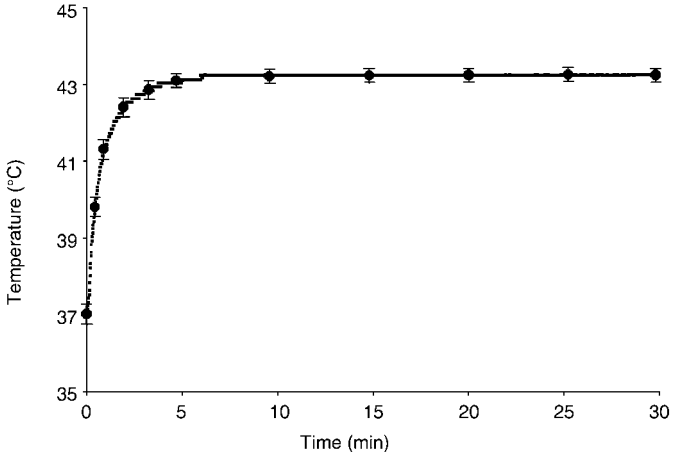

Fig. 2. Hyperthermia induced using ferromagnetic particles (FMPs) *in vitro*. The temperature increased sharply, reaching the Curie temperature (Tc), a transition point at which magnetic materials lose their magnetic properties, within 5 min after exposure of the FMPs to the magnetic field. Thereafter the temperature stabilized at the Tc. Symbols represent means ± SD of five measurements.

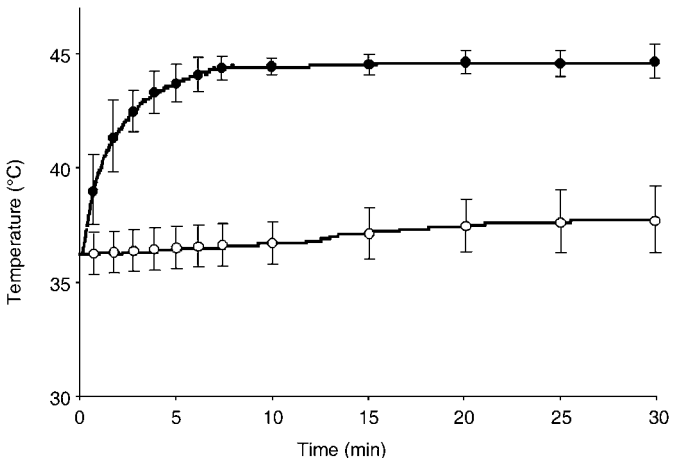

Fig. 3. Hyperthermia induced using ferromagnetic particles (FMPs) *in vivo*. FMPs were injected directly into subcutaneous B16 melanoma tumors in mice and then exposed to a magnetic field for 30 min. Tumor (filled circles) and rectal (open circles) temperatures were measured using a ceramic thermocouple. Symbols represent means ± SD of five mice.

as a control for group III, the tumors grew progressively. Although the mice in group II received one hyperthermia treatment, the tumors also grew progressively. By contrast, in group III, in which the mice received hyperthermia treatment twice a week for 4 weeks, tumor growth was significantly suppressed ($P < 0.05$). All mice were killed at the end of the experimental period. Distribution of FMPs that remained in a stable deposit formation at the injected site was confirmed in all hyperthermia cases, thus allowing repeated magnetic field hyperthermia. Clear toxicity of FMPs was not observed during the experimental period.

To determine whether the effect of hyperthermia reflects an increased incidence of apoptosis, on day 20 tumors were removed from a set of five mice in groups I and III, and the TUNEL method was used to identify apoptotic melanoma cells. We found that the incidence of apoptosis was significantly greater in tumors from group III mice (80.60 ± 13.07%) than in those from group I mice (9.42 ± 2.91%) (Table 2).

Table 1. Temperature distance from the edge of ferromagnetic particles in 1% agar during exposure to a magnetic field. Data represent mean ± SD of five measurements

Distance from ferromagnetic particles (mm)	Temperature (°C)
0	43.8 ± 0.44
5	43.0 ± 0.56
10	42.5 ± 0.32
15	38.2 ± 0.57
20	36.5 ± 0.40
25	36.4 ± 0.41

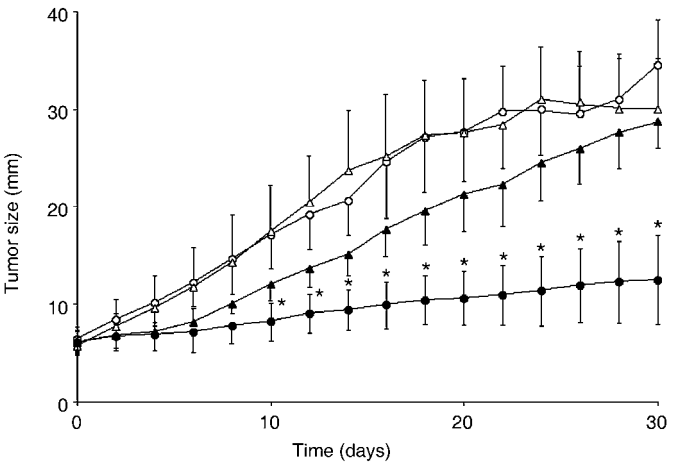

Fig. 4. Time courses of tumor growth in groups I (open circle; control), II (filled triangle; one hyperthermia treatment), III (filled circle; hyperthermia twice a week for 4 weeks), and IV (open triangle; mice injected with vehicle and received alternating magnetic field twice a week for 4 weeks), respectively. Symbols represent means ± SD of 10 mice. *$P < 0.05$ *versus* control.

Table 2. Comparison of terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL)-positive cells in each treatment group. Data represent mean ± SD

Groups	TUNEL-positive cells (%)
Control	9.42 ± 2.91
One hyperthermia treatment	14.71 ± 4.92
Repeated hyperthermia treatments	80.60 ± 13.07*
Vehicle with repeated alternating magnetic fields	9.21 ± 2.78

*$P < 0.05$ *versus* control. Hyperthermia was magnetically induced in B16 melanoma tumors in mice using thermosensitive ferromagnetic particles with low Curie temperature (43°C), sufficient to mediate automatic temperature control.

The survival rates among tumor-bearing mice observed for a period of 120 days are shown in Fig. 5. In groups I (control), II (one hyperthermia treatment), and IV (with injected vehicle received to the alternating magnetic field twice a week for 4 weeks), all of the mice died within 30–45 days from pulmonary metastases and/or the enlarged tumor at the inoculation site. In contrast, the survival rate was significantly prolonged in group III (repeated hyperthermia), and 6 of 10 mice remained alive after 120 days.

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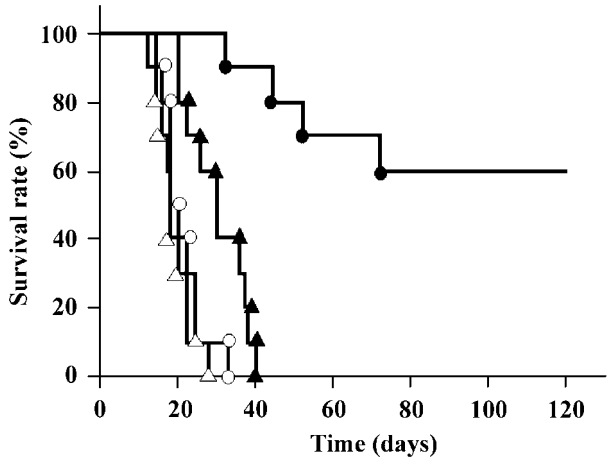

Fig. 5. Survival rates among tumor-bearing mice observed for a period of 120 days after injection of ferromagnetic particles: group I, open circles (control); group II, filled triangles (one hyperthermia treatment); group III, filled circles (hyperthermia twice a week for 4 weeks); group IV, open triangles (mice injected with vehicle and received alternating magnetic field twice a week for 4 weeks). $n = 10$ in each group. Survival in group III was significantly prolonged compared to the other groups ($P < 0.05$ *versus* all other groups).

Discussion

We have developed a hyperthermia treatment protocol that makes use of FMPs designed to produce heat through production of eddy currents and with a Tc of only 43°C. The low Tc enables good automatic temperature control throughout the tumor as a result of the self-regulating nature of the thermosensitive materials. We examined the antitumor effect of hyperthermia induced with these FMPs *in vitro* and *in vivo* in a mouse melanoma model. We found that although the tumor temperature rapidly reached 43–44°C, the rectal temperature remained at approximately 37°C. This means that the FMP-mediated hyperthermia selectively heated the tumor tissue, and that accurate control of tumor temperature could be achieved by setting the appropriate Tc. Although one hyperthermia treatment was insufficient to cause tumor regression, tumor growth was suppressed by repeated hyperthermia treatments with automatic temperature control, which significantly increased survival among mice with malignant melanoma.

At the early stages of tumor growth, problems with regional deep heating using capacitive radiofrequency included excess heating of subcutaneous fat, unexpected heating caused by edge effects, and insufficient penetration.[21,22] These difficulties were largely overcome by applying a surface cooling system involving a pair of large electrodes covered with a water pad, which led to the clinical application of radiofrequency capacitive heating. Still, the time required to heat the tissue to the effective temperature, the so-called temperature accelerating period, was sometimes as long as 30 min, and a heating period of more than 30 min is preferred for killing tumor cells. Such a long heating period is a severe burden for patients. Furthermore, a long temperature accelerating period might result in the malignant tissue becoming heat-tolerant. In the present study, we found that with FMPs the temperature accelerating period required to heat the tissue to 43°C was only approximately 7 min. Thus our hyperthermia system appears effective in terms of both spatial selectivity and speed.

It was previously shown that apoptosis was induced in malignant fibrous histiocytoma cells by heating to 42°C, whereas necrosis was induced when the cells were heated to higher temperatures (>56°C).[23] Although the temperature needed to induce apoptosis or necrosis might vary among cell types, one would expect higher temperatures to result in necrotic cell death, called thermo-ablation. Modern clinical hyperthermia trials focus mainly on the optimization of thermal homogeneity at a moderate temperature (approximately 43°C) in the target volume.[24] These hyperthermia systems are applied to induce apoptosis, not necrosis, and have the advantages of fewer side-effects than thermo-ablation because lower temperatures are used to minimize damage to surrounding normal tissues. Consistent with one of the mechanisms of hyperthermia treatment, TUNEL assays carried out in the present study revealed significant numbers of apoptotic cells in tumors subjected to repeated FMP-mediated hyperthermia.

In our *in vitro* experiment using 1% agar, the temperature increased sharply such that the Tc of 43°C was reached within 5 min, after which the temperature remained stable at approximately 43°C. *In vivo*, by contrast, the tumor temperature increased somewhat less rapidly, reaching the Tc within approximately 7 min, and then stabilized at 43–44°C. The delay in reaching the Tc *in vivo* could reflect heat loss caused by blood perfusion in the tumor-bearing mice. The finding that the stable temperature was approximately 0.5°C higher in tissue than in the agar likely reflects excess heating caused by differences in the conductivity of the body tissues. Tissues with higher conductivity will reach higher temperatures when exposed to the magnetic field.[25] Because the conductivities of skin, fat, and blood are all low (0.1, 0.04, and 0.7, respectively), the excess elevation in temperature during exposure to the magnetic field was minimal. Nevertheless, the conductivity of the body could have had some influence on rectal temperature, which was elevated to a small and variable degree during exposure to the magnetic field, although it remained at approximately 37°C.

Tc was set at 43°C in this hyperthermia method. Therefore re-treatment is possible with minimum invasiveness when tumor re-growth is targeted. For this reason, if Tc was set at a high temperature, like thermo-ablation, the second treatment might also cauterize the once-cured lesion (ferromagnetic particles still remain) that was the target of the previous hyperthermia treatment. A temperature of 43°C has a minimal effect on normal tissue or once-cured lesions, thus allowing repeated hyperthermia treatments in this system.

Repeated hyperthermia suppressed tumor growth, and a key advantage of our hyperthermia system is that it is minimally invasive, requiring only a single injection for repeated treatments with automatic temperature control.

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References

1 Cavaliere R, Ciocatto EC, Giovanella BC *et al*. Selective heat sensitivity of cancer cells. Biochemical and clinical studies. *Cancer* 1967; **20**: 1351–81.
2 Overgaard K, Overgaard J. Investigations on the possibility of a thermic tumor therapy. I. Short-wave treatment of a transplanted isologous mouse mammary carcinoma. *Eur J Cancer* 1972; **8**: 65–78.
3 Lefor AT, Makohon S, Ackerman NB. The effects of hyperthermia on vascular permeability in experimental liver metastasis. *J Surg Oncol* 1985; **28**: 297–300.

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4 Hiraoka M, Jo S, Akuta K, Nishimura Y, Takahashi M, Abe M. Radiofrequency capacitive hyperthermia for deep-seated tumors. I. Studies on thermometry. *Cancer* 1987; **60**: 121–7.
5 van der Zee J. Heating the patient: a promising approach? *Ann Oncol* 2002; **13**: 1173–84.
6 Abe M, Hiraoka M, Takahashi M *et al*. Multi-institutional studies on hyperthermia using an 8-MHz radiofrequency capacitive heating device (Thermotron RF-8) in combination with radiation for cancer therapy. *Cancer* 1986; **58**: 1589–95.
7 Hiraoka M, Nishimura Y, Nagata Y *et al*. Site-specific phase I, II trials of hyperthermia at Kyoto University. *Int J Hyperthermia* 1994; **10**: 403–10.
8 Jordan A, Wust P, Fahling H, John W, Hinz A, Felix R. Inductive heating of ferrimagnetic particles and magnetic fluids: physical evaluation of their potential for hyperthermia. *Int J Hyperthermia* 1993; **9**: 51–68.
9 Shinkai M, Yanase M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study. *Jpn J Cancer Res* 1996; **87**: 1179–83.
10 Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: an *in vivo* study. *Jpn J Cancer Res* 1998; **89**: 463–9.
11 Minamimura T, Sato H, Kasaoka S *et al*. Tumor regression by inductive hyperthermia combined with hepatic embolization using dextran magnetite-incorporated microspheres in rats. *Int J Oncol* 2000; **16**: 1153–8.
12 Jordan A, Scholz R, Maier-Hauff K *et al*. Presentation of a new magnetic field therapy system for the treatment of human solid tumors with magnetic fluid hyperthermia. *J Magnetism Magn Mater* 2001; **225**: 118–26.
13 Ito A, Tanaka K, Kondo K *et al*. Tumor regression by combined immunotherapy and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. *Cancer Sci* 2003; **94**: 308–13.
14 Tanaka K, Ito A, Kobayashi T *et al*. Intratumoral injection of immature dendritic cells enhances antitumor effect of hyperthermia using magnetic nanoparticles. *Int J Cancer* 2005; **116**: 624–33.
15 Johannsen M, Gneveckow U, Eckelt L *et al*. Clinical hyperthermia of prostate cancer using magnetic nanoparticles: presentation of a new interstitial technique. *Int J Hyperthermia* 2005; **21**: 637–47.
16 Wust P, Gneveckow U, Johannsen M *et al*. Magnetic nanoparticles for interstitial thermotherapy – feasibility, tolerance and achieved temperatures. *Int J Hyperthermia* 2006; **22**: 673–85.
17 Jordan A, Scholz R, Maier-Hauff K *et al*. The effect of thermotherapy using magnetic nanoparticles on rat malignant glioma. *J Neurooncol* 2006; **78**: 7–14.
18 Lilly MB, Brezovich IA, Atkinson WJ. Hyperthermia induction with thermally self-regulated ferromagnetic implants. *Radiology* 1985; **154**: 243–4.
19 Kobayashi T, Kida Y, Tanaka T, Kageyama N, Kobayashi H, Amemiya Y. Magnetic induction hyperthermia for brain tumor using ferromagnetic implant with low Curie temperature. I. Experimental study. *J Neurooncol* 1986; **4**: 175–81.
20 Doerschuk CM, Allard MF, Hogg JC. Neutrophil kinetics in rabbits during infusion of zymosan-activated plasma. *J Appl Physiol* 1989; **67**: 88–95.
21 Hahn GM, Kernahan P, Martinez A *et al*. Some heat transfer problems associated with heating by ultrasound, microwaves, or radio frequency. *Ann NY Acad Sci* 1980; **335**: 327–51.
22 Kato H, Hiraoka M, Nakajima T, Ishida T. Deep-heating characteristics of an RF capacitive heating device. *Int J Hyperthermia* 1985; **1**: 15–28.
23 Yonezawa M, Otsuka T, Matsui N *et al*. Hyperthermia induces apoptosis in malignant fibrous histiocytoma cells *in vitro*. *Int J Cancer* 1996; **66**: 347–51.
24 Jordan A, Scholz R, Wust P, Fahling H, Felix R. Magnetic fluid hyperthermia (MFH): cancer treatment with AC magnetic field induced excitation of biocompatible superparamagnetic nanoparticles. *J Magnetism Magn Mater* 1999; **201**: 413–9.
25 Gabriel C, Gabriel S, Corthout E. The dielectric properties of biological tissues. I. Literature survey. *Phys Med Biol* 1996; **41**: 2231–49.

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