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Review

Cancer hyperthermia using magnetic nanoparticles

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Magnetic-nanoparticle-mediated intracellular hyperthermia has the potential to achieve localized tumor heating without any side effects. The technique consists of targeting magnetic nanoparticles to tumor tissue followed by application of an external alternating magnetic field that induces heat through Néel relaxation loss of the magnetic nanoparticles. The temperature in tumor tissue is increased to above 43°C, which causes necrosis of cancer cells, but does not damage surrounding normal tissue. Among magnetic nanoparticles available, magnetite has been extensively studied. Recent years have seen remarkable advances in magnetite-nanoparticle-mediated hyperthermia; both functional magnetite nanoparticles and alternating-magnetic-field generators have been developed. In addition to the expected tumor cell death, hyperthermia treatment has also induced unexpected biological responses, such as tumor-specific immune responses as a result of heat-shock protein expression. These results suggest that hyperthermia is able to kill not only local tumors exposed to heat treatment, but also tumors at distant sites, including metastatic cancer cells. Currently, several research centers have begun clinical trials with promising results, suggesting that the time may have come for clinical applications. This review describes recent advances in magnetite nanoparticle-mediated hyperthermia.

Keywords: Anti-tumor immunity induction · Cancer treatment · Hyperthermia · Magnetic nanoparticles

1 Introduction

Today, the rationale for using hyperthermia in cancer therapy is well established; sustained temperatures above 43°C causes necrosis of cancer cells, which are more heat sensitive than normal tissue. Thus, hyperthermia is a promising approach to cancer therapy, in part, because hyperthermia is a physical treatment that could result in fewer side effects than chemo- or radiotherapy.

A major technical problem with the currently available hyperthermia modalities, including whole-body hyperthermia and radiofrequency capacitance hyperthermia, is the difficulty of heating a local tumor region to the desired temperature without significant damage to normal tissue. High temperatures above 43°C can kill a great number of tumor cells, but normal tissues are also severely damaged under these conventional hyperthermia treatments. Thus, the development of novel hyperthermia systems that are able to heat tumor cells above 43°C without damaging normal tissues is required. Magnetic-nanoparticle-mediated intracellular hyperthermia is a largely experimental modality for hyperthermia application, which has the potential to overcome these shortcomings. This technique consists of targeting magnetic nanoparticles to tumor tissue and then applying an external alternating magnetic field to generate heat in the nanoparticles by hysteresis loss or relaxational loss.

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Abbreviations: AML, antibody-conjugated magnetoliposome; **APC**, antigen-presenting cell; **DC**, dendritic cell; **HSP**, heat-shock protein; **mAb**, monoclonal antibodies; **MCL**, magnetite cationic liposome; **MHC**, major histocompatibility complex; **NPrCAP**, *N*-propionylcysteaminylphenol

2 Characteristics of magnetic nanoparticles for intracellular hyperthermia and in vivo experimental results

Any submicron magnetic particles that can generate heat under an alternating magnetic field can theoretically be used for intracellular hyperthermia. The most important criterion, however, is that the magnetic particles are non-toxic. Because of this requirement, magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) particles have been the focus of most studies. Maghemite is produced by the oxidation of magnetite above 300°C and the steps required to produce magnetite are simpler than those required to produce maghemite. Therefore, most studies of submicron magnetic particles for intracellular hyperthermia have focused on magnetite.

Several types of magnetite nanoparticles have been developed for intracellular hyperthermia and the magnetite core sizes range from 5 to 15 nm. In general, magnetic characteristics depend on particle size and on the preparation methods used to produce the submicron magnetic particles. As the particle size decreases, multi-domain ferromagnetic characteristics change to single-domain ferromagnetic, and finally, to superparamagnetic characteristics [1]. Two types of loss mechanisms are of interest for hyperthermia: hysteresis losses and Néel relaxation loss. Both loss types show a non-monotonic dependence of loss with particle size, that is, there are different optimum particle sizes for each loss mechanism. Multi-domain ferromagnetic particles possess lower hysteresis losses than single-domain ferromagnetic particles. Therefore, single-domain ferromagnetic particles generate more heat due to hysteresis losses. Superparamagnetic particles have no hysteresis losses and generate heat as a result of relaxational losses, mainly Néel relaxation loss.

Magnetic materials suitable for hyperthermia are discussed by Atsumi et al. [2] in terms of their magnetic properties, practical limitations in treatment conditions, and of the instruments used to activate the magnetic particles. By considering the magnetic and biocompatibility of the particles, superparamagnetic magnetite with a diameter of 11–13 nm was considered the most promising by this group.

When magnetic nanoparticles are used in in vivo studies, the lack of colloidal stability of these magnetic nanoparticles is an important issue. To overcome this shortcoming of magnetic colloids, we applied drug-delivery techniques with liposomes to provide intracellular hyperthermia systems using magnetite nanoparticles (with a diameter of 10 nm) [3]. In the case of magnetite cationic liposomes (MCLs), which have a positive surface charge to the liposomal surface, an accumulation of magnetite nanoparticles in tumor cells can be enhanced by conferring a positive surface charge to the liposomal surface (a tenfold higher affinity for glioma cells than neutrally charged magnetoliposomes). By injecting MCLs into tumor tissue, the temperature in tumor tissue increased to the intended temperature above 43°C and complete regression of mammary carcinomas of more than 15 mm was obtained in all mice after frequent, repeated hyperthermic treatments [4].

Furthermore, a significant development in intracellular hyperthermia occurred when antibody-conjugated polyethylene glycol derivatives containing magnetite and antibody-conjugated liposomes containing magnetite nanoparticles (antibody-conjugated magnetoliposomes, AMLs; core diameter, 10 nm) were developed. AMLs were constructed by using mouse G22 monoclonal antibodies (mAb) against human glioma cells [5], mouse G250 mAb against human renal cell carcinomas [6], humanized mAb against human epidermal growth factor receptor-2 (HER2; Herceptin) [7], and mAb against human high-molecular-weight melanoma-associated antigen (HMW-MAA) (unpublished results) and the tumor-specific targeting ability of these AMLs was demonstrated [6].

Jimbow et al. [8] proposed another magnetite nanoparticle system. The melanogenesis substrate, *N*-propionylcysteaminylphenol (NPrCAP), was selectively incorporated into melanoma cells and inhibited their growth by the production of cytotoxic free radicals [8]. Based on the unique biological properties of NPrCAP, they constructed a particle with NPrCAP conjugated onto the surface of magnetite nanoparticles (NPrCAP/M), which selectively led to the disintegration of melanoma cells and generated heat upon exposure to alternating magnetic fields in a B16 mouse melanoma model system.

Since the first in vivo experiment by Gordon et al. [9], many researchers have reported encouraging results using intracellular hyperthermia with magnetic nanoparticles, such as magnetite-core dextran nanoparticles [10] and aminosilane-coated magnetite [11]. One of the most systematic and thorough experiments using MCLs or AMLs was conducted by my group. We demonstrated the efficacy of intracellular hyperthermia using MCLs or AMLs in animals with several types of tumors, including B16 mouse melanoma, MM46 mouse mammary carcinoma, PC3 and LNCaP human prostate cancer cells in athymic mice, spontaneously occurring primary melanoma in transgenic mice, T-9 rat glioma, rat prostate cancer PLS10, Os515 hamster

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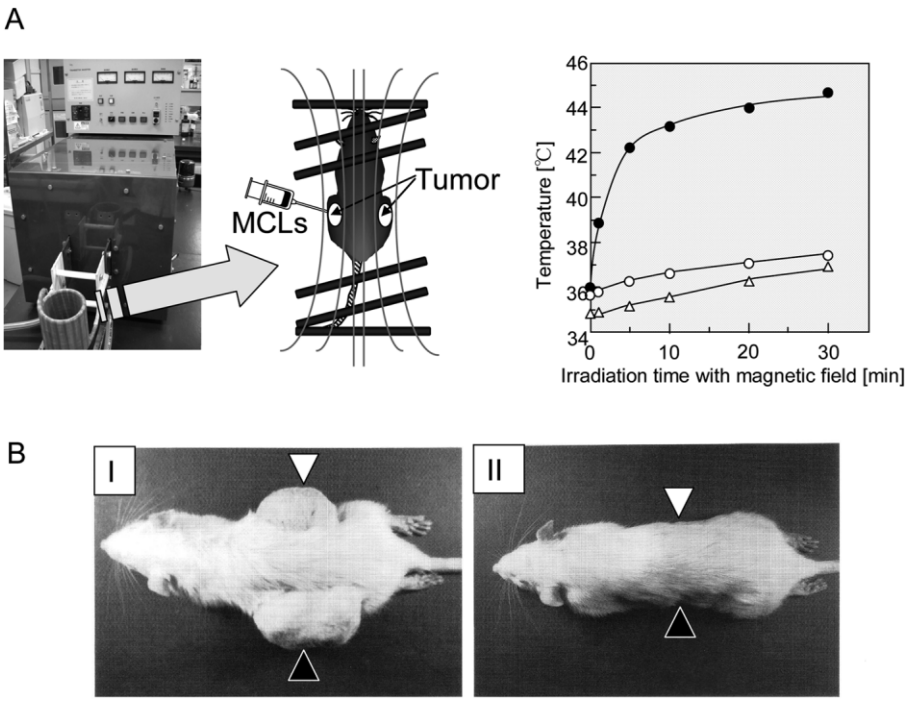

Figure 1. Anti-cancer immune response induced by hyperthermia using magnetite nanoparticles. Rats with tumors on each side of the body were prepared. (**A**) MCLs were injected into the left tumor only and the rats were irradiated with an alternating magnetic field using the apparatus shown (left panel). The temperature of the left tumor, containing MCLs (closed circles), increased specifically, whereas the temperature of the right tumor (open circles) and rectum (open triangles) remained below 37°C (right panel). (**B**) The tumor-specific hyperthermia treatment induced an anti-tumor immune response and both tumors disappeared on the 28th day after hyperthermia treatment. (**I**) Control rat without AML irradiation; (**II**) rat with AML irradiation. Open triangle in (**B**), the side without MCLs; closed triangle in (**B**), the side with MCLs.

osteosarcoma, VX-7 squamous cell carcinomas in rabbit tongue, and human breast cancer BT474 (HER2-positive) cells in nude mice [12]. In these therapeutic experiments, MCLs (net magnetite amount, 3 mg/tumor) were directly injected into solid tumors and the animals were irradiated several times (repeated hyperthermia) for 30 min with an alternating magnetic field of 118 kHz. After irradiation with the alternating magnetic field, the tumor volume decreased markedly and complete tumor regression was observed in 96% (51/53) of the animals in these experiments. These results indicate that MCLs or AMLs can be effective tools for hyperthermia and repeated hyperthermia is a promising approach for cancer therapy.

3 Augmentation of tumor immunogenicity by hyperthermia through HSP expression

Interestingly, we observed anti-tumor immunity resulting from hyperthermia in an experimental T-9 rat glioma model in which T-9 cells were transplanted into each femur of rats used in the experiments (Fig. 1) [13]. Although only one tumor was subjected to hyperthermia, the other tumor also disappeared completely. An immunohistochemical assay revealed that natural killer (NK) cells and $CD8^+$ and $CD4^+$ T cells migrated not only into the heated tumor but also into the unheated counterpart. Also, an in vitro cytotoxicity assay using spleen cells revealed that the cytotoxic T lymphocyte (CTL) activity was selective for T-9 cells. These results suggest that our system is potentially an effective tool for hyperthermic treatment of tumors, because in addition to killing tumor cells with heat, they induce a host anti-tumor immune response. We have performed detailed analysis of the heat-shock proteins (HSPs) upregulated and released from tumor cells during hyperthermia to assess their role in anti-tumor immune responses.

Hyperthermia induces expression of HSPs [14]. As discussed previously [15], the importance of HSPs, such as HSP70, HSP90, and glucose-regulated protein 96 (gp96), in immune responses has been indicated, and Srivastava et al. [14] suggested that HSPs have a chaperone function for tumor antigens. With regard to the mechanism of anti-tumor immunity induced by hyperthermia using MCLs, findings suggest two possible mechanisms of antigen presentation by HSP70 expression during hyperthermia [15].

One possible mechanism is heat-induced enhancement of antigenic peptide presentation through major histocompatibility complex (MHC) class I antigens of tumor cells, and hence, enhanced tumor immunogenicity. Srivastava et al. [14] proposed the following "relay-line model" for tumor antigenic peptide transfer during antigen processing and presentation by HSPs.

(i) The peptides first bind to HSP70 or HSP90, which carry them to the endoplasmic reticu-

lum (ER) through the transporter associated with antigen processing;
(ii) The peptides are transferred to gp96 in the lumen of the ER; and
(iii) In the terminal step, gp96 transfers the peptides to the MHC class I-β_2 microglobulin complexes.

Augmentation of MHC class I antigens on the tumor cell surface by HSP70 expression leads to immune activation [15]. HSP70 expression reached its maximum 24 h after hyperthermia treatment and the augmentation of MHC class I surface expression had a slower response curve in that it started 24 h after heating and peaked after 48 h. In an in vivo experiment, growth of T-9 cells in immunocompetent syngenic rats (F344) was significantly inhibited by hyperthermia, with augmentation of MHC class I antigen surface expression, whereas growth of T-9 cells was not inhibited in nude rats (F344/N Jcl-rnu), suggesting that the effector cells were T lymphocytes. Furthermore, compared with lymphocytes from unimmunized rats or rats injected with unheated T-9 cells, the splenic lymphocytes of rats injected with heated T-9 cells displayed specific cytotoxicity against T-9 cells. These results suggest that HSP70 is an important modulator of tumor-cell immunogenicity during hyperthermia and that CTLs are the effector cells.

An alternative mechanism for recognition of antigens of tumor cells by the host immune system in hyperthermia is cross-presentation of antigenic peptides by dedicated antigen-presenting cells (APCs) [15]. It is possible that HSP-mediated antitumor immunity is caused by a vaccine-like effect of HSP-peptide complexes released from dying tumor cells. The HSP-peptide complex released by the cells is taken up by APCs that express HSP receptors, such as CD91, CD40, and toll-like receptors 2/4 through receptor-mediated endocytosis. The complex then enters the endogenous MHC class I pathway and becomes re-presented on the surface MHC class I of the APC to $CD8^+$ T cells. In addition to receptor-mediated uptake of the HSP-peptide complex, HSP itself is able to directly activate APCs through stimulation of monocyte cytokine secretion and maturation of dendritic cells (DCs) [15]. Because the APC-activating effect of HSP70 is independent of the peptide it chaperones, it suggests that HSP70 is a natural adjuvant.

In summary, it was observed that the hyperthermia system induced very strong anti-tumor immunity. The increased temperature above 43°C induced necrotic tumor cell death, producing a lot of HSPs, which led to anti-tumor immunity. The following scenario in which HSPs function during the successive stages of an anti-tumor response after hyperthermia is proposed:
(i) A sublethal stress response induced by hyperthermia results in elevated levels of intracellular HSP-peptide complexes, enhanced processing of endogenous antigens, and an increase in the density of MHC class I-peptide complexes on the cell surface. These tumor cells are then recognized directly by MHC class I-restricted, tumor-specific T cells;
(ii) Dying tumor cells release their intracellular contents, including HSP-peptide complexes;
(iii) The released HSPs and/or antigenic peptides activate neighboring monocytes to produce proinflammatory cytokines and recruit professional antigen-processing cells, such as DCs; and
(iv) The HSP-peptide complexes are taken up by DCs and in turn presented to tumor-specific T cells by MHC class I molecules.

4 Magnetic field applicators for intracellular hyperthermia

After the magnetite particles have been selectively taken up by tumor cells, external apparatus is applied to increase the temperature of tumor tissues. There are two applicators: a radiofrequency capacitive heat generator and an alternating-magnetic-field generator. Radiofrequency capacitive heating is popular in clinical hospital settings in Japan. In the case of radiofrequency capacitive heating, an alternating electrical current of 8 MHz is applied and the temperature of tissues located between the electrodes increases uniformly. However, when MCLs are injected into tumor tissues, the temperature difference between the tumor tissues and the non-tumor tissues can reach 2 to 3°C [16]. Therefore, intracellular hyperthermia using radiofrequency capacitive heating is one option available for clinical applications. In this case, magnetite particles in the tumor tissues may attract more electrical current than normal tissues. However, the temperature of the non-tumor tissues located between the electrodes inevitably increases in the case of radiofrequency capacitive heating and a target-specific heating system for tumor tissues is desirable.

Alternating-magnetic-field generators have been developed by some companies and universities. In Germany, MagForce Nanotechnologies developed the MFH 300F 100 kHz magnetic-field applicator for intracellular hyperthermia [17]. The magnetic applicator in the MFH 300F instrument is a "gap-type" generator with a C-type toroidal core

and patients are placed inside the aperture. The width of the aperture is 70 cm and the gap size is adjustable from 21 to 45 cm.

In 1996, the Dai-Ichi High Frequency Co. (Tokyo, Japan), together with my group, developed a solenoid-type magnetic applicator [4]. This device generates an alternating magnetic field from a horizontal coil (inner diameter, 7 cm; length, 7 cm) driven by a transistor inverter at a frequency of 118 kHz. In preclinical studies using mice or rats, the animals were placed inside the coil so that the region containing the subcutaneously transplanted glioma tumor was at the center, and exposure to an alternating magnetic field produced strong therapeutic effects [4]. However, it is technically difficult to scale up the coil size in this solenoid-type applicator for clinical use because the very large coil that would be required to accommodate a human body may be accompanied by serious risks associated with the presence of a high voltage between the two ends of the solenoid. Therefore, a new device was recently developed, called a "ferrite-core-inserted solenoid type" [18]. An alternating magnetic field is generated by a solenoid coil (inner diameter, 7 cm; length, 9 cm) driven by a transistor inverter at a frequency of 360 kHz. The ferrite core inside the coil is designed to concentrate the magnetic field generated by the coil, resulting in the emission of the magnetic field from the surface of the device. Using this magnetic applicator, we reported that an agar phantom containing magnetite nanoparticles 10 mm from the outside of the coil showed an increase in temperature of 8°C within 5 min [18], suggesting that the ferrite-core-inserted solenoid was suitable for heating a target positioned outside of the coils.

5 Clinical trials for intracellular hyperthermia

For clinical applications, the toxicity of the particles is an important issue to consider. Toxicity resulting from the systemic administration of MCLs (90 mg, i.p.) in mice was investigated: none of the 10 MCL-treated mice died during the study [19]. Transient accumulation of magnetite was observed in the liver and the spleen, but magnetite nanoparticles were cleared from circulation by hepatic Kupffer cells and/or fixed macrophages in the spleen by 30 days after administration.

The safety of any apparatus must also be guaranteed, as well as the safety of any magnetite agents, before clinical applications can be pursued. Because alternating-magnetic-field generators are electrical appliances that use high voltages and currents, insulators must be carefully placed where any contact with a patient's body will occur. Temperature-monitoring systems are also important for the safe use of intracellular hyperthermia generated by alternating-magnetic-field generators. Generally, temperature monitoring during alternating-magnetic-field irradiation is performed using fiber-optic thermometry probes, which should not be affected by magnetic-field exposure. In experimental animal models, the probes were positioned at tumor surfaces, at the margin of tumors, and/or inserted into the center of the tumors.

In 2005, Johannsen et al. [17] described the first clinical application of magnetite-nanoparticle-mediated hyperthermia in locally recurrent prostate cancer, using the sophisticated MFH 300F apparatus mentioned in the previous section. In this study, the feasibility of hyperthermia was evaluated by using the MFH 300F apparatus and aminosilane-coated magnetite nanoparticles. The particles were prepared by MagForce Nanotechnology Co. (Berlin, Germany), but the method of preparation has not been published. The nanoparticles were injected transperineally into the prostate of a 67-year-old patient under transrectal ultrasound and fluoroscopy guidance. Treatments were performed by using an alternating magnetic field of 100 kHz. In this patient, the maximum and minimum intraprostatic temperatures were 48.5 and 40.0°C during the first treatment and 42.5 and 39.4°C during the sixth treatment.

In October 2007, Toshiaki Saida from Shinshu University, in collaboration with my group, began a clinical application of magnetite-nanoparticle-mediated hyperthermia using AMLs conjugated with mAb against HMW-MAA in melanoma. The magnetic-field applicator (a ferrite-core-inserted solenoid type) was applied with an alternating magnetic field of 110 kHz. The safety of the applicator, as well as that of the AMLs, was investigated before clinical applications. The AMLs were injected into melanoma nodules; treatments were performed for 30 min, and repeated three times at 24-h intervals. The temperatures at the surface of the melanoma nodules were monitored with a fiber-optic thermometer and were maintained between 44 and 46°C. The patients did not suffer from pain derived from the hyperthermia treatments. The clinical trial was executed for three patients without any significant adverse events.

We are now carrying out other clinical trials in collaboration with Dr. Tsuneo Imai at Nagoya University Hospital (Department of Breast and Endocrine Surgery; six patients), and Dr. Hajime Imada at Cancer Treatment Center of Tobata Kyouritsu Hospital (five patients) without any significant adverse events. One of the best results was the clini-

cal trial at the Cancer Treatment Center of Tobata Kyouritsu Hospital. The initial size of the head and neck tumor was 7 cm and the patient experienced complete regression after repeated hyperthermia, radiotherapy, and chemotherapy.

Continuous efforts have been made to construct a safe and effective intracellular hyperthermia system. Finally, some researchers are opening the door to clinical trials, suggesting that the time has come to use this method. Once the door is opened, many potent combination therapies based on intracellular hyperthermia will also be available for clinical trials. I hope that intracellular hyperthermia will provide a novel effective therapy for cancer patients.

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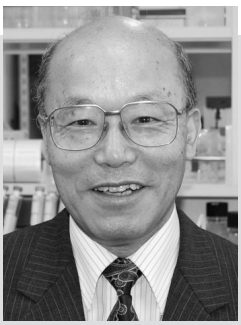

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