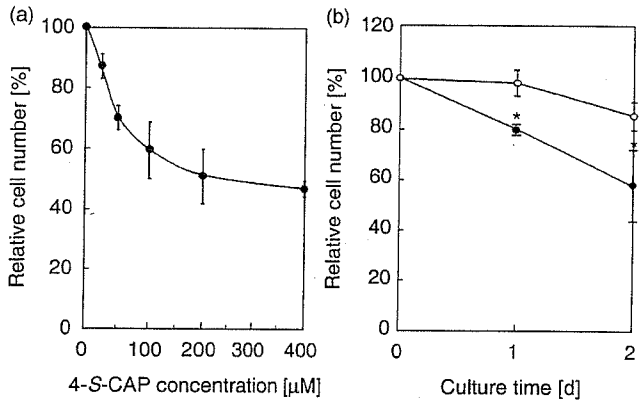

Fig. 4. Antiproliferative activity of 4-*S*-cysteaminylphenol (4-*S*-CAP) mediated by 4-*S*-CAP/magnetite cationic liposomes (MCL). (a) B16 cells were treated with different concentrations of 4-*S*-CAP/MCL (4-*S*-CAP, 0–400 μM). After the 2-day incubation period, antiproliferative effects were assessed as the relative cell number (%). Data and bars are mean ± SD of three independent experiments. (b) B16 (●) and normal human dermal fibroblasts (NHDF) (○) cells were treated with 4-*S*-CAP/MCL at 100 μM 4-*S*-CAP, and the relative cell number was assessed on days 1 and 2. Data and bars are mean ± SD of three independent experiments. $*P < 0.05$.

were incorporated into B16 cells. The uptake of magnetite nanoparticles by B16 cells is shown in Fig. 5a. Magnetite nanoparticles in 4-*S*-CAP/MCL were incorporated into B16 cells, and the amount of uptake was 18.4 ± 1.0 pg magnetite/cell after 24 h, which was comparable to that of MCL (15.0 ± 0.4 pg magnetite/cell).

We then investigated whether AMF irradiation generated heat in B16 cells treated with 4-*S*-CAP/MCL, and observed intracellular hyperthermia *in vitro*. Figure 5b shows the temperature profile of cell pellets treated with 4-*S*-CAP/MCL during AMF irradiation at 360 kHz and 120 Oe. Heat was generated in B16 cells incorporating magnetite nanoparticles. The temperature of these cells rose quickly and reached 43.0°C, which is an effective temperature for hyperthermia treatment, and was then maintained at that temperature for 30 min by controlling the intensity of the AMF. In contrast, in non-treated cells (0 pg magnetite/cell), the temperature increased only 2°C during the AMF irradiation. Figure 5c shows the viable cell number after the AMF irradiation. When the cells were treated with 4-*S*-CAP/MCL alone (without irradiation; 4-*S*-CAP/MCL, 100 μM), the viable cell number decreased to approximately half of that for non-treated cells. Moreover, when the cells were treated with 4-*S*-CAP/MCL plus AMF irradiation, the viable cell number decreased drastically for 1 day and thereafter cells grew again, resulting in an apparently smaller number than with 4-*S*-CAP/MCL alone (without irradiation).

***In vivo* hyperthermic treatment using 4-*S*-CAP/MCL.** For hyperthemic treatment *in vivo*, 4-*S*-CAP/MCL was injected into the tumor and AMF was applied to the whole body of the mouse. Figure 6a shows the tumor surface and rectal temperatures during AMF irradiation. Tumor temperature increased rapidly to 45°C within 3 min and was then maintained for 30 min by controlling the AMF intensity. In contrast, the temperature in the rectum showed only a limited increase during irradiation. These temperature profiles for 4-*S*-CAP/MCL-induced hyperthermia were comparable to those of MCL-induced hyperthermia (data not shown). No serious burns or damage were observed in all mice treated with magnetite-mediated hyperthermia.

For B16 melanoma, the therapeutic effects of the 4-*S*-CAP/MCL-induced combination of chemotherapy and hyperthermia

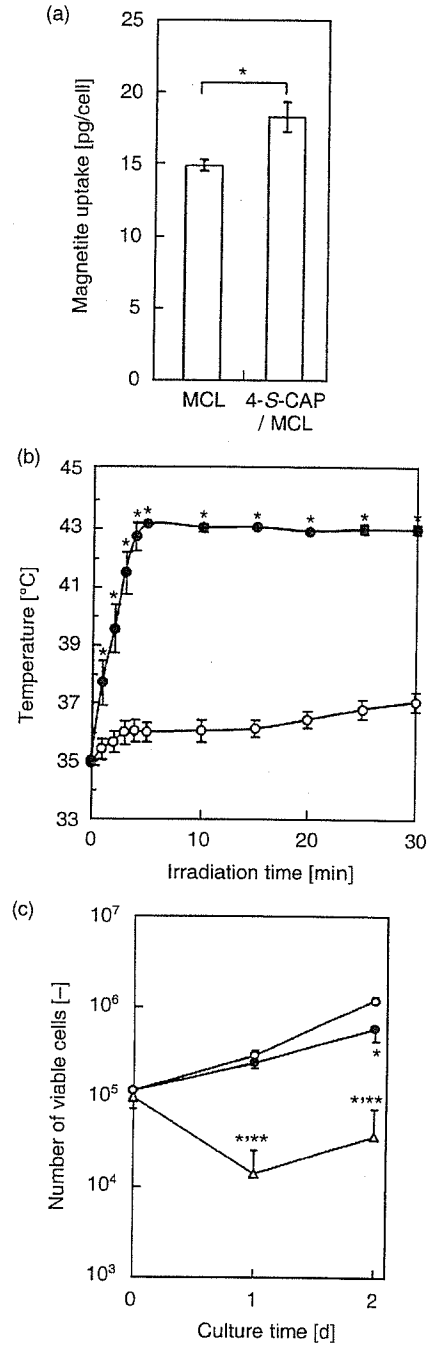

Fig. 5. *In vitro* hyperthermia induced by 4-*S*-cysteaminylphenol (4-*S*-CAP)/magnetite cationic liposomes (MCL). (a) Uptake of magnetite nanoparticles into B16 cells at 24 h after addition of MCL or 4-*S*-CAP/MCL. Percentage magnetite uptake was evaluated. Data and bars are mean ± SD of three independent experiments ($*P < 0.05$). (b) Temperature increase in cell pellets treated with 4-*S*-CAP/MCL during alternating magnetic field (AMF) irradiation. B16 cells with (●) or without (○) 4-*S*-CAP/MCL were irradiated with AMF for 30 min. Data and bars are mean ± SD of three independent experiments ($*P < 0.05$). (c) *In vitro* antiproliferation effects of 4-*S*-CAP/MCL after AMF irradiation. After the AMF irradiation, cells were reseeded and the number of viable cells was measured on the indicated day by the trypan blue exclusion method using a hemocytometer. (○) Control B16 cells; (●) B16 cells with 4-*S*-CAP/MCL; and (△) B16 cells treated with 4-*S*-CAP/MCL and AMF irradiation. Data and bars are mean ± SD of three independent experiments. $*P < 0.05$, significantly different from control group (non-treated B16 cells); $**P < 0.05$, significantly different from 4-*S*-CAP/MCL group (4-*S*-CAP/MCL alone).

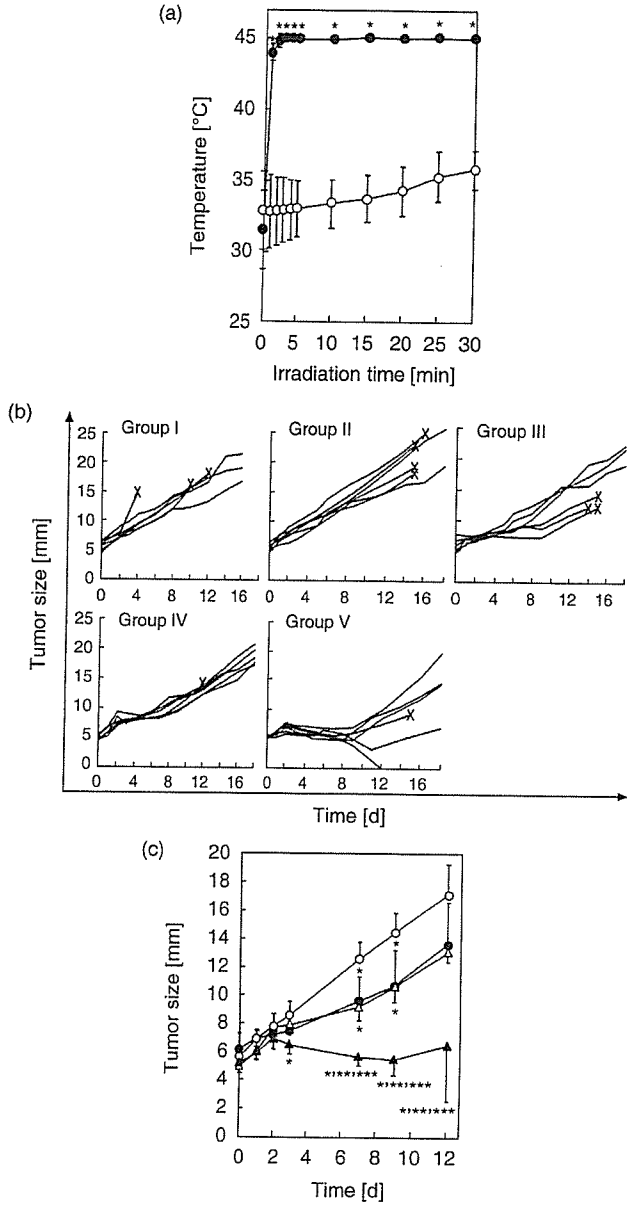

Fig. 6. *In vivo* hyperthermia using 4-*S*-cysteaminylphenol (4-*S*-CAP)/magnetite cationic liposomes (MCL). Schema of the therapeutic experiment. (a) Temperature increase in melanoma nodules treated with 4-*S*-CAP/MCL during alternating magnetic field (AMF) irradiation. 4-*S*-CAP/MCL containing 4 mg magnetite were injected directly into subcutaneous B16 tumors with diameters of 5 mm, which were then irradiated with an AMF for 30 min. Tumor and rectal temperatures were measured using optical fiber probes. (●) Tumor; (○) rectum. Each point represents the mean ± SD of five mice. $*P < 0.01$. (b) Therapeutic effects of 4-*S*-CAP/MCL on B16 tumors. Each line represents tumor growth in a single mouse ($n = 6$). Crosses (×) indicate when each mouse died. (c) Comparison of each group in the first 12 days after treatment. (○) Group II; (●) group III; (△) group IV; (▲) group V. Each point represents the mean ± SD of six mice. $*P < 0.05$, significantly different from group II (MCL alone); $**P < 0.05$, significantly different from group III (4-*S*-CAP/MCL alone); $***P < 0.05$, significantly different from group IV (MCL + AMF).

were assessed. Figure 6b shows the time course of tumor growth in each mouse, and the comparison of tumor growth kinetics in each group in the first 12 days is shown in Fig. 6c. In groups I (control) and II (MCL), tumors grew progressively and some mice died from pulmonary metastases (data not shown). In

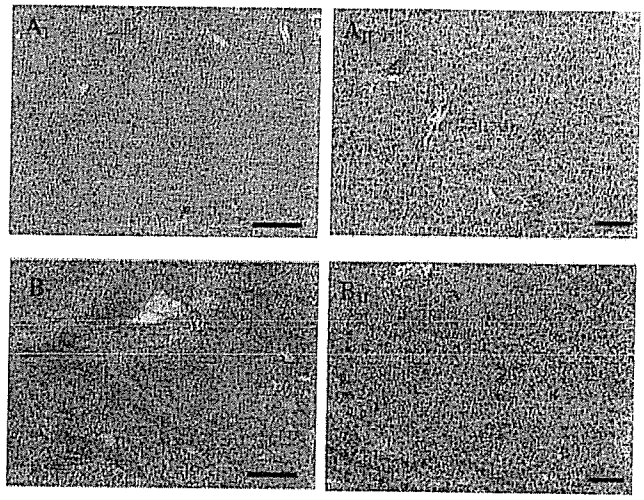

Fig. 7. Pathological features of the tumor in representative mice treated with hyperthermia using 4-*S*-cysteaminylphenol (4-*S*-CAP)/magnetite cationic liposomes (MCL). Tumors of mice in the control group (groups I, A-I and A-II) and 4-*S*-CAP/MCL-induced hyperthermia treatment group (groups IV, B-I and B-II) were stained with hematoxylin and eosin. Scale bars in I and II are 200 μm and 50 μm, respectively.

groups III (4-*S*-CAP/MCL), in which mice received 4-*S*-CAP/MCL alone (without AMF irradiation), and IV (MCL + AMF), in which mice received MCL-induced hyperthermia, tumor growth was slightly but significantly suppressed on days 7 and 9, and thereafter tumors grew progressively. In contrast, in group V (4-*S*-CAP/MCL + AMF), in which mice received 4-*S*-CAP/MCL and AMF irradiation, tumor growth was strongly suppressed in the first 12 days (Fig. 6c), and 17% (1/6) of subcutaneous tumors regressed completely (Fig. 6b). Histological examination of the tumors was done by H&E staining (Fig. 7). Severe necrosis with hemorrhage was observed, and many dead cells with condensed nuclei were seen, as shown in Fig. 7.

Discussion

In the present study, we examined the combination effect of 4-*S*-CAP treatment and hyperthermia on B16 melanoma cells, and demonstrated that the combination therapy has an additive effect (Fig. 1). In normal melanocytes and malignant melanoma cells, the specific enzyme tyrosinase catalyzes the oxidative conversion of L-tyrosine to melanin pigments.[23] 4-*S*-CAP is a good substrate for tyrosinase, and an oxidized form binds to sulphydryl enzymes.[24] With respect to the mechanism of cytotoxicity, dihydro-1,4-benzothiazine-6,7-dione, a metabolite of 4-*S*-CAP, deprives melanoma cells of reduced glutathione and may inactivate SH enzymes essential for DNA synthesis and cell proliferation by covalent binding through their cysteine residues, thereby exerting melanocytotoxicity.[21] Thus, cytotoxicity of 4-*S*-CAP depends mostly on reactive oxygen species (ROS). Similarly to 4-*S*-CAP activities in melanoma cells, hyperthermia can promote the formation of free radicals and related ROS from metabolic pathways, and these ROS may result in some cellular injury.[25] We speculate that ROS played an important role in the additive effect of the combined 4-*S*-CAP treatment and hyperthermia on melanoma cells; therefore, further research is required in order to elucidate the mechanism.

In the case of a superficial tumor, such as melanoma, a simple heat mediator is desirable for the clinical application of hyperthermia. Previously, we confirmed hyperthermic effects on B16

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melanoma using MCL injected directly into the tumor.[26] MCL uptake by cells in the injection site was much higher than that of magnetoliposomes whose liposomal surface had neutral charge, because MCL can electrostatically interact with the negative-charged phospholipid membrane of cells,[7] resulting in retention at the injection site. Thus, in a drug-targeting modality for melanoma, the use of 4-*S*-CAP/MCL is appropriate because superficial tumors can be treated with intratumoral injection of 4-*S*-CAP/MCL. Therefore, we used 4-*S*-CAP/MCL in order to heat the tumoral region and minimize heating of the surrounding healthy tissue. As shown in Fig. 6a, during hyperthermia using 4-*S*-CAP/MCL, tumor temperature reached 45°C rapidly, whereas rectal temperature remained at 38°C. These results indicate that using 4-*S*-CAP/MCL allows the application of hyperthermia to specific tumor tissue and that accurate control of the tumor temperature is possible by manipulating the magnetic field intensity. In this study, we set the tumor temperature at 45°C; however, this was insufficient to completely destroy the malignant melanoma. Our hyperthermia system can be carried out at higher temperatures and can be conducted repeatedly without damaging healthy tissue. For example, complete regression of B16 melanoma was observed in 90% of mice using MCL-induced hyperthermia at 46°C applied once daily for 2 days.[26] In the present study, we set the temperature at 45°C in order to examine the effects of combining hyperthermia with 4-*S*-CAP-madiated chemotherapy.

A major advantage of hyperthermia is that it has few side effects. In contrast to chemotherapy, hyperthermic effects are independent of cell lines and animals.[9–13] Furthermore, hyperthermic effects on cancer cells are caused mainly by physical damage, and differences in sensitivities to hyperthermia are negligible, especially at higher temperatures.[27,28] Therefore, when magnetite nanoparticles are internalized into tumor cells, the hyperthermic effect should be independent of cell type. However, in the case of repeated injection of 4-*S*-CAP/MCL, the toxicity of 4-*S*-CAP/MCL may become an important issue in a clinical trial. For MCL, in our preliminary study, the toxicity of a single administration of MCL solution (33 mg magnetite, intraperitoneally) was investigated. MCL accumulated mostly in the liver and spleen of mice, but none of the five observed mice died after MCL injection.[29] In the present study, 8 mg magnetite was used, which was much less than that used in the preliminary examination (33 mg). The 4-*S*-CAP was synthesized as an antimelanoma agent that is selectively toxic to the *in vivo* melanocytes engaged in melanin synthesis,[30,31] but not to the melanocytes and keratinocytes of albino mice.[31] However, a study by Padgette *et al.* has shown that 4-*S*-CAP is a good substrate of plasma monoamine oxidase, as well as tyrosinase.[19] The cytotoxicity of 4-*S*-CAP to cultured cells may be partly mediated by the aldehyde formed by oxidative deamination.[32] In the present study, non-melanocytic NHDF cells showed a slight decrease in relative cell number (decrease of 15%; Fig. 4b), suggesting that 4-*S*-CAP induced tyrosinase-independent toxicity. However, the mechanism of this tyrosinase-independent toxicity remains to be clarified. Taken together, these findings indicate that 4-*S*-CAP/MCL toxicity should be fully investigated before clinical application.

In conclusion, the promising results of the present study warrant further investigation of new modalities using 4-*S*-CAP/MCL, with the long-term goal of future application in the treatment of malignant melanoma in humans.

Acknowledgments

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SYMPOSIUM IN WRITING

Akira Ito · Hiroyuki Honda · Takeshi Kobayashi

Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novel concept of "heat-controlled necrosis" with heat shock protein expression

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Abstract Heat shock proteins (HSPs) are highly conserved proteins whose syntheses are induced by a variety of stresses, including heat stress. Since the expression of HSPs, including HSP70, protects cells from heat-induced apoptosis, HSP expression has been considered to be a complicating factor in hyperthermia. On the other hand, recent reports have shown the importance of HSPs, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), in immune reactions. If HSP expression induced by hyperthermia is involved in tumor immunity, novel cancer immunotherapy based on this novel concept can be developed. In such a strategy, a tumor-specific hyperthermia system, which can heat the local tumor region to the intended temperature without damaging normal tissue, would be highly advantageous. To achieve tumor-specific hyperthermia, we have developed an intracellular hyperthermia system using magnetite nanoparticles. This novel hyperthermia system can induce necrotic cell death via HSP expression, which induces antitumor immunity. In the present article, cancer immunology and immunotherapy based on hyperthermia, and HSP expression are reviewed and discussed.

Keywords Heat shock proteins · Hyperthermia · Tumor immunity · Magnetite · Glioma · Melanoma

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A. Ito · H. Honda · T. Kobayashi
Department of Biotechnology, School of Engineering,
Nagoya University, Furo-cho, Chikusa-ku,
Nagoya 464-8603, Japan

T. Kobayashi (✉)
School of Bioscience and Biotechnology, Chubu University,
Matsumoto-cho 1200, Kasugai, Aichi 487-8501, Japan
E-mail: takeshi@isc.chubu.ac.jp
Tel.: +81-568-516342
Fax: +81-568-526594

Introduction

Hippocrates (460–370 BC), the father of medicine, stated the following: "the diseases which fever cannot cure, those are to be reckoned wholly incurable." He believed that any diseases could be cured with artificial control of body temperature. Today, hyperthermia is a promising approach to cancer therapy [49]. Worldwide interest in hyperthermia was generated by the first international congress on hyperthermic oncology in Washington in 1975. In the following decade, there was a growing enthusiasm for hyperthermia. Thereafter, interest decreased mainly due to problems with heating systems. Nowadays, there appears to be a renewed interest, due to new developments including improved heating techniques. A major technical problem with hyperthermia is the difficulty of heating the local tumor region to the intended temperature without damaging normal tissue. The rationale underlying hyperthermia is the fact that temperatures over 42.5°C are cytotoxic for tumor cells [7], especially in an environment with a low pO_2 and low pH conditions that are typically found in tumor tissue due to insufficient blood perfusion. Conventional hyperthermia systems are designed to heat tissue to around 42.5–44.0°C. However, higher temperatures can kill greater numbers of tumor cells, and in principle, tumor-specific hyperthermia can kill all kinds of tumor cells.

Using magnetite nanoparticles, we have developed an intracellular hyperthermia system that we believe is the first to achieve artificial local control of temperature within tumors in the human body [12, 34, 44, 52, 53].This novel hyperthermia treatment has produced unexpected biological responses, including overcoming thermotolerance due to specific heating of the tumor at high temperature, and an antitumor immune response induced by expression of heat shock proteins (HSPs). These results suggest that our hyperthermia system can kill not only heated tumors but also nonheated tumors, including metastatic cancer cells. We have investigated

the role of HSP70, which is a well-defined HSP that has immunostimulatory properties [11, 26, 54], in order to elucidate the mechanism of immune induction by hyperthermia [13, 14]. In the present article, the mechanism of the anticancer immune response induced by hyperthermia is reviewed and discussed.

Tumor-specific hyperthermia using magnetite nanoparticles

Hyperthermia is a promising approach to cancer therapy. Various methods have been used to induce hyperthermia, including the use of hot water, capacitive heating, and inductive heating [49]. However, an inevitable technical problem with hyperthermia is the difficulty of uniformly heating only the tumor region to the required temperature without damaging normal tissue. Accordingly, some researchers have proposed the use of "intracellular" hyperthermia, and have developed submicron magnetic particles for this purpose [20, 32]. Intracellular hyperthermia is based on the principle that a magnetic particle can generate heat by hysteresis loss under an alternating magnetic field (AMF). In 1979, Gordon et al. [10] first proposed the concept of intracellular hyperthermia using dextran magnetite (Fe_3O_4) nanoparticles. They administered magnetite nanoparticles to Sprague-Dawley rats bearing mammary carcinomas, and showed that AMF-induced heating occurred in their in vivo experiments. Jordan et al. [21] have conducted several comprehensive in vitro studies of magnetic fluid hyperthermia using dextran magnetite nanoparticles.

As magnetite nanoparticles, including dextran magnetite, have no tumor-specific targeting ability, we have used drug delivery system (DDS) techniques to develop antibody-conjugated liposomes (immunoliposomes) containing magnetite nanoparticles (antibody-conjugated magnetoliposomes, AMLs). The targeting ability of AMLs mainly depends on the specificity of the antibody and the quantity and quality (including homogenous antigen expression) of the antigen on the tumor cell surface. We constructed immunoliposomes using mouse G22 monoclonal antibody (MAb) against human glioma cells [24], mouse G250 MAb against human renal cell carcinomas [35], and humanized MAb against human epidermal growth factor receptor-2 (Herceptin) [15]. We have demonstrated the tumor-specific targeting ability of these immunoliposomes. Also, accumulation of magnetite nanoparticles in tumor cells can be enhanced by conferring a positive surface charge to liposomes. We have developed "magnetite cationic liposomes" (MCLs) with improved adsorption and accumulation properties. MCLs, which have a positive surface charge, have tenfold higher affinity for glioma cells than neutrally charged magnetoliposomes [34]. These two types of magnetite liposomes have a sufficiently high specific absorption rate (SAR), which determines the heat evolution rate in hyperthermia, and a general biocompatibility that is comparable to that of dextran magnetite. We demonstrated the efficacy of hyperthermia using magnetite nanoparticles in animals with several types of tumor, including B16 mouse melanoma [44], MM46 mouse mammary carcinoma [12], T-9 rat glioma [52], Os515 hamster osteosarcoma [27], and VX-7 squamous cell carcinoma in rabbit tongue [28]. The strategy of our hyperthermia system and the two types of liposomes that we have developed containing magnetite nanoparticles are shown in Fig. 1.

Interestingly, we observed antitumor immunity resulting from hyperthermia, induced using magnetite nanoparticles, in an experimental T-9 rat glioma model in which one tumor was transplanted into each femur of a rat (Fig. 2) [53]. Although only one tumor was subjected to hyperthermia, the other tumor also disappeared completely. An immunohistochemical assay revealed that NK cells and CD8- and CD4-positive T cells migrated not only into the heated tumor but also into its nonheated counterpart. Also, an in vitro cytotoxicity assay using spleen cells revealed that the CTL activity was selective for T-9 cells. These results suggest that our therapeutic magnetic particles are potentially effective tools for hyperthermic treatment of tumors,

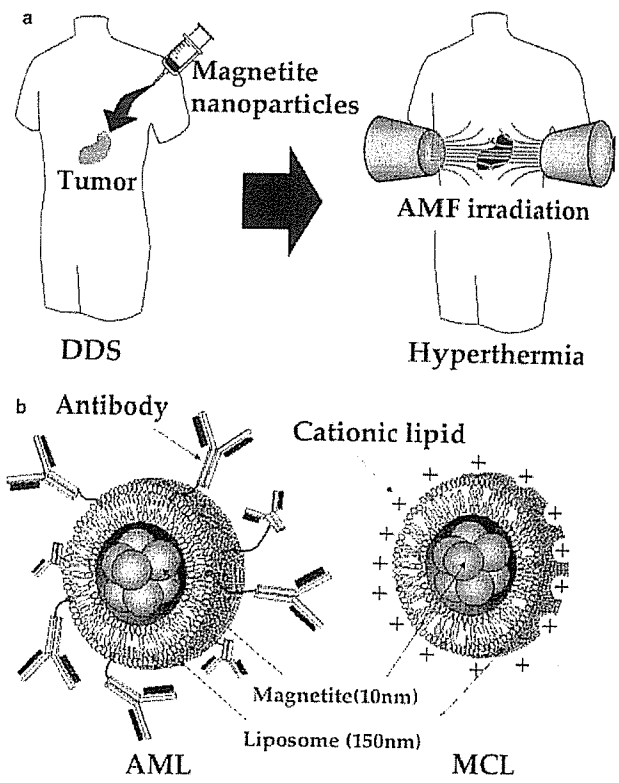

Fig. 1 Hyperthermia using magnetite nanoparticles. **a** Scheme of hyperthermia treatment. Magnetite nanoparticles are concentrated in tumor tissue by the DDS. Then the nanoparticles are irradiated with an AMF produced outside the human body, resulting in tumor-specific hyperthermia. **b** Liposomal drugs containing magnetite nanoparticles for DDS. *Left* antibody-conjugated magnetoliposome (AML); *right* magnetite cationic liposome (MCL)

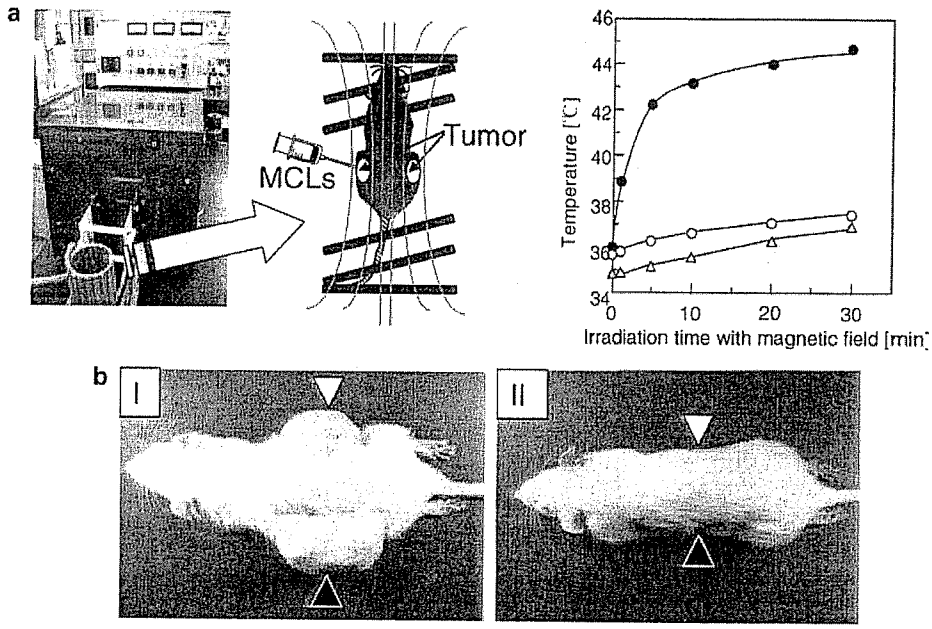

Fig. 2 Anticancer immune response induced by hyperthermia using magnetite nanoparticles. Rats bearing tumors on each side of the body were prepared. MCLs were injected into the left tumor only, and the rats were irradiated with an AMF using the apparatus shown in (**a**, *left*). Temperature of left tumor, containing MCLs (*closed circles*), increased specifically, whereas temperature of right tumor (*open circles*) and rectum (*open triangles*) remained below 38°C (**a**, *right*). The tumor-specific hyperthermia treatment induced an antitumor immune response, and both tumors had disappeared on the 28th day after hyperthermia treatment. *I* Control rat without AMF irradiation; *II* rat with AMF irradiation. *Open triangles* in (**b**), the side without MCLs; *closed triangle* in (**b**), the side with MCLs

because in addition to killing tumor cells with heat, they induce a host immune response. We have performed detailed analysis of the HSPs that were upregulated and released from tumor cells during hyperthermia to assess their role in antitumor immune responses.

Augmentation of tumor immunogenicity by hyperthermia via HSP expression

Hyperthermia induces expression of HSPs [25]. As the expression of HSPs protects cells from heat-induced apoptosis [33], HSP expression has been considered a complicating factor in hyperthermia. Since hyperthermia is a physical treatment, it is likely to have fewer side effects than chemotherapy. Consequently, an advantage of hyperthermia is the feasibility of frequent repeated treatment [12]. However, conventional hyperthermia systems involve treatment only once or twice per week, performed at an interval of more than 48 h to prevent thermotolerance [42, 43] caused by HSP expression.

Recent reports have shown the importance of HSPs in immune reactions, including HSP70, HSP90, and glucose-regulated protein 96 (gp 96); and studies suggest that HSPs chaperone tumor antigens [30, 36]. With regard to the mechanism of antitumor immunity induced by hyperthermia using MCLs, our findings suggest two possible mechanisms of antigen presentation via HSP70 expression during hyperthermia [13, 14]. One possible mechanism is heat-induced augmentation of tumor immunogenicity due to presentation of antigenic peptides via MHC class I antigens of tumor cells. Srivastava et al. [37–39] proposed the following "relay line model" for tumor antigenic peptide transfer during antigen processing and presentation by HSPs (Fig. 3). (1) The peptides are first bound to HSP70 or HSP90, which carry them to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). (2) The peptides are transferred to gp96 in the lumen of the ER. (3) In the terminal step, gp96 transfers the peptides to the MHC class I–β_2 microglobulin complexes. Wells et al. [50, 51] demonstrated that stably transfected B16 melanoma cells which constitutively expressed human HSP70 exhibited significantly increased levels of MHC class I antigens on their surface. We have shown that augmentation of MHC class I antigens on the tumor cell surface via HSP70 expression causes immune induction [13] (the working hypothesis is illustrated in Fig. 4). In that study, HSP70 expression reached its maximum 24 h after heating, and the augmentation of MHC class I surface expression began 24 h after heating and reached its maximum 48 h after heating. The expression of other immunologic mediators, such as intracellular adhesion molecule-1 (ICAM-1), did not increase. 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

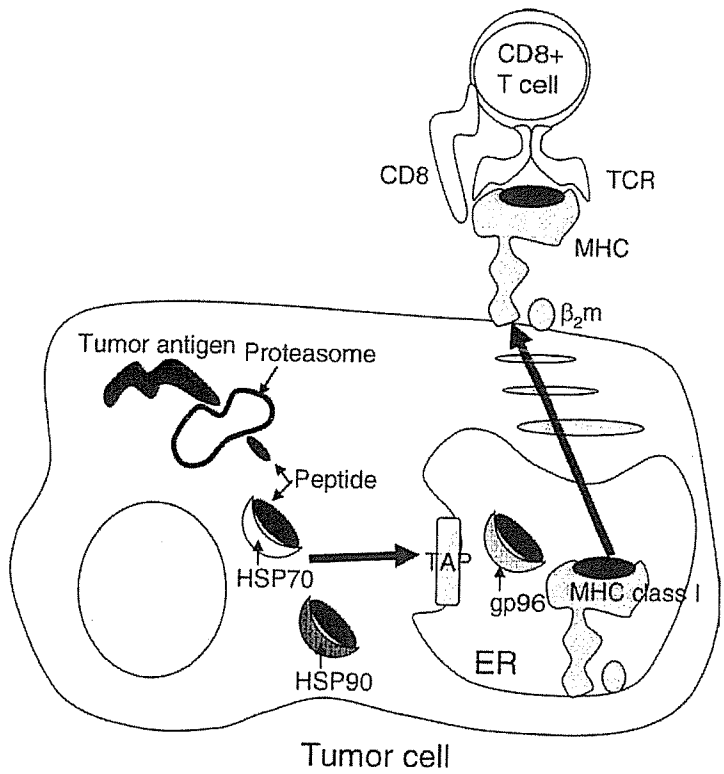

Fig. 3 Relay line model for tumor antigenic peptide transfer during antigen processing and presentation by HSPs. HSP family, including HSP70 and HSP90 in cytoplasm, and gp96 in ER, is involved in peptide transfer to MHC class I molecule

were T lymphocytes. Furthermore, compared with lymphocytes from nonimmunized (injected with PBS) rats or rats injected with nonheated 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.

Thus, we have demonstrated that augmentation of MHC class I antigens on the tumor cell surface via HSP70 expression is a possible mechanism of immune responses induced by hyperthermia treatment. However, the mechanism of enhanced tumor immunogenicity via HSP70 expression has not been fully elucidated. As mentioned above, Wells et al. demonstrated that stably transfected B16 melanoma cells that constitutively expressed human HSP70 exhibited significantly increased levels of MHC class I antigens on their surface [50]. In contrast, Dressel et al. [8] observed that human melanoma cells that overexpressed HSP70 "without" enhanced expression of MHC class I antigens exhibited enhanced susceptibility to CTLs. These results suggest that the effects of HSP70 expression on tumor immunogenicity differ among tumor types. It has recently been reported that sublethal irradiation can alter the phenotype of target tissue by upregulating gene products

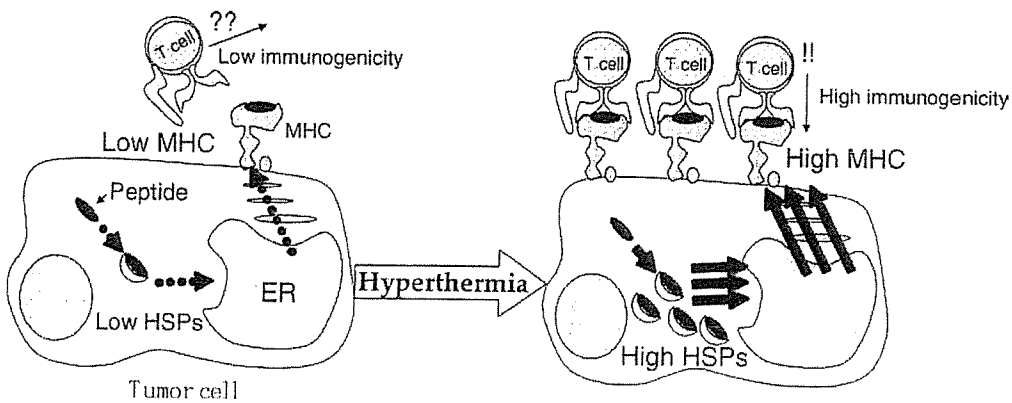

Fig. 4 Mechanism of induction of anticancer immune response by hyperthermia. Augmentation of tumor immunogenicity by an increase of the number of MHC molecules on the surface of cancer cells via inducible HSP expression

that can increase susceptibility of tumor cells to CTLs. Garnett et al. [9] examined responses of 23 human carcinoma cell lines (12 colon, 7 lung, and 4 prostate) to nonlytic doses of radiation, and observed changes in their surface expression of Fas (10/23 tumors were up-regulated), ICAM-1 (14/23), carcinoembryonic antigen (CEA, 16/23), mucin-1 (MUC-1, 8/23), and MHC class I antigens (8/23). We speculate that sublethal heating can alter the phenotype of tumor cells, and that the changes depend on the type of target tumor cell. Further analysis (e.g., using microarrays [22]) is presently being conducted to examine the range of phenotypic changes caused by altered gene expression after hyperthermia treatment. Although hyperthermia has been used in combination with radiotherapy and chemotherapy, little data is available concerning the clinical effects of stimulation of the immune response using such methods. Further investigation of changes in tumor cell characteristics such as immunological phenotype after such combination therapy may lead to development of novel strategies for cancer immunotherapy.

Release of HSP-peptide complex from necrotic cells induced by hyperthermia treatment

An alternative mechanism of 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) [5, 40, 41] (the working hypothesis is illustrated in Fig. 5). HSP-mediated antitumor immunity may be caused by a vaccine-like effect of HSP-peptide complexes released from dying tumor cells. The released HSP-peptide complexes encounter APCs that express receptors such as CD91, CD40, and Toll-like receptors 2/4 [5]. Interaction of HSP-peptide complexes with these receptors leads to receptor-mediated endocytosis, processing of the antigenic peptide by the endogenous MHC class I pathway, and representation on the cell surface to CD8-positive T cell receptors [3]. Additionally, HSP70 functions as a direct activator of APCs, stimulating cytokine secretion from monocytes, and inducing the maturation of dendritic cells (DCs) via CD14 and/or CD91 receptors [1]. This cytokine-like ability of HSP70 to stimulate the innate immune system is independent of the peptides it chaperones, suggesting that HSP70 is a natural adjuvant [40, 41].

We demonstrated that HSP70 expression following hyperthermia using MCLs induced antitumor immunity in rats with T-9 rat glioma [14]. Since MCLs are heated in our hyperthermia system, the distribution of magnetite nanoparticles within tumors is an important issue. When MCLs were repeatedly heated, the surrounding tumor tissues underwent necrosis, and magnetite nanoparticles subsequently expanded into the necrotic area within the tumor, resulting in a wide distribution of magnetite nanoparticles. Thus, the entire tumor area

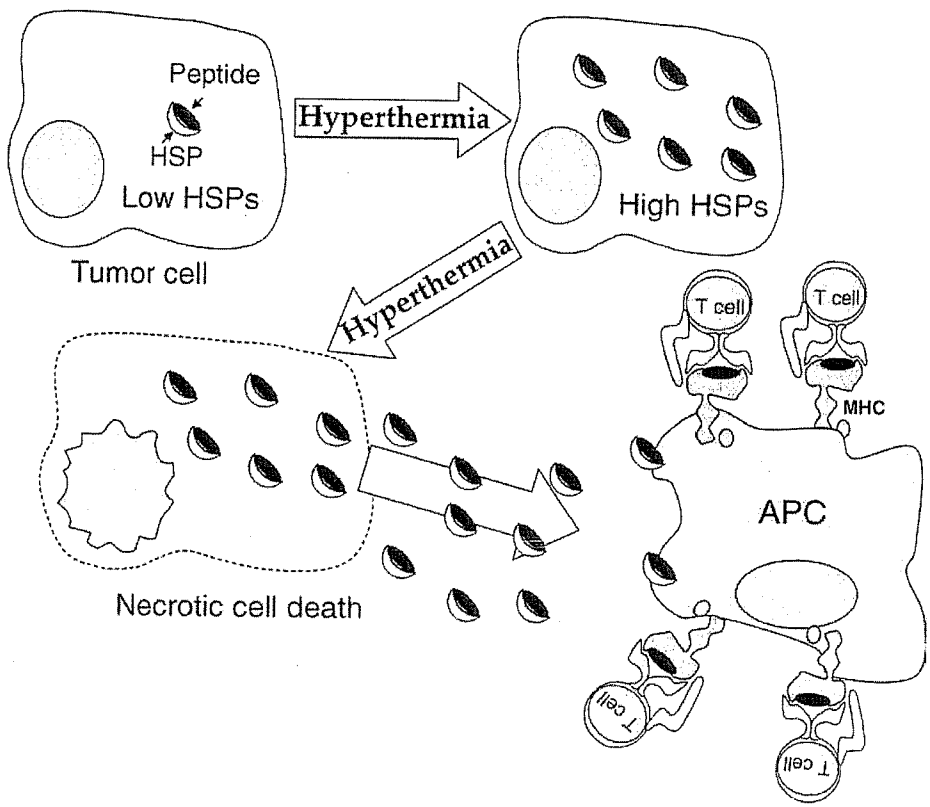

Fig. 5 Mechanism of induction of anticancer immune response by hyperthermia. In-situ vaccination by HSP-peptide complexes released from dying cells via necrosis by hyperthermia treatment

was necrosed by repeated (three times) hyperthermia (with a 24-h interval over 3 days) [14]. The 24-h interval corresponded to the time when HSP70 expression in T-9 cells reached its maximum, and a large amount of HSP70 was detected in the tumor tissue. We previously examined expression of HSP70 in B16 melanoma nodules in mice treated with MCL-induced hyperthermia (once at 43°C for 30 min) 24 h after the treatment, and in nodules of nontreated mice. HSP70 expression in the tumor heated at 43°C was 1.6 ± 0.2 ng/mg-tumor, and the expression in the nontreated mice was 0.17 ± 0.06 ng/mg-tumor [44]. Thus, our hyperthermia system using MCLs overcame thermotolerance and induced necrotic cell death that correlated with HSP70 expression.

Next, we purified the HSP70-peptide complexes obtained from the tumor after hyperthermia, and found that immunization of rats with T-9-derived HSP70 strongly suppressed tumor growth [14]. HSP70-peptide complexes obtained from liver (control) were also purified, and their vaccine effects were examined, but no antitumor effects were observed. These results suggest that HSP70 in tumor cells chaperones some antigenic peptides after hyperthermia. T-9 cells were subjected to hyperthermia, and were then examined using an apoptosis assay. The apoptosis/necrosis assay, which was based on flow cytometric analysis, revealed that necrotic cell death was induced by hyperthermia at 42°C for 30 min. After hyperthermia, HSP70 released into the supernatant from treated cells was detected on a Western blot probed with antibody to HSP70/HSC70. These results suggest that our hyperthermia system induces necrotic cell death via HSP70 expression. Then, in order to investigate the vaccine-like effect of the tumor cells killed via hyperthermia-induced necrosis in rats, a tumor rejection assay was performed after hyperthermia treatment of implanted T-9 cells; tumor growth was strongly suppressed, and 50% of the rats were protected from challenge with T-9 cells.

Our hyperthermia system is designed to induce necrotic cell death. Whether vaccination with apoptotic cells or necrotic cells is more efficient in terms of release of HSPs from cells has been a controversy among cancer immunologists. Apoptosis is programmed cell death, and is also considered "clean" cell death because the contents of the cells (including tumor antigens) are not released into the external environment but get packaged into the apoptotic body. In contrast, necrotic cell death is considered an unprogrammed event that is "not clean," because the cell contents are released into the environment. In necrotic cell death, as mentioned above, HSPs released from necrotic tumors chaperone antigen. HSP-antigen complexes bind to the surface of DCs, thereby transferring the antigens to DCs for MHC-restricted presentation [2]. In apoptosis, apoptotic bodies are engulfed by APCs, followed by antigen processing and presentation of tumor antigens by MHC class I antigens [23]. In fact, appropriate processing by APCs may occur in both apoptosis and necrosis. Apoptotic cell death seems preferable to necrotic cell death, because of the predictability of the results of apoptosis. When the complex program of apoptotic cell death is fully decoded, it will be possible to control activation of a specific cascade of apoptotic events and induce apoptosis in any type of cancer using techniques such as molecular target therapy. However, at present, cancer therapy specifically designed to induce apoptosis does not appear to be feasible, because cancer cells are adapted to escape from "death," especially from apoptotic cell death, due to continual gene mutation. In contrast, it is relatively easy to induce necrotic cell death. Generally, when cells undergo extreme stress, they die in a necrotic manner. Yonezawa et al. [55] examined the manner of cell death induced by hyperthermia. Apoptotic cell death was induced in malignant fibrous histiocytoma cells by mild hyperthermia treatment at 42°C, whereas necrotic cell death was induced by hyperthermia at 44°C. Thus, hyperthermia can easily induce necrotic cell death (in principle, in any type of tumor cell) by heating cells to a sufficiently high temperature.

Our intracellular hyperthermia treatment can heat the tumor specifically via the MCLs. Moreover, the amount of heat generated in the tumor can be controlled by modulating the magnetic field intensity, making it possible to induce necrotic cell death without damage to the surrounding normal tissues. Another reason why necrotic cell death is effective for cancer therapy is that necrotic cell death may strongly induce a danger signal. We have observed that numerous and diverse kinds of immunocytes, such as CD8- and CD4-positive T-cells, NK cells, macrophages, and DCs, infiltrate into the necrotic area of tumors after hyperthermia treatment using MCLs [14, 16, 53]. Todryk et al. [47] observed infiltration of such cells into B16 melanoma nodules transfected with the HSP70 gene, suggesting that HSP70 expression is a danger signal for the recruitment of immunocytes.

Recently, a vaccine consisting of autologous tumor-derived gp96-peptide complexes (HSPPC-96, Oncophage; Antigenics, Inc., Woburn, MA, USA) has entered clinical trials, and the feasibility of its use to treat metastatic melanoma [4] and colorectal cancer [29] has been demonstrated. Since HSP-peptide complexes must be extracted from tumors in the body, surgery is needed in this therapeutic protocol. In contrast, in hyperthermia using MCLs, no surgery or extraction is necessary. Udono et al. [48] reported that the vaccination effect of HSP70-peptide complexes was directly dependent on the dose. The HSPs in the tumor can be regarded as an antigen source, and 1 g (approximately 10^8 cells) of tumor tissue may contain approximately 2 mg HSP70. This represents a much higher dose than that used for the clinical trials. In our hyperthermia system, the expression of HSP70 was enhanced, and tissue lysis via necrosis was observed throughout the tumor. Thus, our hyperthermia system using MCLs increases levels of HSP-peptide complexes (possibly including HSPs such

as HSP90 and gp 96) within tumor cells, and can induce their release via necrosis, resulting in vaccination. Chandawarkar et al. [6] recently reported that low doses of gp96 generated immunity, whereas doses ten times the immunizing dose did not. Further investigations of hyperthermia protocols (e.g., target temperature and heating period) are needed to determine the most suitable conditions for the induction of immune responses via HSP expression and HSP release from dying cells.

These results suggest that our hyperthermia system confers antitumor immunity via release of HSP70-peptide complexes during necrotic tumor cell death in vivo. This phenomenon, which we have termed "in situ vaccination," has important implications for the development of novel antitumor therapies.

Concluding remarks

A proposed scenario in which HSPs function during successive stages of an antitumor response after hyperthermia is summarized and illustrated in Fig. 6. (1) A poor immunogenic tumor cell has a low concentration of intracellular HSP-peptide complexes, decreased function of the endogenous antigen-processing machinery, and a very low level of MHC class I-peptide complexes at the cell surface. (2) A sublethal stress response induced by hyperthermia using MCLs results in increased levels of intracellular HSP-peptide complexes, enhanced processing of endogenous antigens, and an increase in the density of MHC class I-peptide complexes at the cell surface [13]. These tumor cells are then recognized directly by MHC class I-restricted CTLs [8, 50]. (3) Dying tumor cells, which are killed by the CTLs or by lethal hyperthermia treatment, release their intracellular contents, including HSP-peptide complexes [14]. (4) The released HSPs and/or antigenic peptides activate neighboring monocytes to produce proinflammatory cytokines and recruit APCs [1, 47]. (5) The HSP-peptide complexes are taken up by DCs, and are in turn presented to T cells via MHC class I and/or II antigens (cross-priming) [2, 3, 31, 39].

Furthermore, we are currently developing novel cancer immunotherapy based on the mechanism of anticancer immune response via HSP expression. Three key elements may be involved in this mechanism: (1) CTLs as effector cells; (2) APCs as antigen-processing and antigen-presenting cells for HSP-peptide complex released from necrotic cells; and (3) HSPs as natural and powerful immunostimulants. This strategy is based on combinations of hyperthermia using MCLs with cytokines (IL-2 and GM-CSF [16]), heat-inducible TNF-α gene therapy [17], recombinant HSP70 [18], HSP70 gene therapy [19], and DC therapy [45, 46]. We are conducting further studies to establish a novel cancer immunotherapy based on the concept of heat-controlled necrosis with HSP expression.

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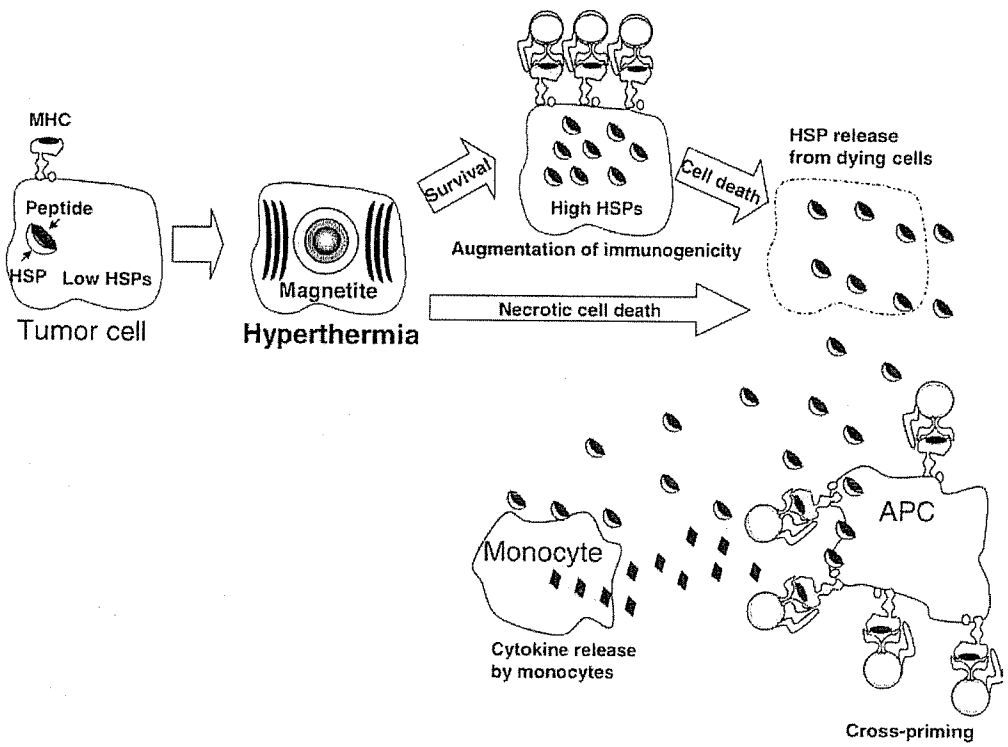

Fig. 6 Proposed scenario of the mechanism of induction of anticancer immune response by hyperthermia

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The Prostate 66:718–727 (2006)

Complete Regression of Experimental Prostate Cancer in Nude Mice by Repeated Hyperthermia Using Magnetite Cationic Liposomes and a Newly Developed Solenoid Containing a Ferrite Core

Noriyasu Kawai,[1]* Akira Ito,[2] Yoko Nakahara,[2] Hiroyuki Honda,[2] Takeshi Kobayashi,[2] Mitsuru Futakuchi,[2] Tomoyuki Shirai,[3] Keiichi Tozawa,[1] and Kenjiro Kohri[1]

[1]Department of Nephro-Urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[2]Department of Biotechnology, School of Engineering, Nagoya University, Nagoya, Japan
[3]Department of Experimental Oncology and Tumor Biology
Nagoya City University Graduate School of Medical Sciences Nagoya, Japan

BACKGROUND. Magnetite cationic liposomes (MCLs) can be used to induce hyperthermia because they generate heat in an alternating magnetic field (AMF). This study aimed at developing more practical method for MCL hyperthermia examining the effect of MCL-induced hyperthermia on human prostate cancer in vivo.

MATERIALS AND METHODS. A newly developed AMF generator incorporating a solenoid with a ferrite core (FC) was used. Human prostate cancer cells (PC-3 and LNCap) were injected subcutaneously into nude mice. MCLs were injected into tumor nodule and the mice were exposed into AMF three times at 24-hr intervals (repeated hyperthermia; RH) until complete tumor regression was observed.

RESULTS. Irradiation with an AMF generated by newly developed device can adequately increase the temperature of tumor tissue. Frequent RH resulted in complete tumor regression in all nude mice.

CONCLUSION. RH using MCLs may be a promising new therapy for hormone-refractory human prostate cancer in the future. *Prostate 66: 718–727, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: **human prostate cancer; hyperthermia; magnetite cationic liposome; complete regression; ferrite core**

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy in Western males and its incidence is increasing rapidly in Japan [1]. This increase is believed to be attributable to longer life expectancy, growing prostate awareness, more-widespread screening, and the more-widespread adoption of Western diets [2,3].

The success of early prostate cancer detection has resulted in an increased number of candidates for therapy. The main treatment options for clinically localized prostate cancer currently consist of surgical extirpation and radiation therapy (external-beam radiation therapy and brachytherapy) [4]. There are instances in which radical prostatectomy is not an acceptable option, such as when the patient is a poor

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*Correspondence to: Dr. Noriyasu Kawai, Department of Nephro-Urology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. E-mail: norikawa@med.nagoya-cu.ac.jp

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risk for surgery or due to the wishes of the patient, in which cases radiation treatment is generally chosen. Hormonal therapy and/or careful observation are also options. Decisions regarding treatment must be made on an individual basis, whilst considering the patient's life expectancy and quality of life, as well as the patient's wishes [5].

Some less-common treatment options for clinically localized prostate cancer include cryotherapy [6–8], high-intensity focused ultrasound [9–11], and hyperthermia. Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients [12]. The most common heating method in clinical settings in Japan is capacitive heating by a radiofrequency electric field [13], but localized heating of tumors using this method is difficult because the heating characteristics are influenced by various factors such as tumor size, the position of electrodes, and adhesion of electrodes at uneven sites. As Kroeze [14] also point out, it may be that pelvic tumors such as prostate cancer are not ideal for treatment with capacitive hyperthermia because of the production of hot spots in normal tissue. Interstitial hyperthermia, in which the tumor is heated by directly injected materials, has been examined for clinically localized prostate cancer to eliminate these problems [15–18]. These interstitial hyperthermia techniques are classified into two types according to the heating mechanism used: microwave hyperthermia and magnetically-mediated hyperthermia (MMH). Interstitial microwave hyperthermia involves heating via a microwave-radiating antenna inserted into the target tissue [15], and MMH is itself divided into two subclasses comprising the insertion of magnetic particles (called intracellular hyperthermia) or seed (called direct-injection hyperthermia) into tumor tissue that are subsequently heated by exposure to an externally applied alternating magnetic field (AMF) [16–18]. The magnetic particles are iron oxide particles with a diameter of 20 nm, and the seed is a columnar piece of metal 1 mm in diameter and 10 mm long. In intracellular hyperthermia, the ferromagnetic particles are modified to facilitate their cellular uptake by the tumor, whereas ferromagnetic seed is injected directly into tumor tissue in direct-injection hyperthermia. In both methods, the subsequent exposure to an AMF results in heat generation within the tumor cells [19].

We have developed magnetite cationic liposomes (MCLs) for inducing intracellular hyperthermia [20,21]. These MCLs have been developed to improve adsorption and accumulation in the tumor cells, and exhibit a tenfold higher affinity for tumor cells than neutrally charged magnetoliposomes [21] due to electrostatic interaction with the negatively-charged cell membrane. The hyperthermic effect of MCLs against certain types of malignant tumor cells has been demonstrated in vivo [22]. Our hyperthermia procedure killed rat prostate cancer cells in vivo not only directly by heating but also by the induction of an immune response [23,24]. However, the AMF-generating machine in the previous study used a solenoid, and for clinical application, it is technically difficult to scale-up the coil size because the very large coil required to accommodate the human body may be accompanied with a serious risk associated with the high voltage between the two ends of the solenoid. Thus, designing a new therapeutic protocol for use in human prostate cancer, including a safe device for AMF generation, is needed before clinical trials can be conducted. In this study, we examined the therapeutic effects of repeated hyperthermia (RH) using MCLs on human prostate cancer tissue growing in athymic mouse subcutis. In the present experiment, we applied a newly-developed device, named the ferrite core (FC)-inserted solenoid (FCIS).

MATERIALS AND METHODS

AMF Generator

A near-lossless MnZn FC was obtained from TDK (Tokyo, Japan), which has a high relative intrinsic permeability, typically 3,000–4,000 at low magnetic field strengths, and a sharp transition from the ferromagnetic to nonmagnetic states. Because the FC was inductively heated by AMF, it was surrounded by a water-cooling jacket (diameter, 6 cm; height, 11.5 cm). An AMF was generated by a vertical coil (inner diameter, 7 cm; length, 9 cm) driven by a transistor inverter (LTG-100-05; Dai-ichi High Frequency, Tokyo, Japan) at a frequency of 360 kHz. The FC was inserted into the vertical coil (Fig. 1A). In the present study, a solenoid without an FC was used as a so-called vacant solenoid (VS; Fig. 1B) in control experiments.

Preparation of MCLs

MCLs were fabricated from colloidal magnetite (a kind gift from Toda Kogyo, Hiroshima, Japan) and a lipid mixture consisting of N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (Sogo Pharmaceutical, Tokyo, Japan), dilauroylphosphatidylcholine, and dioleoylphosphatidyl-ethanolamine (Sigma Chemical, St. Louis, MO) in a molar ratio of 1:2:2, as described previously [20].

In Vitro Experiments Using a Phantom

A 4% agar gel was used for preparing the phantom, with agar purchased from Wako Pure Chemicals (Osaka, Japan). An agar piece containing MCLs was prepared as we described previously [25]. Briefly, the

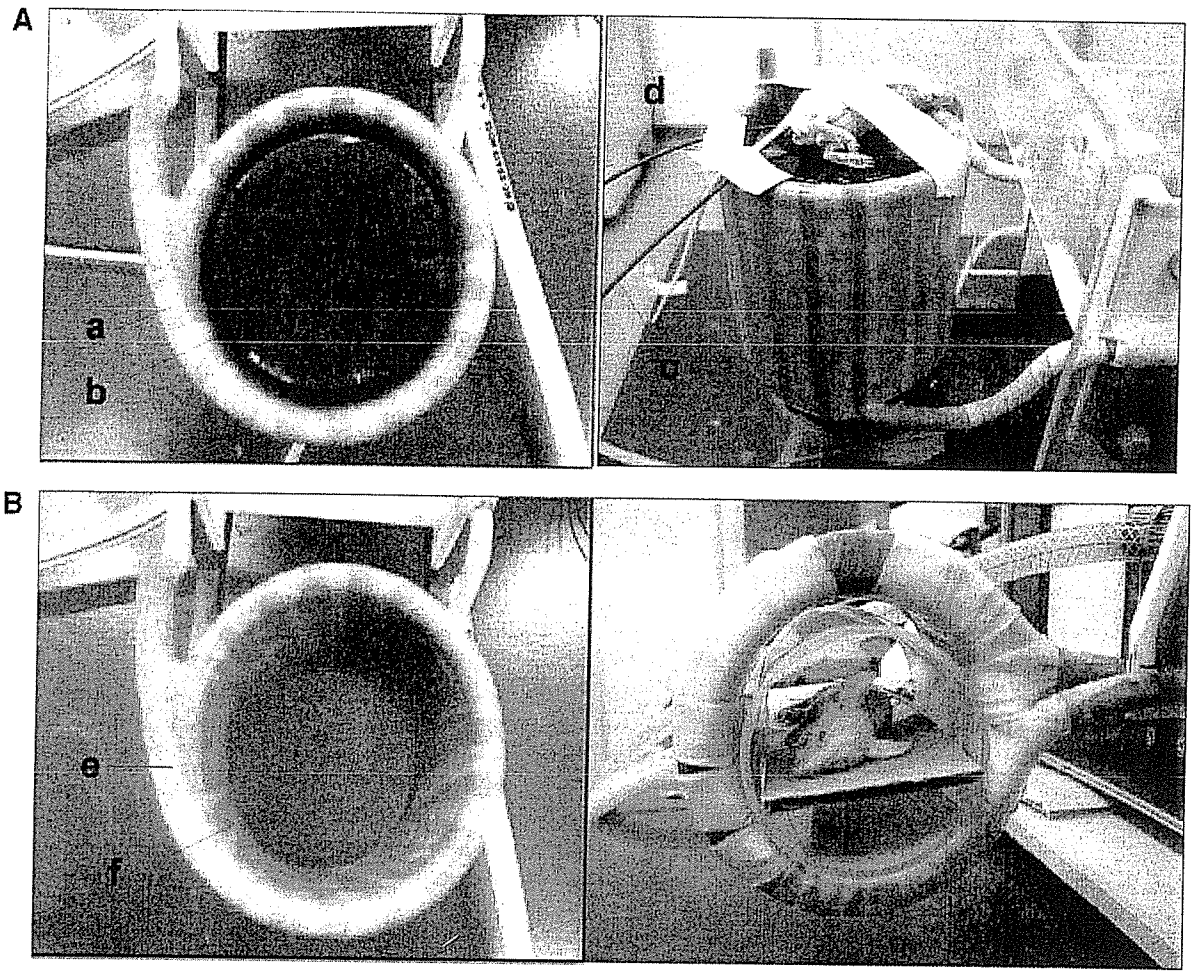

Fig. 1. **A**: Application of MCL-induced hyperthermia in this study. **Left panel**: Top view of the FCIS. Solenoid with an FC (**a**), FC inside the solenoid (**b**). **Right panel**: Oblique top view. The mouse was placed on the surface of the FC in the left lower lateral decubitus position, with the tumor nodule directly touching the surface of the FC. Optical fiber probes were used to measure temperature at the tumor surface (**c**) and inside the rectum (**d**). **B**: Application of our usual method of MCL-mediated hyperthermia using the VS. **Left panel**: Top view of the VS. Solenoid without an FC (**e**), and cavity inside the VS (**f**). **Right panel**: Front view. A tumor-bearing mouse injected with MCLs is placed at the center of the VS and irradiated with an AMF.

MCLs were added to liquid agar at 60°C while stirring with a glass impeller. After mixing for 30 min, the suspension was sonicated for 15 min by a probe-type sonicator operating at 40 W. The sonicated mixture (1 ml) was poured into a 5-ml polypropylene tube (10 mm in diameter; Sarstedt, Nümbrecht, Germany) and cooled rapidly by placing the tube in ice-cold water. The final net concentration of MCLs in the phantom was 3 mg/ml. An agar piece without MCLs was also prepared for control experiments.

To investigate heat generation in the phantom, the phantom with or without MCLs was subjected to AMF for 5 min using the VS or FCIS, with measurement of phantom temperatures using an optical fiber probe (FX-9020; Anritsu Meter, Tokyo, Japan).

Cell Lines

PC-3 (human prostate cancer cell line derived from bone metastatic lesions; androgen insensitive) and LNCap (human prostate cancer cell line derived from lymph node metastatic lesions; androgen sensitive) cells were obtained from the American Type Culture Collection (Rockville, MD). The PC-3 cells were maintained in Dulbecco's modified Eagle's minimal essential medium, and the LNCap cells were maintained in RPMI medium 1640 (Sigma Chemical). All media were supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and a mixture of 0.6% mg/ml glutamine (Gibco), 200 IU/ml penicillin (Gibco), and 200 μg/ml streptomycin (Gibco). FBS was

heat-inactivated for 1 hr at 56°C. All cell cultures were incubated at 37°C in 5% CO_2/95% air, and the media were replaced every 3rd day.

Animal Models

Four-week-old male BALB/c nude mice were purchased from Charles River Japan (Yokohama, Japan). To prepare tumor-bearing animals, cell suspensions containing approximately 1×10^7 PC-3 cells in 100 μl of phosphate buffer (0.05 M sodium phosphate and 0.15 M NaCl, pH 7.4) were injected subcutaneously into the right flank of BALB/c nude mice under short-term anesthesia by intraperitoneal injection (i.p.) of sodium pentobarbital (50 mg/kg body weight). Cell suspensions containing approximately 2.5×10^7 LNCap cells in 100 μl of phosphate buffer mixed with Matrigel (Becton, Dickinson and Company, Franklin Lakes, NJ) were injected in the same way. The PC-3 and LNCap groups each consisted of five mice bearing PC-3 and LNCap tumor nodules, respectively.

The experimental protocol in the present study was approved by the Animal Care Committee of Nagoya City University Medical School. Animal experiments were performed 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 MCLs and Heat Generation by AMF

When tumors became 7 mm in diameter, MCLs were injected into tumor nodule and started to irradiation to AMF as follows. Tumor-bearing mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Under anesthesia, a syringe (26-G needle) containing MCLs was inserted longitudinally into each tumor subcutaneously from the tumor edge to ensure a homogeneous distribution of MCLs. The indicated volumes of MCL solution (20 mg/ml magnetite) were injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL) at 0.2 ml/hr. The mouse was placed at the center of an FCIS, where the magnetic flux was greatest. The temperatures inside the rectum and at the surface of the tumor were measured during AMF irradiation with optical fiber probes (FX-9020; Anritsu Meter). When the tumor surface temperature reached 46°C, this temperature was maintained by controlling the power of the AMF-generating machine. After injection of MCLs, mice were subjected to AMF for 30 min (Fig. 1A).

Therapy Protocol

AMF irradiation was performed three times at 24-hr intervals (referred to as RH). We injected MCLs and performed RH many times until repopulation was not observed, to ensure complete involution of the tumor nodule. The largest and smallest diameters of each tumor were measured using calipers every 3 days. In cases where the tumor increased in size compared to the previous measurement, RH was repeated.

As the treatment group, in both PC-3 and LNCap groups, mice bearing PC-3 or LNCap were irradiated to AMF when the tumor reached 7 mm in diameter but mice in the control group were received injection of MCLs but without AMF irradiation.

RESULTS

Comparison of Temperature Elevation Between the VS and FCIS

Our method of MCL-mediated hyperthermia using the VS needs placing tumor-bearing mice injected with MCLs in the center of the VS and irradiating them with AMF (Fig. 1B). This method can increase the tumor surface temperature to 45°C within 5 min, at which temperature tumor cells are killed. In our new FCIS method, it is also necessary for the tumor surface temperature to increase by 8°C within 5 min from the start of AMF irradiation (assuming that the body temperature before treatment is 37°C). These temperature increases in the phantom with MCLs were possible using either the VS or FCIS (Fig. 2). An AMF-generating-machine power when using the VS of 780 W (58 V, 13.5 A) increased the temperature by 7.8°C at the inside center of the VS. When the phantom with MCLs was placed at the surface of the FCIS, a temperature increase of 7.8°C was achieved using 600 W (50 V, 12 A). In the FCIS, the temperature rose by 7.8°C at the surface of a coil and by 4.7°C at 5 mm above the surface, respectively. In the VS, the temperature rose by 2.6°C at the surface of a coil and by 1°C at 5 mm above the surface for the same AMF power, respectively. These results indicate that the FCIS is more suitable than the VS for heating targets outside the coils.

Heating Ability of the FCIS

The power of the AMF-generating machine to achieve a temperature increase of 8°C was determined when the target (phantom with MCLs) located at the following distances from the FCIS: at the surface of the FCIS (point 0), 5 mm above the surface (point 5), and 10 mm above the surface (point 10). The phantom without MCLs was also examined in this way. The results are shown in Figure 3. In the case of the phantom with MCLs, the powers of the AMF-generating machine required to increase the temperature by 8°C were 600 W (50 V, 12 A) at point 0, 1,220 W (74 V, 16.5 A) at point 5, and 1,500 W (80 V, 18.8 A) at point 10. In

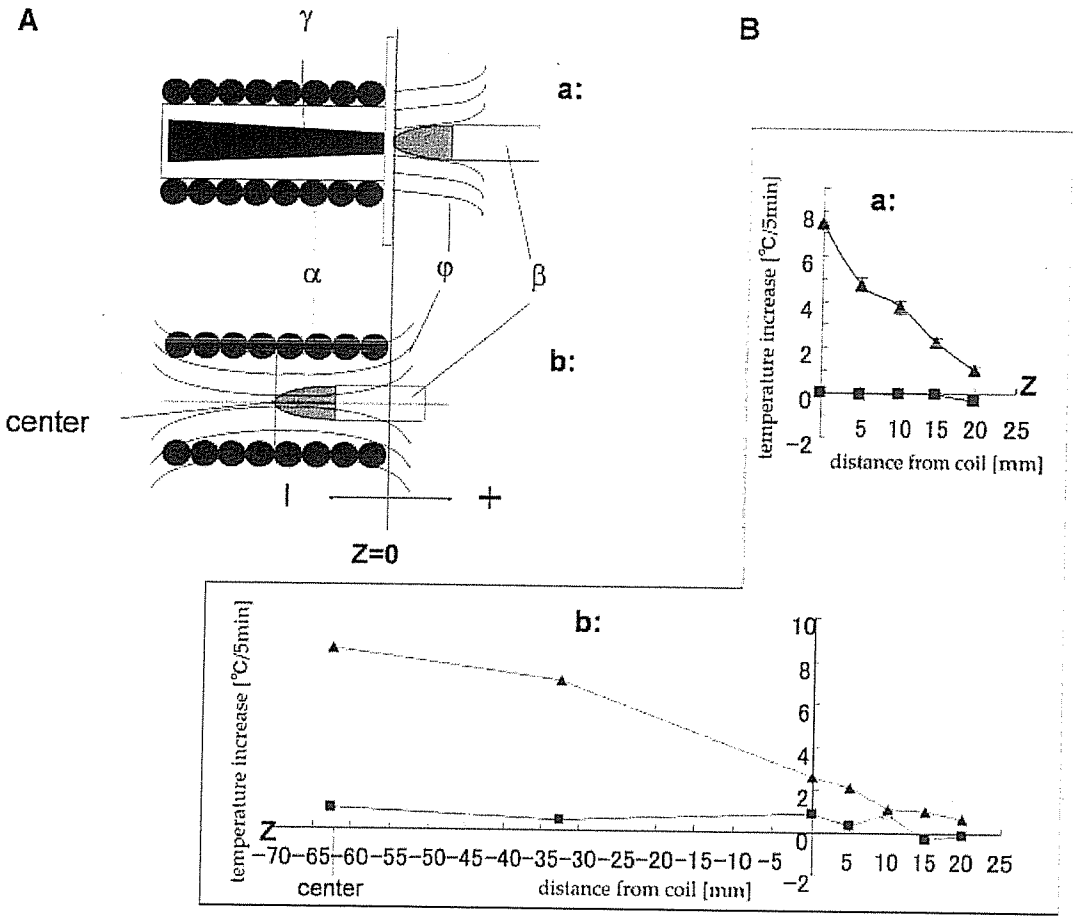

Fig. 2. **A**: Schematic cross-section of the FCIS (**a**) and VS (**b**). α: Solenoid. β: Optical fiber probe for measuring temperature. γ: The FC inside the solenoid. φ: Magnetic flux. Point 0 ($Z = 0$) is set up on the upper edge of the solenoid. Outside point 0 is indicated with the "+" and inside point 0 is indicated with the "−". The center of the VS is indicated by "$Z = -62.5$ cm." **B**: Temperature at various position on phantom irradiated using the FCIS (**a**) and VS (**b**) with (▲) and without (■) MCLs.

contrast, for the phantom without MCLs, the temperature increase was only 1.5°C at point 10 when the power was 1,500 W (80 V, 18.8 A). These results indicate that irradiation with an AMF in the FCIS can adequately increase the temperature of tumor tissue injected with MCLs when it is 10 mm from the surface of the coil.

Heat Generation by MCLs in AMF In Vivo

Tumor-bearing mice were irradiated by an AMF after MCL injection. The tumor surface temperature and the inside of rectum were measured in the PC-3 and LNCap groups. As shown in Figure 4, the tumor surface temperature of both groups rose to 46°C within 5 min from the start of irradiation. During this starting 5 min, the power of the AMF-generating machine was kept between 835 W (60 V, 13.9 A) and 1,500 W (80 V, 18.8 A). After the surface temperature had reached 46°C, it could be maintained accurately for 30 min by fine adjustment of the power of the AMF-generating machine. The temperature inside the rectum increased slightly during this period, but was within the normal range (33.0–36.5°C). These results demonstrate that tumor tissue directly injected with MCLs can be specifically heated by irradiation with an AMF, and that the degree of heating ability can be accurately controlled by adjusting the power of the AMF-generating machine.

Effects of Frequent RH on PC-3 and LNCapTumors

PC-3 group. The scheme of the hyperthermia experiment for the treatment group is shown in Figure 5. To evaluate tumor growth, the tumor long-axis diameters were plotted every 3 days in both control and treatment groups, which are indicated in Figure 6. Tumors in the control group continued to grow progressively (Fig. 6A), whereas those in the treatment group decreased in two steps. The tumor of all mice in the treatment group exhibited resistance to the heating

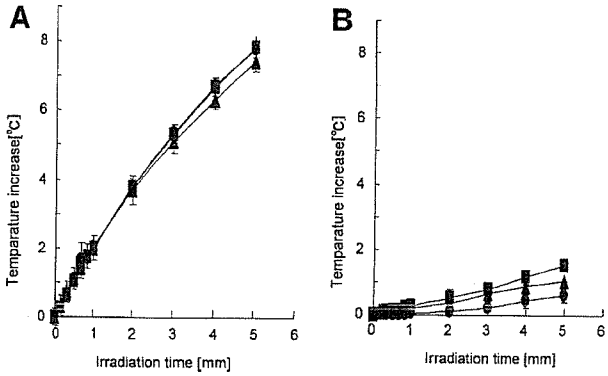

Fig. 3. The relation between the temperature increase of the phantom and distance from the edge of the coil (▲: 0 mm from the edge; ●: 5 mm from the edge; ■: 10 mm from the edge). **A**: A phantom with MCLs. **B**: A phantom without MCLs. The temperature increased equally at all three positions in the phantom with MCLs (A), but not in the phantom without MCLs (B).

induced by frequent RH (first step) until certain points. Resistance periods varied in each mice. However, eventually the tumors suddenly started to decrease, disappearing completely after an additional 3–6 days (second step) (Fig. 6B). In detail, in the treatment group, mouse 1 which received RH on days 0–2, 12–14, 21–23, and 30–32, exhibited sudden complete regression (CR) on day 36; mouse 2 received RH on days 0–2 and 12–14, exhibited CR on day 15; mouse 3 received RH on days 0–2 and 21–23, exhibited CR on day 30; mouse 4 received RH on days 0–2, 6–8, 12–14, and 15–17, exhibited CR on day 21; and mouse 5 received RH on days 0–2, 6–8, 12–14, 21–23, 36, 37, and 48–50, exhibited CR on day 57.

LNCap group. The scheme of the hyperthermia experiment for the treatment group is shown in Figure 7. The tumor long-axis diameters were plotted every 3 days in both control and treatment groups (as for the PC-3 group) and are indicated in Figure 8. The tumors in the control group continued to grow linearly as for the PC-3 groups (Fig. 8A); however, the growth in the treatment group demonstrated a one-step decrease (in contrast to that in the PC-3 group). The tumor decreased in size linearly and then disappeared (Fig. 8B). In detail, in the treatment group, mouse 1 which received RH on days 0–2, 6–8, and 12–14, exhibited CR on day 27; mouse 2 received RH on days 0–2 and 12–14, exhibited CR on day 27; mouse 3 received RH on days 0–2 and 12–14, exhibited CR on day 36; mouse 4 received RH on days 0–2 and 27–29, exhibited CR on day 42; and mouse 5 received RH on days 0–2, exhibited CR on day 9.

DISCUSSION

This study investigated the feasibility of our new AMF-generating device comprising a solenoid coil with an FC, and its capability for hyperthermic treatment in human prostate cancer. FC was used instead of a flat circle electrode, which was used for more-effective hyperthermia of deep tissue [26], because it enabled the magnetic field and eddy currents to be controlled. The FCIS used in this study has an FC inside a solenoid coil that concentrates the magnetic field generated by solenoid coil, resulting in the emission of the magnetic field from the surface of the device. In the present experiment, the following three performance characteristics of the FCIS were carried out: (1) whether it provides sufficient heating for hyperthermia (including a comparison with a VS), (2) the amount of temperature elevation of the target tissue, and (3) whether the induced hyperthermia has the same anticancer effect as for a VS. Characteristics 1

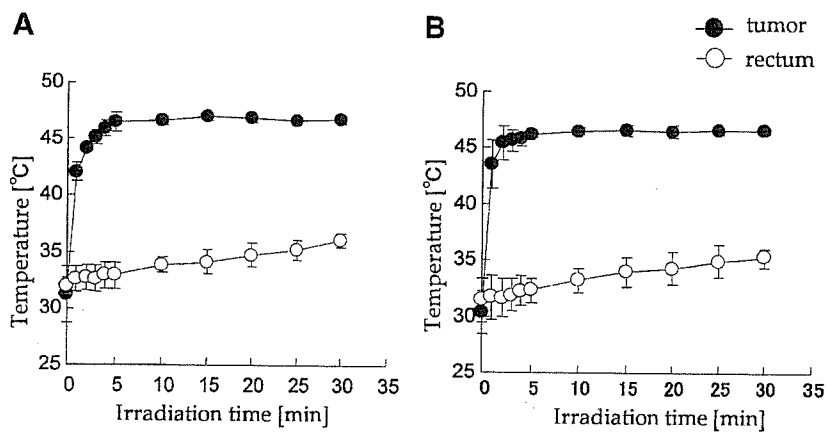

Fig. 4. Temperatures at the tumor surface (●) and inside the rectum (○). **A**: PC-3 group. **B**: LNCap group. Tumor surface. In both the PC-3 and LNCap groups, the tumor surface temperature increases after 5 min, and is maintained at around 46°C. The temperature inside the rectum increased a little, but not above 35°C. Data shown are mean ± SD values from five independent experiments.

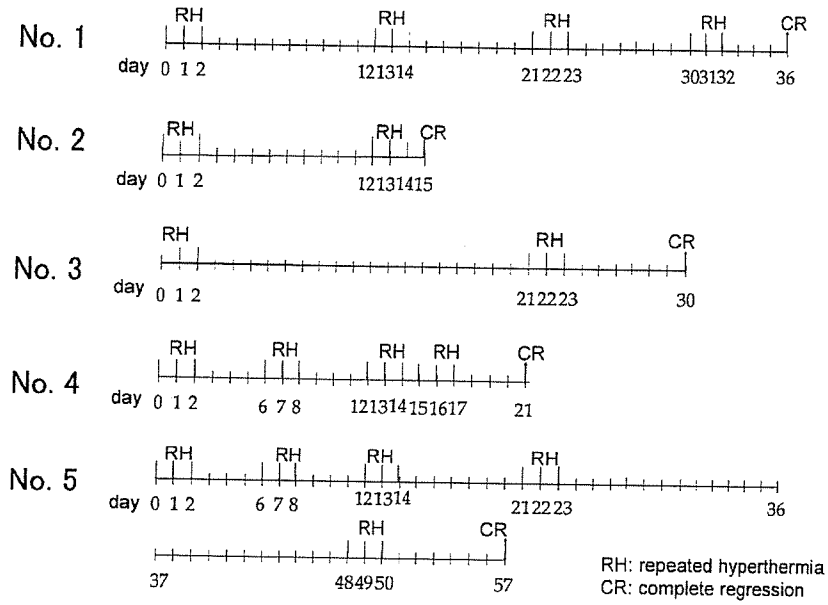

Fig. 5. Scheme of the hyperthermia experiment for the PC-3 group, indicating the various patterns of RH among five mice. The RH was repeated from two to five times.

and 2 were examined using a phantom with MCLs in the place of a living body. The results indicated that the FCIS was suitable for heating a target positioned outside the coils (Fig. 2). In the case of the phantom with MCLs, a phantom 10-mm distant from the FCIS could be increased in temperature by 8°C within 5 min. Using the AMF-generating machine at the same position, the temperature of the phantom without MCLs did not increase (Fig. 3). These results demonstrate that our new hyperthermic procedure with the FCIS has the ability to specifically heat the tumor tissue injected with MCLs up to 45°C (given that normal body temperature is 37°C).

Characteristic 3 was examined by assessing the therapeutic effect in human prostate cancer cell nodules. We reported previously the therapeutic inhibiting effect of the VS on the tumor growth of rat prostate cancer, but CR was not observed [24]. And we also reported that frequent RH with a VS-induced CR of mouse mammary carcinomas with a diameter of larger than 15 mm [27]. To confirm therapeutic effect of our

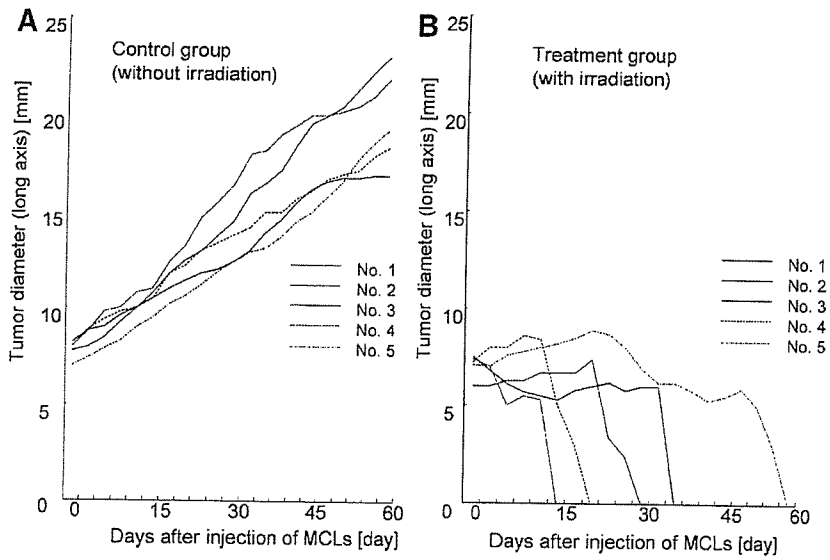

Fig. 6. Tumor long axis of the PC-3 group in both the control group (**A**) and the treatment group (**B**). Tumor size increased linearly in the control group. However, a two-step decrease and CR were demonstrated in the treatment group.