

Tumor regression by combined immunotherapy and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma

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Immunotherapy (IT) has become an accepted therapeutic modality. We previously reported that intracellular hyperthermia (IH) using magnetic nanoparticles induces antitumor immunity. We undertook these studies in order to study the combined effects of IT and IH on melanoma. Magnetite cationic liposomes (MCLs) have a positive surface charge and generate heat in an alternating magnetic field (AMF) due to hysteresis loss. MCLs were injected into a B16 melanoma nodule in C57BL/6 mice, which were subjected to AMF for 30 min. The temperature at the tumor reached 43°C and was maintained by controlling the magnetic field intensity. At 24 h after IH, interleukin-2 (IL-2) or granulocyte macrophage-colony stimulating factor (GM-CSF) was injected directly into the melanoma. Mice were divided into six groups: group I (control), group II (IH), group III (IL-2), group IV (GM-CSF), group V (IH+IL-2), and group VI (IH+GM-CSF). Complete regression of tumors was observed in mice of groups V and VI (75% (6/8) and 40% (4/10) of the mice, respectively), while no tumor regression was observed in mice of the other groups. This study supports the combined use of IT and IH using MCLs in patients with advanced malignancies. (*Cancer Sci* 2003; 94: 308–313)

Hyperthermia has been used for many years to treat a wide variety of tumors both in experimental animals and patients.¹ The most commonly used heating method in clinical settings is capacitive heating using a radiofrequency (RF) electric field.² However, specifically heating tumors by capacitive heating using an RF electric field is difficult, because the heating characteristics are influenced by various factors, such as tumor size, position of electrodes, and adhesion of electrodes at uneven sites. From a clinical point of view, a simple heat mediator is preferable for superficially seated tumors such as cutaneous melanoma. Magnetic nanoparticles have been applied to generate hyperthermia in an attempt to overcome these disadvantages.^{3,4} These magnetic nanoparticles generate heat in an alternating magnetic field (AMF) due to hysteresis loss.⁵ We have developed “magnetite cationic liposomes” (MCLs) for intracellular hyperthermia (IH).^{6,7} MCLs were developed to show improved adsorption and accumulation in tumor cells and have a ten-fold higher affinity for tumor cells than for neutrally charged magnetoliposomes due to electrostatic interaction with the negatively charged cell membrane.⁶ In our *in vitro* experiments, 55% of MCLs were incorporated into cells and the intracellular magnetic nanoparticles could generate heat under AMF.⁶ We have also demonstrated the efficacy of IH using MCLs against T-9 rat glioma in an *in vivo* study.⁸

We previously reported that our IH system induced antitumor immunity.⁹ Hyperthermia is known to induce heat shock proteins (HSPs).¹⁰ Because expression of HSP70 protects cells from heat-induced apoptosis,¹¹ HSP70 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, in

immune reactions.^{12,13} HSP-mediated antitumor immunity was reported to cause a vaccine effect due to HSP-peptide complexes purified from human melanoma cells.¹⁴ With regard to the mechanism of antitumor immunity induced by IH, we demonstrated that HSP70 expression occurs during IH-induced antitumor immunity against the T-9 rat glioma.^{15,16} Our hyperthermia system leads to vaccination with HSP70-peptide via necrotic tumor cell death *in vivo*, resulting in antitumor immunity. This phenomenon, which may be called *in situ* vaccination, has numerous implications in the development of novel antitumor therapies based on IH.

Immunotherapy (IT) has become an accepted therapeutic modality. Interleukin-2 (IL-2) is a potent stimulator of lymphocyte proliferation and augments the activity of cytotoxic T lymphocytes (CTLs).¹⁷ IL-2 has a broad range of immunologic effects, such as inducing specific T helper cells, natural killer cells and lymphokine-activated killer cells.¹⁸ Due to these effects, IL-2 is widely used in cancer therapy to enhance cellular immunity and the cytotoxic activity of effector cells.¹⁹ Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor that stimulates proliferation and differentiation of granulocytic and monocytic progenitor cells.^{20–22} Because of this effect on hematopoietic cells, GM-CSF has been used to counter myelosuppression induced by high-dose chemotherapy, to bolster host defense in immunocompromised patients, and to accelerate recovery after bone marrow transplantation.^{23–26} It has also been reported that GM-CSF plays an important role in the activation of antigen-presenting cells (APCs).²⁷ Dranoff *et al.*²⁸ reported that vaccination with irradiated GM-CSF-transduced murine melanoma cells induced a potent systemic immune response against the melanoma cells.

Based on these results, we studied combination therapy using IT (with IL-2 or GM-CSF) and IH. In the present paper, we examined the feasibility of combined IH and IT for malignant melanoma. To our knowledge, this is the first time that the combination of local injection of GM-CSF and hyperthermia has been shown to exhibit an obvious antitumor effect against malignant melanoma.

Materials and Methods

Cell line and animal model. Mouse B16 melanoma cells (Riken Cell Bank, Tsukuba) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin). Mouse T-lymphoid EL4 cells were grown in RPMI 1640 containing 10% FBS and antibiotics (100 U/ml penicillin G and 0.1 mg/

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ml streptomycin). These cells were grown at 37°C in an atmosphere containing 5% CO₂. Female C57BL/6 mice (age, 4 weeks) were purchased from Charles River Japan, Inc. (Yokohama). To prepare tumor-bearing animals, cell suspensions including approximately 1×10⁶ melanoma cells in 0.1 ml of phosphate-buffered saline (PBS; 0.05 M sodium phosphate and 0.15 M NaCl, pH 7.4) were injected subcutaneously into the right flank of C57BL/6 mice, which were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Melanoma nodules that had grown to 6 mm in diameter were used for the experiments. Tumor diameter was measured every 3 days. The size was determined by applying the following formula,

$$\text{Tumor size} = 0.5 \times (\text{length} + \text{width})$$

where length and width are measured in millimeters.

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.

Preparation of MCLs. Magnetic particles were kindly donated by Toda Kogyo Co. (Hiroshima; average size of magnetite: 10 nm). MCLs were prepared using the previously described sonication method, with a slight modification.⁶⁾ Briefly, 1 ml of colloidal magnetite (net 20 mg magnetite) was coated with a lipid membrane that consisted of *N*-(α -trimethylammonioacetyl)dodecyl-D-glutamate chloride (Sogo Pharmaceutical Co., Tokyo), dilaurylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical Co., St. Louis, MO) in molar ratios of 1:2:2. Magnetite concentration was measured using the potassium thiocyanate method.²⁹⁾

Injection of MCLs and heat generation in AMF. After melanoma nodules had grown to 6 mm in diameter, a syringe (26 G needle) containing MCLs was inserted longitudinally into each melanoma nodule subcutaneously from the nodule edge. MCLs (2.0 mg) were injected using an infusion pump (SP100i; World Precision Instruments, Inc., Sarasota, FL) for 30 min. Mice were then separated into six groups. Each group contained 8–10 mice. Mice in groups I (control), III (IL-2), and IV (GM-CSF) were not subjected to AMF. After injection of MCLs, mice in groups II (IH), V (IH+IL-2), and VI (IH+GM-CSF) were subjected to AMF for 30 min. AMF was generated by a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency Co., Tokyo). The magnetic field frequency was 118 kHz. The mouse was placed inside the coil such that the nodule was positioned at the center. Temperature in the tumor and rectum during AMF irradiation was measured with an optical fiber probe (FX-9020; Anritsu Meter Co., Tokyo). The tumor was kept at 43.0°C by controlling the magnetic field intensity.

Preparation of specimens for immunohistochemical staining. On the day after hyperthermic treatment, tumors were removed and specimens for immunohistochemical staining were prepared. For immunostaining of HSP70 and Mac-3, tumors were resected 24 h after hyperthermia, fixed in 10% formalin solution, and embedded in paraffin. Thin (4 μ m) slices of paraffin-embedded specimens were deparaffinized in xylene and rehydrated with a series of ethanol washes. Autoclave treatment at 120°C for 10 min in 10 mM sodium citrate buffer (pH 6.0) was then employed for antigen retrieval. For immunostaining of CD8, tumor tissue was immediately frozen at -80°C, embedded in and fixed with O.C.T compound (Sakura Finetechnical Co., Tokyo). Thin (4 μ m) slices were air-dried for 30 min and fixed with cold acetone for 10 min. Paraffin-embedded sections were incubated at 37°C for 60 min with mouse monoclonal antibody preparations (MAbs) against HSP70 (clone: W27, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antigens and rat MAbs against macrophage/dendritic cell³⁰⁾ (Mac-3, clone: M3/84, BD

PharMingen, San Diego, CA) antigens. Frozen sections were incubated at 37°C for 60 min with rat MAbs against CD8a (clone: 53-6.7, BD PharMingen, San Jose, CA) antigens. These sections were subsequently incubated at 37°C for 60 min with biotinylated goat anti-mouse IgG (DAKO, Kyoto) or biotinylated mouse anti-rat IgG (DAKO). Specimens were incubated at 37°C for 30 min with alkaline phosphatase or peroxidase-conjugated streptavidin (DAKO). Each step was followed by washing with PBS. Alkaline phosphatase and peroxidase activity were visualized by New Fuchsin Substrate (DAKO) and diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxide, respectively. All slides were counterstained with hematoxylin. For negative controls, primary antibodies were replaced with either unrelated monoclonal antibodies or PBS.

Analysis of major histocompatibility complex (MHC) class I antigen on cell surface. Melanoma cells in the logarithmic growth phase were heated at 43°C for 30 min by direct immersion of cell culture dishes in a temperature-controlled water bath. The temperature of the medium increased quickly and reached 43°C within 5 min. The temperature in the medium was monitored with the fiber optic thermometer probe. Heated cells or non-heated cells were harvested at 24 h after heating and were washed in cold PBS, after which the cell density was adjusted at 1×10⁶ cells/ml in PBS. For flow cytometric analysis of MHC class I antigen, B16 and EL4 cells were washed twice with cold PBS containing 2% fetal calf serum and then incubated with the FITC-conjugated anti-mouse H-2K^b MAbs (clone: AF6-88.5, BD PharMingen) for 40 min at 4°C. Cells were washed three times with cold PBS and the stained cells were analyzed by flow cytometry (Paltec, Münster, Germany).

Injections of IL-2 and GM-CSF. Mice in groups III (IL-2) and V (IH+IL-2) were injected with recombinant human IL-2 (5×10⁴ U, Wako Pure Chemical Industries Co., Osaka). Mice in groups IV (GM-CSF) and VI (IH+GM-CSF) were injected with recombinant human GM-CSF (1×10⁴ U, Wako Pure Chemical Industries Co.). At 24 h after hyperthermia, IL-2 or GM-CSF was injected directly into the melanoma nodules. Injection of cytokines was carried out once daily for 2 days.

Results

Hyperthermia by means of MCLs. Fig. 1 shows the temperature in the tumor and rectum during AMF irradiation. AMF was ap-

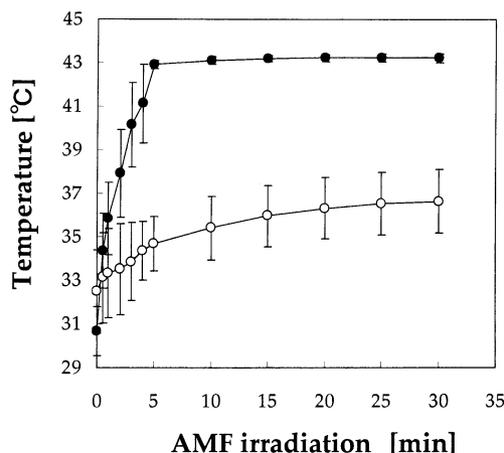


Fig. 1. Hyperthermia using MCLs *in vivo*. MCLs were injected directly into subcutaneous B16 tumors of mice, which were then irradiated with an alternating magnetic field (AMF) for 30 min. Tumor and rectal temperatures were measured with optical fiber probes. Closed circles, tumor; open circles, rectum. Each point represents the mean±SD of 10 mice.

plied for 30 min. The tumor temperature reached 43°C within 5 min, and was maintained at 43°C by controlling the magnetic field intensity. In contrast, the rectal temperature remained below 37°C. These results indicate that our hyperthermia system using MCLs is able to heat the tumor specifically without damaging healthy tissue. The tumor temperature was also maintained very precisely within a small standard deviation, thus demonstrating the ease of temperature control within the tumor by adjusting the magnetic field intensity.

Immunohistochemistry of B16 tumors after hyperthermia with MCLs.

Expression of HSP70 in B16 melanoma was investigated and the results are shown in Fig. 2A. Without AMF irradiation, staining for HSP70 was negative (Fig. 2, A-I). However, after irradiation, HSP70 was strongly expressed in the viable cells around the necrotic area, as shown in Fig. 2A-II. To investigate which cells mediate the antitumor effects of IH on the melanoma, the immune infiltrates of B16 melanoma with or without IH were compared. In agreement with the results obtained in T-9 rat gliomas,¹⁶⁾ melanoma tumors treated with IH were heavily infiltrated with CD8-positive T cells (Fig. 2B) and Mac3-positive macrophages/dendritic cells (Fig. 2C) when compared to untreated tumors.

MHC class I expression on the surface of B16 cells. To examine whether the CD8-positive T cells infiltrating into tumors after hyperthermia (Fig. 2B) were effective for tumor immunity, MHC class I expression on the surface of B16 cells was studied. Flow cytometric analysis using the anti-mouse H-2K^b antibody revealed that B16 cells expressed no MHC class I antigen or a lower amount compared with that of EL4 cells (Fig. 3A). On the other hand, a significant increase of MHC class I antigen on the surface of the heated B16 cells was observed, as shown in Fig. 3B. The mean fluorescence intensity for peaks of H-2K^b in heated and non-heated B16 cells was 10.0 and 6.5, respectively.

Effects of intracellular hyperthermia plus immunotherapy on tumor growth. HSP70 expression and infiltration by CD8-positive T cells and Mac3-positive macrophages/dendritic cells into B16 tumors were observed after IH, and thus immunotherapy with IL-2 or GM-CSF, which may activate the immune cells, was

combined in order to investigate whether the antitumor effects were enhanced. Fig. 4 shows the time courses of tumor growth. After melanoma nodules had grown to 6 mm in diameter, combination therapy with IT and IH was carried out. In group I (control), tumors grew progressively. Although the mice in group II received hyperthermia treatment at 43°C, the tumors grew progressively. Groups III and IV received injections of IL-2 or GM-CSF alone, respectively, but no tumor regression was observed. On the other hand, some of the tumors com-

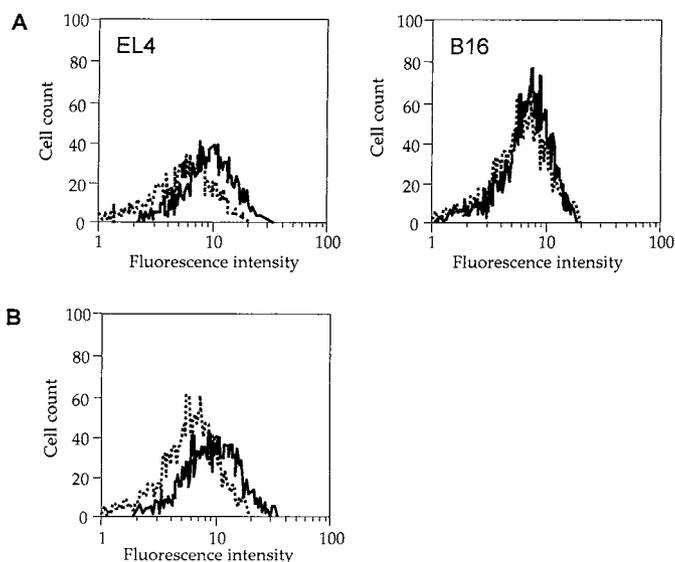


Fig. 3. Augmentation of MHC class I surface antigen on melanoma cells by hyperthermia. Flow cytometric analyses were carried out using anti-H-2K^b antibody. A. H-2K^b expression of EL4 (left) and B16 cells (right). Dotted line, isotype control; solid line, FITC-conjugated anti-mouse H-2K^b antibody. B. H-2K^b expression of heated or non-heated B16 cells at 24 h after heating. Dotted line, non-heated; solid line, 43°C, 30 min.

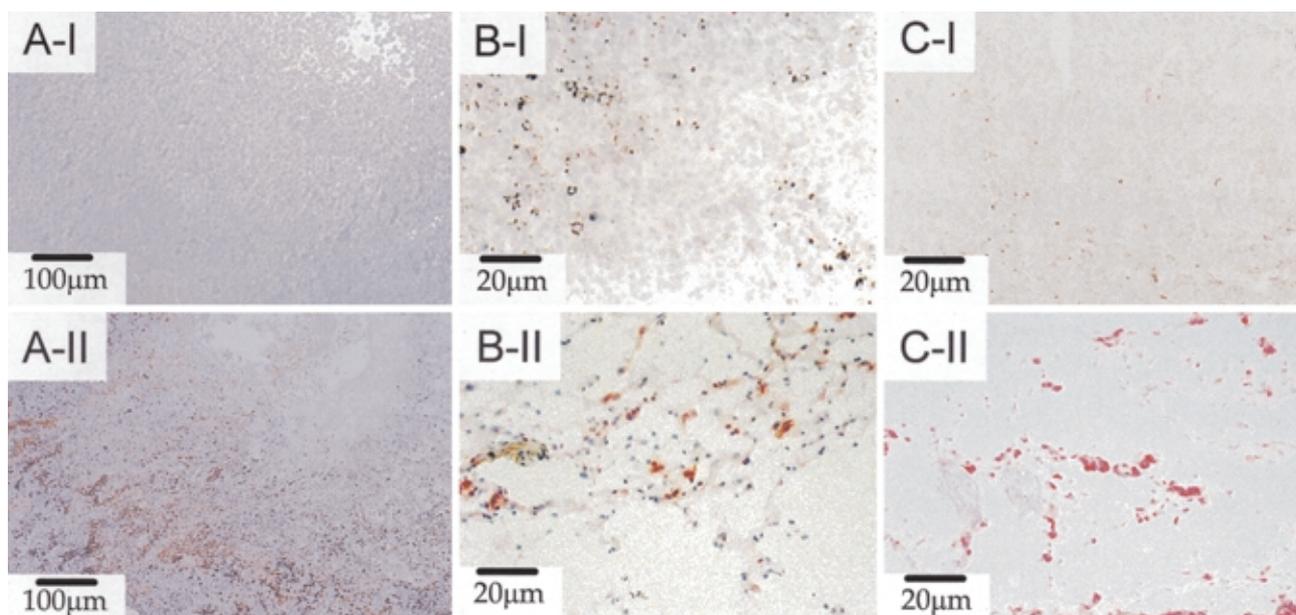


Fig. 2. Immunohistochemical staining for HSP70 and immunocytes. Tumors were resected at 24 h after hyperthermia and were immunohistochemically stained with anti-HSP70 (A), anti-CD8 (B), and anti-Mac-3 (C) antibody. I, without AMF irradiation; II, 30 min AMF irradiation. Brown areas in A are MAb-stained area visualized by diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxide. Red cells in B and C are MAb-stained cells visualized by New Fuchsin Substrate.

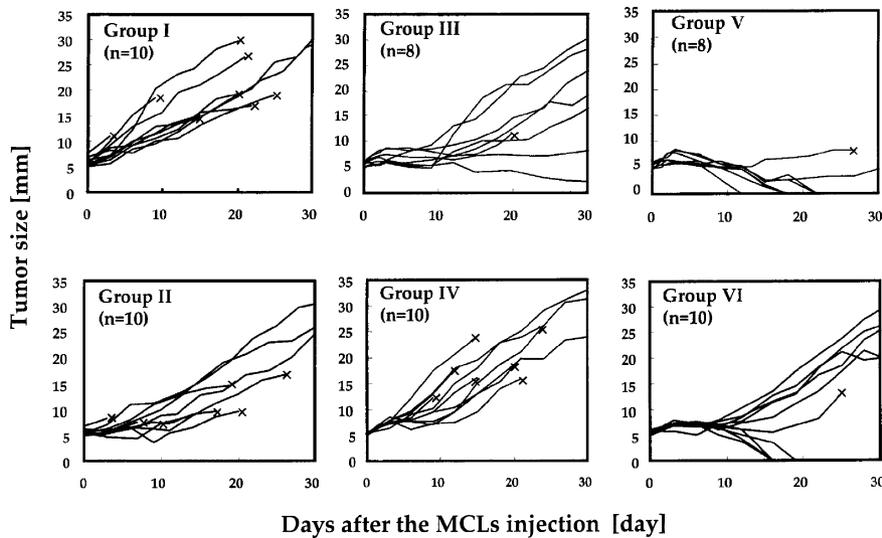


Fig. 4. Time course of tumor growth in each group after hyperthermia. Group I (control, $n=10$), group II (IH, $n=10$), group III (IL-2, $n=8$), group IV (GM-CSF, $n=10$), group V (IH+IL-2, $n=8$), and group VI (IH+GM-CSF, $n=10$). Each line represents tumor growth kinetics in a single mouse. Crosses (x) indicate when each mouse died.

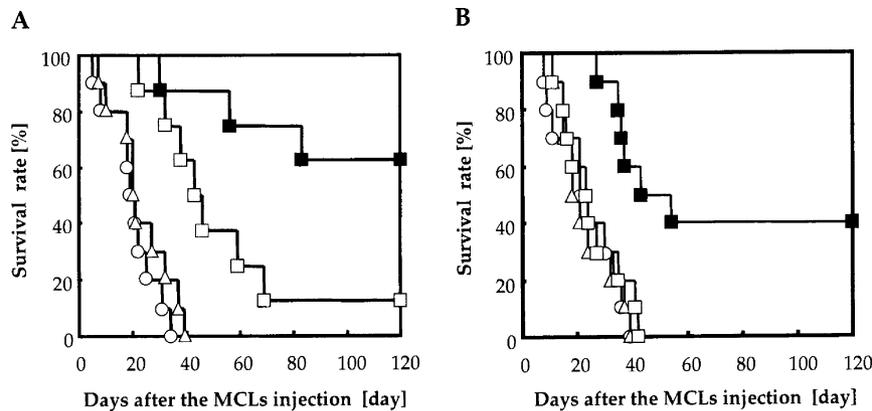


Fig. 5. Percentage survival of tumor-bearing mice observed over a period of 120 days after hyperthermia. A. IL-2. Open circles, group I (control, $n=10$); open triangles, group II (IH, $n=10$); open squares, group III (IL-2, $n=8$); and closed squares, group V (IH+IL-2, $n=8$). B. GM-CSF. Open circles, group I (control, $n=10$); open triangles, group II (IH, $n=10$); open squares, group IV (GM-CSF, $n=10$); and closed squares, group VI (IH+GM-CSF, $n=10$).

pletely regressed in groups V (IH+IL-2) and VI (IH+GM-CSF) after combination therapy with IH using MCLs and IT using IL-2 or GM-CSF. In group V, 75% (6/8) of subcutaneous tumors showed complete regression, while in group VI, 40% (4/10) showed complete regression. Furthermore, these tumors did not begin growing again for the remainder of the experimental period (120 days).

Effects of intracellular hyperthermia plus immunotherapy on survival rate of melanoma-burdened mice. The survival rates of tumor-bearing mice observed for a period of 120 days after injection of MCLs are shown in Fig. 5. In groups I and II, all mice died from pulmonary metastases and/or an enlarged tumor at the inoculated site within 40 days. Survival was slightly prolonged in group III (IL-2), and 1 of 8 mice survived for 120 days, while in group V (IH+IL-2), 62.5% (5/8) of mice survived for 120 days (Fig. 5A). For GM-CSF alone, no significant effect on survival was observed in group IV (GM-CSF), while 40% (4/10) of mice in group VI (IH+GM-CSF) survived for 120 days (Fig. 5B). These results suggest that survival of melanoma-burdened mice was prolonged by combination therapy with IH using MCLs and IT using IL-2 or GM-CSF.

Discussion

In the case of a superficial tumor such as melanoma, a simple heat mediator is desirable for the clinical application of hyperthermia. However, it is difficult to specifically heat a superficial tumor by capacitive heating using an RF electric field. We used MCLs in order to heat the tumoral region and minimize heating of the surrounding healthy tissue. Fig. 1 shows the temperature in the tumor and in the rectum during IH using MCLs. The tumor temperature rapidly reached 43°C, while the rectal temperature remained at 38°C. These results indicate that IH using MCLs is able to specifically heat 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 43°C, though this was insufficient to destroy the malignant melanoma. Our hyperthermia system can generate 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 our hyperthermia system at 46°C applied once daily for 2 days.³¹⁾ In the present study, we set the temperature at 43°C in order to examine the effects of combining IH with IT.

Matzinger *et al.*^{32,33} proposed that the immune system has evolved to detect “danger” by employing professional APCs as sentinels of tissue distress, and according to this hypothesis, HSPs act as one of these danger signals. Todryk *et al.*³⁴ reported that induction of HSP70 expression led to infiltration by T cells, macrophages, and dendritic cells into the tumor and enhanced immunogenicity through a T cell-mediated mechanism. In the present study, we observed HSP70 expression and infiltration by CD8-positive T cells and Mac-3-positive macrophage/dendritic cells into the B16 tumor after IH, as shown in Fig. 2. Because self antigens expressed by tumors do not efficiently stimulate T cells,³⁵ one of the most effective ways to stimulate antitumor immunity is to promote the cross-priming of host professional APCs.³⁶ Suto *et al.*³⁷ elucidated the mechanism of cross-priming by exogenous antigens chaperoned by HSPs, a pathway that is functional in a subset of macrophages. Our hyperthermia system killed the tumor via expression of HSP70-tumor antigen complexes at the tumor site, and recruitment of immune effector cells, including APCs (macrophages and dendritic cells), into the tumor subsequently occurred. Based on these results, we propose that IH using MCLs is an *in situ* vaccination therapy for cancer.

B16 mouse melanoma cells are known to express a lower amount of H-2 antigen, as shown in Fig. 3A. Efficient recognition of tumor cells by CTLs is often dependent on the presentation of cytosolic peptides in the context of MHC class I molecules. This process may be influenced by various molecular chaperons such as HSPs. Wells *et al.* reported that stably transfected clones of B16 which constitutively expressed human Hsp70 exhibited significantly increased levels of MHC class I antigens on their surface.³⁸ This Hsp70-mediated up-regulation of surface MHC class I antigen represented an increase in the amount of functional MHC-peptide complexes as measured by conformation-dependent antibodies and recognition by MHC class I-restricted CTL. We previously showed the augmentation of MHC class I antigen presentation via HSP70 expression by hyperthermia.¹⁵ The T-9 rat glioma cell surface presentation of MHC class I antigen increased in tandem with increased HSP70 expression and the immunogenicity of the cells was enhanced by hyperthermia. In the present study, we showed the augmentation of H-2K^b presentation in B16 melanoma cells by hyperthermia (Fig. 3B). We previously demonstrated that the IH induced an antitumor immunity specific to B16 cells.³¹ In the present study, although we have not eluci-

dated the roles of IL-2 and GM-CSF in combination with IH, we suppose that the antitumor immunity specific to B16 melanoma induced by IH was enhanced by the treatment with IL-2 and GM-CSF. Since the H-2K^b expression on non-heated B16 cells is low, the role of other effector cells such as the natural killer cells remains to be investigated.

When applying IH as a tumor vaccine, administration of cytokines may supplement the vaccine-induced response against tumor cells. In the present study, we used IL-2 and GM-CSF for this purpose. As mentioned above, we believe that our IH system should be regarded as a vaccination therapy. In vaccination therapy, professional APCs play an important role in eliciting a primary immune response to tumor antigens. Enhancement of APC function by cytokines can potentially occur at two levels; directly on APCs during generation and maturation, and indirectly by acting on the elicited T cell response. The most important finding from our studies was that GM-CSF, a cytokine playing an important role in the maturation and function of professional APCs, proved to be a powerful immunostimulant after hyperthermia. IL-2 is a cytokine known for its ability to elicit T cell response, and has been used in APC-based tumor vaccines.³⁹ Survival was prolonged in group III (IL-2), whereas no significant effect on survival was observed in group IV (GM-CSF), as shown in Fig. 5. Because IL-2 has a broad range of immunologic effects, an effect on survival was observed even without vaccination by IH. On the other hand, injection of GM-CSF alone had no effect on survival. When treatment with GM-CSF was combined with IH, 40% (4/10) of mice survived for 120 days, as shown in Fig. 5B. These results suggest that the antitumor effects of GM-CSF may require (or be enhanced by) vaccination by IH. The roles of IL-2 and GM-CSF in combination with IH, and the involvement of specialized APCs and elicited effector cells in this therapy, remain to be fully elucidated.

In summary, complete regression of tumors was observed in mice after combination therapy with IH and IT. This study suggests the efficacy of combined IT (IL-2 and GM-CSF) and IH using MCLs in patients with advanced malignancies.

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