This means that 4 mg of magnetite was sufficient to achieve a tumor temperature of 45°C. No serious burning on the skin was observed in these mice. In addition, rectal temperature increased only slightly during the irradiation, thus suggesting tumor-specific hyperthermia.

The therapeutic effects of RH of the melanoma nodules in MT/*ret* transgenic mice were then examined. A melanoma nodule with a size of 5-7 mm in diameter was injected with MCLs, and AMF irradiation was carried out three times at 24-h intervals. We previously reported that an iteration of RH was effective for malignant cancers (e.g. mouse mammary carcinoma of size > 15 mm)[13]; in the present study, this protocol was applied to MT/*ret* transgenic mice. Mice were treated with RH, as shown in Fig. 2, until complete tumor regression was achieved. Complete regression of melanoma nodules in three mice (mice no. 1, 2, 4) were achieved by single round of RH. For mice nos. 3 and 5, additional MCLs were injected and RH was again conducted because partial tumor regrowth occurred after the first RH treatment, and complete tumor regression was achieved after 2 or 3 rounds of RH treatment for mice nos. 3 and 5, respectively. Fig. 3 shows the time course for tumor size. In

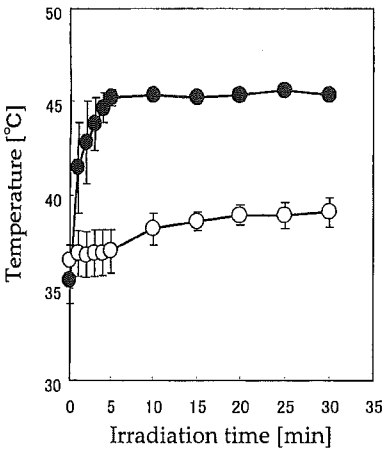

Fig. 1. MCL-mediated hyperthermia in MT/*ret* transgenic mice. MCLs were injected directly into the tumors of mice, which were then irradiated with an alternating magnetic field (AMF) for 30 min. Tumor and rectal temperatures were measured by optical fiber probes. Closed circles: tumor; open circles: rectum. Each point represents the mean±SD of 5 mice.

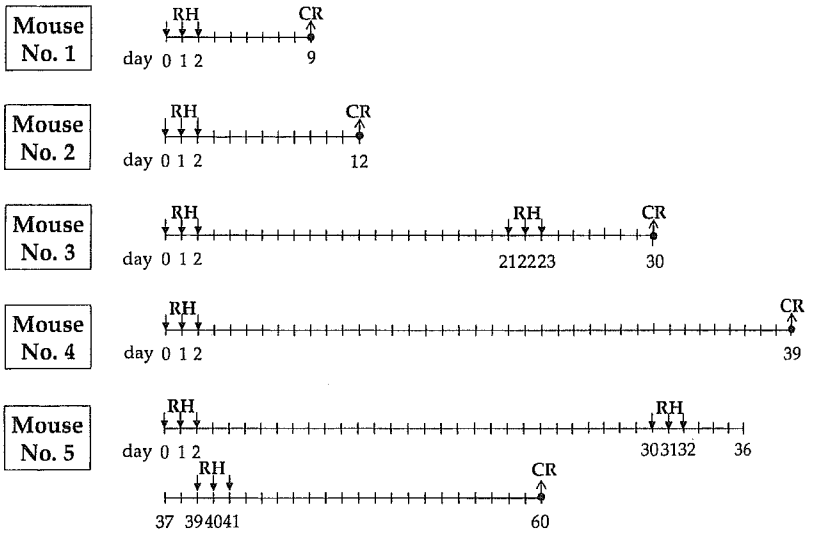

Fig. 2. Protocol of repeated hyperthermia (RH) for MT/*ret* transgenic mice. MCLs (4 mg magnetite) were injected directly into the tumors, which were then irradiated with an AMF for 30 min. AMF irradiation was repeated three times at 24-h intervals (RH). If tumors began to regrow, RH treatment was carried out frequently after injection of MCLs (4 mg magnetite), until complete regression (CR) was achieved.

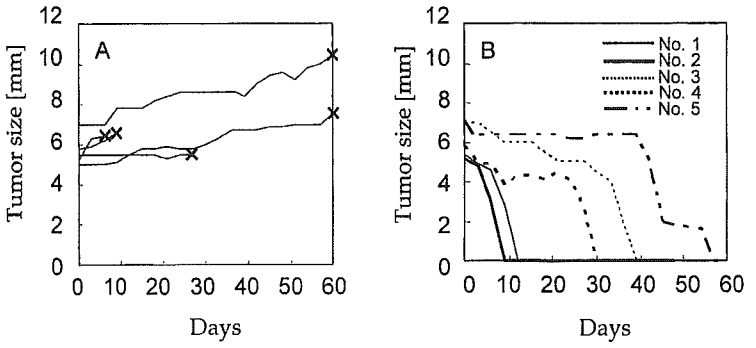

Fig. 3. Time course of changes in tumor size in control mice (*A*) and in mice undergoing repeated hyperthermia (RH) treatment (*B*). Each line represents growth of a tumor of interest in a single mouse.

the control group, tumors grew for 60 d, and all mice died within 60 d. Moreover, 3 mice died within 30 d despite the relatively small tumor size, as shown in Fig. 3A. However, in the hyperthermia group, all tumors completely regressed after several rounds of RH and no mice died within 60 d, as shown in Fig. 3B. Furthermore, tumors successfully treated with RH did not undergo regrowth throughout the lifespan of the mice.

Survival data for a period of 120 days after hyperthermia is shown in Fig. 4. The tumors in MT/*ret* transgenic mice metastasize to the lymph nodes, lung, brain, kidney, liver, and spleen[26]. This distribution pattern of metastasis corresponds well to that in human skin malignant melanomas in which the lung and lymph nodes are the most common sites of distant metastasis[33]. In the present study, the lung was extirpated from all dead mice and examined for pulmonary metastases. In the control groups, all mice died within 60 d, and pulmonary metastases were observed in two of five mice. On the other hand, three of five mice in the hyperthermia group survived to 120 days, and significant prolongation of overall survival was observed compared with control group ($p=0.024$).

Because multiple tumors (4.5 tumors on average) developed in the skin, the size of all tumors, including heated and non-heated tumors were measured. Previously, we observed a 'bystander effect' resulting from MCL-mediated hyperthermia in an experimental T-9 rat glioma model in which one tumor was transplanted into each femur of a rat ; although only one tumor was subjected to hyperthermia, the other tumor also disappeared completely[34]. In the present study, in order to investigate whether a bystander effects could be induced after RH in MT/*ret* transgenic mice, the sizes of all the tumors were measured. Representative data are shown in Figure 5. Each mouse (control group, Fig. 5A ; hyperthermia group, Fig. 5B) had eight tumors

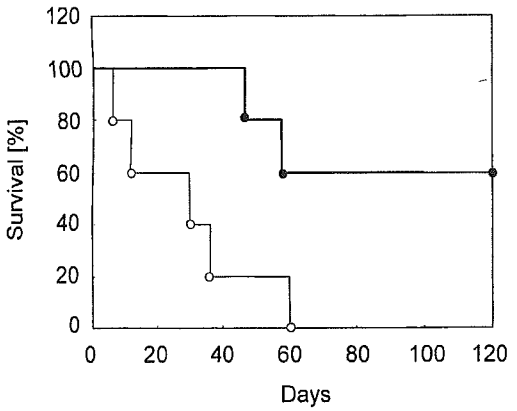

Fig. 4. Survival of MT/*ret* transgenic mice for a period of 120 d after the first hyperthermia treatment. Open circle, control mice ($n=5$) ; closed circle, RH-treated mice ($n=5$).

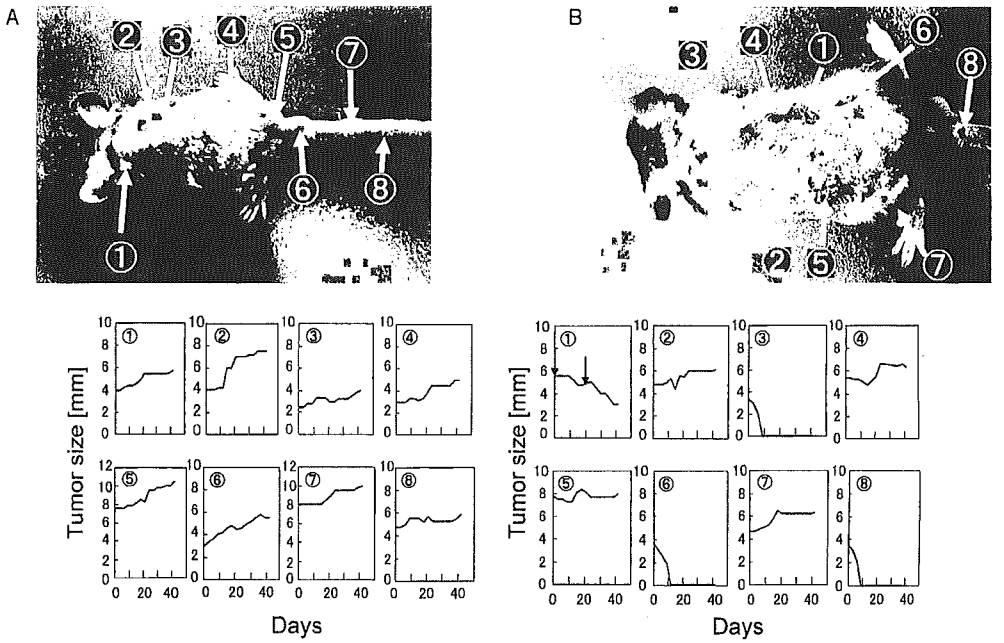

Fig. 5. Bystander effects of MCL-mediated hyperthermia on MT/*ret* transgenic mice. Mice bearing 8 tumors in the skin were used as representatives from the control group (*A*) and the hyperthermia group (*B*). Each tumor was measured over 40 days. For the hyperthermia group, MCLs (4 mg magnetite) were injected directly into a tumor (tumor no. 1 in *B*), which was then irradiated with an AMF for 30 min on day 0. AMF irradiation was repeated three times at 24-h intervals (RH). Because the tumor began to regrow, RH treatment was carried out after injection of MCLs (4 mg magnetite) on day 20. The timing of RH treatment is indicated by arrows in tumor no. 1 in *B*.

with sizes ranging from 2 to 8 mm, which were located in the back and tail. In the control mice, all tumors grew and no tumor regression was observed, as shown in Fig. 5A. In the hyperthermia group (Fig. 5B) on the other hand, although only one tumor (number 1 in Fig. 5B) was treated twice with RH, the other three tumors (two on the back and one on the tail) also regressed and disappeared.

Discussion

In the case of superficial tumors, such as melanoma, a simple heat mediator is desirable for the clinical application of hyperthermia. However, it is difficult to heat a superficial tumor specifically with a capacitive heating method using an RF electric field. Microwave hyperthermia causes severe injury, because the temperature of the surrounding epidermic tissue becomes substantially higher than that of tumor tissue. We used MCLs in order to heat the tumoral region and minimize heating of surrounding healthy tissue. The results shown in Figure 1 suggest that hyperthermia using MCLs allows tumors to be heated specifically. Tumor temperature was maintained very precisely within a small standard deviation, thus demonstrating the ease of temperature control by manipulating the magnetic field intensity. We previously demonstrated the efficacy of hyperthermia using MCLs against animals having several types of tumor, including B16 mouse melanoma[11]. In these cases, our hyperthermia system could specifically generate heat at the tumor site with magnetite during AMF irradiation, and no heat was

generated and no histological change was observed by only AMF irradiation in tissues without magnetite.

Although the thermal dose-response depends on the correlation between cell lines and microenvironmental factors, such as pH[35], we believe that, in principle, any type of tumor can be killed using MCL-mediated hyperthermia at higher temperatures. In the present study, the therapeutic effects of MCL-mediated hyperthermia on hereditary melanoma in MT/*ret* transgenic mice were investigated, and complete regression of all treated tumors was observed using an RH protocol. Although parts of the tumor containing sufficient amounts of MCLs were killed by heat, other parts of the tumor without MCLs or with insufficient amounts of MCLs, particularly at the tumor edge, may continue to grow. RH is effective in such cases of insufficient heating. In mouse no. 5, it took 3 rounds of RH to achieve complete tumor regression (Fig. 2). In a previous study, we showed that in MM46 mouse mammary carcinoma of size 7 mm, all tumors (5/5) disappeared when treated once with RH[13]. Because melanoma nodules in MT/*ret* transgenic mice, which generated spontaneously, had uneven shape when compared with MM46 tumors, differences in the number of rounds of RH treatment for complete regression were probably due to tissue shape.

This protocol can be clinically applied numerous times because of its ability to specifically heat the targeted region. However, in the case of repeated injection of MCLs, MCL toxicity may become an important issue. In a preliminary study, the toxicity of a single administration of MCL solution (33 mg of magnetite, i.p.) was investigated[36]. MCLs accumulated in the liver and spleen of mice, but none of the five observed mice died after MCL injection (unpublished results). In the present study, a total of 12 mg of magnetite was used in mouse no. 5 (Fig. 2), which was less than that used in the preliminary examination (33 mg). However, MCL toxicity should be fully investigated before the clinical application of RH.

Interestingly, bystander effects of MCL-mediated hyperthermia in hereditary melanoma were observed in MT/*ret* transgenic mice (Fig. 5B). Several investigators have demonstrated that hyperthermia treatment induces bystander effects. Matsumoto *et al.*[37] reported that nitric oxide (NO), which is known to be a multifunctional physiological substance, released from donor cells after hyperthermia induced p53 accumulation in the co-cultivated NO-recipient cells through intercellular signal transduction without cell-to-cell interactions, such as gap junctions, which led to bystander effects. On the other hand, we previously reported that the bystander effects induced by hyperthermia were caused by an antitumor immune response[34]. Heat shock proteins (HSPs) are highly conserved proteins whose syntheses are induced by a variety of stresses, including heat stress[38]. Recent reports have shown the importance of HSPs, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), in immune reactions[39]. In our previous study[11], expression of HSP70 was examined in the B16 melanoma nodules of both the hyperthermically treated mice (46°C for 30 min) and the non-treated mice on the next day after the treatment. HSP70 expression in the tumor tissue heated was 1.3 ± 0.1 ng/mg-tumor tissue, while 0.17 ± 0.06 ng/mg-tumor tissue was observed in the non-treated mice. As a mechanism for recognition of tumor antigens by the host immune system, we have proposed HSP-mediated antitumor immunity[40–42]; dying tumor cells killed by MCL-mediated hyperthermia release their intracellular contents, including tumor antigen peptides chaperoned by HSPs, and the HSP-peptide complexes are taken up by antigen presenting cells such as dendritic cells, and are in turn presented to T cells via MHC

class I and/or II antigens. The mechanism of these bystander effects in MT/*ret* transgenic mice is particularly important because MT/*ret* transgenic mice represent a hereditary cancer model that is thought to be good approximation of cancer patients, and we are now investigating the mechanism. These results suggest that MCL-mediated hyperthermia is potentially effective for malignant melanoma, because in addition to directly killing tumors with heat, they induce a bystander effect on distant metastases.

In summary, the stable tumoral treatment temperatures, therapeutic effects, and survival benefits demonstrate the feasibility of RH using MCLs in a hereditary melanoma model. The efficacy of bystander effects remains to be confirmed in further experiments.

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正電荷リポソーム包埋型マグネタイトを用いた繰り返しハイパーサーミアによるマウス遺伝性メラノーマの完全退縮

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要　旨：正電荷リポソーム包埋型マグネタイト（MCL）は交番磁場中で発熱することから，細胞内加温法のための発熱素子として利用されている．今までに，様々なガンの移植モデルで高い治療効果が得られているが，全て可移植性腫瘍における検討であり，実際のガン患者の状態に近い遺伝性腫瘍を用いた検討は行っていない．本研究では，metallothionein-I／*ret* を導入したトランスジェニックマウスの遺伝性メラノーマに対する MCL を用いた温熱療法の抗腫瘍効果を調べた．MCL を 5-7mm 径の腫瘍に投与して，交番磁場を 30 分間照射した．腫瘍温度は 5 分で 45°C に上昇し，そこからは磁場強度を調節することで 45°C に一定に保った．温熱療法は 24 時間間隔で 3 回繰り返して行い（repeated hyperthermia, RH），RH は腫瘍が完全に退縮するまでに行った．腫瘍の完全退縮は 1 回から 3 回の RH を行うことで達成された．さらに，RH で完全退縮した腫瘍は，治療 120 日後まで再増殖せず，また，有意な生存の延長がみられた．これらの結果は，MCL を用いた温熱療法はメラノーマに対する強力な治療法になることを示唆している．

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Heat Immunotherapy Using Magnetic Nanoparticles and Dendritic Cells for T-Lymphoma

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Dendritic cells (DCs) are potent antigen-presenting cells that play important roles in regulating immune responses in cancer. Immunotherapy using these immunocytes has become an accepted therapeutic modality. We previously reported that hyperthermia using magnetic nanoparticles induces antitumor immunity, which could be activated by adjuvant including cytokines. In the present study, we investigated the therapeutic effects of hyperthermia combined with DC immunotherapy on mouse EL4 T-lymphoma. 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 an EL4 nodule in C57BL/6 mice, which were subjected to AMF for 30 min. The temperature at the surface of the tumor reached 45°C and was maintained by controlling the magnetic field intensity. Hyperthermia treatment was repeated twice with 24 h intervals. After hyperthermia, immature DCs were directly injected into the EL4 nodule. As a result, complete regression of tumors in 75% (6/8) of the mice was observed, while the percentage of complete regression of tumors was 12.5% (1/8) in the case of mice treated by hyperthermia alone. This novel cancer therapy, which we have termed "heat immunotherapy", may be applicable to patients with advanced malignancies.

[**Key words:** cancer therapy, dendritic cell, immunotherapy, hyperthermia, magnetite cationic liposome]

Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients (1). Since magnetite nanoparticles generate heat in an alternating magnetic field (AMF) due to hysteresis loss, we have developed magnetite cationic liposomes (MCLs) for intracellular hyperthermia (2–4). MCLs were developed in order to improve adsorption by and accumulation in tumor cells due to their electrostatic interaction with the cell membrane (2).

Hyperthermia is often used in multi-modality therapy, including in combination with immunotherapy with interleukins (5) or interferons (6). Some researchers have reported that heat treatments themselves enhance the immunogenicity of cancer cells (7, 8). We have also earlier reported on the antitumor immunity induced by our hyperthermia system (9–11) and antitumor effect of combination therapy using hyperthermia and immunotherapy with cytokines such as interleukin-2 (IL-2) and granulocyte macrophage-colony stimulating factor (GM-CSF) (12). Thus, increasing knowledge concerning the mechanisms involved in immune reactions against cancer cells has lead to the development of novel immunotherapeutic approaches for the treatment of malignant cancer patients. In the present study, we employed mouse EL4 T-lymphoma in order to investigate the immunotherapeutic effect. The EL4 cell line, isolated from a chemically-induced lymphoma over 50 years ago, has been extensively utilized in immunological research.

Dendritic cells (DCs) are potent antigen presenting cells (APCs) and can induce an immune response to tumors via antigen uptake and maturation (13). Mature DCs stimulate $CD4^+$ T cells, $CD8^+$ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells via direct cell–cell interactions and/or cytokine production (14, 15). The efficient isolation and preparation of both human and murine DCs is now possible (16, 17). However, the use of mature DCs requires efficient methods to incorporate antigens into DCs because mature DCs lose their ability to take up antigens. To date, several methods for induction of antitumor immunity by DCs have been investigated. The major methods for antigen loading into DCs are pulsing DCs with proteins or peptides extracted from tumor cells (18), transfecting DCs with gene encoding tumor antigens (19), fusing DCs with tumor cells (20), or culturing DCs with tumor cells (21). Although some of these methods require primary culture of tumor cells from patients, primary cultures often fail to give the re-

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quired number of cells, or expanded tumor cells often show different antigens from those expressed in tumor specimens due to changes in immunological characteristics over the long culture period for expansion. In addition, it is difficult to repeatedly obtain tumor specimens from patients with cancer. One of the methods to overcome this problem is not to rely on antigen loading of DCs *in vitro* but on that *in situ* by intratumoral injection of immature DCs. It has been reported that intratumoral injection of immature DCs induced antitumor effects (22, 23). Based on these results, we directly injected immature DCs into tumor after hyperthermia to activate antitumor immunity induced by hyperthermia and investigated the antitumor effect as a novel combination therapy comprising hyperthermia with MCLs and immunotherapy with DCs.

Immature DCs were cultured from the bone marrow of C57BL/6 mice (Charles River Japan, Yokohama) according to a protocol described by Inaba *et al.* with slight modification (24). Briefly, bone marrow was harvested from the femur and tibia of sacrificed mice. To eliminate erythrocytes, dead cells, and debris from murine bone marrow, cells were collected and suspended in RPMI 1640 medium, the same volume of Lympholyte-M (Cedarlane, Ontario, Canada) in the medium was underlaid, and this was centrifuged at 1200×*g* for 20 min at room temperature. After centrifugation, cells including DCs at the interface were collected and washed with medium. The cells were then plated in 100-mm cell culture dishes at a concentration of 10^7 cells in 10 ml of RPMI 1640 medium supplemented with 10 ng/ml recombinant mouse (rm) GM-CSF (Peprotech, Rocky Hill, NJ, USA) and 10 ng/ml rmIL-4 (Peprotech). Medium exchange was carried out every 2 d by gently swirling the plates, aspirating 75% of the medium, and adding fresh medium with rmGM-CSF and rmIL-4. On day 7, non-adherent and loosely adherent cells were collected and used as immature DCs.

Mouse T-lymphoid EL4 cells were grown in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS), 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G. Cells were grown at 37°C in an atmosphere containing 5% CO_2 and 95% air. To prepare tumor-bearing mice, 1×10^6 EL4 cells were injected subcutaneously into the right flank of C57BL/6 mice (age, 4 weeks), which were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). EL4 nodules that had grown to 6 mm in diameter were used for experiments. Tumor diameter was measured every 3 d and the average 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 Prime Minister of Japan.

MCLs were prepared from a colloidal magnetite and lipid mixture consisting of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (Sogo Pharmaceutical, Tokyo), dilauroylphosphatidylcholine, and dioleoylphosphatidylethanolamine (Sigma Chemical, St. Louis, MO, USA) in a 1:2:2 molar ratio, as described previously (2).

Tumor-bearing mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). Under anesthesia, a syringe (26 G needle) containing MCLs was inserted longitudinally into each tumor subcutaneously from the tumor edge. MCLs (net magnetite amount, 2 mg) were injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL, USA) for 30 min. Tumor-bearing mice were then separated into four groups. Mice in groups I (control) and III (DC therapy) were not subjected to an alternating magnetic field (AMF). After injection of MCLs, mice in groups II (hyperthermia) and IV (hyperthermia + DC therapy) were subjected to AMF for 30 min once daily for 2 d in a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency, Tokyo). Magnetic field frequency and intensity were 118 kHz and 384 Oe, respectively. Tumor and rectal temperatures were measured by optical fiber probe (FX-9020; Anritsu Meter). One day after irradiation, the tumors of mice in groups III (DC therapy) and IV (hyperthermia + DC therapy) were injected with 2×10^6 immature DCs.

Tumor temperature increased rapidly to 45°C in 5 min and was then maintained for 30 min by controlling AMF intensity (data not shown). In contrast, the temperature in the rectum increased only slightly during AMF irradiation. This suggests that our hyperthermia system using MCLs is able to heat the tumor specifically without damaging healthy tissue. The tumor temperature was maintained very precisely, thus demonstrating the ease of temperature control within the tumor by adjusting the power of the AMF generator. Moreover, thermographic images of the mice during hyperthermia revealed that the whole tumor area was heated with an accuracy of less than 1°C (data not shown).

The therapeutic effects of MCL-induced hyperthermia with immature DC therapy were assessed *in vivo*. Figure 1 shows the time courses of tumor growth. In group I (control), tumors grew progressively. In group II, in which mice received only hyperthermic treatment, a tumor in only 1 of 8 mice regressed completely, while the tumors in the other mice grew. When mice were treated with immature DCs (group III), tumors grew progressively and no complete regression was observed. On the other hand, when MCL-induced hyperthermia was combined with immature DC injection (group IV), tumor growth was suppressed and 75% (6/8) of the subcutaneous tumors regressed completely.

EL4 is known to be a highly metastatic cancer and mice tend to die from metastases in lymph nodes after tumor inoculation. The survival rates of tumor-bearing mice over a period of 100 d after injection of MCLs are shown in Fig. 2. In groups I and III, all mice died from pulmonary metastases and/or an enlarged tumor at the inoculated site within 32 d. Survival was slightly prolonged in group II (hyperthermia), and 12.5% of the mice survived for about 70 d. In group IV (hyperthermia + DC therapy), survival was prolonged more than in mice in other groups, and 25% of the mice survived over 100 d without a metastasis. These results indicated that the antitumor effects of hyperthermia using MCLs were enhanced by DC therapy.

Although hyperthermia induces cell death via necrosis (in

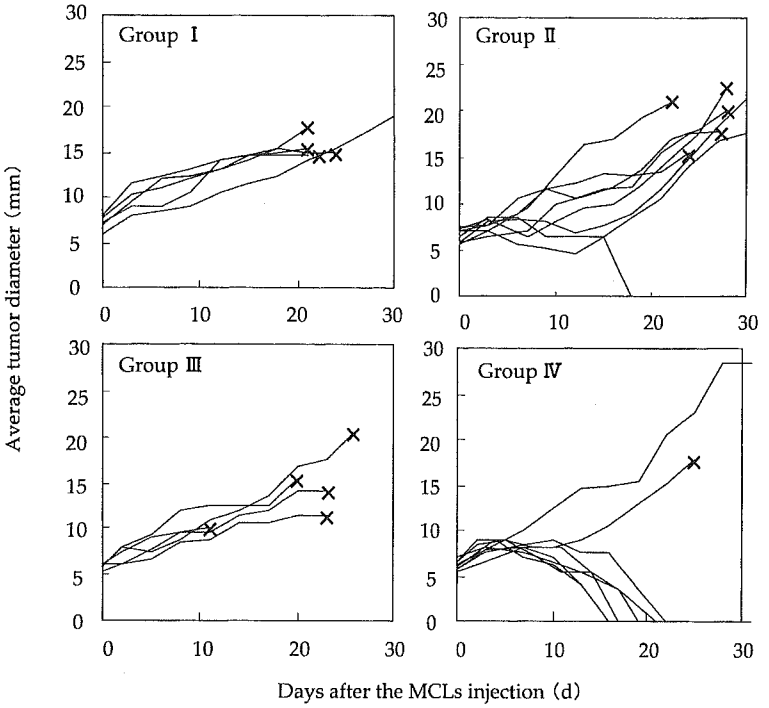

FIG. 1. Antitumor effects of combination therapy of MCL-induced hyperthermia with immature DC therapy. Mice in each group were treated as described in Materials and Methods. Group I, Control, n=5; group II, hyperthermia, n=8; group III, DC therapy, n=5; and group IV, hyperthermia + DC therapy, n=8. Each line represents tumor growth kinetics in a single mouse. Crosses indicate when each mouse died.

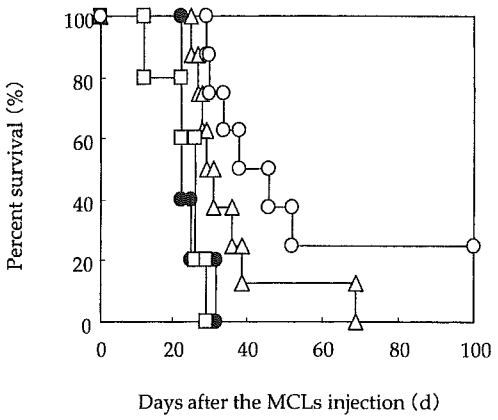

FIG. 2. Percent survival of tumor-bearing mice over a period of 100 d after hyperthermia. Closed circles, Group I (control, n=5); open triangles, group II (hyperthermia, n=8); open squares, group III (DC therapy, n=5); and open circles, group IV (hyperthermia + DC therapy, n=8).

principle, in any type of tumor cell), the heating process is generally quite mild because of potential adverse effects on non-target organs or tissues. On the other hand, our intracellular hyperthermia treatment was able to heat the tumor specifically by means of the MCLs. Moreover, the degree of heat generated in the tumor can be controlled via the magnetic field intensity, which makes it possible to induce necrotic cell death without damaging the surrounding normal tissues. In the present study, we set the tumor temperature at 45°C, but this was insufficient to destroy the lymphoma nodules. Our hyperthermia system can achieve higher temperatures and can be applied repeatedly without damaging healthy tissue. For example, complete regression of B16 melanoma was previously observed in 90% of mice using our hyperthermia system once daily for 2 d at 46°C (25). In the present study, the treatment temperature was set to 45°C in order to examine the effects of combining hyperthermia with immature DC therapy.

The mechanism of synergy in combination therapy of hyperthermia and immature DC injection has not been fully elucidated and we are now investigating the mechanism. It is known that necrotic tumor cells induced DC maturation (26). Yonezawa *et al.* reported that heating tumor cells at higher temperatures resulted in necrotic cell death (27). We previously demonstrated that MCL-induced hyperthermia resulted in tumor necrosis (10, 11), because our hyperthermia system is able to heat the tumor specifically at higher temperatures. We speculate that the dying tumor cells, which are killed by hyperthermia treatment, release their intracellular contents, including tumor antigen peptides, and the released antigenic peptides are taken up by immature DCs injected directly into the tumor and are in turn presented to T cells via MHC class I and/or II antigens by the DCs.

In the present study, we have described a novel combination therapy comprising hyperthermia with MCLs and immunotherapy with DCs. We showed the antitumor effect of hyperthermia was improved by intratumoral injection of immature DCs. This study has important implications for the application of MCL-induced hyperthermia plus DC therapy

as a treatment for malignant cancer patients and the prevention of cancer recurrence.

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Screening of Cytokines to Enhance Vaccine Effects of Heat Shock Protein 70-Rich Tumor Cell Lysate

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Heat shock proteins (HSPs) have been recognized as significant participants in immune reactions. We have previously reported that heat-treated cells expressing HSP70 can mediate potent antitumor immune responses. As successful immunotherapy is dependent on the host immune system, the present study evaluated whether systemic administration of immunocyte stimulatory and growth promoting cytokines could enhance heat-treated cell lysate vaccine (HCLV) immunization to further promote the antitumor immunity. After heating mouse melanoma B16 cells (43°C, 30 min) to elicit increased HSP70 expression, cells were lysed by freeze thawing to prepare HCLV. In approaches using a poorly immunogenic melanoma B16, the effects of various cytokines (IL-1β, -2, -4, -6 and -12, IFN-β and -γ, GM-CSF and TNF-α) were assessed in combination with HCLV. Syngenic C57BL/6 mice were immunized subcutaneously with HCLV twice, on days −14 and −7, while cytokines were injected intraperitoneally on day −7. Subcutaneous B16 cell challenge was performed on day 0. IL-12 significantly enhanced the efficacy of HCLV, compared to non-heated cell lysate vaccine (CLV) and non-vaccination. Systemic administration of recombinant IL-12 augmented the efficacy of HCLV, inducing protective immunity against tumor challenge and enhancing cytotoxicity assessed in primed splenocytes against B16 cells in treated mice. These results suggest that IL-12 represents an important modulator of antitumor immune responses induced by HCLV, and may facilitate further efforts to develop novel cancer immunotherapies based on HSP70-mediated vaccination.

[**Key words:** cancer vaccine, interleukin-12, heat shock protein, cancer immunotherapy, melanoma]

Cancer vaccines may represent a promising new approach for cancer immunotherapy. However, preclinical and clinical evidence has shown that therapeutic effects can be polarized by vaccination strategies, and no anticancer vaccine can currently be recommended outside of clinical trials (1). Novel strategies for cancer vaccines are thus highly desirable, and new targets for cancer vaccines may involve two important characteristics: high immunogenicity; and multivalency.

Heat shock proteins (HSPs) are highly conserved proteins the synthesis of which is induced by various stressors, including heat stress (2). HSPs act as chaperones for peptides, including peptides derived from tumor-associated antigens (3, 4). Tumor-derived HSPs, such as HSP70, HSP90 and glucose-regulated protein 96, have been shown to elicit cancer immunity (5, 6). HSPs can be isolated and used as a multivalent, autologous vaccine preparation with undefined tumor-associated antigens (7), removing the need to identify the epitopes of tumor-associated antigens. In addition, HSP70 itself acts as a cytokine, activating polymorphonuclear cells to produce proinflammatory cytokines (8). This suggests that HSPs are natural adjuvants involved in innate immunity. HSP-peptide complexes thus offer the possibility of highly immunogenic and multivalent cancer vaccines.

Some researchers have reported that heat treatments themselves enhance the immunogenicity of cancer cells in vaccine preparations (9–11). We have previously investigated the role of a single defined HSP, inducible HSP70, and shown that the mechanism of immunogenic augmentation against cancer after heat treatment is associated with HSP70 expression (12, 13). We and others have previously shown that HSP70 upregulation in tumor cells or HSP70-rich cell lysates, through heat- or drug-induced stress or by gene transfection, increases tumor immunogenicity and protects animal models from challenge by wild-type tumor (14–17). The present study aimed to extend our previous study using a vaccine prepared from HSP70-expressing cells by attempting to combine immunocyte stimulatory and growth promoting cytokines as a novel strategy for HSP70-mediated cancer vaccines.

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MATERIALS AND METHODS

Cell lines and mice Mouse B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G. Mouse T-lymphoid EL4 cells were grown in RPMI 1640 medium (Gibco BRL) containing 10% fetal calf serum, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G. Cells were grown at 37°C in an atmosphere containing 5% CO_2 and 95% air. Female C57BL/6 mice (4 weeks old) were purchased from Charles River Japan (Yokohama).

Heat treatment Heat treatment of cultured cells was performed as previously reported (18). Briefly, B16 cells in the logarithmic growth phase were heated by direct immersion of cell culture dishes in a temperature-controlled water bath. The temperature of the medium was rapidly increased, reaching the required temperature (37–45°C) within 5 min. The temperature of the medium was monitored using an FX-9020 fiberoptic thermometer probe (Anritsu Meter, Tokyo).

Assay for HSP70 expression After heat treatment, cells were collected by detachment with trypsin/EDTA, washed 3 times in PBS and resuspended at 5×10^5 cells in 0.1 ml of PBS. Disruption of cells was performed using 5 cycles of freeze thawing with vigorous mixing using a vortex mixer (Scientific Industries, Bohemia, NY, USA), resulting in the cell lysate. Inducible HSP70 in cell lysates was assayed using an HSP70 enzyme immunoassay (EIA) Kit (StressGen Biotechnologies, British Columbia, Canada), which can detect and quantify levels of inducible HSP70 in both human and mouse samples. ELISA was performed according to the manufacturer's instructions.

Inducible HSP70 expression was also investigated using Western blotting analysis. Cell lysates of B16 cells (5×10^5) were subjected to SDS–PAGE on a 7.5% polyacrylamide gel. Protein blots transferred onto a nitrocellulose membrane were incubated with mouse monoclonal anti-HSP70 antibody (clone, C92F3A-5; StressGen Biotechnologies) specific to the inducible form of HSP70 (HSP72), for 2 h at 37°C, probed with peroxidase-labeled goat anti-mouse IgG for 1 h at 37°C, and then visualized using ECL™ (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Vaccine preparation and immunization For preparation of the heat-treated cancer cell vaccine, B16 cells were heated at 43°C for 30 min. After a 12-h incubation at 37°C, heated or nonheated B16 cells were collected following detachment with trypsin/EDTA, washed 3 times in PBS and resuspended at the specified cell number in 0.1 ml of PBS. Cell lysates prepared by freeze thawing as described above were referred to as heat-treated cell lysate vaccine (HCLV) and non-heated cell lysate vaccine (CLV), respectively.

For prophylactic vaccinations, mice were immunized subcutaneously in the right flank with HCLV, CLV or PBS (control), once at 14 d before tumor challenge (day −14), and once on day −7. Tumor challenge was performed by subcutaneous injection of B16 or EL-4 cells (5×10^5 cells in 0.1 ml PBS) in the left flank on day 0.

Cytokines Recombinant mouse cytokines, such as interleukin (IL)-1β, -4 and -6, interferon (IFN)-γ, granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)-α were purchased from PeproTech EC (London, UK). Recombinant human IL-2, mouse IL-12, and mouse IFN-β were purchased from Wako Pure Chemical Industries (Osaka), Techne (Minneapolis, MN, USA), and PBL Biomedical Laboratories (Piscataway, NJ, USA), respectively. Cytokines were intraperitoneally injected into mice at a concentration of 4 μg in 0.1 ml of PBS on day −7.

Assessment of vaccine efficacy After subcutaneous tumor challenge, tumor take was defined when a palpable tumor of >2 mm could be measured using a caliper. Percentages of tumor-free mice were compared with the log-rank test using WinSTAT software (Light Stone International, Tokyo) and values of $P<0.05$ were considered significant.

In order to assess systemic immune responses, an *in vitro* cytotoxicity assay was performed 14 d after the first immunization (day 0). Splenocytes were isolated from mice using the Medimachine System (Dako A/S, Glostrup, Denmark). Erythrocytes in splenic lymphocyte preparations were depleted using 0.75% ammonium chloride. Cytotoxic activity was determined using a lactate dehydrogenase (LDH; Promega, Madison, WI, USA) release assay according to the instructions of the manufacturer. Briefly, target cells (5×10^4 B16 cells) were mixed with effector cells at an effector: target ratio of 50:1, and co-cultured for 4 h at 37°C in an atmosphere containing 5% CO_2. Release of LDH was then evaluated. Cytotoxicity was calculated as follows:

$$\text{cytotoxicity (\%)} = [(OD_{\text{experiment}} - OD_{\text{effector spontaneous}} - OD_{\text{target spontaneous}})/(OD_{\text{target maximum}} - OD_{\text{target spontaneous}})] \times 100$$

Animal experiments were performed according to the principles outlined in the Guide for the Care and Use of Laboratory Animals prepared under the direction of the Office of the Prime Minister of Japan.

RESULTS

Generation of HCLV Inducible HSP70 expression was examined in B16 melanoma cells that were subjected to heat shock. Figure 1A shows the results from the ELISA for HSP70 in cell lysates using different temperatures (Fig. 1A-I), heating times (Fig. 1A-II) and incubation periods after heating (Fig. 1A-III). B16 cells expressed the largest amount of HSP70 after heating at 43°C for 30 min with a 12-h incubation period, and expression of HSP70 was also detected by Western blot analysis (Fig. 1B). Conversely, inducible HSP70 expression in non-heated cells was barely detected by ELISA or Western Blot analysis. Consequently, for preparation of HCLV in the subsequent experiments, B16 melanoma cells were heated at 43°C for 30 min with a 12-h incubation period. CLV was used as a control.

Figure 1C shows the efficacy of HCLV against challenge with B16 cells. For mice vaccinated with CLV, all mice (n=5) displayed tumor take within 10 d. For mice vaccinated with HCLV, no significant delay in tumor take was observed compared to mice vaccinated with CLV, suggesting that vaccination with HCLV alone does not significantly activate immune responses against B16 melanoma.

Cytokine panel for screening HCLV-oriented immunomodulators Successful immunotherapy depends on the activation of host immune cells. Thus, we next investigated whether systemic administration of immunocyte stimulatory and growth promoting cytokines could enhance HCLV efficacy, to generate a novel cancer vaccine comprising an HSP70-rich whole-cell lysate and specific cytokines. To facilitate rapid screening of various cytokines for their influence on the vaccine effects of HCLV, a cytokine panel was used (comprising nine cytokines at doses of 4 μg in 0.1 ml PBS; intraperitoneal; Fig. 2). Mice were immunized with HCLV, CLV or PBS (control) on days −14 and −7, and were also administered cytokines on day −14. When live B16 cells (5×10^5 cells) were inoculated subcutaneously into mice on day 0, all mice treated with PBS displayed tumor take

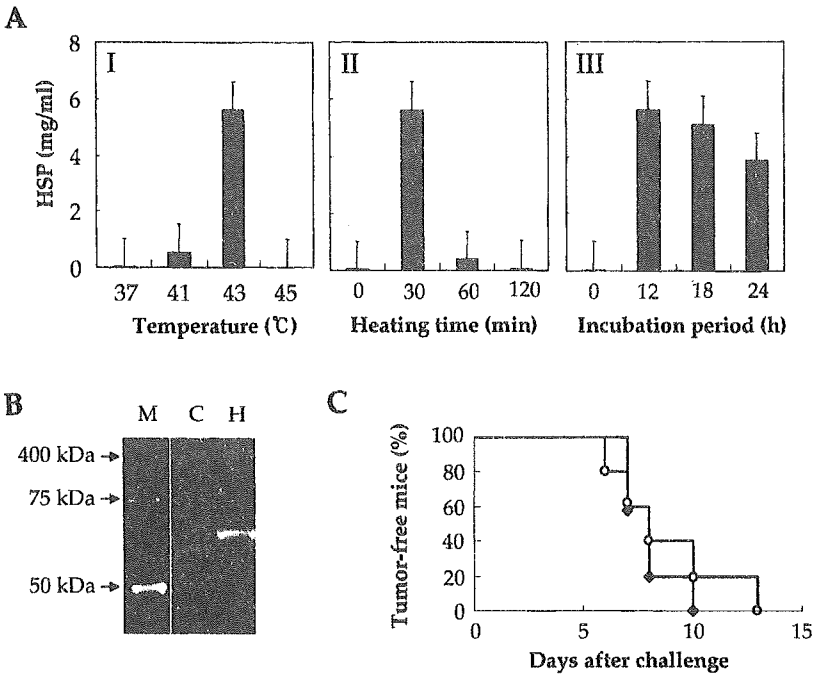

FIG. 1. Generation of heat-treated cell lysate vaccine (HCLV). B16 cells were heated by direct immersion of cell culture dishes in a temperature-controlled water bath. The temperature was varied from 37°C to 45°C (A_I). After heating at the indicated temperature for 30 min, cells were cultured for 12 h, then mechanically disrupted and subjected to ELISA for inducible HSP70. Heating time was varied from 0 to 120 min (A_{II}). Here, temperature and incubation period after heating were fixed at 43°C and 12 h. The incubation period after heating at 43°C for 30 min was also varied from 0 to 24 h (A_{III}). Data represent the mean and standard deviation of three independent experiments. Western blotting analysis was performed after heat treatment of B16 cells at 43°C for 30 min with a 12-h incubation period (B). M, Marker; C, unheated cells (control); H, heat-treated cells. (C) Vaccine effect of HCLV against challenge by B16 cells. Unheated cell lysate vaccine (CLV, closed rhombuses) or HCLV (open circles) was injected subcutaneously into the right flank of mice on days −14 and −7, and B16 tumor challenge was performed in the left flank of immunized mice on day 0.

within 10 d. When mice were immunized with cell lysate vaccines (HCLV or CLV) plus IL-1β, IL-4, IL-6 or IFN-γ, 20–60% of mice remained tumor-free by day 25. However, log-rank testing indicated that these delays in tumor formation were generally not significant, and only mice treated with HCLV plus IL-12 (P=0.012) or IL-2 (P=0.012) showed significant improvements compared to mice treated with PBS (Table 1). Furthermore, only mice treated with IL-12 showed significant improvement with HCLV compared to CLV (P=0.038), suggesting that IL-12 conferred some protective benefits against tumorigenesis on hosts treated with an HSP70-mediated vaccine.

TABLE 1. Evaluation of significant differences in percent tumor-free mice

Cytokine	P value	
	HCLV vs PBS	HCLV vs CLV
IL-12	0.012[a]	0.038[a]
IL-2	0.012[a]	0.790
GM-CSF	0.107	0.385
IL-6	0.251	0.606
IL-4	0.285	0.528
IL-1β	0.409	0.828
IFNγ	0.480	0.701
IFNβ	0.509	0.414
TNFα	0.951	0.787

[a] Significant difference by log rank test, P<0.05.

Vaccine effects of HCLV plus IL-12 Only IL-12 resulted in significantly delayed tumor take in hosts vaccinated with HCLV, but not with CLV. As a result, the vaccine effects of HCLV plus IL-12 were further investigated. Figure 3 shows the effects of vaccination dose on tumor rejection. IL-12 alone did not confer any systemic protection. However, when IL-12 administration was combined with a vaccine (CLV or HCLV), dose-dependent increases were observed in the percentage of tumor-free mice on day 25. For each vaccination dose tested in the present study (cell lysates comprising 5×10^5, 1×10^6, and 5×10^6 heated or unheated B16 cells), percentages of tumor-free mice on day 25 were higher for mice treated using HCLV plus IL-12 than for mice treated using CLV plus IL-12. Moreover, when mice were treated with HCLV prepared using 5×10^6 cells and IL-12, 100% of mice (5/5) were protected from tumor formation.

Immune responses were examined in the splenic cells of mice that received vaccination with HCLV (5×10^6 cells) plus IL-12. Splenic lymphocytes from mice treated with HCLV plus IL-12 showed high cytotoxic activity against B16 melanoma cells, approximately 3-fold greater than splenic cells from mice treated with CLV plus IL-12 (Fig. 4A). To investigate whether tumor-specific immune responses were activated in mice treated with HCLV plus IL-12, irrelevant live mouse syngenic T-lymphoid EL4 cells were inoculated into mice that received HCLV plus IL-12. While B16 cells were

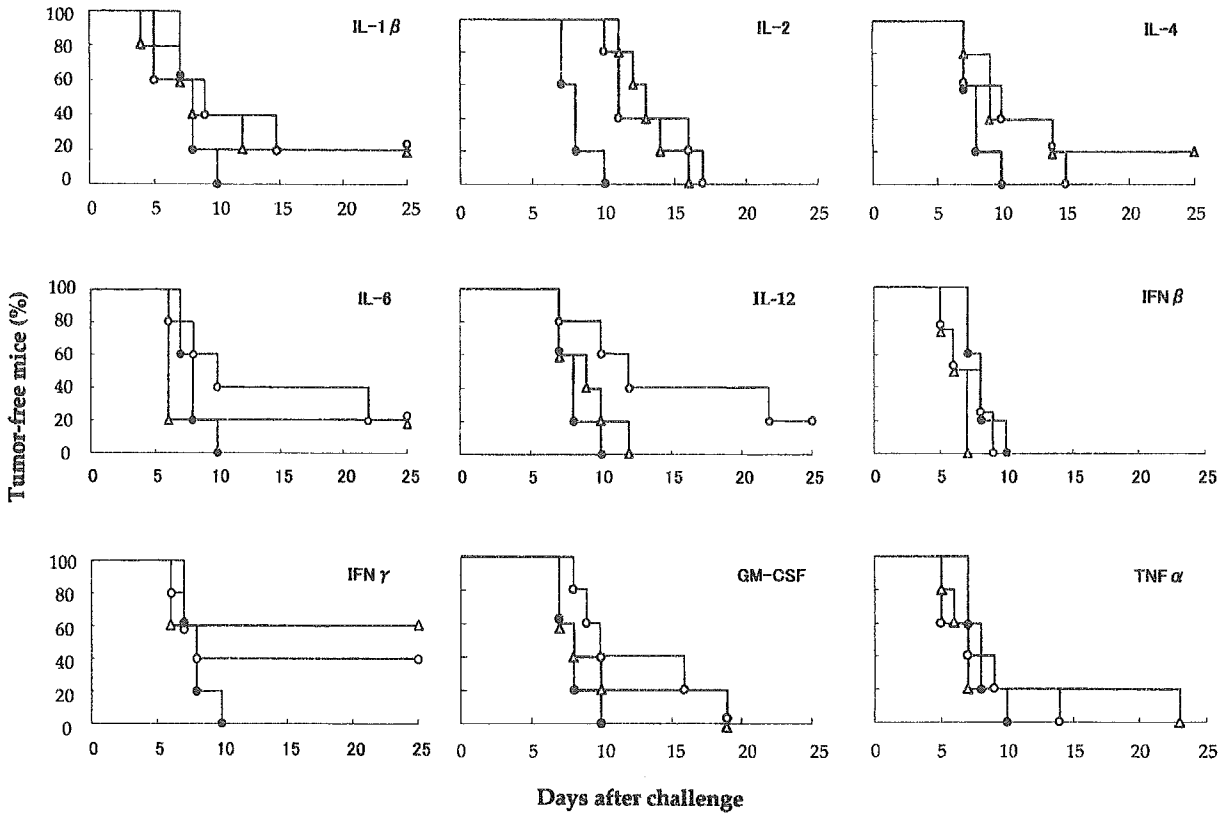

FIG. 2. Panel analysis for screening of cytokines effective in combination with HCLV from among nine candidate cytokines. Subcutaneous immunization was performed in the right flank of mice with HCLV (open circles) or CLV (open triangles) on days −14 and −7. Recombinant cytokines (IL-1β, -2, -4, -6 or -12, IFN-β or -γ, GM-CSF, or TNF-α, at 4 μg in 0.1 ml of PBS) were intraperitoneally injected into mice on day −14. Subcutaneous tumor challenge by B16 cells (5×10^5 cells) was performed into the left flank on day 0. As a non-vaccination control, mice were injected with PBS on days −14 and −7 (closed circles). Each group contained five mice.

rejected in 100% of these mice (5/5), EL-4 cells were rejected in only 20% (1/5) as of day 25, suggesting that immunization with HCLV plus IL-12 induced tumor-specific antitumor immunity (Fig. 4B).

DISCUSSION

The present study was motivated by the large number of recent studies showing that HSP-based cancer vaccines, including HSP-peptide complexes, and heat-treated cells or lysates, could activate antitumor immune responses in both animals and humans. We have previously shown augmentation of immunogenicity via HSP70 expression following heat treatment, and sole administration of heat-treated T-9 rat glioma cells resulted in activation of the systemic immune response (12). In the present study, however, sole administration of HCLV did not activate immune responses against B16 melanoma cells (Fig. 1C). B16 mouse melanoma cells are known to be poorly immunogenic due to the expression of low amounts of the H-2 antigen, and are thus widely utilized in immunological experiments to assess immunotherapeutic effects (19–21). We have previously studied the amounts of H-2K^b on B16 cells using flow cytometry (22). Flow cytometric analysis using anti-mouse H-2K^b antibody revealed that the B16 cells used in the present study express very low amounts of MHC class I antigen. The present study used poorly immunogenic B16 cells to examine the preclinical feasibility of cancer vaccines.

Evidence is accumulating regarding the important role of HSPs in initiating immune responses against cancer. Some researchers have suggested that the mechanisms of tumor cell death *in vivo* may be directly related to the induction of the host immune response, and that HSP expression may provide just such an immunological induction signal (23–25). HSP70-peptide complexes released from dying tumor cells activate and transfer antigenic peptides to professional antigen-presenting cells (APCs) (26). Released HSP-peptide complexes encounter APCs that express receptors such as CD91 (27), CD40 (28), and Toll-like receptors 2 and 4 (29). The interaction of HSP-peptide complexes with these receptors leads to receptor-mediated endocytosis, processing of antigenic peptides by the endogenous MHC class I pathway, and re-presentation on the cell surface to CD8-positive T-cell receptors (30). In addition, HSP70 acts to directly activate APCs, stimulating cytokine secretion from monocytes and inducing the maturation of dendritic cells (DCs) via CD14 and CD91 receptors (8). These findings indicate that successful immunotherapy based on HSP-mediated vaccines depends on host immune cells, including DCs and cytolytic T lymphocytes (CTLs), so immunocyte stimu-

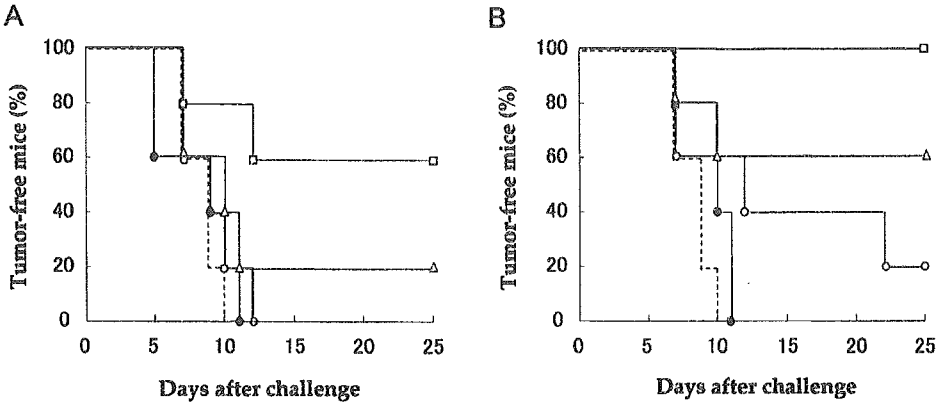

FIG. 3. Vaccination dose-dependent effects of HCLV plus IL-12 on tumor protection. On days −14 and −7, subcutaneous immunization was performed into the right flank of mice with CLV (A) or HCLV (B) at 5×10^5 cells (open circles), 1×10^6 cells (open triangles) or 5×10^6 cells (open squares). Recombinant IL-12 (4 μg in 0.1 ml of PBS) was intraperitoneally injected into mice on day −7. Subcutaneous tumor challenge using B16 cells (5×10^5 cells) was performed in the left flank on day 0. As cell lysate-free controls, mice were injected with PBS on days −14 and −7 and IL-12 was injected into mice on day −7 (IL-12 alone, closed circles) or mice were injected with PBS on days −14 and −7 (non-immunization, dotted line). Each group contained five mice.

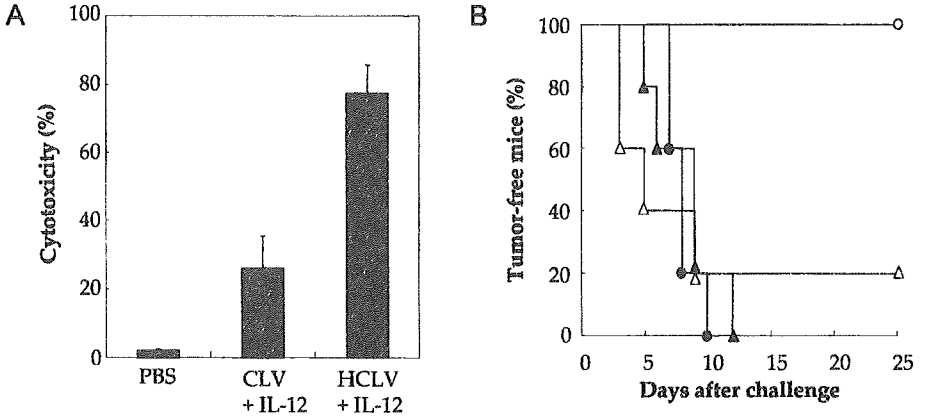

FIG. 4. Systemic immune responses induced by vaccination with HCLV plus IL-12. *In vitro* cytotoxicity of splenic lymphocytes from C57BL/6 mice injected with PBS, CLV plus IL-12, or HCLV plus IL-12 on days −14 and −7 (A). Splenic lymphocytes from mice were prepared on day 0, and cytotoxicity against B16 cells at an effecter-to-target ratio of 50:1 was assayed. Data represent mean and standard deviation (n=3). Tumor-specific protection by vaccination of HCLV plus IL-12 was investigated using irrelevant syngenic mouse EL-4 cells (B). On days −14 and −7, subcutaneous immunization was performed into the right flank of mice with HCLV containing 5×10^6 B16 cells. Recombinant IL-12 (4 μg in 0.1 ml of PBS) was intraperitoneally injected into mice on day −7. Subcutaneous tumor challenge using B16 cells (5×10^5 cells, open circles) or EL-4 cells (5×10^5 cells, open triangles) was performed into the left flank on day 0. As non-vaccination controls, mice were injected with PBS on days −14 and −7 and B16 cells (5×10^5 cells, closed circles) or EL-4 cells (5×10^5 cells, closed triangles) were injected into mice on day 0. Each group contained five mice.

latory and growth promoting cytokines should enhance the antitumor effects induced by HSP-mediated vaccines. However, since much preclinical and clinical evidence has shown that the immune system can be polarized by several vaccination strategies, specific strategies for HSP-mediated vaccines are required. To the best of our knowledge, no previous studies have compared the relative activity of different cytokines in combination with an HSP-mediated vaccine. The present investigation involved the construction of a cytokine panel comprising nine cytokines, and comparison of the ability of these cytokines to enhance antitumor activities against mouse B16 melanoma cells (Fig. 2). Our previous study combined immunotherapy using GM-CSF or IL-2 with hyperthermia to enhance vaccine-like effects induced by hyperthermia (22). Both GM-CSF and IL-2 were found to be effective in that combination therapy. GM-CSF reportedly plays an important role in the activation of APCs (31). Dranoff *et al.* reported that vaccination with irradiated GM-CSF-transduced B16 melanoma cells induced a potent systemic immune response against B16 cells (32). In the present study, only a slight delay in tumor take for HCLV-treated mice was observed with GM-CSF (Fig. 2), and log-rank testing revealed that GM-CSF was ranked third among the nine tested cytokines (Table 1). For IL-2, although a significant improvement compared to unvaccinated mice (PBS injection) was obtained, no significant difference was identified between mice treated with HCLV or CLV ($P=0.79$, Table 1). Since IL-2 displays a broad range of immunological effects, such as inducing CTLs, specific T helper cells, natural killer (NK) cells and lymphokine-activated killer

cells (33), the immunostimulatory effects of both HCLV and CLV were presumably exerted.

The most important finding from the present investigation was that IL-12 represented the most effective immunostimulant for HCLV among the nine cytokines tested. IL-12, a cytokine with important immunoregulatory functions, stimulates T cells and NK cells to produce IFN-γ, and increases cytotoxic activity by NK cells and CTLs (34, 35). In addition, a recent report suggests that IL-12 is an effective immune adjuvant for vaccination therapy. Portielje *et al.* reported that the inflammatory properties of IL-12 play important roles in the adjuvant effect to evoke Th1 cellular immune response (36). These effects are thought to result from the activation of APCs, and DCs in particular. Todryk *et al.* reported that HSP70 induced Th1 cytokines and targeted immature DC precursors to enhance antigen uptake (26). These reports suggest that augmentation of systemic immune response induced by HCLV plus IL-12, which showed greater cytotoxic activity of splenic lymphocytes than CLV plus IL-12 (Fig. 4A), and tumor-specific protection from tumor challenge (Fig. 4B) resulted from powerful immune induction due to HSP70 and IL-12 stimulation. In the present study, effector cells in cancer immunity induced by HCLV plus IL-12 have not been identified yet. Efficient recognition of tumor cells by cytolytic T lymphocytes (CTL) is often dependent on the presentation of cytosolic peptides in the context of MHC class I molecules. Since B16 mouse melanoma cells are known to express a lower amount of H-2 antigen (22), the role of other effector cells such as the natural killer cells, the activity of which is known to be stimulated by IL-12, remains to be investigated.

The present investigation screened various potent cytokines for enhancement of the vaccine effects of HSP-rich melanoma cell lysate, and revealed IL-12 as an important modulator of antitumor immune response induced by the HSP-rich cell lysate. IL-12 administration resulted in significant protection against tumor challenge and augmented splenic lymphocyte activity in hosts vaccinated with the HSP-rich cell lysate, but not with unheated cell lysate. Use of IL-12 may thus facilitate further efforts to develop novel cancer immunotherapies based on HSP70-mediated immune responses.

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REVIEW

Medical Application of Functionalized Magnetic Nanoparticles

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Since magnetic particles have unique features, the development of a variety of medical applications has been possible. The most unique feature of magnetic particles is their reaction to a magnetic force, and this feature has been utilized in applications such as drug targeting and bioseparation including cell sorting. Recently, magnetic nanoparticles have attracted attention because of their potential as contrast agents for magnetic resonance imaging (MRI) and heating mediators for cancer therapy (hyperthermia). Magnetite cationic liposomes (MCLs), one of the groups of cationic magnetic particles, can be used as carriers to introduce magnetite nanoparticles into target cells since their positively charged surface interacts with the negatively charged cell surface; furthermore, they find applications to hyperthermic treatments. Magnetite nanoparticles conjugated with antibodies (antibody-conjugated magnetoliposomes, AMLs) are also applied to hyperthermia and have enabled tumor-specific contrast enhancement in MRI via systemic administration. Since magnetic nanoparticles are attracted to a high magnetic flux density, it is possible to manipulate cells labeled with magnetic nanoparticles using magnets; this feature has been applied in tissue engineering. Magnetic force and MCLs were used to construct multilayered cell structures and a heterotypic layered 3D coculture system. Thus, the applications of these functionalized magnetic nanoparticles with their unique features will further improve medical techniques.

[**Key words:** magnetic nanoparticle, magnetic resonance imaging, hyperthermia, drug delivery system, tissue engineering]

Magnetic particles ranging from the nanometer to micrometer scale are being used in an increasing number of medical applications. The important properties of magnetic particles for medical applications are nontoxicity, biocompatiblilty, injectability, and high-level accumulation in the target tissue or organ; the most important property among those mentioned above is nontoxicity. Ferrous or ferric oxide is the main constituent of magnetic particles, although metals such as cobalt and nickel are used in other fields of application. Magnetic particles are attracted to high magnetic flux density; this feature is used for drug targeting and bioseparation including cell sorting. Currently, magnetic nanoparticles are attracting attention because of their potential use as contrast agents for magnetic resonance imaging (MRI) and heating mediators for cancer thermotherapy (hyperthermia). Furthermore, a novel application of magnetic nanoparticles and magnetic force for tissue engineering, termed "magnetic force-based tissue engineering (Mag-TE)" has been proposed. This review mainly focuses on the medical applications of magnetic nanoparticles in MRI, hyperthermia, and Mag-TE.

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I. MAGNETIC SEPARATION

The processes of isolation and separation of specific molecules are used in almost all areas of biosciences and biotechnology, and are the most documented and currently the most useful application of magnetic particles. Various magnetic particles have been developed as magnetic carriers in separation processes including purification and immunoassays (1–9). Liu *et al.* (10) also demonstrated that the population of nitrifying bacteria increased in the water treatment process in which magnetic particles conjugated with a positively charged polymer matrix were used. The binding of specific ligands to magnetic particles is the same as with other carriers comprising a polymer matrix. Separation techniques are extremely important in process engineering such as bioprocessing.

Automatic DNA/RNA purification apparatuses are commercially available for laboratory use. With respect to medical applications, magnetic cell sorting was developed for use in cellular therapy. In the removal of a specific cell population from blood by conventional immunoaffinity column