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PRECLINICAL STUDY

Anti-cancer effect of hyperthermia on breast cancer by magnetite nanoparticle-loaded anti-HER2 immunoliposomes

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Abstract *Background* We have constructed anti-HER2 immunoliposomes containing magnetite nanoparticles (HML) that generate heat in an alternating magnetic field (AMF). The effective targeting and cytocidal abilities of HML have been achieved using cell culture models. This study aimed to investigate feasibility of this modality for breast cancer treatment using tumor-bearing mouse models. *Material and methods* The subcutaneous cancer nodules of BT474 (high HER2 expression) or SKOV3 (low HER2 expression) cells in nude mice were employed as models. HMLs were injected into these cancer nodules and were then exposed to an AMF for 30 min twice at 24 h intervals. Accumulation of magnetite and tumor growth rates were examined. Histological findings of the thermal effect were also examined. *Results* HMLs accumulated in only BT474 tumors. The tumor temperature increased to 45°C whereas the body temperature remained at around 38°C. Tumor regression was observed in the hyperthermic group and was sustained for 10 weeks after hyperthermia. *Conclusion* These results suggest that hyperthermia using HML is an effective and specific therapy for breast cancer over-expressing HER2. This therapy may provide an alternative way to treat recurrent cancer refractory to other modalities.

Keywords Breast cancer · HER2/neu · Hyperthermia · Liposome · Magnetite nanoparticles · Trastuzumab

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Introduction

Breast cancer is one of the leading causes of death in females. Many treatment modalities for primary and recurrent breast cancer have been developed. However, once recurrent breast cancer becomes refractory to conventional therapies (e.g. hormonal therapy, chemotherapy, and irradiation), there are few modalities to alleviate symptoms. Even as a palliative therapy, another modality which exerts other anti-cancer mechanisms is desired.

Hyperthermia is a promising approach to cancer therapy because it not only kills cancer cells directly, but it also activates anti-cancer immunity as an indirect effect [1, 2]. The intrinsic technical problem with hyperthermia is the difficulty in focusing heat on the intended region without damaging the surrounding healthy tissue. Various hyperthermia techniques have been developed to treat breast cancer [3, 4]. The most commonly used heating method in clinical settings is capacitive heating using a radiofrequency (RF) electric field [5]. 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. Other modalities such as radiofrequency ablation [6] and high-intensity focused ultrasound [7] have been reported. These modalities have some disadvantages in targeting ability and controllability.

Magnetite nanoparticles have been used for hyperthermia treatment in an attempt to overcome these obstacles [8, 9]. If magnetite nanoparticles can be made to accumulate only in tumor tissue, cancer-specific hyperthermia is attainable by generating heat in an alternating magnetic field (AMF) due to hysteresis loss [10]. We have developed magnetite cationic liposomes (MCLs) as mediators of intracellular hyperthermia [11]. These cationic liposomes

exhibit improved adsorption and incorporation into tumor cells than neutrally charged magnetoliposomes. We previously demonstrated the efficacy of MCL-mediated hyperthermia in animals with several types of tumors, including B16 mouse melanoma [12], T-9 rat glioma [13], and VX-7 sequamous cell carcinoma in rabbit tongue [14].

Trastuzumab (Herceptin®) is a humanized monoclonal antibody against human epidermal growth factor receptor-2 (HER2). It has been demonstrated that Trastuzumab has remarkable binding ability to HER2 [15] and efficacy in treating HER2-overexpressing breast cancer [16]. To achieve improved targeting ability and long-acting effect of magnetoliposomes, we developed Herceptin®-conjugated liposomes containing magnetite nanoparticles (HMLs) (Fig. 1). We previously reported anti-cancer effect of HMLs and demonstrated their tumor-specific targeting ability using cell culture models [17]. In the present study, we established a subcutaneous tumor model of breast cancer in nude mice and investigated retention ability of HML and the hyperthermic effects of HMLs on the subcutaneous tumors.

Material and methods

Animals and tumor cells

Female Balb/c nu/nu mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan). BT474 human breast cancer cells with high HER2 expression were obtained from American Type Culture Collection (ATCC) and were maintained in McCoy's 5a medium (Gibco BRL, Gaithersburg, MD) supplemented with 1.5 mM L-glutamine, 10% fetal bovine serum (FBS), and antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin). SKOV3 human ovarian cancer cells with low HER2 expression were obtained from ATCC and were maintained in the same medium as for BT474. Cells were grown at 37°C in an atmosphere containing 5% CO_2. These two cell lines have been extensively characterized for HER2 expression by flow cytometry, ELISA, and immunohistochemistry [18]. HER2 expression in the subcutaneous tumor of each cell line was reexamined with Herceptest® (Dako, Carpinteria, CA) following the instruction manual provided by the manufacturer (Fig. 2).

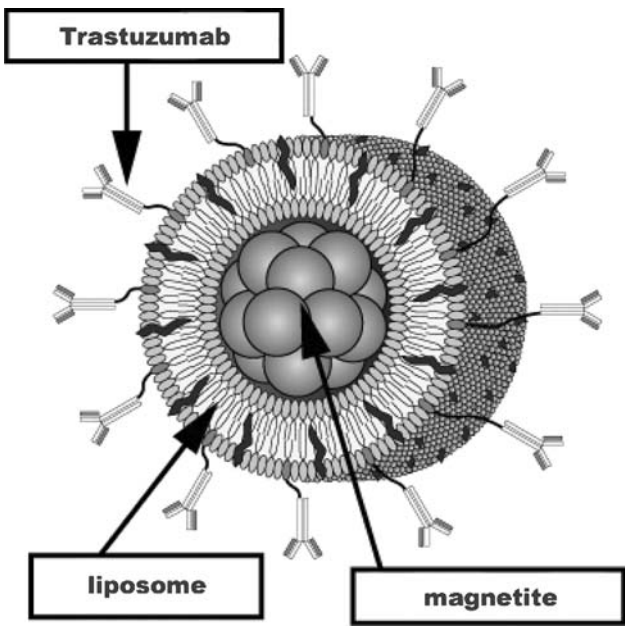

Fig. 1 Schematic illustration of Herceptin®-conjugated magnetoliposome (HML). Liposome containing magnetite nanoparticles on which Herceptin® is covalently conjugated is shown

To prepare tumor-bearing animals, cell suspensions including approximately 1×10^7 BT474 or SKOV3 cells in 100 µl Matrigel® (Becton, Dickinson and Company, Tokyo, Japan) or above-mentioned medium were injected subcutaneously into the left flank of nude mouse under short-term anesthesia by inhalation of ethyl ether. For BT474-bearing mice, 0.05 ml of oily estrogen suspension (Pelanin depot®, Mochida Pharmaceutical Co Ltd., Tokyo, Japan) was injected into the muscle of the thigh once a month. Subcutaneous cancer nodules that had grown to a diameter of about 8–12 mm were used for the experiments. The tumor diameter was measured every week with vernier caliper. The tumor volume was approximated as tumor volume = 0.5 × major axis × minor axis × depth. Due to the variation of initial tumor volume, change of tumor volume is expressed as relative value.

The experimental protocol in the present study was approved by the Animal Care Committee of Nagoya University School of Medicine. 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 HMLs

The magnetic particles were kindly provided by Toda Kogyo (Hiroshima, Japan) and had an average diameter of 10 nm. HMLs were prepared by sonication as described previously [17]. HMLs were diluted with distilled water to yield 80 mg/ml of iron concentration. In order to investigate specificity of HML, magnetite-loaded liposomes in which Trastuzumab was replaced with cysteine (ML) were also prepared.

Injection of HMLs/MLs

After the subcutaneous cancer nodules had grown to 8–12 mm in diameter, a 27-G needle with microsyringe (Hamilton Company, Reno, Nevada) containing HML or ML was inserted longitudinally into the cancer nodules while the animals

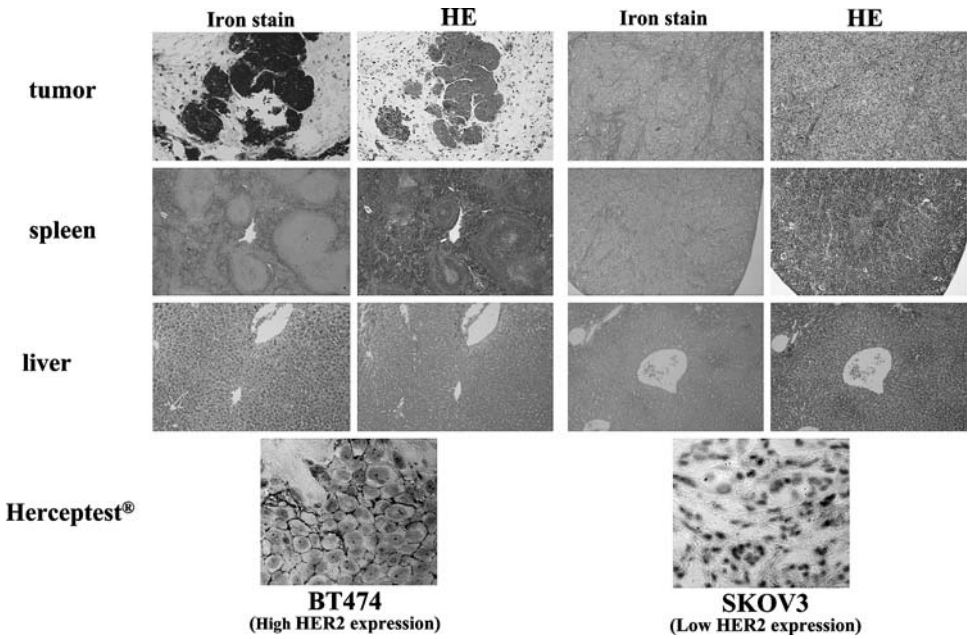

Fig. 2 Iron (*left* column) and HE (hematoxylin and eosin) stains (*right* column) of subcutaneous tumors. Dark dots indicate iron deposit. Bottom: Immunohistochemical analysis of HER2 expression in the subcutaneous tumor of each cell line. Cell surface was stained in BT474 cell tumor

were under short-term anesthesia by intraperitoneal injection of pentobarbital solution (Nembutal®, Dainippon Sumitomo Pharma, Japan). Total volume of injected HML or ML solution was one tenth of tumor volume. The solution was divided into several portions and injected very slowly into the tumor. Resultant iron concentration of the tumor was 8 mg/cm^3 of tumor volume.

Heat generation in an AMF

In consideration of clinical application, hyperthermia was carried out on the same day of injection. The injected mice were separated into two groups: Group I mice were not exposed to an AMF (control group), whereas Group II mice were subjected to hyperthermia for 30 min after injection of the HML. Hyperthermia was performed twice with a 24 h interval. For ML, in order to eliminate the effect of mechanical retention, injected mice were left for 48 h before hyperthermia was carried out.

The AMF was created using vertical coil with a transistor inverter (LTG-100–05, Dai-Ichi High Frequency, Tokyo, Japan) operating at 118 kHz. The magnetic field intensity was adjusted to maintain the temperature of overlaying skin to be approximately 45°C.

Preparation of specimens for histology and iron-content measurement

Forty-eight hours after HML or ML injection, mice were sacrificed and the carcinoma and organs were removed to assess for accumulation of magnetite. The quantity of adsorbed magnetite was measured by the iron content, as per our previous method [19]. To examine thermal effect, the treated subcutaneous tumors were removed under general anesthesia 14 days after hyperthermia. Pathological specimens of 6 μm in thickness were prepared, stained with Berlin Blue to visualize the location of the magnetite, and counterstained with Kernechtrot or hematoxylin and eosin to examine the hyperthermia effect.

Temperature measurement

Surface temperature was measured with infrared thermography (TVS-200ME, Nippon Avionics Co., Ltd. Tokyo) at 1 min intervals. The temperature of overlaying skin should be lower than the underlying, internal tissue containing the magnetite when the mice were irradiated with the magnetic field. Therefore, we measured the surface temperature of the tumor to evaluate the utility of this technique.

Results

In vivo retention of HMLs in carcinoma

Retention of HML at 48 h after injection was investigated in an in vivo experiment. Figure 2 shows histological sections of the carcinoma, liver, spleen and kidney removed from mice into which the HML was injected. Many magnetite particles accumulated in the BT474 tumor, while few magnetite particles accumulated in SKOV3 tumor. There was no evidence of accumulation of HML except for physiologic iron in liver, spleen and kidney in both tumor-bearing mice.

As indicated in Fig. 3, almost the entire injected amount of magnetite accumulated in the tumor of BT474 cells,

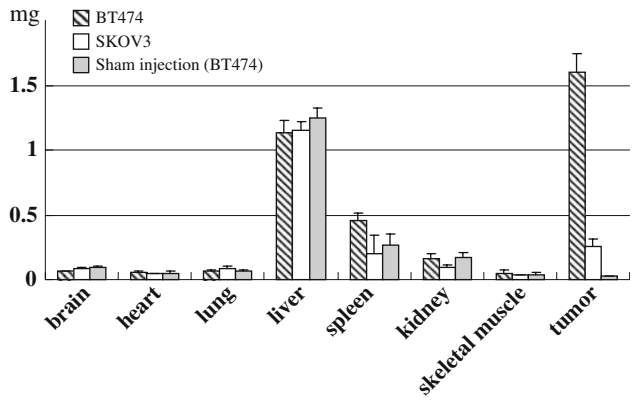

Fig. 3 Iron content of the tumor and various organs. Data and bars are shown as mean ± SD of three independent mice

whereas only trace amount of magnetite accumulated in the tumor of SKOV3 cells, while total accumulation amount in other organs was almost the same between BT474 and SKOV3-bearing mice. These results indicate that HML-retention depends on HER2 expression and Trastuzumab conjugated with magnetoliposme, and that unbound HMLs are rapidly removed from tissue.

Hyperthermia

Irradiation with the AMF heats only magnetic particles in this experiment. The temperature at the BT474 tumor surface was elevated rapidly by the AMF irradiation and reached 45°C within 5 min, as shown in Fig. 4a. During irradiation, the surface temperature was maintained around 45°C by controlling the power of the irradiation apparatus. After cessation of irradiation, the surface temperature dropped rapidly to normal range while the temperature of the gluteal skin was elevated slightly to approximately 38°C. In another experiment, rectal temperature did not rise significantly during hyperthermia (data not shown). In contrast, the temperature of ML-injected BT474 tumor surface did not or slightly elevate under the same conditions (Fig. 4b). These experiments were repeatedly performed on at least five mice.

Figure 5 shows the time-course of macroscopic findings of representative tumor-bearing mice treated by hyperthermia. The BT474 tumors treated by hyperthermia appeared to be necrotic on the 2nd day of hyperthermia; thereafter the tumors gradually shrank, leaving slight scars or tiny skin ulcers after 14 days. Figure 6 shows histological images of the tumors removed at 14 days after hyperthermia. Some necrotic tissues were observed in subcutaneous tissue and almost no tumor cells remained.

To investigate the effect of AMF irradiation itself, tumor-bearing mice were subjected to irradiation without injection of HML. Irradiation did not affect tumor growth (data not shown). Also, to investigate the effect of Trastuzumab conjugated to HML, injected mice without

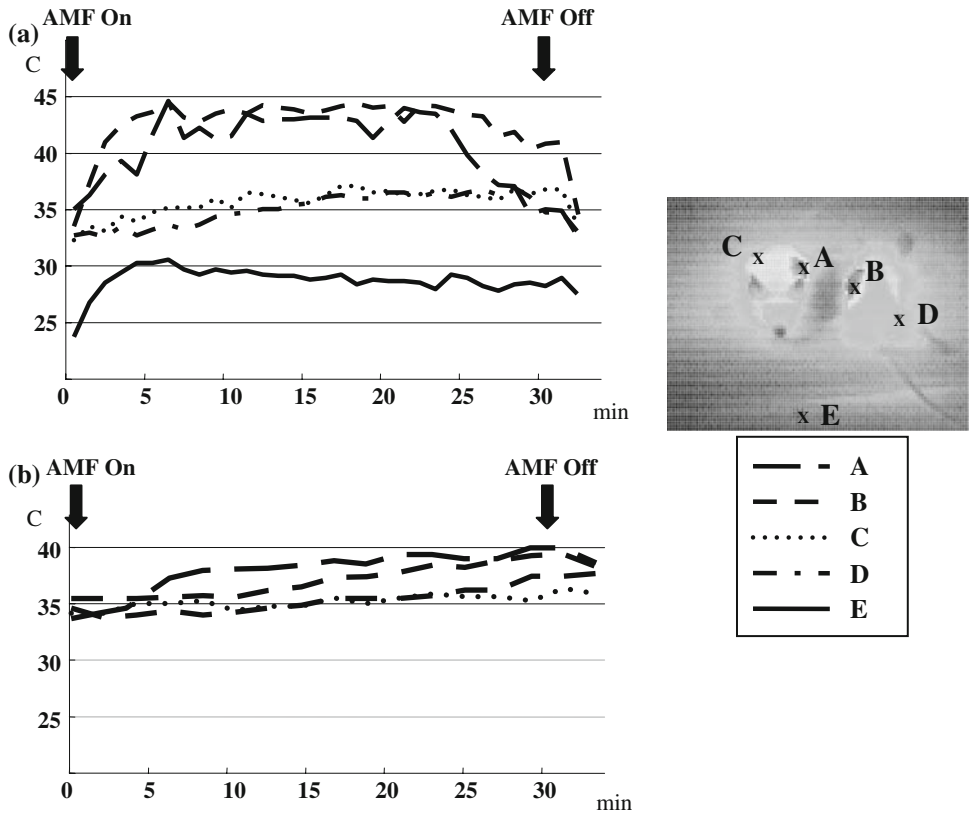

Fig. 4 (**a**) Temperature trends of the tumor and gluteal skin of HML-injected mice during AMF (alternating magnetic field) irradiation. (**b**) Temperature trends of the tumor and gluteal skin of ML-injected mice during AMF irradiation. A, B: tumors; C, D: gluteal skin; E: surface of the machine; (inset) mice captured with thermography on which measure points were indicated

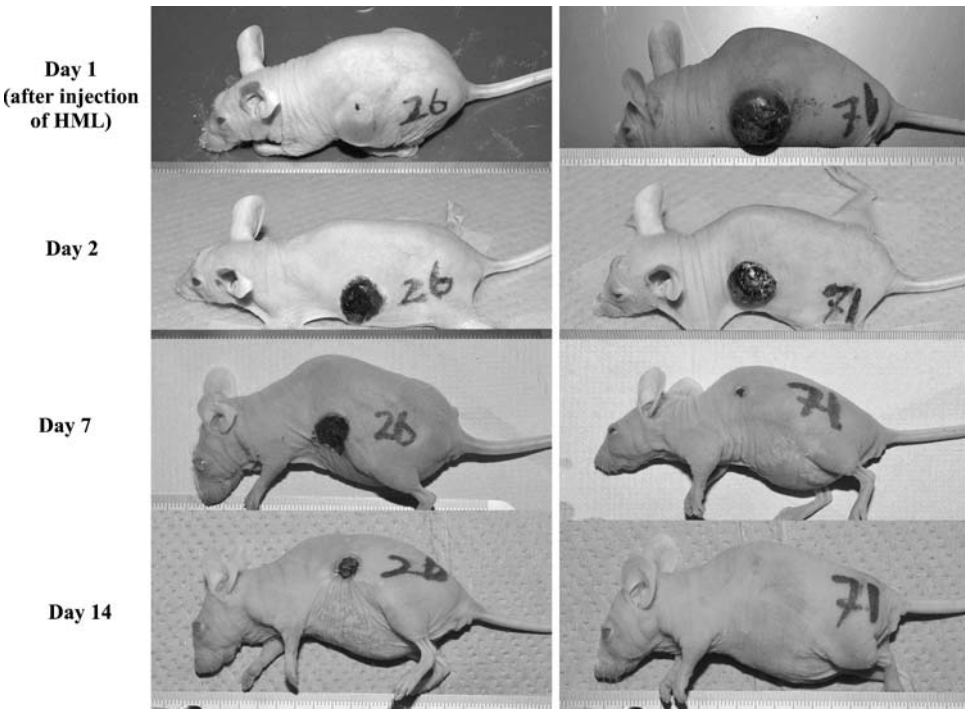

Fig. 5 Photographs of representative mice treated with HML-mediated hyperthermia. Photographs were taken on the day of injection and on days 2, 7, and 14 following injection

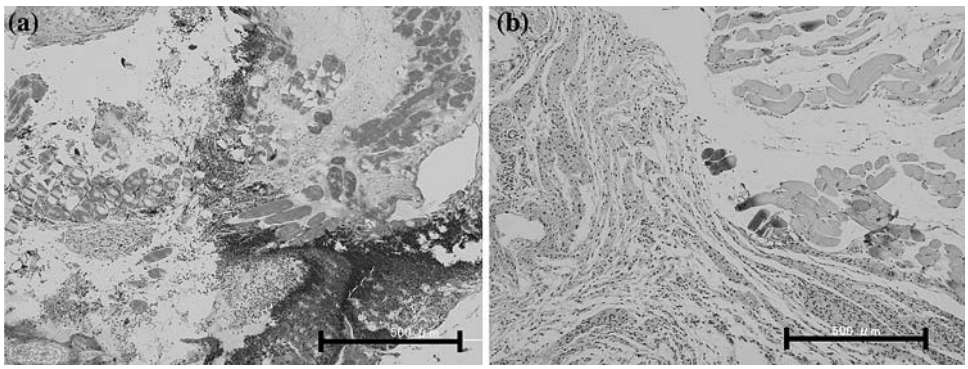

Fig. 6 Histological findings of the tumor or scar removed at 14 days after hyperthermia treatment. Tumor cells showed necrosis (**a**) or complete elimination (**b**). Bar indicates 500 μm

irradiation (group I) were examined. Injection of HML did not affect tumor growth (data not shown).

Long-term effect of hyperthermia

In order to investigate the long-term effect of HML-mediated hyperthermia, the tumors treated with HML-mediated hyperthermia (n = 5) were monitored for 10 weeks. There was no indication of regrowth of tumors (Fig. 7).

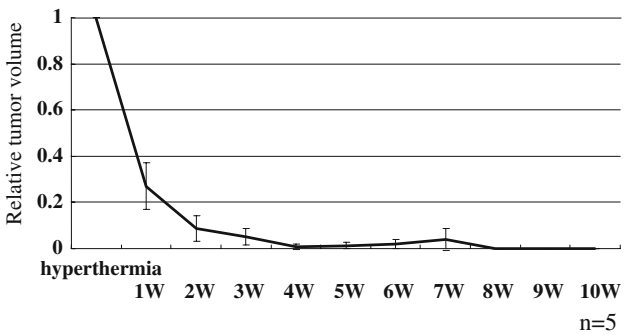

Fig. 7 Long-term effect of hyperthermia. Time course of changes in tumor size of mice undergoing hyperthermia. The graph represents the mean relative tumor volume after hyperthermia. Tumor volume is exhibited as relative value (tumor volume prior to hyperthermia is represented as 1) and the vertical bars represent standard deviation

Discussion

This study investigated the feasibility of our new modality for targeting HER2-overexpressing tumor and its capability for hyperthermic treatment in human breast cancer.

As shown previously, HMLs can specifically bind to HER2-overexpressing cells and the binding depends on HER2/Trastuzumab interaction in vitro. Here, we demonstrated that HMLs can specifically bind to HER2-overexpressing tumors in animal models. Almost all injected magnetite were retained in the tumors after 48 h of

injection into HER2-overexpressing tumors, whereas in HER2-negative tumors, injected magnetite were virtually cleared in the same period. This indicates that retention of magnetite in the tumors is dependent on conjugation to Trastuzumab. We believe that retention of HML enabled the remarkable hyperthermic effects to be achieved.

HML contains only a very small amount of Trastuzumab, i.e. 1.1 mg of Trastuzumab is conjugated to HML containing 20 mg magnetite. However, HML is injected directly into the tumor; therefore the concentration of Trastuzumab in the tumor could be high enough to enhance anti-cancer effects.

In contrast to conventional hyperthermic techniques, hyperthermia using HMLs allowed the temperature to reach the target of 45°C without any substantial damage to the surrounding tissue. Unlike hyperthermia using relatively low temperature (e.g. 42°C), in which the treated tumor undergoes apoptosis, tumor treated with relatively higher temperature (e.g. 45°C) undergoes necrosis. 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. We previously demonstrated that during hyperthermia using magnetite nanoparticle, heat shock proteins (HSPs) were released and stimulated host anti-tumor immune system [20, 21]. In the present study, we used nude mice as a model, therefore we could not investigate involvement of the immune system during hyperthermia.

The present results clearly demonstrate the major advantage of hyperthermia, i.e. the fact that the hyperthermic effect is independent of the type of cancer cell was reconfirmed. We succeeded in demonstrating the hyperthermic effect using melanoma [12], glioma [13], and squamous carcinoma [14]. The present results prove that hyperthermia is a reliable and effective cancer therapy across species and cell types.

Since HMLs are heated in our hyperthermic system, the distribution of magnetite nanoparticles within the tumors is an important issue. We injected HMLs into several points in the tumor. Although injected HMLs could disperse slowly and be incorporated into the cancer cells, they could not distribute evenly within the tumor. Thus, uniform hyperthermia could not be achieved. However, according to our previous reports [22], repeated hyperthermia induced complete tumor necrosis. We speculated that when magnetite nanoparticles were repeatedly heated, they subsequently diffused beyond the necrotic area within the tumor, resulting in a wide distribution of magnetite nanoparticles and necrosis of the surrounding tumor tissues as well. Thus the entire tumor area was necrosed by repeated hyperthermia.

Another important issue is safety of this modality. HMLs comprise of Trastuzumab, lipid, and magnetite. Each component has already been introduced into clinical use. However, the major concern about this modality is whether repeated injection of HMLs induces iron-accumulation in the organs. The iron content of the organs (i.e., liver, spleen, brain, heart, etc) of HML-injected mice was the same as that of the control mice. In rat model, repeated subcutaneous administration of HML causes no specific pathologic change in liver, spleen, heart, and brain (data not shown) nor has any effect on survival. These results indicate that there is virtually no possibility of iron-deposition in vital organs due to repeated administration of HMLs. From the clinical view point, the iron content of HML used for hyperthermia is much smaller than the dosage for iron-deficiency anemia treatment. For example, the iron content of HML used for a 2 cm-diameter tumor is approximately 32 mg. This value is much smaller than the iron dosage for mild iron-deficiency anemia (e.g. several hundred mg of iron).

The promising results of this study warrant further investigation of hyperthermia using HMLs with consideration for future application in the treatment of refractory breast cancer in humans. In future work, involvement of anti-tumor immunity should also be investigated.

In summary, we have developed tumor-specific antibody-conjugated magnetoliposomes, which can target the HER2 on human breast cancer cells in vivo, and allow efficient application of hyperthermia to the tumor. The growth of the tumor was almost entirely suppressed by just two hyperthermia treatments. This study indicates that HML-mediated hyperthermia is effective as an alternative approach for refractory breast cancer.

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