Complete Regression of Mouse Mammary Carcinoma with a Size Greater than 15 mm by Frequent Repeated Hyperthermia Using Magnetite Nanoparticles

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Magnetite cationic liposomes (MCLs) have a positive surface charge and are used as a heating mediator for hyperthermia, because they generate heat in an alternating magnetic field (AMF) due to hysteresis loss. In our previous paper, hyperthermia using MCLs was applied to animals having several types of tumors in mice, rats, hamsters, and rabbits, and a strong anticancer effect was observed. For mice, complete tumor regression was observed when the tumor size was smaller than 5 mm. However, a protocol for large tumors is necessary for the clinical application. In the present paper, a protocol for tumors with a size greater than 15 mm in mice was investigated. MCLs were injected into an MN46 tumor (size, 15 mm) in C3H/HeN 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 (repeated hyperthermia; RH), and RH was carried out until complete tumor regression was observed. Complete tumor regression was achieved in all mice treated once, twice or six times with RH, and mice acquired antitumor immunity. This protocol, which is termed frequent RH, is a potent approach for cancer therapy.

[Key words: hyperthermia, magnetite, mammary carcinoma, liposome, tumor size]

Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients (1). The most commonly used heating method in clinical settings in Japan is capacitive heating using a radio-frequency (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, the position of electrodes, and adhesion of electrodes at uneven sites. Magnetite nanoparticles have been applied to hyperthermia treatment in an attempt to overcome these disadvantages (3, 4). Magnetite nanoparticles generate heat in an alternating magnetic field (AMF) due to hysteresis loss (5). We have developed magnetite cationic liposomes (MCLs) for hyperthermia (6, 7). MCLs were developed in order to improve adsorption by and accumulation in tumor cells due to their electrostatic interaction with the cell membrane (6). We previously demonstrated the efficacy of hyperthermia using MCLs in animals with several types of tumors, such as B16 mouse melanoma (8, 9), T-9 rat glioma (10), Os515 hamster osteosarcoma (unpublished results), and VX-7 squamous cell carcinoma in rabbit tongue (11). Although hyperthermia using MCLs was found to be very effective for inducing complete regression of tumors with diameters of less than 5 mm in mice, tumors with diameters larger than 10 mm were very difficult to treat using the same protocol, because this protocol was optimized for the smaller tumors. Therefore, development of a new protocol is required for larger tumors. In the present study, we use 15-mm tumors in mice as a terminal cancer model, because the 15-mm tumors (1 cm³) are obviously a huge volume compared with the body of mice (20 cm³). To the best of our knowledge, no researchers have reported the complete regression of such huge tumors in mice.

The rationale for hyperthermia is based on the direct cell-killing effect observed at temperatures above 42.5°C, and this effect is enhanced at higher temperatures (12). Because hyperthermia using MCLs can specifically heat the tumor without damaging healthy tissue, tumors can be heated to higher temperatures than are possible using capacitive heating by an RF electric field. We previously showed that subcutaneous B16 melanoma nodules could be heated to 46°C using MCLs, and that serious burning on the skin did not occur (8). Complete tumor regression was observed in 30% of mice after a single heat treatment at 46°C, whereas no nodule regression was observed after heat treatment at 43°C.

The hyperthermic effect is also enhanced by iteration of heating. The advantage of hyperthermia using MCLs is that it can be applied numerous times because tumor tissue is heated specifically without damaging surrounding healthy tissue. The more frequently AMF irradiation is applied, the
greater the hyperthermic effect is obtained. The observed hyperthermic effect should be independent of cell lines and animals, in contrast to chemotherapy. When repeated hyperthermia (RH) was conducted at 46°C, the subcutaneous melanoma nodules in 90% of mice disappeared, whereas only 30% of nodules regressed completely after a single heat treatment (8). Heat shock proteins (HSPs) such as HSP70 are involved in acquiring thermotolerance (13, 14) and are also recognized as significant participants in immune reactions (15, 16). We previously showed that RH using magnetite nanoparticles at high temperatures was able to completely regress tumors greater than 15 mm in size in mice, and confirmed the antitumor immunity (17) and demonstrated that HSP70 expression after RH was the mechanism for this induced antitumor immunity in T-9 rat glioma (18, 19).

These results prompted the development of a new protocol, which is based on two concepts: higher temperature and frequent RH. In the present paper, we studied whether frequent RH using magnetite nanoparticles at high temperatures was able to completely regress tumors greater than 15 mm in size in mice, and confirmed the antitumor immunity induced by RH.

MATERIALS AND METHODS

Animals and tumors Male C3H/HeN mice at 6 weeks of age were purchased from Charles River Japan (Yokohama). MM46 mammary carcinoma was maintained in C3H/HeN mice by weekly passage. To prepare tumor-bearing animals, cell suspensions including approximately 2×10^6 cells of MM46 mammary carcinoma in 0.2 ml phosphate buffer saline (PBS) were inoculated subcutaneously into C3H/HeN mice. Tumor diameter was measured every 3 d. The largest and smallest diameters of each tumor were measured using calipers and the average diameter (mm) was calculated. The MM46 tumors with the largest diameters of 7 mm and 15 mm were obtained after about 7 d and 22 d of inoculation, respectively.

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

Injection of MCLs and heat generation by AMF Tumor-bearing animals were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Under anesthesia, a syringe (26 G needle) containing MCLs was inserted longitudinally into each tumor subcutaneously from the tumor edge. The indicated volumes of MCL solution (20 mg-magnetite/ml) were injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL, USA) for 30 min. After injection of MCLs, mice 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, Tokyo). The magnetic field frequency was 118 kHz. The mouse was placed inside the coil such that the tumor was positioned at the center. Temperatures in the rectum and at the surface of the tumor during AMF irradiation were measured with an optical fiber probe (FX-9020; Anritsu Meter, Tokyo).

Rechallenge of cured mice with MM46 cells Completely cured mice were challenged with MM46 cells 120 d after the first hyperthermic treatment. MM46 cells (4×10^6 cells) were injected subcutaneously into C3H/HeN mice. Tumor sizes were measured every 3 d, and the mice were judged to be tumor free when they did not bear palpable tumors (less than about 2 mm in diameter).

RESULTS AND DISCUSSION

Hyperthermia using MCLs for tumors of various sizes

The effects of tumor size on temperature increase during hyperthermia using MCLs in MM46 tumors was investigated. After MM46 tumors had grown to 7 mm or 15 mm in diameter, 0.1 ml of MCL solution (net magnetite weight: 2 mg) was injected into the center of tumors, and mice were subjected to AMF for 30 min. Figures 1a and 1b show the temperature at the surface of tumors with diameters of 7 and 15 mm during AMF irradiation, respectively. The temperature profiles were fairly similar. Tumor temperature with both tumor sizes increased rapidly, reaching 45°C within 5 min, and was maintained at 45°C by controlling the magnetic field intensity. This means that 2 mg of magnetite was sufficient to achieve a tumor temperature of 45°C, even when the tumor size was 15 mm. No serious burning on the skin was observed in these mice. In addition, rectal temperature remained below 38°C in mice with tumors of 7 and 15 mm in diameter.

Although capacitive heating using an RF electric field has been applied to superficial tumors, such as mammary carcinoma, it is difficult to specifically heat a superficial tumor. We used MCLs in order to heat the tumoral region and minimize heating of the surrounding healthy tissue. The results shown in Fig. 1 suggest that hyperthermia using MCLs allows the tumor 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.

Comparison of the therapeutic effects of single RH in various tumor sizes

We then examined the therapeutic effects of a single round of RH treatment in MM46 tumors of various sizes. AMF irradiation was carried out three times at 24-h intervals. Figure 2 shows the time course of MM46 carcinomas of various sizes. Tumors of size 7 mm in the control group grew progressively (Fig. 2a). On the other hand, all tumors of size 7 mm (5/5) disappeared within 18 d after RH, as shown in Fig. 2b. For tumors of size 15 mm, tumor growth was suppressed while one of the five tumors regressed completely, as shown in Fig. 2c. However, the remaining four tumors began to regrow 10 d after RH.

We previously reported that a single round of RH (three times at 24-h intervals) was effective for several tumors. In the present study, this protocol was effective for MM46 tumors with a diameter of 7 mm. With 15-mm tumors, however, complete tumor regression was not achieved in 80% (4/5) of mice. We previously reported that the necrotic area was enlarged and spread to the whole tumor after RH. When the tumor was irradiated using AMF, the temperature of the
MCLs increased and the surrounding tumor cells were killed. After three irradiations, the MCLs diffused and spread, and the necrotic area also spread throughout the whole tumor (10, 19). In the present study, MCLs were spread throughout the whole MM46 tumor at a diameter of 7 mm by RH, but MCLs did not spread to the tumor edges when the diameter was 15 mm (data not shown). For larger tumor diameters, such as 15 mm, it was difficult to uniformly heat the whole tumor using MCLs because of their uneven shape, and tumor cells in viable areas continued to grow. We therefore propose a new protocol, which is termed frequent RH, for 15-mm tumors in the next experiment.

**Effects of frequent RH on MM46 tumors with diameters of 15 mm** Mice with MM46 tumors with diameters of 15 mm were frequently treated with RH as follows, until complete tumor regression was achieved. In view of MCL distribution, the amount of MCLs in the protocol was modified from 2 mg, which resulted in complete regression of 7-mm tumors (Fig. 2b), to 8 mg in proportion to tumor size. After MM46 tumors had grown to 15 mm in diameter, 0.4 ml of MCL solution (net magnetite weight: 8 mg) was divided into two and injected at two points in each tumor. Mice were then subjected to AMF for 30 min. The tumor was heated to 45°C within 5 min, and kept at 45°C by controlling the magnetic field intensity, and the temperature was the same as shown in Fig. 1. AMF irradiation for RH was carried out twice at 24-h intervals. Moreover, if partial tumor regrowth occurred after RH, a further 0.1 ml of MCL solution (net magnetite weight: 2 mg), which was sufficient to regress the 7-mm tumor as shown in Fig. 2b, was injected into the tumor and RH was again conducted.

Figure 3 shows the time course for the average MM46 tumor size. Tumors in the control group grew progressively, as shown in Fig. 3a. On the other hand, all 15-mm tumors completely regressed after several rounds of RH, as shown in Fig. 3b. Mice were treated once (mouse no. 4), twice
COMPLETE TUMOR REGRESSION BY REPEATED HYPERTHERMIA

(mice no. 2, 3 and 5) or 6 times (mouse no. 1) with RH. Furthermore, tumors successfully treated by frequent RH did not undergo regrowth for 120 days post-treatment and no mice died (Fig. 3c). On the other hand, all control mice (5/5) died from an enlarged tumor at the inoculated site within 52 days. Figure 4 shows representative mice with (mouse no. 3, Fig. 4c) and without RH treatment (Fig. 4b) on day 45. In the control mouse, the subcutaneous tumor grew substantially, as shown in Fig. 4b. On the other hand, the subcutaneous tumors completely disappeared and no serious damage was observed in the mice treated with frequent RH (Fig. 4c).

Although the thermal dose-response relationship varies between cell lines and depends on microenvironmental factors such as pH (21), we believe that in principle tumors of any size or type can be killed using frequent RH with higher temperatures. In the present study, mouse MM46 carcinoma was investigated and complete regression of tumors with greater sizes was observed using a novel hyperthermia protocol, which should be termed frequent RH. In mouse no. 1, it took six rounds of RH to achieve complete tumor regression. Although parts of the tumor containing sufficient amounts of MCLs were killed by heat, other parts of the tumor without MCLs, particularly at tumor edges, may continue to grow. Differences in the number of rounds of RH treatment for complete regression are 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, the toxicity of MCLs may become an important issue. In our preliminary study, the toxicity of a single administration of MCL solution (33 mg of magnetite, i.p.) was investigated. MCLs largely 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 maximum of 18 mg of magnetite was used (mouse no. 1) and all treated mice survived until day 120 (Fig. 3c). This amount of magnetite was less than that used in the preliminary examination.

FIG. 3. Therapeutic effects of frequent RH on MM46 tumors of 15 mm in diameter. MCLs (8 mg magnetite) were injected directly into subcutaneous MM46 tumors with diameters of 15 mm, which were then irradiated with an AMF for 30 min. AMF irradiation was repeated twice at 24-h intervals (RH). After injection of MCLs (2 mg magnetite), RH treatment was carried out frequently if tumors began to regrow. Time course of changes in tumor size in control mice (a) and in mice undergoing frequent RH treatment (b). Each line represents tumor growth in a single mouse. (c) Percentage survival of tumor-bearing mice over a period of 120 days after hyperthermia. Closed circles, Control mice (n=5); open circle, frequent RH-treated mice (n=5).

FIG. 4. Photographs of representative mice treated with frequent RH. (a) Before AMF irradiation; (b) control mouse on day 45; (c) cured mouse (mouse no. 3 in Fig. 3b) after frequent RH treatment on day 45.
induced previously showed that hyperthermia by means of MCLs
mors. These results suggest that even viable cells in the
systemic antitumor immunity, even in mice with large tu-
tumors was observed after several rounds of RH (Fig. 3), sug-
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minated immunotherapy. On the other hand, recent reports
have shown the importance of HSPs, such as HSP70, HSP90,
and glucose-regulated protein 96, in immune reactions (15,
16). HSP70-mediated antitumor immunity was reported to
cause a vaccine effect due to HSP70-peptide complexes pu-
rified from human cancer cells (23). Our hyperthermia sys-
term RH leads to vaccination with HSP70-peptide via necrotic
tumor cell death in vivo, resulting in antitumor immunity. In order to confirm whether antitumor immunity
was induced by frequent RH, mice exhibiting complete re-
gression after frequent RH treatment were challenged with
MM46 cells at 120 d after the first hyperthermic treatment.
As shown in Fig. 5, all of the naive mice subsequently
formed tumors within 12 d. In contrast, 60% of the cured
mice rejected the MM46 cells and MM46 tumors did not
appear in these mice for up to 50 d after inoculation (Fig. 5).

In the present study, complete regression of 15-mm tu-
mors was observed after several rounds of RH (Fig. 3), sug-
gest that frequent RH was able to kill the large tumors even if thermotolerance via HSP70 expression occurred. These results prompted us to examine whether frequent RH can induce antitumor immunity, even in mice with large tumors. As shown in Fig. 5, frequent RH was able to induce systemic antitumor immunity, even in mice with large tumors. These results suggest that even if viable cells in the tumor survive hyperthermia, they become targets for an anti-
tumor immune reaction and complete regression will even-
entially occur. In the present study, MM46 was used as a
representative of cancer cells, but frequent RH is basically
applied for any cancer cells. Therefore, frequent RH may
become a very potent cancer therapy. MM46 is a relatively
antigenic carcinoma and has been used for many immuno-
ological experiments (24, 25). Before the clinical application
of frequent RH, it should be investigated whether this anti-
tumor immunity is induced in many tumors. Even if a strong
antitumor immunity is not induced due to the low antigenic-
ity of human cancer, the combination of immunotherapy us-
ing a cytokine such as interleukin 2 with RH will enhance
the immune induction caused by HSPs (9).

In summary, complete regression of tumors with sizes up
to 15 mm was observed in all mice after one, two or six
rounds of RH treatment, and mice acquired antitumor immu-
ity. This study indicates that the frequent RH using
MCLs is a potent approach for cancer therapy.

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FIG. 5. Time course of tumor growth after rechallenge at 120 d
after first hyperthermia treatment (on day 120). Mice cured by fre-
quent RH (open circles) and naive mice (closed circles) were chal-
enged with MM46 cells. Each group included five mice.


