Anticancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes
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The hyperthermic effect of magnetic particles was examined in an in vivo study of mouse B16 melanoma. Magnetite cationic liposomes (MCLs) have a positive surface charge and generate heat under an alternating magnetic field (AMF) by hysteresis loss. MCLs were injected into melanoma nodules, which were then subjected to an AMF. The mice were divided into four groups: group I (control), group II (hyperthermia at 43°C for 30 min, once), group III (hyperthermia at 46°C for 30 min, once) and group IV (hyperthermia at 46°C for 30 min, twice). Complete tumour regression was observed in 90% of the mice in group IV, while no mice in groups I and II and only 40% in group III showed regression. To examine whether hyperthermia caused immune induction in B16 melanoma, in vitro cytotoxicity assays and rechallenge experiments were performed. Cytotoxic activity was observed in the spleen cells of the cured mice in group IV. In the rechallenge experiment, 66% of the cured mice rejected melanoma cells. These results suggest that hyperthermia using MCLs is an effective therapy for melanoma, since this treatment can kill the tumour cells not only by heat but also by inducing an immune response. Melanoma Res 13:129–135 © 2003 Lippincott Williams & Wilkins.

Introduction
Malignant melanoma is the most malignant tumour among the malformation neoplasms of the skin. In addition, the increase in the incidence of malignant melanoma is greater than that of any other cancer [1]. Various therapies are commonly employed in the treatment of melanoma patients, including surgery, chemotherapy, radiotherapy and immune therapy. None of these has proved to be sufficiently effective, however, and a more effective protocol for the prevention and therapy of melanoma is urgently needed.

The use of hyperthermia is based on the fact that tumour cells are more sensitive to heat than healthy tissue. Popular heating methods that have been used clinically for melanoma are capacitive heating of tumours using a radiofrequency (RF) electric field or microwaves [2,3]. However, it is difficult to heat tumours specifically with capacitive heating using an RF electric field, since its heating ability is influenced by various factors such as tumour size, the position of the electrodes, and adhesion of the electrodes at uneven sites. From the clinical point of view, a simple heat mediator is desirable for superficial tumour such as melanoma. Compared with the capacitive heating method using an RF electric field, microwaves can be used to heat tumours more specifically. However, microwave hyperthermia can cause considerable injury to the patient and cannot treat a large tumour. In addition, the use of hyperthermia has the universal technical problem of providing uniform heating of only the tumour region until the required temperature has been reached, without damaging normal tissue.

In order to overcome these disadvantages, some researchers have investigated the use of submicron-sized magnetic particles to produce intracellular hyperthermia [4,5]. These magnetic particles generate heat under an alternating magnetic field (AMF) by hysteresis loss [6]. We have developed 'magnetic cationic liposomes' (MCLs) for the production of intracellular hyperthermia [7,8]. MCLs were developed to improve adsorption and accumulation in the tumour cells, and show a 10-fold higher affinity for tumour cells than neutrally charged magnetoliposomes [8]. This higher affinity is the result of an electrostatic interaction with the negatively charged cell membrane. The hyperthermic effect of MCLs in T9 rat glioma cells was examined in an in vivo study [9]. A significantly high temperature was generated intracellularly, and tumour cells were killed not only by heat but also by the induction of an immune response [10].

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In the present study we examined the hyperthermic effect of the MCLs in an in vitro study of mouse B16 melanoma, and demonstrated that our hyperthermia protocol can induce an antitumour immune response.

**Materials and methods**

**Tumour cell line and animal model**

Mouse B16 melanoma cells (Riken Cell Bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, Maryland, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 0.1 μg/ml streptomycin). Melanoma cells were grown at 37°C in an atmosphere containing 5% CO₂. Female C57BL/6 mice, 4 weeks old, were purchased from Japan SLC (Hamamatsu, Japan) or Charles River Japan Inc. (Yokohama, Japan). To prepare tumour-bearing animals, cell suspensions consisting of approximately \(1 \times 10^7\) melanoma cells in 100 μl phosphate buffer (0.05 M sodium phosphate and 0.15 M NaCl, pH 7.4) were injected subcutaneously into the right flank of C57BL/6 mice under short-term anaesthesia (sodium pentobarbital 50 mg/kg body weight intraperitoneally). Melanoma nodules that had grown to approximately 5–6 mm in diameter were used for the experiments (10 mice in each group). The tumour diameter was measured every 3 days.

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**

The magnetic particles (magnetites) were kindly donated by Toda Kogyo Co. (Hiroshima, Japan) (average size 10 nm). MCLs were prepared by the sonication method as described previously [7], with partial modification. Briefly, 1 ml of the colloidal magnetite (net 20 mg magnetite) was coated with a lipid membrane that consisted of N-(α-trimethylammonioacetyl) didodecyl-d-glutamate chloride (Sogo Pharmaceutical Co., Tokyo, Japan), dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical Co., St Louis, Missouri, USA) in molar ratios of 1:2:2. The magnetite concentration was measured by the potassium thiocyanate method [11].

**Injection of MCLs and heat generation within an AMF**

After the melanoma nodules had grown to 5–6 mm in diameter, the 26 gauge needle of the syringe containing the MCLs was inserted longitudinally into each melanoma nodule subcutaneously from the nodule’s edge, and 2.0 mg of MCLs were injected using an infusion pump (SP100H, World Precision Instruments Inc., Sarasota, Florida, USA) over 30 min. The mice were then separated into four groups, with 10 mice in each group. Group I mice were not subjected to an AMF (control group). After injection of the MCLs, group II and III mice were subjected to hyperthermia for 30 min once at 43.0°C and 46.0°C, respectively. On the fifth day after the first hyperthermia, an additional 2.0 mg of MCLs were injected into the mice in group IV and they were again subjected to an AMF. Therefore, group IV mice were subjected to hyperthermia at 46.0°C for 30 min twice (on days 1 and 5). The AMF was created using a horizontal coil (inner diameter 7 cm, length 7 cm) with a transistor inverter (L/TG-100-05, Dai-ichi High Frequency Co., Tokyo, Japan). The magnetic field frequency was 118 kHz. Each mouse was placed inside the coil such that the nodule was positioned at the centre of the coil. Temperatures at the outside skin of the tumour centre and at 0.5 cm from the tumour edge were measured during the application of an AMF using an optical fibre probe (FX-9020, Anritsu Meter Co., Tokyo, Japan). The temperature at the outside skin of the tumour centre was kept at 43.0°C or 46.0°C by altering the magnetic field intensity.

**In vitro cytotoxicity assay**

Spleen cells were obtained from the cured mice (group IV) 1 month after the hyperthermic treatment using the Medimachine System (DAKO A/S, Glostrup, Denmark). Naïve mice that had been born at almost the same time as the heat-treated mice were used as controls. Spleen cells were cultured for 48 h in RPMI 1640 supplemented with 10% fetal bovine serum and 2-mercaptoethanol (5 × 10^-5 M). Mouse recombinant interleukin-2 was added at a concentration of 5 U/ml. Cytotoxicity was determined using a long-term cytotoxicity assay [12]. Melanoma cells (1 × 10^5 cells) were cultured in a 24-well flat plate containing DMEM. After 24 h, the medium was replaced with RPMI 1640. Spleen cells were added into each well. The wells were washed with phosphate buffer after incubation for 48 h at 37°C in an atmosphere containing 5% CO₂. After incubation, 0.3 ml of RPMI 1640 and 0.03 ml of cell counting kit-8 (Dojindo Co., Kumamoto, Japan) solution were added to the wells, and the cells were further incubated for 1 h at 37°C in an atmosphere containing 5% CO₂. The absorbance of each well was measured at 405 nm using a spectrophotometer (V- 530, Jasco Co., Tokyo, Japan). The experiments were performed in triplicate at effector-to-target ratios of 100:1, 50:1 and 25:1. Cytotoxicity was calculated using the following equation:

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\text{Cytotoxicity} \% = \left[1 - \frac{\text{absorbance of target cells treated with effector cells}}{\text{absorbance of target cells only}}\right] \times 100
\]
Rechallenge of the cured mice with B16 melanoma cells

The completely cured mice in group IV (n = 6) were challenged with B16 melanoma cells 1 month after the hyperthermic treatment. B16 melanoma cells (1 × 10^6 cells) were transplanted subcutaneously into the left flank as described above. Melanoma cells (1 × 10^6 cells) were also transplanted into naïve control mice (n = 6) that had been born at almost the same time as the cured mice.

Measurement of heat shock protein 70 expression

On the day after hyperthermic treatment, tumours were removed and homogenized using the Medimatrix System. Heat shock protein 70 (HSP70) in the tumour extract was assayed using the Hsp70 FIA Kit (StressGen Biotechnologies, Victoria, British Columbia, Canada), which detects and quantifies inducible HSP70.

Results

Heat generation by MCLs in an AMF

Figure 1 shows the temperature increases at the outside skin of the tumour centre and at 0.5 cm from the tumour edge. The AMF was applied for 30 min. The temperature at the tumour centre reached 43.0°C or 46.0°C within 2 min, and was kept at the same temperatures by varying the magnetic field intensity. In contrast, the temperature 0.5 cm from the tumour edge remained below 38°C. When the AMF was removed, the temperature at the tumour centre decreased rapidly, reaching body temperature within 5 min. The temperature at the tumour centre was maintained very accurately, with only a small standard deviation. The temperature difference between the tumour centre and 0.5 cm from the tumour edge was very large, as shown in Figure 1. The standard deviation of the temperature 0.5 cm from the tumour edge was approximately 2–3°C. However, the diameters of the optical fibre probe and the tumour at the start of the hyperthermic treatment were about 1 mm and 5–6 mm, respectively, and it was therefore not easy to position the optical fibre probe exactly 0.5 cm from the tumour edge. The highest temperature recorded 0.5 cm from the tumour was 38°C, and no damage was observed in that area. These results indicate that with hyperthermia using MCLs it is feasible to heat only the tumour and not damage healthy tissues.

Monitoring tumour growth after hyperthermia

Figure 2 shows the time course of tumour growth in the 10 mice in each group. After the melanoma nodules had grown to 5–6 mm in diameter, hyperthermia was carried out. In group 1 (control), tumour growth was linear. In group II (hyperthermia at 43°C, once), growth of the subcutaneous tumour was suppressed for about 1 week, and then the tumour grew linearly. In group III (hyperthermia at 46°C, once), the subcutaneous tumours in four of the 10 mice disappeared. However, the tumours in the other mice began to grow several days after the hyperthermia treatment. In group IV, hyperthermia was performed twice (days 1 and 5 at 46°C); the subcutaneous tumours in nine of the 10 mice disappeared and did not begin to grow again.

Figure 3 shows photographs of typical mice from groups 1 and IV 15 days after the MCL injection. In group I, the subcutaneous tumour grew large, whereas
Antitumour immunity induction by hyperthermia using MCLs
To examine whether hyperthermia caused cytotoxic activity against melanoma cells, an *in vitro* long-term cytotoxicity assay was performed. Spleen cells were obtained from the cured mice (group IV) and from naive mice. As shown in Figure 5, spleen cells from the cured mice showed cytotoxic activity against B16 melanoma cells that was approximately two times greater than that of spleen cells from naive mice.

To examine whether tumour-specific immunity had been induced, the mice cured by hyperthermia treatment were challenged with melanoma cells. Melanoma cells were transplanted into the cured mice and into naive mice 1 month after treatment. All of the naive mice subsequently displayed melanoma nodules within 6 days. In contrast, 66% of the cured mice rejected the
Fig. 3

Typical appearance of group I and group IV mice on the 15th day after MCL injection.

Fig. 4

Percentage survival of tumour-bearing mice observed for a period of 120 days after hyperthermia treatment.

Discussion
The hyperthermic effect of magnetic particles was examined in an in vivo study of mouse B16 melanoma. In the case of a superficial tumour such as melanoma, a simple heat mediator is desirable for clinical applications. Figure 1 shows that the temperature at the outside skin of the tumour reached 43.0°C or 46.0°C very rapidly, while the temperature 0.5 cm from the tumour edge remained under 38°C. These results indicate that the hyperthermia using MCLs can heat tumour tissue specifically, and that accurate control of the tumour temperature is possible by changing the magnetic field intensity.

For deep-seated tumours or smaller tumours such as metastatic tumours, drug delivery techniques should be developed since it is difficult to inject MCLs directly into such tumours. Use of stick-type carboxymethylcellulose (CMC)-magnetite could improve the distribution

melanoma cells, and melanoma nodules did not appear in these mice up to 1 month after transplantation.

HSP70 protein expression after hyperthermic treatment
Expression of HSP70 was examined in the tumour tissues of the hyperthermally treated mice (43°C and 46°C) and the non-treated mice on the day after treatment. HSP70 expression (mean ± SD) in the tumour tissue heated at 43°C and 46°C was 1.6 ± 0.2 and 1.3 ± 0.1 ng/mg tumour tissue, respectively, and was 0.17 ± 0.06 ng/mg tumour tissue in the non-treated mice. HSP70 expression in tumour tissue heated at 43°C was greater than at 46°C, suggesting that most of the tumour cells heated to 46°C were necrosed by the hyperthermia.
of magnetic particles and enable safe heating of deep-seated tumours [13,14]. We have also developed magnetic particles that are coated with a liposomal membrane and conjugated with an antibody: ‘antibody-conjugated magnetoliposomes’ (ACMs) [15]. These are able to target renal cell carcinoma cells in vitro and in vivo, and have been efficiently used in the hyperthermic treatment of carcinoma. Melanoma antigens are well known and anti-melanoma antibody is available. Therefore, ACMs using anti-melanoma antibody would be expected to be effective for metastatic tumours derived from melanoma.

Hyperthermia is known to induce HSPs [16]. Until now, the expression of HSPs was considered to be an unwanted side effect of hyperthermia because they cause thermotolerance in tumour tissue [17]. HSP-mediated antitumour immunity has been reported to cause a vaccine effect via HSP–peptide complexes released from human melanoma cells [18]. In addition, it has been reported that expression of HSP70 was increased after exposure to 45°C [19]; the hyperthermia in the present study also induced HSP70.

HSP-mediated augmentation of MHC class I surface expression and endogenous antigen presentation has been reported [20]. We also demonstrated heat-induced expression of MHC class I antigen on the cells as a possible mechanism for the antitumour immunity induced by hyperthermia [21,22]. Even if viable cells are still present in a tumour after hyperthermia, they will become targets for the antitumour immune reaction produced by the augmentation of the heat-induced expression of MHC class I antigen, and complete tumour regression will eventually occur. Abrogation of the metastatic capacity of B16 melanoma cells has been achieved by transferring an MHC class I-deficient vector into class I-deficient B16 melanoma clones [23]. This suggests that hyperthermia also has the potential to abrogate metastatic capacity via antitumour immunity mediated by the release of HSP–peptide complexes and the expression of MHC class I antigen. The complete tumour regression seen in some mice (Fig. 2) should be particularly noted, and further improvement of the treatment protocol is expected to result in an even more effective therapy.

The present study shows that hyperthermia using MCLs is an effective therapy for the treatment of melanoma, since this treatment can kill the tumour cells not only by heat but also by inducing an immune response.

References


