Targeted hyperthermia using magnetite cationic liposomes and an alternating magnetic field in a mouse osteosarcoma model

Y. Shido, Y. Nishida, Y. Suzuki, T. Kobayashi, N. Ishiguro

From Nagoya University Graduate School of Medicine, Nagoya, Japan

We undertook a study of the anti-tumour effects of hyperthermia, delivered via magnetite cationic liposomes (MCLs), on local tumours and lung metastases in a mouse model of osteosarcoma. MCLs were injected into subcutaneous osteosarcomas (L8) and subjected to an alternating magnetic field which induced a heating effect in MCLs. A control group of mice with tumours received MCLs but were not exposed to an AMF. A further group of mice with tumours were exposed to an AMF but had not been treated with MCLs. The distribution of MCLs and local and lung metastases was evaluated histologically. The weight and volume of local tumours and the number of lung metastases were determined. Expression of heat shock protein 70 was evaluated immunohistologically. Hyperthermia using MCLs effectively heated the targeted tumour to 45°C. The mean weight of the local tumour was significantly suppressed in the hyperthermia group (p = 0.013). The mice subjected to hyperthermia had significantly fewer lung metastases than the control mice (p = 0.005). Heat shock protein 70 was expressed in tumours treated with hyperthermia, but was not found in those tumours not exposed to hyperthermia.

The results demonstrate a significant effect of hyperthermia on local tumours and reduces their potential to metastasise to the lung.

Osteosarcoma is the most common primary malignant tumour of bone.1,2 Despite the use of adjuvant chemotherapy and adequate margins of resection of the primary tumour, a significant proportion of patients develop metastatic disease from which they die. Occasionally osteosarcoma arises in the axial skeleton at a site where complete resection cannot be achieved, or develops a local recurrence in conjunction with distant metastases, in which case complete resection might not be indicated. In such cases, there is a need for new therapeutic approaches.

Hyperthermia is one promising approach to cancer therapy. The most commonly used heating method in the clinical setting is capacitive heating using a radiofrequency electric field.3 However, a major technical problem with hyperthermia is the difficulty of heating the targeted tumour to the desired temperature without damaging the surrounding tissues, as the electromagnetic energy must be directed to the targeted tumour.4,5 Other hyperthermia modalities, including radiofrequency ablation and ultrasound hyperthermia, have been reported,6 but the efficacy of these modalities depends on the size and depth of the tumour, and disadvantages include the ability to target the tumour and control the exposure.

Magnetic nanoparticles have therefore been applied to hyperthermia in an attempt to overcome these disadvantages.6-8 Magnetic nanoparticles generate heat in an alternating magnetic field (AMF) as a result of the hysteresis and relaxational losses, which results in heating the tissue in which magnetic nanoparticles accumulate.8 We have developed magnetite cationic liposomes (MCLs) as an intracellular heating transducer, and to improve the absorption and accumulation of magnetoliposomes in tumour cells. They have a tenfold higher affinity for cells than neutrally charged magnetoliposomes owing to the electrostatic interaction with the negatively charged cell membrane.9 Although magnetite particles can migrate passively into the reticuloendothelial system, being found in cells such as the Kupffer cells of the liver and spleen, it has been reported in one study that 40% to 60% of injected MCLs accumulated in tumour tissue, whereas only 20% to 25% of the neutral magnetoliposomes will accumulate.10 Hyperthermia has also been reported to enhance anti-tumour immunity. Heat shock protein (HSP)-mediated antitumour immunity...
has been reported in rat glioma\(^{11}\) and human melanoma.\(^ {12}\) Given that the most critical event in most patients with osteosarcoma is the progression of generally lethal distant metastases, therapeutic tools to reduce the progression of distant metastases, such as the induction of autoimmunity, would be of great benefit.

In this study we analysed the retention and heating ability of MCLs in an AMF using a subcutaneous tumour model of osteosarcoma. We also studied the anti-tumour effect of hyperthermia using MCLs on both local tumours and their potential to metastasise to lung, in addition to evaluating the expression of HSP, which reflects induced autoimmunity.

**Materials and Methods**

**Cell culture.** Murine osteosarcoma cells of the tumour line LM8 (donated by Dr A. Uchida, University of Mie, Tsu, Japan) were maintained at 37°C in a humidified 5% CO\(_2\) atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical, St Louis, Missouri) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin sulphate prior to subcutaneous implantation in mice.

**Animal models.** The cultures of LM8 used in this study were tumourigenic when injected subcutaneously into syngeneic hosts, consistently forming local tumour masses and lung metastases.\(^ {13}\) We purchased 20 five-week-old male C3/He mice from Chubu Kagaku Shizai Co. Ltd, Nagoya, Japan. LM8 cells (5 × 10\(^6\)) suspended in 100 μl of the culture medium described above were injected subcutaneously into the dorsal buttck of these mice. The subcutaneous tumour nodules that developed up to 6 mm to 10 mm in diameter were used for the experiments. The experimental protocol was approved by the Animal Care Committee of Nagoya University School of Medicine and the experiments were performed according to the principles laid down in the "Guide for the Care and Use of Laboratory Animals"\(^ {14}\)

**Preparation of MCLs.** These were prepared using the sonication method.\(^ {15}\) Briefly, 1 ml of colloidal magnetite containing 20 mg magnetite with an average diameter of 10 mm (provided by Toda Kogyo, Hiroshima, Japan) was coated with a lipid membrane consisting of N-(α-trimethylammonionoacetyl) didodecyl-d-glutamate chloride (Sogo Pharmaceutical Co., Tokyo, Japan), dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical Co.) at a molar ratio of 1:2:2.

**Alternating magnetic field (AMF) generation.** An AMF was generated by a vertical coil with an inner diameter of 7 cm, driven by a transistor inverter (LTG-100-05, Dai-ichi High Frequency, Tokyo, Japan) at a frequency of 118 kHz.

**Hyperthermia treatment with MCLs in AMF.** After the subcutaneous tumour nodules had grown to the appropriate size the mice were anaesthetised with an intra-peritoneal injection of 0.1 mg pentobarbital sodium and a syringe (26-needle) containing 200 μl of MCL (containing 4 mg of magnetite) was inserted longitudinally into each nodule and the solution was injected in a number of small aliquots. The mice receiving the MCLs were then separated into two groups. The seven mice in the hyperthermia group were subjected to AMF, whereas six mice in the control group were not. Hyperthermia was applied at 45°C for 30 minutes on three occasions at 24-hour intervals by adjusting the magnetic field intensity under anaesthesia. During AMF application the temperature of the skin overlying the subcutaneous tumour was measured with the infra-red thermography (TVS-200; NEC Avio Infrared Technologies Co., Kanagawa, Japan) at one-minute intervals. The temperature within the tumour tissue was also measured by FX-9020 optical fibre probes (Anritsu Meter, Kyoto, Japan). In order to evaluate the effects of AMF alone, seven mice that had not been injected with MCLs also received this. The tumour diameter was measured every week using calipers until four weeks after the hyperthermia treatment had been concluded. The tumour volume was estimated by the formula 0.5 × length × width\(^2\).

**Histological analysis of local and metastatic tumours.** At four weeks after hyperthermia treatment the mice in all three groups (MCL and AMF = 7, AMT alone = 7, MCL alone = 6) were killed, and the treated subcutaneous tumours and lungs were excised to evaluate the thermal effect both locally and systemically. The tumours were weighed immediately after excision and the tumours and lungs were fixed with formalin, embedded in paraffin, and sections of 6 μm thick were stained with haematoxylin and eosin. The number of lung metastases was counted under light microscopy on coronal sections (magnification × 200). Sections were also subjected to immunohistochemical analysis for the expression of HSP70. Briefly, sections were incubated with a blocking serum solution in PBS for one hour, followed by incubation overnight with primary rabbit anti-HSP70 antibody (Clone C92F3A-5, StressGen Biotechnologies Corporation, Victoria, British Columbia) at a dilution of 1:200 at 4°C. Antigen-antibody complexes were detected by an avidin-biotin-peroxidase technique (Vectastain ABC Kit; Vector Laboratories, Burlingame, California) with diaminobenzidine (DAB) used as a substrate.

**Statistical analysis.** Differences between the groups for mean tumour weight and the mean number of metastases were analysed with the Mann-Whitney U test using Statview J 5.0 software (SAS Institute Inc., Cary, North Carolina). A p-value of < 0.05 was considered statistically significant.

**Results**

**Temperature responses.** During AMF the temperature of the skin over the MCL-injected tumours reached a mean of 45°C (43°C to 46°C) within five minutes and was maintained at this temperature for 30 minutes by adjusting the AMF power, as shown in Figure 1. In contrast, the temperature of the skin over the non-MCL-injected tumours did not increase during AMF. The temperatures within the tumour measured by the fibre probes almost coincided with those measured by thermography, reaching a mean of 45°C.
Magnetite cationic liposome (MCL)-induced hyperthermia in LM8 tumour-bearing mice. These were injected directly into subcutaneous LM8 tumours of mice, then subjected to irradiation with an alternating magnetic field for 30 minutes. a) graph showing temperatures, measured by thermography, of the skin overlying the tumour (black line) and normal tissues (grey dotted line). b) thermogram indicating that the temperature of skin overlying the targeted tumour increased (black arrowhead) compared to the overlying normal tissues (white arrow).

(43°C to 46°C) for the hyperthermia group. Although no skin defects occurred, three of the seven mice in the hyperthermia group suffered skin burns. No temperature changes were observed within the tumours of the mice that did not receive MCL injections.

**Tumour volume.** The tumour volume in the control group increased heterogeneously among individual mice, albeit in a time-dependent manner. In contrast, tumour growth in the hyperthermia group was well suppressed until two weeks after the treatment (Fig. 2). Three of seven mice in the hyperthermia group showed complete regression of the tumours. In the remaining four mice, although the subcutaneous tumours slowly regrew, the weights of the tumours were significantly lower than those in the control group (Table I, p = 0.013). Interestingly, regrowth in the hyperthermia group was slower than that in the control group. Histological examination of a representative subcutaneous tumour in the hyperthermia group at four weeks after the exposure to hyperthermia demonstrated necrosis (Fig. 3). Histological analysis of a tumour in the hyperthermia group that regrew slowly three weeks after the completion of the exposure to heat showed that the MCLs were not dispersed in the tumour but were sited at the periphery, suggesting that uneven initial distribution of MCLs resulted in ineffective hyperthermia.
Table I. Local tumour weight and the number of lung metastases in the control and hyperthermia groups

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<th>Mean tumour weight (mg)</th>
<th>Mean number of lung metastases</th>
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<tr>
<td>Control (range)</td>
<td>2870 (1000 to 4600)</td>
<td>56.8 (31 to 78)</td>
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<tr>
<td>Hyperthermia (range)</td>
<td>260 (0 to 650)</td>
<td>17.6 (0 to 40)</td>
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<tr>
<td>Mann-Whitney U test</td>
<td>p = 0.013</td>
<td>p = 0.005</td>
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Histology of tumour tissues in the hyperthermia group, showing that a) Necrotic tissue was present at the centre of the specimen. There was prominent intracellular magnetic cationic liposome (MCL) deposition (white arrowhead) (haematoxylin and eosin stain, × 200) and b) A case with regrowth of the tumour. MCLs (black arrowhead) were localised peripherally in the viable tumour (haematoxylin and eosin stain, × 20), and c) Expression of heat shock protein (HSP70) was immunohistochemically determined. HSP70 expressed in necrotic cells (black arrowhead) next to MCLs deposition (black arrow) Counterstain with haematoxylin and eosin × 200.

The group of mice treated with AMF but without injection of MCLs showed no alteration in the development of their tumours, suggesting that AMF had no therapeutic effect.

**Lung metastases.** The mean number of lung metastases in the control group was 56.8 (31 to 78) and 17.6 (0 to 40) in the hyperthermia group (Table I, p = 0.005).

**Heat shock protein.** Immunohistochemical analysis localised HSP70 in the necrotic tumour cells and surrounding viable tumour cells in the hyperthermia group, whereas it was not expressed in the control group (Fig. 3c).

**Discussion**

Various methods have been employed to induce hyperthermia, including hot water, capacitative heating and induction heating. A major problem with the application of hyperthermic treatment is the difficulty of heating the target tissue without damaging the surrounding tissue. In the clinical setting, external hyperthermia using a radiofrequency electric field or ultrasound energy is common. However, because these methods also heat normal tissues, heating a tumour to the desired 45°C to induce cell death is problematic. Intracellular hyperthermia using MCL has the important advantage of heating the target tumour to the desired temperature without heating the surrounding tissues, as shown in Figure 1. Our results showed that hyperthermia using MCLs exerts a suppressive effect in an osteosarcoma model, and has also been shown to be effective with B16 mouse melanoma, T-9 rat glioma, and VX-7 squamous cell carcinoma in rabbit tongue.

The prognosis of patients with osteosarcoma has improved markedly following the introduction of effective chemotherapy. However, the prognosis remains poor in the presence of an unresectable primary tumour arising in the axial skeleton or presenting with distant metastases. Hyperthermia using MCLs exhibited suppressive effects on both local tumours and their metastatic potential to the lung in this study. It has been reported that hyperthermia delivered with ultrasound or a polarised near-infra-red source can suppress locally implanted osteosarcoma cells. However, the efficacy of heating with these modalities depends on the depth of the tumour, and they cannot be sufficiently focused on the targeted tumour to avoid damaging the surrounding normal tissues. One previous study reported hyperthermia using MCLs for hamster osteosarcoma, with the temperature elevated to 42°C, which may have induced apoptosis rather than necrosis. That study did not analyse the effect of hyperthermia using MCLs on the metastatic potential of the local tumours. Given that it is the metastatic disease that accounts for the dismal prognosis of advanced osteosarcoma, the effects of hyperthermia using MCL on both local tumours and their potential to metastasise to lung should be examined.

In this study, expression of HSP70 was stimulated in the necrotic tumour cells in the hyperthermia group. Because expression of HSP70 protects cells from heat-induced apoptosis, it is considered to be a complicating factor in hyperthermia. However, several reports have noted that HSP70 expression can induce strong antitumour immu-
death, one possible interpretation is that hyperthermia in tents including tumour antigens are not released from the necrotic cells. Released HSP chaperones antigens, and the complex stimulates macrophages and dendritic cells to secrete cytokines, thereby activating antigen-presenting cells. As shown in Figure 3, expression of HSP70 was observed in necrotic tissue and surrounding viable tumour cells in the hyperthermic group, suggesting an anti-tumour effect of hyperthermia via HSP70 expression. This might explain the slower re-growth of the MCLs and AMF-treated tumours that of control tumours. The mean number of lung metastases was significantly smaller in the hyperthermic group than in the control group. This might be due to the limitation in the size of the local tumours before they could form any distant metastases. However, the hyperthermia may have induced a vaccine-like effect caused by necrotic cell death via HSP70 expression, thereby having a systemic action. A previous study demonstrated HSP70 induction in osteosarcoma cells at 42°C, but the analysis was confined to a monolayer culture. However, osteosarcoma has a characteristic extracellular matrix requiring the efficacy of hyperthermia and expression of HSP to be studied in an in vivo system.

A major concern about the therapeutic application of the method of hyperthermia we describe is safety. The iron content of various organs such as the spleen, liver and brain of MCL-injected mice has been shown to be the same as that of the control mice, and repeated subcutaneous administration of MCL caused no specific pathological change in any of the organs examined, nor did it show any adverse effect on survival. Another limitation was re-growth of the tumour in four mice, but it seems likely that this was due to uneven distribution of magnetite nanoparticles within the tumours. Given that hyperthermia can induce complete tumour necrosis, one can speculate that MCLs would subsequently diffuse beyond the necrotic area within the tumour, resulting in a wide distribution of magnetite nanoparticles and a more widespread tissue destruction. An improved method of targeted delivery of MCLs needs to be devised.

We recognise the limitation of our animal model of osteosarcoma with the tumour carried in soft tissue. It is difficult to obtain stable implantation of LMS osteosarcoma cells in bone. Therefore, the behaviour of MCLs in inducing necrosis of tumour cells in bone needs to be studied in the bone micro-environment.

In conclusion, our results demonstrated that hyperthermia using MCLs effectively heated the targeted tumour tissues to 45°C, induced necrosis in local tumours of osteosarcoma present in soft tissue, and suppressed their metastatic potential to lung. However, several tumours re-grew albeit at a reduced rate, after hyperthermia treatment, probably owing to the uneven distribution of MCLs. Despite the heating effect being localised to the tumour, several burns affected the overlying skin. Further investigation of this treatment for advanced osteosarcoma is warranted.

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References


