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Hyperthermia using magnetite cationic liposomes for hamster osteosarcoma

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Published: 25 March 2004

Received: 20 February 2004

BioMagnetic Research and Technology 2004, **2**:3

Accepted: 25 March 2004

This article is available from: <http://www.biomagres.com/content/2/1/3>

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Abstract

Background: We have developed magnetite cationic liposomes (MCLs) and applied them to local hyperthermia as a mediator. MCLs have a positive charge and generate heat under an alternating magnetic field (AMF) by hysteresis loss. In this study, the effect of hyperthermia using MCLs was examined in an *in vivo* study of hamster osteosarcoma.

Method: MCLs were injected into the osteosarcoma and then subjected to an AMF.

Results: The tumor was heated at over 42°C, but other normal tissues were not heated as much. Complete regression was observed in 100% of the treated group hamsters, whereas no regression was observed in the control group hamsters. At day 12, the average tumor volume of the treated hamsters was about 1/1000 of that of the control hamsters. In the treated hamsters, no regrowth of osteosarcomas was observed over a period of 3 months after the complete regression.

Conclusion: These results suggest that this treatment is effective for osteosarcoma.

Background

Magnetite nanoparticles have been used in biological and medical applications, such as the separation of biological materials using magnetically labeled beads [1], drug delivery and medicine [2], or cell sorting, based on the fact that high magnetic flux density attracts magnetically labeled cells [3-5]. We previously developed "magnetite cationic liposomes" (MCLs), which are cationic liposomes containing 10-nm magnetite nanoparticles, in order to improve the accumulation of magnetite nanoparticles in target cells using the electrostatic interaction between MCLs and the cell membrane [6]. Currently, we have developed a tissue engineering technique using MCLs [7-

9]. Mesenchymal stem cells (MSCs), which can differentiate into multiple mesodermal tissues, can be isolated from bone marrow in a small number. Magnetically labeled MSCs were easily prepared, because our MCLs exhibited no toxicity against the MSCs in proliferation and differentiation, and then the MSCs were enriched into the localized area that the magnetic force can reach. Cell growth was promoted and a five-fold increase in cell number was obtained at 7 days after cell seeding [7]. To establish 3D *in vivo*-like tissues consisting of various types of cells, we applied MCLs to the co-culture system of rat hepatocytes and human aortic endothelial cells (HAECs). Magnetically labeled HAECs accumulated onto

hepatocyte monolayers by magnetic force to form a heterotypic, layered construct with tight and close contact. Albumin secretion by hepatocytes was about three times higher than that of the control co-culture system without magnetic force [8]. MCLs were also applied to construct multilayered keratinocyte sheets. On a 24-well ultra-low-attachment plate, a 5-layered keratinocyte sheet was first produced and a 10-layered epidermal sheet was formed in a high-calcium medium. The sheet formed ordinarily was detached from the bottom of the plate when the magnet was removed, and transplantation to the patient was easily performed [9]. We have termed this culture methodology as "magnetic force-based tissue engineering (Mag-TE)". We have also used MCLs as a heating mediator for cancer hyperthermia, because magnetite nanoparticles generate heat under an alternating magnetic field (AMF) [10-13].

Hyperthermia is one of the promising approaches in cancer therapy, and various methods have been employed in hyperthermia [14,15]. The most commonly used heating method in clinical settings is capacitive heating using a radiofrequency (RF) electric field [16]. However, heating tumors specifically 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. From a clinical point of view, a simple heat mediator is more desirable not only for superficially located tumors but also for deep-seated tumors. Some researchers have proposed inductive heating methods, using submicron magnetic particles, for hyperthermia [17,18]. We have also developed MCLs for intracellular hyperthermia [6,10,11], which showed a ten-fold higher affinity for the tumor cells than neutrally charged magnetoliposomes [10]. Based on this feature, MCLs can be highly superior heating mediators. We previously demonstrated the efficacy of MCL-mediated hyperthermia in animals with several types of tumors, including B16 mouse melanoma [19,20], T-9 rat glioma [11,21], renal cell carcinoma [22], and VX-7 squamous cell carcinoma in rabbit tongue [23]. We also reported complete regression of mouse mammary carcinoma, larger than 15 mm in size, by frequent repeated hyperthermia [24]. Although MCL-mediated hyperthermia was found to be very effective for inducing complete regression of tumors, no studies have investigated the biodistribution of MCLs after local injection.

Osteosarcoma is a primary malignant tumor of the bone that mostly occurs in growing children and young adults [25]. Effective systemic adjuvant chemotherapy on the primary tumor and improvements in surgical resection techniques have improved the survival rate. However, these have not proved to be sufficiently effective, and a

more effective protocol for the prevention and treatment of osteosarcoma is needed.

Therefore, in the present paper, our hyperthermia system was applied to hamster osteosarcoma and its hyperthermic effect was investigated.

Materials and methods

Animals and osteosarcoma models

Three-week-old Syrian female hamsters were purchased from Japan SLC, Inc., Shizuoka, Japan, and used for the animal study.

Experimentally transplantable osteosarcoma Os515, induced by the BK virus, was used [25]. Since it was very difficult to culture the cells in a liquid medium using a dish, the osteosarcoma tissue of Os515 was maintained by the transplanting it in the Syrian hamsters.

Preparation of tumor-bearing hamsters

Mined osteosarcoma fragments (100 μ l) were transplanted into the subcutaneous layer of the back of Syrian female hamsters, which were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Tumor size was measured every 3 days. The volume and the ratio of tumor volume were determined by the following formulas [10]:

$$\text{Sarcoma volume} = 0.5 \times (\text{length} \times \text{width}^2)$$

$$\text{Ratio of tumor volume} = (\text{sarcoma volume on each day}) / (\text{sarcoma volume before hyperthermia}),$$

where the unit of length and width is expressed in centimeters.

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

Preparation of MCLs

Magnetic particles were kindly donated by Toda Kogyo Co. (Hiroshima, Japan; average particle size of magnetite: 10 nm). MCLs were prepared using the previously described sonication method, with slight modification [6]. Briefly, 1 ml of 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), dilaurylphosphatidylcholine, and dioleoylphosphatidylethanolamine (Sigma Chemical Co., St. Louis, MO, USA) in a molar ratio of 1:2:2. Magnetite concentration was measured using the potassium thiocyanate method [22].

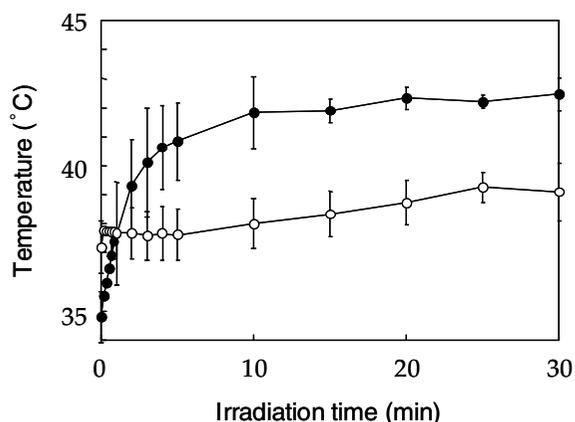


Figure 1

Temperature increase at outside of the tumor center (closed circles) and in the rectum (open circles). The temperature at the outside of the tumor center was maintained at 42°C. Data points and bars represent the mean and SD of the 3 independent experiments, respectively.

Hyperthermia treatment

After the tumors had grown to about 10 mm in diameter, a syringe (25 G needle) containing MCLs was longitudinally inserted into each osteosarcoma nodule, subcutaneously from the nodule edge. MCLs (0.4 ml, net magnetite weight: 3 mg) were injected using an infusion pump (SP100i; World Precision Instruments Inc., Sarasota, FL, USA) for 30 min. The hamsters were then separated into control ($n = 4$) and treatment ($n = 4$) groups. The hamsters in group I (control) were not subjected to AMF. In group II (treatment group), 24 h after the injection, the hamsters were subjected to the first hyperthermia treatment after being anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). A magnetic field was created using a horizontal coil (inner diameter, 7 cm; length, 7 cm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency Co., Tokyo). The magnetic field frequency was 118 kHz. The hamster was placed inside the coil such that the nodule was positioned at the center of the coil. Temperatures at the surface of the tumor tissue and in the rectum during AMF were measured by an optical fiber probe (FX-9020; Anritsu Meter Co., Tokyo). The treatment was carried out for 30 min, three times at 24 h intervals.

Results

Heat generation by MCLs in an alternating magnetic field

Figure 1 shows the temperature increase at the outside skin covering the osteosarcoma and in the rectum. The temperature of the osteosarcoma was rapidly elevated by

magnetic heating and reached over 42°C after 10 min; it was maintained at the same temperature by controlling the magnetic field intensity. In contrast, the temperature in the rectum remained between 37–39°C. These results indicate that using MCLs for hyperthermia makes it feasible to heat only tumor, and not damage healthy tissues.

Monitoring tumor growth after hyperthermia

Figure 2 shows the time courses of osteosarcoma growth in the control and treatment groups. In the control group, the growth ratio of osteosarcoma in each hamster steadily increased with no evidence of regression. In contrast, complete regression of osteosarcoma was observed in 100% of the hamsters in the treatment group. In two cases, the osteosarcoma volume increased until day 4 or 10, after which it began to decrease and finally disappeared. Figure 3 shows photographs of typical hamsters from the treatment (A) and control (B) groups, at day 20 after the MCL injection. Osteosarcomas in the treatment group hamsters clearly disappeared and the skin was also quite normal.

In the treatment group, all osteosarcomas disappeared by day 15 and no regrowth of osteosarcomas was observed over a period of 3 months. As shown in Table 1, tumor volume was compared at day 12 of the MCL injection, because two control hamsters died on day 12. At day 12 of the MCL injection, 75% of the osteosarcomas disappeared, and the average tumor volume of the treated hamsters was about 1/1000 that of the control hamsters. Although there were only four hamsters in each group, it has been proven that our hyperthermia was a significantly effective treatment for osteosarcoma.

Discussion

The treatment of osteosarcoma usually involves the administration of anticancer drugs or surgery or a combination of both. In such cases, several side effects of drugs and the mutilation of arms or legs cause patients additional pain. Hyperthermia is a promising approach for the treatment of osteosarcoma. Although radiofrequency capacitive heating has performed well as a heating method, electric waves cannot be focused on the tumor tissue and may affect, and thereby damage, normal bone tissue. Therefore, although several researchers have proposed simulation of heating to prevent such side effects [26,27], it is not a fundamental solution. Inductive heating has a great advantage of hyperthermia for osteosarcoma in bone because the magnetic field used in this therapy does not cause bone decay. Takegami *et al.* have proposed a ferromagnetic bone cement as a thermoseed to generate heat by hysteresis loss under an AMF [28]. The heat-generating ability was investigated using rabbit and human cadaver tibiae in which this thermoseed was implanted. By applying a magnetic field with a maximum

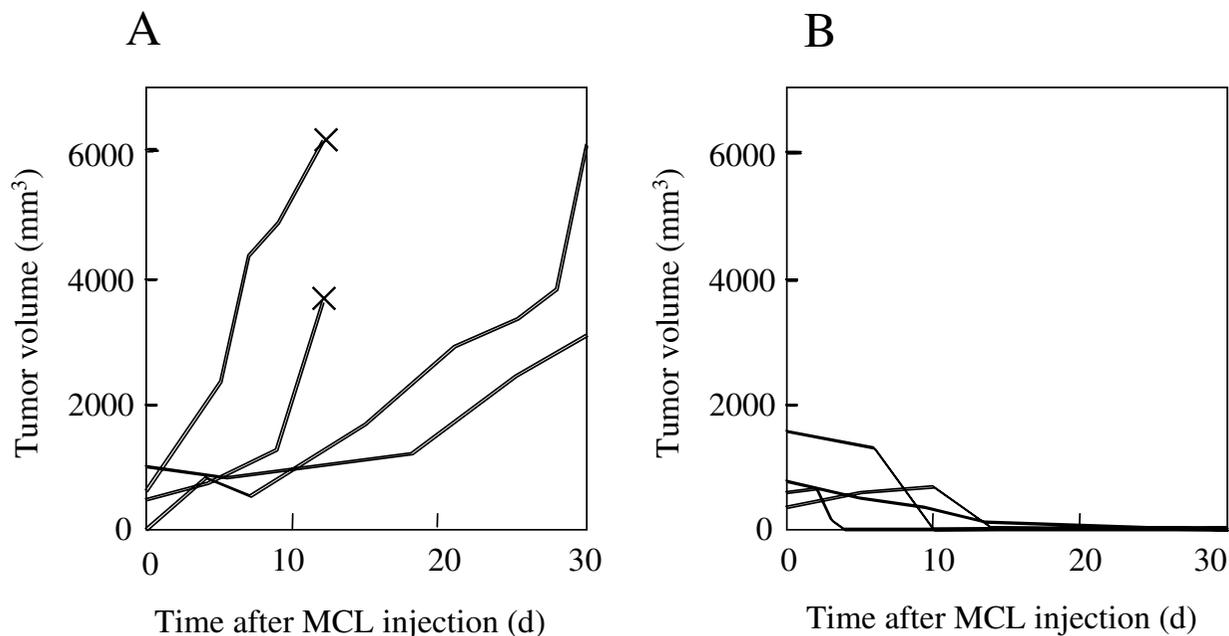


Figure 2

Time courses of tumor growth after the MCL injection for control (A) and treatment (B) groups. Tumor regrowth was not observed within 3 months in hamsters showing complete regression. ×: The day the hamsters died.

of 300 Oe and 100 kHz, it was found that the temperature increase of the thermoseed implanted bone was beyond 50°C. However, implanted magnetic cement remains in patient bone. Therefore, it should be carefully investigated whether long-term deposits of magnetite affects patient health, that is, acute and/or chronic toxicity by excess absorption of Fe ions, e.g., hemochromatosis [29]. In our previous study, the magnetic particles administered were completely cleared from the body within 10 days in the case of mice [30].

We have performed hyperthermia using magnetic particles against brain tumor, tongue cancer, kidney cancer, and a malignant melanoma. In the present study, the effect of hyperthermia was investigated against a hamster osteosarcoma. Although it is an elementary investigation using subcutaneous tumor, the anti-tumor effect against

the osteosarcoma was confirmed. Moreover, it should be noted that the treatment temperature was only 42°C. In the case of melanoma-bearing mice, a more efficient treatment effect was observed when the treatment at 46°C was carried out and complete regression was obtained [19]. In addition, mouse mammary carcinoma, larger than 15 mm in size, was also completely regressed. Furthermore, all large tumors disappeared within 70 days when frequent repeated treatment involving MCL injection was carried out, whereas all control mice died within 52 days [24]. Therefore, sufficient treatment effect can be also expected for large osteosarcomas and/or malignant invasive osteosarcoma.

From the present results, it can also be stated the essential advantage of hyperthermia, i.e., the fact that the hyperthermic effect is independent of the type of cancer cell,

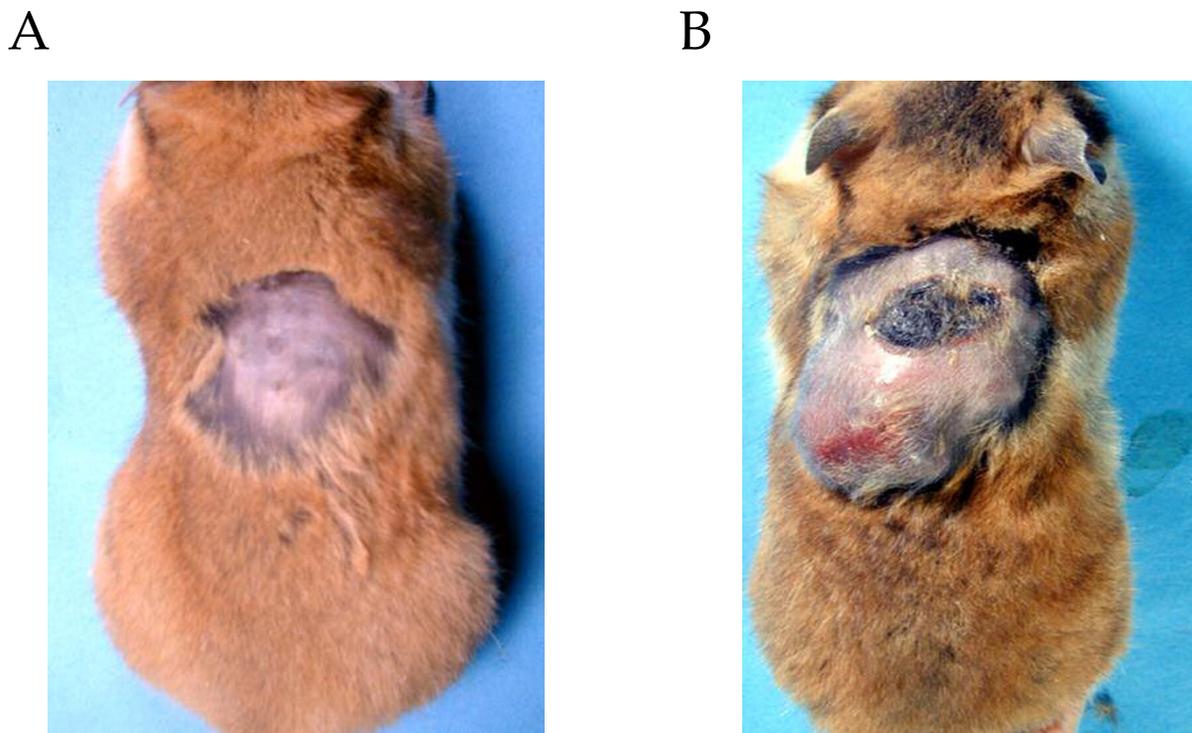


Figure 3
 Typical photographs of hamsters on day 20 after the MCL injection. These photographs show one hamster of the treatment (A) group and one of the control group (B).

Table 1: Hyperthermic effect on subcutaneous tumor 12 days after the magnetite cationic liposomes injection

Group	Number of rats tested	Average tumor volume ^a (mm ³)	Relative tumor volume ^b	Complete regression ^c (%)
Control	4	3006	1	0
Treatment	4	27	0.009	75 (100% at 15 days)

a: Tumor volume = $0.5 \times (\text{length} \times \text{width}^2)$ b: Relative tumor volume = (average tumor volume in treatment group) / (average tumor volume in control group) c: hyperthermic effect in two groups was compared at 12 days after MCL injection since two control hamster died at 12 days. At 15 days, all treated hamsters cured completely.

was reconfirmed. We succeeded in demonstrating the hyperthermic effect using a hamster model in addition to mouse, rat, and rabbit. The present results prove that hyperthermia is a reliable and effective cancer therapy across species.

In future work, it is necessary to study the medical effects against osteosarcomas that originate in bone cells, such as in the femur. In this case, it will be necessary to monitor the distribution of magnetic particles in the tumor and/or bone marrow. When a dense magnetic particle solution is flowed to the bone marrow, the toxic effect to the systemic

immune system should be carefully investigated. If particles are localized into the tumor tissues in the bone, it will be easy to heat the tumor because heat quenching by the blood flow is ignored and a high hyperthermic effect will be expected.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (No. 13853005, 12558106) from the Ministry of Education, Science, Sports and Culture of Japan.

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