Original Contribution

Complete Regression of Hereditary Melanoma in a Mouse Model by Repeated Hyperthermia Using Magnetite Cationic Liposomes

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Abstract: Magnetite cationic liposomes (MCLs) have a positive surface charge, and have been used as heating mediators for intracellular 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 tumor, and a strong antitumor effect was observed in those animal models using transplantable tumor cell lines. In the present study, our protocol was applied to a hereditary melanoma model; primary skin melanoma developing in a metallothionein (MT)-I/ret transgenic mouse line. MCLs were injected into a melanoma nodule (size, 5-7 mm) in MT/ret transgenic mice, which were subjected to AMF for 30 min. The temperature at the surface of the tumor reached 45°C and was maintained by adjusting the magnetic field intensity. Hyperthermia treatment was repeated three times at 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 three times with RH. Furthermore, tumors successfully treated by RH did not undergo regrowth for 120 d post-treatment, and significant elongation of survival was observed. These results suggest that MCL-mediated hyperthermia is a potent approach to treat malignant melanoma.

Key words: hyperthermia, liposome, magnetite, melanoma, metallothionein-I/ret transgenic mouse

Introduction

Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients[1]. In Japan, the most commonly used heating method in clinical settings is
capacitive heating using a radiofrequency (RF) electric field. 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. Hyperthermia produced by heating mediators is a promising approach for specifically heating tumors without damaging normal tissues. Previously, studies have examined inductive heating methods in which the heating mediators are sub-micron magnetic particles. We have developed magnetite cationic liposomes (MCLs) as mediators of intracellular hyperthermia. These cationic liposomes exhibit improved adsorption and accumulation in tumor cells, and have 10-fold higher affinity for tumor cells than neutrally charged magnetoliposomes, thus suggesting that MCLs are superior mediators of hyperthermia. We previously demonstrated the efficacy of MCL-mediated hyperthermia in animals with several cell lines, including B16 mouse melanoma, T-9 rat glioma, Os515 hamster osteosarcoma, MM46 mouse mammary carcinoma, PLS 10 rat prostate cancer, and VX-7 squamous cell carcinoma in rabbit tongue.

Although MCL-mediated hyperthermia was found to be very effective for inducing complete tumor regression in transplantable tumor models, no studies have used a hereditary cancer model, which is thought to be good approximation of cancer patients.

To date, various types of transgenic mouse have been produced to investigate the function of oncogenes in the process of cell differentiation of cells in vivo. The ret oncogene was activated by DNA rearrangement of the ret proto-oncogene with other cellular sequences during NIH3T3 transfection assay. The ret proto-oncogene encodes a receptor-type tyrosine kinase and is often expressed in human cell lines or tumors of neuroectodermal origin, such as neuroblastoma, pheochromocytoma and thyroid medullary carcinoma. Previously, in order to further investigate the action of the ret protein in various tissues, the mouse metallothionein (MT)-I promoter, which is known to function in almost all tissues, was used. Surprisingly, four independent transgenic lines showed hyperpigmented skin due to aberrant melanogenesis, and the melanocytic tumor that developed in MT/ret transgenic mice of line 304 finally progressed to melanoma accompanied by distant metastasis. In this transgenic mouse line, tumors developed mainly in the skin.

An effective protocol for melanoma therapy is urgently needed because of the recent reduction in the Earth’s ozone layer, which blocks the sun’s ultraviolet rays and the increasing incidence of melanoma, which is occurring at a greater rate than that of any other cancer. In addition, a relatively high percentage of melanoma is hereditary (6-14%). In the present study, we assessed the feasibility and potential of MCL-mediated hyperthermia to treat hereditary melanoma in a MT/ret transgenic mouse line.

Materials and Methods

Preparation of MCLs

Magnetite particles were kindly donated by Toda Kogyo Co. (Hiroshima; average particle size, 10 nm). MCLs were prepared using a previously described sonication method, with slight modification. Briefly, 1 ml of colloidal magnetite was coated with a lipid membrane consisting of N- (a-trimethylammonioacetyl) didodecyl-D-glutamate chloride (Sogo Pharmaceutical Co., Tokyo), dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical Co., St. Louis,
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MO) at a molar ratio of 1:2:2. Magnetite concentration was measured using the potassium thiocyanate method. In the present paper, MCL concentration is expressed as net magnetite concentration. Concentration of MCLs was adjusted to 20 mg/ml using phosphate-buffered saline (PBS).

MCL-mediated hyperthermia in MT/ret transgenic mice

MT/ret transgenic mice had no tumors at birth. Thereafter, multiple tumors with the histologic appearance of typical melanoma developed in the skin throughout the body, and tumor size reached 5-7 mm in diameter at an average of 5.4 months after birth. MT/ret transgenic mice with tumor sizes of 5-7 mm were randomly divided into two groups; a control group, which received no treatment, and hyperthermia group, which received MCL-mediated hyperthermia.

For the hyperthermia group, tumor-bearing mice 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. MCL solution (0.2 ml, 20 mg-magnetite/ml) was injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL, USA) for 30 min. After injection of MCLs, mice were subjected to an alternating magnetic field (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). Hyperthermia treatment was repeated three times at 24 h intervals (repeated hyperthermia; RH), and RH was carried out until complete tumor regression was achieved; if partial tumor regrowth occurred after RH, a further 0.2 ml of MCL solution was injected into the tumor and RH was again conducted. Tumor diameter was measured every 3 days, and size was determined by the following formula; Tumor size = 0.5 × (length + width), where length and width are measured in millimeters.

Statistical analysis

For survival analysis, differences in survival rates were analyzed by log-rank test in WinSTAT (Light Stone International, Tokyo). A P value of < 0.05 was considered to indicate statistical significance.

Animal experiments were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” prepared under the direction of the Office of the Prime Minister of Japan.

Results

The temperature increase during hyperthermia using MCLs was investigated. After melanoma nodules had grown to 5-7 mm in diameter, 0.2 ml of MCL solution (net magnetite weight: 4 mg) was injected into the center of tumors, and mice were subjected to AMF for 30 min. Fig. 1 shows the temperature profile at the surface of tumors during AMF irradiation. Tumor temperature increased rapidly, reaching 45°C within 5 min, and was maintained at 45°C by adjusting the magnetic field intensity.
This means that 4 mg of magnetite was sufficient to achieve a tumor temperature of 45°C. No serious burning on the skin was observed in these mice. In addition, rectal temperature increased only slightly during the irradiation, thus suggesting tumor-specific hyperthermia.

The therapeutic effects of RH of the melanoma nodules in MT/ret transgenic mice were then examined. A melanoma nodule with a size of 5-7 mm in diameter was injected with MCLs, and AMF irradiation was carried out three times at 24-h intervals. We previously reported that an iteration of RH was effective for malignant cancers (e.g. mouse mammary carcinoma of size > 15 mm); in the present study, this protocol was applied to MT/ret transgenic mice. Mice were treated with RH, as shown in Fig. 2, until complete tumor regression was achieved. Complete regression of melanoma nodules in three mice (mice no. 1, 2, 4) were achieved by single round of RH. For mice nos. 3 and 5, additional MCLs were injected and RH was again conducted because partial tumor regrowth occurred after the first RH treatment, and complete tumor regression was achieved after 2 or 3 rounds of RH treatment for mice nos. 3 and 5, respectively. Fig. 3 shows the time course for tumor size. In

![Fig. 1. MCL-mediated hyperthermia in MT/ret transgenic mice. MCLs were injected directly into the tumors of mice, which were then irradiated with an AMF for 30 min. Tumor and rectal temperatures were measured by optical fiber probes. Closed circles: tumor; open circles: rectum. Each point represents the mean±SD of 5 mice.](image)

![Fig. 2. Protocol of repeated hyperthermia (RH) for MT/ret transgenic mice. MCLs (4 mg magnetite) were injected directly into the tumors of mice, which were then irradiated with an alternating magnetic field (AMF) for 30 min. AMF irradiation was repeated three times at 24-h intervals (RH). If tumors began to regrow, RH treatment was carried out frequently after injection of MCLs (4 mg magnetite), until complete regression (CR) was achieved.](image)
the control group, tumors grew for 60 d, and all mice died within 60 d. Moreover, 3 mice died within 30 d despite the relatively small tumor size, as shown in Fig. 3A. However, in the hyperthermia group, all tumors completely regressed after several rounds of RH and no mice died within 60 d, as shown in Fig. 3B. Furthermore, tumors successfully treated with RH did not undergo regrowth throughout the lifespan of the mice.

Survival data for a period of 120 days after hyperthermia is shown in Fig. 4. The tumors in MT/ret transgenic mice metastasize to the lymph nodes, lung, brain, kidney, liver, and spleen. This distribution pattern of metastasis corresponds well to that in human skin malignant melanomas in which the lung and lymph nodes are the most common sites of distant metastasis. In the present study, the lung was extirpated from all dead mice and examined for pulmonary metastases. In the control groups, all mice died within 60 d, and pulmonary metastases were observed in two of five mice. On the other hand, three of five mice in the hyperthermia group survived to 120 days, and significant prolongation of overall survival was observed compared with control group (p = 0.024).

Because multiple tumors (4.5 tumors on average) developed in the skin, the size of all tumors, including heated and non-heated tumors were measured. Previously, we observed a 'bystander effect' resulting from MCL-mediated hyperthermia in an experimental T-9 rat glioma model in which one tumor was transplanted into each femur of a rat; although only one tumor was subjected to hyperthermia, the other tumor also disappeared completely. In the present study, in order to investigate whether a bystander effects could be induced after RH in MT/ret transgenic mice, the sizes of all the tumors were measured. Representative data are shown in Figure 5. Each mouse (control group, Fig. 5A; hyperthermia group, Fig. 5B) had eight tumors.

![Fig. 3. Time course of changes in tumor size in control mice (A) and in mice undergoing repeated hyperthermia (RH) treatment (B). Each line represents growth of a tumor of interest in a single mouse.](image)

![Fig. 4. Survival of MT/ret transgenic mice for a period of 120 d after the first hyperthermia treatment. Open circle, control mice (n = 5); closed circle, RH-treated mice (n = 5).](image)
with sizes ranging from 2 to 8 mm, which were located in the back and tail. In the control mice, all tumors grew and no tumor regression was observed, as shown in Fig. 5A. In the hyperthermia group (Fig. 5B) on the other hand, although only one tumor (number 1 in Fig. 5B) was treated twice with RH, the other three tumors (two on the back and one on the tail) also regressed and disappeared.

**Discussion**

In the case of superficial tumors, such as melanoma, a simple heat mediator is desirable for the clinical application of hyperthermia. However, it is difficult to heat a superficial tumor specifically with a capacitive heating method using an RF electric field. Microwave hyperthermia causes severe injury, because the temperature of the surrounding epidermic tissue becomes substantially higher than that of tumor tissue. We used MCLs in order to heat the tumoral region and minimize heating of surrounding healthy tissue. The results shown in Figure 1 suggest that hyperthermia using MCLs allows tumors 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. We previously demonstrated the efficacy of hyperthermia using MCLs against animals having several types of tumor, including B16 mouse melanoma\(^1\)). In these cases, our hyperthermia system could specifically generate heat at the tumor site with magnetite during AMF irradiation, and no heat was
generated and no histological change was observed by only AMF irradiation in tissues without magnetite.

Although the thermal dose-response depends on the correlation between cell lines and microenvironmental factors, such as pH\(^35\)), we believe that, in principle, any type of tumor can be killed using MCL-mediated hyperthermia at higher temperatures. In the present study, the therapeutic effects of MCL-mediated hyperthermia on hereditary melanoma in MT/ret transgenic mice were investigated, and complete regression of all treated tumors was observed using an RH protocol. Although parts of the tumor containing sufficient amounts of MCLs were killed by heat, other parts of the tumor without MCLs or with insufficient amounts of MCLs, particularly at the tumor edge, may continue to grow. RH is effective in such cases of insufficient heating. In mouse no. 5, it took 3 rounds of RH to achieve complete tumor regression (Fig. 2). In a previous study, we showed that in MM46 mouse mammary carcinoma of size 7 mm, all tumors (5/5) disappeared when treated once with RH\(^19\)). Because melanoma nodules in MT/ret transgenic mice, which generated spontaneously, had uneven shape when compared with MM46 tumors, differences in the number of rounds of RH treatment for complete regression were 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, MCL toxicity may become an important issue. In a preliminary study, the toxicity of a single administration of MCL solution (33 mg of magnetite, i.p.) was investigated\(^38\)). MCLs 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 total of 12 mg of magnetite was used in mouse no. 5 (Fig. 2), which was less than that used in the preliminary examination (33 mg). However, MCL toxicity should be fully investigated before the clinical application of RH.

Interestingly, bystander effects of MCL-mediated hyperthermia in hereditary melanoma were observed in MT/ret transgenic mice (Fig. 5B). Several investigators have demonstrated that hyperthermia treatment induces bystander effects. Matsumoto et al.\(^37\)) reported that nitric oxide (NO), which is known to be a multifunctional physiological substance, released from donor cells after hyperthermia induced p53 accumulation in the co-cultivated NO-recipient cells through intercellular signal transduction without cell-to-cell interactions, such as gap junctions, which led to bystander effects. On the other hand, we previously reported that the bystander effects induced by hyperthermia were caused by an antitumor immune response\(^39\)). Heat shock proteins (HSPs) are highly conserved proteins whose syntheses are induced by a variety of stresses, including heat stress\(^39\)). Recent reports have shown the importance of HSPs, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), in immune reactions\(^39\)). In our previous study\(^11\)), expression of HSP70 was examined in the B16 melanoma nodules of both the hyperthermally treated mice (46°C for 30 min) and the non-treated mice on the next day after the treatment. HSP70 expression in the tumor tissue heated was 1.3 ± 0.1 ng/mg-tumor tissue, while 0.17 ± 0.06 ng/mg-tumor tissue was observed in the non-treated mice. As a mechanism for recognition of tumor antigens by the host immune system, we have proposed HSP-mediated antitumor immunity\(^40-42\)); dying tumor cells killed by MCL-mediated hyperthermia release their intracellular contents, including tumor antigen peptides chaperoned by HSPs, and the HSP-peptide complexes are taken up by antigen presenting cells such as dendritic cells, and are in turn presented to T cells via MHC.
class I and/or II antigens. The mechanism of these bystander effects in MT/ret transgenic mice is particularly important because MT/ret transgenic mice represent a hereditary cancer model that is thought to be good approximation of cancer patients, and we are now investigating the mechanism. These results suggest that MCL-mediated hyperthermia is potentially effective for malignant melanoma, because in addition to directly killing tumors with heat, they induce a bystander effect on distant metastases.

In summary, the stable tumoral treatment temperatures, therapeutic effects, and survival benefits demonstrate the feasibility of RH using MCLs in a hereditary melanoma model. The efficacy of bystander effects remains to be confirmed in further experiments.

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References


正電荷リポソーム包埋型マグネタイトを用いた
繰り返しハイパーサーミアによる
マウス遺伝性メラノーマの完全退縮

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要旨: 正電荷リポソーム包埋型マグネタイト (MCL) は交番電場中で発熱することから、細胞内加温法のための発熱素子として利用されている。今までに、様々なガンの移植モデルで高い治療効果が得られているが、全て可移植性腫瘍における検討であり、実際のガン患者の状態に近い遺伝性腫瘍を用いた検討は行っていない。本研究では、metallothionein-I/retを導入したトランスジェニックマウスの遺伝性メラノーマに対するMCLを用いた温熱療法の抗腫瘍効果を調べた。MCLを5-7mm径の腫瘍に投与し、交番電場を30分間照射した。腫瘍温度は5分で45℃に上昇し、そこからは電場強度を調節することで45℃に一定に保った。温熱療法は24時間間隔で3回繰り返して行い (repeated hyperthermia, RH), RHは腫瘍が完全に退縮するまでに行った。腫瘍の完全退縮は1回から3回のRHを行うことで達成された。さらに、RHで完全退縮した腫瘍は、治療120日後まで再発せず、また、有意な生存の延長がみられた。これらの結果は、MCLを用いた温熱療法はメラノーマに対する強力な治療法になることを示唆している。