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Targeting Hyperthermia for Renal Cell Carcinoma Using Human MN Antigen-specific Magnetoliposomes

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Magnetoliposomes (MLs) conjugated with an antibody fragment to give specificity to a tumor were applied to hyperthermia for cancer. The Fab′ fragment of the G250 antibody, which binds to MN antigen on many types of human renal cell carcinoma, was cross-linked to *N*-(6-maleimidocaproyloxy)-dipalmitoyl phosphatidylethanolamine (EMC-DPPE) in liposomal membrane. The targetability of the G250-Fab′ fragment-conjugating MLs (G250-FMLs) was investigated using the mouse renal cell carcinoma (mRCC) and MN antigen-presenting cell, MN-mRCC. The amount of G250-FMLs uptake reached 67 pg/cell against MN-mRCC cells in an *in vitro* experiment using plastic dishes and this value was about 6 times higher than that in the case of MLs. In an *in vivo* experiment using MN-mRCC-harboring mice, 1.5 mg of the FMLs per carcinoma tissue accumulated (tumor weight was 0.19 g), which corresponded to approximately 50% of the total injection. This value was 27 times higher than that of the MLs. After injection of the FMLs, mice were exposed to intracellular hyperthermia using alternating magnetic field irradiation. The temperature of tumor tissue increased to 43°C and the growth of the carcinoma was strongly arrested for at least 2 weeks. These results indicate the G250-FMLs could target renal cell carcinoma cells *in vitro* and *in vivo*, and are efficiently applicable to the hyperthermic treatment of carcinoma.

Key words: Hyperthermia — Magnetic particle — Drug delivery — Antibody — Renal cell carcinoma

Hyperthermia is a therapy based on the fact that tumor cells are more sensitive to temperature in the range of 42–45°C than normal tissue cells.[1–3] Unlike chemotherapy and radiotherapy, hyperthermia itself has few side effects. However, almost all recent hyperthermia methods heat not only the tumor, but also normal tissue. Therefore, a new method that can heat only the desired area and a new heating mediator for this purpose are needed. Some researchers have been investigating submicron magnetic particles as the heating mediator.[4–7] If magnetic particles could be accumulated only in tumor tissue, they could generate heat by hysteresis loss under a high frequency magnetic field to afford cancer-specific heating.

We have developed 'magnetite cationic liposomes' (MCLs) and proven that the high affinity of magnetite to the cells allowed complete killing of tumors.[8, 9] However, administration of MCLs is limited to direct injection into the tumor tissue. From this background, we developed Fab′-conjugating magnetoliposomes (FMLs) that can specifically bind to human glioma cell lines[10] and applied them to subcutaneous tumor.

In the present paper, we prepared FMLs conjugated with Fab′ fragments of human MN antigen-specific antibody, and investigated their affinity to MN antigen-presenting mouse renal cell carcinoma *in vitro* and *in vivo*. Moreover, their hyperthermic effects were confirmed using a mouse renal cell carcinoma model.

MATERIALS AND METHODS

Materials The magnetite particles were prepared by the nitrite oxidation method as reported previously.[11] Phosphatidylcholine (PC) was purchased from Wako Pure Chemicals Co. (Osaka) and phosphatidylethanolamine (PE) from Tokyo Chemical Industry Co. (Tokyo). Dipalmitoyl phosphatidylethanolamine, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and pepsin (1:10 000, porcine stomach mucosa) were purchased from Sigma Chemicals Co. (St. Louis, MO). *N*-(6-Maleimidocaproyloxy)succinimide (EMCS) was from Dojin Laboratories Co. (Kumamoto). *N*-(6-Maleimidocaproyloxy)dipalmitoyl phosphatidylethanolamine (EMC-DPPE) was prepared by our method.[10] All other chemicals were purchased from Wako Pure Chemicals.

Cell culture and antibody A mouse renal cell carcinoma (mRCC) and MN-mRCC were used. MN-mRCC is a stable transformant of mRCC with human MN-antigen cDNA. Human MN antigen is a cell-surface antigen expressed in clear-cell type renal cell carcinoma, the most

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frequent form of human RCC, gastrointestinal mucosa and intestinal epithelium, but not detected in normal kidney.[11] These cells were maintained at 37°C in a 5% CO_2 atmosphere in RPMI1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 5 m*M* nonessential amino acids, and antibiotics (100 U/ml penicillin G, and 100 mg/liter streptomycin). Geneticin (500 mg/liter) was added in the medium for MN-mRCC. The G250 monoclonal antibody F(ab′)$_2$ fragment, which specifically binds to the human MN antigen, was prepared by the following method. The whole antibody (1 mg/ml) was incubated with pepsin (5 mg/liter) in 0.1 *M* sodium citrate buffer (pH 3.8) for 15 h at 37°C. Digestion was terminated by adding 3 *M* Tris (pH 8.0) to a final concentration of 80 m*M*. After centrifuging (10 000*g*, 30 min, 4°C), the resultant mixture was separated to F(ab′)$_2$ and Fc fractions using a Protein G column (MABTrap G II, Amersham Pharmacia Biotech AB, Uppsala, Sweden). The F(ab′)$_2$ fraction was dialyzed with 0.1 *M* Tris-HCl buffer (pH 7.6) for the following step. F(ab′)$_2$ fragments (1 mg/ml) were reduced with 0.1 *M* Tris-HCl buffer containing 30 m*M* cysteine (pH 7.6) for 15 min at 37°C to obtain the Fab′ fragment. Then, cysteine was removed by gel filtration (Econopack 10DG column, Bio-Rad Laboratories, Inc., Hercules, CA) with 0.1 *M* sodium phosphate buffer containing 5 m*M* EDTA (pH 6.0).

Immobilization of antibodies on magnetoliposomes (MLs) Procedures for preparation of the G250 Fab′-conjugating MLs (G250-FMLs) and the MLs were previously described.[10, 12] To prepare the G250-FMLs, the Fab′ fragment (70 mg/liter) was used with 1 mg/ml of net magnetite. Antibody concentration was determined by protein assay (BCA Protein Assay Reagent, Pierce, Rockford, IL).

FMLs uptake by carcinoma cells *in vitro* MN-mRCC cells (2×10^5 cells/ml) were cultivated in 35 mm tissue culture dishes with 2 ml of the medium. After 24 h, the medium was replaced with G250-FMLs, MLs or MCLs containing the medium (net magnetite: 100 pg/cell) and incubated at 37°C, under gentle shaking by a reciprocating shaker (SHK-320, Asahi Techno Glass Co., Tokyo, 70 rpm). After incubation for 1, 4 or 8 h, the cells were washed with 1 ml PBS twice and harvested with a rubber policeman. The quantity of adsorbed magnetite was measured by our reported method.[13]

Preparation of carcinoma-bearing mice The MN-mRCC or mRCC cells (1×10^7 cells) were transplanted under the outer membrane of kidney of female 6-week-old Balb/c mice using a cylinder with a needle (needle size: 26 G). The transplanted tumors had grown to 0.19 g approximately 1 week after transplantation.

***In vivo* experiment** Mice were separated into three groups. Animals in group I, the first control group, were transplanted with MN-mRCC and MLs were injected. Group II animals, the second control group, were transplanted with mRCC and the G250-FMLs were injected. Group III animals were transplanted with MN-mRCC and the G250-FMLs were injected. A 0.4 ml aliquot of G250-FMLs or MLs (net magnetite: 3 mg) containing 0.3 mol% Tween 20 was injected via the heart with a needle (needle size: 26 G). Some mice in all groups were sacrificed and the carcinoma and organ were removed to assess accumulation of magnetite at 48 h after G250-FMLs or MLs injection. Removed blood from each animal was separated into blood cells and blood plasma by centrifugation (1000 rpm, 5 min). The quantity of adsorbed magnetite was measured by our previous method.[9] Other mice were subjected to magnetic field irradiation three times. At 48 h after the injection, the mice were anesthetized and subjected to magnetic field irradiation, which was created by using a horizontal coil (inner diameter, 7 cm; length, 7 cm) with a transistor inverter (LTG-100-05 (5.0 kW, 118 kHz); Dai-Ichi High Frequency Co., Tokyo). Anesthetized mice were laid inside the coil such that the tumor region was at the center. The magnetic field frequency and intensity were 118 kHz and 30.6 kA/m (384 Oe), respectively. The heat evolution rate of all types of MLs used in the present paper was 96 W/g under this condition. Treatment was repeated three times at 24-h intervals. An optical fiber probe (FX-9020; Anritsu Meter Co., Tokyo) was used to measure temperatures at the carcinoma surface and rectum. The temperature at the surface of the carcinoma was lower than that in the inner part where the magnetite exited when the mice were irradiated with the magnetic field. Therefore, we measured the surface temperature of the tumor to evaluate the utility of this technique.

Pathological specimens 6 μm in thickness were prepared, stained with Berlin blue to visualize the location of the magnetite and counterstained with Kernechtrot, or stained with hematoxylin and eosin.

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.

RESULTS

***In vitro* uptake of antibody-conjugated MLs by tumor cells** The uptake of G250-FMLs by tumor cells *in vitro* is shown in Fig. 1. The G250-FMLs were quickly incorporated into MN-mRCC cells, and the maximum amount of uptake was 67 pg/cell after 12 h. This value was 5 times higher than that by mRCC cells. The uptake of G250-FMLs was 1.5 times higher than that of the MCLs. The MCLs uptake by both carcinoma cells was almost the same.

***In vivo* uptake of antibody-conjugated MLs by carcinoma cells** Uptake of G250-FMLs or MLs at 48 h after

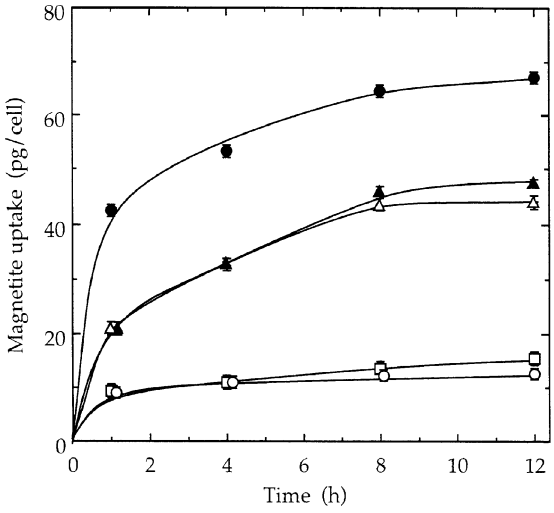

Fig. 1. Time courses of magnetite uptake by MN-mRCC or mRCC cells *in vitro*. Closed symbols indicate data for MN-mRCC cells and open symbols indicate data for mRCC cells. Circles, triangles, and squares indicate data for G250-FMLs, MCLs, and MLs, respectively. Data points and bars are means and SDs of 5 independent experiments.

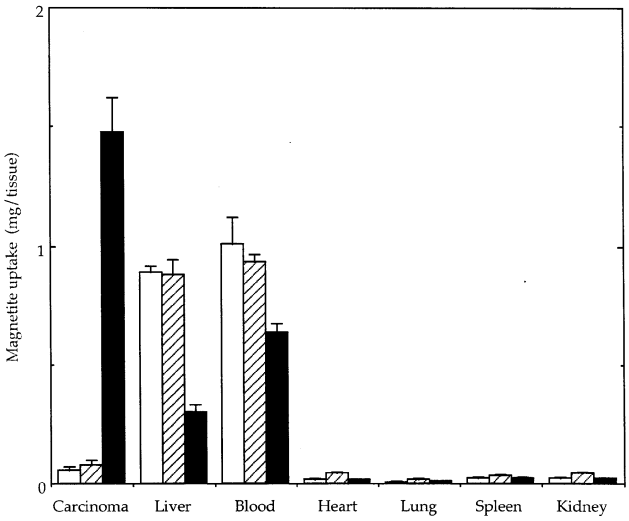

Fig. 2. Magnetite uptake by carcinomas and various organs. MN-mRCC cells were used. Open, shaded, and closed symbols indicate data for group I (MN-mRCC/MLs), group II (mRCC/G250-FMLs), and group III (MN-mRCC/G250-FMLs), respectively. Data and bars are means and SDs of 5 independent experiments.

injection was investigated in an *in vivo* experiment (Fig. 2). An amount of 1.5 mg per tissue of the G250-FMLs was found to accumulate in the MN-mRCC (group III). This amount corresponded to 50% of the total injected amount, and its value was approximately 27 times higher than that of the MLs. In the case of the MLs, only 0.054 mg per tissue was found to accumulate in the MN-mRCC (group I); however, the accumulation ratio of the magnetite was 32% in the liver and 33% in the blood cells, which were 2.5 times and 1.4 times higher than those of the G250-FMLs. In the case of group II, G250-FMLs did not accumulate in the mRCC but accumulated in the liver, as in group I. The accumulation of G250-FMLs in the other organs was almost the same as that of the MLs.

Fig. 3 shows microscopic sections of the carcinoma, liver, spleen and kidney removed from mice into which the G250-FMLs or the MLs were injected. As shown in Fig. 3a, many G250-FMLs accumulated in the MN-mRCC tissue, while few G250-FMLs accumulated in liver from group III animals. There was no evidence of accumulation of the G250-FMLs in normal kidney and spleen from group III animals, or in stomach, intestine, lung, or heart tissue (data not shown). Total accumulation amount in the liver was higher than in the other organs, as shown in Fig. 2, but concentration per tissue was low because of the relatively large volume of the liver. Therefore, only a few magnetite particles were stained in the liver, as shown in Fig. 3a. On the other hand, relatively more magnetite particles (MLs for group I animals) were observed in the liver and the spleen as shown in Fig. 3b. They were barely observed in normal kidney, and never observed in carcinoma. In the case of group II animals, almost the same results as in group I animals were obtained. The G250-FMLs were observed in the liver and the spleen (data not shown).

Hyperthermia Irradiation with the alternating magnetic field heats only magnetic particles in this experiment. The temperature at the carcinoma surface of group III animals was elevated rapidly by the magnetic field and reached 43°C after 10 min, as shown in Fig. 4a. When the magnetic field irradiation was extended to 30 min, the temperature was maintained over 43°C. On the other hand, the temperature of the rectum was elevated to around 37°C. Fig. 4b shows the temperature of group II animals. The carcinoma surface and the rectum were not heated, because the G250-FMLs did not accumulate in the mRCC cells. The temperatures of the carcinoma surface and the rectum in group I animals also did not increase (data not shown).

Fig. 5 shows specimens of carcinoma-transplanted kidney removed at 24 h after hyperthermia treatment. Some necrotic areas were observed only in the carcinoma tissue from group III animals (Fig. 5a). The necrotic area of the carcinoma in the group III animals coincided with the area of the FMLs accumulation. Although the G250-FMLs were also accumulated in the liver as shown in Fig. 2, no necrotic cells were histologically detected in liver (data not shown). The temperature in the liver seems not to have

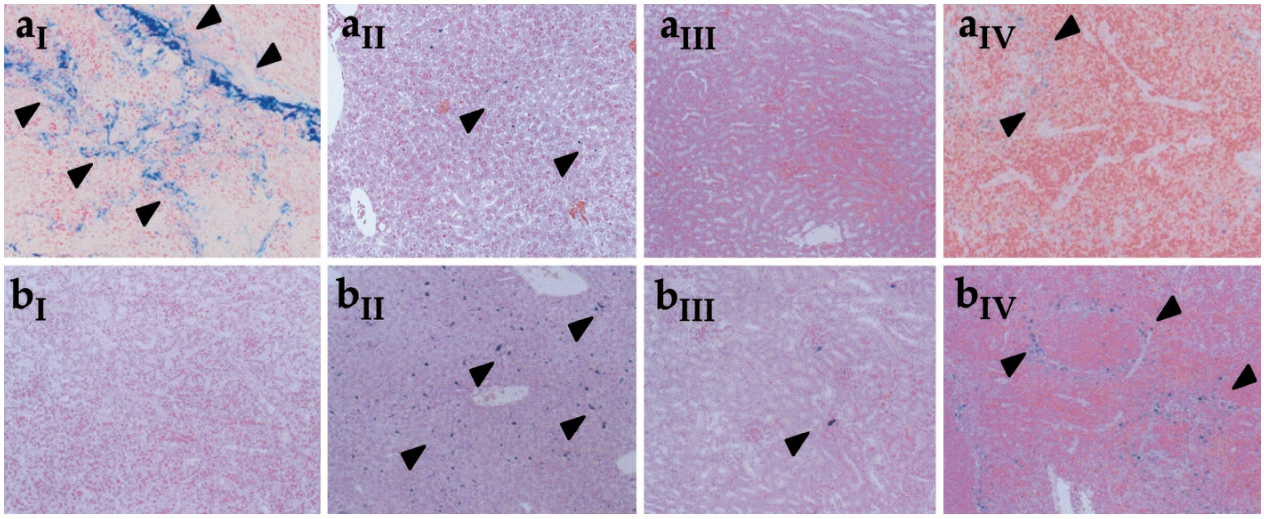

Fig. 3. Light microscopic sections of the carcinoma, liver and kidney stained with iron (Berlin blue) and counterstained with Kernechtrot. The G250-FMLs or MLs were injected via the heart and each tissue was removed at 48 h after the injection. Magnetite reagents appear as a blue color. a, group III (MN-mRCC/G250-FMLs); b, group I (MN-mRCC/MLs); I, carcinoma; II, liver; III, normal kidney; IV, spleen.

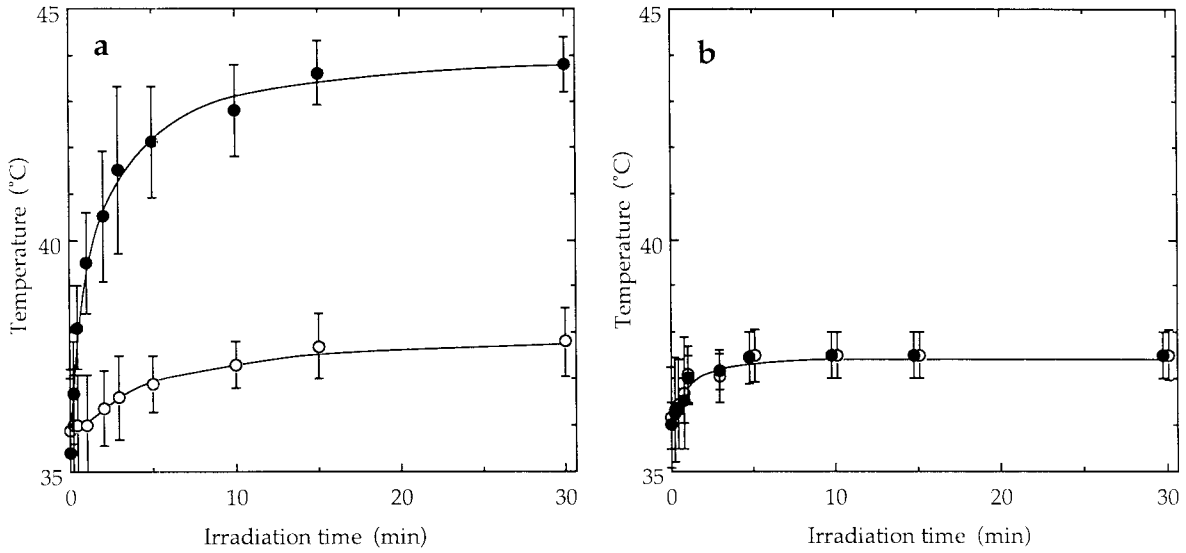

Fig. 4. Temperature increases at the carcinoma surface and in the rectum during magnetic field irradiation. The G250-FMLs were injected into group III animals (MN-mRCC bearing mice) (a) and group II animals (mRCC-bearing mice) (b) via the heart. Closed circle, at carcinoma surface; open circle, in rectum. Data points and bars are means and SDs of 5 independent experiments.

increased to 43°C since the concentration of G250-FMLs in this organ was low and they existed sporadically, and so the generated heat may have been removed by the blood flow. As shown in Fig. 5b, no necrotic cells were histologically detected in the carcinoma in group II animals. This was also the same in group I animals (data not shown).

Fig. 6 shows the carcinoma-transplanted kidneys at 14 days after the last hyperthermia treatment. In group III, the growth of the carcinoma was almost wholly suppressed, as shown in Fig. 6a. Carcinomas of group III were only observed on the surface of the kidney. On the other hand, carcinomas of group II became bigger than the original

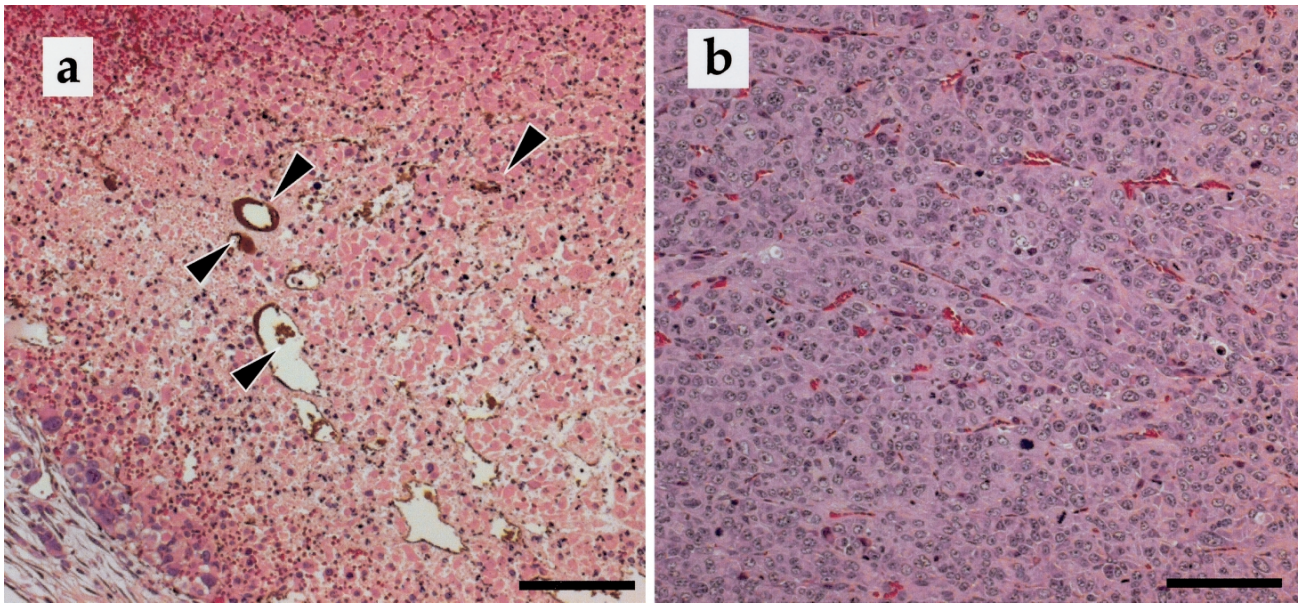

Fig. 5. Light microscopic sections stained with HE of carcinomas at 24 h after hyperthermia (scale bar, 100 μm). Arrow heads indicate magnetite. a, group III (MN-mRCC/G250-FMLs); b, group II (mRCC/G250-FMLs).

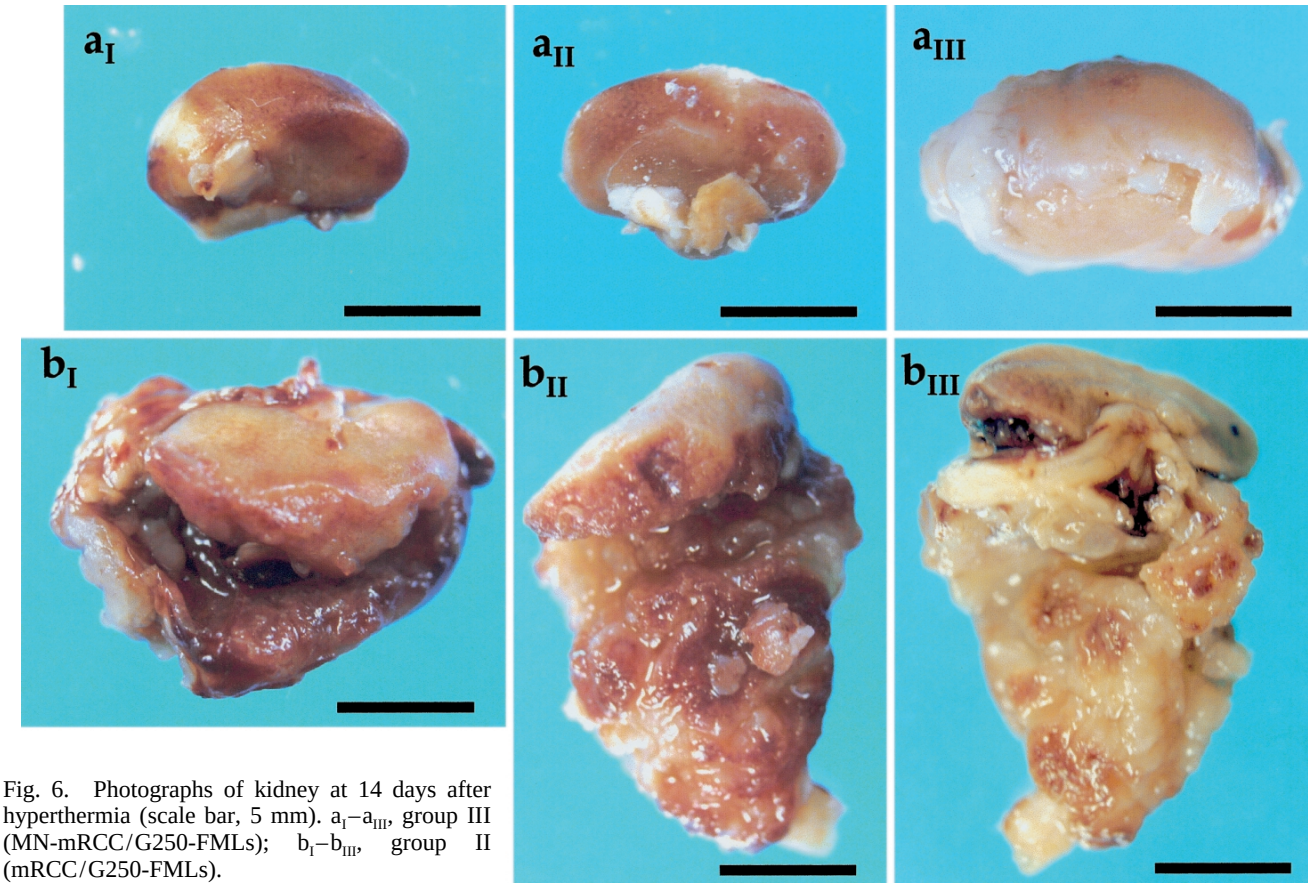

Fig. 6. Photographs of kidney at 14 days after hyperthermia (scale bar, 5 mm). a_I–a_{III}, group III (MN-mRCC/G250-FMLs); b_I–b_{III}, group II (mRCC/G250-FMLs).

Table I. Carcinoma Weight 14 Days after Hyperthermia Treatment

Group	Mean weight±SD (g)
II	1.52±0.19
III	0.27±0.12

Carcinoma weight = (Total weight of carcinoma-transplanted kidney) − (Mean weight of normal kidney).
Initial carcinoma weight = 0.19 g.
Data are means and SDs of 5 independent experiments.
Group II: mRCC-bearing mice injected G250-FMLs.
Group III: MN-mRCC bearing mice injected G250-FMLs.

kidneys as shown in Fig. 6b. These data are summarized in Table I. The mean initial carcinoma weight was 0.19 g. In the case of group II, carcinomas grew vigorously and became 7 times heavier during 14 days. In the case of group III, however, the mean weight gain of carcinomas was only 0.08 g. As a result of hyperthermia treatment three times, a large part of the carcinoma could be killed. Fig. 7 and Table II show the survival curve and the mean survival time of all groups. Mean survival of the two control groups I and II was about 35 days in spite of magnetic field irradiation. This time was the same as that of the non-irradiated group III mice (data not shown). On the other hand, the survival time of irradiated group III was extended by 25 days. Therefore, it is clear that the tumor was specifically heated by the G250-FMLs and the hyperthermia using the G250-FMLs was effective. Furthermore, no severe side effects such as unusual behavior or hematuria were observed, and magnetic field irradiation itself did not influence survival time.

DISCUSSION

As shown in Figs. 1 and 2, the G250-FMLs can specifically bind to MN-mRCC cells and the binding depends on MN antigen/G250 antibody interaction. The amount of specific magnetite uptake was higher than that in our previous method[12] using the MCLs and was sufficient to heat the carcinoma as shown in Fig. 4. *In vitro* experiments revealed that the uptake of G250-FMLs was 1.5 times higher than that of the MCLs. The MCLs have positive charge and normally adsorb negatively charged cells. In the present work, the MCLs uptakes by both carcinoma cells were almost the same, which means that the electrostatic interaction between the MCLs and MN-mRCC or mRCC is dominant and the MN antigen does not affect the uptake of magnetite. We have applied the MCLs for hyperthermia experiments and have obtained complete regression of rat glioma cell T-9.[8] As described above, the uptake of the G250-FMLs was 1.5 times higher than that of the MCLs for MN-mRCC, which means G250-FMLs is an excellent mediator for hyperthermic treatment of carcinomas.

In vivo experiments also revealed that G250-FMLs were adsorbed on the carcinoma cell surface at the MN-antigen, as shown in Fig. 2. About 30−50% of the injected G250-FMLs or the MLs were gradually trapped by blood cells such as macrophages. The other particles were delivered to each organ or to the carcinoma. It should be considered that the affinities to normal tissues of G250-FMLs and the MLs were the same, because their binding activity is not specific and depends on phagocytes of the reticuloendothelial system, such as Kupffer cells in the liver. Therefore, the G250-FMLs seemed to bind to the MN-mRCC at an early period due to the antigen/antibody interaction and their accumulation in the liver was reduced. On the other hand, both the MLs and the G250-FMLs were observed in the liver and spleen in groups I and II. However, total accumulation in the spleen was low, and differences of accumulation among the groups were very small, as shown in Fig. 2.

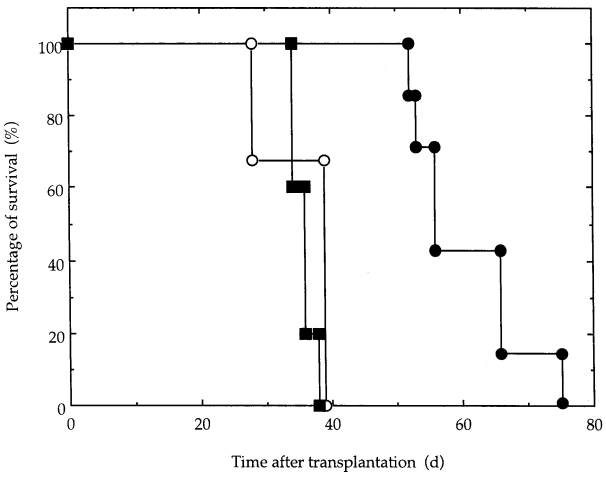

Fig. 7. Survival after transplantation of each carcinoma. Open circles, group I (MN-mRCC/MLs); closed squares, group II (mRCC-bearing mice); closed circles, group III (MN-mRCC/G250-FMLs).

Table II. Mean Survival Time of Each Group

Group	Mean survival time (days)
I	35.6±1.7
II	34.6±6.0
III	60.6±8.6

Data are means and SDs of 5 independent experiments for groups I and II, and 7 independent experiments for group III.
Group I: MN-mRCC-bearing mice injected MLs.
Group II: mRCC-bearing mice injected G250-FMLs.
Group III: MN-mRCC bearing mice injected G250-FMLs.

Matsumura and Maeda[14] reported that drug-polymer conjugates could be delivered to a tumor by utilizing the enhanced permeability and retention (EPR) concept. Drug-containing liposomes can also be delivered. According to this concept, the MLs should also accumulate in both MN-mRCC and the parental mRCC, and the G250-FMLs should accumulate in the parental mRCC. However, we could not observe this. Duncan and Sat[15] suggested that tumor size, but not tumor type or macromolecular form, determines the localization pattern of the administered dose. Tumor size may be one reason why the MLs did not accumulate in the carcinoma, but the critical size is unclear. In the previous paper, the MLs were directly injected into a 1 cm-diameter human glioma subcutaneously transplanted in nude mouse, but only 10% of the injected MLs accumulated.[10] In the present case, antibody reaction kept the MLs in the tumor. We have found that an electrostatic force or magnetic force can keep magnetic particles in a tumor.[8, 16] Therefore, the involvement of an EPR effect in the present case is not clear.

Temperature was measured at the surface of the tumor to evaluate the hyperthermia, as shown in Fig. 4. If we could measure the actual temperature in the tumor, severer hyperthermic conditions could be applied and better results than those shown in Fig. 6 might be obtained. The amount of G250-FMLs accumulated in MN-mRCC is known, and the heat evolution rate can be calculated under our experimental conditions. Therefore, the temperature in the tumor could be simulated if the blood flow rates in the tumor and the surrounding tissues were known.

As shown in Table II, we succeeded in extending the survival time of carcinoma-bearing mice. This extension of the survival time was due to the hyperthermic effect only, because the survival time of non-irradiated group III mice was not extended. As shown in Fig. 4, the irradiation time was 30 min in this experiment, although the irradiation was carried out three times (once per day). In order to optimize the heating conditions and maximize the hyperthermic effect, the effects of duration of the irradiation time and the number of times of irradiation should be studied.

In the present paper, the G250 Fab′ fragment which binds human MN antigen was immobilized on the surface of the MLs. The MN antigen is a general one observed in over 90% of human renal cell carcinomas. This antigen is also observed on gastrointestinal mucosa and intestinal epithelium. The delivery of the G250-FMLs was performed by injection via the heart, so that the G250-FMLs were not accumulated in such tissues. Therefore, G250-FMLs should be suitable for clinical application to human renal cell carcinoma.

In summary, we have developed tumor-specific antibody (Fab′ fragment)-conjugated MLs, which can target the human MN antigen on mouse kidney cancer cells *in vitro* and *in vivo*, and allow efficient application of hyperthermia to the tumor. The growth of the carcinoma was almost entirely suppressed by hyperthermia treatment three times.

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