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Intracellular Hyperthermia for Cancer Using Magnetite Cationic Liposomes: *In vitro* Study

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'Magnetite cationic liposomes (MCL)' were developed as a means to generate intracellular hyperthermia. Affinity of the MCL to glioma cells was ten times higher than that of magnetite 'neutral' liposomes due to the electrostatic interaction based on the positive charge of the MCL. Heat generation of the MCL was studied using agar phantoms and small pellets of rat glioma cells. When a high-frequency magnetic field, 118 kHz, 384 Oe was applied to glioma cells in the presence of MCL, the glioma cell pellet of 80 μl (5.4 mm in diameter) was heated to over 43°C and all the cells died after 40 min irradiation owing to the hyperthermic effect. The terminal temperature of the cell pellet was proportional to the pellet volume when other parameters were constant. It thus appears that the MCL can heat a tumor of more than 80 μl in volume to above 42°C.

Key words: Cationic liposome — Magnetite — Magnetoliposome — Intracellular hyperthermia — Glioma cell

Various methods have been reported to achieve cancer hyperthermia,[1,2] but almost all of these heat not only the tumor, but also normal tissue, which will be damaged by nonspecific heating. Intracellular hyperthermia using small heating elements has been proposed in order to overcome this disadvantage.[3] We have developed new 'magnetoliposomes (ML)' with the ability to target and heat cancer intracellularly, and reported their magnetic properties and suitability for generating hyperthermia in previous papers.[4,5] The ML possessed excellent properties as regards dispersibility in aqueous solution, affinity to the cell and heat generation. Those properties can be modulated by variation of the magnetite core size. The magnetite core of 40 nm gave the maximum heating level. The MLs were also conjugated with antibody, allowing them to be adsorbed specifically by human glioma cells and to be incorporated into the cells. As a result of simulation with a concentric spherical model, a heat evolution rate of 0.2 W/ml-tissue was found to be necessary to heat tumor tissue of 6 cm in diameter to above 42°C, which is the temperature required for cancer hyperthermia. However, further improvements in the adsorption and accumulation of the ML in tumors were necessary to kill smaller tumors. In the present paper, we report novel magnetic particles, 'magnetite cationic liposomes (MCL).' Their adsorption on rat glioma cells, heating properties and hyperthermic efficacy were investigated.

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MATERIALS AND METHODS

Materials Dilauroylphosphatidylcholine (DLPC) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-(α - Trimethylammonioacetyl)didodecyl - D - glutamate chloride (TMAG) was purchased from Sogo Pharmaceutical Co., Ltd. (Tokyo). All other chemicals were purchased from Wako Pure Chemicals Co., Ltd. (Osaka).

Cell culture The rat glioma cell line T-9 was used. The cells were maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 90 μg/ml of streptomycin sulfate).

Preparation of magnetite and magnetite cationic liposomes Magnetite (Fe_3O_4), the core of the MCL, was prepared by the nitrite oxidation method as reported previously.[6] We selected magnetite with a mean diameter of 35 nm for the present study, because its hysteresis loss was high.[4,7] The heat evolution rate of this magnetite was 140 W/g at 384 Oe. The magnetite precipitate obtained was washed with distilled water for the removal of remaining ions. The washing was continued until a stable dispersion of magnetite particles in water was obtained. This magnetite colloid was added to lipid in a round-bottomed flask containing TMAG, DLPC and DOPE in a molar ratio of 1 : 2 : 2. For preparation of the 'neutral' ML, DLPC and DOPE were used in 1 : 1 molar ratio. The flask was vigorously shaken by vortexing, then the

solution was adjusted for both pH and salt concentration (pH 7.0, 0.05 *M* Na phosphate+0.15 *M* NaCl) by addition of ten-fold concentrated phosphate-buffered saline. The mixture of lipid and magnetite colloid was sonicated for 40 min (28 W, cycles of 1 min on and 30 s interval). After sonication, well-dispersed MCL (or ML) were obtained. All MCL and ML concentrations are expressed as net magnetite concentration.

MCL uptake by glioma cells T-9 cells (1×10^6) were seeded into 100 mmϕ tissue culture dishes (Iwaki Glass Co., Ltd., Funabashi) with 10 ml of medium. After 24 h of incubation, the medium was replaced by MCL-containing medium, in which the net magnetite concentration was 20 μg/ml and incubated again. For the assay of magnetite uptake using MCL or ML, the cells were sampled at constant intervals and the iron concentration and cell number were measured by the potassium thiocyanate method[8] and the dye-exclusion method with trypan blue, respectively. Each experiment was repeated 3 times.

Heat generation using MCL gel phantom Agarose solution (1%) was mixed with the MCL at various concentrations. MCL-agarose solution (100 μl) was hardened in 1.5-ml micro centrifuge tubes. The size of the MCL-agarose gel (MCL gel) was about 6 mm in diameter. Then the tube was filled with medium above the MCL gel (Fig. 1). The micro tube was placed in a glass jacket in which water (37°C) was circulating to protect the sample from temperature increase owing to radiation from the coil. The remainder of the glass jacket was filled with agarose gel and the jacket was placed inside the coil. A transistor inverter (LTG-100-05, 5.0 kW, 118 kHz, Dai-ichi High Frequency Co., Ltd., Tokyo) was used to apply a high-frequency magnetic field to the MCL gel. The coil was 85 mm in diameter with 21 turns. An optical fiber thermometer (FX-9020, Anritsu Meter Co., Ltd., Tokyo) was used for temperature measurement at the center of the MCL gel.

Hyperthermia *in vitro* with glioma cell pellets T-9 cells (1×10^6) were seeded into 100 mmϕ tissue culture dishes and incubated for 24 h. After replacement of the medium with MCL-containing medium as mentioned above, the cells were again incubated for 8 h. The cells were washed with PBS under gentle shaking and detached by trypsin treatment. The suspended cells were collected as cell pellets in 1.5-ml micro tubes to form pellets by centrifugation. These cell pellets were placed in the glass vessel as described above. After magnetic irradiation for various periods, the cells were reseeded into a 100 mmϕ tissue culture dish and incubated. After 24 h, surviving cells were counted by trypan blue exclusion. Each experiment was repeated 3 times and each data point is a mean of 3 independent experiments.

RESULTS AND DISCUSSION

MCL uptake by glioma cells The glioma cells were incubated with various concentrations of MCL in order to investigate the toxicity of the MCL in the medium (Fig. 2). At the concentration of 20 μg-magnetite per milliliter of medium, growth inhibition was not observed. However, cell growth was inhibited at higher concentra-

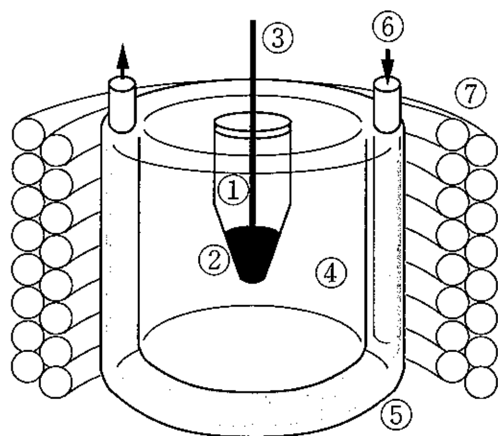

Fig. 1. Experimental set-up for phantom heating and cell pellet heating. ①, medium; ②, magnetite gel or cell pellet; ③, optical fiber thermometer probe; ④, agarose gel; ⑤, jacket; ⑥, circulating water (37°C); ⑦, induction coil (connected to transistor inverter).

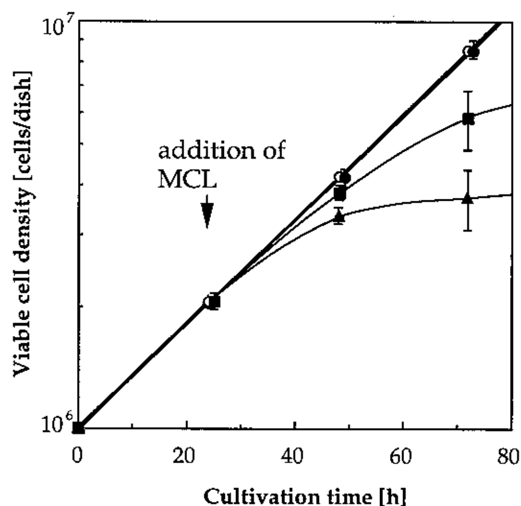

Fig. 2. Toxicity of MCL. Symbols; ○, MCL concentration was 0 μg/ml; ●, 20 μg/ml; ■, 40 μg/ml; ▲, 80 μg/ml. Each data point and bar are the mean and standard deviation (SD) of 3 independent experiments, respectively.

tions. When empty liposomes were added with the same concentration of lipid, the cell growth was not inhibited (data not shown). From these results, a magnetite concentration above 20 μg/ml seems to be toxic.

The MCL and ML uptakes by the glioma cells are shown in Fig. 3. The MCL uptake started rapidly and reached 80% of the maximum at 1 h after addition. The maximum uptake (55 pg/cell) was achieved after 4 h, and was ten times higher than that of the ML. Since in the case of the MCL, the positively charged lipids are anchored in the magnetoliposome membrane, the MCL are able to achieve a high adsorption rate through electrostatic interaction with the negatively charged phospholipid membrane of cells.[9] Due to this property, the MCL seem to be adsorbed not only by glioma cells, but also by normal cells such as fibroblasts or glia cells. Moreover, it seems that the MCL can not pass through the blood brain barrier (BBB). Therefore, intratumoral injection would be necessary to deliver the MCL to the tumor, as has been reported in our previous paper[10] on cationic liposomal gene transfection. In the case of intratumoral injection, cationic liposomes can be incorporated in glioma cells regardless of the BBB. In order to improve the selectivity against glioma cells, we are also studying anti-glioma monoclonal antibody-conjugated MCL.

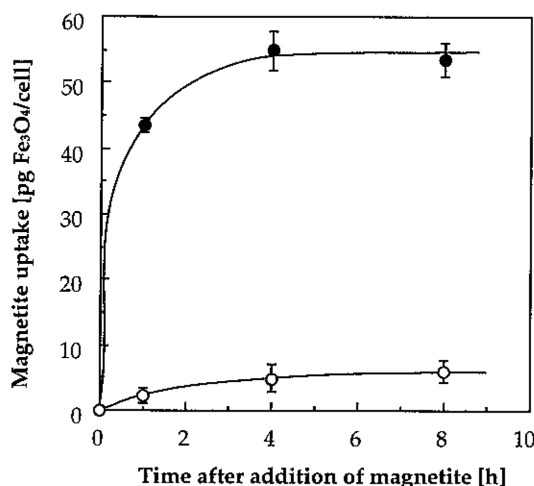

Fig. 3. Comparison of magnetite uptake between MCL and ML. Symbols; ●, magnetite cationic liposomes (MCL); ○, magnetoliposomes (ML). Each data point and bar are the mean and SD of 3 independent experiments, respectively.

Heat generation by MCL in a high-frequency magnetic field First, heat generation of MCL was studied with agarose phantoms (Fig. 4). The magnetic field frequency and intensity were 118 kHz and 30.6 kA/m (384 Oe), respectively, in all experiments. Temperature at the center of the MCL gel increased quickly due to the magnetic field. In the case of a concentration of 5 mg magnetite/ml in MCL, the temperature at the center increased to 40.6°C after 1 min and to 42°C after 20 min of irradiation. The temperature finally reached 42.6°C and then remained constant due to the balance of heat generation and heat transfer to the surroundings. For the MCL concentrations of 7.5 and 10 mg magnetite/ml, the

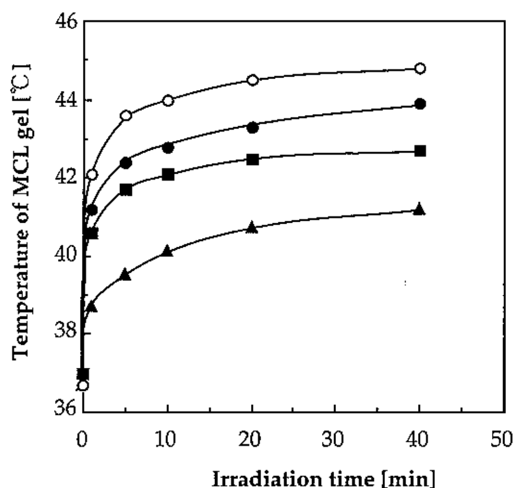

Fig. 4. Temperature increase of magnetite gel in magnetic field. Symbols; ▲, magnetite concentration was 2.5 mg/ml; ■, 5.0 mg/ml; ●, 7.5 mg/ml; ○, 10 mg/ml. Magnetic field intensity: 384 Oe.

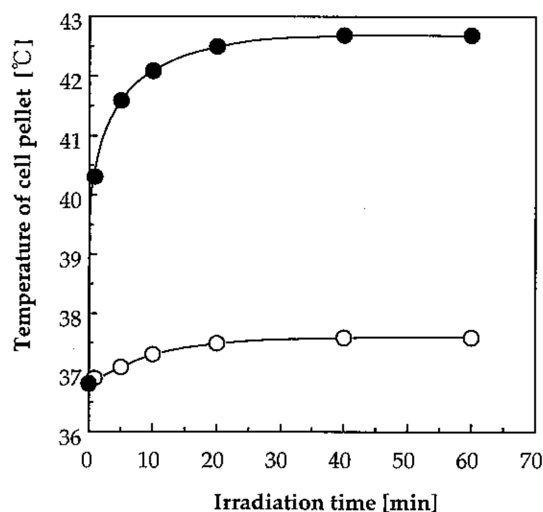

Fig. 5. Temperature increase of cell pellet in magnetic field. Symbols; ●, cells containing MCL; ○, MCL-free medium only. Pellet volume, 80 μl; magnetic field intensity, 384 Oe.

temperature plateaus were at 43.9°C and 44.8°C, respectively. These values are sufficient to kill cancer cells. In the case of 2.5 mg/ml, the temperature did not rise above 42°C. From these results, it is suggested that for inducing hyperthermia of small tumors about 100 ml in volume, at least 5 mg of magnetite in MCL per 1 ml of tissue is necessary.

Heat generation in glioma cells was then studied (Fig. 5). Instead of the MCL gel phantoms, glioma cell pellets were used for this experiment. The volume of the cell pellets was 80 μl (5.4 mm in diameter) and the cell concentration of all pellets was 1.6×10^8 cell/ml; this value is similar to the cell concentration in tumor tissue. The magnetite concentration in the pellet was 7.2 mg/ml. Temperature elevation occurred quickly, as in the phantom experiments. The temperature at the center of the pellet reached 42.6°C after 20 min of irradiation and became constant. This terminal temperature was lower than that of the phantoms (MCL concentration: 7.5 mg magnetite/ml) due to the slightly lower MCL concentration and smaller pellet size. However, these conditions seemed to be sufficient for hyperthermia, as shown by the study of the cell killing effect (Fig. 6). Indeed, when a magnetic field was applied to cells incorporating MCL, the cell viability decreased quickly, depending on the duration of the magnetic field irradiation. The viable cell count decreased to about one-thirtieth of that of the control after 20 min of irradiation, and after 40 min of irradiation, no viable cells remained. Magnetic field irradiation for 60 min did not change the viable cell number in the case of the control experiments. If the cell death rate is expressed in terms of an Arrhenius equation with a constant inactivation energy, the survival curve (such

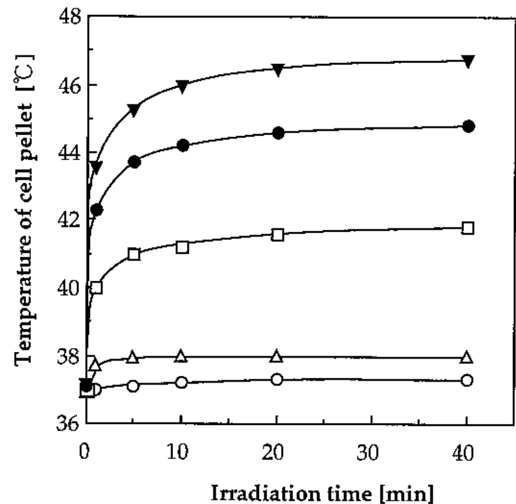

Fig. 7. Temperature increase of cell pellet in a magnetic field of varying intensity. Symbols; △, 95 Oe; □, 192 Oe; ●, 288 Oe; ▼, 384 Oe; ○, MCL-free medium only at 384 Oe. Pellet volume, 150 μl.

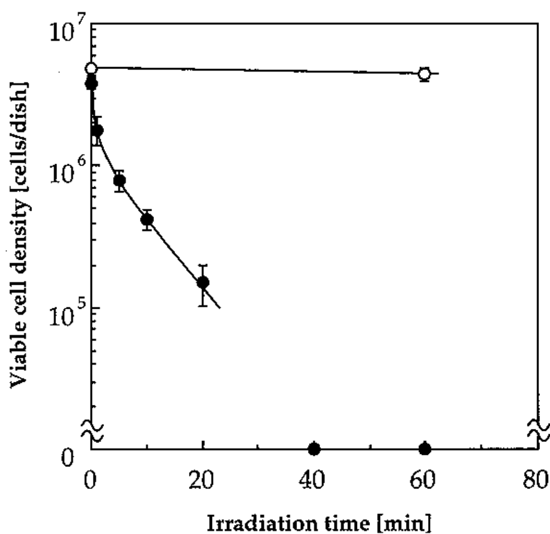

Fig. 6. Viable cell density as a function of magnetic field irradiation. Symbols; ●, cells containing MCL; ○, MCL-free cells. Pellet volume, 80 μl; magnetic field intensity, 384 Oe. Each data point and bar are the mean and SD of 3 independent experiments, respectively.

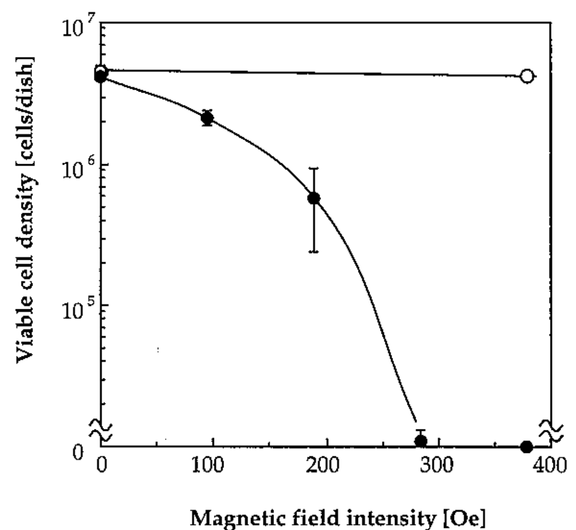

Fig. 8. Viable cell density after magnetic field irradiation (effect of magnetic field intensity). Symbols; ●, cells containing MCL; ○, MCL-free cells. Pellet volume, 150 μl; duration, 40 min. Each data point and bar are the mean and SD of 3 independent experiments, respectively.

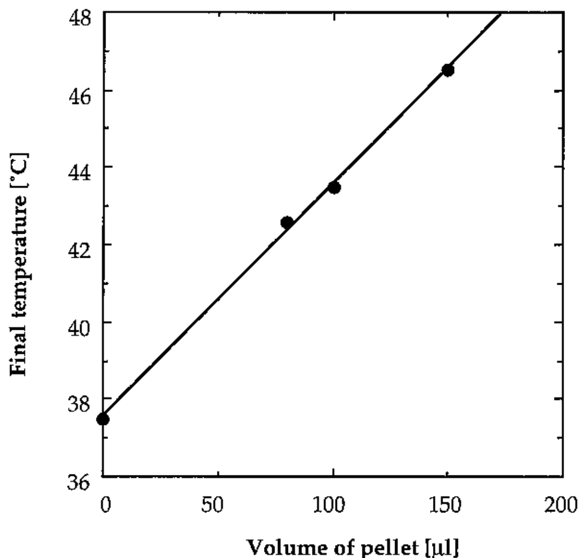

Fig. 9. Variation in the terminal temperature of the pellet in relation to its volume. Magnetic field intensity, 384 Oe.

as Fig. 6) should be convex, because at the beginning of the irradiation the temperature of the pellet did not increase above 42°C, as shown in Fig. 5. In practice, however, the survival curve was concave. Magnetite was incorporated in the glioma cells and local regions of the cell pellet seemed to be temporarily heated over the critical temperature.

The cell killing effects under several intensities of the magnetic field were also studied (Figs. 7 and 8). In this study, cell pellets of 150 μl in volume (6.7 mm in diameter) were used. Fig. 7 shows the temperature profiles of the cell pellets under the magnetic field irradiation. In the case of 384 Oe, the cell pellets were heated to 46.5°C after 20 min irradiation. This value was higher than the previous result (Fig. 5), because these pellets had twice the volume of those used in Fig. 5. Fig. 8 shows that the viable cell numbers after magnetic field irradiation of over 192 Oe for 40 min greatly decreased. However, many cells survived due to insufficient heating below this intensity. This is consistent with the temperature profile of Fig. 7, since the 192 Oe induction gave 41.8°C as the terminal temperature. A higher temperature is necessary to kill the glioma cells.

Fig. 9 shows the correlation between the terminal temperature of the pellet and its volume. The temperature of the medium without MCL is plotted at the origin of the curve. The terminal temperature was proportional to the pellet volume when other parameters were constant. It is clear that the MCL can heat tumors larger than 80 μl in volume to above 42°C.

In conclusion, the MCL had ten times higher affinity for glioma cells than that of the ML, because of the positive charge on the surface. Due to this high affinity for the cells, the MCL incorporated in cells were able to heat a glioma cell pellet of 80 μl in volume (5.4 mm in diameter) to above 43°C and kill the cells by hyperthermic effect.

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