Complete Regression of Experimental Prostate Cancer in Nude Mice by Repeated Hyperthermia Using Magnetite Cationic Liposomes and a Newly Developed Solenoid Containing a Ferrite Core

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BACKGROUND. Magnetite cationic liposomes (MCLs) can be used to induce hyperthermia because they generate heat in an alternating magnetic field (AMF). This study aimed at developing more practical method for MCL hyperthermia examining the effect of MCL-induced hyperthermia on human prostate cancer in vivo.

MATERIALS AND METHODS. A newly developed AMF generator incorporating a solenoid with a ferrite core (FC) was used. Human prostate cancer cells (PC-3 and LNCap) were injected subcutaneously into nude mice. MCLs were injected into tumor nodule and the mice were exposed into AMF three times at 24-hr intervals (repeated hyperthermia; RH) until complete tumor regression was observed.

RESULTS. Irradiation with an AMF generated by newly developed device can adequately increase the temperature of tumor tissue. Frequent RH resulted in complete tumor regression in all nude mice.

CONCLUSION. RH using MCLs may be a promising new therapy for hormone-refractory human prostate cancer in the future.

KEY WORDS: human prostate cancer; hyperthermia; magnetite cationic liposome; complete regression; ferrite core

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy in Western males and its incidence is increasing rapidly in Japan [1]. This increase is believed to be attributable to longer life expectancy, growing prostate awareness, more-widespread screening, and the more-widespread adoption of Western diets [2,3].

The success of early prostate cancer detection has resulted in an increased number of candidates for therapy. The main treatment options for clinically localized prostate cancer currently consist of surgical extirpation and radiation therapy (external-beam radiation therapy and brachytherapy) [4]. There are instances in which radical prostatectomy is not an acceptable option, such as when the patient is a poor

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risk for surgery or due to the wishes of the patient, in
which cases radiation treatment is generally chosen.
Hormonal therapy and/or careful observation are also
options. Decisions regarding treatment must be made
on an individual basis, whilst considering the patient’s
life expectancy and quality of life, as well as the
patient’s wishes [5].

Some less-common treatment options for clinically
localized prostate cancer include cryotherapy [6–8],
high-intensity focused ultrasound [9–11], and
hyperthermia. Hyperthermia has been used for many
years to treat a wide variety of tumors in both
experimental animals and patients [12]. The most
common heating method in clinical settings in Japan
is capacitive heating by a radiofrequency electric field
[13], but localized heating of tumors using this method
is difficult because the heating characteristics are
influenced by various factors such as tumor size, the
position of electrodes, and adhesion of electrodes at
uneven sites. As Kroeze [14] also point out, it may be
that pelvic tumors such as prostate cancer are not ideal
for treatment with capacitive hyperthermia because of
the production of hot spots in normal tissue. Interstitial
hyperthermia, in which the tumor is heated by directly
injected materials, has been examined for clinically
localized prostate cancer to eliminate these problems
[15–18]. These interstitial hyperthermia techniques are
classified into two types according to the heating
mechanism used: microwave hyperthermia and mag-
netically-mediated hyperthermia (MMH). Interstitial
microwave hyperthermia involves heating via a micro-
wave-radiating antenna inserted into the target tissue
[15], and MMH is itself divided into two subclasses
comprising the insertion of magnetic particles (called
intracellular hyperthermia) or seed (called direct-
 injection hyperthermia) into tumor tissue that are
subsequently heated by exposure to an externally
applied alternating magnetic field (AMF) [16–18].
The magnetic particles are iron oxide particles with a
diameter of 20 nm, and the seed is a columnar piece
of metal 1 mm in diameter and 10 mm long. In
intracellular hyperthermia, the ferromagnetic particles
are modified to facilitate their cellular uptake by the
tumor, whereas ferromagnetic seed is injected directly
into tumor tissue in direct-injection hyperthermia. In
both methods, the subsequent exposure to an AMF
results in heat generation within the tumor cells [19].

We have developed magnetite cationic liposomes
(MCLs) for inducing intracellular hyperthermia
[20,21]. These MCLs have been developed to improve
adsorption and accumulation in the tumor cells, and
exhibit a tenfold higher affinity for tumor cells than
neutrally charged magnetoliposomes [21] due to
electrostatic interaction with the negatively-charged
cell membrane. The hyperthermic effect of MCLs
against certain types of malignant tumor cells has
been demonstrated in vivo [22]. Our hyperthermia
procedure killed rat prostate cancer cells in vivo not
only directly by heating but also by the induction of an
immune response [23,24]. However, the AMF-generat-
ing machine in the previous study used a solenoid, and
for clinical application, it is technically difficult to scale-
up the coil size because the very large coil required to
accommodate the human body may be accompanied
with a serious risk associated with the high voltage
between the two ends of the solenoid. Thus, designing a
new therapeutic protocol for use in human prostate
cancer, including a safe device for AMF generation, is
needed before clinical trials can be conducted. In this
study, we examined the therapeutic effects of repeated
hyperthermia (RH) using MCLs on human prostate
cancer tissue growing in athymic mouse subcutis. In
the present experiment, we applied a newly-developed
device, named the ferrite core (FC)-inserted solenoid
(FCIS).

MATERIALS AND METHODS

AMF Generator

A near-lossless MnZn FC was obtained from TDK
(Tokyo, Japan), which has a high relative intrinsic
permeability, typically 3,000–4,000 at low magnetic
field strengths, and a sharp transition from the
ferromagnetic to nonmagnetic states. Because the FC
was inductively heated by AMF, it was surrounded by
a water-cooling jacket (diameter, 6 cm; height, 11.5 cm).
An AMF was generated by a vertical coil (inner
diameter, 7 cm; length, 9 cm) driven by a transistor
inverter (LTG-100-05; Dai-ichi High Frequency, Tokyo,
Japan) at a frequency of 360 kHz. The FC was inserted
into the vertical coil (Fig. 1A). In the present study, a
solenoid without an FC was used as a so-called vacant
solenoid (VS; Fig. 1B) in control experiments.

Preparation of MCLs

MCLs were fabricated from colloidal magnetite (a
kind gift from Toda Kogyo, Hiroshima, Japan) and a
lipid mixture consisting of N-(ß-trimethylammonioace-
tyl)-didodecyl-D-glutamate chloride (Sogo Pharma-
aceutical, Tokyo, Japan), dilauroylphosphatidylethanolamine, and
dioleoylphosphatidyl-ethanolamine (Sigma Chemical,
St. Louis, MO) in a molar ratio of 1:2:2, as described
previously [20].

In Vitro Experiments Using a Phantom

A 4% agar gel was used for preparing the phantom,
with agar purchased from Wako Pure Chemicals
(Osaka, Japan). An agar piece containing MCLs was
prepared as we described previously [25]. Briefly, the
MCLs were added to liquid agar at 60°C while stirring with a glass impeller. After mixing for 30 min, the suspension was sonicated for 15 min by a probe-type sonicator operating at 40 W. The sonicated mixture (1 ml) was poured into a 5-ml polypropylene tube (10 mm in diameter; Sarstedt, Nümbrecht, Germany) and cooled rapidly by placing the tube in ice-cold water. The final net concentration of MCLs in the phantom was 3 mg/ml. An agar piece without MCLs was also prepared for control experiments.

To investigate heat generation in the phantom, the phantom with or without MCLs was subjected to AMF for 5 min using the VS or FCIS, with measurement of phantom temperatures using an optical fiber probe (FX-9020; Anritsu Meter, Tokyo, Japan).

Cell Lines

PC-3 (human prostate cancer cell line derived from bone metastatic lesions; androgen insensitive) and LNCap (human prostate cancer cell line derived from lymph node metastatic lesions; androgen sensitive) cells were obtained from the American Type Culture Collection (Rockville, MD). The PC-3 cells were maintained in Dulbecco’s modified Eagle’s minimal essential medium, and the LNCap cells were maintained in RPMI medium 1640 (Sigma Chemical). All media were supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and a mixture of 0.6% mg/ml glutamine (Gibco), 200 IU/ml penicillin (Gibco), and 200 μg/ml streptomycin (Gibco). FBS was
heat-inactivated for 1 hr at 56°C. All cell cultures were incubated at 37°C in 5% CO₂/95% air, and the media were replaced every 3rd day.

**Animal Models**

Four-week-old male BALB/c nude mice were purchased from Charles River Japan (Yokohama, Japan). To prepare tumor-bearing animals, cell suspensions containing approximately 1 x 10⁷ PC-3 cells in 100 μl of phosphate buffer (0.05 M sodium phosphate and 0.15 M NaCl, pH 7.4) were injected subcutaneously into the right flank of BALB/c nude mice under short-term anesthesia by intraperitoneal injection (i.p.) of sodium pentobarbital (50 mg/kg body weight). Cell suspensions containing approximately 2.5 x 10⁷ LNCap cells in 100 μl of phosphate buffer mixed with Matrigel (Becton, Dickinson and Company, Franklin Lakes, NJ) were injected in the same way. The PC-3 and LNCap groups each consisted of five mice bearing PC-3 and LNCap tumor nodules, respectively.

The experimental protocol in the present study was approved by the Animal Care Committee of Nagoya City University Medical School. 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.

**Injection of MCLs and Heat Generation by AMF**

When tumors became 7 mm in diameter, MCLs were injected into tumor nodule and started to irradiation to AMF as follows. 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 to ensure a homogeneous distribution of MCLs. The indicated volumes of MCL solution (20 mg/ml magnetite) were injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL) at 0.2 ml/hr. The mouse was placed at the center of an FCIS, where the magnetic flux was greatest. The temperatures inside the rectum and at the surface of the tumor were measured during AMF irradiation with optical fiber probes (FX-9020; Anritsu Meter). When the tumor surface temperature reached 46°C, this temperature was maintained by controlling the power of the AMF-generating machine. After injection of MCLs, mice were subjected to AMF for 30 min (Fig. 1A).

**Therapy Protocol**

AMF irradiation was performed three times at 24-hr intervals (referred to as RH). We injected MCLs and performed RH many times until repopulation was not observed, to ensure complete involution of the tumor nodule. The largest and smallest diameters of each tumor were measured using calipers every 3 days. In cases where the tumor increased in size compared to the previous measurement, RH was repeated.

As the treatment group, in both PC-3 and LNCap groups, mice bearing PC-3 or LNCap were irradiated to AMF when the tumor reached 7 mm in diameter but mice in the control group were received injection of MCLs but without AMF irradiation.

**RESULTS**

**Comparison of Temperature Elevation Between the VS and FCIS**

Our method of MCL-mediated hyperthermia using the VS needs placing tumor-bearing mice injected with MCLs in the center of the VS and irradiating them with AMF (Fig. 1B). This method can increase the tumor surface temperature to 45°C within 5 min, at which temperature tumor cells are killed. In our new FCIS method, it is also necessary for the tumor surface temperature to increase by 8°C within 5 min from the start of AMF irradiation (assuming that the body temperature before treatment is 37°C). These temperature increases in the phantom with MCLs were possible using either the VS or FCIS (Fig. 2). An AMF-generating-machine power when using the VS of 780 W (58 V, 13.5 A) increased the temperature by 7.8°C at the inside center of the VS. When the phantom with MCLs was placed at the surface of the FCIS, a temperature increase of 7.8°C was achieved using 600 W (50 V, 12 A). In the FCIS, the temperature rose by 7.8°C at the surface of a coil and by 4.7°C at 5 mm above the surface, respectively. In the VS, the temperature rose by 2.6°C at the surface of a coil and by 1°C at 5 mm above the surface for the same AMF power, respectively. These results indicate that the FCIS is more suitable than the VS for heating targets outside the coils.

**Heating Ability of the FCIS**

The power of the AMF-generating machine to achieve a temperature increase of 8°C was determined when the target (phantom with MCLs) located at the following distances from the FCIS: at the surface of the FCIS (point 0), 5 mm above the surface (point 5), and 10 mm above the surface (point 10). The phantom without MCLs was also examined in this way. The results are shown in Figure 3. In the case of the phantom with MCLs, the powers of the AMF-generating machine required to increase the temperature by 8°C were 600 W (50 V, 12 A) at point 0, 1,220 W (74 V, 16.5 A) at point 5, and 1,500 W (80 V, 18.8 A) at point 10.
contrast, for the phantom without MCLs, the temperature increase was only 1.5°C at point 10 when the power was 1,500 W (80 V, 18.8 A). These results indicate that irradiation with an AMF in the FCIS can adequately increase the temperature of tumor tissue injected with MCLs when it is 10 mm from the surface of the coil.

Heat Generation by MCLs in AMF In Vivo

Tumor-bearing mice were irradiated by an AMF after MCL injection. The tumor surface temperature and the inside of rectum were measured in the PC-3 and LNCap groups. As shown in Figure 4, the tumor surface temperature of both groups rose to 46°C within 5 min from the start of irradiation. During this starting 5 min, the power of the AMF-generating machine was kept between 835 W (60 V, 13.9 A) and 1,500 W (80 V, 18.8 A). After the surface temperature had reached 46°C, it could be maintained accurately for 30 min by fine adjustment of the power of the AMF-generating machine. The temperature inside the rectum increased slightly during this period, but was within the normal range (33.0–36.5°C). These results demonstrate that tumor tissue directly injected with MCLs can be specifically heated by irradiation with an AMF, and that the degree of heating ability can be accurately controlled by adjusting the power of the AMF-generating machine.

Effects of Frequent RH on PC-3 and LNCap Tumors

PC-3 group. The scheme of the hyperthermia experiment for the treatment group is shown in Figure 5. To evaluate tumor growth, the tumor long-axis diameters were plotted every 3 days in both control and treatment groups, which are indicated in Figure 6. Tumors in the control group continued to grow progressively (Fig. 6A), whereas those in the treatment group decreased in two steps. The tumor of all mice in the treatment group exhibited resistance to the heating
induced by frequent RH (first step) until certain points. Resistance periods varied in each mice. However, eventually the tumors suddenly started to decrease, disappearing completely after an additional 3–6 days (second step) (Fig. 6B). In detail, in the treatment group, mouse 1 which received RH on days 0–2, 12–14, 21–23, and 30–32, exhibited sudden complete regression (CR) on day 36; mouse 2 received RH on days 0–2 and 12–14, exhibited CR on day 27; mouse 3 received RH on days 0–2 and 12–14, exhibited CR on day 30; mouse 4 received RH on days 0–2, 6–8, 12–14, and 15–17, exhibited CR on day 21; and mouse 5 received RH on days 0–2, 6–8, 12–14, 21–23, 36, 37, and 48–50, exhibited CR on day 57.

LNCap group. The scheme of the hyperthermia experiment for the treatment group is shown in Figure 7. The tumor long-axis diameters were plotted every 3 days in both control and treatment groups (as for the PC-3 group) and are indicated in Figure 8. The tumors in the control group continued to grow linearly as for the PC-3 groups (Fig. 8A); however, the growth in the treatment group demonstrated a one-step decrease (in contrast to that in the PC-3 group). The tumor decreased in size linearly and then disappeared (Fig. 8B). In detail, in the treatment group, mouse 1 which received RH on days 0–2, 6–8, and 12–14, exhibited CR on day 27; mouse 2 received RH on days 0–2 and 12–14, exhibited CR on day 27; mouse 3 received RH on days 0–2 and 12–14, exhibited CR on day 36; mouse 4 received RH on days 0–2 and 27–29, exhibited CR on day 42; and mouse 5 received RH on days 0–2, exhibited CR on day 9.

**DISCUSSION**

This study investigated the feasibility of our new AMF-generating device comprising a solenoid coil with a FC, and its capability for hyperthermic treatment in human prostate cancer. FC was used instead of a flat circle electrode, which was used for more-effective hyperthermia of deep tissue [26], because it enabled the magnetic field and eddy currents to be controlled. The FCIS used in this study has an FC inside a solenoid coil that concentrates the magnetic field generated by solenoid coil, resulting in the emission of the magnetic field from the surface of the device. In the present experiment, the following three performance characteristics of the FCIS were carried out: (1) whether it provides sufficient heating for hyperthermia (including a comparison with a VS), (2) the amount of temperature elevation of the target tissue, and (3) whether the induced hyperthermia has the same anticancer effect as for a VS. Characteristics 1...
characteristic 3 was examined by assessing the therapeutic effect in human prostate cancer cell nodules. we reported previously the therapeutic inhibiting effect of the vs on the tumor growth of rat prostate cancer, but cr was not observed [24]. and we also reported that frequent rh with a vs-induced cr of mouse mammary carcinomas with a diameter of larger than 15 mm [27]. to confirm therapeutic effect of our specifically heat the tumor tissue injected with mcl s up to 45°C (given that normal body temperature is 37°C). and 2 were examined using a phantom with mcl s in the place of a living body. the results indicated that the fcis was suitable for heating a target positioned outside the coils (fig. 2). in the case of the phantom with mcl s, a phantom 10-mm distant from the fcis could be increased in temperature by 8°C within 5 min. using the AMF-generating machine at the same position, the temperature of the phantom without mcl s did not increase (fig. 3). these results demonstrate that our new hyperthermic procedure with the fcis has the ability to
new hyperthermic procedure, we applied it whether RH could induce the CR of human prostate cancer cell lines (PC-3 and LNCap) in nude mice models. CR was induced in all the tumors in the PC-3 and LNCap groups (Figs. 6 and 8).

PC-3 is a cell line derived from bone metastatic lesions and is androgen-independent [28], whereas LNCap is derived from lymph node metastatic lesions and is androgen-sensitive [29]. In other words, these two prostatic cancer cell lines have different biological characters. In this study, our new hyperthermia method produced CR in all of the tumor nodules in the PC-3 and LNCap groups, although the rates of disappearance differed. In the LNCap group, linear involution started after just one round of RH, with CR occurring after one to three rounds of RH (Figs. 7 and 8). The PC-3 cancer nodules exhibited greater resistance to hyperthermia. In many cases of the PC-3 group, hyperthermia had no effect on tumor nodules during the first and second rounds, with the last tumors starting involution and exhibiting sudden CR after several RH rounds. This resulted in the total amount of RH rounds being greater for the PC-3 group than for the LNCap group (Figs. 5 and 6). The differing hyperthermic effects between PC-3 and LNCap can be explained as follows: when cultured at 43°C, the PC-3 cell population decreased according to cell-growth inhibition, whereas the LNCap cell population decreased according to a cell-killing effect. This difference is attributable to PC-3 cells possessing heat resistance in which hyperthermic stimulation stops the cell cycle, whereas LNCap cells are more sensitive to hyperthermia because the cell cycle does not change [30]. The huge variation in the time until CR (15–60 days in PC-3, 7–42 days in LNCap: Figs. 6 and 8) can be explained as follows: The distribution of magnetite nanoparticles within tumors must be considered in our use of a hyperthermia system to heat MCLs. When the MCLs were heated, the surrounding tumor tissues underwent necrosis, and magnetite nanoparticles subsequently expanded into the necrotic area within the tumor, resulting in a wide distribution of magnetite nanoparticles [21–23]. In the present study, CR of human prostate cancer cell nodules in nude mice was observed using a hyperthermia protocol, which should in fact be termed frequent RH. For LNCap in mouse 5, it took five rounds of RH to achieve CR. Although parts of the tumor containing sufficient amounts of MCLs were killed by heat, other parts of the tumor without MCLs—
particularly at the tumor edges—may continue to grow. Therefore, differences in the number of rounds of RH treatment for CR are probably due to variations in the tumor shape.

It is well known that there is no reliable therapy for hormone-refractory prostate cancer [31,32]. The use of frequent RH in the present study resulted in CR of PC-3 tumor nodules, which are androgen-independent. This result suggests that frequent RH could be used as one of treatment for hormone-refractory prostate cancer. Since our new hyperthermia technique can induce CR in cancer tissue only by heating, it may be a useful therapy in various types of cancer. From the perspective point of view for clinical use, our new MCL-induced hyperthermia technique will be especially useful if irradiation with an AMF in the FCIS can adequately increase the temperature of tumor tissue injected with MCLs positioned 50 mm above the surface of the coil. The major tumor mass is peripheral in location in clinical stage T2 carcinomas and in 85% of nonpalpable tumors diagnosed on needle biopsy (stage T1c) [33–35], and can be heated by an FCIS attached to the perineal portion since the magnetic flux reaches the peripheral zone of the prostate. In the remaining cases, tumors are predominantly located in the transition zone, and adenocarcinoma of the prostate is multifocal in more than 85% of cases [34]. The development of an endoscopic AMF-generation machine would enable tumors in the transition zone and other multifocal tumors to be treated. The further development of the FCIS will allow our new MCL-induced hyperthermia technique to become a new treatment method for hormone-refractory prostate cancer.

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