Anticancer Effect of Hyperthermia on Prostate Cancer Mediated by Magnetite Cationic Liposomes and Immune-Response Induction in Transplanted Syngeneic Rats

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BACKGROUND. The hyperthermic effect of magnetic particles was examined in rat prostate cancer in vivo. Magnetic cationic liposomes (MCLs) have a positive surface charge and generate heat in an alternating magnetic field (AMF) due to hysteresis losses.

METHODS. Rat prostate cancer cells (PLS 10; androgen independent) were injected subcutaneously into the flank of F344 rats. MCLs were injected into rat prostate cancer nodules that had grown to 5–6 mm in diameter, and were then exposed to an AMF. Tumor growth rates were measured. To examine whether hyperthermia caused immune induction for PLS 10, cytotoxicity assays and immunohistochemical staining for CD3, CD4, CD8, and Heat Shock Protein (HSP) 70 were performed.

RESULT. The tumor temperature increased to 45°C whereas the body temperature remained at around 38°C. Tumor regression was observed in the hyperthermic group. CD3, CD4, and CD8 immunocytes were present in the tumor tissues of the rats exposed to hyperthermia, but they were not detected in any of the tumor tissue of untreated rats. HSP70 also appeared in the viable area at its boundary with the necrotic area. The cytotoxic activity of tumor-transplanted rats for PLS 10 cells increased in hyperthermic-treatment rats.

CONCLUSION. These results suggest that hyperthermia using MCLs is an effective therapy for prostate cancer, since this treatment appears to kill the prostate cancer cells not only directly by heating but also by inducing an immune response. This therapy may cure not only the primary lesion but also metastatic lesions.

KEY WORDS: prostate cancer; hyperthermia; magnetite cationic liposome; immune-response induction

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, and more intense screening [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/or brachytherapy) [4]. There are instances in which radical prostatectomy is not an
option, such as when the patient is a poor risk for surgery or due to the wishes of the patient, in which cases radiation treatment becomes an option. Hormonal therapy, careful observation, or combination therapy with these methods are also options. Decisions regarding treatment must be made on an individual basis, with consideration for the patient’s life expectancy and quality of life, as well as the patient’s wishes [5].

Some other treatment options are less popular: cryotherapy [6–8], high-intensity focused ultrasound [9–11], and hyperthermia. The preliminary data suggest that high-intensity focused ultrasound represents a valid alternative treatment strategy for patients with localized prostate cancer who are unsuitable for surgery [9–11].

Cryosurgical ablation of the prostate is one approach to the treatment of localized prostate cancer. Third-generation cryosurgery uses gas-driven probes that enable the use of probe diameters of only 17 gauge, and this minimally invasive technique appears to be well tolerated. Cryosurgery has reemerged as an evolving technology and a minimally invasive treatment option. In 1996 the American Urological Association recognized cryoablation as a therapeutic option for prostate cancer in its position statement and removed the “investigational” label from this procedure.

Adding hyperthermia to conventional radiotherapy may also improve local control in prostate carcinoma. Hyperthermia is known to enhance the effect of radiation on prostate cancer cells in vitro [12]. Various hyperthermia techniques can be used to treat prostate carcinoma. Transurethral and/or transrectal hyperthermia produce a relatively uncontrollable heat distribution because of the limited heat penetration depth [13], and Algan et al. [14] reported no improvement in treatment outcome when using transrectal hyperthermia. The feasibility of interstitial [15] and regional hyperthermia for locally advanced prostate carcinoma has been reported [16–18], but these techniques require further development before they can be applied clinically.

Hyperthermia is based on the fact that tumor cells are more sensitive to heat than is healthy tissue [19]. However, it is difficult to specifically heat tumors with hyperthermia because the heating effects are influenced by various factors such as tumor size and the position of electrodes. In addition, the inevitable technical problem with hyperthermia is the difficulty of uniformly heating only the tumor region until the required temperature has been reached whilst not damaging normal tissue. Some researchers have investigated the application of submicron-sized magnetic particles for intracellular hyperthermia in order to overcome these disadvantages [20,21]. These magnetic particles generate heat under an alternating magnetic field (AMF) due to hysteresis losses [22]. We have developed “magnetite cationic liposomes” (MCLs) for inducing intracellular hyperthermia [23,24]. MCLs have been developed to improve adsorption and accumulation in the tumor cells, and have shown a tenfold higher affinity for the tumor cells than neutrally charged magnetoliposomes [24] due to electrostatic interaction with the negatively charged cell membrane. The hyperthermic effect of MCLs against some malignant tumor cells has been demonstrated in vivo [25]. Our hyperthermia procedure kills tumor cells not only directly by heating but also by the induction of an immune response [26].

In the study described in the present paper, we examined the hyperthermic effect of MCLs on rat prostate cancer in vivo, and demonstrated that our hyperthermic protocol can induce an antitumor immune response against rat prostate cancer.

**MATERIALS AND METHODS**

**Tumor Cell Type and Animal Model**

Cells were obtained from the rat prostate cancer cell line PLS 10, which has been established at Department of Experimental Oncology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences [27] (Fig. 1A). This cell line has been established from 3,2’-dimethyl-4-aminobiphenyl plus testosterone-induced carcinomas in the dorsal prostate of male F344 rats. This cell line forms well-differentiated adenocarcinomas with abundant connective tissue stroma. PLS 10 cells are immunohistochemically negative for androgen receptors, and the growth of PLS 10 cells is androgen independent.

Four-week-old male F344 rats were purchased from Charles River Japan (Yokohama, Japan). To prepare tumor-bearing animals, cell suspensions including approximately $1 \times 10^6$ PLS 10 in 100 μl of phosphate buffer (0.05M sodium phosphate and 0.15M NaCl, pH 7.4) were injected subcutaneously into the right flank of F344 rat under short-term anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Prostate cancer nodules that had grown to a diameter of about 5–6 mm were used for the experiments. The tumor diameter was measured every 3 days. The tumor volume was determined as Tumor volume = $0.5 \times (length \times width)^2$.

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. In this experiment, prostate cancer derived from the prostate of F344 rats...
are transplanted into syngeneic rats, which differs considerably from the experiment in which human tumor tissue is transplanted into nude mice. This experiment therefore represents an excellent model for evaluating host immune responses to tumor tissue.

**Preparation of MCLs**

The magnetic particles were kindly donated by Toda Kogyo (Hiroshima, Japan), and had an average diameter of 10 nm. MCLs were prepared by sonication as described previously with some modifications [23]. One milliliter of the colloidal magnetite (net 20 mg of magnetite) was coated with a lipid membrane comprising N-(α-trimethylammonioacetyl) didodecyl-D-glutamate chloride (Sogo Pharmaceutical, Tokyo) and dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical, St. Louis, MO) at a molar ratio of 1:2:2, respectively. The magnetite concentration was measured by the potassium thiocyanate method [28].

**Injection of MCLs and Heat Generation in an AMF**

After the prostate cancer nodules had grown to 5–6 mm in diameter, a 26-G syringe needle containing MCLs was inserted longitudinally into each prostate cancer nodule subcutaneously from the nodule edge (Fig. 2A). Two grams of MCLs was injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL) for 30 min (Fig. 1B). The rats were then separated into two groups: Group I mice were not exposed to an AMF (control group), whereas after injection of the MCLs, Group II rats were subjected once to hyperthermia for 30 min at 45°C. The AMF was created using a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05;...
Dai-ichi High Frequency, Tokyo) operating at 118 kHz. The magnetic field intensity was 30.6 kA/m (384 Oe). The rat was placed inside the coil such that the nodule was positioned at the center of the coil (Fig. 2B). Temperatures at the center of the tumor and inside the rectum (representative of the rat body temperature) during AMF were measured by optical fiber probes (FX-9020; Anritsu Meter, Tokyo). The temperature at the center of the tumor was maintained at around 45°C by controlling the magnetic field intensity.

**Preparation of Specimens for Immunohistochemical Staining**

On the 30th day after the MCL injection, tumors of the two groups were removed and specimens for immunohistochemical staining were prepared as follows. Blood was flushed out with phosphate buffer, then the tumor tissues were extracted, and fixed with Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo) at −20°C. The frozen tumor tissues were sectioned at 10 μm. Tissue sections were air dried for 30 min and fixed with cold acetone for 15 min. These sections were incubated with 5% normal goat serum and 1% skim milk at 37°C for 30 min to block background staining. They were then incubated at 37°C for 60 min with mouse antirat CD3 (1F4), antirat CD4 (W3/25), antirat CD8 (MRC OX-8) monoclonal antibodies (Serotec, Oxford, UK), and antirat HSP70 (W27) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200, and at 37°C for 60 min with goat antimouse monoclonal antibody-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA) at a dilution of 1:200. Each step was followed by washing with the phosphate buffer. Peroxidase activity was visualized by treatment at room temperature for 10 min with 0.02% diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxidase. All sections were also stained with hematoxylin.

**In Vitro Cytotoxicity Assay**

Spleen cells were derived from the Group II rats at 1 month after the hyperthermic treatment using the Medimachine System (DAKO, Glostrup, Denmark). Naive rats that had been born at a similar time to the Group II rats were used as the controls. The spleen cells were cultured for 48 hr in RPMI 1640 supplemented with 10% fetal bovine serum and 2-mercaptoethanol (5 × 10−5M). Mouse recombinant interleukin-2 was added once at a concentration of 5 U/ml. The cytotoxicity of spleen cells to prostate cancer cells was determined by a long-term cytotoxicity assay [29]. Rat prostate cancer cells (PLS 10, 1 × 105 cells) were cultured in a 24-well flat plate containing DMEM. After 24 hr, the medium was replaced with RPMI 1640. Spleen cells were added to each well. The wells were washed with phosphate buffer after 48 hr of incubation at 37°C in an atmosphere containing 5% CO2. After incubation, 0.3 ml of RPMI 1640 and 0.03 ml of cell-counting solution (kit-8; Dojindo, Kumamoto, Japan) were added to the wells and the cells were incubated for 1 hr at 37°C in an atmosphere containing 5% CO2. The absorbance of each well was measured at 405 nm with a spectrophotometer (V-530; Jasco, Tokyo). Cytotoxicity was calculated using the following equation: cytotoxicity (%) = [1 − (absorbance of target cells treated with effector cells)/(absorbance of target cells only)] × 100.

**Statistical Analysis**

Levels of statistical significance in the tumor-growth experiments were evaluated using the Mann–Whitney rank sum test.

**RESULTS**

**Heat Generation by MCLsin AMF**

Figure 3 shows the temperature increases at the center of the tumor and inside the rectum of the rats during application of an AMF for 30 min. The temperature at the center of the tumor reached 45°C within 5 min, and was maintained at this temperature by controlling the magnetic field intensity. In contrast, the temperature inside the rectum remained below 38°C.

Fig. 3. Temperature increases at the center of the tumor (solid line), and after 10 min is maintained at around 45°C. Temperature inside the rectum (dotted line) increased a little but remained at around 38°C. Values shown are the mean ± SD of five independent experiments.
whereas the temperature difference between the center of the tumor and the rectum was very large (Fig. 3). These results demonstrate that hyperthermia using MCLs makes it feasible to heat only the tumor whilst not damaging healthy tissues.

**Monitoring Tumor Growth After Hyperthermia**

Figure 4 shows the time course of the tumor-volume growth in five rats in each group. Hyperthermia was induced once the rat prostate cancer nodules had grown to 5–6 mm in diameter. In all rats of Group I (control group), the tumor volume increased linearly. In all rats of Group II (hyperthermic treatment group), the tumor volume increased linearly for 12 days and then was suppressed from days 15 to 21 (and was significantly smaller than that of Group I), after which it did not increase further.

**Immunohistological Features of Tumors After Hyperthermic Treatment**

To examine whether immunocytes were present in tumor tissue, the tissues of rats with and without hyperthermic treatment were stained immunohistochemically. CD3-, CD4-, and CD8-positive lymphocytes were detected in the tumor tissues of the rats exposed to hyperthermia, but they were not detected in any of the tumor tissue of untreated rats (Fig. 5). HSP70 was expressed in viable tissue at its boundary with necrotic areas (Fig. 6).

**In Vitro Immune Response Using Spleen Cells From Rats Cured by Hyperthermic Treatment**

To examine further the mechanisms underlying the antitumor activity of the hyperthermic treatment, we evaluated its effect on the generation of cytotoxic T lymphocyte (CTL)-killing PLS 10 cells. As shown in Figure 7, the CTL activity for PLS 10 cells was approximately twofold higher in spleen cells of Group II rats than in those of naive rats.

**DISCUSSION**

Hyperthermia treatment is divided into two methods, regional and systemic, with the former being generally selected. Heating methods of regional hyperthermia are divided into external and internal heating. Capacitive hyperthermia with radiofrequency (RF) irradiation is the most common clinical technique, and this represents a type of external, regional hyperthermia treatment. However, RF capacitive hyperthermia is associated with the complication of raising the

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Fig. 4. Time course of tumor-volume growth in each group of five rats: Group I, no treatment (control); Group II, treatment with the AMF. In Group I, the tumor volume increased steadily. In Group II, the growth of all the tumors was suppressed after 15 days.
body temperature which exerts a massive burden on the patient—it is difficult to heat a tumor specifically with a capacitive heating method using an RF electric field. Kroeze et al. [30] used modeling of capacitive hyperthermia of the prostate to reveal the difficulty of heating deep-seated tumors in the pelvic area. We have developed MCLs for implementing intracellular hyperthermia in vivo without the disadvantage of

Fig. 5. Immunohistochemical staining for immunocytes. **A**: Tumor tissue of a rat of Group I (control, ×400); no immunocytes appeared. **B, C, D**: Each solid arrows indicates CD3 (B), CD4 (C), and CD8 (D) immunocytes around viable cancer tissue but not necrotic tissue (×400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6. **A**: Histology of tumor tissue for a rat of Group II. Necrotic tissue was present at the specimen center (**), and viable cancer tissue was present around the necrotic tissue (**). H-E stain, ×200. **B**: Light-microscopy section of anti-HSP70 antibody staining at the boundary between necrotic and viable areas at the same position of A. HSP70 appeared in the viable area but not in the necrotic area. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
RF capacitive hyperthermia [23,24]. According to the criteria of hyperthermia, our hyperthermia method using MCLs in an AMF (MCL-induced hyperthermia) represents a type of internal, regional hyperthermia treatment. For the purpose of applying MCL-induced hyperthermia to the future clinical treatment of patients with prostate cancer, in the present study this technique was applied to rat prostate cancer in vivo. This study investigated the following four factors: (1) whether the temperature of rat prostate cancer nodules increases, (2) whether body temperature also increases, (3) the effect on rat prostate cancer regression, and (4) the presence of mechanisms other than heating. In this experiment, prostate cancer derived from the prostate of F344 rats are transplanted into syngeneic rats, which differs significantly from the experiment in which human tumor tissue is transplanted into nude mice. This experiment therefore represents an excellent model for evaluating host immune responses to tumor tissue.

In this study, the temperature at the center of the tumor reached 45°C within 5 min, and was maintained for 20 min at this level by controlling the magnetic field intensity. In contrast, the temperature inside the rectum, which is representative rat body temperature, remained below 38°C. These results demonstrate that MCL-induced hyperthermia is able to increase the tumor temperature whilst not also increasing the overall body temperature.

The tumor-volume growth rate demonstrated that the growth of prostate cancer nodules was clearly suppressed by the heating of MCLs with an AMF. However, the prostate cancer nodules did not disappear completely under the present experimental conditions. This is the first examination of MCL-induced hyperthermia for rat prostate cancer and hence the injection of an adequate amount of MCLs into the optimal position for prostate cancer would make it possible to cure prostate cancer by heating with AMF. And one of the significant points of our MCL-induced hyperthermia method is specific MCLs injection to tumor tissue. It is difficult to inject MCLs into lesions that are located deep in the body or lesions with hypervascularity such as in hepatic cancer and renal tumors. However, the technique of injection of MCLs can be readily applied to the treatment of prostate cancer. Three-dimensional computerized tomography or transrectal ultrasound techniques, as used to guide interstitial brachytherapy for prostate cancer [31–33], could also be applied for the accurate injection of MCLs to tumor tissue.

In addition to heating, our MCL-induced hyperthermia technique is suggested to induce an immune response. In this study, CD3-, CD4-, and CD8-positive lymphocytes appeared around the border between necrotic tissue and viable tumor tissue. CD4 cells recognize antigen-presenting cells (such as macrophages) which, when activated, play roles in the activation of B cells to antibody-producing cells and in the activation of CD8 cells to CTLs. On the other hand, CD8 cells recognize antigen-bearing cells such as virus-infected cells and tumor cells, and themselves change into CTLs. Both cell types were observed in the present study, suggesting that CD8 cells were activated to CTLs by CD4 cells. Our results clearly demonstrate that antitumor T lymphocytes were amplified by the induction of hyperthermia, and that this amplification would lead to destruction of the tumor. Both CD8 and CD4 T lymphocytes were detected in the tumor tissue of rats subjected to the hyperthermic treatment, while no immunocytes was observed in untreated rats. These results suggest that activation of T lymphocytes at the tumor site after hyperthermia is attributable to tissue necrosis.

In contrast, HSP70 also appeared in viable tissue at its boundary with necrotic tissue. Hyperthermia is known to induce HSPs [34]. Until now, the expression of HSPs was considered an unwanted side effect of hyperthermia because HSPs cause thermotolerance in tumor tissue [35]. However, HSP-mediated antitumor immunity was reported to cause a vaccine effect of HSP-peptide complexes released from tumor cells [36–38]. Blom et al. [39] reported that the expression of HSP70 was increased after exposure of melanoma cells to a temperature of 45°C. Our MCL-induced hyperthermia also induced HSP70 in a viable area of tumor tissue. The progression of T lymphocytes was also caused by the
elevated expression of HSPs, which is characteristic of heat treatment [40,41].

A CTL assay was therefore used to investigate the in vivo immune response of rats after MCL-included hyperthermia, and an approximately twofold higher activity of CTL was induced in spleen cells. These data demonstrate that the immune response was induced specifically in PLS 10 cells and that even if viable cells remain in a tumor after hyperthermia, they will become targets for the antitumor immune reaction and complete tumor regression may eventually occur.

The present study shows that our MCL-induced hyperthermia technique is an effective therapy for the treatment of prostate cancer, since this treatment kills tumor cells not only directly by heating but also by inducing an immune response. That the new technique described here represents an epoch-making therapy having the possibility to treat both primary and metastatic tumors.

There are many problems that should be resolved before the application of our MCL-induced hyperthermia technique to clinical cases. These include the development of an AMF irradiation device suitable for the human body and a safety investigation of the use of MCLs in the human body. The injection of MCLs into tumor tissue, however, is made easier with the assistance of three-dimensional computerized tomography or transrectal ultrasound techniques. MCL-induced hyperthermia therefore does not have the disadvantage associated with RF hyperthermia. Moreover, by inducing an immune response, our technique opens the possibility of treating both primary and metastatic lesions, and hence should be considered a useful future therapy for prostate cancer.

REFERENCES


