Localized Hyperthermia Combined with Intratumoral Dendritic Cells Induces Systemic Antitumor Immunity

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Abstract

Prostate adenocarcinoma, treated with localized tumor hyperthermia (LTH), can potentially serve as a source of tumor antigen, where dying apoptotic/necrotic cells release tumor peptides slowly over time. In addition, LTH-treated cells can release heat shock proteins that can chaperone antigenic peptides to antigen-presenting cells, such as dendritic cells. We attempted to discern whether sequential LTH and intratumoral dendritic cell and/or systemic granulocyte macrophage colony-stimulating factor (GM-CSF) would activate antitumor immune response in a syngeneic murine model of prostate cancer (RM-1). Palpable RM-1 tumors, grown in the distal appendage of C57BL/6 male mice, were subjected to LTH (43.7°C for 1 hr) × 2, separated by 5 days. Following the second LTH treatment, animals received either PBS or dendritic cells (2 × 10^6) intratumorally (every 3 days for three injections). Separate cohorts also received i.v. injection of recombinant adenovirus-expressing murine GM-CSF (AdGMCSF), 1 day after LTH. Control animals received AdenolAcZ or AdenoGFP. Intratumoral dendritic cell injection induced tumor-specific T-helper cell activity (IFNγ ELISPOTS) and CTL activity, which was further augmented by AdGMCSF, indicating amplification of tumor-specific TH1 immunity. The combination of LTH, AdGMCSF, and intratumoral dendritic cell injection resulted in significant tumor growth delays when compared with animal cohorts that received LTH alone. These results support an in situ autovaccination strategy where systemic administration of GM-CSF and/or intratumoral injection of autologous dendritic cells, when combined with LTH, could be an effective treatment for local and systemic recurrence of prostate cancer. [Cancer Res 2007;67(16):7798–806]

Introduction

Prostate cancer is the most common malignancy in American men and the second most common cause of death. Although surgery and radiation therapy are curative treatment options for early, organ-confined disease, treatment of locally advanced prostate cancer has been suboptimal. Radiation therapy cures few patients. Frequently, adjuvant hormonal therapy is used to improve the efficacy of radiation therapy. However, a subpopulation of androgen-independent cells will not respond to hormonal therapy and ~25% to 60% of patients will show elevated prostate-specific antigen (PSA), which is a surrogate for future clinical recurrence. This translates to roughly 100,000 patients per year facing the possibility of recurrent prostate cancer after initial local treatment (1). Persistence of local disease after radiation is a significant issue in the management of this disease, as there remains a continuing potential for symptomatic local recurrence or metastatic seeding. Currently, there is no curative treatment for recurrent and metastatic prostate cancer.

Hyperthermia has been used effectively as a radiation sensitizer in the treatment of locally advanced and recurrent solid tumors, including breast, head and neck, and prostate cancer (2–4). Although hyperthermia has been evaluated as a definitive treatment in combination with external beam radiation therapy for locally advanced prostate cancer, the inability to achieve high temperature uniformly within the tumor limits its application (2). It is possible to expose parts of the tumor to high temperatures (>40°C) using hyperthermia, which is sufficient to induce cell death in prostate cancer cells by both nonapoptotic (>43°C) and apoptotic mechanisms (>43°C; ref. 5). Despite these problems, local tumor hyperthermia (LTH) could be considered as a palliative treatment in the management of locally advanced and recurrent prostate cancer.

LTH increases the release of heat-shock proteins (HSP) from dying tumor cells, whereby HSPs in the extracellular milieu can act simultaneously as a source of antigen due to their ability to chaperone intracellular peptides and as a maturation signal for immune cells (6). The maturation step enables dendritic cells to present antigens to T cells. Thus, the central defect in inducing tumor-specific immunity against prostate cancer is inefficient antigen presentation by tumor cells. Various immunotherapeutic approaches are being evaluated for patients with metastatic prostate cancers (8–10) by inducing systemic immunity to antigens such as PSA expressed by prostate cancer. Prostate cancer cells are inefficient in their antigen-presenting capacity, as they frequently do not express molecules important for antigen processing and presentation, such as the antigen transporter gene product, TAP-2, and class I MHC molecules (11, 12). In addition, they lack T-cell costimulatory molecules (CD80 and CD86) and, therefore, could potentially induce anergy of T cells. Thus, the central defect in inducing tumor-specific immunity against prostate cancer is inefficient antigen presentation by tumor cells. Various studies have shown that the potency of whole-cell tumor vaccines were further enhanced by cytokines, especially granulocyte macrophage colony-stimulating factor (GM-CSF) in murine tumor models (13) and in patients with...
Melanoma (14), lung cancer (15), and prostate cancer (9). GM-CSF increases circulating dendritic cells in the blood, which are available to process and present antigens that are released from irradiated tumor cells in the whole-cell vaccine. Because dendritic cells are thought to be the major antigen-presenting cells involved in triggering primary T-cell responses in vivo, dendritic cell–based tumor vaccination approaches are being increasingly tested (16). These protocols use defined tumor antigens and peptides, whole-cell extracts, apoptotic and/or necrotic tumor cells, and tumor-derived DNA and RNA (reviewed in ref. 16), and have been shown to induce significant antitumor immune response in animal tumor models. Sipuleucel-T (Provenge; APC8015; Dendreon Corp.), a novel immunotherapeutic agent, which includes autologous dendritic cells pulsed ex vivo with a recombinant fusion protein (PA2024) consisting of GM-CSF and prostatic acid phosphatase, has shown significant activity in two phase II trials in men with androgen-dependent biochemically relapsed prostate cancer with a decrease in PSA and prolongation in PSA doubling time (17). Data from two phase III trials in men with asymptomatic, metastatic hormone-refractory prostate cancer showed an improved median overall survival in men who received sipuleucel-T compared with placebo (17, 18). These approaches, however, require additional ex vivo manipulation to grow tumor cells and/or to prepare tumor antigenic extracts from resected tumor specimens and the results from early clinical trials have pointed to a need for additional improvement of dendritic cell–based vaccines before this therapy could be added to existing cancer strategies. As an alternative approach, intratumoral injection of immature (19) or cytokine-transduced (20–22) dendritic cells has been shown to induce strong antitumor immune response in various models of murine and human tumors. This approach depends on the capture and processing of tumor-derived antigens by intratumoral dendritic cells for presentation to T lymphocytes. Although the exact mechanisms of tumor antigen acquisition by dendritic cell are still unclear, available data suggest a role for HSPs released from dying malignant cells and for the internalization of tumor-derived apoptotic bodies.

Because LTH induces a temperature-dependent apoptotic or necrotic cell death in tumors, which is associated with the release of HSPs, intratumoral injection of dendritic cell could induce a strong tumor-specific immune response. We hypothesized that LTH-treated prostate tumor cells could potentially serve as a source of tumor antigens in vivo, where dying tumor cells would release tumor antigens and HSP slowly over time, thus providing a depot of tumor-derived antigenic peptides and "danger" signal for dendritic cell maturation. To examine our hypothesis, we evaluated our comprehensive in situ tumor vaccination strategy using LTH followed by intratumoral dendritic cell injection and GM-CSF cytokine therapy in a nonimmunogenic mouse prostate cancer model for the induction of systemic tumor-specific immune response and therapeutic efficacy.

Materials and Methods

Cell Lines, Medium, Cytokines, and Other Reagents

Mouse prostate cancer cell line, RM-1 (a gift from Dr. T.C. Thompson, Baylor College of Medicine, Houston TX) originally isolated from a ras + myc-transformed mouse prostate carcinoma (23, 24) were cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals), 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 µg/mL streptomycin, and 100 units/mL penicillin (Life Technologies Invitrogen Corporation). Recombinant adenoviruses expressing murine GM-CSF, β-galactosidase, and green fluorescent protein (GFP) were obtained from the Gene Therapy Core of the Albert Einstein College of Medicine (AECOM).

Animal Model

Five- to 6-week-old male C57Bl/6 mice (Jackson Laboratory) were maintained in the animal facility, and all animal studies were done under the guidelines and protocols of the Institutional Animal Care and Use Committee of AECOM. RM-1 tumor cells (10^6) were inoculated s.c. into the dorsum of the foot of male C57Bl/6 mice. Tumor dimensions were measured by vernier caliper on alternate days and the average tumor volume was determined by 4/3 × 3.14 × (L/2 × W/2 × H/2), where L is length, W is width, and H is height. Palpable tumors, with ≥5 mm in diameter, were used for these studies.

In vitro Studies with RM-1 Cells

Hyperthermia treatment. Exponentially growing RM-1 cells (10^6) were immersed into a temperature-controlled Haake water bath maintained at 42°C, 43.7°C, and 45°C for 1 h. Because optimal cell killing and release of HSP70 was seen with 43.7°C, further LTH studies were done at this temperature.

Apoptosis/necrosis detection assay. One day after hyperthermia treatment, cells were assayed for necrosis or apoptosis by Annexin V–FITC/propiodium iodide detection kit (BD Biosciences) according to the manufacturer’s protocol. Briefly, 10^6 to 10^7 cells were analyzed using FACScan flow cytometry (Becton Dickinson, Pharmigen) and analyzed using the FLOWJO program.

ELISA for HSP70 release. Release of HSP70 by hyperthermia-treated cells was assessed using an HSP70 ELISA kit (StressGen Biotechnologies), according to the manufacturer’s instructions.

Culture of immature dendritic cell. Bone marrow cells from the tibia and femur of C57Bl/6 mice were harvested following an established protocol (25) and the cells were plated at 10^6/mL of culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 µg/mL streptomycin, 100 units/mL penicillin (Life Technologies, Inc.), and recombinant murine GM-CSF, 10 ng/mL (specific activity, 5 × 10^6 units/mg), and interleukin-4 (IL-4; 10 ng/mL, specific activity, 2.8 × 10^8 units/mg; Peprotech). Immature dendritic cells were cultured for 5 days with cytokine supplementation on alternate days.

Engulfment assay of dendritic cell. RM-1 cells (10^6) and bone marrow–derived immature dendritic cells (10^6) were incubated with carboxylfluorescein diacetate, succinimidyl ester (CFDA-SE; Invitrogen-Molecular Probes) and PKH 26 (Sigma Chemical) for labeling according to the manufacturer’s instructions. One day after hyperthermia treatment, CFDA-SE–stained RM-1 cells were cultured with 10^6 PKH26-stained resting dendritic cells for 1 to 2 days, followed by harvesting and staining with FITC-conjugated monoclonal antibody against CD80 (BD Pharmingen). The dendritic cells were analyzed by FACScan flow cytometry.

Reverse transcriptase-PCR of CCR7. Autologous dendritic cells from 5-day cultures of bone marrow cells were cocultured with heat-treated RM-1 cells for 24 and 48 h. After incubation, cells were harvested and total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed using the Superscript First Strand Synthesis System (Invitrogen). CCR7 expression was then evaluated by reverse transcription-PCR (RT-PCR).

In vivo Experiments

LTH treatment. Palpable RM-1 tumors (21–25 mm³) were treated with LTH (43.7°C) for 1 h, using a temperature-controlled (Haake Circulator) water bath. Animals were anesthetized with i.p. ketamine and xylazine (7:1 mg/mL for 100 µL/mouse) and immobilized in a lucite jig from which the legs projected through. The foot bearing tumor was immersed into the water heated at 43.7°C and the tumor-bearing legs were maintained...
in that position for 1 h. A second LTH treatment was delivered, 5 days after the first LTH treatment.

**Intratumoral dendritic cell and recombinant adenovirus injection.**

Ten cohorts were used for these studies as follows: (a) untreated; (b) adenovirus-expressing GM-CSF (AdGMCSF) alone; (c) intratumoral dendritic cell; (d) intratumoral dendritic cell + AdGFP; (e) intratumoral dendritic cell + AdGMCSF; (f) LTH alone; (g) LTH + control AdGFP or AdLacZ [8 × 10^0 plaque-forming unit (pfu)]; (h) LTH + AdGMCSF [8 × 10^5 pfu]; (i) LTH + intratumoral dendritic cell and (j) LTH + AdGMCSF [8 × 10^6 pfu] + intratumoral dendritic cell. On day 1 after the first LTH treatment, recombinant adenoviruses [8 × 10^0 pfu in 100 μL PBS] containing either murine GM-CSF or control GFP was i.v. injected via tail vein. On day 1 after the second LTH treatment, 2 × 10^6 bone marrow–derived dendritic cells were injected intratumorally in separate cohorts of AdGMCSF- and AdGFP-treated animals. A total of three dendritic cell injections were administered on 3-day intervals.

**Cell death assay after LTH in vivo.** One day after LTH, tumor cells were harvested from tumors grown in LTH-treated and untreated mice and cells were prepared for Annexin V–FITC/propidium iodide staining according to the manufacturer’s protocol (BD Biosciences) and analyzed by Cell Quest program. ELISPOT assay. Splenocytes were harvested from C57Bl/6 mouse spleens 21 days after the first LTH treatment according to standard procedures (25). The ELISPOT assays were done following established protocols (14, 26). Briefly, 96-well nitrocellulose-based microtiter plates were coated overnight at room temperature with anti-IFN-γ monoclonal antibodies (BD Biosciences-PharMingen), diluted in PBS. After the plate was washed with PBS, all wells were blocked with PBS + 1% bovine serum albumin for 2 h at 37°C. The splenocytes [10^6/50 μL] from mice of various cohorts were mixed with LTH-treated RM-1 cells [10^5/50 μL] and cocultured in RPMI 1640 with 10% FCS for 30 to 40 h at 37°C under 5% CO2. Following this incubation, the wells were washed in PBS-Tween 20 and incubated with biotinylated anti-IFN-γ monoclonal antibodies for 1 h at 37°C, followed by incubation with streptavidin–horseradish peroxidase conjugate for 1.5 h. Spots representing a single cytokine secreted by individual cells were developed by using the peroxidase substrate 3-amino-9-ethylcarbazole and analyzed using the ELISPOT analyzer. Mean numbers of spot-forming cells were calculated from the triplicate assays. The results are presented as means ± SDs for three separate experiments.

**CTL assay.**

For CTL assay, splenocytes were prepared after the same protocol as described in the ELISPOT assay and stimulated with LTH-treated RM-1 cells in 5-day cultures. The cells were then analyzed for cytotoxic activity using a nonradioactive CTL assay [lactate dehydrogenase (LDH) release assay] method (CytoTox 96 Assay-Promega), according to the manufacturer’s protocol. Briefly, the assay was done in a 96-well round-bottomed plate using RM-1 cells as the target cells. CTL assays were done at lymphocyte effector/target (E/T) ratios of 5:1 and 10:1. The results were expressed according to the following formula:

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\% \text{ Specific analysis} = \left( \frac{\text{Experimental release}}{\text{Maximum release} - \text{Spontaneous release}} \right) \times 100
\]

**Results.**

To evaluate the effect of LTH treatment and dendritic cell–based immunotherapy, we chose the RM-1 murine prostate cancer model. The RM-1 cells were originally derived from a mouse prostate reconstitution model where the ras and myc oncogenes were retrovirally introduced into the urogenital sinus (23). The mouse prostate reconstitution model consistently produces androgen-independent, poorly differentiated prostate adenocarcinoma and the derivative cell lines, such as RM-1 prostate cancer cells, have been used to examine the efficacy of a variety of gene, chemotherapeutic, immunotherapeutic, and hormonal manipulation strategies (27, 28).

![Figure 1](image-url) Induction of cell death 24 h after LTH in vitro (A and B) and in vivo (C and D). RM-1 cells or tumor-bearing foot pads were treated either with LTH (43.7°C for 1 h; B) or incubated in control temperature (37°C for 1 h; A). Twenty-four hours after treatment, tumor cells were harvested and stained for fluorescence-activated cell sorting with propidium iodide (PI) and Annexin V–FITC. Flow cytometry (representative of three experiments) of Annexin V–FITC and propidium iodide–stained RM-1 cells shown below. Note the presence of early apoptotic (5.5%, Annexin+/propidium iodide–), late apoptotic (31.4%, Annexin+/propidium iodide+), and necrotic (21.7%, Annexin–/propidium iodide+) RM-1 cell population after heat treatment in vitro (B), whereas in vivo (C, D) LTH treatment primarily induced apoptosis (93.1%, Annexin+/propidium iodide+; D).
Heat treatment induces both apoptosis and necrosis and the release of HSP70 from RM-1 cells cultured in vitro and in vivo.

To determine the type of cell death induced by heat treatment, RM-1 cells were treated with hyperthermia (43.7°C) for 1 h, followed by incubation for 24 h. In contrast to unheated control cells that had <8% apoptotic/necrotic cell population, 50% to 58% of heat-treated RM-1 cells exhibited either apoptosis or necrosis (Fig. 1A and B). As displayed in Fig. 1B, Annexin V–FITC/propidium iodide staining showed the presence of early apoptotic (5.53%, Annexin+/propidium iodide–), late apoptotic (31.4%, Annexin+/propidium iodide+), and necrotic (21.7%, Annexin–/propidium iodide+) RM-1 cell population after heat treatment. We next treated mice with 2- to 3-week-old palpable tumors with LTH, 43.7°C for 1 h. One day after LTH, there was significant increase in late apoptotic cells (Annexin V+/propidium iodide+) in RM-1 tumors (90 ± 5%), compared with control animals (<1%; Fig. 1C and D). Interestingly, in contrast to heat treatment in vitro, pure necrotic cell population was less prominent after LTH treatment in vivo. This could be due to an increase in tumor tissue temperature during our LTH treatment in vivo, when apoptosis dominates with higher temperatures (>43–44°C), as supported by previous studies in human prostate cancer cells (5). Alternatively, necrotic cells may have been discarded as debris during the harvesting of tumor cells from foot tumors.

To examine whether HSP70 was released from heat-treated RM-1 cells, ELISA was done to quantitate HSP70 protein levels in the cell culture medium of heat-treated RM-1 cells and in serum of LTH-treated mice. There was significant release of HSP70 (range 1–8 ng/mL) in culture medium of heat-treated 10⁶ RM-1 cells and in serum (1–5 ng/mL) of LTH-treated mice, indicating the induction of HSP70 protein expression and subsequent release from dying tumor cells.

Imature dendritic cells engulfed hyperthermia-treated RM-1 cells followed by maturation. Immature dendritic cells have the highest capacity of engulfing apoptotic/necrotic cells and

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**Figure 2.** Engulfment of heat-treated RM-1 cells by immature dendritic cells. Confocal microscopy showing CFDA-SE–stained apoptotic RM-1 cell (A), PKH 26–stained immature dendritic cell (B), and dendritic cell engulfing RM-1 cells (nucleus stained with 4',6-diamidino-2-phenylindole; C and D).

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**Figure 3.** Maturation of dendritic cells after coculturing with treated RM-1 cells. fluorescence-activated cell sorting (A–C) demonstrating an increase in CD80 cell surface expression in dendritic cells, 24 h after cocultivation with (A) untreated, (B) heat-treated RM-1, and (C) freeze-thawed RM-1 cells; (D) RT-PCR showing an increase in expression of CCR7 mRNA in dendritic cells cocultured with LTH-treated RM-1 cells for 48 h.
dendritic cells lose the efficiency of antigen uptake upon maturation. Therefore, immature dendritic cells were purified from bone marrow cultured for 5 days in medium containing GM-CSF and IL-4 and used for these studies. Twenty four hours after heat treatment, CFDA-SE (green)–stained RM-1 cells were cocultured with PKH26 (red)–stained dendritic cells for 4 h for examining whether dendritic cells can engulf heat-treated RM-1 cells. Confocal microscopy showed that green debris of RM-1 cells were in the vesicles within the PKH26-stained red dendritic cells, indicating dendritic cells have phagocytosed necrotic or apoptotic debris from heat-treated RM-1 cells (Fig. 2). When dendritic cells were cocultured with heat-treated RM-1 cells for 24 to 48 h, flow cytometry revealed an increase in expression of T-cell costimulatory molecule, CD80 (B7-1), on the dendritic cell surface (Fig. 3B). Because dendritic cell maturation results in an increase in the gene expression for chemokine receptor, CCR7 mRNA, we did RT-PCR to evaluate the expression of CCR7 mRNA. Figure 3D shows an increase in CCR7 gene expression by 48 h of incubating dendritic cells with heat-treated RM-1 cells. Furthermore, there was minimal induction of CD80 in dendritic cells that were cocultured with cell lysate from necrotic RM-1 cells after repeated freeze-thaws (Fig. 3C). These experiments indicated that dendritic cells could engulf cell fragments of heat-treated RM-1 cells, followed by maturation with induction of CD80 and CCR7.

**Systemic TH1-type immune response is induced after LTH and intratumoral dendritic cell injection.** Next, we examined whether injection of immature dendritic cells in LTH-treated RM-1 tumors could stimulate T cells and induce RM-1–specific systemic immune response *in vivo*. ELISPOT assays showed that splenocytes from mice that received LTH + dendritic cell or LTH + AdGMCSF contained significantly (*P > 0.001*) higher frequencies of IFNγ-secreting T cells (117 and 93 colonies per 10^6 cells, respectively) than mice that received LTH alone (29 colonies per 10^6 cells). A combination of systemic AdGMCSF and intratumoral dendritic cell induced the highest frequency of IFNγ-secreting splenocytes (223 colonies per 10^6 cells; Fig. 4A). When splenocytes were stimulated with a nonspecific, C57Bl/6-derived, heat-treated, 3LL (Lewis lung adenocarcinoma) cells, the IFNγ-secreting T cells were consistently below baseline values (Fig. 4A).

**Induction of CTL response.** We next investigated whether the combination of LTH of RM-1 primary tumor and intratumoral dendritic cell injection induced CTLs that can lyse tumor cell targets. LDH release assay showed that intratumoral dendritic cell injection induced tumor-specific CTL activity (LDH release 17–20% versus 5% in control). Comparable levels of CTL activity was also noted in splenocytes of mice that received AdGMCSF + LTH (approximately 19% LDH release), indicating that systemic administration of GM-CSF could substitute intratumoral dendritic cell
injection. However, the addition of systemic administration of AdGMCSF to intratumoral dendritic cell injection in LTH-treated RM-1 tumors augmented the CTL response to a maximum of 36.3% (range, 30–44%), indicating that systemic GM-CSF could maximize the CTL response induced by intratumoral dendritic cell injection (Fig. 4B). These results show that a combination of LTH, intratumoral injection of dendritic cells and systemic administration of AdGMCSF induces IFN-γ-secreting, TH1 T cells and RM-1–specific CTLs, suggesting that LTH treatment of RM-1 tumors provides a source of tumor antigens and maturation signals for dendritic cell activation, which, in turn, induces a strong tumor-specific systemic immune response.

**Systemic AdGMCSF and/or intratumoral dendritic cell injection augments the tumor growth retardation of LTH.** To determine whether induction of tumor-specific TH1-type immune response in LTH-treated animals resulted in greater tumor control, we compared the treatment efficacy of LTH alone or in combination with dendritic cell–based immunotherapy. Two weeks after inoculation of RM-1 cells, palpable tumors were treated with LTH (43.7°C for 1 h) in two fractions separated by 5 days. LTH (n = 18) significantly inhibited (P < 0.001) tumor growth (Fig. 5A; Table 1). Because GM-CSF increases the amount of circulating dendritic cells in peripheral blood, we examined whether systemic administration of a recombinant adenovirus expressing murine GM-CSF can enhance the tumor growth delay in LTH-treated tumors and made comparisons to control viruses (AdLacZ or AdGFP). These results are summarized in Table 1 and are representative of three separate experiments. Twenty-four hours after the first LTH treatment, animals received either i.v. AdGMCSF or control viruses, AdLacZ, or AdGFP (8 x 10⁹ particles/mouse). Animals receiving control adenoviruses showed growth progression (Fig. 5A) similar to LTH-alone group (P = 0.67). In contrast, AdGMCSF (n = 17) significantly (P ≤ 0.01) delayed LTH-treated RM-1 tumor progression within the 2nd week of treatment (Fig. 5A; Table 1). We then determined whether the tumor growth delay is further enhanced by combining intratumoral dendritic cell injection with LTH + AdGMCSF treatment. Intratumoral dendritic cell inoculation, administered every 3 days after the second LTH treatment for three injections, augmented the tumoricidal effects of LTH (P ≤ 0.01; Fig. 5B; Table 1). The tumor growth inhibition was maximal (P ≤ 0.0001) in animals receiving LTH + AdGMCSF + intratumoral dendritic cell injection (n = 13; Fig. 5B). Intratumoral
dendritic cell injection alone or in combination with AdGMCSF and/or AdGFP without LTH had only a marginal tumoricidal effect, indicating that LTH is necessary for volume reduction in established tumors (Table 1). Thus, although dendritic cell–based immunotherapy is ineffective in local tumor control, it can enhance the tumoricidal effects of LTH and induce a systemic immune response against prostate cancer.

**Discussion**

Currently, there is no satisfactory salvage treatment for locally recurrent prostate cancer, after failure of radiation therapy. Reirradiation of prostate cancer is not possible because of limiting toxicities of normal tissues to radiation therapy. Hyperthermia could be used as a salvage therapy for patients presenting with evidence of clinical and biochemical failure, whereby it not only helps local control but also provides a source of tumor antigens for intratumoral dendritic cells for induction of tumor-specific immune response. LTH has been used in clinical trials in patients with locally advanced prostate cancer (1–3) as a radiosensitizing adjuvant therapy in combination with radiation therapy. A number of methods, such as transurethral microwave thermotherapy, radiofrequency interstitial tumor ablation, and high-frequency ultrasound have been safely used in the clinic to treat locally recurrent prostate cancer with LTH. LTH can elevate tumor temperatures due to the inability to rapidly dissipate heat because of tortuosity of tumor blood vessels and tumor avascularity. This results in an increase in cell killing when compared with attendant normal tissues, which have the abilities to dissipate heat by vascular dilation.

Our studies show that heat treatment (42–43.7°C) induces apoptosis and necrosis of RM-1 cells resulting in release of HSPs in the culture medium and serum. Interestingly, we found subtle differences between *in vitro* and *in vivo* effects of LTH on the percentage of apoptotic or necrotic death of RM-1 cell populations. Elevated temperatures (43.7°C for 1 h) *in vitro* resulted in cells that were both apoptotic and necrotic, whereas, *in vivo* it induced primarily apoptosis of RM-1 cells. The predominance of one form of cell death over other can be due to a difference in tumor tissue temperature *in vivo* versus that in culture conditions. The nature of cell death induced by LTH and the dependence on a critical temperature varies between cell types and tumor tissues. Li et al. (5) described apoptosis as the primary mode of cell death in human prostate cancer cells at higher temperatures (>43°C), whereas Yonezawa et al. (29) showed that apoptotic cell death was induced in malignant fibrous histiocytoma cells at 42°C and necrosis at higher (44°C) temperatures. Sauter et al. (30, 31) showed that although dendritic cells can engulf both apoptotic and necrotic cell, the maturation signal for dendritic cells, necessary for efficient cross-presentation of antigens to T cells, is provided by necrotic cells. Several groups have shown that the endogenous “danger” signal in necrotic cells, necessary for dendritic cell maturation, is HSP (32, 33). Our results show that HSPs are released from both LTH-treated apoptotic and necrotic RM-1 cells. Additionally, incubation of heat-treated RM-1 cells with immature dendritic cells resulted in induction of CD80 and CCR7 molecules, indicating maturation of dendritic cells. Similar results have been reported with tumor lysates derived from UV-irradiated apoptotic and necrotic tumor cells (34, 35). The induction of CCR7 is a hallmark of dendritic cell maturation because CCR7 is the “homing receptor” for antigen-loaded dendritic cells to migrate into draining lymph nodes, where they would present the processed antigens to T cells for the induction of adaptive immune response (36–38).

HSPs are highly conserved, abundant intracellular proteins and function as molecular chaperones that guide several steps during synthesis, transport, and degradation of proteins (39). They bind to peptides that are generated by the proteosomal degradation of heat-denatured intracellular proteins (26). The release of HSP-peptide complex from dying tumor cells could induce anticancer immune responses by targeting tumor-derived antigenic peptides to the professional antigen presenting cells, the dendritic cells, whereby dendritic cells endocytose HSP-peptide complex actively via several HSP receptors (CD91 for gp96 and CD14 for HSP70; refs. 32, 40). Once endocytosed, the HSP-peptide complexes are routed to the endogenous antigen pathway and cross-presented in MHC class I molecules, where it is recognized by class I-dependent CD8+ T cells. HSPs not only present a set of comprehensive tumor-derived peptide

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**Table 1. Tumor volumes in treatment cohorts**

<table>
<thead>
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<th>Groups</th>
<th>Day 0</th>
<th>Day 18</th>
<th>Day 22</th>
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<td></td>
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<td>698 ± 40.5</td>
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<td>DC ± AdGFP</td>
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<td>26.2</td>
<td>685 ± 18.6</td>
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<td>10</td>
<td>24.8</td>
<td>621 ± 54.6</td>
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<tr>
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<td>219 ± 158*</td>
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<tr>
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</tr>
<tr>
<td>LTH + AdGMCSF + DC</td>
<td>13</td>
<td>23.9</td>
<td>158 ± 19.5*†</td>
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NOTE: The volumes are averaged from three experiments.

*P ≤ 0.01.
†P ≤ 0.05.
‡P ≤ 0.005.
§P ≤ 0.001.
∥P ≤ 0.0001.
Antigens to the dendritic cells, but also provide the maturation signal for these cells. Thus, HSP proteins noncovalently bound to antigenic peptides are able toviccinate against tumors and infectious agents (39). In addition, HSP fusion proteins have been shown to induce CTL responses (41).

Random mutations in cancer cell generate unique tumor antigens, which if identified would be a logical choice for tumor vaccine specifically designed for individual patients. Because individual mutations in cancer cells are not identified, autologous tumor-derived HSP-peptide complexes has been proposed as a logical personalized approach that may obviate the need to identify the unique antigens contained in the individual vaccine (42, 43). Thus, autologous HSP vaccines are undergoing testing in clinical trials and preliminary data show some promise (44). However, this strategy requires isolation and purification of HSP-peptide complexes from selected tumor specimens. This report shows that LTH-treated primary tumor can be harnessed as an autologous in situ tumor vaccine, whereby the LTH-treated tumor cells provide a depot of tumor antigens in vivo and by inducing the expression and release of HSP-peptide complex from dying tumor cells, LTH treatment of prostate cancer cells could provide the very essential “danger” signals for dendritic cell maturation and induction of tumor-specific adaptive immune response. Thus, the combination of LTH and intratumoral dendritic cell inoculation induced TH1 type immune response with an increase in IFNγ-secreting T cells and CTLs resulting in enhancement of tumor growth retardation with the combination treatment. Interestingly, systemic administration of AdGM-CSF induced similar levels of RM-1–specific immune response and augmented tumor growth retardation, suggesting that systemic GM-CSF administration could bypass the requirement for intratumoral dendritic cell injection. This is not surprising because GM-CSF increases the number of circulating dendritic cells in the blood, thus enabling circulating dendritic cells to infiltrate LTH-treated tumors for antigen engulfing and processing. The combination of LTH with AdGM-CSF and intratumoral dendritic cell was found to be the most efficacious in inducing antitumor immunity and tumor growth retardation. In this study, Ad-GM-CSF was used as an inexpensive and effective means to administer GM-CSF in laboratory mice. However, in the clinic, one could envision using s.c. injection of recombinant GM-CSF to increase the number of circulating dendritic cells. When feasible, autologous dendritic cells can be harvested from patients for direct intratumoral injection into LTH-treated primary tumors. Previous studies by Alfieri et al. (45) have shown that LTH can induce cell-mediated immunity in immunogenic tumors. However, we now show that immunomodulation of LTH with dendritic cell–based immunotherapy can induce cell-mediated immunity in a nonimmunogenic RM-1 tumor model. This is in concordance with our earlier studies with another nonimmunogenic tumor model (3L lung adenocarcinoma), where we combined Flt3L therapy with radiation therapy to induce systemic protective antitumor immunity (46, 47).

Various approaches of prostate cancer immunotherapy, such as recombinant tumor antigen vaccines delivered by viral and plasmid vectors, peptide vaccines, and HSP-peptide vaccines, are being tested in clinical studies. Although vaccination with defined tumor antigens present by dendritic cell has obvious appeal, natural immune variation, MHC polymorphism, and emergence of antigen-loss variants would require an ever-changing mixture of potential tumor antigens in vaccine formulations. Attempts have been made to treat tumors with irradiated whole-cell vaccines transduced with the GM-CSF gene in murine tumor models (13) and in patients with melanoma (14), lung cancer (15), and prostate cancer (9). As mentioned before, such a strategy requires growing tumor cells ex vivo, which may not be possible in most cases of recurrent and metastatic prostate cancer. Instead of these whole-cell vaccines, our in situ vaccination approach combines local prostate hyperthermia to release prostate cancer antigens with dendritic cell–activating cytokine, GM-CSF, to induce a strong tumor-specific immune response. Immunomodulation by hyperthermia with dendritic cell–stimulating cytokine therapy would be beneficial in treating locally advanced and recurrent prostate cancer and hopefully, successfully eradicate micrometastatic tumor foci. In the clinic, prostate hyperthermia can be delivered by a variety of procedures, such as high-energy transurethral thermotherapy (48), transperineal radiofrequency interstitial tumor ablation (49), and high-frequency ultrasound (50). The experiments, reported here, would serve as a basis of future clinical trials of hyperthermia and dendritic cell–based immunotherapy in patients with recurrent and metastatic prostate cancer.

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This article is dedicated to the memory of Dr. Joseph Mendecski whose research focused on the applications of hyperthermia in the treatment of cancer.

References


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Localized Hyperthermia Combined with Intratumoral Dendritic Cells Induces Systemic Antitumor Immunity

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