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## Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles

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**Abstract** In this study we demonstrated that heat shock protein (HSP) 70 expression by hyperthermia induced antitumor immunity in the T-9 rat glioma. Our hyperthermic system using magnetic nanoparticles induced necrotic cell death that correlated with HSP70 expression. We purified the HSP70-peptide complexes from the tumor after hyperthermia to investigate whether HSP70 was involved in the antitumor immunity, and we found that in the F344 rats immunized with T-9-derived HSP70 the tumor growth of T-9 was significantly suppressed. Tumor rejection assay after hyperthermic treatment of implanted T-9 cells with incorporated magnetite cationic liposomes (MCL) was performed to investigate whether antitumor immunity was induced by release of HSP70 from the necrotic cells in the F344 rat. Tumor growth was strongly suppressed in the rats subjected to hyperthermia of implanted T-9 cells, and 50% of rats were protected from challenge with T-9 cells. Immunogenicity was enhanced when the HSP70-overexpressing T-9 cells were killed via necrosis in rats by hyperthermia, after which all rats were completely protected from challenge with T-9 cells. Our hyperthermic system produces vaccination with HSP70-peptide via necrotic tumor cell death *in vivo*, resulting in antitumor immunity. This phenomenon, which may be termed in

situ vaccination, has important implications for the development of novel antitumor therapies.

**Keywords** Antitumor immunity · Cationic liposome · HSP70 · Hyperthermia · Magnetite

### Introduction

Hyperthermia is one of the promising approaches in cancer therapy [7, 17, 36]. However, the inevitable technical problem with hyperthermia is the difficulty of heating only the local tumor region until the intended temperature has been reached without damaging normal tissue. To circumvent this problem, we have developed magnetite cationic liposomes (MCL) as an intracellular heating mediator [24]. The hyperthermic effect of the MCL was examined in an *in vivo* study, and complete tumor regression was observed [40]. Some researchers have reported that heat treatment itself can enhance the immunogenicity of cancer cells [8, 14, 15, 20]. We have previously reported on the antitumor immunity induced by hyperthermia of T-9 rat glioma cells *in vivo* [41]. This induced immunity continued for an extended period of time, and the rats treated by hyperthermia completely rejected a rechallenge of T-9 cells as a metastasis model. However, the underlying mechanism has not been elucidated.

Heat shock proteins (HSP) are highly conserved proteins whose synthesis is induced by a variety of stresses, including heat stress [23]. Because expression of the HSP70 protects cells from heat-induced apoptosis [18], HSP70 expression has been considered to be a complicating factor in hyperthermia. On the other hand, recent reports have shown the importance of HSP, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), in immune reactions [12, 29]. A possible mechanism by which HSP might influence tumor cell immunogenicity may be associated with the processing and presentation of endogenous tumor antigens directly to tumor-specific T-cells [38]. Thus Wells et al. reported HSP70-mediated

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augmentation of MHC class I surface expression and endogenous antigen presentation [37]. We demonstrated heat-induced expression of major histocompatibility complex (MHC) class I antigen on the cells as a possible mechanism for antitumor immunity induced by hyperthermia [6]. Alternatively, the HSP-mediated antitumor immunity may be caused by a vaccine-like effect of HSP-peptide complexes released from dying tumor cells. It has been demonstrated that tumor-derived HSP-peptide complexes can elicit cancer immunity [21, 33, 34]. To elucidate how tumor antigens of T-9 glioma cells are recognized by the host immune system in hyperthermia, we tested the hypothesis that an HSP-antigen complex released via necrotic tumor cell death functions as a tumor vaccine, because the expression of several HSP is always associated with hyperthermic treatment.

In the present study, we examined the role of a single defined HSP, HSP70, in the mechanism of antitumor immunity induced by intracellular hyperthermia, and demonstrated the possibility of *in situ* vaccination.

## Materials and methods

### Cell lines

T-9 rat glioma cells were cultured in minimum essential medium (MEM), supplemented with 10% fetal calf serum (FCS), 10 mM nonessential amino acids, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G.

The pCMVhygro.HSP70 plasmid was kindly provided by Dr. Kenzo Ohtsuka (Aichi Cancer Center, Japan). This plasmid contains the human inducible *hsp70* cDNA and the hygromycin resistance gene. The *hsp70* cDNA was located under the CMV promoter. The pCMVhygro plasmid was constructed by blunt-end ligation at *Xba*I and *Kpn*I sites, resulting in the exclusion of the *hsp70* cDNA in the pCMVhygro.HSP70 plasmid. The T-9 clones were constructed by transfection with the pCMVhygro.HSP70 plasmids (T-9/HSP70 cells) and the pCMVhygro plasmids (T-9/hygro cells) using the cationic liposomes that included the plasmids, as reported earlier by us [6]. The transfectants were cultured in MEM plus 0.5 mg/ml of hygromycin B (Wako Pure Chemical Industries, Osaka, Japan), and hygromycin-resistant T-9 clones were obtained. All cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### Preparation of MCL

MCL as prepared with the colloidal magnetite and a lipid mixture that consisted of *N*-( $\alpha$ -trimethylammonioacetyl)-didodecyl-D-glutamate chloride (Sogo Pharmaceutical, Tokyo) dilauroylphosphatidylcholine and dioleoylphosphatidyl-ethanolamine (Sigma Chemical, St. Louis, Mo.) in the molar ratios 1:2:2 according to our previously described method [24].

### In vivo hyperthermic treatment and immunohistochemical staining

To prepare tumor-bearing rats,  $1 \times 10^7$  T-9 glioma cells were injected into the subcutaneous (s.c.) space of 6-week-old female Fisher 344 rats (Japan SLC, Shizuoka, Japan). When the s.c. tumors had reached about 10 mm in diameter, 400  $\mu$ l of MCL (net magnetite weight: 3 mg) was injected into the tumor via a syringe (needle size: 25 G) attached to an infusion pump (SP100i; World Precision Instruments, Sarasota, Fla.). Subsequently, 24 h after injection, rats were subjected to the first hyperthermic treatment. A magnetic

field was created by using a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency, Tokyo) [40]. The magnetic field frequency and intensity were 118 kHz and 30.6 kA/m (386 Oe), respectively. Tumor and rectal temperatures were measured by optical fiber probe (FX-9020; Anritsu Meter, Tokyo). The treatment was carried out for 30 min once, twice or three times at 24-h intervals.

For immunohistochemical staining, the tumor was resected and fixed in a 10% formalin solution 24 h after hyperthermia. The tumor was sectioned longitudinally into 6- $\mu$ m thick slices. These sections were incubated with 10% skim milk at 37°C for 10 min to block background staining. They were then incubated at 37°C for 60 min with mouse monoclonal antibody preparations (mAb) for macrophage (clone ED2; Biosource International, Camarillo, Calif.), HSP70 (clone W27; Santa Cruz Biotechnology, Santa Cruz, Calif.), or CD8 (clone MRC OX-8; Serotec, Oxford, U.K.) antigens, and then at 37°C for 60 min with biotinylated goat anti-mouse IgG. Following this step, the slices were incubated at 37°C for 30 min with peroxidase-conjugated streptavidin (Dako, Kyoto, Japan). Each step was followed by washing with phosphate buffer. Peroxidase activity was visualized by treatment at room temperature for 10 min with 0.02% diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxide. Double staining with anti-HSP70 and anti-macrophage antibodies was also performed. In this case, peroxidase activity of HSP70 was stained blue. All sections, except for the double staining, were also stained with hematoxylin. For the negative control, the primary antibodies were replaced with either irrelevant mAb or PBS.

### Purification of the HSP70-peptide complex

The HSP70-peptide complex was purified by a published method [22]. Briefly, 5-g samples of tumor tissue from hyperthermia-treated rats, or 5 g of liver tissue from naive F344 rats were homogenized in 40 ml of hypotonic buffer [10 mM NaHCO<sub>3</sub>, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.1], and a 100,000 *g* supernatant was obtained. The sample buffer was changed to buffer D (20 mM Tris-acetate, 20 mM NaCl, 15 mM  $\beta$ -mercaptoethanol, 3 mM MgCl<sub>2</sub>, 0.5 mM PMSF, pH 7.5) on a PD-10 column (Sephadex G-25; Pharmacia Biotech, Piscataway, N.J.). The eluted sample was applied directly to an ADP-agarose column (Sigma Chemical, St. Louis, Mo.) equilibrated with buffer D. The column was incubated with buffer D containing 3 mM ADP (Sigma Chemical) at room temperature for 30 min and subsequently eluted with the same buffer. The eluate buffer was changed on a PD-10 to a FPLC buffer (20 mM sodium phosphate, 20 mM NaCl, pH 7.0). Then the protein fractions were resolved on an FPLC system (Vision work station; Applied Biosystems, Foster City, Calif.) and eluted with a 300–600 mM NaCl gradient. All protein was quantified with the Bradford assay, and BSA was used as the standard (Biorad, Richmond, Calif.).

### SDS-PAGE and immunoblotting

SDS samples were heated at 100°C for 6 min and centrifuged at 18,000 *g* for 5 min, and then the supernatants were subjected to SDS-PAGE on a 7.5% polyacrylamide gel. After electrophoresis, proteins were either silver-stained or transferred onto a polyvinylidene difluoride (PVDF) membrane. The transferred PVDF membrane was incubated with mouse mAb for HSP70/HSC70 (clone N27F3-4; StressGen Biotechnologies, Victoria, B.C., Canada) for 2 h at 37°C, subsequently probed with peroxidase-labeled goat anti-mouse IgG for 1 h at 37°C, and then visualized by ECL (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

### Immunization with HSP70-peptide complex and tumor challenge study

Rats were immunized by s.c. injections of 20  $\mu$ g HSP70-peptide preparations (in 200  $\mu$ l PBS). Three immunizations were performed

at weekly intervals. The rats were challenged by s.c. injection of T-9 cells ( $1 \times 10^6$  cells in 100  $\mu$ l PBS) 1 week after the last immunization. Tumor size was measured every 3 days, and was determined by the following formula: Tumor volume =  $0.5 \times (\text{length} \times \text{width}^2)$  where the units of length and width are in millimeters.

#### In vitro hyperthermia study

T-9 cell pellets were prepared by the previously described method [39]. One ( $1 \times 10^7$ ) T-9 cells were incubated for 24 h with the MCL-containing medium (100  $\mu$ g/ml). The pellets were irradiated by the magnetic field and the temperature was maintained at 42°C for 30 min. After irradiation and centrifugation, the supernatant of pellets was applied to SDS-PAGE and, after electrophoresis, analyzed by immunoblot for the released HSP70. The pellets were analyzed by the Vybrant apoptosis assay kit no. 4 (Molecular Probes, Eugene, Ore.): The pellets were washed in cold PBS and then incubated with 1  $\mu$ l of the green fluorescent dye Yo-Pro-1, which can enter apoptotic cells, and 1  $\mu$ l of propidium iodide (PI). As soon as possible after the incubation period, the stained cells were analyzed by flow cytometry (Paltec, Munster, Germany).

#### Tumor rejection assay after hyperthermia treatment

The T-9 cell pellets ( $1 \times 10^7$  cells in 150  $\mu$ l PBS) including MCL, as mentioned above, were implanted s.c. into the left femoral region of the rats and irradiated in the magnetic field. The temperature of the implanted site was maintained at 42°C for 30 min. This treatment was repeated 3 times at weekly intervals. The rats were challenged by s.c. injection of T-9 cells ( $1 \times 10^6$  cells in 100  $\mu$ l PBS) into the right femoral region 1 week after the last treatment. Tumor sizes were measured every 3 days.

#### Statistical analysis

Levels of statistical significance in the tumor growth experiments were evaluated using the Mann-Whitney rank sum test [16]. Animal experiments were performed according to the principles laid down in the "Guide for the Care and Use of Laboratory Animals" prepared under the directions of the Office of the Prime Minister of Japan.

## Results

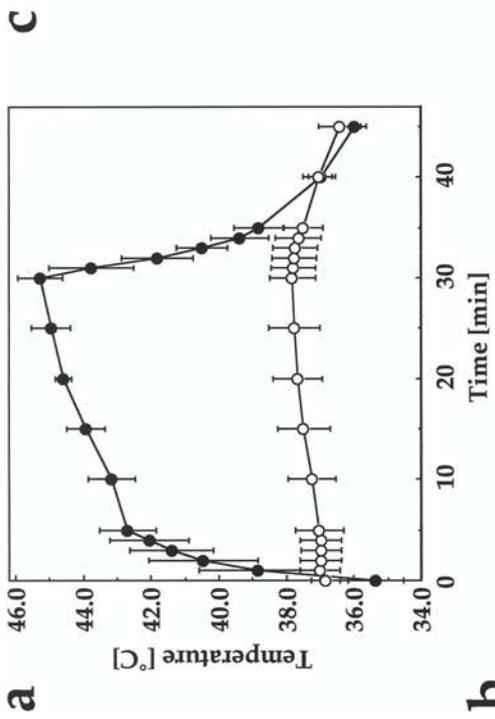
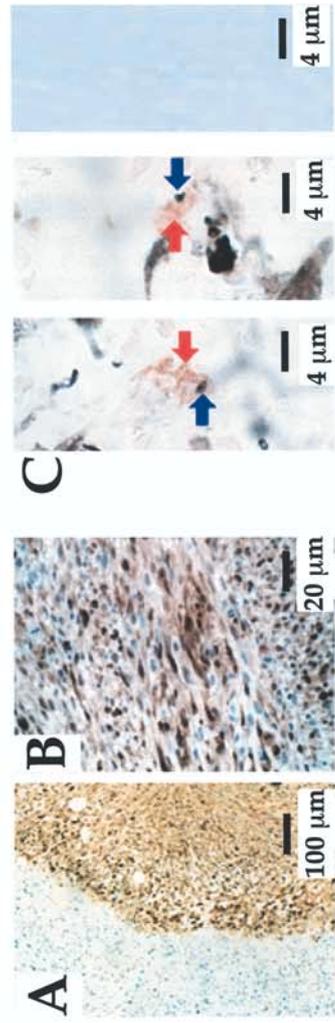
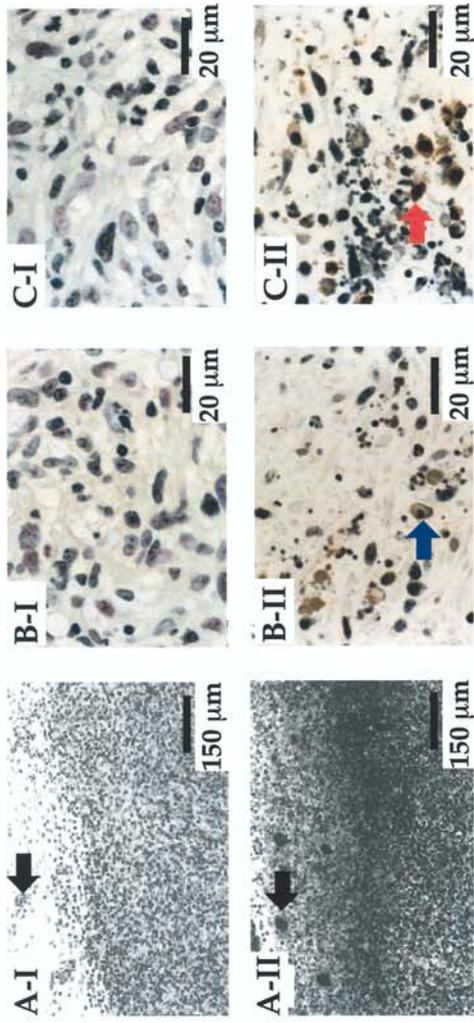
### MCL-induced hyperthermia

One day after MCL injection into the tumor, an alternating magnetic field (AMF) was applied to the whole body of the mouse. Tumor temperature increased rapidly to 42°C in 4 min and was then allowed to increase for 30 min (Fig. 1a). When the AMF irradiation was stopped, the tumor temperature decreased to 36°C within 15 min. In contrast, the temperature in the rectum did not increase over 38°C during the entire irradiation period. This suggests that the MCL injected into the tumor were heated specifically by the AMF. Fig. 1b shows MCL distribution (part A), necrotic area as hematoxylin and eosin (HE) staining (part B), and immunohistochemistry for HSP70 (part C) in a tumor specimen. Without irradiation, the injected MCL remained in the center of the tumor (Fig. 1b; part A–I). When the tumor was irradiated with the AMF, the temperature of the tumor was specifically increased, as shown in Fig. 1a, and the MCL diffused and spread to all areas of the tumor

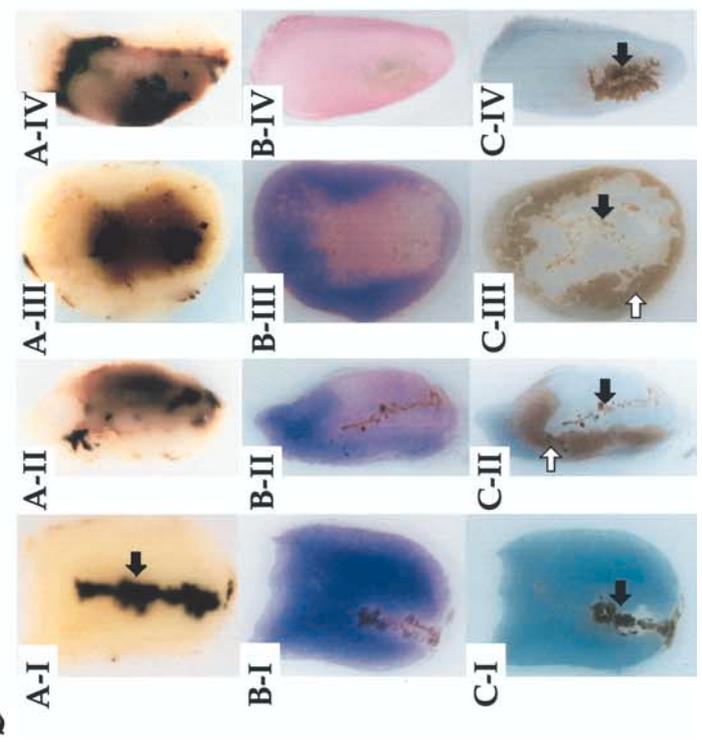
after 3 irradiations (Fig. 1b; part A–IV). According to this distribution, the necrotic areas also spread to the whole tumor (Fig. 1b; part B–IV). Fig. 1b; part C shows the HSP70 expression in the tumor. Without irradiation, HSP70 was not strongly stained (Fig. 1b; part C–I). By the repeated irradiation, HSP70 was strongly expressed in the viable cells around the necrotic area and it was observed like a belt, as shown in Figs. 1b; parts C–II and III. As the necrotic area spread widely following repeated irradiation, the HSP belt was found to spread toward the peripheral region of the tumor. In the case of 3 irradiations, the HSP70 belt was not observed (Fig. 1b; part C–IV), because the necrotic area had spread throughout the tumor. Apoptosis detection assay by TdT-mediated dUTP nick end-labeling (TUNEL) was performed, but no significant numbers of apoptotic cells were observed (data not shown).

Fig. 1c shows recruiting of immune effector cells to the tumor. A great number of inflammatory cells such as neutrophils infiltrated the tumor tissue after hyperthermia produced with 2 irradiations (Fig. 1c; part A–II) compared to the tumor without hyperthermia (Fig. 1c; part A–I). Moreover, immunohistochemical staining showed that macrophages and CD8<sup>+</sup> T lymphocytes were also observed in the necrotic area of the tumor after hyperthermia, as shown in Figs. 1c; part B–II and 1c; part C–II, respectively. These cells were also observed after 1 and 3 irradiations (data not shown). The HSP70 belt was observed in the viable area around the necrotic area (Fig. 1d; part A). The morphology of the cells in the HSP70 belt was seriously damaged, as shown in Fig. 1d; part B. We performed double staining with anti-macrophage antibody and anti-HSP70 antibody, and HSP70 engulfed by macrophages were observed, as shown in Fig. 1d; part C.

**Fig. 1a–d** Hyperthermia using MCL in vivo. MCL were injected directly into s.c. tumors in rats. One day after MCL injection, the rats were irradiated with an AMF for 30 min. **a** Temperature during AMF irradiation. Tumor and rectal temperatures were measured by optical fiber probes. *Closed circles*: tumor; *open circles*: rectum. Each point represents the mean  $\pm$  SD of 5 rats. **b** Photographs of tumor specimens. Tumors were resected at 24 h after hyperthermia and were *A* paraffinized; *B* histologically stained by HE; and *C* immunohistochemically stained by anti-HSP70 antibody. *I* without AMF irradiation; *II* irradiated once for 30 min; *III* irradiated twice for 30 min; and *IV* irradiated three times for 30 min. The *black and white arrows* indicate the MCL and cells expressing HSP70, respectively. **c** Immunohistochemical staining for immunocytes. The tumors were resected at 24 h after hyperthermia. *A* HE staining; *B* anti-macrophage antibody staining; and *C* anti-CD8 antibody staining. *I* without irradiation; *II* irradiated twice in the necrotic area. The *black, blue, and red arrows* indicate a blood vessel, a macrophage and a CD8<sup>+</sup> cell, respectively. **d** Histological analysis of HSP70 and APC. The tumors were resected at 24 h after hyperthermia. Light microscopic sections of *A* anti-HSP70 antibody staining in the boundary between necrotic and viable areas; *B* the HSP70-stained area in *A* under higher magnification; *C* double staining with anti-macrophage antibody and anti-HSP70 antibody (*left and center*) and isotype control antibody (IgG<sub>1</sub>) staining (*right*). The *blue and red arrows* indicate HSP70 and a macrophage, respectively. Here, no detectable staining was evident for all negative controls



d



## Immunogenicity of tumor-derived HSP70-peptide complexes

We purified HSP70 produced in tumor tissue after hyperthermia to investigate whether the HSP70 expression by hyperthermia was involved in antitumor immunity. The HSP70-peptide complexes from T-9 rat glioma and liver tissue of F344 rats were purified (Fig. 2a), as described in Materials and methods. F344 rats were immunized with 20  $\mu\text{g}$  of the purified HSP70-peptide complexes 3 times at weekly intervals. The rats were challenged with  $1 \times 10^6$  T-9 cells 1 week after the third immunization, and tumor growth was monitored (Fig. 2b). Tumors grew progressively in all non-immunized rats (PBS-injected), but the growth rate was significantly reduced in tumor-derived HSP70-immunized rats ( $P < 0.01$ ), and one out of 5 rats rejected a tumor completely. HSP70-peptide complexes from liver were also tested, because HSP70 is a ubiquitous protein present in normal tissues. No significant effect of HSP70 derived from normal tissue was observed.

### Release of HSP70 from necrotic tumor cell death

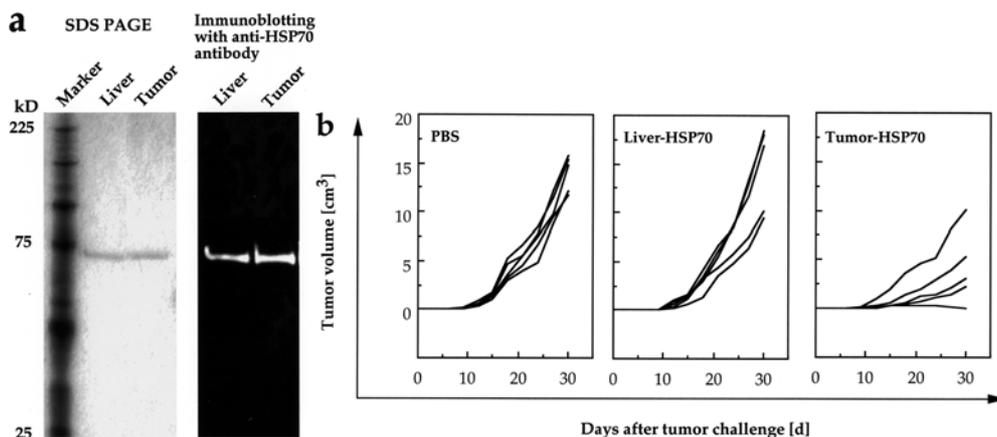
Next we investigated whether tumor cell death by hyperthermia caused a release of HSP70. Two T-9 clones, T-9/hsp70 cells and T-9/hygro cells, were established as described in Materials and methods. The T-9/hsp70 cells

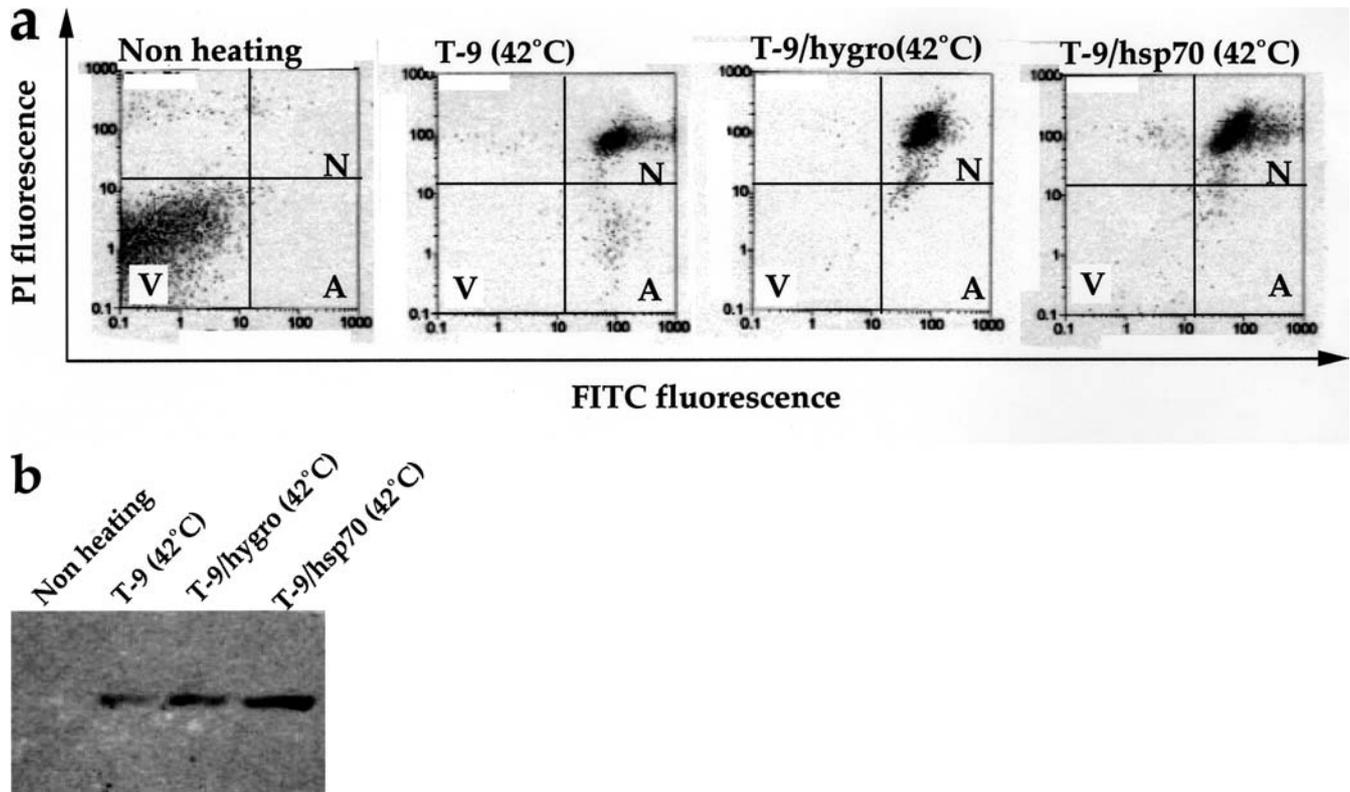
were constructed by transfection with the pCMVhygro.HSP70 plasmids to investigate the effect of HSP70 expression level in antitumor immunity caused by hyperthermia. The T-9/hsp70 cells stably expressed 100.3 pg/cell of HSP70/HSC70, whereas T-9 and the T-9/hygro expressed 25.1 and 22.5 pg/cell, respectively, under non-heating conditions. These T-9 clones were subjected to hyperthermia and then examined by the apoptosis assay. The apoptosis/necrosis assay, using flow cytometric analysis, revealed that necrotic cell death was induced in the 3 types of T-9 cell lines by hyperthermia at 42°C for 30 min (Fig. 3a). After hyperthermia, HSP70 released into the supernatant from treated cells was detected on a western blot probed with antibody to HSP70/HSC70. From densitogram analysis, the amount released of HSP70 from the T-9/hsp70 clone was found to be about 2.5 times higher than that released from the other 2 clones (Fig. 3b). Without hyperthermia, more than 99.5% of the cells were viable with or without MCL addition, and HSP70 was never detected in the supernatants of the 3 clones.

### Tumor rejection after hyperthermic treatment

Tumor rejection assay after hyperthermic treatment was performed to investigate whether antitumor immunity was induced via tumor cell death by hyperthermia. The three types of cell lines (T-9, T-9/hygro, T-9/hsp70) with incorporated MCL were implanted s.c. into rats. In the magnetic field irradiation, the temperature of the implanted site was maintained at 42°C for 30 min. Afterwards, no tumors were formed, since the T-9 cells had died via necrosis as shown in Fig. 3a. The rats were challenged by s.c. injection of T-9 cells 1 week after the last treatment, and tumor growth was monitored (Fig. 4). Tumors grew progressively in all non-treated rats, whereas tumor growth was strongly suppressed in the rats subjected to hyperthermia. The rats acquired the most significant antitumor immunity using the HSP70-overexpressing clone, T-9/hsp70, which released an about 2.5 times higher amount of HSP70 than the other 2 clones (Fig. 3b). After hyperthermic treatments of T-9/hsp70 cells, complete protection in all rats (8/8) was

**Fig. 2a, b** Immunogenicity of tumor-derived HSP70-peptide complexes. **a** Silver-stained SDS-polyacrylamide gels and immunoblots with the anti-HSP70 antibody of purified HSP70-peptide complexes from T-9 tumor and liver. **b** Antitumor effect of tumor-derived HSP70-peptide complexes. Rats were immunized with 20  $\mu\text{g}$  of HSP70-peptide complexes derived from T-9 tumor and liver as indicated. The nonimmunized group was injected with PBS. Immunization was carried out s.c. in 200- $\mu\text{l}$  volumes three times at weekly intervals. Rats were challenged with  $1 \times 10^6$  T-9 cells s.c. 1 week after the third immunization. Each line represents the kinetics of tumor growth in a single rat.  $P < 0.01$  for nonimmunized versus tumor-derived HSP70-treated groups.  $P < 0.02$  for liver derived HSP70-treated versus tumor-derived HSP70-treated groups.  $P = 0.6$  for nonimmunized versus liver derived HSP70-treated groups





**Fig. 3a, b** Necrotic cell death and release of HSP70. T-9, T-9/hygro, and T-9/hsp70 cells were subjected to hyperthermia *in vitro*. **a** Apoptosis/necrosis detection assay by flow cytometry of the cells after heating at 42°C for 30 min. *V*: viable cells, *N*: necrotic cells, *A*: apoptotic cells. **b** Released HSP70 assay. Supernatants of the heated cells ( $1 \times 10^7$  cell equivalents) were collected after hyperthermia, resolved on SDS-PAGE, immunoblotted, and probed with anti-HSP70 antibody

observed, whereas 50% (4/8) of rats were protected by the treatment of T-9 and T-9/hygro cells. Taken together, the necrotic cell death of T-9 cells in F344 rats was caused by hyperthermia-induced antitumor immunity, and the immunity was enhanced by a high-level expression of HSP70 in hyperthermia.

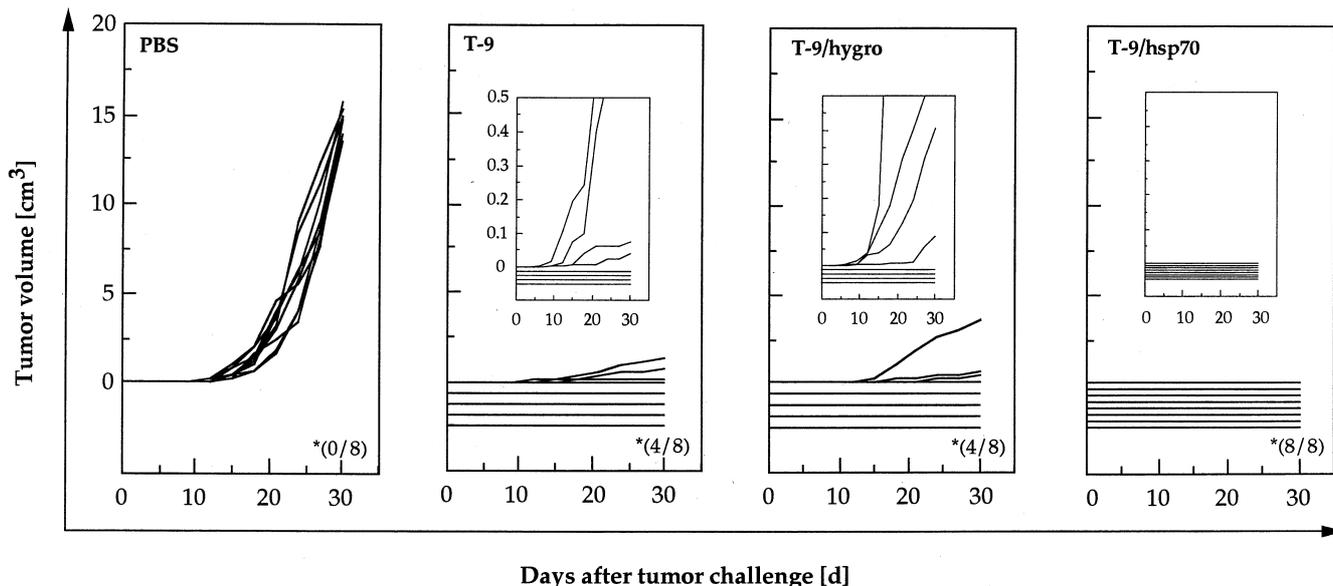
## Discussion

Intracellular hyperthermia using MCL induced necrotic cell killing via HSP70 release

In general, hyperthermia is effective under mild conditions such as 42°C, which aim to induce apoptosis in cancer cells rather than necrosis. The mild hyperthermic conditions would cause minimal, if any, damage to surrounding normal tissues. However, it may not be efficient in terms of antitumor immunity. Melcher et al. compared the immunogenicity of murine melanomas undergoing necrotic versus apoptotic cell death after chemotherapy *in vivo* [11]. Tumors that responded to chemotherapy by necrotic cell death expressed HSP70, whereas tumors that experienced apoptotic cell death

did not express HSP70. Only animals carrying tumors expressing HSP70 that underwent necrotic cell death after chemotherapy exhibited resistance to subsequent tumor challenge. Yonezawa et al. compared the manner of cell death induced by hyperthermia [42]. Apoptotic cell death was induced in malignant fibrous histiocytoma cells by hyperthermic treatment at 42°C, whereas necrotic cell death was induced by hyperthermia at 44°C. Thus hyperthermia easily induces necrotic cell death (in principle, in any type of tumor cell) by heating to a higher temperature. Our intracellular hyperthermic treatment can heat the tumor specifically by means of MCL. Moreover, the degree of heat generation in the tumor can be controlled by the magnetic field intensity, which makes it possible to induce the necrotic cell killing without any damage to the surrounding normal tissues. Several reports have suggested that HSP expression induces thermotolerance [9, 30, 31], which would negatively affect hyperthermic treatment. We have shown that our hyperthermic system could break the thermotolerance and enlarge the necrotic area, as seen in Fig. 1. The enlargement of the necrotic area can cause the induction of the immune response, as mentioned below. These results have never been obtained by conventional hyperthermia, as far as we know. As a result, our hyperthermic system should be considered as a very effective treatment of solid tumors.

Basu et al. reported that necrotic, but not apoptotic, cell death leads to the release of biologically potent HSP from cells [3]. In the present paper, the release of HSP70 from necrotic tumor cells by hyperthermia induced antitumor immunity, which was enhanced by overexpression



**Fig. 4** Antitumor immunity induced via tumor cell death by hyperthermia. T-9, T-9/hygro, and T-9/hsp70 cells were treated with MCL and implanted s.c. into rats, and irradiated with a magnetic field. The temperature of the injected sites was maintained at 42°C for 30 min. This hyperthermic treatment was carried out three times at weekly intervals. The nonimmunized group was injected with PBS. Rats were challenged with  $1 \times 10^6$  T-9 cells s.c. 1 week after the last immunization. Each line represents the kinetics of tumor growth in a single rat and inset figures represent a smaller scale. \*Animals cured of tumor, n

of HSP70 in T-9/hsp70, as shown in Figs. 3 and 4. Udono et al. reported the HSP70 vaccination effect for tumor treatment and showed that this effect was directly dependent on the dose of HSP70 [35]. When the HSP70 in the tumor is regarded as an antigen source, 1 g (approximately  $10^8$  cells) of T-9 tumor tissue may contain approximately 2 mg HSP70, since T-9 cells expressed 20 pg/cell of HSP70 as measured by ELISA. This represents a much higher dose than that used in any *in vivo* study in murine models reported previously [1]. In our hyperthermic system, the expression of HSP70 was enhanced, and the tissue lysis via necrosis was observed throughout the tumor, as shown in Fig. 1. Our hyperthermic system, using MCL, can promote the release of HSP (possibly including HSP such as HSP90 and gp 96), resulting in vaccination therapy.

It has not been elucidated whether the biological activities as vaccines of the inducible HSP are different from those of their constitutive counterparts. Srivastava et al. reported that the constitutive HSP70 served as an effective vaccine [26, 27, 28]. On the other hand, Ménoret et al. obtained the variant which showed an increased capacity for inducible HSP70 synthesis and demonstrated that inducible but not constitutive HSP70 was involved in immunogenicity of tumors [13]. We previously showed the augmentation of MHC class I antigen presentation via HSP70 expression by hyperthermia [6]. The tumor cell surface presentation of MHC class I antigen was increased in tandem with increased

HSP70 expression and the immunogenicity of the cells was enhanced by hyperthermia. These results suggest that the increased HSP70 may chaperon the tumor antigenic peptides and enable an increased number of them to transfer to MHC class I molecules in the endoplasmic reticulum. We therefore suppose that the inducible HSP70 by hyperthermia should serve as a vaccine.

Without hyperthermia, HSP70 in T-9 tumor was not strongly stained (Fig. 1b; C-I). Nakajima et al. reported that expression of HSP70 was frequent partial loss in tumor cells and the reduction of HSP70 expression was significantly correlated with poor prognosis in esophageal squamous cell carcinoma [19]. Generally, glioma tumor cells express very little MHC class I antigen. We previously showed that T-9 rat glioma cells displayed very little MHC class I antigen on their surface and were poorly immunogenic [6]. We propose a scenario of an antitumor immunity induced by hyperthermia: 1) a poorly immunogenic tumor cell with a low concentration of intracellular HSP; 2) stress response induced by hyperthermia results in raised levels of intracellular HSP; 3) dying tumor cells release their intracellular HSP; 4) these HSP activate the immune system.

Matzinger et al. proposed that the immune system has evolved to detect "danger" by employing professional antigen-presenting cells (APC) as sentinels of tissue distress, and in this hypothesis one of the danger signals is one or more HSP [4, 10]. HSP70 itself has also a role as a cytokine that activates polymorphonuclear cells to produce proinflammatory cytokines and recruit APC [2]. In our study, a large number of immune effector cells, such as macrophages or  $CD8^+$  T cells, were recruited to the tumor by hyperthermia, as described in Fig. 1c. Self antigens expressed by tumors do not stimulate T cells efficiently [25]. Therefore, one of the most effective ways to stimulate antitumor immunity is to promote the cross-priming of host professional APC [5]. Suto et al. reported the mechanism of the cross-priming

by exogenous antigens chaperoned by HSP, a pathway that is functional in a subset of macrophages [32]. We demonstrated the uptake of the HSP70 by macrophages, as shown in Fig. 1d; part C. Our hyperthermia system thus killed the tumor via release of HSP70-tumor antigen complexes at the tumor site, and the recruitment of immune effector cells, including APC, to the tumor subsequently occurred as a consequence of the inflammation. Based on these results, we propose that intracellular hyperthermia by means of MCL is an *in situ* vaccination therapy for cancer. This antitumor immune response has important implications for the development of novel antitumor therapies based on hyperthermia.

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