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ORIGINAL ARTICLE

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Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia

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Abstract Heat shock proteins are recognized as significant participants in immune reactions. In this study, we have demonstrated that the cell surface presentation of MHC class I antigen was increased in tandem with increased heat shock protein 70 (HSP70) expression and the immunogenicity of rat T-9 glioma cells was enhanced by hyperthermia. T-9 cells showed growth inhibition for 24 h after the heat treatment at 43 °C for 1 h in vitro, but then resumed a normal growth rate. HSP70 expression reached a maximum at 24 h after heating. Flow cytometric analysis revealed a significant increase in MHC class I antigen on the surface of the heated cells. The augmentation of MHC class I surface expression started 24 h after heating and reached a maximum 48 h after heating. The expression of other immunologic mediators, such as intracellular adhesion molecule-1 (ICAM-1) and MHC class II antigens, did not increase. In an in vivo experiment using immunocompetent syngeneic rats (F344), growth of the heated T-9 cells, with augmentation of MHC class I antigen surface expression, was significantly inhibited, while the cells grew progressively in nude rats (F344/N Jcl-*rnu*). Furthermore, compared with lymphocytes from non-immunized (PBS only injection) rats or rats injected with non-heated T-9 cells, the splenic lymphocytes of the rats in which the heated T-9 cells were injected displayed specific cytotoxicity against T-9 cells. These results suggest that HSP70 is an important modulator of tumor cell immunogenicity, and that hyperthermic treatment of tumor cells can induce the host antitumor immunity via the expression of HSP70. These results may benefit further efforts on developing novel cancer immunotherapies based on hyperthermia.

Keywords Hyperthermia · Heat shock protein · Tumor immunogenicity · MHC · glioma

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Introduction

Hyperthermia is one of the promising approaches in cancer therapy [10] and is often used in multi-modality therapy, including in combination with immunotherapy with interleukins [7, 29] or interferons [1]. Some researchers have reported that heat treatments themselves enhance the immunogenicity of cancer cells [14, 16, 20]. We have earlier reported on the antitumor immunity induced by hyperthermia of T-9 rat glioma cells in vivo [30]. This induced immunity continued for an extended period of time, and the rats treated with hyperthermia rejected completely a rechallenge of T-9 cells as a metastasis model. To elucidate how tumor antigens of T-9 glioma cells are recognized by the host immune system, we proposed that heat shock protein (HSP)-mediated augmentation of major histocompatibility complex (MHC) class I surface expression and antigen presentation causes antitumor immunity, because antitumor immunity is always associated with expression of several HSPs in the cells treated with hyperthermia.

Heat shock proteins are highly conserved proteins, whose synthesis is induced by a variety of stresses, including heat stress [21]. HSPs play a major role in

protecting cell proteins against heat denaturation. Recent results have shown the importance of HSPs in immune reactions [15]. Thus, it has been demonstrated that tumor-derived HSPs, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), can elicit cancer immunity [25, 26], and investigators have suggested that HSPs chaperon tumor antigens. On the basis of these studies, Srivastava et al. proposed the following relay line model for tumor antigenic peptide transfer during antigen processing and presentation by HSPs [22, 23, 24]. (1) The peptides are first bound to HSP70 (or HSP90), which are responsible for carrying them to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). (2) The peptides are transferred to gp96 in the lumen of the ER. (3) In the terminal step, gp96 transfers the peptides to the MHC class I/β2 microglobulin complexes. Wells et al. demonstrated that stably transfected B16 melanoma cells that constitutively expressed the human HSP70 exhibited significantly increased levels of MHC class I antigens on their surface. However, the expression of HSP70 did not improve the antigen presentation in cells lacking the activity of the TAP [28].

In the present study, we examined the role of a single defined HSP, the inducible HSP70, and show that the mechanism of antitumor immunity induced by hyperthermia is associated with HSP70 expression.

Materials and methods

Rats, cell lines and antibodies

Female F344 rats (Japan SLC, Shizuoka, Japan) and F344/N Jcl-*rnu* nude rats (Clea Japan, Tokyo, Japan) were used in experiments at 6 weeks of age. T-9 rat glioma cells and malignant fibrous histiocytoma (MFH) cells were cultured in minimum essential medium (MEM), supplemented with 10% fetal calf serum, 10 mM non-essential amino acids, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Two mouse monoclonal antibody preparations (mAbs) for HSP70/HSC70 were purchased: clone BRM-22 from Sigma Chemicals (St. Louis, Mo.) and clone N27F3-4 from StressGen Biotechnologies (Victoria, B.C., Canada), and used for an enzyme-linked immunosorbent assay (ELISA) and Western blotting analysis, respectively. These mAbs recognize both the inducible (HSP70) and constitutive (heat shock cognate protein, HSC70) types of HSP70 from human and rat. The MAb for HSP70 (clone C92F3A-5; StressGen Biotechnologies), recognizing the human and rat inducible HSP70, was used for a Western blotting analysis. The mAbs against rat MHC class I (clone OX-18), mouse and rat I-A MHC class II (clone OX-6), and rat CD54 (clone 4C9) recognize the rat MHC class I, mouse and rat MHC class II and intracellular adhesion molecule-1 (ICAM-1, CD54). These antibodies were used for flow cytometric analyses and were purchased from Immunotech (Marseille Cedex, France).

Construction of stable T-9 cell transfectants that constitutively express inducible HSP70 and evaluation of in vivo immunogenicity

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. T-9 cells (5×10^6 cells) were transfected by the lipofection method, using cationic liposomes including the plasmid, as previously reported [8]. Transfectants were cultured in MEM plus 0.5 mg/ml of hygromycin B (Wako Pure Chemical Industries, Osaka, Japan) and the hygromycin-resistant T-9 clones were obtained.

The T-9 cell transfectants (1×10^6 cells) were injected subcutaneously into the left femoral region of F344 rats. The tumor size in the animals was examined every 3 days until the tumors became palpable. Tumors that were established from the primary inoculation were excised when the size was approximately 1.0×1.0 cm. The rats were then rechallenged with a subcutaneous (s.c.) injection of parental T-9 cells (1×10^6 cells) on the opposite side of the femoral region. Rats that had rejected the initial inoculation of tumor cells were also rechallenged at the same time.

Flow cytometric analysis and DSG treatment

The apoptosis assay was performed using the Vybrant apoptosis assay kit #4 (Molecular probes, Eugene, Ore.). Heated cells or non-heated cells were harvested at 6 h, 24 h, 48 h and 72 h after heating and were washed in cold PBS, after which the cell density was adjusted at 1×10^6 cells/ml in PBS. Cells were incubated with 1 μl of the green fluorescent dye YO-PRO-1, which can enter apoptotic cells, and 1 μl of propidium iodide (PI). As soon as possible after the incubation period, the stained cells were analyzed by flow cytometry (Paltec, Münster, Germany).

For flow cytometric analysis of surface proteins, adherent T-9 cell monolayers were harvested in PBS containing 1 mM EDTA, washed in complete MEM, and then incubated with the appropriate mAb (10 μl or 20 μl mAb/10^6 cells) for 30 min at 4 °C. Cells were washed twice with cold PBS containing 2% fetal calf serum and incubated with 1 μl of FITC-conjugated goat anti-mouse IgG antibody (Immunotech, Marseille Cedex, France) for 30 min at 4 °C.

Deoxyspergualin (DSG) was kindly supplied by Nippon Kayaku Co. (Tokyo, Japan). It was dissolved in complete MEM, with 10% horse serum substituted for 10% FBS, to a final concentration of 20 μg/ml. Cells were routinely replenished with fresh medium containing DSG every 24 h.

Hyperthermic treatment of cultured cells and evaluation of viable cells

Hyperthermic treatment of cultured cells was performed as previously reported [8]. Briefly, T-9 cells in the logarithmic growth phase were heated at 43 °C for 1 h by direct immersion of cell culture dishes in a temperature-controlled water bath. The temperature of the medium increased quickly and reached 43 °C within 5 min. The temperature in the medium was monitored with a fiber optic thermometer probe (FX-9020; Anritsu Meter Co., Tokyo, Japan). The number of viable cell was evaluated by the trypan blue dye-exclusion method using a hemocytometer.

Assay for HSP70

To determine the concentration of both inducible and constitutive HSP70 (HSP70/HSC70) in cells, we performed an ELISA [9]. Briefly, heated cells or non-heated cells were lysed by freezing and thawing in 50 mM sodium carbonate buffer (pH 9.6) and centrifuged at 10,000 rpm for 10 min. The supernatants, or highly purified HSP70 protein (from bovine brain; Sigma Chemicals, St. Luis, Mo.) as a standard, were added to a 96-well microplate and incubated overnight at 4 °C. Each well was washed with phosphate-buffered saline (PBS) three times and then incubated with blocking solution (PBS/1%BSA) for 1 h at room temperature. Wells were washed with PBS and 50 μl of the mAb against HSP70 (1,000-fold dilution in PBS/1% BSA) was added and incubated for

1 h at room temperature. Wells were again washed with PBS, three times, and 50 µl of POD-conjugated anti-mouse IgG (1,000-fold dilution in PBS/1%BSA) was added and incubated for 1 h at room temperature. Wells were washed with PBS/0.01%Triton and 50 µl of substrate was added. After incubation for a few minutes at room temperature, the reaction was stopped with 50 µl of 1 N sulfuric acid, and the optical density at 492 nm was measured.

We also investigated the HSP70/HSC70 and HSP70 expression by Western blotting analysis [31]. SDS samples of T-9 cells (1×10^6) 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. The protein blots, transferred onto a nitrocellulose membrane, were incubated with the first antibodies (as indicated) for 2 h at 37 °C, subsequently probed with peroxidase-labeled goat anti-mouse IgG for 1 h at 37 °C, and then visualized using an ECL™ (Amersham Pharmacia Biotech, Buckinghamshire, UK).

In vivo immunogenicity assay and in vitro cytotoxicity assay on heated T-9 cells

The heated or the non-heated T-9 cells (1×10^7) were injected subcutaneously into the left femoral region of F344 rats or F344/ N Jcl-*rnu* nude rats 24 h after the heating. Tumor sizes were measured every 3 days. The tumor volume was determined by the following formula [32]:

$$\text{Tumor volume} = 0.5 \times (\text{length} \times \text{width}^2)$$

where length and width are in millimeters.

In vitro cytotoxicity assay was undertaken 12 days after the transplantation of the heated or the non-heated T-9 cells. Naive rats that had been born at almost the same time as the tested rats were used as a control. Splenocytes were harvested from the rats, and the parental T-9 cells or cells of another rat tumor cell line, MFH, were used as target cells. The cytotoxicity was determined by a long-term cytotoxicity assay [2]. Briefly, tumor cells (1×10^5 cells) were cultured in a 24-well flat plate in 1 ml of MEM. After 24 h, the medium was replaced with RPMI-1640 medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum, 2 mM fresh L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The spleen cells were added into each well. The wells were washed with phosphate buffer after 48 h and fixed with 10% formaldehyde solution for 20 min. The wells were then washed with water, and 0.2% crystal violet solution was added into each well for 5 min. The washing with water was repeated until the blank wells became clear. Then plates were dried at room temperature overnight and the remaining dye was dissolved in 70% ethanol solution. The absorbance of each well was measured at 570 nm in a spectrophotometer (V-530, JASCO, Tokyo), and cytotoxicity was calculated by the following equation:

$$\text{Cytotoxicity}\,(\%) = [-(\text{absorbance of tumor cells treated with effector} \times \text{cells})/(\text{absorbance of control})] \times 100$$

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

Enhanced immunogenicity by HSP70 overexpression

We constructed a stable transfectant of T-9 (T-9/pCMVhygro.HSP70) cells that constitutively express the inducible HSP70 and investigated whether the immunogenicity of T-9 cells was enhanced by HSP70 overexpression. The T-9/pCMVhygro.HSP70 cells expressed stably about 100 pg/cell of HSP70/HSC70, whereas the T-9 cell clone transfected with the control vector (T-9/pCMVhygro) expressed about 22.5 pg/cell of HSP70/HSC70. Flow cytometric analysis using the anti-MHC class I antibody revealed a significant increase of MHC class I antigen on the surface of the HSP70-overexpressing cells (Fig. 1A). In contrast, other immunologic mediators, such as ICAM-1 (Fig. 1A) and MHC class II (data not shown), did not increase.

DSG is an immunosuppressive agent that binds specifically to HSP70 [18, 19]. The drug can enter cells, where it interacts with endogenous HSP70 and blocks the trafficking of the peptides destined for loading the cell surface MHC class I molecules. MHC class I/β2 microglobulin complexes devoid of peptides are unstable on the cell surface and the number of MHC class I molecules decreases [11]. Since the treatment of cells with DSG results in it binding to HSP70 and abrogates the ability of cells to present antigenic peptides through MHC class I molecule, DSG has been used as a reagent to probe the involvement of HSP70 in MHC class I antigen processing [5]. In the present paper, we used DSG to examine whether the augmentation of MHC class I antigen on the surface of cells overexpressing

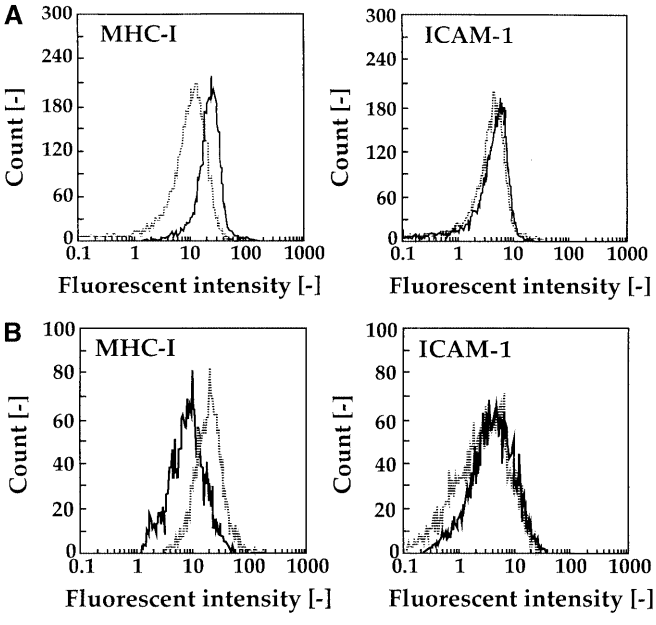

Fig. 1A, B Flow cytometric analysis of MHC class I surface antigen on T-9 cells stably transfected with pCMVhygro-hsp70 or pCMVhygro. Anti-MHC class I or anti-ICAM-1 mAb was used in the analysis. **A** The MHC class I antigen expression on the two transfectants was compared. *Dotted line* T9/pCMVhygro cells. The mean fluorescence intensities (MFI) for peaks of MHC-I and ICAM-1 were 11.7 and 5.0, respectively. *Solid line* T9/pCMVhygro-hsp70 cells. The MFI for peaks of MHC-I and ICAM-1 were 21.5 and 6.0, respectively. **B** T-9/pCMVhygro.HSP70 cells were incubated with or without DSG before reaction with antibodies. *Dotted line* without DSG. The MFI for peaks of MHC-I and ICAM-1 were 20.0 and 6.0, respectively. *Solid line* with 20 µg of DSG. The MFI for peaks of MHC-I and ICAM-1 were 10.0 and 4.8, respectively

HSP70 was based on trafficking of peptides to MHC class I molecules via HSP70. Although HSP70 transfection augmented the surface expression of MHC class I antigen (Fig. 1A), DSG treatment of the T-9/pCMVhygro.HSP70 clone led to a twofold reduction in the number of MHC class I-peptide complexes on the cell (Fig. 1B). ICAM-1 surface expression was also monitored and found to be unaffected by DSG treatment.

The tumorigenicity of 1×10^6 T-9/pCMVhygro.HSP70 cells was monitored following injection subcutaneously into F344 rats and compared with the tumorigenicity of unmodified T-9 cells and T-9/pCMVhygro cells. Five rats were prepared in each of three groups. T-9 and T-9/pCMVhygro tumors grew in all animals 3 days after the injection, as shown in Fig. 2A. The T-9/pCMVhygro.HSP70 tumors grew initially in all animals after 9 days (Fig. 2A), but they subsequently regressed, and 60% of the animals were free of tumor at 21 days. Animals in which primary tumors developed underwent surgery to remove the primary tumors, and all animals were subsequently rechallenged with the parent T-9 cells. Sixty percent of animals in which the primary tumor had expressed the *hsp70* gene (T-9/pCMVhygro.HSP70 cells) resisted the rechallenge with the parental cells, whereas almost all animals in other groups could not reject the tumor (Fig. 2B). These results suggest that the immunogenicity of the T-9 cells was enhanced by HSP70 expression.

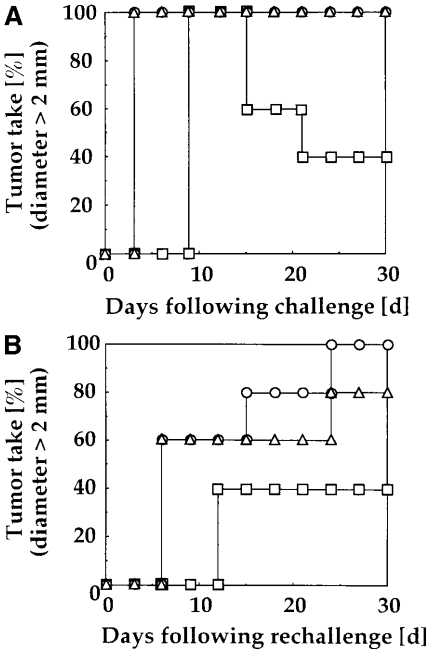

Fig. 2A, B Enhanced immunogenicity by HSP70 overexpression. **A** Effect of HSP70 expression on primary tumorigenicity of T-9 cells (○ T-9, Δ T-9/pCMVhygro, □ T-9/pCMVhygro.HSP70). **B** Effect of HSP70 expression on immunogenicity of T-9 cells. Rats, which had been exposed to an inoculum of T-9 (○), T-9/pCMVhygro (Δ), or T-9/pCMVhygro.HSP70 (□) cells, had their primary tumors surgically removed when they were palpable and all groups were rechallenged with the parent T-9 cells. Each group included five rats

Heat shock damage and HSP70 expression in T-9 rat glioma cells

We next examined whether tumor immunogenicity was enhanced by hyperthermia. In order to ensure that potential apoptotic or necrotic cell death would not interfere with immunogenicity studies, we first assessed the

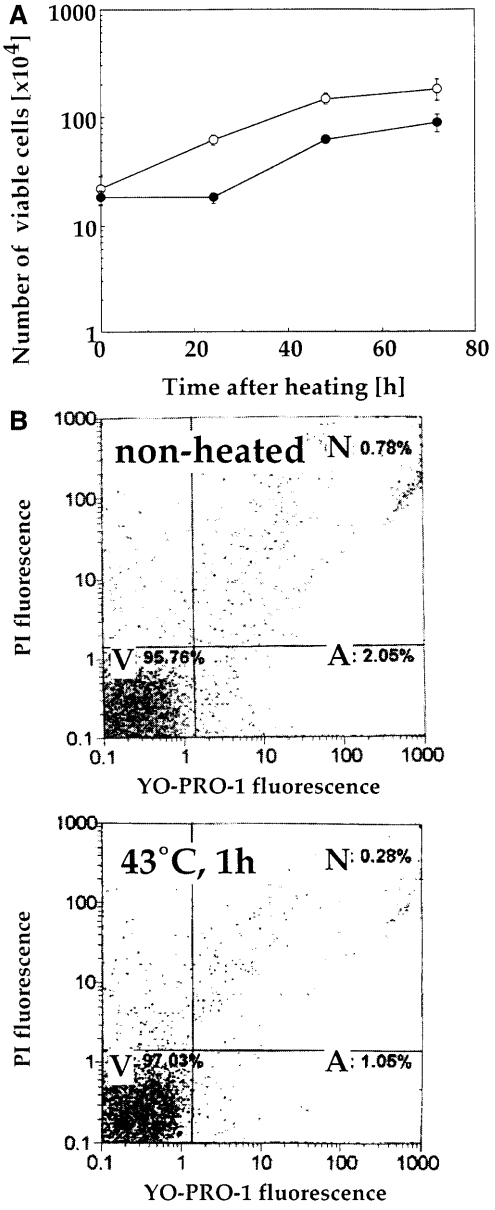

Fig. 3A, B Heat shock effect on rat glioma cells after 43 °C for 1 h. **A** Number of viable cells after heating (○ non-heated, ● 43 °C for 1 h). Data are the means ± SD of 3 experiments. **B** Apoptotic detection assay by flow cytometry of heated and non-heated T-9 cells at 6 h after heating. *V* viable cells, *N* necrotic cells, *A* apoptotic cells

heat shock damage in T-9 rat glioma cells in vitro at various temperatures. To assess the damage after heating for 1 h at 43 °C, the growth inhibition of T-9 cells was observed for 24 h after the heat treatment (Fig. 3). After the 24-hour lag period, the growth rate recovered, and cells proliferated until 72 h after heating, as shown in Fig. 3A. Few apoptotic or necrotic cells were detected in the heated cells (Fig. 3B), so we concluded that these heating conditions had only a cytostatic effect on T-9 cells. Therefore, we chose to analyze the immunogenicity of T-9 cells after a hyperthermic treatment of 43 °C for 1 h.

Figure 4 shows HSP70 expression in T-9 cells. Monoclonal antibody for HSP70/HSC70, which recognized both the inducible (HSP70) and constitutive (HSC70) types of HSP70, was used in the experiment. For non-heated cells, about 20 pg/cell of constitutive HSP70/HSC70 concentration was observed throughout this experiment. When the cells were heated at 43 °C for 1 h, the concentration of HSP70/HSC70 increased about 4-fold compared with the concentration on non-heated cells. After that, the concentration declined to that of non-heated cells. Western blot analysis revealed that the elevated concentration of HSP70 that was produced by hyperthermic treatment was of the inducible type, as shown in Fig. 4B.

MHC class I expression on the surface of heated cell

Flow cytometric analysis using the anti-MHC class I antibody revealed a significant increase of MHC class I antigen on the surface of the heated cells (Fig. 5A). The augmentation of MHC class I surface expression started from 24 h after heating and reached a maximum (two-fold increase) at 48 h after heating and then decreased to the expression level of non-heated cells at 72 h. In contrast, other immunologic mediators, such as ICAM-1 (Fig. 5A) and MHC class II (data not shown), did not increase. It should be noted that the transient increase of MHC class I surface expression started at the time (24 h after heating) when the HSP70 expression reached the maximum (Fig. 4A). Subsequently, as the cell growth recovered, the MHC class I surface expression decreased in tandem with the decreasing HSP70 expression (Figs. 4A and 5A).

Treatment of the heated cells with DSG led to a 50% reduction in the number of MHC class I-peptide complexes on the cells (Fig. 5B), contrasting with the augmentation of MHC class I surface expression observed after hyperthermic treatment without DSG (Fig. 5A). ICAM-1 surface expression was unaffected by DSG treatment.

These results suggest that the transient augmentation of MHC class I surface expression by hyperthermia was caused by the peptide-trafficking role of the heat-induced HSP70.

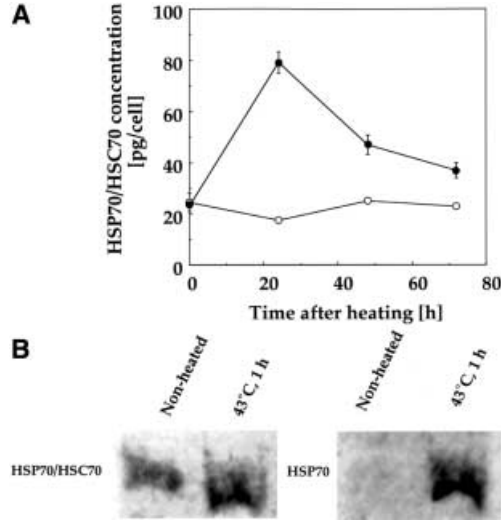

Fig. 4A, B Enhanced expression of HSP70 in T-9 cells after heating at 43 °C for 1 h. **A** Heated or non-heated cells were harvested at the indicated times and assayed for HSP70 by ELISA (○ non-heated, ● 43 °C for 1 h). Data are the means ± SD of 3 experiments. **B** Western blotting analysis of cells at 24 h after hyperthermia treatment. *Left* mAb for HSP70/HSC70, recognizing both constitutive and inducible types of HSP70; *right* mAb for HSP70, recognizing only inducible HSP70

Enhanced immunogenicity of heated tumor cells in vivo

We investigated whether the hyperthermic treatment could enhance the immunogenicity. The heated or non-heated T-9 cells were transplanted along the right flank of immunocompetent F344 rats or nude rats (F344/N Jcl-*rnu*) at 24 h after heating (Fig. 6A). As mentioned above, the heated cells restarted to proliferate (Fig. 3A) 24 h after heating, which was also at the maximal level of HSP70 expression in vitro (Fig. 4A) and at the beginning of the augmentation of the MHC class I surface expression (Fig. 5A). The heated T-9 cells were tumorigenic, but a delay in tumor growth was observed in vivo compared with the tumor growth of the non-heated cells (Fig. 6A). The tumor size of the heated cells at 30 days after transplantation into F344 rats was 2.7 ± 1.2 cm^3, while that of the non-heated cells was 8.9 ± 2.4 cm^3. In contrast to the kinetics of their growth in immunocompetent F344 rats, both heated and non-heated tumors grew progressively in athymic rats, which are devoid of T lymphocytes. This result indicates that the T lymphocytes played an important role in the immunogenicity of heated cells. For further investigation of this immunogenicity, the in vitro cytotoxicity of the host effector cells against tumor cells was assessed. The heated or the non-heated T-9 cells were injected into F344 rats at 24 h after heating and splenic lymphocytes of the hosts were prepared 12 days after transplantation and used as effector cells in cytotoxicity assays against T-9 cells or MFH (Fig. 6B). Splenic lymphocytes of rats immunized with heated T-9 cells displayed enhanced

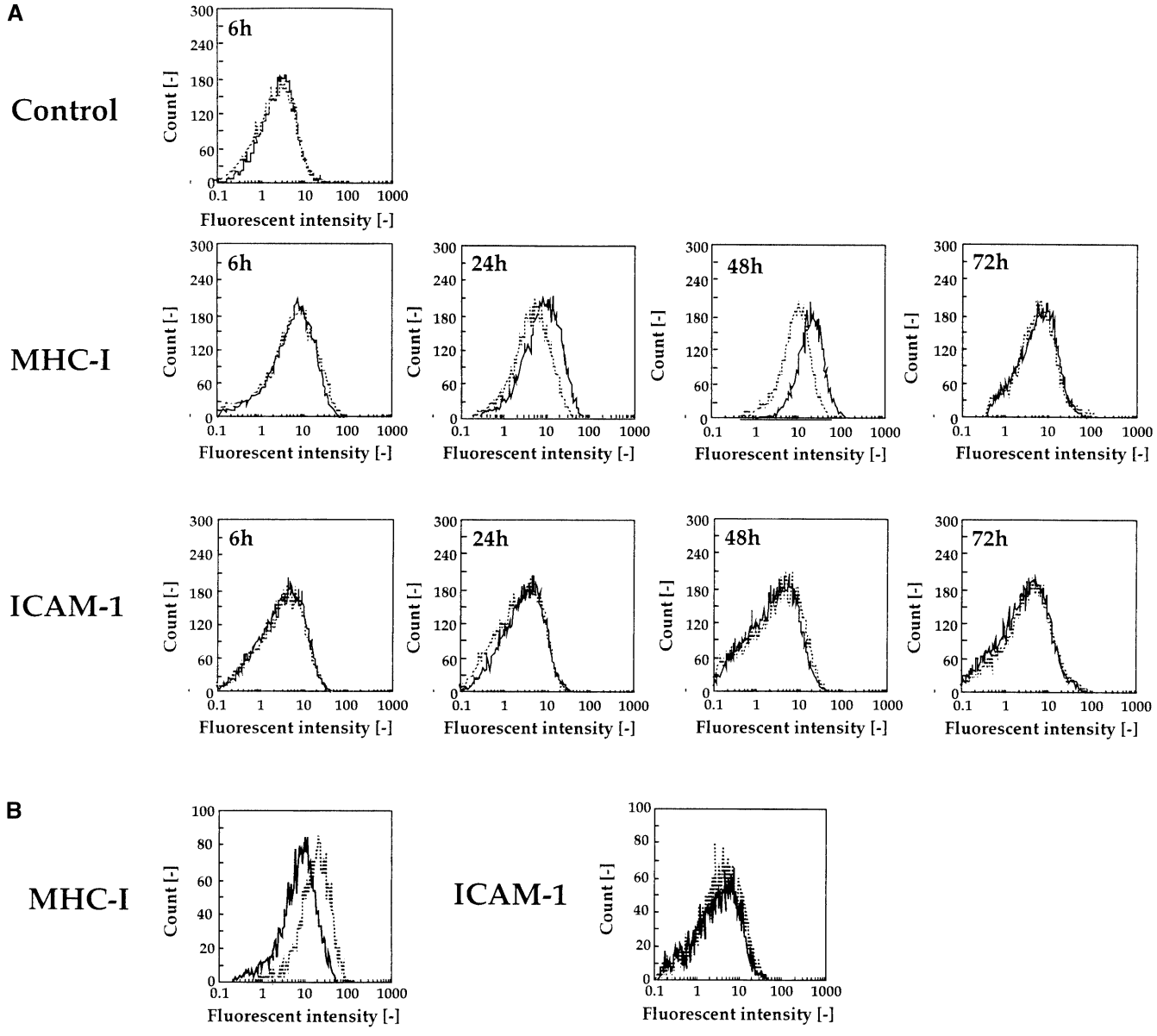

Fig. 5A, B Augmentation of MHC class I surface antigen on heated cells. **A** Heated or non-heated cells were harvested at 6, 24, 48 and 72 h after heating, and flow cytometric analyses were carried out using isotype control antibody (*Control*), anti-MHC class I (*MHC-I*), or anti-ICAM-1 antibody (*ICAM-1*). *Dotted line* non-heated; *solid line* 43 °C, 1 h. **B** Heated cells were treated with DSG and harvested at 48 h. Flow cytometric analyses of MHC-I and ICAM-1 on the heated cells were carried out. *Dotted line* cells incubated without DSG; *solid line* cells incubated with 20 μg of DSG

cytotoxic effect against T-9 cells compared with lymphocytes from non-immunized (PBS only injection) rats or rats immunized with non-heated T-9 cells. In contrast, no cytotoxicity was observed against MFH cells by lymphocytes from rats immunized with heated T-9 cells. This finding therefore suggests that the immunity induced by injection of heat-treated T-9 cells was specific for these cells.

These results thus show that the inducible form of HSP70 enhances the MHC class I surface expression and significantly enhances the immunogenicity of T-9 cells, indicating that HSP70 is responsible for the enhanced immunogenicity of the tumor treated by hyperthermia.

Discussion

Recently, cancer immunologists have suggested that the mechanism of tumor cell death in vivo may be directly related to induction of the host immune response and that heat shock protein expression may provide such an immunological induction signal [6, 12, 13]. We applied this idea to hyperthermia and have, for the first time, as far as we know, demonstrated the association of hyperthermia with increased antitumor

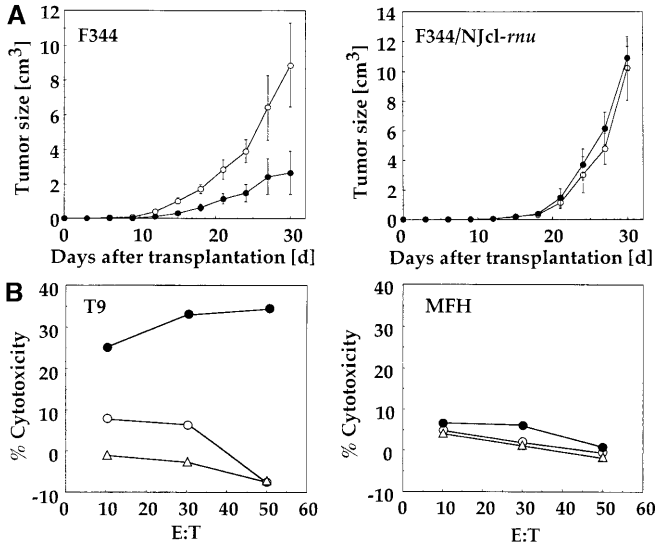

Fig. 6A, B Enhanced tumor cell immunogenicity after hyperthermic treatment. **A** Tumor size in rats injected with heated-T-9 cells. Heated cells (43 °C, 1 h) or non-heated cells were injected into F344 rats (*left*) or F344 nude rats (*right*) at 24 h after heating (○ non-heated, ● 43 °C, 1 h). Data are the means ± SD from five rats per group. **B** In vitro cytotoxicity of splenic lymphocytes from F344 rats injected with heated T-9 cells. Heated T-9 cells (43 °C, 1 h ●), non-heated cells (○), or PBS (Δ) were injected into F344 rats at 24 h after heating and splenic lymphocytes of the rats were prepared at 12 days after the injection. The cytotoxicity against parental T-9 cells (*left*) or another rat tumor, MFH (*right*), was assayed (*E:T* effector to target cell ratio)

immunity via HSP70 expression. Because expression of the HSP70 protects cells from heat-induced apoptosis [17], HSP70 expression has been considered a complicating factor in hyperthermia. In our studies, T-9 rat glioma cells, which expressed induced-HSP70 after heating at 43 °C, did not die by apoptosis, but resumed proliferation after a lag period (Fig. 3B). At the same time, an immune response to the heated cells and a tumor growth delay were observed in the in vivo experiments (Fig. 6). These results suggest that HSP70 expression in hyperthermia have an important role in tumor immunity.

Generally, glioma tumor cells express very little MHC class I antigen. The flow cytometric analysis revealed that T-9 rat glioma cells displayed less MHC class I antigen on their surface than did the negative control (Fig. 5) and were poorly immunogenic, as demonstrated by the experiment using the splenic lymphocytes as effector cells (Fig. 6B). MHC class I antigen expression on T-9 cells was enhanced by a stable *hsp70* gene expression (Fig. 1A), but the antigen augmentation was reduced by DSG treatment (Fig. 1B). Our results correspond to the observation of Binder et al., who showed that HSP70 is necessary for transport of antigenic peptides in the cytosol and that DSG interferes with this step [5]. 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 ER. Heated T-9 cells showed the augmentation of MHC class I surface antigen to the same degree as the stable HSP70 transfectants (Fig. 5A). As in the stable transfectants, the heat-induced augmentation of MHC class I antigen on T-9 cells was also reduced by DSG treatment (Fig. 5B). Since DSG functionally blocks a trafficking role of HSP70, we concluded that this augmentation was caused by hyperthermia through the heat-induced HSP70 expression.

We further found that hyperthermic treatment of the usually poorly immunogenic T-9 cells can make them immunogenic through heat-induced HSP70 expression and provoke antitumor immunity in transplanted, immunocompetent rats. We assume that this antitumor immunity was due to influencing the capacity of a tumor cell to process and present endogenous antigens directly to T cells, but the mechanism in vivo is still unclear. Wells and Malkovsky proposed some possible mechanisms by which HSPs might influence tumor cell immunogenicity [27]. One of the mechanisms is that HSP70-peptide complexes released from dying tumor cells transfer antigenic peptides to professional antigen presenting cells (APC) and activate them [4]. HSP70 itself has also a role as cytokine that activates polymorphonuclear cells to produce proinflammatory cytokines and recruit APC [3]. According to our preliminary results, a great number of immune effector cells, such as $CD8^+$ T cells and $CD4^+$ T cells, infiltrated to tumor site after hyperthermia, and we speculate that the proposed mechanisms also occur subsequent to the tumor cell death by direct recognition of T cells in the present case.

In the present paper, we have investigated the presentation of MHC class I antigen associated with hyperthermic induction of HSP70 expression. With regard to clinical use of hyperthermia, we suggest that HSP70 expression can be an important modulator of tumor immunogenicity and that this antitumor immune response may have important implications for the development of novel antitumor therapies.

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References

1. Anjum A, Fleischmann WR Jr (1992) Effect of hyperthermia on the antitumor actions of interferons. J Biol Regul Homeost Agents 6: 75
2. Aruga A, Aruga E, Chang AE (1997) Reduced efficacy of allogeneic versus syngeneic fibroblasts modified to secrete cytokines as a tumor vaccine adjuvant. Cancer Res 57: 3230
3. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK (2000) HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperon and cytokine. Nat Med 6: 435

4. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol 12: 1539
5. Binder RJ, Blachere NE, Srivastava PK (2001) Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. J Biol Chem 276: 17163
6. Fuchs EJ, Matzinger P (1996) Is cancer dangerous to the immune system? Semin Immunol 8: 271
7. Geehan DM, Fabian DF, Lefor AT (1995) Combined local hyperthermia and immunotherapy treatment of an experimental subcutaneous murine melanoma. J Surg Oncol 59: 35
8. Ito A, Shinkai M, Bouhon IA, Honda H, Kobayashi T (2000) Induction of *TNF-α* gene expression by heat inducible promoter *gadd* 153. Jpn J Hyperthermic Oncol 16: 91
9. Kikuchi A, Itoh YH, Seki K, Morita M, Kobayashi M, Aihata M, Oguri T (1996) A new quantitative assay of heat shock protein 70 based on an enzyme-linked immunosorbent assay method. Rinsho Byori 44: 351
10. Kobayashi T (1993) Hyperthermia for brain tumors. Jpn J Hyperthermic Oncol 9: 245
11. Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hoglund P, Heemels MT, Bastin J, Schumacher TN, Townsend A, Karre K, et al. (1990) Empty MHC class I molecules come out in the cold. Nature 346: 476.
12. Matzinger P (1994) Tolerance, danger, and the extended family. Annu Rev. Immunol 12: 991
13. Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile G (1998) Tumor immunogenicity is determined by mechanism of cell death via induction of heat shock protein expression. Nat Med 4: 581
14. Mise K, Kan N, Okino T, Nakanishi M, Satoh K, Teranuma Y, Yamasaki S, Ohgaki K, Tobe T (1990) Effect of heat treatment on tumor cells and antitumor effector cells. Cancer Res 50: 6199
15. Ménoret A, Chandawarkar R (1998) Heat-shock protein-based anticancer immunotherapy: an idea whose time has come. Semin Immunol 25: 654
16. Mondovi B, Santoro AS, Strom R, Faiola R, Fanelli AR (1972) Increased immunogenicity of Ehrlich ascites cells after heat treatment. Cancer 30: 885
17. Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B (2000) The chaperon function of hsp 70 is required for protection against stress-induced apoptosis. Mol Cell Biol 20: 7146
18. Nadeau K, Nadler SG, Saulnier M, Tepper MA, Walsh CT (1994) Quantitation of the interaction of the immunosuppressant deoxyspergualin and analogs with Hsc70 and Hsp90. Biochemistry 33: 2561
19. Nadler SG, Tepper MA, Schacter B, Mazzucco CE (1992) Interaction of the immunosuppressant deoxyspergualin with a member of the hsp70 family of heat shock proteins. Science 258: 484
20. Ozdemirli M, Akdeniz H, el-Khatib M, Ju ST (1991) A novel cytotoxicity of $CD4^+$ Th1 clones on heat-shocked tumor targets. I. Implications for internal disintegration model for target death and hyperthermia treatment of cancers. J Immunol 147: 4027
21. Schlesinger MJ (1990) Heat shock proteins. J Biol Chem 265: 12111
22. Srivastava PK, Maki RG (1991) Stress-induced proteins in immune response to cancer. Curr Top Microbiol Immunol 167: 109
23. Srivastava PK, Heike M (1993) Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? Semin Immunol 3: 57
24. Srivastava PK, Udono H, Blachere NE, Li Z (1994) Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics 39: 93
25. Tamura Y, Peng P, Liu K, Daou M, Srivastava PK (1997) Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. Science 278: 117
26. Udono H, Srivastava PK (1993) Heat shock protein 70-associated peptides elicit specific cancer immunity. J Exp Med 178: 1391
27. Wells AD, Malkovsky M (2000) Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view. Immunol Today 21: 129
28. Wells AD, Rai SK, Salvato MS, Band H, Malkovsky M (1998) Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. Int Immunol 10: 609
29. Wu B, Shen RN, Wang WX, Broxmeyer HE, Lu L (1993) Antitumor effect of interleukin 7 in combination with local hyperthermia in mice bearing B16a melanoma cells. Stem Cells 11: 412
30. Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T (1998) Antitumor immunity induction by intracellular hyperthermia using magnetite cationic liposomes. Jpn J Cancer Res 89: 775
31. Yokoyama N, Hirata M, Ohtsuka K, Nishiyama Y, Fujii K, Fujita M, Kuzushima K, Kiyono T, Tsurumi T (2000) Co-expression of human chaperone Hsp70 and Hsdj or Hsp40 co-factor increases solubility of overexpressed target proteins in insect cells. Biochim Biophys Acta 1493: 119
32. Yoshida J, Mizuno M, Yagi K (1991) Secretion of human *β*-interferon into the cystic fluid of glioma transfected with the interferon gene. J Clin Biochem Nutr 11: 123