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ORIGINAL ARTICLE

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Hsp110 over-expression increases the immunogenicity of the murine CT26 colon tumor

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Abstract Several studies have suggested a positive correlation between heat shock protein (hsp) expression and tumor immunogenicity. Independently, many studies have shown that hsp purified from tumors can be used as a tumor-specific vaccine. In this study, we have explored the connection between hsp expression and anti-tumor immunity by transducing murine CT26 colon carcinoma cells with the cDNA of a major hsp, i.e. hsp110. We have shown that over-expression of hsp110 has no effect on CT26 tumor cell growth in vitro, and does not inhibit their anchorage-independent growth capacity. However, in situ, hsp110 over-expressing CT26 tumor (CT26-hsp110) grew at a significantly reduced rate as compared to the wild-type CT26 tumor in immunocompetent mice. Moreover, immunization of mice with inactivated CT26-hsp110 cells significantly inhibited the growth of wild-type CT26 tumor. This immunity was associated with an increased frequency of tumor-specific T cells after vaccination. An in vivo antibody depletion assay demonstrated that inactivated CT26-hsp110 cells elicited anti-tumor responses involving $CD8^+$ T cells and natural killer (NK) cells, but not $CD4^+$ T cells. Lastly, the effect of the addition of granulocyte–macrophage colony stimulating factor (GM–CSF) to these vaccine formulations was determined. Mice immunized with irradiated CT26-hsp110 cells combined with GM–CSF-producing bystander cells revealed a complete inhibition of CT26 tumor growth, indicating a synergy between inactivated CT26-hsp110 vaccine activity and GM–CSF. These observations demonstrate that manipulation of hsp110 expression in tumors, specifically when combined with GM–CSF, represents a potentially powerful approach to cancer vaccine formulation.

Keywords Gene therapy · GM–CSF · Heat shock protein · Tumor Immunogenicity · Vaccine

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Introduction

Heat shock proteins (hsp) have been demonstrated to be essential in many cellular housekeeping functions such as protein synthesis, folding and transport across intracellular membranes as well as protein degradation [25, 60]. It has long been recognized that the major hsp of mammalian origin include the hsp25, hsp60, hsp70, hsp90 and hsp110 families. While the hsp25, 60, 70 and 90 families have been studied for many years, the hsp110 family has been almost entirely ignored [10, 22].

In addition to their numerous functions within the cell, hsp have also been recognized in recent years to have important extracellular functions and activities in mediating the host's immune response. Many reports have now shown that tumor-derived hsp–peptide complexes can serve as effective vaccines to generate anti-tumor immunity [4, 50, 54, 55, 58]. The molecular basis for this immunogenicity is the capacity of hsp to function as molecular chaperones in binding denatured peptide chains including antigenic peptides [20, 36, 53, 61]. These hsp–peptide complexes can be internalized by antigen-presenting cells (APC) through receptor-mediated endocytosis, and are eventually introduced to endogenous antigen-processing pathways [2, 6, 9]. In addition, several studies have indicated that exogenous hsp trigger immunomodulatory signals and stimulate macrophages

and dendritic cells (DC) to secret proinflammatory cytokines [3, 5, 7, 33]. Lastly, various reports have demonstrated a connection between hsp expression and tumor immunogenicity [31, 35, 29, 59, 65].

Although most tumor cells are not sufficiently immunogenic to trigger an effective immune response in vivo, various approaches have been developed to manipulate the immune system to attack and eliminate tumor. It has been clearly demonstrated that a variety of genes transfected into the autologous cells will enhance an anti-tumor immune response. Those genes that are able to target the host's immune system against a tumor include genes for many cytokines, growth factors, co-stimulatory molecules such as CD80, CD86 as well as major histocompatibility complex (MHC) class I and II. Consequently, a number of clinical protocols have been developed to treat cancer patients with genetically modified cancer cell vaccines utilizing one or more of these genes.

In the present study, we established a murine CT26 tumor model which over-expresses hsp110 and studied the effects of this over-expression on tumor immunogenicity. We also examined the immunological mechanisms of the anti-tumor activity obtained by this novel hsp and evaluated the potential use of hsp110 over-expressing tumor cells as whole cell cancer vaccines.

Materials and methods

Mice and cell lines

Six to 8-week-old female BALB/c mice purchased from Taconic (Germantown, N.Y.) were housed under pathogen-free conditions. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of Roswell Park Cancer Institute. CT26 is a carcinogen-induced syngeneic BALB/c colon adenocarcinoma [12]. B78H1 is a C57BL/6-derived B16 tumor variant that has lost MHC class I expression. B78H1-GM–CSF is a B78H1 cell line transfected with the granulocyte–macrophage colony stimulating factor (GM–CSF) gene [23]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, N.Y.), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Antibodies

Rabbit anti-asialo GM1 antibody [17] was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-L3T4 hybridoma (GK1.5 cells) [63] and anti-Lyt 2.2 hybridoma (2.43 cells) [42] were purchased from the American Type Culture Collection (Rockville, Md.). The hybridomas were inoculated i. p. into SCID mice, and monoclonal antibodies (mAb) were purified from ascites using standard procedures. Rabbit polyclonal hsp110 antibody was generated in this laboratory. Mouse monoclonal hsc70/hsp70 antibody (SPA-820), which recognizes both hsc70 and hsp70, was purchased from StressGen Biotechnologies (Victoria, B.C., Canada).

Generation of stably transduced hsp110 over-expressing cell

The full-length cDNA (2.5 kb) encoding murine hsp110 was inserted into a mammalian expression vector pCI-neo (5.4 kb) purchased from Promega (Madison, Wis.). The inserted cDNA is under the transcriptional control of the enhancer/promoter sequence from the human cytomegalovirus (HCMV) immediate-early gene. CT26 cells were transfected with this plasmid by Lipofectin reagent (Life Technologies, Grand Island, N.Y.), and selected with 1 mg/ml Geneticin (Life Technologies). By limiting dilution, a clone that expressed hsp110 at a high level was isolated. As a control, the CT26 clone transfected with the empty vector was prepared in the same way.

Western blot analysis

Equivalent protein samples were subjected to 7.5% to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto Immobilon-P membrane (Millipore, UK) [57]. Membranes were blocked with 5% non-fat milk in TBST (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, and then incubated for 1 h with primary antibodies diluted at 1:1,000 in TBST. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG diluted at 1:2,000 in TBST at room temperature for 1 h. The protein was visualized using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham, Arlington Heights, Ill.).

Tumor growth assay

Mice were inoculated s.c. with 5×10^4 CT26 cells, empty vector or hsp110 cDNA transduced CT26 tumor cells in the right flank. Tumor development was followed every other day. Tumor size was determined by measurements of the shortest diameter (A) and the longest diameter (B) with a caliper every 2 days. The volume was calculated using the formula $V=(A^2B)/2$. For prophylactic assay using irradiated tumor cells, mice were immunized s.c. with 2×10^5 irradiated wild-type or hsp110-transfected CT26 tumor cells in the left flank, and challenged with 2×10^5 live parental tumor cells in the right flank 2 weeks later.

Chromium ^{51}Cr release assay

Splenocytes were harvested 2 weeks following immunization and stimulated in vitro with irradiated CT26 cells for 5 days. Then splenocytes were serially diluted in 96 V-bottomed well plates (Costar, Cambridge, Mass.) in triplicate at varying E:T ratios. ^{51}Cr-labeled tumor cells (1×10^4) were added to a final volume of 200 μl/well. Wells containing target cells only with either culture medium or 0.5% Triton X-100 served as spontaneous or maximal release controls respectively. After 4 h incubation at 37°C, 150 μl of supernatant was analyzed for radioactivity in a gamma counter (Packard, Downers Grove, Ill.) and the percentage of specific lysis was calculated by the formula: percentage specific lysis $=100\times$(experimental release–spontaneous release)/(maximum release–spontaneous release). In some experiments, the re-stimulated effector cell populations were incubated with the anti-CD8 antibodies (20 μg/ml) for 30 min at 4°C to block $CD8^+$ T cells before cytotoxicity assays [63].

ELISpot analysis

The ELISpot assay was used to determine tumor-specific interferon-gamma (IFN-γ) secreting T cells [32]. The 96-well filtration plates (Millipore, Bedford, Mass.) were coated with 10 μg/ml rat anti-mouse IFN-γ (Pharmingen, San Diego, Calif.) in 50 μl of phosphate-buffered saline (PBS). After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% FBS. Splenocytes (1×10^6/well) isolated from the mice 2 weeks after vaccination were added to the wells along with interleukin-2 (IL-2; 10 IU/ml). Cells were incubated at 37°C for

24 h either with or without irradiated target cells (1×10^4/well). Plates were washed and then incubated with 5 μg/ml biotinylated rat anti-mouse IFN-γ (Pharmingen, San Diego, Calif.) in 50 μl PBS at 4°C overnight. After washing to remove unbound antibodies, 0.2 μg/ml avidin–alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was added, and plates were incubated for 2 h at room temperature. After washing, spots were developed by adding 50 μl of 5-bromo-4-chloro-3-indolyl phosphatase/nitro blue tetrazolium (Sigma Chemical Co.), and incubated at room temperature for 20 min. The spots were counted using a dissecting microscope.

In vivo antibody depletion

In vivo antibody depletion experiments were carried out as previously described [23]. The depletion of $CD4^+$, $CD8^+$ T cell subsets and natural killer (NK) cells was carried out by i.p. injections of 200 μg GK1.5 (anti-$CD4^+$), 2.43 (anti-$CD8^+$) or 40 μl anti-asialo GM1 antiserum (for the depletion of NK cells) respectively, given every other day for 6 days before vaccination or tumor challenge. Depletion of cell subsets was verified by FACS analysis of splenocytes 1 day after the third injection, and maintained by continuing the antibody injections twice weekly for the duration of the tumor challenge experiment. Isotype-matched antibodies were also used as control, and no effect on the tumor growth was observed.

Data analysis

All experiments were repeated a minimum of three times. The data in each figure was taken from one representative experiment. Each group included at least five mice. The unpaired Student's *t*-test was performed for statistical analysis and data were presented as mean ± standard error, as indicated in the figure legends. Values of $P < 0.05$ were considered statistically significant.

Results

Generation and characterization of hsp110 over-expressing CT26 cells

To construct the hsp110 expression plasmid, the full-length cDNA of mouse hsp110 was subcloned into multi-cloning sites of the eukaryotic expression vector pCI-neo. The recombinant hsp110 plasmid was designated pCI-hsp110, which contains a human cytomegalovirus (HCMV) promoter for high-level transcription in mammalian cells, and a neomycin resistance gene neo for drug selection. CT26 tumor cells were transfected with either pCI-neo or pCI-hsp110 respectively, and G418-resistant clones were isolated and amplified after G418 selection (1 mg/ml). Cells transfected with pCI-hsp110 were designated as CT26-hsp110. Empty vector pCI-neo transfected cells, designated as CT26-vector, together with the parental cell line served as the controls for the experiments.

To measure the level of hsp110 in transfected cells, we performed an immunoblot analysis using an anti-hsp110 antibody. It was demonstrated that the hsp110 protein level in the transfected cells was about 3.5-fold higher compared to control cells (Fig. 1). To determine whether the introduction of the hsp110 gene into these cells alters the expression of other hsp, we also examined hsc70/hsp70 expression by immunoblot. Using an antibody recognizing both hsc70 and hsp70, we found that hsp70 was not detectable in either non-transfected or transfected CT26 cells, while endogenous expression of hsc70 remained unchanged. Thus, hsp110 over-expression did not appear to have an effect on the expression of other major hsp such as hsp70.

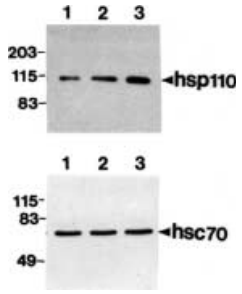

Fig. 1. Immunoblotting analysis of hsp110 expression in transfected cells. Equivalent protein samples from CT26 (*lane 1*), CT26-vector (*lane 2*) and CT26-hsp110 (*lane 3*) were subjected to 10% SDS–PAGE and transferred onto Immobilon-P membrane. Membranes were probed with antibodies for hsp110 (*top panel*) and hsc/hsp70 (*bottom panel*)

Given the demonstrated elevation in hsp110 expression level, we then addressed the question as to whether the over-expression of hsp110 was capable of affecting cell proliferation in vitro and transformation phenotype of CT26 tumor cells. It was found that there was no difference in the in vitro growth of hsp110-transduced cells (CT26-hsp110) compared to wild-type CT26 cells or vector-transduced cells (CT26-vector), and transfection of the empty vector or hsp110 did not significantly affect the average number of colonies formed in soft agar as compared with their parental cells (data not shown). Thus, hsp110 over-expression did not affect the anchorage-independent growth of CT26 cells in vitro.

Hsp110 over-expression increases tumor cell immunogenicity

The tumorigenicity of CT26-hsp110 cells was examined following tumor inoculation in syngeneic mice. Subsequent tumor growth was determined and compared to parental cells as well as to CT26-vector cells. As shown in Fig. 2A, parental and vector-transfected CT26 cells grew rapidly, forming tumors within 6–8 days. However, the over-expression of hsp110 significantly delayed tumor formation and correspondingly resulted in an increased survival time in all of the animals.

To directly assess the effect of hsp110 over-expression on the immunogenicity of CT26 cells, we next examined whether hsp110 over-expressing cells that were inactivated by irradiation would be capable of inducing systemic anti-tumor immunity. Mice were immunized with irradiated CT26-vector cells, irradiated CT26-hsp110 cells, or left untreated. After 2 weeks, all mice were

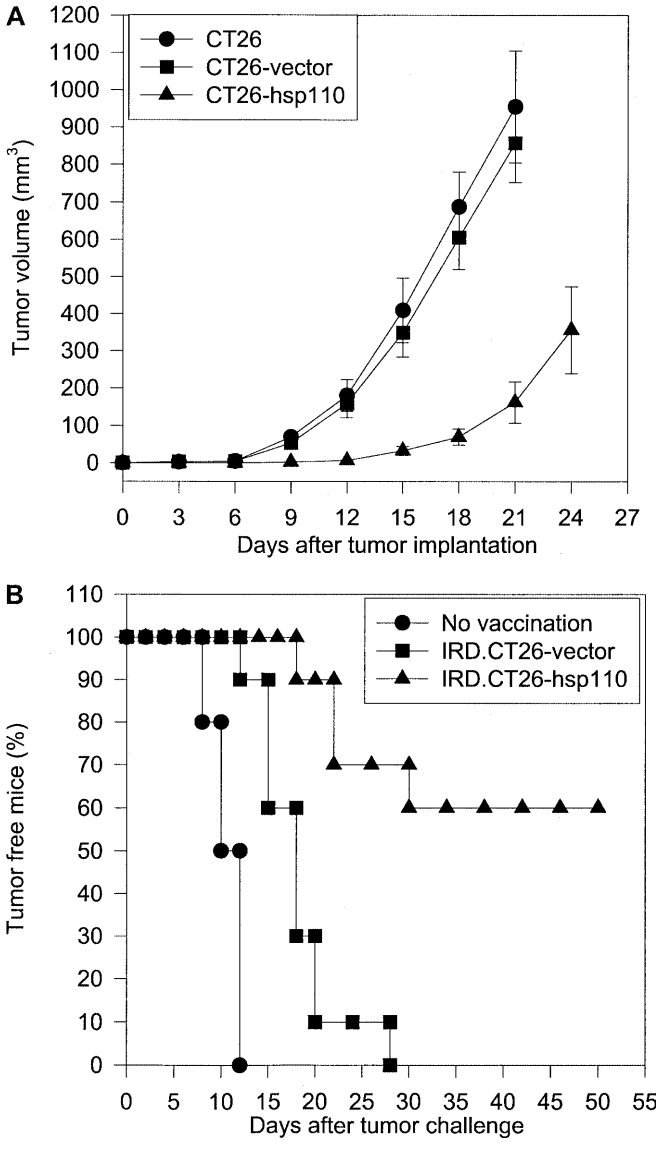

Fig. 2. A, B Hsp110 over-expression reduces tumorigenicity and increases immunogenicity of CT26 tumor. **A** In vivo growth of wild-type CT26 tumor, CT26-vector tumor and CT26-hsp110 tumor. 5×10^4 cells were inoculated s.c. into the flank area of BALB/c mice. Tumor growth was recorded twice a week by measuring two diameters with a caliper. **B** Vaccination with irradiated CT26-hsp110 tumor cells elicited systemic immunity against wild-type CT26 tumor. Mice were immunized s.c. with 2×10^5 irradiated (9,000 rad) CT26-vector cells, CT26-hsp110 cells in the left flank or left untreated. Two weeks later, mice were challenged in the right flank with 2×10^5 wild-type CT26 cells

challenged with wild-type CT26 cells (Fig. 2B). It was observed that all non-immunized mice developed rapidly growing tumors by day 14. However, vaccination with irradiated CT26-hsp110 cells led to a potent anti-tumor response, with 60% of mice remaining tumor-free. In those cases with tumor development, the tumor grew more slowly than did tumors in the control group. Immunization of irradiated CT26-vector cells only minimally inhibited tumor growth. The systemic immunity observed was specific in that irradiated CT26-hsp110 cells did not protect mice from challenge with an unrelated syngeneic tumor cell line, i.e. Meth A fibrosarcoma cells (data not shown).

Characterization of the immune response stimulated by hsp110 over-expressing cells

$CD8^+$ T lymphocytes are one of the most important antitumor effectors [30]. To better define the CT26-specific cytotoxic T lymphocyte (CTL) response generated by irradiated CT26-hsp110 vaccines, CTL assay was carried out after vaccination. As shown in Fig. 3A, splenocytes isolated from irradiated CT26-hsp110 immunized mice demonstrated a tumor-specific lytic activity against CT26 cells. This specific killing was completely inhibited by blocking $CD8^+$ T cells with an anti-CD8 antibody, indicating that $CD8^+$ T cells were responsible for the observed CTL response. It was also found that CTL were able to lyse both wild-type CT26 and CT26-hsp110 cells equivalently (data not shown). Furthermore, CT26-specific IFN-γ-producing T cells were also examined in an ELISpot assay using splenocytes derived from control and vaccinated mice (Fig. 3B). Mice vaccinated with irradiated CT26-hsp110 cells generated the highest number of CT26-specific IFN-γ-secreting T-cell precursors.

Identification of the effector cell subsets responsible for anti-tumor activity

To determine which cells were critical for systemic immunity, mice were depleted of $CD4^+$, $CD8^+$ or NK cells by the administration of antibodies in vivo. They were subsequently inoculated with CT26-hsp110 tumor cells. Depletion of lymphocyte subsets was assessed on the day of tumor injection and weekly thereafter by flow cytometry analysis of spleen cells. More than 99% depletion of the appropriate subset was achieved without affecting the normal levels of the other subsets (data not shown). As shown in Fig. 4A, CT26-hsp110 tumor grew aggressively in all mice depleted of $CD8^+$ T cells or NK cells, indicating that both cell subsets were involved in the in vivo growth inhibition of CT26-hsp110 cells. However, mice depleted of only $CD4^+$ T cells as well as the "non-depleted" mice developed slowly growing tumors, indicating that $CD4^+$ T cells were not involved in the tumor growth delay described above. Parallel studies were also performed in the mice, which were first vaccinated with irradiated CT26-hsp110 cells 1 day after in vivo antibody depletion, then challenged with wild-type CT26 cells 2 weeks after immunization (Fig. 4B). Again, depletion of $CD8^+$ T cells and NK cells abrogated the anti-tumor effect of vaccination. All naive mice and mice depleted of $CD8^+$ T cells or NK cells developed aggressive growing tumors within 14 days after tumor challenge. In contrast, all mice depleted of $CD4^+$ T cells developed tumors

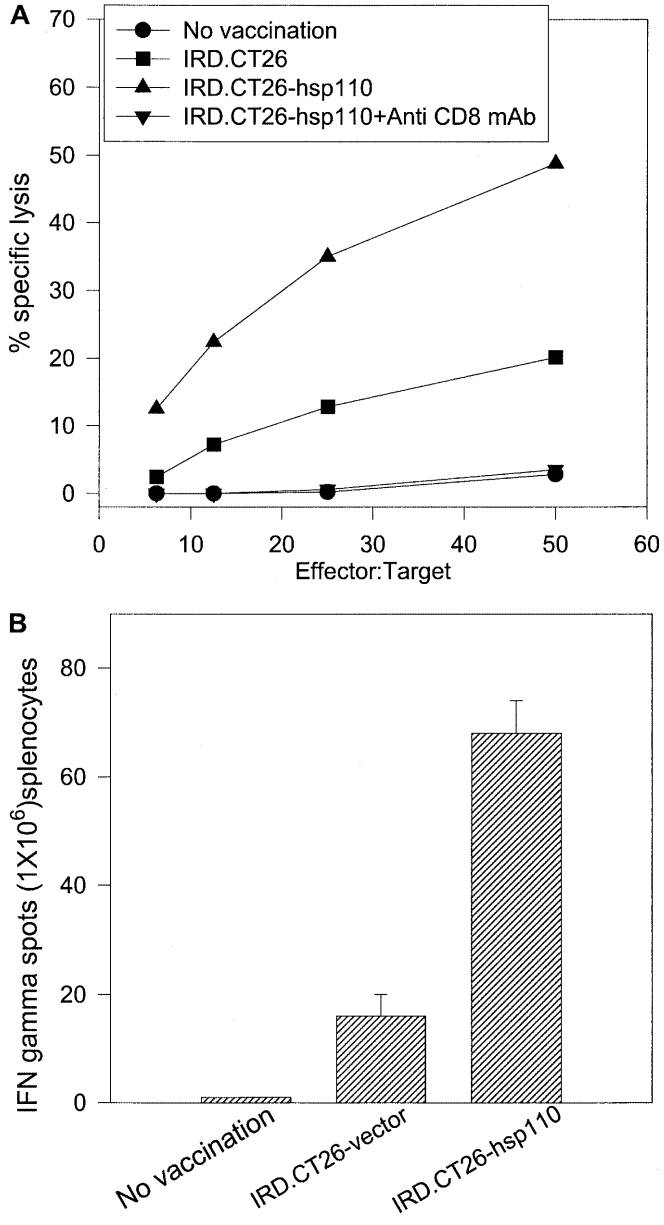

Fig. 3. A, B Tumor specific CTL response elicited by immunization with irradiated CT26-hsp110 tumor cells. **A** Mice were vaccinated with 2×10^5 irradiated (9,000 rad) CT26-vector or CT26-hsp110 cells s.c. or left untreated. Two weeks later, splenocytes were isolated as effector cells and re-stimulated with irradiated wild-type CT26 in vitro for 5 days. The lymphocytes were analyzed for cytotoxic activity using ^{51}Cr-labeled CT26 as target cells. For $CD8^+$ mAb inhibition, effector cells were pre-incubated for 30 min with 20 μg/ml of the CD8-blocking antibody 2.43. **B** Vaccination with irradiated CT26-hsp110 increased the number of tumor-specific T cells producing IFN-γ. Mice were immunized with irradiated CT26-vector cells, CT26-hsp110 cells or left untreated. Splenocytes were prepared 2 weeks after immunization. ELISpot analysis was used to detect the production of IFN-γ. Results shown here are representative of three experiments

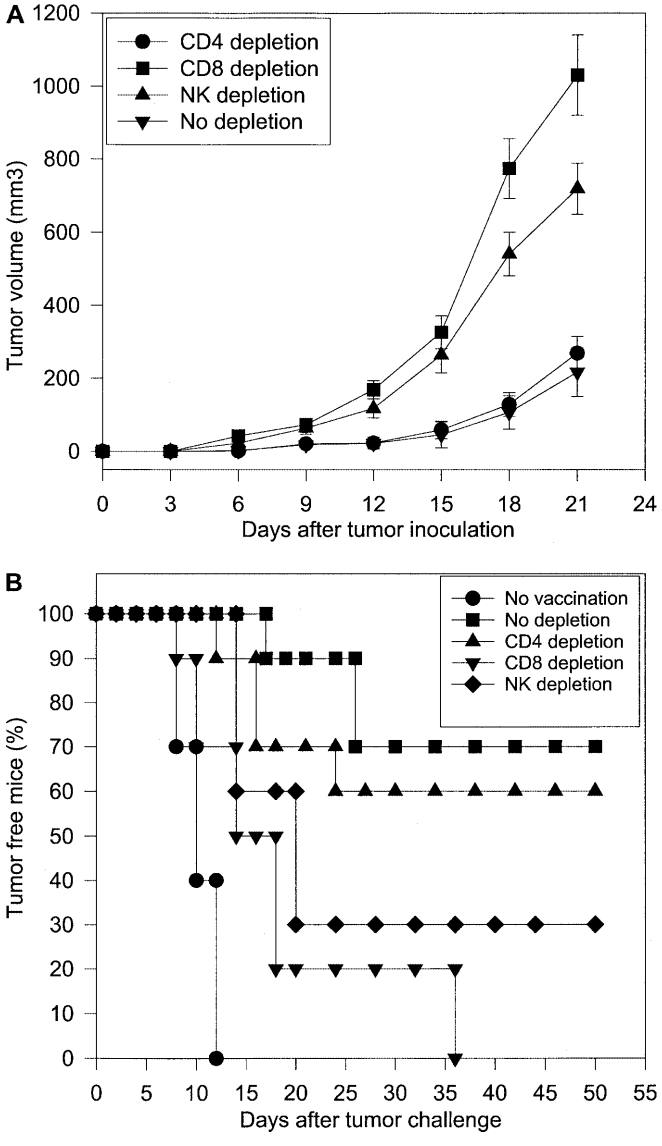

Fig. 4. A, B Identification of the effector cell subsets involved in the immunogenicity of CT26-hsp110 cells. **A** Effect of cell depletion on the growth of CT26-hsp110 tumor in mice. $CD4^+$, $CD8^+$ or NK cells were depleted 1 week before tumor cell inoculation, and maintained by weekly i. p. injections of anti-CD4 antibody (GK1.5), anti-CD8 antibody (2.43), anti-NK antibody (anti-asialo GM1). 5×10^4 CT26-hsp110 tumor cells were inoculated after verification of lymphocyte depletion. Tumor growth was followed by measuring the size of the tumor. **B** Effect of cell depletion on the vaccination with irradiated CT26-hsp110 cells. In vivo depletion was accomplished by i. p. antibody injection 1 week before vaccination with irradiated CT26-hsp110 tumor cells. Two weeks after immunization, mice were challenged with 2×10^5 wild-type CT26 tumor cells and monitored for tumor formation

with kinetics that were similar to those seen in the non-depleted control animals. These results suggest that both $CD8^+$ T cells and NK cells are required for an effective anti-tumor response.

Enhanced anti-tumor effects after vaccination using irradiated hsp110 over-expressing CT26 cells combined with GM–CSF

In the above experiments, we found that immunization with irradiated CT26-hsp110 cells did not lead to complete rejection of CT26 tumor in all mice. We hypothesized that this might be due to insufficient presentation

of tumor antigens by host APC, and that better presentation might enhance the anti-tumor effect. In order to enhance the anti-tumor efficacy of the vaccine, GM–CSF-producing bystander cells were included in the vaccination protocol. Presumably, GM–CSF production at the site of vaccination site might attract host APC and enhance their functions in vivo [16, 46], which would improve the delivery and presentation of antigenic peptides chaperoned by hsp110.

Mice were first immunized with irradiated CT26-hsp110 cells mixed with GM–CSF-producing cells (2:1). Then mice were challenged with wild-type CT26 tumor cells 2 weeks later (Fig. 5). It was observed that administration of irradiated CT26-hsp110 cells combined with GM–CSF-producing bystander cells was sufficient to reject tumors in all mice, indicating that combined vaccination had a greater effect than vaccination with irradiated CT26-hsp110 cells alone. For the group of mice which received irradiated CT26-hsp110 cells in the left flank and irradiated GM–CSF-producing cells in the right flank, no enhanced vaccine effect was observed.

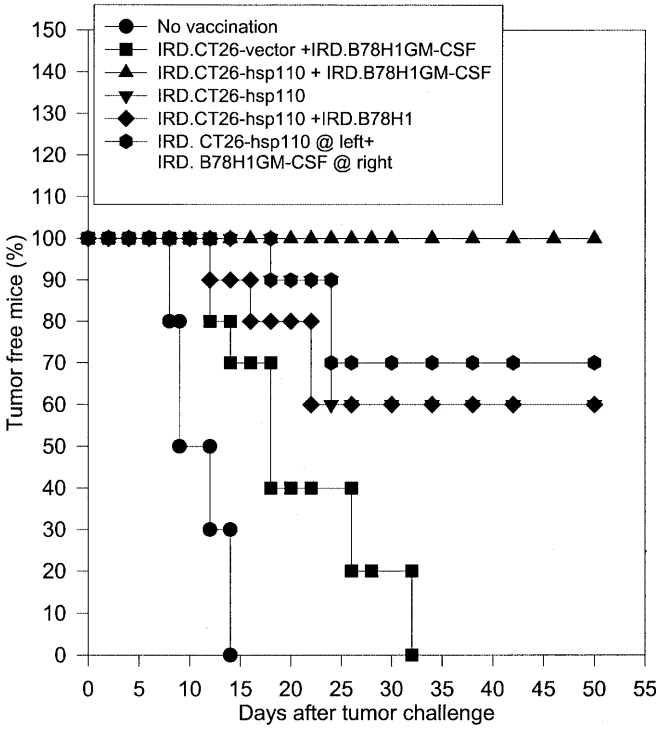

Fig. 5. Combined immunization with irradiated CT-hsp110 tumor vaccine and GM–CSF-producing bystander cells protects mice against the challenge of wild-type CT26 tumor cell. Mice were immunized s.c. with vaccine formula containing irradiated 2×10^5 tumor cells as follows: CT26-vector cells, CT26-vector cells plus B78H1GM-CSF cells (2:1 ratio), CT26-hsp110 cells plus B78H1GM-CSF cells (2:1), CT26-hsp110 cells, CT26-hsp110 plus B78H1 cells (2:1), CT26-hsp110 in the left flank and B78H1GM-CSF in the right flank. Two weeks later, mice were challenged with wild-type CT26 tumor cells and monitored every other day for tumor development. Each group has ten mice. Results are representative of three experiments

This suggests that the synergy of the combined therapy requires co-localization of GM–CSF and tumor antigens. To determine whether mice cured from the initial challenge of CT26 had developed immunity against re-challenge, surviving mice received a second challenge of wild-type CT26 tumor cells 3 months after the primary challenge. None of the re-challenged mice developed palpable tumors, indicating that memory responses were generated in mice cured by combined therapy (data not shown).

Discussion

Induction of a heat shock response as well as transfection with hsp genes have long been known to confer a cytoprotective effect against further cellular stress conditions such as heat shock or apoptotic stimuli [21, 24, 34, 37, 47, 48]. It has also been reported that gene transfer of bacterial hsp into tumor cells enhances their immunogenicity [26, 27]. However, these bacterial hsp would be expected to be highly immunogenic themselves.

In the present study, we first demonstrated that stable transfection of autologous hsp110 in the CT26 tumor cells significantly enhanced the immunogenicity of these tumor cells. This suggests that an increased level of hsp110 provides an immunostimulatory signal in vivo which helps in breaking tolerance to tumor antigens. This result with murine hsp110 is analogous to studies showing that tumor immunogenicity was observed to co-segregate with the expression of inducible hsp70 [31]. Moreover, stable transfection of B16 and CMT93 tumor cells with murine hsp70 significantly enhanced the immunogenicity of these tumors [29, 62]. In addition to gene manipulation as used here, hsp levels can also be enhanced simply by hyperthermia. It has been shown that mild whole-body hyperthermia of either severe combined immunodeficient (SCID) mice bearing human breast tumor xenografts or BALB/c mice bearing syngeneic CT26 tumor causes a tumor growth delay [8] and the induction of hsp in the tumor (Wang et al., unpublished data). In another study tumor cells subjected to a non-lethal heat shock stress in vitro, which also induced a high level of hsp expression, were unable to form tumors in syngeneic mice [11]. Both studies indicated a correlation between the induction of hsp and tumor immunogenicity.

The present study demonstrated that vaccination with irradiated hsp110 over-expressing tumor cells elicited a strong tumor-specific CTL response and generated a high frequency of IFN-γ-secreting T cells. Hsp110 may function to enhance the presentation of endogenous tumor-associated antigen in vivo, as suggested by the capacity of CT26-hsp110 cells, but not control CT26 cells, to induce an immune response capable of recognizing and rejecting wild-type CT26 cells. Others have described the up-regulation of MHC class I molecules secondary to the expression of the human hsp72 gene in

B16 cells [26]. We did not detect any up-regulation of MHC class I surface expression in CT26-hsp110 cells in our study (data not shown). However, we cannot exclude the possibility that hsp110 over-expression increases opportunities for the binding of hsp110 to tumor antigenic peptides, enhancing the capacity of the tumor cells to present antigens through other unknown mechanisms and/or altering the repertoire of MHC-binding antigens.

Based on the well-defined functions of hsp as molecular chaperones which naturally complex antigenic peptides generated within the tumor cells [44, 45, 49], it is tempting to suggest that antigen presentation is mediated through cross-priming where hsp110/antigenic peptide complexes are released from the hsp110 over-expressing cells during cell lysis, then taken up and processed by APC via MHC class I-restricted pathways [1, 40]. Although immunoblot analysis on supernatant from CT26-hsp110 cells failed to detect secretion of hsp110 in vitro (data not shown), hsp110 release from CT26-hsp110 tumor after lysis in vivo is still an obvious possibility.

A recent report has shown that tumor hsp70 induction attracts T cells, macrophages and DC which infiltrate into the tumor microenvironment, inducing Th1 cytokine secretion and a specific anti-tumor response. More importantly, hsp70 released from tumor cells was seen to be internalized directly into DC and to enhance the capability of DC to take up proteins/peptides [51]. A similar response has recently been shown using purified preparations of hsp. Exposure of DC to gp96 resulted in the secretion of the cytokines IL-12 and tumor necrosis factor-α (TNF-α), and in the up-regulation of MHC class II as well as CD86 molecules [43]. Exogenous hsp70 was also found to up-regulate the expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 in human monocytes [3]. Most recently, it has been shown that targeting of gp96 onto the tumor cell surface can induce DC maturation and antitumor immunity [65]. Thus, hsp may not only serve as a vehicle for the delivery of antigenic peptides, but also provide a danger signal stimulating the innate immune system when released from stressed cells [5, 28, 52].

To determine which effector cell subsets mediate the therapeutic antitumor activity of hsp110-overexpressing cells, we performed an in vivo depletion study. Depletion studies revealed that both $CD8^+$ T cells and NK cells were involved in tumor growth inhibition. While it has been proposed that a requirement for NK cells might be related to the hsp stimulated release of IL-12 and other cytokines by APC [38, 50], the precise role of NK cells requires further investigation. In our studies, it was found that $CD8^+$ T cells were activated in a CD4-independent fashion, supporting the model in which hsp can provide a potential adjuvant effect, bypassing the need for $CD4^+$ T cell help by directly or indirectly activating or affecting the maturation state of DC [18, 19, 64].

In an attempt to generate a more potent antitumor effect, hsp110 over-expression and GM–CSF production have been combined. It was found that vaccination using irradiated CT26-hsp110 tumor cells and GM–CSF-producing cells resulted in a significantly better protection from tumor challenge than either treatment alone, i.e. all animals in the group which received combined treatment remained tumor-free. The concept of combining these two factors was based on the hypothesis that DC play an important role as primary APC in initiating and maintaining antigen-specific T-cell responses [16, 46], and GM–CSF produced from bystander cells facilitates DC at the vaccine site to take up and present hsp110-chaperoned tumor antigens.

It has been found that impaired APC activity could be partially responsible for defective immune responses observed in tumor-bearing hosts [41]. Although the mechanism by which GM–CSF mediates anti-tumor immunity is still not completely understood, it has been demonstrated to be a potent modifier of the immune response in multiple tumor vaccine models [13]. This is likely due to its unique ability to promote DC differentiation and maturation at the vaccine site [39]. By administering hsp110-over-expressing cells and GM–CSF-producing cells together, both tumor antigens and GM–CSF are targeted to the DC. There is presently sufficient laboratory evidence to support using other forms of combined gene therapy as a first-line clinical intervention. For example, IL-2/B7.1 and CTLA-4/GM–CSF have produced promising results [15, 56]. Given the highly diverse phenotypes of tumor cells, no single therapy should be expected to efficiently eliminate most tumors. This study shows that increased expression of hsp110 is associated with tumor immunogenicity, and manipulation of relevant hsp, like hsp110, in suitable situations might help boost the immune response and be therapeutically beneficial. The combined therapy studied here, i.e. inactivated CT26-hsp110 cells and GM–CSF, holds significant promise for use in future clinical trials.

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