Potent Tumor-Specific Immunity Induced by an In vivo Heat Shock Protein-Suicide Gene–Based Tumor Vaccine

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ABSTRACT

Tumor cells harbor a repertoire of unique, mutated antigens and shared self-antigens but generally are incapable of provoking an effective immune response, likely because of inadequate antigen presentation by professional antigen-presenting cells. Heat shock proteins (HSPs) play important roles in eliciting innate and adaptive immunity by chaperoning peptides for antigen presentation and providing endogenous danger signaling. Although effective in inducing tumor-specific immune responses in mice and in some clinical trials, tumor-derived HSPs have many limitations like vaccines, such as the technical difficulty of ex vivo preparation of adequate quantities of HSPs from the resected tumors of individual patients. Here we have developed an in vivo HSP-suicide gene tumor vaccine by generating a recombinant replication-defective adenovirus (Ad-HT) that coexpresses HSP70 and a herpes simplex virus thymidine kinase suicide gene. The combination of HSP70 overexpression in situ and tumor killing by thymidine kinase/ganciclovir treatment, but neither strategy alone, provoked potent systemic antitumor activities after intratumor injection of Ad-HT. Tumor-specific CD4+ and CD8+ T-cell responses were induced by Ad-HT intratumor injection. CD11c+ dendritic cells (DCs) isolated from mice treated with Ad-HT were able to prime tumor-specific CTLs. Collectively, these results indicate that the combination of tumor killing by activation of a suicide gene to release tumor antigens and in situ HSP70 overexpression to enhance DC antigen presentation overcomes host immune tolerance to tumor antigens, leading to the induction of potent antitumor immunity. Our findings may have broad relevance to the use of the in vivo HSP-suicide gene tumor vaccine in therapy for human solid tumors.

INTRODUCTION

Increasing evidence indicates that heat shock proteins (HSPs) play important roles in eliciting innate and adaptive immunity. The ability of HSPs to chaperone peptides and to activate dendritic cells (DCs) seems to be the common feature associated with all the major HSPs, including HSP110, gp96, HSP90, and HSP70 (1). HSPs complex with antigenic peptides and gain access to the MHC class I and class II antigen-processing pathways in DCs via receptor-mediated uptake (2, 3), leading to CD8+ and CD4+ T-cell responses (4–14). Generating antitumor immune responses against tumor-associated self-antigens and mutated antigens requires endogenous nonmicrobial danger molecules that activate antigen-presenting cells (APCs). Besides their ability to chaperone antigenic peptides, HSPs may function as endogenous nonmicrobial danger molecules (1, 15, 16) that can up-regulate the expression of cosstimulatory and antigen-presenting molecules on DCs (2, 8, 17). They also stimulate the secretion of proinflammatory cytokines such as interleukin 12 (IL-12), leading to the activation of natural killer (NK) cells and other immune cells (2, 18–22).

Tumor-derived HSPs provide effective tumor vaccines in mouse models (1), and the ability of human melanoma-derived HSP70 to stimulate autologous melanoma-specific T cells also has been shown in vitro (23). Clinical trials of tumor-derived HSPs have been conducted in patients with a broad range of malignancies, including lymphoma, renal cell carcinoma, melanoma, colorectal cancer, gastric cancer, pancreatic cancer, and breast cancer (1). In a recent phase II clinical trial, vaccination of metastatic melanoma patients with autologous gp96 proteins induced clinical and tumor-specific T-cell responses in a substantial proportion of patients (24). There were no unacceptable side effects or autoimmune reactions associated with gp96 vaccination.

Because each tumor harbors a unique repertoire of mutated antigenic peptides, these HSP vaccines have been tailored to the properties of tumor cells in individual patients (1), but this approach has many limitations. For example, when HSP peptide vaccines represent a unique array of tumor antigens, it is difficult to establish a reproducible therapeutic standard. Moreover, the quantity of HSP used for tumor immunotherapy is strictly limited by the size of the surgically resected tumor mass, and some antigenic peptides are inevitably lost during HSP purification. Even if HSP vaccines could be produced, they could not be expected to destroy bulky tumors by themselves, thus limiting their utility.

We previously developed an HSP-mediated oncolytic tumor (“HOT”) vaccine by combining the versatile ability of HSPs to induce tumor-specific immune responses with the oncolytic activity of viruses (25), but enthusiasm for this strategy was tempered by concern regarding the safety of using replication-competent viruses in the clinic. Thus, we developed an in vivo HSP tumor vaccine by generating a recombinant replication-defective adenovirus (Ad-HT) that coexpresses HSP and a herpes simplex virus thymidine kinase suicide gene (HSV-TK). The combination of in situ HSP70 overexpression and tumor killing by thymidine kinase (TK)/ganciclovir (GCV) treatment after intratumor injection of Ad-HT, but not in situ HSP70 overexpression or tumor killing alone, provoked potent systemic antitumor immune responses. We also found that Ad-HT intratumor injection promoted tumor infiltration of DCs. The results described here suggest the broad therapeutic potential of this in vivo HSP suicide vaccination approach against solid tumors.

MATERIALS AND METHODS

Cell Lines and Antibodies. The human tumor cell line Hep3B (hepatocellular carcinoma) was maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Murine mammary sarcoma tumor cells EMS6 were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 11.25 μg/mL t-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO2 atmosphere (26). Antibodies to human-inducible HSP70 were purchased from StressGen Biotechnologies, Inc. (Victoria, British Columbia, Canada). Dr. Sumers (Yale University, New Haven, CT) provided antibody to HSV-TK. Antimouse CD11c, CD8α, CD45RA (B220), and CD205 antibody conjugates and matched isotype controls were from BD PharMingen (San Diego, CA). Antimouse INF-γ antibodies were purchased from Mabtech, Inc. (Mariemont, OH).

Generation of Recombinant Replication-Defective Virus Ad-HT. An AdEasy system (E1 and E3 deletion; Quantum Biotechnologies Inc., Palo Alto,
CA) was used to construct and generate replication-defective adenoviruses. The HSV-TK gene was generated by PCR using the Ad-TK vector (27) as a template with a pair of primers (5'-ACTGCTAGCTACGCTTGGTCG-3' and 5'-AGCTGCTAGCTACGCTTGGTCG-3'). The resulting HSV-TK fragment contained an NheI restriction site at the 5'-end and XhoI at the 3'-end. The shuttle vector Ad-HT was constructed by inserting NheI/XhoI-digested HSV-TK into an NheI/XhoI-digested Ad-HE virus, replacing the EIA fragment. The recombinant replication-competent Ad-HE virus containing the human HSP70 and EIA linked with an encephalomyocarditis virus IRES sequence under control of the CMV promoter was constructed as described previously. PCR and DNA sequencing confirmed the insertion of HSP70 and HSV-TK. The recombinant adenovirus Ad-HT, which harbors the HSP70 and HSV-TK expression cassette under transcription control of the human CMVIE promoter in the E1 region, subsequently was generated according to the manufacturer’s instructions. The replication-defective virus, Ad-H, expressing human-inducible HSP70, was generated earlier (25). PCR and DNA sequencing confirmed the insertion of HSP70 and HSV-TK. The recombinant adenovirus Ad-HT, which harbors the HSP70 and HSV-TK expression cassette under transcription control of the human CMVIE promoter in the E1 region, subsequently was generated according to the manufacturer’s instructions. The replication-defective virus, Ad-H, expressing human-inducible HSP70, was generated earlier. Dr. Alan Davis (Baylor College of Medicine, Houston, TX) provided a replication-defective Ad-TK, HSV-TK expression vector. Recombinant adenoviruses were produced and titrated in 293 cells according to the manufacturer’s instructions (Quantum Biotechnologies Inc., Palo Alto, CA).

MTT Assay. Tumor cells were infected in triplicate with adenovirus at multiplicity of infection (MOI) of 0, 1, 10, 100, and 1000 in a 96-well plate. Serum-free infection medium was replaced with complete culture medium containing 10% fetal bovine serum after 2 hours of infection. At 24 hours after infection, all of the cells were exposed to GCV at 30 μg/mL. Viability of the cell was quantitated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Roche Laboratories, Inc., Nutley, NJ), at day 5 after treatment with GCV.

Animal Study. Five- to 6-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), housed in a specific pathogen-free facility at Baylor College of Medicine, and treated according to institutional guidelines. EMT6 cells (5 × 105) were injected subcutaneously into the right flank of syngeneic BALB/c mice to establish a tumor model. Palpable tumors started to develop after 4 days, and their volumes were measured twice a week with a caliper until the experiment was completed. Tumor volume (mm³) was calculated by the formula: length × width² × 0.52. When tumor sizes reached 60 to 70 mm³, all of the mice were randomly divided into groups of mice and injected with 50 μL of Ad-HT, Ad-HSP, or Ad-TK virus (2.5 × 10⁴ PFUs) (endotoxin-free; Sigma, St. Louis, MO) or PBS control via intratumor injection. GCV (Roche) (50 mg/kg) dissolved in 0.5 mL PBS (endotoxin-free) was administered intraperitoneally twice a day for 5 consecutive days, beginning at 48 hours after injection of virus. All of the tumors were resected when tumor diameters in the PBS group reached 10 mm. In the tumor challenge experiment, animals were inoculated with 1 × 10³ EMT6 cells on the contralateral flank 5 days after the viral treatment.

To examine the effect of intratumor injection on the growth of lung metastasis, we injected mice with 5 × 10⁵ EMT6 cells via the tail vein 2 weeks after viral intratumor injection. Lungs were harvested 2 weeks later and fixed in Bouin’s solution. The resultant tissues were cut into 5-μm sections representing the front, middle, and back portions of the lung; each consecutive section was separated from the next by 500 μm. Sections were stained with hematoxylin and eosin, and lung metastases were quantified by counting the number of nodules in the lung sections.

CTL and Natural Killer Assay. At different times after intratumor injection of virus, mice in each treatment group were sacrificed. The splenocytes isolated from each group were assessed for the activity of NK cells or stimulated with EMT6 cell lysate (per 10⁵ splenocytes stimulated with 10⁵ cell lysate) in the presence of 20 units/mL of human IL-2 for 7 days to assess the activity of CTLs. Tumor cell lysate was prepared by five freeze-thaw cycles with 5 × 10⁵ tumor cells resuspended in 2 mL of serum-free DC medium. The cells were sonicated for 10 minutes and then centrifuged at 15,000 × g for 30 minutes (4°C). Supernatant fluids were recovered, divided into aliquots, and stored at −80°C until further use (28). The cytotoxic activity of the effector cells was determined in a standard 4-hour ⁵¹Cr-release assay (25). To measure NK cell activity, splenocytes from treated mice were tested for cytotoxic activity against ⁵¹Cr-labeled YAC-1 cells in the presence of 20 units/mL of human IL-2.

**INF-γ Enzyme-Linked Immunospot Assay.** The enzyme-linked immunospot assay was carried out as described previously (25, 29). CD4+ and CD8+ T cells were isolated from splenocytes by using MACS CD4 (L3T4) or MACS CD8 (Ly-2) microbeads (Miltenyi Biotec, Auburn, CA).

In vitro Priming of T Cells by Dendritic Cells. CD11c+ DCs from the splenocytes of tumor-bearing BALB/c mice treated with recombinant adenoviruses were purified with CD11c+ microbeads (MiniMacs; Miltenyi Biotec) and used for subsequent experiments. Splenocytes (2.5 × 10⁶ cells/mL) from naive BALB/c mice were cocultured with DCs (2.5 × 10⁵ cells/mL) isolated from the spleens of treated mice in complete RPMI 1640 medium in six-well plates for 1 week with human IL-2 (20 units/mL) and then tested for CTL activity.

**Statistical Analysis.** Student’s t test was used to calculate the significance of statistical comparisons. The overall significance level was set at 5%. Results are presented as mean ± SE.

**RESULTS**

Generation and Characterization of a Replication-Defective Adenovirus Coexpressing HSP70 and HSV-TK (Ad-HT). An adenovirus AdEasy vector system was used to generate the recombinant replication-defective adenovirus (Ad-HT) that coexpresses human-inducible HSP70 and HSV-TK (Fig. 1A). Restriction enzyme digestion and DNA sequencing confirmed the insertion of HSP70 and HSV-TK genes in Ad-HT. The replication-defective virus Ad-HSP, which expresses human HSP70 only, and the replication-defective virus Ad-TK, which expresses HSV-TK only, were generated previously (27, 31). All of the recombinant viruses were produced in 293A cells, purified with CsCl gradients, and titrated on 293A cells with a commercial kit (BD Biosciences).

We first tested the ability of the recombinant adenovirus Ad-HT to...
enhance HSP70 expression because adenovirus infection alone also can increase HSP70 expression via responses to cellular stress. Mouse tumor cells (EMT6) that are susceptible to human adenovirus infection (data not shown) were seeded on 12-well plates (1 × 10^5 cells per well) in triplicate and were infected with Ad-HT, Ad-HSP, or Ad-TK at MOI 1 or mock infected for 2 hours and then replaced with fresh medium. At 48 hours after infection, the cell lysates were subjected to ELISA (HSP70 kits; StressGen Inc.). The results are presented as means of triplicate determinations from one of two experiments. High levels of HSP70 protein (mean, 106.2 or 99.2 ng/10^5 cells) were expressed in Ad-HT- or Ad-HSP-infected cells, whereas only low levels (mean, 14 or 8.16 ng/10^5 cells) were expressed in Ad-TK- or mock-infected cells, representing a 7.5-fold increase of HSP70 expression in Ad-HT– compared with Ad-TK–infected cells. We also tested whether Ad-HT retained TK-mediated cytotoxicity. Murine tumor cells were infected in triplicate with Ad-HT or control adenoviruses at a range of MOIs. Five days after virus infection in the presence of GCV (30 μg/mL), the percentage of viable cells in each well was determined by an MTT assay. At MOIs ranging from 10 to 1000, the cell death rates associated with Ad-HT and Ad-TK were

Fig. 2. Eradication of local tumor and systemic antitumor activity induced by intratumor injection of Ad-HT. A, activity of Ad-HT against local tumor in immunocompetent mice. EMT6 mouse tumors growing subcutaneously in BALB/c mice (six mice per group) were treated with Ad-HT, Ad-HSP, Ad-TK (5 × 10^8 pfu/mouse), or PBS by intratumor injection once a day for two consecutive days, followed by intraperitoneal injection with GCV, 50 mg/kg twice a day for 5 consecutive days. The values are mean ± SE tumor volumes. *P < 0.01, Ad-HT versus Ad-TK or Ad-HSP. These experiments were repeated twice with similar results. B, prevention of secondary tumor growth by Ad-HT intratumor injection. EMT6 tumor cells were inoculated subcutaneously into the right flank of BALB/c mice. When tumor volumes reached ≥40 mm^3, groups of six to eight randomly assigned mice were treated with Ad-HT, Ad-TK, or Ad-HSP (5 × 10^8 pfu/injection), or PBS by intratumor injection once a day for two consecutive days, followed by 5 days of GCV treatment. Mice were rechallenged with EMT6 tumor cells (1 × 10^5 per mouse) in the left flank 5 days after the beginning of viral injection. Percentages of tumor-free mice 3 weeks after rechallenge are shown. The experiment was repeated once with similar results.

IN vivo HSP-SUICIDE GENE–BASED TUMOR VACCINE Research.
essentially the same and clearly exceeded those in cultures infected with Ad-HSP or Ad-GFP (Fig. 1B). A comparable human tumor cell killing was observed in Ad-HT– or Ad-TK–infected human tumor cells (data not shown).

**Intratumor Injection of Ad-HT Eradicates Primary Tumor and Prevents the Growth of Secondary Tumor.** We next examined the ability of Ad-HT to inhibit local tumor growth in vivo. Four groups of BALB/c mice bearing syngeneic EMT6 murine mammary sarcoma tumors were intratumorally injected with Ad-HT, Ad-HSP, Ad-TK (5 × 10⁸ plaque-forming units (pfu)/mouse), or PBS (26). After Ad injections on two consecutive days, all of the mice received GCV at 50 mg/kg twice a day for 5 days. As shown in Fig. 2A, the mice treated with Ad-HSP or Ad-TK had only a temporary delay in tumor growth, whereas Ad-HT uniformly eradicated tumors (P < 0.01). Because Ad-HT and Ad-TK showed comparable cytotoxic activity in vitro, whereas Ad-HSP lacked apparent cytotoxicity, we attribute the enhanced ability of Ad-HT to eradicate primary tumors and the tumor inhibitory effect of Ad-HSP in the immunocompetent mice to HSP-mediated antitumor immune responses.

To test our hypothesis that intratumoral injection of Ad-HT coexpressing HSP70 and HSV-TK can induce a systemic antitumor response, we examined the susceptibility of mice to tumor rechallenge after treatment with Ad-HT or other adenoviruses. EMT6-bearing mice from different groups were intratumorally injected with Ad-HT, Ad-HSP, Ad-TK (5 × 10⁸ pfu/mouse), or PBS, followed by GCV treatment. All of the mice then were rechallenged with EMT6 cells on the contralateral side 5 days after the viral injection. The mice treated with Ad-HT were completely resistant to rechallenge with EMT6 cells, whereas those treated with Ad-HSP or Ad-TK were largely susceptible to such rechallenge (Fig. 2B). The resistance of the mice treated with Ad-HT was EMT6 specific because they were susceptible to rechallenge with a different murine mammary tumor (4T1) of the same genetic background (data not shown). Ad-HT–treated mice also remained resistant to EMT6 cells 2 months later, attesting to the durability of the antitumor response. We further used a lung metastasis model to test whether intratumor injection of Ad-HT could prevent the growth of metastatic tumors. Mice bearing EMT6 tumors were treated intratumorally with different Ad vectors twice and challenged with 5 × 10⁴ EMT6 cells via tail vein injection 2 weeks after the intratumor injection of adenoviruses. As shown in Fig. 3, none of the mice treated with Ad-HT developed lung metastases in contrast to large numbers of lung lesions in mice treated with Ad-HSP or Ad-TK. Collectively, these results indicate that intratumor injection of Ad-HT induces a more potent systemic antitumor activity than either Ad-HSP or Ad-TK treatment.

**Both In situ HSP Overexpression and Cytotoxicity Are Required to Induce Potent Tumor-Specific T-Cell Responses.** To determine whether a systemic antitumor immune response is induced by intratumor injection of Ad-HT, we assessed the T-cell responses in mice treated with different Ad vectors using enzyme-linked immunospot assays. As shown in Fig. 4A, the splenocytes from mice treated with Ad-HT responded vigorously to EMT6 lysate-pulsed DCs, whereas the responses from mice treated with Ad-HSP or Ad-TK were significantly weaker (P < 0.01). To analyze the contribution of CD4⁺ and CD8⁺ T lymphocytes to the observed responses, we isolated CD4⁺ T cells and CD8⁺ T cells from the spleens of immu-

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Fig. 4. Enzyme-linked immunospot assays of tumor-specific CD4⁺ and CD8⁺ T cells induced by Ad-HT. Splenocytes from BALB/c mice bearing EMT6 tumors treated with Ad-HT, Ad-TK, Ad-HSP, or PBS once a day for two consecutive days were harvested and pooled 10 days after the second intratumor injection. The number of IFN-γ-producing T-cell precursors in the splenocyte population (A), isolated CD4⁺ T cells (B), or CD8⁺ T cells (C) was determined with the enzyme-linked immunospot assay (200,000 cells/well). Results are the mean numbers of spots (±SE) observed after stimulation with EMT6 lysate-pulsed BALB/c mouse bone marrow-derived DCs (P < 0.01, Ad-HT versus Ad-HSP, Ad-TK, or PBS group). The numbers of IFN-γ CD4⁺ or CD8⁺ T cells from Ad-HT–treated mice stimulated with DCs pulsed with EMT6 lysate or irrelevant tumor lysate (CT26) also are shown (P < 0.01) (D). Data are representative of three independent experiments.
nized mice using the anti-CD4 or -CD8 microbeads (Miltenyi Biotec). When stimulated with EMT6 lysate-pulsed DCs, significantly more CD4+ and CD8+ T cells from the Ad-HT treatment group secreted IFN-γ compared with results for the remaining groups (Fig. 4B and C; P < 0.01 for all of the comparisons between Ad-HT and other groups). The CD4+ and CD8+ T-cell responses were tumor specific because the CD4+ and CD8+ T cells from the Ad-HT–treated mice responded to EMT6 lysate-pulsed DCs but not to DCs pulsed with the cell lysate from an irrelevant murine tumor (CT26; Fig. 4D).

We further examined the CTL activity in mice treated with Ad-HT. Splenocytes from Ad-HT mice showed significantly higher CTL activity against the target EMT6 tumor cells than did splenocytes from the Ad-HSP–, Ad-TK–, or PBS-treated mice (Fig. 5A). Splenocytes from the Ad-HT mice lacked any apparent CTL activity against irrelevant murine tumor cells, including JC and CT26 (Fig. 5B), indicating that the CTL activity was tumor specific. Antibody blocking assays showed that T lymphocytes were the major effector cells responsible for tumor cell killing (Fig. 5C). We also tested the NK cell status of treated mice by chromium release assay. The data showed that NK cells from Ad-HT–treated mice performed a significantly higher YAC-1 cell killing efficiency (Fig. 5D). Collectively, these results indicate that the combination of HSP70 overexpression in situ and tumor killing by TK/GCV treatment postinjection of Ad-HT, but not either mechanism alone, can elicit potent tumor-specific CD8+ CTL, CD4+ T-helper, and NK cell responses.

**DISCUSSION**

Tumor cell populations represent clonal expansions of cells bearing largely random genetic mutations (~11,000 mutations per tumor cell)
either eradication of local tumor or control of metastasis. In this study – is safe and produces local tumor killing via direct and bystander cancer patients. The results of clinical trials indicate that this therapy induce antitumor immune responses (38). Intratumor injection of HSP expression vectors or expresses HSPs has been used before to induce tumor-specific immune responses (36, 37). Suicide gene therapy as DC maturation and antigenic presentation. Because our strategy uses suicide molecules and the ability of overexpressed HSPs to promote DC maturation and antigenic presentation. Because our strategy uses and shared antigens (32). Antigenic peptide repertoires derived from mutated antigens and shared antigens (33, 34) could potentially induce specific immunity against tumor cells. However, the host immune system largely ignores or tolerates these tumor antigens, most likely because of inadequate antigen presentation by professional APCs such as DCs (35). In this study, we show that the in situ HSP/suicide gene tumor vaccine can effectively induce systemic tumor-specific immune responses that control the growth of primary and metastatic tumors in immunocompetent mice, suggesting that such a vaccine may have broad therapeutic potential for the management of human solid tumors.

Transfer of tumor cells with HSP expression vectors to overexpress HSPs has been used before to induce tumor-specific immune responses (36, 37). Intratumor injection of HSP expression vectors or enhancement of endogenous HSP expression has been reported to induce antitumor immune responses (38–40). Suicide gene therapy as exemplified by HSV-TK/GCV treatment also has been investigated in cancer patients. The results of clinical trials indicate that this therapy is safe and produces local tumor killing via direct and bystander effects (41–44). However, suicide gene therapy has not resulted in either eradication of local tumor or control of metastasis. In this study we found that in situ overexpression of HSP70 or TK/GCV-mediated tumor killing alone was insufficient to provoke potent antitumor immune responses. Our data indicate that the combination of TK/GVC-mediated cytotoxicity that facilitates tumor antigen releases and in situ HSP overexpression that promotes DC antigen presentation are required to break the body’s tolerance of self or mutated tumor antigens, leading to the induction of potent tumor-specific immune responses.

Injection of tumors with recombinant viruses expressing various cytokines, including IL-12, granulocyte macrophage colony-stimulating factor, or other immune-enhancing molecules, such as B-7, has been exploited to induce tumor-specific immune responses (45–47). The HSP/suicide gene tumor vaccine we describe is aimed at enhancing DC antigen presentation and maturation, a key step in provoking antitumor immune responses, by combining the cytolytic activity of suicide molecules and the ability of overexpressed HSPs to promote DC maturation and antigenic presentation. Because our strategy uses HSP-mediated antigen presentation to induce tumor-specific immune responses, we predict that it would readily complement other previously described approaches and may even produce synergistic effect overall (45–47). In summary, the results of this study indicate that the combination of tumor killing by activation of a suicide gene to release tumor antigens and in situ HSP70 overexpression to enhance DC antigen presentation overcomes host immune tolerance to tumor antigens, leading to the induction of potent antitumor immunity. Our findings may have broad relevance to the use of the in vivo HSP/suicide gene tumor vaccine in therapy for human solid tumors.

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