A Broadly Applicable, Personalized Heat Shock Protein-Mediated Oncolytic Tumor Vaccine

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Abstract

Each tumor harbors unique repertoire of mutated antigenic peptides that are immunogenic and potentially can induce tumor-specific immune responses. Because heat shock proteins (HSPs) have the promiscuous ability to chaperone and present a broad repertoire of tumor antigens to antigen presenting cells, HSP tumor vaccine has been tested in clinical trials. However, this vaccine has many limitations, including individual preparation of HSP vaccines from each tumor and quantity of HSPs for therapy strictly limited by the size of the resected tumor mass. Hence, we developed a novel HSP-mediated oncolytic tumor vaccine, referred to as HOT vaccine, by combining the versatile ability of overexpressed HSPs to chaperone antigenic peptides and induce immune responses against a broad array of mutated tumor antigens, with the oncolytic activity of viruses. The results of this study demonstrate that intratumor vaccination with a recombinant oncolytic adenovirus overexpressing the HSP70 protein can eradicate primary tumors, as well as inhibit the growth of established metastatic tumor in mice. Because of its capacity to induce individual tumor-specific immune responses, this HSP-mediated oncolytic tumor vaccine might become a universally applicable, personalized vaccine against any type of solid tumor.

Introduction

Tumor vaccines afford an attractive approach to the development of systemic cancer therapy (1, 2). Because tumors are clonal cell populations that contain thousands of largely random genetic mutations (~11,000 mutations/cell; Ref. 3), the antigenic peptide repertoires derived from the mutated proteins are immunogenic and potentially can induce individual tumor-specific immunity. However, tumor cells are generally incapable of stimulating a robust immune response, probably because of inadequate antigen presentation by professional antigen-presenting cells such as DCs (4, 5). Thus, by improving tumor antigen presentation by antigen-presenting cells, one might significantly boost antitumor immune responses (4).

HSPs have the promiscuous ability to chaperone and present a broad repertoire of tumor antigens to DCs (6–8). HSPs first form complexes with antigenic peptides and then gain access to the MHC class I and class II antigen-processing pathways in DCs via HSP complexes with antigenic peptides and then gain access to the MHC class I and class II antigen-processing pathways in DCs via HSP receptors-mediated uptake (9), leading to prime CD8+ and CD4+ T-cell responses (8, 10). These proteins also elicit innate immunity, characterized by the release of cytokines from DCs and the activation of NK and other types of immune cells (11). Furthermore, HSPs deliver maturation signals to DCs by up-regulating the expression of costimulatory and antigen-presenting molecules, including B7-1, B7-2, and MHC class II molecules (8, 12, 13), in addition to functioning as cytokines that attract DCs and T cells to tumors (14).

HSP protein vaccines have been extensively tested in animals and more recently in clinical trials (8, 15). However, this approach, in which HSPs are purified from tumors and then are administered back to the same cancer patient, has many limitations. First of all, the unique repertoire of mutated antigenic peptides within each tumor dictates that HSP vaccines be produced individually from tumor specimens ex vivo, making it difficult and expensive to reproduce results, and set a therapeutic standard. Second, 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. Third, bulky tumors probably could not be destroyed with HSP vaccines alone.

In this study, we have developed a novel HSP-mediated oncolytic tumor vaccine, referred to as HOT vaccine, by combining the versatile ability of HSPs to chaperone antigenic peptides and induce immune responses against a broad array of mutated tumor antigens, with the oncolytic activity of viruses. The HOT vaccine, a recombinant replicative Ad overexpressing HSP proteins, possesses dual functions: oncolytic activity to kill local tumor and release tumor antigens complexed with HSPs and HSP-mediated immune responses against a broad array of mutated tumor antigens, leading to potent systemic immunity against metastatic tumor. The results described here suggest the potential of this vaccination strategy against diverse types of solid tumors.

Materials and Methods

Cell Lines and Antibodies. The murine tumor cell lines, TRAMP-C2 (16), and B16 (melanoma; Ref. 17), were of C57Bl/6 origin. TRAMP-C2 cells were maintained in complete RPMI 1640 supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin. B16 cells were maintained in DMEM. Human tumor cell lines (Hep3B, DU145, A549, and C33A) and a human primary fibroblast line WI38 (ATCC) and a normal lung cell line MRC-5 (ATCC) were maintained in DMEM supplemented with 10% FCS (18). Antibodies to human-inducible HSP70 were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Antibodies to Ad E1A were purchased from Oncogene. Anti mouse CD4 and antitumor CD8 antibodies were purchased from BD PharMingen (San Diego, CA). Anti-tumor NK antibodies (PK136) were purified from the culture media of the hybridoma (ATCC).

Generation of Recombinant Oncolytic Ad-HE Virus. A modified human Ad-Easy system (E1 and E3 deletion) was used to construct and generate replication-competent Ad vectors by insertion of the E1A gene, as described by Freytag et al. (19). We constructed a replication-competent Ad vector, Ad-E, containing the Ad E1A region from bases 559 to 2262 generated by PCR, using Ad Ad1055 as the template with a pair of primers (A90: 5′-AGTCTCGAGTAAGCCACCTATACATTCATTCC-3′; and A91: 5′-ATACGAGCTTATGAGACATATTATCTGCCAC-3′). The resultant 1715-bp E1A fragment contained a HindIII restriction site at the 5′-end and XhoI at the 3′-end and two
mutations at bases 2253 (C→T) and 2262 (G→T), generating premature translation stop codons in the 55-kDa E1B reading frame at codons 79 and 82, respectively. The PCR fragment was digested with HindIII/Xhol and cloned into the HindIII/Xhol-digested Ad vector (∆E1 region) to generate the Ad-E virus, which resembles the E1B-deleted ONYX-015 virus (19). To generate the oncolytic Ad-expressing HSP70, designated Ad-HE, we constructed an Ad shuttle vector containing the CMV-HSP70-ires-E1A-PolyA expression cassette and used it to generate the recombinant Ad-HE virus. Specifically, the human-inducible HSP70 gene was generated by PCR, using pCMV-HSP70 (unpublished results) as the template with a pair of primers (A92: 5'-ATCGCTAGCATGAGACATATTATCTGCCA-3' and A90). The E1A DNA fragment was digested and cloned into the Xhel/Xhol-digested PBS-ires vector (unpublished data) to generate PBS-ires-E1A. The IRES-E1A fragment was cut by Xhel/Xhol digestion and gel purified. The Ad vector Ad-HE was generated by three-piece ligation of HindIII/Sphl-digested HSP70, SpeI/Xhol-digested IRES-E1A, and HindIII/Xhol-digested Ad vector. The resultant Ad-HE virus harbors the E1A region and HSP70 expression cassette under the transcriptional control of the human CMV-IE promoter in the ∆E1 region. The E1A gene was transcriptionally linked to the HSP70 gene via an encephalomyocarditis virus IRES (Fig. 1B). Insertion of the HSP70 and E1A genes in the Ad-HE virus was confirmed by PCR analysis and DNA sequencing. A replication-defective Ad-null virus (E1 and E3 deletion), a replication-defective Ad-GFP virus, and a replication-defective Ad-HPSE expressing human-inducible HSP70 were also generated. D15250/ONYX-015 virus was kindly provided by Dr. Arnold Berk at University of California at Los Angeles (18). A replication-defective Ad-TK expressing HSV TK was kindly provided by Dr. Alan Davis (20). Recombinant Ads were produced and titrated in 293 cells by the Adenovirus Core at Baylor College of Medicine (Houston, TX).

**Cell Viability Assays.** Tumor cells were infected in duplicate or triplicate with Ad at an MOI of 0.0, 0.1, 1, 10, 100, or 1000 pfu/cell. Serum-free infection medium was removed after 2 h and replaced with 2 ml of 5% fetal bovine serum growth medium. The cell viability at different days after infection was quantitatively measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Promega).

**Animal Experiments.** C57Bl/6 (H-2b) female and male mice (4–8 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Some experiments used 4–8 week-old athymic nude mice obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice were maintained in the mouse facilities at Baylor College of Medicine and treated according to institutional guidelines. Mice were age- and sex-matched for individual experiments. Tumor cells were injected intratumorally once a day for 3 consecutive days (5 × 10^6 pfu/mouse) when the tumor size reached certain sizes. For all injections, materials were suspended in a 50-μl volume of PBS. Tumor growth was monitored three times each week, and mice were sacrificed when their tumors severely ulcerated or reached 1.5 cm in diameter. Tumor volume was determined with the simplified formula of a rotational ellipsoid (w × l^2/2).

**IFN Enzyme-Linked Spot Assay.** MultiScreen-HA plates (Millipore, Bedford, MA) were coated overnight at 4°C with 10 μg of the antihuman IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden) in coating buffer [carbonate-bicarbonate buffer (pH 9.6)]. Plates were blocked with RPMI 1640 and 10% human antibody serum (C-SIX Diagnostics, Inc., Germantown, WI) for at least 2 h at 37°C. Splenocytes from treated mice were seeded at 10^5/well and cocultured with different numbers of irradiated tumor cells in triplicate. Plates were incubated at 37°C in a humidified 5% CO₂ incubator for 16 h, and cells were then removed by six washes with PBS-0.05% Tween 20 (Sigma). Biotinylated antihuman IFN-γ antibody 7-B6-1 (Mabtech) at a concentration of 1 μg/ml PBS-0.5% human antibody serum was added, and plates were incubated for 2 h at 37°C. Streptavidin-conjugated alkaline phosphatase (Mabtech) was added for an additional hour. Cytokine-producing cells were detected after a 5–10-min reaction with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Life Technologies, Inc.). The results were evaluated in a blinded fashion by ZeillNet Consulting, Inc. (New York, NY) with an automated ELISPOT reader system (Carl Zeiss, Inc., Thornwood, NY), using KS ELISPOT 4.3 software.

**CTI Assay.** At different days after intratumor Ad injections, mice were sacrificed, and splenocytes from mice bearing TRAMP-C2 tumors treated with different viruses were harvested. The splenocytes were restimulated in vitro with TRAMP-C2 cells in 24-well plates for 6 days. Target cells (TRAMP-C2), taken together with control B16 or CT26 cells, were labeled with 300 μCi of ^51^Cr for 90 min. Effector cells were plated in 96-well plates at various E:T cell ratios in triplicate. After 4 h of incubation of E:T cells, supernatant was collected and counted with a gamma counter (Beckman, Fullerton, CA). The percentage of specific lysis was calculated as follows: percentage of lysis = (sample cpm × spontaneous cpm)/(total cpm × spontaneous cpm) × 100 (21). In some experiments, anti-CD4, anti-CD8, or anti-NK antibodies (30 μg/well; BD PharMingen) were added into the cultures of E:T cells to block CD4+, CD8+, T cells, or NK cells.

**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 means ± SE.

**RESULTS**

**Generation and Characterization of a Replicative Ad-Expressing HSP70 (Ad-HE).** A modified Adenovirus-Easy system (E1 and E3 deletion) was used to construct a replication-competent Ad by insertion of the E1A gene, as described by Freytag et al. (19). A recombinant replication-competent (E1B 55 kDa-deleted) Ad-expressing HSP70 (Ad-HE) was then generated that contained the E1A and HSP70 genes linked by an IRES sequence, under the transcriptional control of the human CMV-IE promoter (Fig. 1A). The human-inducible HSP70 gene was used because the antitumor activity of its protein has been demonstrated in mouse models (7, 8, 22, 23). The Ad E1A region from bases 559 to 2262 with translation stop codons was generated by PCR with Ad Ad1055 used as a template. A control replication-competent Ad (Ad-E) that expresses the E1A protein and resembles the E1B 55-kDa deleted ONYX-015 virus (19, 24) was also generated. Recombinant Ads were produced in 293 cells, purified on CsCl2 gradients, and titrated on 293 cells. Incorporation of the HSP70 and E1A genes into the recombinant Ad-HE virus was detected by PCR analysis (Fig. 1A) and confirmed by DNA sequencing of the entire insert expression cassette.

Fig. 1B demonstrates that recombinant Ad-HE viruses can infect and express HSP70 in human tumor cells. High levels of HSP70 and E1A proteins were detected in human hepatocellular carcinoma cells (Hep3B) infected with Ad-HE, by use of radiolabeling and an immunoprecipitation assay with antibodies against human-inducible HSP70 and the adenoviral E1A protein. By contrast, only low-level HSP70 expression was detected in tumor cells infected with Ad-E and ONYX-015 (18). It was noted that E1A proteins were preferentially assembled into oligomers in Ad-HE-infected cells. Because Ad infection may induce cellular stress responses (25), we compared HSP70 expression levels in cells infected with Ad-HE at various MOIs with those achieved using the Ad-E control virus. Ad-E virus infection did not significantly induce expression of HSP70, whereas high levels of HSP70 expression were consistently detected in Hep3B tumor cells infected with Ad-HE at different times after infection. Similar results were observed in other human lung tumor cells (A549) infected with Ad-HE or Ad-E (data not shown). By quantifying the intensity of radiolabeled HSP70 bands on SDS-PAGE gels with a PhosphorImager (Molecular Dynamics), we found ~80–100-fold increases of HSP70 expression in Ad-HE-infected compared with Ad-E-infected tumor cells (data not shown). We additionally compared HSP70...
expression levels in the cells infected with Ad-H and those infected with control Ad-TK using ELISA (StressGen Biotechnologies). Ad-TK virus infection did not significantly induce the expression of inducible HSP70, whereas high levels of inducible HSP70 expression were consistently detected in the Hep3B tumor cells infected with Ad-HE (2127 ng of HSP70 in 10^5 Ad-HE-infected cells versus 16 ng of HSP70 in 10^5 Ad-TK-infected cells; Fig. 1C).

**Eradication of Local Tumor by Ad-HE in Immunocompetent Mice.** A major limitation of oncolytic Ad therapy is its inability to eradicate local tumor, even after repeated high-dose injection (26, 27). We postulated that this HSP-mediated oncolytic tumor vaccine might be able to eradicate local tumor by inducing antitumor immune responses by overexpressed HSP in immunocompetent hosts in addition to the oncolytic activity. The Ad-HE virus and the control Ad-E virus were shown to retain the selective killing of various human tumor cells (data not shown). Although human Ads usually do not replicate in mouse cells, they do produce an abortive although lytic infection (28, 29). We therefore selected immunocompetent mice as models for evaluation of the recombinant Ad-HE virus, recognizing that results obtained with this murine
system would underestimate the virus’ oncolytic potential in humans.

We first used a human Ad-GFP to demonstrate that human Ad viruses can efficiently infect murine tumor cell lines, including TRAMP-C2 (16, 30; Fig. 2A) and B16 (melanoma; Ref. 17) demonstrated by using Ad-βGal because melanin in B16 interferes with GFP. High levels of HSP70 expression were also observed in TRAMP-C2 and other mouse tumor cells infected with Ad-HE (data not shown). Additionally, we found that Ad-HE viruses were cytotoxic in many types of murine tumor cells, including TRAMP-C2, B16 (Fig. 2B), and others. Interestingly, Ad-HE was more cytotoxic to some tumor cells such as TRAMP-C2, probably because of the HSP70 expression.

We next tested whether Ad-HE could inhibit or eradicate weakly immunogenic mouse tumors (B16 and TRAMP-C2), which are syngeneic with C57/BL6 mice. It was found that immunization of radiated B16 and TRAMP-C2 cells (4 × 10⁶ cells/mouse) did not protect the mice from the growth of subsequently rechallenged tumors (data not shown). Groups of mice were injected s.c. in the right flank with B16 tumor cells, and when the tumors reached >40 mm³ in diameter, they were treated with Ad-HE (5 × 10⁸ pfu/mouse), Ad-E (5 × 10⁸ pfu/mouse), or PBS once a day for 3 consecutive days. All mice receiving Ad-HE became tumor free after treatment, whereas in mice receiving Ad-E, the tumors continued to grow, although their growth was inhibited by comparison with PBS-treated control mice (Fig. 3, A and B). The replication-defective Ad-HSP70 and Ad-GFP viruses had only minor antitumor effects (Fig. 3C). Similar results were obtained when the experiments were repeated in mice bearing primary B16 tumors injected with Ad-HE or Ad-E (Fig. 3D). However, both Ad-E and Ad-HE were effective in inhibiting local TRAMP-C2 tumors when tumor sizes were smaller (30–60 mm³; Fig. 4B).

Although Ad-HE clearly produced potent effects against local B16 and TRAMP-C2 tumors, the contribution (if any) of immune responses to these effects was unclear. We therefore tested the same treatment strategy in nude mice (Nu/nu) bearing primary B16 tumors. As shown in Fig. 3E, Ad-HE failed to eradicate tumors in nude mice, suggesting a significant antitumor contribution from immune responses induced by Ad-HE in immunocompetent animals.
Similar results were obtained when the experiments were performed in nude mice bearing TRAMP-C2 tumors injected with Ad-HE or Ad-E (5 × 10⁸ pfu/mouse) or PBS by intratumor injection once a day for 3 consecutive days. A, mean ± SE tumor volumes of each group. P < 0.01 versus Ad-E or PBS group. These experiments were repeated three times with similar results. B, the picture shows different groups of mice bearing B16 tumor on day 11 after treatment (two mice in PBS group were already sacrificed after tumors reached 1.5 cm in diameter). C, B16 mouse tumors growing s.c. in C57/B (five to six mice/group) were treated with replication-defective Ad-HSP70, Ad-GFP, or the replication-competent Ad-HE (5 × 10⁸ pfu/mouse) by intratumor injection once a day for 3 consecutive days. Mean tumor volumes of each group are shown. D, TRAMP-C2 mouse tumors growing s.c. in C57/B mice (five to seven/group) were treated with Ad-HE or Ad-E (5 × 10⁸ pfu/mouse) or PBS by intratumor injection once a day for 3 consecutive days. Mean ± SE tumor volumes of each group. TRAMP-C2 tumors were eradicated in all mice treated with Ad-HE, P < 0.01 versus Ad-E or PBS group. These experiments were repeated twice with similar results. E, inhibition, but not eradication, of primary B16 tumors in nude mice by Ad-HE. B16 mouse tumors growing s.c. in nude mice (five to seven mice/group) were treated with Ad-HE, Ad-E (5 × 10⁸ pfu/mouse), or PBS by intratumor injection once a day for 3 consecutive days. The results are mean ± SE tumor volumes for each group. *, P < 0.05, compared with Ad-E group. All mice in PBS group were sacrificed on day 9 after treatment when their tumors reached the size of 1.5 cm in diameter.

**Systemic Antitumor Effect Induced by Intratumor Injection of Ad-HE.** We additionally tested whether intratumor injection with Ad-HE could induce systemic immune responses that would prevent tumor growth after rechallenge. Mice were injected s.c. in the right flank with B16 tumor cells. When tumor volumes reached >40 mm³, we injected Ad-HE or Ad-E (5 × 10⁸ pfu/mouse) or PBS into tumors of different groups of mice and surgically removed all primary tumors once they reached 1.2 cm in diameter. The mice were then rechallenged with B16 tumor cells in the left flank at 7 days after surgery. As shown in Fig. 4A, mice treated with Ad-HE were resistant to rechallenge with B16 tumor, whereas those treated with Ad-E or PBS were mostly still susceptible. Similar results were observed in other mouse tumor cell lines such as CT26 (data not shown). Moreover, mice treated with replication-defective Ad-HSP70 viruses were also mostly susceptible to rechallenge with the same tumor (Fig. 4A), suggesting that the tumor killing also plays a role in induction of antitumor responses. These results indicate that local intratumor therapy with Ad-HE induces systemic immune responses that prevent subsequent tumor growth.

We then tested whether intratumor injection with Ad-HE could induce systemic immune responses that are potent enough to control well-established secondary tumors. The slow-growing mouse TRAMP-C2 tumor, which allows sufficient time for immune responses to develop (16, 30), was used for this study. After s.c. injection of mice in both the left and right flanks with TRAMP-C2 tumor cells, followed by tumor growth to 30–60 mm³ in both flanks, we then injected Ad-HE or Ad-E (5 × 10⁸ pfu/mouse) or PBS once
a day for 3 consecutive days into tumors in the right flank only. By comparison with the PBS control group, mice receiving Ad-HE injections showed a significant delay in tumor growth on the untreated, contralateral flank, whereas in mice injected with Ad-E, the growth of established tumors on the contralateral flank was essentially unimpeled (\(P < 0.05; \text{Fig. 4B}\)). Repeat experiments yielded similar results. Thus, intratumor injection of Ad-HE induces potent systemic antitumor responses that can inhibit the growth of well-established distant (metastatic) tumors.

**Tumor-Specific Immune Responses Induced by Ad-HE.** The contribution of cellular immunity to the systemic antitumor activity of the Ad-HE vaccine was primarily assessed in nude mice injected s.c. with TRAMP-C2 tumor cells on the right and left flanks. Only tumors in the right flank were injected with Ad-HE, Ad-E, or PBS. In contrast to results in wild-type mice, the growth rates of tumors in the contralateral (untreated) flanks of nude mice were similar in all groups of mice treated with different constructs (Fig. 4C), suggesting that T cells are largely responsible for the systemic antitumor activity elicited by Ad-HE in immunocompetent mice. To demonstrate if tumor-specific immune responses are indeed induced by intratumor injection of Ad-HE, we used ELISPOT assays. Splenocytes from mice bearing B16 tumors treated with Ad-HE, Ad-E1, or PBS were tested for their ability to respond to B16 stimulation by secreting IFN-\(\gamma\), whereas those from mice treated with Ad-E or PBS responded only weakly (Fig. 5A).

We additionally evaluated the cytolytic activity of splenocytes from treated mice to target tumor cells using a chromium release assay. As shown in Fig. 5B, there was significant cytotoxic activity against TRAMP-C2 target cells by splenocytes from mice bearing Ad-HE-treated TRAMP-C2 tumors, whereas only low levels of cytotoxicity were detected with splenocytes from mice treated with Ad-E. The cytotoxic activity was specific because the splenocytes of the mice treated with Ad-HE were unable to lyse irrelevant target tumor cells with the same (H-2\(^d\), B16) or different (H-2\(^d\), CT26) genetic backgrounds (data not shown). To additionally define the contributions of
CD4\(^+\) and CD8\(^+\) T cells and NK cells, we performed antibody blocking experiments. Fig. 5C shows that the anti-CD8 antibody drastically inhibited the cytotoxic effect of the splenocytes from Ad-HE-treated mice (\(P < 0.01\)), whereas anti-CD4 and anti-NK cell (PK136) antibodies inhibited this effect only modestly. These results indicate that CD8\(^+\)-cytotoxic T-lymphocyte responses are primarily responsible for the potent antitumor activity elicited by the Ad-HE vaccine.

**DISCUSSION**

The HOT vaccine developed in this study not only eradicated primary tumors after intratumor injection in immunocompetent mice but also induced systemic tumor-specific immune responses that controlled the growth of established distal (metastatic) tumors. These results support our hypothesis that the HOT vaccine, a recombinant oncolytic Ad-expressing HSP, is capable of eliciting potent antitumor immune responses by combining the versatile ability of overexpressed HSPs to chaperone antigenic peptides and induce immune responses against a broad array of mutated tumor antigens, with the oncolytic activity of viruses (Fig. 6).

Given that HSPs are abundant in normal and tumor cells and their expression is induced by Ad infection and other stress-inducing conditions (6–8, 25), can one realistically expect overexpressed HSPs to enhance antitumor immunity? It is apparent that the host’s antitumor
immune responses, even when tumors are necrotic, are usually very weak. We postulate that overexpression of HSPs by Ad-HE-infected tumor cells, taken together with simultaneous tumor killing and release of antigenic peptides complexed with HSPs, will provide both danger signals and antigenic peptides to DCs, leading to strong tumor-specific immune responses. Indeed, there is a >80-fold increase of inducible HSP70 expression driven by the CMV promoter in Ad-HE-infected tumor cells compared with the control oncolytic virus Ad-E or Ad-TK-infected tumor cells. The oncolytic Ad-HE virus vaccine is more potent than the oncolytic virus Ad-E to induce systemic antitumor immune responses. Consistent with our finding, it was reported that immunization with tumor cells, which were genetically modified to express and secret HSP-Fc fusion molecules, generated potent antitumor responses in mice (31).

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 portion of patients (32). However, this vaccine requires individual production from surgically resected tumor specimens ex vivo, and the quantity of HSPs is strictly limited by the size of the resected tumor mass. Antigen-HSP fusion DNA or protein vaccines may overcome some of these obstacles (33, 34). However, these strategies can only enhance immune responses to a limited number of shared antigens that may be tolerated by the immune system, not against a broad array of unique mutated antigens, resulting in suboptimal efficacy. In addition, there are studies of in situ tumor therapy with viruses that express cytokines (35) and the TK gene (20).

The HOT vaccine described here, however, may be superior to the HSP or other tumor vaccines now in various phases of clinical and preclinical investigation, as follows: (a) This HOT vaccine with its unique capacity to induce individual tumor-specific immune responses against a broad array of mutated tumor antigens may be a universally applicable, personalized vaccine against any type of solid tumor, obviating the need to prepare HSP proteins from individual tumors. (b) This vaccine may be more potent than HSP protein or recombinant HSP-Ag tumor vaccines. For example, overexpression of HSPs by tumors infected with the vaccine should additionally enhance the presentation of tumor antigenic peptides and antitumor responses, in contrast to current HSP vaccines, the efficacy of which is limited by the availability of surgically resected tumor tissues. The oncolytic activity of the vaccine leads to the release of immunologically hidden tumor antigens that are complexed with HSPs. The HSPs released from killed tumor cells provide maturation signals to DCs, activating both innate and adaptive immunity, and may function as cytokines to recruit immune cells to tumor sites (7, 8). Furthermore, the Ad-HE virus was found to not only retain the selective killing of various human tumor cells but also unexpectedly have enhanced oncolytic activity, probably because of the enhanced virus replication by E1A assembly facilitated by overexpressed HSPs (unpublished data).

Intratumor injection of HOT vaccine can induce immune responses
against mutated tumor-specific antigens, as well as normal self-antigens. Immune responses to self-antigens presented by HSPs may thus produce damage to normal tissues. However, the reported lack of toxic side effects in mouse models and clinical trials with HSP proteins isolated from tumors (7, 8, 32), argues against this outcome, probably because of the very low frequencies and affinity of T-cell precursors for self-antigens (1, 2, 5).

Because every tumor contains mutated antigens (2, 3), intratumor vaccination with this HOT vaccine should elicit individual tumor-specific immune responses against any type of solid tumor. How, then, might this vaccine be used to advantage in cancer patients? One possibility would be to treat cancer patients before surgery to kill primary tumor and prime individual tumor-specific immune responses against mutated tumor antigens, which could reduce the need for surgery and the rate of tumor recurrence. A second application might involve repeated treatment of recurrent disease to control metastases by provoking strong recall tumor-specific immune responses. Presently, we are evaluating the therapeutic potency and in a broad range of experimental tumor models.

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