Enhancement of DNA Vaccine Potency by Linkage of Antigen Gene to an HSP70 Gene1

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ABSTRACT

Nucleic acid vaccines represent an attractive approach to generating antigen-specific immunity because of their stability and simplicity of delivery. However, there is still a need to increase the potency of DNA vaccines. Using human papillomavirus type 16 E7 as a model antigen, we evaluated the effect of linkage to Mycobacterium tuberculosis heat shock protein 70 (HSP70) on the potency of antigen-specific immunity generated by naked DNA vaccines. We found that vaccines containing E7-HSP70 fusion genes increased the frequency of E7-specific CD8+ T cells by at least 30-fold relative to vaccines containing the wild-type E7 gene. More importantly, this fusion converted a less effective vaccine into one with significant potency against established E7-expressing tumors. Surprisingly, E7-HSP70 fusion vaccines exclusively targeted CD8+ T cells; immunological and antitumor effects were completely CD4-independent. These results indicate that fusion of HSP70 to an antigen gene may greatly enhance the potency of DNA vaccines via CD8-dependent pathways.

INTRODUCTION

DNA vaccines have become an attractive approach for generating antigen-specific immunotherapy. The naked plasmid DNA is safe, has low immunogenicity, and can be repeatedly administered. DNA vaccines can be easily prepared in large scale with high purity and are highly stable relative to proteins and other biological polymers (for review, see Ref. 1–3). One of the concerns about DNA vaccines is their limited potency. Several strategies have been applied to increase the potency of DNA vaccines, for example, targeting antigens for rapid intracellular degradation (4, 5), directing antigens to APCs by fusion to ligands for APC receptors (6), fusing antigens to chemokines (7) or to a pathogen sequence, such as fragment C of tetanus toxin (8), coinjecting cytokines (9, 10), costimulatory molecules (11), and co-administration with CpG oligonucleotides (12).

Linkage of antigens to HSP represents a potential approach for increasing the potency of DNA vaccines. In the past few years, immunization with HSP complexes isolated from tumor or virus-infected cells has been shown to be able to induce potent antitumor (13–19) or antiviral immunity (20, 21). The immunogenic HSP-peptide complexes can also be reconstituted in vitro by mixing the peptides with HSPs (22, 23). The HSP-based protein vaccines can also be administered by fusing antigens to HSPs (24, 25). These experiments demonstrate that (1) HSP-peptide complexes derived from tumor cells or virus-infected cells, but not from normal tissue, can stimulate tumor or virus-specific immunity; (2) the specificity of this immune response is caused by tumor-derived peptides that are bound to the HSPs, not by the HSPs themselves, and (3) the immune response can be induced in mice with MHC either identical or different to the MHC of donor HSPs (26, 27). These investigations have made HSPs more attractive for use in immunotherapy. However, all of the HSP vaccines tested are in the form of protein-based vaccines. To date, HSPs have not been used in the form of chimeric DNA vaccines.

We chose human HPV-16 E7 as a model antigen for vaccine development because HPVVs, particularly HPV-16, are associated with most cervical cancers. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and coexpressed in most HPV-containing cervical cancers. Vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. In our present study, we investigated whether genes linking full-length E7 to HSPs can enhance the potency of DNA vaccines. We compared DNA vaccines containing wild-type HPV-16 E7 with DNA vaccines containing full-length E7 fused to Mycobacterium tuberculosis HSP70 for their immune response generation and their ability to protect animals against the HPV-16 E7-expressing murine tumors (28). We show that linkage of E7 to HSP70 dramatically increases expansion and activation of E7-specific CD8+ T cells, completely bypassing the CD4 arm. This enhanced CD8 response results in potent antitumor immunity against established tumors.

MATERIALS AND METHODS

Plasmid DNA Constructs and Preparation. DNA fragment encoding M. tuberculosis HSP70 was obtained from pK70 (24). For the generation of HSP-expressing plasmid (pDNA3-HSP), the HSP70 was subcloned from pK70 into the unique BamHI and HindIII cloning sites of the pcDNA3.1(−) expression vector (Invitrogen, Carlsbad, CA) downstream of the cytomegalovirus promoter. For the generation of HPV-16 E7-expressing plasmid (pDNA3-E7), E7 DNA was amplified by PCR using primers designed to generate BamHI and HindIII restriction sites at the 5' and 3' ends of the amplified fragments, respectively. The amplified E7 DNA was then cloned into the unique BamHI and HindIII cloning sites of the pcDNA3.1. For the generation of E7-HSP70 chimera (pDNA-E7-HSP70), E7 DNA was amplified by PCR using primers designed to generate BamHI restriction sites at both 5' and 3' ends of the amplified fragments. The E7 DNA was then subcloned to the 5' end of pDNA3-HSP. The accuracy of these constructs was confirmed by DNA sequencing. Plasmid DNA with HSP, E7, or E7HSP70 gene insert and the “empty” plasmid vector were transfected into subcloning efficient DH5 (TM cells; Life Technologies). The DNA was then amplified and purified using double cesium chloride purification (BioServe Biotechnologies, Laurel, MD). The integrity of plasmid DNA and the absence of Escherichia coli DNA or RNA were checked in each preparation using 1% agarose gel electrophoresis. DNA concentration was determined by the absorbance measured at 260 nm. The presence of the inserted E7 fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

DNA Vaccination. Gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad, Hercules, CA) according to the protocol provided by the manufacturer. Briefly, DNA-coated gold particles were prepared by combining 25 mg of 1.6 μm of gold microcarriers (Bio-Rad, Hercules, CA) and 100 μl of 0.05 μL spermidine (Sigma, St, Louis, MO).
Plasmid DNA (50 µg) and 1.0 M CaCl₂ (100 µl) were added sequentially to the microcarriers while mixing by vortex. This mixture was allowed to precipitate at room temperature for 10 min. The microcarrier/DNA suspension was then centrifuged (10,000 rpm for 5 s) and washed three times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg/ml; Bio-Rad, Hercules, CA) in absolute ethanol. The solution was then loaded into tubing and allowed to settle for 4 min. The ethanol was gently removed, and the microcarrier/DNA suspension was evenly attached to the inside surface of the tubing by rotating the tube. The tube was then dried by 0.4 liters/min of flowing nitrogen gas. The dried tubing coated with microcarrier/DNA was then cut to 0.5-inch cartridges and stored in a capped dry bottle at 4°C. As a result, each cartridge contained 1 µg of plasmid DNA and 0.5 mg of gold. The DNA-coated gold particles (1 µg of DNA/bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 p.s.i.

ELISPOT Assay. The ELISPOT assay described by Miyahira et al. (29) and Murali-Krishna et al. (30) was modified to detect HPV-16 E7-specific CD8⁺ T cells. The 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 µg/ml rat antinmouse IFN-γ antibody (clone R4-6A2, Pharmingen, San Diego, CA) in 50 µl of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. Different concentrations of fresh isolated spleen cells from each vaccinated mice group, starting from 1 × 10³/well, were added to the well along with 15 IU/ml IL-2. Cells were incubated at 37°C for 24 h either with or without 1 µg/ml E7-specific H-2Dᵇ CTL epitope (E7, amino acids 49–57). After culture, the plate was washed and then followed by incubation with 5 µg/ml biotinylated IFN-γ antibody (clone XMG1.2, Pharmingen) in 50 µl in PBS at 4°C overnight. After washing six times, 1.25 µg/ml avidin-alkaline phosphatase (Sigma, St. Louis, MO) in 50 µl of PBS were added and incubated for 2 h at room temperature. After washing, spots were developed by adding 50 µl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Boehringer Mannheim, Indianapolis, IN) and incubated at room temperature for 1 h. The spots were counted using a dissecting microscope.

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis. Splenocytes from naïve or vaccinated groups of mice were incubated either with the E7 peptide (amino acids 49–57) that contains the MHC class I epitope (31) or the E7 peptide (amino acids 30–67) that contains the MHC class II peptide (32). The E7 peptide was added at a concentration of 2 µg/ml for 20 h. To detect E7-specific CD8⁺ T-cell precursors and E7-specific CD4⁺ T-helper cell responses, CD8⁺ CTL epitopes amino acids 49–57 and amino acids 30–67 were used, respectively. Golgistop (PharMingen, San Diego, CA) was added 6 h before harvesting the cells from the culture. Cells were then washed once in FACSscan buffer and stained with phycoerythrin-conjugated monoclonal rat antimouse CD8 or CD4 antibody (PharMingen, San Diego, CA). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer’s instructions (PharMingen). FITC-conjugated anti-IFN-γ or anti-IL-4 antibodies and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from PharMingen. Analysis was done on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

ELISA for Cytokines. Splenocytes (4 × 10⁶) were harvested 2 weeks after the last vaccination and cultured with 10 µg/ml E7 protein in a total volume of 2 ml of RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 2 mM nonessential amino acids in a 24-well tissue culture plate for 72 h. The supernatants were harvested and assayed for the presence of IFN-γ and IL-4 using ELISA kits (Endogen) according to the manufacturer’s protocol.

Anti-E7 ELISA. The anti-HPV 16 E7 antibodies in the sera were determined by a direct ELISA as previously described (33). A 9-microwell plate was coated with 100 µl of 10 µg/ml bacteria-derived HPV-16 E7 proteins and incubated at 4°C overnight. The wells were then blocked with PBS containing 2% fetal bovine serum. Sera were prepared from the mice on day 14 postimmunization, serially diluted in PBS, added to the ELISA wells, and incubated on 37°C for 2 h. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit antimus-e IgG antibody (Zymed, San Francisco, CA) at room temperature for 1 h. The plate was washed six times, developed with 1-Step Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1 M H₂SO₄. The ELISA was monitored twice a week for determination of the appropriate lymphocyte subset were depleted with a normal level of other lymphocyte subsets. Depletion was terminated on day 40 after the tumor challenge.

RESULTS

Vaccination with E7-HSP70 Fusion DNA Enhances E7-Specific CD8⁺ T-Cell-mediated Immune Responses. CD8⁺ T lymphocytes are one of the most crucial components among antitumor effectors (38). To determine the E7-specific CD8⁺ T-cell precursor frequencies generated by E7-HSP70 DNA vaccines, ELISPOT assays and intracellular cytokine stains were used. Both ELISPOT assay and intracellular cytokine staining are sensitive functional assays used to measure IFN-γ production at the single-cell level, which can thus be applied to quantify antigen-specific CD8⁺ T cells (30). As shown in Fig. 1A, 435 IFN-γ spot-forming CD8⁺ T cells specific for the immunodominant Dᵇ-restricted E7 peptide were detected per 10⁶ splenocytes derived from the E7-HSP70 DNA vaccinated mice, compared to only 14 E7-specific IFN-γ spot-forming CD8⁺ T cells/10⁶ splenocytes derived from the E7 DNA-vaccinated mice. Subtracting the background produced by the pcDNA-3 vector alone (3 spots/10⁶ splenocytes) yielded 11 and 432 E7-specific IFN-γ spot-forming CD8⁺ T cells for the E7 and E7-HSP70 DNA vaccines, respectively. Similarly, the quantity of E7-specific CD8⁺ T-cell precursors can also be determined by flow cytometry analysis using double staining for CD8 and intracellular IFN-γ (30). Values from this assay (Fig. 1B) correlated closely with the ELISPOT results presented in Fig. 1A. As shown in Fig. 1B, mice vaccinated with E7-HSP70 DNA generated the highest number of E7-specific IFN-γ⁺ CD8⁺ T-cell precursors.
E7-specific CD4+ T-cell precursors in C57BL/6 mice immunized with E7-HSP70 DNA vaccines. C57BL/6 mice were immunized with empty plasmid (pcDNA3), HSP70 DNA (HSP), E7 DNA (E7), E7-HSP70 DNA (E7/HSP), or E7 DNA mixed with HSP70 DNA (E7+HSP) via a gene gun or received no vaccination. For vaccinated mice, 2 µg of DNA/mouse were given twice. Splenocytes were harvested 10 days after the last DNA vaccination. A, ELISPOT assay. The number of IFN-γ-secreting E7-specific CD8+ T-cell precursors was determined using the ELISPOT assay (see text for the detailed method). The spot numbers were the mean of triplicates ± SE in each vaccinated group. Mice vaccinated with E7-HSP70 DNA generated the highest IFN-γ+ spot numbers. Results shown here are E7-specific spot-forming cells (subtracting the spot numbers without adding the E7 CTL peptide). B, flow-cytometry analysis. Splenocytes from vaccinated mice were cultured in vitro with the E7 peptide (amino acids 49–57) overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ-secreting CD8+ T-cell precursors in mice immunized with various recombinant DNA vaccines was analyzed by flow cytometry. Mice vaccinated with E7-HSP70 DNA generated the highest IFN-γ+ CD8+ double-positive T cells. The numbers of CD8+ IFN-γ+ double-positive T cells in 3 × 10^6 splenocytes are indicated in the upper right corner. The data of ELISPOT and intracellular cytokine staining shown here are one representative experiment of two performed.

Fig. 2. Flow-cytometry analysis of IFN-γ-secreting E7-specific CD4+ T cells in mice vaccinated with various recombinant DNA vaccines. C57BL/6 mice were immunized as described in Fig. 1. Splenocytes from vaccinated mice were cultured in vitro with the E7 peptide (amino acids 30–67) overnight and were stained for both CD4 and intracellular IFN-γ. The number of IFN-γ-secreting CD4+ T cells was analyzed by flow cytometry. Mice vaccinated with E7-HSP70 DNA generated comparable CD4+ IFN-γ+ double-positive cells when compared to mice vaccinated with wild-type E7 DNA. The numbers of CD4+ IFN-γ+ double-positive T cells in 3 × 10^6 splenocytes are indicated in the upper right corner. The data of intracellular cytokine staining shown here are one representative experiment of two performed.

Vaccination with E7-HSP70 Fusion DNA Does Not Generate E7-specific Antibodies. The quantity of anti-HPV 16 E7 antibodies in the sera of the vaccinated mice was determined by a direct ELISA 2 weeks after the last vaccination. No anti-E7 antibodies could be detected in the sera of mice of any vaccinated group (Fig. 3). The commercial anti-E7 monoclonal antibody (Zymed, San Francisco, CA) and sera from mice vaccinated with vaccinia virus containing the Sig/E7/LAMP-1 chimera (33) were used as positive controls to ensure the success of anti-E7 ELISA for this study. This result is consistent with the complete absence of apparent E7-specific CD4 stimulation by either E7 DNA or E7-HSP70 DNA vaccines.

Vaccination with E7-HSP70 Fusion DNA Enhances Protection of Mice Against the Growth of TC-1 Tumors. To determine whether vaccination with the E7-HSP70 DNA construct protects mice against E7-expressing tumors, two in vivo tumor protection experiments were performed using different doses of DNA vaccines. For the
first experiment, mice were vaccinated with 2 μg of naked DNA/mouse via a gene gun and boosted with the same dose 1 week later. For the second experiment, mice were vaccinated with 2 μg of naked DNA/mouse via a gene gun without a further booster. The mice were then challenged with 5 × 10^4 TC-1/mouse s.c. in the right leg 7 days after the last vaccination. For the mice receiving vaccination with a booster, 100% of those receiving E7-HSP70 DNA vaccination remained tumor-free 60 days after the TC-1 challenge, whereas only 40% of mice receiving E7 DNA vaccination remained tumor-free. In contrast, all of the unvaccinated mice and mice receiving empty plasmid or HSP DNA developed a tumor growth within 15 days after the tumor challenge (Fig. 4A). For the mice receiving vaccination once without a booster, 100% of those receiving E7-HSP70 DNA vaccination remained tumor-free 60 days after the TC-1 challenge, whereas all of the unvaccinated mice and mice receiving empty plasmid or HSP DNA developed tumor growth within 15 days after the tumor challenge (Fig. 4B).

We compared E7-HSP70 DNA to E7-DNA mixed with HSP70 DNA for their ability to generate the antitumor immunity. We pre-mixed the E7 DNA with HSP70 DNA before the bullet preparation. Thus, a single bullet would contain both E7 DNA and HSP70 DNA. We performed the immunological assays and a tumor protection experiment (without a vaccination booster). As shown in Fig. 1, only E7-HSP70 DNA could enhance the E7-specific CD8+ T-cell-mediated immune responses. Mixing HSP70 DNA with E7 DNA did not enhance the CD8+ T-cell-mediated immune responses. For the tumor protection experiment, all of the mice vaccinated with E7-HSP70 DNA remained tumor-free 60 days after the tumor challenge. In contrast, all of the unvaccinated mice or mice vaccinated with E7 DNA mixed with HSP70 DNA developed tumor growth within 15 days after the tumor challenge (Fig. 5).

In summary, these results indicated that E7-HSP70 fusion DNA could significantly enhance the antitumor immunity against the growth of TC-1 tumors, especially tested under more stringent conditions (no booster).

**Therapeutic Vaccination with E7-HSP70 Fusion DNA Cures Mice with Established E7-expressing Tumors.** To test the efficacy of DNA vaccines in eradicating established TC-1 tumors, two in vivo tumor treatment experiments were performed using different doses of DNA vaccines. TC-1 cells were first injected into C57BL/6 mice s.c. at a dose of 2 × 10^4 cells/mouse in the right leg. Three days later, each mouse was treated with 2 μg of either control plasmid DNA, HSP70 DNA, wild-type E7 DNA, or E7-HSP70 DNA intradermally via a gene gun. For the first experiment, mice were boosted with the same vaccine dose 7 days after priming. For the second experiment, mice did not receive further booster after priming. As shown in Fig. 6A, for mice receiving a boosted DNA vaccination, the TC-1 tumor was eliminated from 80% of mice receiving the E7-HSP70 DNA vaccination, whereas all of the unvaccinated mice and mice receiving empty plasmid, HSP70 DNA, or E7 DNA developed a tumor growth within 20 days after the tumor challenge. For the mice receiving a vaccine once without a booster, 60% of those receiving E7-HSP70 DNA vaccination remained tumor-free 70 days after the TC-1 challenge, whereas all of the unvaccinated mice and mice receiving empty plasmid, HSP70 DNA, or E7 DNA developed a tumor growth within 20 days after the tumor challenge (Fig. 6B). In summary, these results showed that vaccination with wild-type E7 DNA failed to eradicate the previously inoculated E7-expressing tumors in mice, whereas vaccination with E7-HSP70 DNA could eradicate the established E7-expressing tumors. This indicated that E7-HSP70 DNA significantly enhanced antitumor immunity.

**CD8+ T Cells but Not CD4+ T Cells or NK Cells Are Essential for the Antitumor Effect Generated by DNA Vaccine with E7 Fused to HSP70.** To determine the subset of lymphocytes that are important for the rejection of E7-positive tumor cells, we performed in vivo antibody depletion experiments. The antibody depletion was started 1 week before the tumor challenge and terminated on day 40 after the tumor challenge. As shown in Fig. 7, all naïve mice and all of the mice depleted of CD8+ T cells grew tumors within 14 days after the tumor challenge. In contrast, all of the nondepleted mice and all of the mice depleted of CD4+ T cells or NK1.1 cells remained tumor-free 50 days after the tumor challenge. These results suggest that CD8+ T cells that are activated in a CD4-independent fashion are essential for the antitumor immunity generated by the E7-HSP70 DNA vaccine.

**DISCUSSION**

In this study, we demonstrated that *M. tuberculosis* HSP70 can dramatically enhance the potency of HPV-16 E7-expressing DNA vaccines.
vaccines. DNA vaccines with HSP70 fused to HPV-16 E7 elicited strong E7-specific cellular immunity (at least a 30-fold increase in the E7-specific CD8+ T-cell precursor frequencies) and generated significant CD8+ T-cell-dependent preventive and therapeutic effects against HPV-16 E7-expressing murine tumors.

Our data demonstrated that HSP70 can preferentially enhance CD8+ T-cell responses of E7 DNA vaccines. In contrast, CD4+ T-cell responses were not detectably enhanced by HSP70 linkage. This was demonstrated by a failure to induce HSP-specific CD4+ T cells by flow cytometry and a failure to induce E7-specific antibodies. One of the possible mechanisms for the enhancement of CD8+ T-cell responses is the generation of HSP-specific CD4+ T cells. Although E7-specific CD4+ T cells were not detected in E7-HSP70 vaccinated mice, it is possible that HSP-specific CD4+ T cells were generated and contribute to the generation and expansion of CD8+ T cells. An alternative mechanism for the enhancement of CD8+ T-cell responses is the chaperone effect of HSP70. Ballistic DNA delivery can introduce DNA directly into dermal precursors. The E7-HSP70 DNA-transfected DCs expressed HSP70. HSP70 is a cytosolic HSP that has been shown to play multiple roles in protein folding, transport, and degradation (48). It has also been proposed to be involved in processing MHC class I restricted antigens (49, 50). We are presently determining whether E7-HSP fusion products are indeed targeted more efficiently for proteasomal processing.

Our in vivo antibody depletion experiment was consistent with the concept that CD8+ T cells are the key players in gene gun-mediated E7-HSP70 DNA vaccination. Our data showed that CD8+ T cells are required in the effector phase of antitumor immunity. In contrast, the depletion of CD4+ or NK1.1+ cells did not decrease the antitumor immunity generated by E7-HSP70 DNA. Our finding is in contrast to approaches using protein-based HSP vaccines, which showed that CD4+ and CD8+ T cells and NK cells are required in the effector phases of antitumor immunity using gp96 preparations from tumor cells (17, 18). The exact reason for such a discrepancy is unknown, but it could be related to different effects due to different HSPs or to differences between DNA-based versus protein-based HSP-containing vaccines.

HSP complexes taken up by professional APCs are supposed to play an important role in introducing HSP-associated peptides into the MHC-I antigen presentation pathway (17, 21). It has been suggested that HSP complexes can enter into professional APCs via receptor-mediated endocytosis (51). These findings provide a possible explanation for the “cross-priming” of HSP/peptide complexes where the HSP can lead exogenous proteins to the MHC-I restricted antigen presentation pathway. The mammalian uptake of HSP70 is reported to be cell-type specific. Only activated B cells and mononuclear cells can uptake HSP70, whereas activated T cells do not transport HSP70 (52). Although the receptor-mediated uptake of HSP is important for HSP/peptide complex protein vaccines, perhaps it does not play a major role in the gene gun-mediated E7-HSP70 DNA vaccines. It has been shown that direct priming of CD8+ T cells by gene-transfected dendritic cells is the key event in gene gun-mediated DNA immunization (53, 54), whereas cross-priming of DCs is not an major mechanism for gene gun-mediated DNA vaccination (53, 54). It is therefore quite possible that the E7-HSP70 fusion gene was sent directly into DCs via a gene gun, bypassing the need for receptor-mediated endocytosis. However, we cannot completely rule out the possibility of cross-priming because E7-HSP70 might be released from other cell types, such as keratinocytes (which were also transfected by gene gun vaccination), and then enter the DCs via receptor-mediated endocytosis.
The observation that the fusion of HSP70 to E7 enhances E7-specific CD8+ T-cell-mediated immune responses and antitumor effect is consistent with previous reports using malaria peptide (NANP)40 (55), HIV-1 p24 (24), ovalbumin (25), or influenza nucleoprotein (56) as model antigens. The presence of HSP70 makes E7 more immunogenic. It has been shown that CTL responses can be enhanced by adding T-help epitopes to the CTL epitopes (39). If the E7-HSP70 DNA vaccine generates HSP-specific CD4+ T cells, these cells may contribute to the generation and expansion of E7-specific CD8+ T cells. It has been suggested that a high precursor frequency of HSP70-reactive T cells exists due to the continual exposure of the immune system to HSP70 from commensal or pathogenic organisms (40). In this regard, vaccination with HSP70 DNA can further expand the pool of HSP70-reactive T cells. These HSP70-reactive T cells can exert a strong helper effect by reacting to conjugated peptides (41). This may contribute to the increase in E7-specific CD8+ T-cell precursors observed in mice vaccinated with the E7-HSP70 DNA vaccine.

The nonspecific immune response of HSPs probably did not play a major role in the enhancement of the E7 DNA vaccine. It has been demonstrated that HSPs may exert their immune-enhancing effect via a nonspecific response. HSPs may directly activate T cells in vivo and in vitro via an antigen-independent mechanism. Breloer et al. (42) demonstrated that in the absence of antigenic peptides, HSP can induce the secretion of TNF-α and IFN-γ of the antigen-specific CTL clones. Chen et al. (43) showed that the human HSP-60 can act as a danger signal to the innate immune system. HSP60 can induce a T-helper 1 proinflammatory response. However, in our present study, we did not observe a significant increase in the numbers of E7-specific CD4+ T cells in mice vaccinated with HSP70 DNA alone or E7 plasmid mixed with HSP70 plasmids.

γδ T cells may also contribute to the HSP-associated antitumor immunity. Wei et al. (44) demonstrated that γδ T cells could kill the heat-treated autologous tumor cells through recognition of HSP70 on the target cells. Laad et al. (45) also showed that γδ T cells isolated from the peripheral blood of oral cancer patients have the ability to lyse oral tumor cells via recognition of HSP60 on the surface of oral tumor cells. Whether γδ T cells are activated and participate in the antitumor effect generated by E7-HSP70 DNA vaccines needs further investigation.

Although E7-HSP70 generates potent CD8+ T-cell responses through enhanced MHC class I presentation, other constructs that target antigen to MHC class II presentation pathways may provide enhanced CD4+ T-cell responses. This realization raises the notion of coadministration of vaccines that directly enhance MHC class I and class II restricted pathways. We have previously developed a chimeric Sig/E7/LAMP-1 DNA vaccine that uses the LAMP-1 endosomal/lysosomal targeting signal for enhancing the MHC class II presentation pathway of E7 (33). The E7-HSP70 vaccine described here in conjunction with a MHC class II-targeting vaccine such as Sig/E7/LAMP-1 may activate multiple arms of the immune system in a synergistic fashion, leading to significantly enhanced CD4+ and CD8+ T-cell responses and potent antitumor effects.

Although the E7-HSP70 vaccine holds promise for mass immunization, three safety issues need to be resolved. First, the DNA may integrate into the host genome, resulting in the inactivation of tumor suppressor genes or the activation of oncogenes. This may lead to a malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation.
and integration should not pose any real risk (46). The second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei (47). Thus, the presence of E7 in host cells may lead to an accumulation of genetic aberrations and eventual malignant transformation in the host cells. The oncogenicity of E7 can be eliminated by introducing mutations into E7 DNA so that the resulting E7 protein cannot bind with pRB (57) but still maintains most of its antigenicity. The third issue is the concern over the generation of autoimmunity that may be caused when CTL clones specific for mycobacterial HSP cross-react to host HSP. A previous study by Steinhoff et al. (58) demonstrated the induction of intestinal inflammation following transfer of HSP-60-reactive CD8+ T cells into mice. Inflammatory reactions were MHC class I-dependent and developed primarily in the small intestine. In our study, we performed pathological examination of the vital organs in the E7-HSP-vaccinated mice, including the intestines. We did not observe pathology similar to that described in the study by Steinhoff et al. (58).

In summary, our results indicate that the fusion of HSP70 to the HPV-16 E7 gene can generate stronger E7-specific CD8+ T-cell-mediated immune responses and antitumor effects against HPV-16 E7-expressing murine tumors generated by E7 DNA vaccines. Our results indicate that fusion of HSP70 to an antigen gene may greatly enhance the potency of DNA vaccines and can potentially be applied to other cancer systems with known tumor-specific antigens.

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