

# Recombinant Adeno-Associated Virus Expressing Human Papillomavirus Type 16 E7 Peptide DNA Fused with Heat Shock Protein DNA as a Potential Vaccine for Cervical Cancer

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**In this study, we explore a potential vaccine for human papillomavirus (HPV)-induced tumors, using heat shock protein as an adjuvant, a peptide vaccine for safety, and adeno-associated virus (AAV) as a gene delivery vector. The tumor vaccine was devised by constructing a chimeric gene which contained HPV type 16 E7 cytotoxic T-lymphocyte (CTL) epitope DNA (M. C. Feltkamp, H. L. Smits, M. P. Vierboom, R. P. Minnaar, B. M. de Jongh, J. W. Drijfhout, J. ter Schegget, C. J. Melief, and W. M. Kast, *Eur. J. Immunol.* 23:2242–2249, 1993) fused with the heat shock protein gene as a tumor vaccine delivered via AAV. Our results demonstrate that this vaccine can eliminate tumor cells in syngeneic animals and induce CD4- and CD8-dependent CTL activity in vitro. Moreover, studies with knockout mice with distinct T-cell deficiencies confirm that CTL-induced tumor protection is CD4 and CD8 dependent. Taken together, the evidence indicates that this chimeric gene delivered by AAV has potential as a cervical cancer vaccine.**

Cervical carcinoma remains one of the most common malignancies worldwide, with 500,000 new cases diagnosed each year (5) and 200,000 cervical cancer deaths annually (21). Human papillomavirus type 16 (HPV-16) is the predominant etiologic agent of cervical cancer and carries three transforming oncogenes, E5, E6, and E7 (10, 20, 45). Their products are thus unique tumor antigens and can be ideally used as tumor vaccines (7). Because E6 and E7 oncoproteins are consistently retained and expressed, the E6 and E7 oncogenes become more attractive targets for T-cell-based immunotherapy of cervical cancer. Evidence for the value of HPV antigen-directed immunotherapy against cervical cancer comes from the experimental and natural papillomavirus-associated tumors that can be controlled by immunization with E7 antigen. Previous studies have used different modes of immunization, such as (i) recombinant E7 vaccinia viruses (2, 3, 16, 24, 28, 42), (ii) syngeneic cells transfected with E7 (8, 9), (iii) E7 protein-pulsed dendritic cells (12, 37), (iv) peptides corresponding to a cytotoxic T-lymphocyte (CTL) epitope in E7 with incomplete Freund's adjuvant (13), (v) E7 vaccine based on papillomavirus-like particles (17, 25, 31), and (vi) *Salmonella enterica* serovar Typhimurium expressing E7 epitope inserted into hepatitis B virus core (26). These studies demonstrate that CTLs are likely to be the most effective immunological effector mechanisms.

Adeno-associated virus (AAV), a single-stranded virus, has been studied as a vector for gene therapy (29, 41). Classified as a defective human parvovirus, AAV has many natural features that are attractive for a human gene therapy vector, such as nonpathogenicity, targeted integrating capability, and a broad host range (human, simian, murine, canine, and avian). In

sharp contrast to other viral vectors that have been used in vaccination, such as vaccinia virus or adenovirus, AAV vectors do not express any viral genes. The only viral DNA that must be included in an AAV vector is the 145-bp inverted terminal repeat. Since naked DNA is used for immunization, the only gene expressed by AAV vectors is that for the antigen itself.

Since HPV-16 E7 is a transforming oncoprotein (20), dangerous side effects such as transformation are not anticipated with a protein vaccine. Peptide vaccination with a CTL epitope to prevent the outgrowth of a tumor is a safe and effective immunotherapeutic method (13). However, a peptide vaccine combined with a toxic adjuvant such as incomplete Freund's adjuvant sometimes can lead to rapid tumor growth through specific T-cell tolerance induction (36). Recently, *Mycobacterium tuberculosis* heat shock protein 70 (hsp70) has been used as an adjuvant-free carrier to stimulate the humoral and cellular response to a full-length human immunodeficiency virus p24 (33) that is covalently linked to hsp. Mycobacterial hsp as an adjuvant has also been reported to enhance the induction of cellular immunity by an ovalbumin peptide vaccine (34).

In this study, we have accordingly taken advantage of the safety of a peptide vaccine with hsp as an adjuvant and AAV as a gene delivery vector. Thus, we devised the tumor vaccine by constructing a chimeric gene containing the HPV-16 E7 CTL epitope (13, 35) and hsp. The efficacy of protection against tumors was investigated through use of the vector AAV against syngeneic tumors. Results show that intramuscular (i.m.) vaccination with this chimeric gene via AAV vector could efficiently eliminate tumor cells, indicating that vaccination with this gene could be a therapeutic treatment for cervical cancer containing HPV-16 E7.

**Construction and generation of recombinant AAV (rAAV) encoding E7 CTL epitope fused to hsp DNA.** The DNA fragment containing the HPV-16 E7 amino acid 44 to 62 coding sequence (35) was synthesized by PCR using the plasmid HPV-16 E7/pCEP4 as a template, which contained the se-

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quence nucleotide 540 to 885 of HPV-16. The forward primer used in the PCR was 5'-GATGGTgaattcATGCAAGCAGAA CCG-3', which not only spanned the region covering the initiation codon (boldface) and HPV-16 E7 amino acid 44 to 47 coding sequence but also contained an *EcoRI* site (lowercase) at the 5' end. The reverse primer was 5'-AAACCGAAGggt ccGTCACACTTGCA-3', which contained a *BamHI* site (lowercase) immediately after the HPV-16 E7 amino acid 62 coding sequence.

The plasmid vector pKS70 (33), which was created by subcloning the *M. tuberculosis* hsp 70 gene into the expression vector pT7-7 at the *BamHI* and *HindIII* sites, was a gift from Richard A. Young. The E7 epitope PCR product as described above was transferred into pGEM-T vector (Promega, Madison, Wis.) to generate pGEM-T/E7CTL and then subcloned into pKS70 at *EcoRI* and *BamHI* sites to generate pKS70/E7CTL-hsp. The plasmid pKS70/E7CTL-hsp was sequenced by the dideoxynucleotide termination method, and the HPV-16 E7 amino acid 44 to 62 coding sequence was confirmed to be in frame with the hsp70 gene. Then the DNA fragment of E7CTL-hsp was isolated by digesting *EcoRI* and *HindIII* from the plasmid pKS70/E7CTL-hsp, subcloned into SK(+) vector (Stratagene), and named pSK/E7CTL-hsp. The *SmaI* and *XhoI* DNA fragment from pSK/E7CTL-hsp was transferred into pXX-UF1 (23), which contains the green fluorescent protein gene under the regulation of the human cytomegalovirus immediate-early promoter and flanked by AAV 145-bp inverted terminal repeat sequences. The pXX-UF1 plasmid was digested with *PvuII* and *SalI* to remove the green fluorescent protein gene, and then the E7CTL-hsp DNA fragment was placed downstream of the transcriptional regulation of human cytomegalovirus immediate-early promoter and named pXX/E7CTL-hsp.

Preparation of rAAV E7CTL-hsp viral vector was done by cotransfection according to published protocols with modifications (30, 40). Briefly, a total of 49  $\mu\text{g}$  of plasmid DNA (16  $\mu\text{g}$  of pXX/E7CTL-hsp plasmid plus 8  $\mu\text{g}$  of pXX2, which encoded Rep and Cap proteins, and 25  $\mu\text{g}$  of pXX6, which encoded adenovirus gene products) was used to transfect 293 cells in each 15-cm-diameter dish using a modified calcium phosphate precipitation method. Cells from 80 dishes were harvested 48 h posttransfection, then cell mixtures were homogenized, and CsCl was added to a final density of 1.37 g/ml. The virus particles were then purified by CsCl density gradient centrifugation as previously published (39). Titers of rAAV E7CTL-hsp were determined by slot blot analysis to calculate the relative concentration of viral particles. The vector titers were in the range of  $1 \times 10^{12}$  to  $5 \times 10^{12}$  viral particles per ml (data not shown).

**Northern blot analysis of chimeric E7 gene expression through AAV vector in 293 cells.** To determine whether the chimerically constructed E7CTL-hsp gene can be expressed via AAV delivered to cells, we injected  $10^9$  viral particles of rAAV E7CTL-hsp into 293 cells. Two days later, the cellular RNA was extracted and the expression of the E7CTL-hsp chimeric gene was analyzed by Northern blotting. Figure 1 shows that the chimeric E7CTL-hsp RNA (lane 2) could be expressed in rAAV encoding this chimeric construct but not in the control cells which were infected with rAAV *lacZ* (lane 1).

**Efficient transduction of muscle cells with AAV vector.** A previous report demonstrated that AAV vector can deliver the gene efficiently into muscle and establish long-term gene transduction (39). In this study, we introduced this chimeric tumor vaccine into muscle by delivery by AAV vector. Hence, we first tested the efficiency of AAV vectors encoding the *lacZ* reporter gene that harbors a nuclear localization signal under the

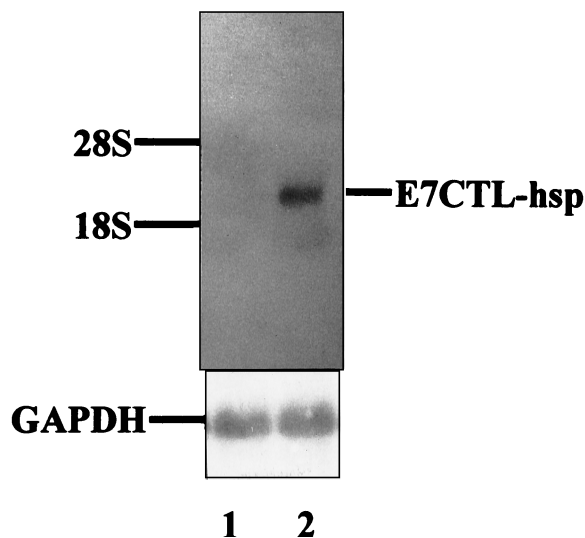


FIG. 1. Northern blot analysis. RNA (20  $\mu\text{g}$ ) was fractionated by agarose gel electrophoresis, blotted, and hybridized with  $^{32}\text{P}$ -labeled E7CTL-hsp DNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA separately. A GAPDH probe was used to ensure equal RNA loading. Lane 1, 293 cells infected with rAAV *lacZ*; lane 2, 293 cells infected with rAAV E7CTL-hsp.

regulation of the cytomegalovirus immediate-early promoter by delivering the vector to muscle. C57BL/6 (*H-2<sup>b</sup>*) mice were anesthetized with 2.5% Avertin intraperitoneally. Thirty microliters of rAAV *lacZ* ( $3 \times 10^{10}$  viral particles) was injected into the hind leg tibialis anterior muscles percutaneously. After 2 weeks, mice were sacrificed, and muscle tissues were cryo-sectioned and stained for  $\beta$ -galactosidase activity, using X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) as a substrate. Figure 2A shows  $\beta$ -galactosidase nuclear staining within most of the myotubes (marked by arrow), indicating efficient gene transduction by the AAV vector.

**Vaccination with rAAV encoding E7CTL-hsp generates tumor elimination.** TC-1 was an E7-expressing tumorigenic cell line. It was established from primary lung epithelial cells of C57BL/6 mice immortalized by HPV-16 E6 and E7 and then transformed with an activated *ras* oncogene (38). To assess treatments of an established tumor, 10 C57BL/6 mice of each group were subcutaneously injected in the left flank with  $5 \times 10^4$  TC-1 or B16F1 cells. B16F1 was a melanoma cell line derived from C57BL/6 mice (ATCC CRL 6322) and served as non-E7 expression syngeneic cells. After 1 week of tumor cell injection, they were immunized with  $5 \times 10^{10}$  viral particles of rAAV encoding E7CTL-hsp, rAAV *lacZ*, or normal saline (mock) into the hind leg tibialis anterior muscles. Then the tumor volume was measured once a week. As shown in Fig. 3, vaccination with rAAV E7CTL-hsp had significantly retarded the tumor growth induced by E7 expression cells (TC-1) but not that induced by non-E7 expression cells (B16F1), while inoculation with rAAV *lacZ* or mock vector had no effect on tumor elimination.

**Cellular immune response in mice immunized with rAAV E7CTL-hsp.** To elucidate the mechanism of protection against TC-1 tumors, we determined whether a CTL response was induced in mice immunized with rAAV encoding E7CTL-hsp. Spleen cells from C57BL/6 mice immunized with rAAV encoding E7CTL-hsp, rAAV *lacZ*, or phosphate-buffered saline were isolated and stimulated in vitro with E7 amino acid 49 to 57 synthetic peptide, which was identified as a CTL epitope (13). These stimulated spleen cells were then tested for recog-



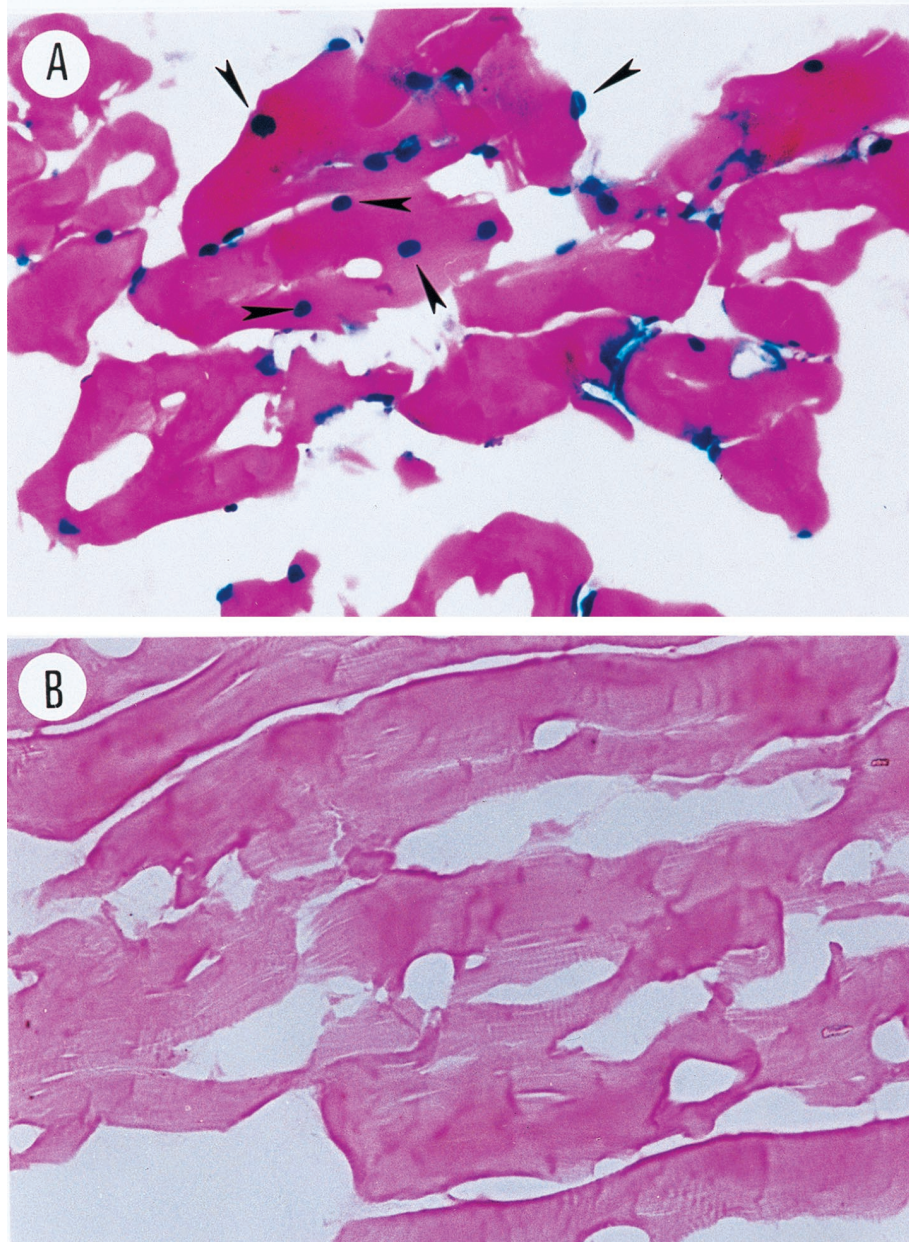


FIG. 2. X-Gal staining of the cryostat sections of the muscle injected with rAAV *lacZ*. (A) Five-week-old mice were injected with rAAV *lacZ* ( $3 \times 10^{10}$  viral particles) in the tibialis anterior muscle. After 14 days, the cryostat sections were stained with X-Gal and eosin. (B) Control mice without rAAV *lacZ* injection.

niton and lysis of  $^{51}\text{Cr}$ -labeled target cells including TC-1 (Fig. 4A); B16F1, which was a syngeneic C57BL/6 cell line (Fig. 4B); B16F1 pulsed with E7 peptide 49 to 57 (Fig. 4C); and B16F1 pulsed with nonspecific peptide HPV-16 E5 peptide 6 to 13 (Fig. 4D). As shown in Fig. 4, TC-1 and syngeneic B16F1 cells loaded with E7 49 to 57 peptide were lysed, whereas B16F1 cells alone or B16F1 cells pulsed with nonspecific peptide were not. The CTL response to TC-1 cells was significantly higher than that to B16F1 cells in the rAAV E7CTL-hsp-vaccinated mice by Student's *t* test ( $P < 0.05$ ), and the CTL lysis activity for E7 peptide-pulsed B16F1 cells was also higher than that for E5 peptide-pulsed cells ( $P < 0.05$ ). Moreover, we assayed for stimulation of E7 44 to 62-specific major histocompatibility complex (MHC) class II restricted proliferation response (35).

Figure 5 shows that spleen cells from mice vaccinated with rAAV E7CTL-hsp could be stimulated by E7 peptide 44 to 62, but cells from rAAV *lacZ*-vaccinated and mock-vaccinated mice could not. Also, the spleen cells from mice vaccinated with rAAV E7CTL-hsp could not be stimulated by nonspecific peptide (E5 peptide 6 to 13 [data not shown]). Taken together, these results indicate that vaccination with rAAV encoding E7CTL-hsp induces CD4- and CD8-dependent CTL response to retard tumor growth.

**Vaccination-induced tumor protection involves both CD4 and CD8 T cells.** To understand the relative roles of CD4 and CD8 T cells in rAAV E7CTL-hsp vaccine-induced tumor protection, mice deficient in CD4 and CD8 T cells as a result of targeted gene disruption at  $\beta 2m$  and *MHC-II*, respectively,

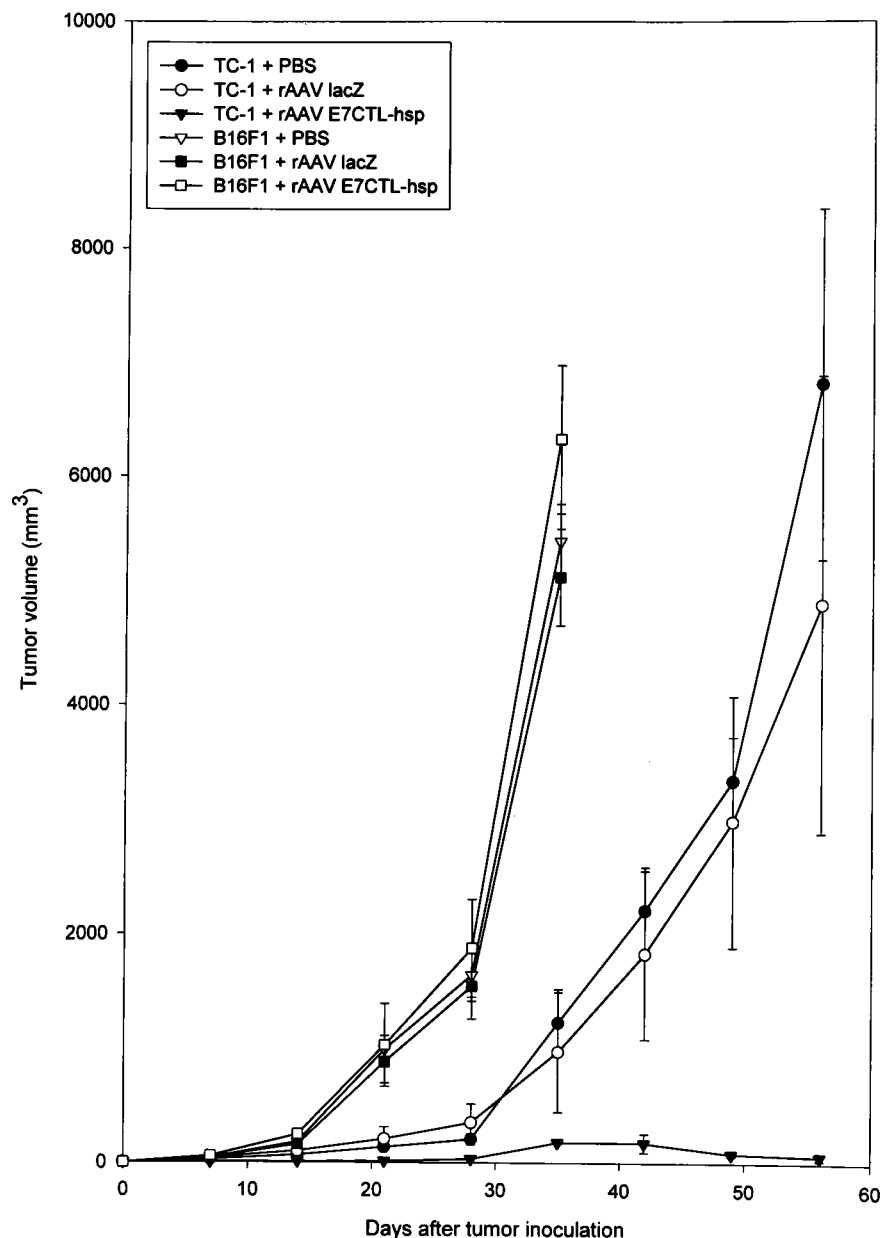


FIG. 3. In vivo tumor elimination assay. Groups of 10 mice were injected subcutaneously with  $5 \times 10^4$  TC-1 or B16F1 tumor cells and, after 1 week, were immunized with  $5 \times 10^{10}$  viral particles of rAAV E7CTL-hsp or rAAV lacZ or phosphate-buffered saline (PBS) (mock). The tumor volume was monitored once a week. The data are represented as means and standard errors of each group.

were studied. The source of CD8 and CD4 T-cell-deficient mice was  $\beta 2m^{-/-}$  and MHC-II $^{-/-}$  mice on a C57BL/6 background, respectively (18, 22, 44). Breeders for  $\beta 2m^{-/-}$  and MHC-II $^{-/-}$  mice were kindly provided by B. J. Fowlkes (National Institutes of Health, Bethesda, Md.), and mice were bred under specific-pathogen-free conditions in the Institute of Molecular Biology Animal Facility of the Academia Sinica. Groups ( $n = 6$ ) of CD4 (KOII) and CD8 (KOI) T-cell-deficient mice were injected with  $5 \times 10^4$  TC-1 cells, followed 1 week later by vaccination with rAAV E7CTL-hsp or control rAAV lacZ. The tumor growth of TC-1 cells in the immunocompromised mice (KOI and KOII) was faster than that in the immunocompetent mice (C57BL/6). We monitored the tumor size in KOI and KOII mice for only 5 weeks after TC-1 cell

injection into the immunocompromised mice as compared to the 8 weeks of observation for the immunocompetent mice (Fig. 3). Figure 6 shows clearly evident tumor growth in both CD4 and CD8 T-cell-deficient groups. It was not statistically significantly different between the rAAV E7CTL-hsp- and rAAV lacZ-vaccinated mice, implying participation of both CD4 and CD8 T cells in our rAAV E7CTL-hsp vaccine-induced tumor protection.

As described above, vaccination with E7 by different strategies can inhibit tumor growth (2, 3, 8, 9, 12, 16, 24, 28, 37, 42). The response to tumor inhibition is mediated by CTLs. It has been reported that this CTL response is CD4 and CD8 dependent (35, 38). However, CTL response induced by virus-like-particle-based E7 vaccine is CD8 dependent but CD4 indepen-

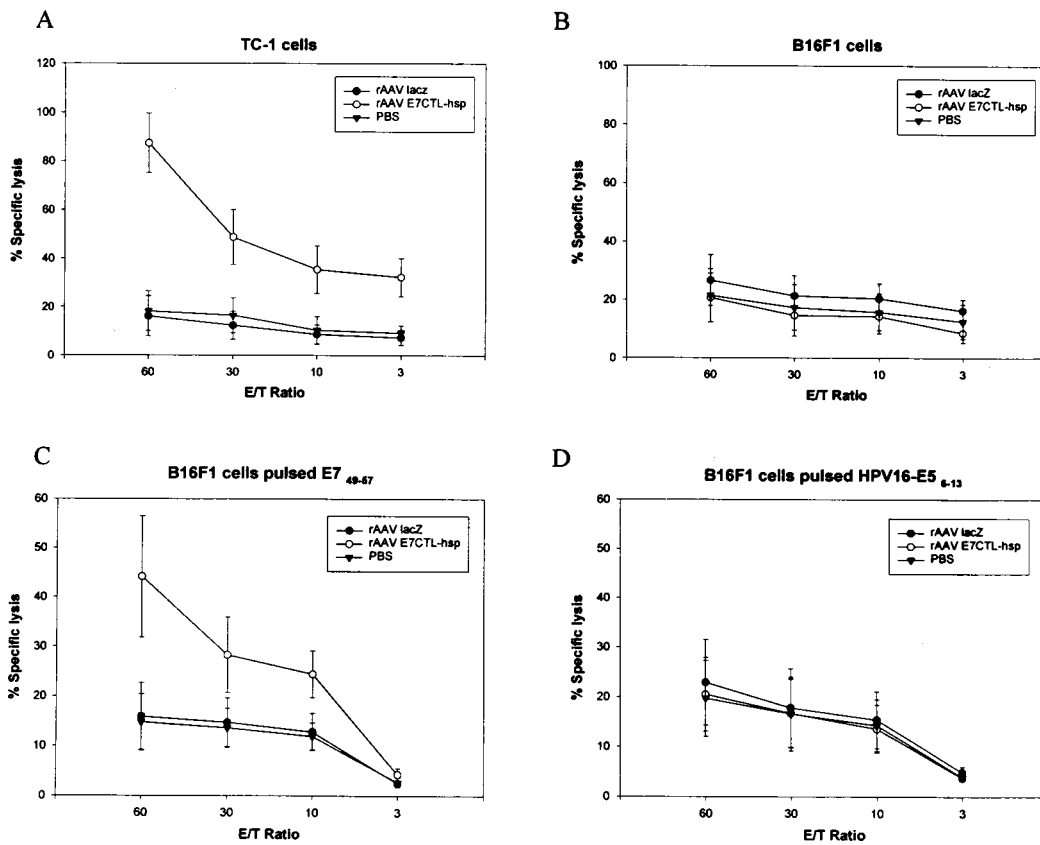


FIG. 4. HPV-16 E7 49 to 57 specific CTL responses induced by immunization with rAAV E7CTL-hsp. C57BL/6 mice were i.m. immunized with rAAV E7CTL-hsp. Four weeks after the vaccination, the spleens were collected and analyzed for in vitro CTL assay. (A) TC-1 cells; (B) B16F1 cells; (C) B16F1 cells pulsed with E7 peptide 49 to 57, (D) B16F1 cells pulsed with HPV-16 E5 peptide 6 to 13. The data were the averages of five vaccinated mice. E/T, effector/target. PBS, phosphate-buffered saline.

dent (17, 25, 37). Our results demonstrate that E7 CTL epitope fused with hsp as a vaccine can enter the MHC class I and class II processing pathways in antigen-presenting cells and stimulate the production of CD8 CTLs. It may be the reason that *M.*

*tuberculosis* hsp 70 is an especially powerful antigen containing multiple B- and T-cell epitopes (34). Previously, *M. tuberculosis* hsp70 has been used as an adjuvant-free carrier to stimulate the humoral and cellular response to a full-length human immunodeficiency virus p24 protein (33) or ovalbumin peptide (34) that is covalently linked to the hsp. The mechanisms by which hsp70 enables covalently linked polypeptide fusion partners to enter into the MHC class I and II antigen-presenting pathway and to elicit CD8 CTLs have been proposed to be (i) hsp70's ability to assist protein folding (15, 43) and to facilitate the translocation of proteins into subcellular compartments (4, 11), (ii) hsp's ability to facilitate the breakdown of intracellular proteins (32), and (iii) the high frequency of T cells directed against mycobacterial hsp70. Our study further supports the idea that hsp can act as an adjuvant to facilitate E7 peptide antigen induction of CD4- and CD8-dependent CTL responses to E7-containing tumors.

Our study demonstrates that AAV vector, which has all viral coding sequences (96% of genome) removed (1, 14, 39), represents a promising alternative for delivering tumor vaccine (27). The first clinical trial of a live recombinant vaccinia virus expressing the E6 and E7 proteins of HPV-16 and -18 has been evaluated elsewhere (2). For gene therapy, AAV vector may be superior to other viral vectors that have been used in vaccination, such as vaccinia virus and adenovirus, since AAV vectors do not express viral genes. As with immunization with naked DNA, the only expressed gene carried by AAV vectors is the cloned gene itself. Using vaccinia virus or adenovirus as a vector to deliver vaccine may offer some advantages in the stimulation of the immune system; however, the advantages

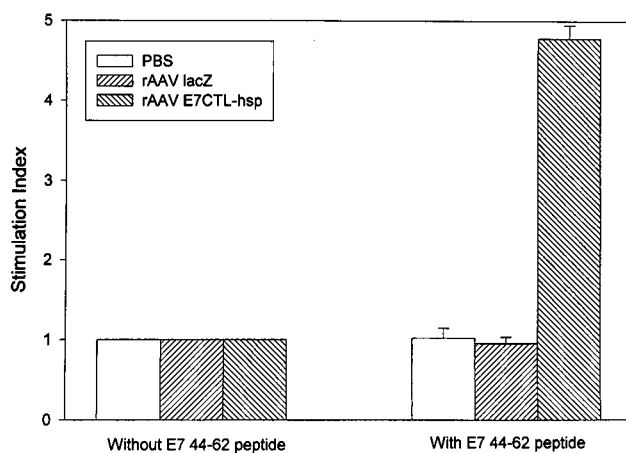


FIG. 5. Proliferation of rAAV E7CTL-hsp-vaccinated lymphocytes in response to E7 peptide 44 to 62. C57BL/6 mice were i.m. immunized with rAAV E7CTL-hsp, rAAV lacZ, or mock. Four weeks after the vaccination, the spleens were collected and analyzed for T-cell proliferation measured by incorporation of [<sup>3</sup>H]thymidine. The data were the averages of five vaccinated mice of each group. The error bars represent standard errors. Stimulation index was defined as the ratio of mean counts per minute of incorporated [<sup>3</sup>H]thymidine for cells with antigen (E7 peptide 44 to 62) to mean counts per minute for cells without any added antigen. PBS, phosphate-buffered saline.

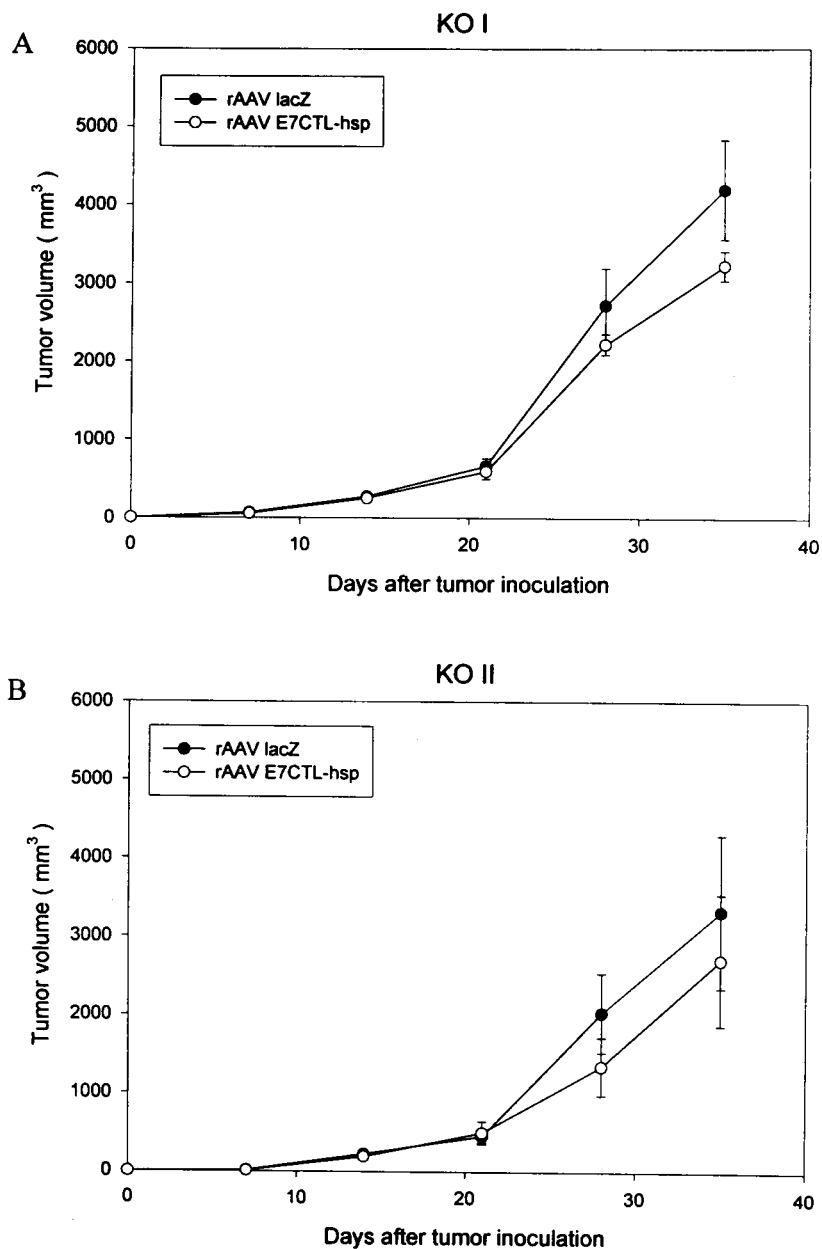


FIG. 6. CD4- and CD8-dependent lymphocytes in tumor eradication by rAAV E7CTL-hsp vaccination. KOI (CD8-deficient) (A) and KOII (CD4-deficient) (B) mice were subcutaneously injected with  $5 \times 10^4$  TC-1 tumor cells. One week later, they were divided into two groups for treatments with  $5 \times 10^{10}$  viral particles of rAAV lacZ or rAAV E7CTL-hsp. Each group consists of six mice. Tumor volume was checked once a week.

are probably outweighed by the significant risks associated with virus infection, especially for immunodeficient patients (6, 19). In addition, it was previously reported that AAV vector does not induce strong cellular immune responses to the transduced cells, allowing the persistence of gene expression. The gene expression in skeletal muscle transduced by AAV has been shown to persist for more than 1.5 years (39). Although AAV vector can elicit a humoral immune response which results in neutralizing activity after a second administration, repeated dosing appears not to be necessary given that the originally transduced cells can escape a CTL response and persist in the long term. AAV may thus be useful for the viral immunization of humans. Up to now, for vaccination, AAV vectors represent the combination of the best properties of viral and nonviral

vectors. Recently, a study of vaccine development targeted at herpes simplex virus infection has demonstrated that AAV-mediated immunization can prime specific CTL and antibody responses (27). Hence, our study provides a potential vaccine for HPV-induced tumors.

In this study, we successfully developed rAAV encoding HPV-16 E7 CTL peptide DNA fused with hsp DNA as a tumor vaccine. It is a potential vaccine for cervical cancer treatment using hsp as a carrier protein and delivery by rAAV vector.

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