Cervical cancer is one of the leading cancers in the world. Human papillomavirus type 16 (HPV-16) is the predominant type of virus identified in cervical cancers. It carries three transforming oncogenes—E5, E6, and E7 (24, 65). Thus, they are unique tumor antigens and serve as ideal materials for a tumor vaccine. Because E6 and E7 oncoproteins are consistently retained and expressed, these two oncogenes are attractive targets for T-cell-based immunotherapy of cervical cancer. Previous studies have used different modes of E6 and/or E7 immunization to both experimental and natural papillomavirus-associated tumors, such as (a) recombinant vaccinia viruses expressing HPV-16 E6 and E7; (b) recombinant adenovirus- associated tumors, such as (a) recombinant vaccinia viruses expressing HPV-16 E6 and E7; (b) recombinant adenovirus encoding HPV-16 E6 and E7 (21). Hence, in premalignant lesions, including SILs and condylomata, when E5 is still expressed, using E5 as a vaccine to target E5-expressing cells may be a good strategy to prevent premalignant lesions from progressing into invasive cervical cancers. However, the potential of E5 protein as a tumor vaccine candidate has not been identified. Hence, in this study, we evaluated the HPV-16 E5 protein delivered by an adenovirus vector as a tumor vaccine for cervical lesions. The results demonstrate that a single intramuscular injection of the recombinant adenovirus carrying the HPV-16 E5 gene into syngeneic animals can reduce the growth of tumors which contain E5 gene expression. Moreover, the E5 vaccine-induced tumor protection occurs through CD8 T cells but not through CD4 T cells in in vitro assays. In addition, our studies using knockout mice with distinct T-cell deficiencies confirm that cytotoxic T-lymphocyte-induced tumor protection is CD8 dependent but CD4 independent. Hence, HPV-16 E5 can be regarded as a tumor rejection antigen.

**MATERIALS AND METHODS**

**Construction of recombinant adenovirus vector containing HPV-16 E5 gene.** To generate replication-deficient recombinant viruses carrying the HPV-16 E5 gene, we isolated a 0.3-kb BamHI fragment from HPV-16 E5/pCEP4 which contained the influenza virus hemagglutinin 1 (HA1) epitope tagged at the 5' terminal end of the HPV-16 E5 gene and ligated it with pAdECMV/pA (30), and it was named pXCMVHA16E5 (Fig. 1A). The HA1 epitope tag is an 11-amino-acid sequence (Met-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser) from the HA1 protein of influenza virus, against which a highly reactive monoclonal antibody was raised. After restriction enzyme mapping, a plasmid containing the E5 gene was cotransfected with pJM17 into 293 cells to generate recombinant viruses (30). The genomic structure of the recombinant adenoviruses containing the E5 gene was confirmed by PCR. For large-scale virus production, the recombinant viruses were harvested from 20 plates of 293 cells grown on a P-150 dish after 36 h of infection and subjected to two cycles of CsCl gradient ultracentrifugation (31). After overnight dialysis, the stock of viruses was aliquoted and stored at −80°C until use. The average titers of viral stocks were determined by a plaque assay in triplicate.

**Cells.** TC-1 is an E6- and E7-expressing tumorigenic cell line which came from primary lung epithelial cells of C57BL/6 mice immortalized by HPV-16 E6 and E7 and then transformed with an activated ras oncogene (57). To establish a C57BL/6 syngeneic mouse tumor model containing the E5 gene, TC-1 cells were transected using a liposome method with the HPV-16 E5/pHOOK plasmid, which contained the HPV-16 E5 gene with a S'-tagged HA1 epitope in the plasmid vector pHOOKs (Invitrogen, Carlsbad, Calif.). The E5 gene expression was driven by a minimal human cytomegalovirus (hCMV) early promoter. Three or 4 weeks following transfection, at least 80 to 100 Kaezin-resistant colonies were selected, pooled, and named TC-1/E5. TC-1 and TC-1/E5 cells were maintained...
in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin-streptomycin (50 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (2 mM), G418 (0.4 mg/ml), and hygromycin (0.2 mg/ml). They were grown at 37°C in a 5% CO2 atmosphere.

Animals. C57BL/6 (H-2b) mice were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and maintained in our institute under specific-pathogen-free conditions. The mice were used at 7 to 10 weeks of age. Knockout I (KO I) mice are devoid of functional CD8+ T lymphocytes, which fail to express MHC-I molecules on the cell surface and thus are virtually devoid of functional CD8+ T lymphocytes.

Northern blot analysis. Total cytoplasmic RNA was prepared from cells and analyzed by Northern blot hybridization. Filters were washed to remove nonspecifically bound probes, air dried, and then exposed to Kodak XAR film with Dupont Lightning-plus intensification screens.

Analysis of E5 protein expression. Cell extracts were prepared as described previously (9). An antibody to the HA1 epitope (ATCC 12CA5) was added to the extract, which was then incubated for 2 h at 4°C. After rotation at 4°C for 2 h, 50 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia) in TBS-BSA (10 mM Tris-HCl pH 7.4; 165 mM NaCl; 0.1% [wt/vol] bovine serum albumin) was added, and the mixture was rotated again for 45 min at 4°C. The beads were pelleted and washed five times with cold radioimmunoprecipitation buffer with 2% [vol/vol] Triton X-100 and 1% sodium dodecyl sulfate (SDS–15% PAGE). Gels were subjected to immunoblot analysis with HA1 antibody. Then, they were electrophoresed by SDS-PAGE and analyzed with the HA1 antibody by using Western blotting.

RESULTS

Expression of HPV-16 E5 protein in the adenovirus-transduced E5 gene. The plasmid pXCMVHA16E5, which carries the HPV-16 E5 gene, was constructed by inserting the E5 gene into the adenovirus vector pAdE1CMV/pA (Fig. 1A). The replication-defective recombinant adenoviruses (rAd-E5) were generated as described in Materials and Methods. E5 gene expression in rAd-E5 was monitored by Northern blot analysis and immunoprecipitation or Western blot analysis. Figures 2A and B, lane 1, show E5 RNA and E5 protein in TC-1/E5 cells, respectively, but not in TC-1/V cells (lane 2, containing the vector only).

Vaccination with rAd-E5 generates tumor prevention and protection functions against challenge with TC-1/E5 tumor cells. To assess the degree of prevention of tumor cell growth, 10 C57BL/6 mice of each group were vaccinated with 5 × 1010 PFU of either rAd-E5, rAd-lacZ, or PBS (mock) i.m. One week after vaccination, the mice were injected s.c. with 5 × 104 TC-1/E5 or TC-1 tumor cells. The tumor volume was measured once a week. As shown in Fig. 3, vaccination with rAd-E5 significantly retarded TC-1/E5 cell-induced tumor development.

Cell-mediated lymphocyte cytotoxicity. Cell-mediated cytotoxicity was measured using a 51Cr release assay and performed using standard protocols (57). Splenocytes were harvested from mice that were vaccinated with rAd-E5, rAd-lacZ, or PBS 2 weeks previously. The splenocytes were cocultured with mitomycin-C-treated TC-1/E5 cells or a combination of E5 peptides which cover the whole E5 protein (stimulators) (5, 21, 29) for 6 days. Various numbers of stimulator-treated splenocytes (effector) were added to 105 α25CrO4-labeled target cells (TC-1/E5) in 100 μl of culture medium in 96-well U-bottom plates. After a 4-h incubation at 37°C, 25 μl of culture supernatant was collected for gamma radiination counting. To characterize the roles of CD4 and CD8 T lymphocytes in E5-induced cytotoxicity the anti-CD4 monoclonal antibody (GK1.5) or anti-CD8 monoclonal antibody (2.43) was mixed with effector cells, respectively, before being added to target cells in a final concentration of 50 μg/ml to block CD4+ or CD8+ T lymphocytes. The mean percentage of specific lysis of triplicate wells was calculated as follows, where cpm is counts per minute; % specific lysis = [(cpm of experimental release − cpm of spontaneous release) (cpm of maximum (1% Triton X-100) release − cpm of spontaneous release)] × 100%.
ment while inoculation of rAd-lacZ or PBS had no effect, but it could not prevent TC-1 cell-induced tumor growth.

To evaluate the tumor treatment effect of the rAd-E5 vaccination, 10 C57BL/6 mice of each group were injected s.c. with $5 \times 10^4$ TC-1/E5 cells. One week following the tumor cell injection, they were immunized i.m. with $5 \times 10^1$ PFU of either rAd-E5, rAd-lacZ, or PBS (mock). Then, the tumor volume was measured once a week. As shown in Fig. 4, vaccination with rAd-E5 significantly eliminated TC-1/E5 cell-induced tumor growth while inoculation of rAd-lacZ or PBS had no effect, but it could not reduce TC-1 cell-induced tumor growth.

From the data of Fig. 3 and 4, we observed small-volume tumors in the E5-vaccinated mice which might express no or low E5. This may be the reason that E5-specific CTLs cannot eradicate them. In the future, we will look into the differential gene expression of E5 in the tumor and investigate the cytolytic effects by vaccination.

Cellular immune response in mice immunized with rAd-E5. To elucidate the mechanism of protection against TC-1/E5 tumors, we determined whether a CTL response was induced in rAd-E5-immunized mice. Spleen cells from C57BL/6 mice were isolated and stimulated in vitro with mitomycin-treated TC-1/E5 cells (Fig. 5C and D) or the combination of E5 peptides which cover the whole E5 protein (Fig. 5A and B) (5, 21, 29). These stimulated spleen cells were then tested for recognition and lysis of $^{51}$Cr-labeled target cells, including the TC-1/E5 tumor cells expressing the E5 gene (Fig. 5A and C) and B16F1, which was a syngeneic C57BL/6 cell line lacking E5 gene expression (Fig. 5B and D). As shown in Fig. 5, spleen cells from rAd-E5-immunized animals had CTL activity to lyse TC-1/E5 target cells (Fig. 5A and C) but not B16F1 cells (Fig. 5B and D). Cells from rAd-lacZ- or mock-immunized mice had no effect. In addition, since the HA1 epitope tagged the 5’ end of the E5 gene, we also assayed CTL activity by using the HA1 peptide as a stimulator in E5-vaccinated mice to rule out the possibility that the response was induced by HA1 instead of E5. Figure 5E and F show that HA1-specific T cells could not lyse TC-1/E5 and B16F1 cells, respectively. Taken together, it is evident that rAd-E5 vaccine-induced tumor protection is through E5-specific CTL cells.

CD8-dependent immunity on tumor protection by vaccination with rAd-E5. To understand the relative roles of CD4 and CD8 T cells in rAd-E5 vaccine-induced tumor protection, mice deficient in CD4 and CD8 T cells as a result of targeted gene disruption at β2m and MHC-II, respectively, were studied. The sources of CD8 and CD4 T-cell-deficient mice were β2m−/− mice on a C57BL/6 background, respectively (23, 32, 64). β2m−/− mice were kindly provided by B. J. Fowlkes (National Institutes of Health, Bethesda, Md.) and were bred under specific-pathogen-free conditions. Groups (n = 6) of CD4 (KO II) and CD8 (KO I) T-cell-deficient mice were injected with $5 \times 10^4$ TC-1/E5 cells, followed by vaccination with either rAd-E5 or control rAd-lacZ 1 week later. Figure 6 shows evident tumor growth in CD8 T-cell-deficient groups, but not in CD4 T-cell-deficient mice.
Furthermore, we blocked CD8 T cells or CD4 T cells by coculturing effector cells with anti-CD8 or anti-CD4 antibody, respectively, and assayed the in vitro CTL response by rAd-E5-immunized mice. As shown in Fig. 7, the lysis of E5-stimulated splenocytes (effector cells) to target cells (TC-1/E5) significantly dropped when effector cells were cocultured with anti-CD8 antibody, but not with anti-CD4 antibody or PBS (mock). Taken together, these data suggest that CD8 T cells, but not CD4 cells, participate in rAd-E5 vaccine-induced tumor reduction.

**DISCUSSION**

This is the first demonstration that HPV-16 E5 can be regarded as a tumor vaccine to suppress tumor growth. Previous studies have reported that recombinant vaccinia virus expressing the E5 gene of bovine papillomavirus type 1 (BPV-1) can immunize against BPV-1 tumor cells (43), but vaccination with the recombinant vaccinia virus expressing the HPV-16 E5 protein fails to influence tumor development (42). Such a failure to eradicate tumors by using a vaccinia virus delivery system may be due to the fact that they cannot detect the E5 gene expression in tumor cells, or perhaps the vaccinia virus, unlike the adenovirus, cannot assist the E5 protein to enter the MHC-I or -II pathway for antigen presentation. However, our study manifests that vaccination with rAd-E5 can reduce the growth of tumors via CTL activity. While investigating the roles of CD4 and CD8 T lymphocytes in rAd-E5 vaccine-induced tumor protection, we found that CD8 knockout mice vaccinated with rAd-E5 lost tumor-reducing activity, but CD4 knockout mice did not lose tumor-reducing activity (Fig. 6). This was further confirmed by an in vitro E5-specific CTL assay using incubation with anti-CD4 or anti-CD8 antibody to block CD4 or CD8 cell function (Fig. 7). Our observation means that CTL activity is caused only by CD8 T cells activated by vaccination with the rAd-E5, and not by CD4 T cells.

In this study, we demonstrated that E5 vaccine delivered by adenovirus vectors can induce tumor reduction. The potential for tumor vaccine development using adenovirus vectors has been explored widely. Previous studies have shown that mice vaccinated with a recombinant adenovirus encoding the tumor-specific antigen p815A present on mouse mastocytomas can induce an anti-p815A CTL response (55) and eradicate tumors. A recombinant adenovirus encoding β-Gal, administered with exogenous interleukin-2 (IL-2), can lead to a reduction of an established β-Gal-expressing CT26 murine colorectal cancer (8). Similarly, immunization with a recombinant adenovirus encoding the melanoma-associated antigen (gp100) can protect mice from intradermal challenge with murine B16 melanoma cells via CD8 T cells (61). In addition, an adenovirus vector as a vaccine against virus challenges has also been developed. For example, cattle immunized with a recombinant adenovirus encoding the structural proteins of the foot-and-mouth disease virus (FMDV) develop booster responses against challenge with wild-type FMDV virus (8). The ability of adenoviruses to efficiently deliver recombinant DNA vaccines may be due to their ability to escape from the MHC-I pathway for antigen presentation, allowing for efficient uptake by antigen-presenting cells (8).
mouth disease virus can produce significant protection against viral challenge (48). In mice, protection has been demonstrated against subsequent challenge by a variety of viruses by prior immunization with an appropriate recombinant adenovirus-mediated viral gene expression. Examples of such viruses include rabies virus (46), tick-borne encephalitis virus (27), rotavirus (2), herpes simplex virus (19), murine hepatitis virus (56), measles virus (15, 16), and simian immunodeficiency virus (SIV) (14). All these studies demonstrate that an adenovirus vector can help a transgene elicit a CTL response in mice against antigen-specific tumors (8, 55, 61) and induce both humoral and cellular immunity against subsequent virus challenges (2, 14–16, 19, 27, 46, 56).

In this study, we chose a single injection of rAd-E5 for vaccine delivery. Recombinant adenoviruses are efficient carriers for vaccination, as described above (26, 62). It is usually not efficient to reintroduce an adenovirus vector for a booster response. This is mainly due to the adenovirus-induced neutralizing antibodies which are directed against the fiber and hexon of adenovirus in infected mice (12, 59), rats (39), cotton rats (60), and rhesus monkeys (28) and which can particularly affect secondary entry and delivery of the vector. But, no adenovirus immunity to transgene expression has been reported. However, one recent report showed that preexisting immunity to the adenovirus does not prevent antitumor protection following intratumoral administration of an IL-12-expressing adenovirus vector (4). Thus, the influence of immunogenicity from the adenovirus on vaccine efficacy is still mysterious. But if humoral immune responses reveal certain limitations of the adenovirus vectors that may affect its potency and readministration for gene therapy of cancer, then single immunization may overcome this booster effect, in which a neutralizing anti-adenovirus antibody abolishes the vector-directed gene expression (16, 18).

The importance of HPV as a necessary but insufficient component in the development of cervical cancers has been well established (24, 65). Numerous cofactors can explain the imbalance between the very high prevalence of HPV infection and the relatively low incidence of anogenital cancers in the United States (17, 44). The high prevalence of HPV-associated SILs in human immunodeficiency virus (HIV)-infected individuals implies that the host immune response may play a significant role in the development of HPV-associated cancers (40, 52). The higher rates of HPV infection and SILs in HIV-infected women are thought to be attributed specifically to a decrease in CD4 T cells that causes the immune system to be impaired (33, 40, 47, 52, 54). HIV infection adversely affects the synthesis of Th1 cytokines by CD4 T cells, but not gamma interferon (INF-γ) synthesis by CD8 T cells of women with active HPV infection (34). The increase in IFN-γ γ+ CD8 T cells is a phenotype consistent with CTLs. These unaffected INF-γ γ+ CD8 T cells are less likely to be HPV specific as there is a higher incidence of HPV-related cervical SIL for HIV-positive, HPV-positive women than for HIV-negative, HPV-positive women (54). In this study, we demonstrated that the E5 vaccine-induced CTL response is CD8 dependent but CD4 independent. Accordingly, HIV patients with higher HPV loads have CD8 T-cell counts similar to those of healthy women but lack CD4 T cells. Thereafter, E5 as a therapeutic vaccine may have the capacity to stimulate CD8 cells into E5-specific CTLs to eradicate E5-expressing dysplasia cells; thus, it may have a higher chance of preventing SILs progressing into invasive cervical cancers in both HPV infection alone and HPV-HIV infection.

In summary, our study demonstrates that a single i.m. injection of recombinant adenovirus carrying the HPV-16 E5 gene into syngeneic animals could reduce tumor growth. It also shows that the E5 vaccine-induced tumor protection is through a CD8-dependent and CD4-independent CTL response. Hence, HPV-16 E5 can be regarded as a tumor rejection antigen.

ACKNOWLEDGMENTS

We are grateful to T. C. Wu for providing TC-1 cells, B. J. Fowlkes for providing β2m−/− and MHC-II−/− mice, and Judy Perry for proofreading the manuscript. This work was supported by National Science Council grant NSC 87-2312-B106-003.

REFERENCES

9. Conrad, M., V. J. Bubb, and R. Schlegel. 1993. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate...