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 oncoproteins 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 (1, 3, 20, 35, 42, 63), (b) recombinant adeno-associated virus type 5 genes 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.

The potential of the E5 protein as a tumor vaccine candidate has not been explored yet. In this study, we evaluate the human papillomavirus type 16 (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 a 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.
in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin-
streptomycin (50 U/ml), l-glutamine (2 mM), sodium pyruvate (1 mM), noness-
tial 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 Breed 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
T lymphocytes. The mean percentage of specific lysis was calculated as follows, where cpm is counts per minute; % specific lysis =

\[ \frac{\text{cpm of experimental release} - \text{cpm of spontaneous release}}{\text{cpm of maximum (1% Triton X-100 release)} - \text{cpm spontaneous release}} \] \times 100%.

RESULTS

Expression of HPV-16 E5 protein in the adenovirus-trans-
duced E5 gene. The plasmid pXCMVA1E65, 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. Figure 1B shows that only rAd-E5-infected
cells could express the 12.5-kDa E5 protein.

Establishment of E5 syngeneic cells. We have established
C57BL/6 syngeneic cells containing the E5 gene (TC-1/E5) as
described in Materials and Methods. E5 gene expression in
TC-1/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, respec-
tively, but not in TC-1/V cells (lane 2, containing the vector
only).

Vaccination with rAd-E5 generates tumor prevention and
protection 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 \times 10^{10}
PFU of either rAd-E5, rAd-lacZ, or PBS (mock) i.m. One
week after vaccination, the mice were injected s.c. with 5 \times 10^4
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 develop-
ment 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 \mu g/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 =

\[ \frac{\text{cpm of experimental release} - \text{cpm of spontaneous release}}{\text{cpm of maximum (1% Triton X-100 release)} - \text{cpm spontaneous release}} \] \times 100%.

of TC-1/E5 cells (lane 1), but not in TC-1/V cells (lane 2), by immunoprecipitation
and Western blot analysis with the HA1 antibody.
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 × 10⁴ TC-1/E5 or TC-1 tumor cells. Then, the tumor volume was monitored once a week. The data are the means and standard errors of each group.

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 immunized with either rAd-E5, rAd-lacZ, or PBS 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 ⁵¹Cr-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⁻/⁻ and MHC-II⁻/⁻ mice on a C57BL/6 background, respectively (23, 32, 64). β2m⁻/⁻ and MHC-II⁻/⁻ 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 × 10⁴ 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 (MDV) showed reduced viremia, as well as the dissemination of MDV to lymph nodes and the lungs (59).
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 eliciting 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 a single immunization may overcome this booster effect, in which a neutralizing antiadenovirus 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+ CD8 T cells is a phenotype consistent with CTLs. These unaffected INF-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 (34). 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.

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FIG. 7. CTL response is CD8 dependent and CD4 independent. C57BL/6 mice were i.m. injected with rAd-E5. Two weeks after the vaccination, the splenocytes were collected and cocultured with anti-CD4 antibody, anti-CD8 antibody, or PBS and then were analyzed by an in vitro CTL assay. Target cells and stimulators were TC-1/E5 cells and mitomycin-treated TC-1/E5 cells, respectively. The data are from three independent experiments.


