Fusion Protein Vaccine by Domains of Bacterial Exotoxin Linked with a Tumor Antigen Generates Potent Immunologic Responses and Antitumor Effects

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Abstract

Antigen-specific immunotherapy represents an attractive approach for cancer treatment because of the capacity to eradicate systemic tumors at multiple sites in the body while retaining the requisite specificity to discriminate between neoplastic and nonneoplastic cells. It has been shown that certain domains of bacterial exotoxins facilitate translocation from extracellular and vesicular compartments into the cytoplasm. This feature provides an opportunity to enhance class I and/or II presentation of exogenous antigen to T lymphocytes. We investigated previously whether the translocation domain (domain II) of Pseudomonas aeruginosa exotoxin A with a model tumor antigen, human papillomavirus type 16 E7, in the context of a DNA vaccine could enhance vaccine potency. We then attempted to determine whether this chimeric molecule could also generate strong antigen-specific immunologic responses and enhance the potency of cancer vaccine in the protein format. Our results show that vaccination with the PE(ΔIII)-E7-KDEL3 fusion protein enhances MHC class I and II presentation of E7, leading to dramatic increases in the number of E7-specific CD8+ and CD4+ T-cell precursors and markedly raised titers of E7-specific antibodies. Furthermore, the PE(ΔIII)-E7-KDEL3 protein generates potent antitumor effects against s.c. E7-expressing tumors and pre-established E7-expressing metastatic tumors. Further, mice immunized with PE(ΔIII)-E7-KDEL3 protein vaccine also retained long-term immunologic responses and antitumor effects. Our results indicate that retrograde-fusion protein via the delivery domains of exotoxins with an antigen greatly enhances in vivo antigen-specific immunologic responses and represents a novel strategy to improve cancer vaccine potency.

Introduction

Cervical cancer is the second leading cause of death in women worldwide, with an annual global incidence of ~500,000 cases, of which about one-third are fatal (1). Human papillomavirus (HPV) has been recognized as the primary cause of cervical cancer, and HPV DNA can be detected in ~90% of all cases (2). HPV 16, which is the most prevalent strain, is detected in >50% of cervical cancer patients (3). The E7 protein interacts with the retinoblastoma tumor suppressor protein, impairing its function as a cell growth regulator (4). Because E7 expression is closely associated with neoplasia, and several lines of evidence suggest that cell-mediated immunity is important for control of both HPV infection and HPV-associated neoplasia, the E7 protein is considered a potential target for cancer therapy.

Ideally, cancer treatment should eradicate systemic tumors at multiple sites in the body while having the specificity to discriminate between neoplastic and nonneoplastic cells. In this regard, activation of antigen-specific T-cell-mediated immune responses permits elimination of tumors associated with a specific antigen (5, 6). Recently, many strategies, such as vaccines based on peptides (7, 8), proteins (9, 10), DNA (11–13), naked RNA (14, 15), and recombinant viruses (16, 17), have emerged to be applied in the development of cancer treatment and immunotherapy. Protein-based vaccines are reportedly capable of generating CD8+ T-cell responses in vaccinated humans (18, 19). However, one of the limitations of peptide and protein-based vaccines is their potency due to poor immunogenicity, especially against some tumor antigens, such as E7 (20). Development of novel strategies that enhance protein vaccine potency is important for generation of effective cancer vaccines and immunotherapies. One potential strategy for enhancement of antigen presentation through the MHC class I pathway to CD8+ T cells is the use of the translocation features of certain bacterial toxins, such as exotoxin (21). Exotoxins are one of a class of several secreted bacterial toxins that are able to covalently modify particular proteins in mammalian cells through the translocation of bacterial toxin. Exotoxin domain II is responsible for translocation to the cytosol (22), and its has been used to engineer a chimeric multidomain protein to deliver DNA into the cytosol (23, 24). Our previous work has shown that a DNA vaccine encoding domain II of an exotoxin linked to the model antigen HPV 16 E7 enhances MHC class I presentation of E7 antigen to CD8+ T cells and thereby vaccine potency (11). The aim of this study was to test if the retrograde-delivery domains, including the I, II, and COOH-terminal KDEL domains of exotoxin A of Pseudomonas aeruginosa [PE(ΔIII)-KDEL3], could also enhance antigen-specific immunologic response and improve vaccine potency when linked with the same E7 antigen in the fusion protein format. We also attempted to distinguish any differences between the antitumor mechanisms of PE(ΔIII)-KDEL3 when linked with the E7 antigen in naked DNA and protein formats.

Our data indicate that vaccination with the PE(ΔIII)-E7-KDEL3 fusion protein enhances MHC class I and II presentation of E7, leading to a dramatic increase in the numbers of E7-specific CD8+ and CD4+ T-cell precursors and raised titers of E7-specific antibodies. Furthermore, the PE(ΔIII)-E7-KDEL3 fusion protein...
vaccine generated potent antitumor effects against s.c. E7-expressing tumors and preestablished E7-expressing metastatic lung tumors. Further, mice immunized with PE(ΔIII)-E7-KDEL3 fusion protein vaccine also contained long-term immunologic responses and antitumor effects. These results indicate that fusion protein by exotoxin domains with an antigen greatly enhances in vivo antigen-specific immunologic response and represents a novel strategy for improvement of vaccine potency.

Materials and Methods

Preparation of various DNA constructs. E7 is a gene encoding a 98-amino acid nucleocapsid protein in HPV 16 (HPV NC001526). The gene sequence was obtained from the National Center Biotechnology Information Web site.4 The modified E7 polynucleotide sequence, with additional EcoRI-SalI and XhoI restriction sites at two site arms, was produced by running multiple-step PCR. The PCR products were purified using 5% polyacrylamide gel; then, the bands corresponding to each product were cut out for elution. The E7 PCR fragment was inserted into pET and pPE(ΔIII) vectors as detailed in our previous reports (25, 26). The E7 fragment was inserted into the EcoRI/XhoI cloning site of the pET15 or pET15-PE(ΔIII) plasmid to generate the plasmid encoding E7 or PE(ΔIII)-E7 (named pE7 and pETA-E7, respectively). For increased antigenicity, the pE7-E7-encoded E7 dimer was generated by insertion of an E7 DNA fragment cutting with SalI and PstI from the pE7 into another pET containing the E7 DNA fragment and cutting with XhoI and PstI. The DNA fragment containing the polynucleotide encoding EcoRI-SalI restriction enzyme site, KDEL3-ΔKDEL-KDEL-stop codon-XhoI sequence (named pKDEL3 encoding KDEL3 signal), was generated using PCR with the primers: 5’-AGAATTCCGTGACACCTCAAAAAAGACGAACTGAGAGATGAACTG-3’ (forward primer) and 5’-GTCGGTGGCTGCTGAGATTACAGTTCGTCTTTCAGTTCATCTCTCAGTT-3’ (reverse primer). These two primers were able to anneal at the complement sequences of the forward and reverse primers 5’-AAGAGCTAGATGACGTAAGC-3’ and 5’-GAGGCTACCATTCATCTCACTCTCAGTT-3’ (reverse primer). The KDEL3 DNA fragment could be generated by running PCR reactions without an additional template. The PCR product of the KDEL3 sequence was further inserted into pET23 using the recombinant technique. For generation of PE(ΔIII)-E7-KDEL3, the DNA fragment containing pKDEL3 was cut with PstI and XhoI and then cloned into the PstI and XhoI sites of pETA-E7. The accuracy of all of the constructs was confirmed by DNA sequencing.

Generation and preparation of various protein vaccines. To induce the expression and production of the various recombinant proteins, the host-vector system in Escherichia coli BL21(DE3)plys was used as described previously (25). The proteins were then purified under the His-Tag system in the denatured condition according to the manufacturer’s manual (Novagen). The denatured samples in 8 mol/L urea were loaded into a column packed with a NTA-Ni2+-bound agarose resin. The bound proteins were then eluted with multiple-step PCR. The PCR products were purified using 5% polyacrylamide gel; then, the bands corresponding to each product were cut out for elution. The E7 PCR fragment was inserted into pET and pPE(ΔIII) vectors as detailed in our previous reports (25, 26). The E7 fragment was inserted into the EcoRI/XhoI cloning site of the pET15 or pET15-PE(ΔIII) plasmid to generate the plasmid encoding E7 or PE(ΔIII)-E7 (named pE7 and pETA-E7, respectively). For increased antigenicity, the pE7-E7-encoded E7 dimer was generated by insertion of an E7 DNA fragment cutting with SalI and PstI from the pE7 into another pET containing the E7 DNA fragment and cutting with XhoI and PstI. The DNA fragment containing the polynucleotide encoding EcoRI-SalI restriction enzyme site, KDEL3-ΔKDEL-KDEL-stop codon-XhoI sequence (named pKDEL3 encoding KDEL3 signal), was generated using PCR with the primers: 5’-AGAATTCCGTGACACCTCAAAAAAGACGAACTGAGAGATGAACTG-3’ (forward primer) and 5’-GTCGGTGGCTGCTGAGATTACAGTTCGTCTTTCAGTTCATCTCTCAGTT-3’ (reverse primer). These two primers were able to anneal at the complement sequences of the forward and reverse primers 5’-AAGAGCTAGATGACGTAAGC-3’ and 5’-GAGGCTACCATTCATCTCACTCTCAGTT-3’ (reverse primer). The KDEL3 DNA fragment could be generated by running PCR reactions without an additional template. The PCR product of the KDEL3 sequence was further inserted into pET23 using the recombinant technique. For generation of PE(ΔIII)-E7-KDEL3, the DNA fragment containing pKDEL3 was cut with PstI and XhoI and then cloned into the PstI and XhoI sites of pETA-E7. The accuracy of all of the constructs was confirmed by DNA sequencing.

Preparation and vaccination of protein vaccines. The stock E7, PE(ΔIII), PE(ΔIII)-E7, and PE(ΔIII)-E7-KDEL3 were diluted with PBS (1:10) 10 times and incubated for 2 hours in 37°C. The activated proteins were further mixed with different buffers (pH 8.0, 7.0, 6.5, 6.0, 5.0, 4.0, and 3.5) containing 4 mol/L urea, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl, and 20 mmol/L phosphate buffer. After purification, the protein elution fractions were analyzed for purity and quantification by SDS-PAGE analysis as described previously (26).

Immunologic response of E7 and PE(ΔIII)-E7-KDEL3 proteins by titration assay. Protein titration assays were done to test the ability of the E7 and PE(ΔIII)-E7-KDEL3 proteins to induce the E7-specific immunologic response. The E7-specific CD8+ T cell line (kindly provided by Dr. T.C. Wu, Johns Hopkins University, Baltimore, MD) was used for the effector cells. Mice marrow–derived dendritic cells (BMDC) were generated from murine bone marrow cells in the presence of granulocyte macrophage colony-stimulating factor as described previously (15). The BMDCs pulsed with various concentrations of E7 peptide (amino acids 49-57), E7 protein, or PE(ΔIII)-E7-KDEL3 fusion protein and incubated at 37°C for 16 hours were used as feeder cells. Then, the E7-specific CD8+ T cells and peptide or protein-pulsed BMDCs were cocultured for a further 24 hours. Golgistop was added 6 hours before harvesting the cells from the culture. Cell surface marker staining for CD8+ and intracellular cytokine staining for IFN-γ as well as FACScan analysis were done using conditions described previously (27).

Tumor cell line. The production and maintenance of TC-1 cells has been described previously (28). On the day of tumor challenge, tumor cells were harvested by trypsinization, washed twice with 1× HBSS, and finally resuspended in 1× HBSS to the designated concentration for injection.

Mice. Six- to 8-week-old female C57BL/6j mice were purchased from the National Taiwan University (Taipei, Taiwan) and bred in the animal facility of the National Taiwan University Hospital. All animal procedures were done according to approved protocols and in accordance with recommendations of the Committee for the Proper Use and Care of Laboratory Animals.

Cell surface marker staining and flow cytometric analysis. In the first experiment, mice were immunized with 0.1 mg/mouse E7, PE(ΔIII), PE(ΔIII)-E7, or PE(ΔIII)-E7-KDEL3 with 10% ISA206 adjuvant as described earlier. These animals were then boosted s.c. 1 and 2 weeks later using the same regimen. One week after the last immunization, the mice were sacrificed and the splenocytes were prepared as described previously (29). Before intracellular cytokine staining, 3.5 × 105 pooled splenocytes from each vaccinated group were incubated for 16 hours with either 1 μg/mL E7 peptide (amino acids 49-57) containing a MHC class I epitope (30) for detecting E7-specific CD8+ T-cell precursors or 10 μg/mL E7 peptide (amino acids 30-67) containing a MHC class II epitope (16) for detecting E7-specific CD4+ T-cell precursors. Cell surface marker staining for CD8+ or CD4+ and intracellular cytokine staining for IFN-γ as well as FACScan analysis were done as described earlier.

In the second experiment, mice were immunized with PE(ΔIII)-E7-KDEL3 fusion protein vaccine once, twice, or thrice as described earlier. One week after the last immunization, the mice were sacrificed and the splenocytes were prepared, stained, and analyzed as described earlier.

In the third experiment, the mice were immunized with PE(ΔIII)-E7-KDEL3 thrice as described earlier. The mice were then sacrificed 7, 14, 21, 30, or 60 days after the last vaccination. The splenocytes were prepared, stained, and analyzed as described earlier.

ELISA for anti-E7 antibody. In the first experiment, mice were vaccinated with various protein vaccines (five animals per group) a total of three times as described earlier. Sera were prepared from the mice 14 days after the last immunization. E7-specific antibody titers were detected by direct ELISA as described previously (16).

In the second experiment, mice were immunized s.c. with PE(ΔIII)-E7-KDEL3 fusion protein once to thrice. Sera were prepared 14 days after the last immunization. E7-specific antibody titers were also detected using ELISA.

In vivo tumor protection experiments. For the first experiment, mice were vaccinated s.c. with 0.1 mg of various protein vaccines (five animals per group) and then boosted 1 and 2 weeks later using the same regimen. One week after the last vaccination, the mice were challenged with 5 × 104 TC-1 tumor cells by s.c. injection in the right leg. Naive mice received the same number of TC-1 cells to assess natural tumor growth control. Tumor growth was monitored by visual inspection and palpation twice weekly until 60 days after the tumor challenge.

For the second experiment, mice were vaccinated with PE(ΔIII)-E7-KDEL3 fusion protein once to thrice. One week after the last vaccination, the mice were challenged with 5 × 104 TC-1 tumor cells injected s.c. Tumor growth was monitored as described.

For the third experiment, the mice were vaccinated with PE(ΔIII)-E7-KDEL3 fusion protein a total of three times. The mice were then challenged.
with $5 \times 10^4$ TC-1 tumor cells injected s.c. 7, 30, or 60 days after the last vaccination. Tumor growth was monitored as described earlier.

**In vivo antibody depletion experiments.** In **vivo** antibody depletions were done as described previously (31). Briefly, mice (five per group) were vaccinated s.c. with 0.1 mg/mouse of PE(ΔIII)-E7-KDEL3 fusion protein, boosted 1 and 2 weeks later, and challenged with $5 \times 10^3$ TC-1 tumor cells per mouse 1 week after the last immunization. Depletion was started 1 week before tumor challenge, with monoclonal antibodies GK1.5, 2.43, and PK136 used for CD4+, CD8+, and NK1.1 depletion, respectively. Depletion was terminated on day 40 after the tumor challenge.

**In vivo tumor treatment experiments.** In **vivo** tumor treatment experiments were done using a previously described lung hematogenous spread model (14). In the first experiment, the animals (five per group) were challenged with $5 \times 10^4$ TC-1 tumor cells per mouse via the tail vein. Two days after tumor challenge, the animals received 0.1 mg/mouse of E7, PE(ΔIII), PE(ΔIII)-E7, or PE(ΔIII)-E7-KDEL3 protein vaccines s.c. every 7 days for 2 more weeks (a total of three times; 0.3 mg protein). Unvaccinated mice were used as negative controls. The animals were sacrificed and their lungs were explanted on day 30. Pulmonary tumor nodules in each mouse were evaluated and enumerated by experimenters blinded to sample identity.

In the second experiment, mice were challenged with TC-1 tumor cells and then received 0.1 mg PE(ΔIII)-E7-KDEL3 protein once to thrice as described earlier. The animals were sacrificed on day 30 and pulmonary tumor nodules were evaluated and enumerated as described earlier.

**Statistical analysis.** All data are expressed as mean ± SE. Statistical Package for Social Sciences software (SPSS 9.0, SPSS, Inc., Chicago, IL) was used for ANOVA to compare the experimental groups. Differences were considered significant if $P < 0.05$.

**Results**

**Generation and characterization of the PE(ΔIII)-E7-KDEL3 fusion protein vaccine.** A schematic of the domains of full-length exotoxin and the constructs for E7, chimeric PE(ΔIII)-E7, and PE(ΔIII)-E7-KDEL3 are presented in Fig. 1A. The SDS-PAGE of PE(ΔIII), PE(ΔIII)-E7, PE(ΔIII)-E7-KDEL3, and E7 dimer are shown in Fig. 1B.

PE(ΔIII)-E7-KDEL3 fusion protein stimulates E7-specific CD8+ T cells secreting IFN-γ more efficiently than E7. Firstly, we evaluated the relative efficacy of the cross-priming effects of PE(ΔIII)-E7-KDEL3 and E7. E7-specific CD8+ T cells were cocultured with BMDCs pulsed with various concentrations of E7 or PE(ΔIII)-E7-KDEL3 protein, stained with the anti-IFN-γ antibody, and analyzed with flow cytometry as described in Materials and Methods. As shown in Fig. 2, there were more IFN-γ-secreting CD8+ T cells where high to low concentrations of PE(ΔIII)-E7-KDEL3 fusion protein were used for pulsing in comparison with the E7 protein.

Our results show a more potent E7-specific CD8+ T-cell response with PE(ΔIII)-E7-KDEL3 fusion protein compared with E7 protein.

PE(ΔIII)-E7-KDEL3 fusion protein increases the quantities of E7-specific CD4+ and CD8+ T-cell precursors and raises the titers of the anti-E7 antibodies. The ability of the PE(ΔIII)-E7-KDEL3 fusion protein to influence in vivo E7-specific immunologic responses was then evaluated. The numbers of E7-specific CD4+ and CD8+ T-cell precursors for each group are presented in Fig. 3A. The number of E7-specific IFN-γ-secreting CD4+ T-cell precursors in the PE(ΔIII)-E7-KDEL3 group was the highest of all the vaccinated and naive groups (Fig. 3B). Further, the number of E7-specific IFN-γ-secreting CD8+ T-cell precursors in the PE(ΔIII)-E7-KDEL3 group was also higher than all the other groups (Fig. 3C). Mice vaccinated with PE(ΔIII)-E7-KDEL3 fusion protein generated ~20- and 40-fold increases in the number of E7-specific

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**Figure 1.** Chimeric PE(ΔIII)-E7-KDEL3 DNA construct and characterization of PE(ΔIII)-E7-KDEL3 fusion protein expression. A, schematic diagram showing the constructs for full-length exotoxin and PE(ΔIII), PE(ΔIII)-E7, and PE(ΔIII)-E7-KDEL3-3 genes. B, SDS-PAGE for various proteins. Lane 1, bovine serum albumin; lane 2, PE (exotoxin); lane 3, PE(ΔIII); puJ9; lane 4, PE(ΔIII)-E7; lane 5, PE(ΔIII)-E7-KDEL3; lane 6, E7-E7, E7 dimer.
IFN-γ/CD4+ and IFN-γ/CD8+ T-cell precursors compared with mice vaccinated with the E7 protein. We further evaluated whether the PE(ΔIII)-E7-KDEL3 fusion protein could also enhance the anti-E7 antibody titer. The animals vaccinated with the PE(ΔIII)-E7-KDEL3 fusion protein generated the highest sera anti-E7 antibody titers compared with the other vaccinated groups (Fig. 3D).

The data for our intracellular cytokine staining and anti-E7 antibodies showed that PE(ΔIII)-E7-KDEL3 fusion protein can enhance various E7-specific immunologic responses. Vaccination with PE(ΔIII)-E7-KDEL3 fusion protein generates tumor protection in mice challenged with E7-expressing tumor cells. In vivo tumor protection experiments were done to determine if the observed enhancement in E7-specific CD4+ and CD8+ T-cell–mediated immunity translated to a significant E7-specific antitumor effect. All the mice receiving PE(ΔIII)-E7-KDEL3 fusion protein remained tumor-free 60 days after the TC-1 challenge (Fig. 4A). In contrast, the unvaccinated E7, PE(ΔIII), and PE(ΔIII)-E7 groups developed tumors within 15 days of the tumor challenge. Further, all of the mice receiving E7 mixed with PE(ΔIII) protein also suffered tumorigenesis (data not shown).

CD4+ T, CD8+ T, and natural killer cells are essential for the antitumor effect generated by the PE(ΔIII)-E7-KDEL3 fusion protein vaccine. To determine the subset of lymphocytes that are important for the rejection of E7-positive tumor cells, in vivo antibody depletion experiments were done. As shown in Fig. 4B, all naive mice and all those depleted of CD8+ T cells grew tumors within 14 days of tumor challenge. Further, all mice depleted of CD4+ T cells grew tumors within 40 days of challenge. Forty percent of the animals depleted of NK1.1 cells grew tumors within 60 days of tumor challenge. By contrast, all of the nondepleted mice remained tumor-free for 60 days after the tumor challenge.

These results suggest that CD4+ T, CD8+ T, and natural killer (NK) cells are all essential for the antitumor immunity generated by the PE(ΔIII)-E7-KDEL3 fusion protein vaccine.

Treatment with PE(ΔIII)-E7-KDEL3 fusion protein leads to significant reduction in pulmonary tumor nodules in C57BL/6 mice. The therapeutic potential of each protein vaccine was assessed by performing an in vivo tumor treatment experiment using a previously described lung hematogenous spread model (29). The representative figures of pulmonary tumor nodules for various protein-vaccinated groups are shown in Fig. 4C. The mean numbers of pulmonary tumor nodules for mice treated with PE(ΔIII)-E7-KDEL3 fusion protein were also significantly lower than those for the animals treated with the E7 protein (Fig. 4D).

These results indicate that PE(ΔIII)-KDEL3-to-E7 fusion is required for antitumor immunity and prevention of E7-expressing TC-1 tumor cells and that treatment with PE(ΔIII)-E7-KDEL3 fusion protein could control established E7-expressing tumors in the murine lung.

Multiple immunization of PE(ΔIII)-E7-KDEL3 fusion protein enhances E7-specific immunologic responses. We further evaluated whether the number of immunizations with PE(ΔIII)-E7-KDEL3 protein would influence generation of E7-specific immunologic responses. The numbers of IFN-γ-secreting CD4+ and CD8+ T-cell precursors and the titers of anti-E7 antibodies increased significantly in mice vaccinated with PE(ΔIII)-E7-KDEL3 fusion protein once to thrice (Fig. 5A-C).

Our data showed that increasing numbers of vaccinations with PE(ΔIII)-E7-KDEL3 fusion protein can also enhance E7-specific immunologic responses.

Further immunization with PE(ΔIII)-E7-KDEL3 protein vaccine improves antitumor effects in mice. In vivo prevention and treatment experiments using mice immunized once to thrice with PE(ΔIII)-KDEL3 fusion protein were done (as described earlier) to evaluate if the antitumor effects were reflected in the E7-specific immunologic profiles of the different subgroups. All of the naive mice and the animals immunized once with the PE(ΔIII)-KDEL3 fusion protein grew tumors within 14 days of the tumor challenge. By contrast, however, 60% or 100% of the mice immunized twice or thrice, respectively, were tumor-free 60 days after the tumor challenge (Fig. 5D).

Similar results were also observed in the tumor treatment experiments. The number of pulmonary tumor nodules decreased significantly after one to three injections of PE(ΔIII)-KDEL3 fusion protein (Fig. 5E).
Our results indicate that multiple PE(ΔIII)-KDEL3 fusion protein vaccinations are needed to generate the effectively preventative and therapeutic antitumor effects against E7-expressing tumor cells. Mice immunized with PE(ΔIII)-E7-KDEL3 fusion protein vaccine retain long-term immunologic responses and antitumor effects. Finally, the mice were immunized with PE(ΔIII)-E7-KDEL3 fusion protein vaccine and long-term immunologic response was evaluated by comparing the various immunologic assays (7-60 days after immunization) as described in Materials and Methods. The number of E7-specific CD4+ and CD8+ T-cell precursors peaked at 7 days after immunization (Fig. 6A). However, the number of E7-specific CD4+ T-cell precursors decreased gradually, whereas the numbers of E7-specific CD8+ T-cell precursors dropped dramatically 14 days after immunization. The titers of E7-specific antibodies increased from 14 days after immunization, with this elevation persisting as far out as 60 days after immunization (Fig. 6C).

We further tested the relationship between long-term immunologic response and in vivo antitumor effect. Mice were immunized with PE(ΔIII)-E7-KDEL3 fusion protein thrice and then challenged with TC-1 tumor cells 7, 30, or 60 days after the last immunization. All of the mice receiving the PE(ΔIII)-E7-KDEL3 fusion protein 7 or 30 days after, when challenged with TC-1, remained tumor-free 60 days later (Fig. 6D). Only 60% of mice receiving the PE(ΔIII)-E7-KDEL3 fusion protein 60 days after their last immunization remained tumor-free 60 days after TC-1 challenge.

Our results show that PE(ΔIII)-E7-KDEL3 fusion protein induces various E7-specific immunologic responses that persist for different periods after immunization. Additionally, PE(ΔIII)-E7-KDEL3 fusion protein seems to protect mice from tumor growth, at least partially, for an extended interval after vaccination.

Discussion

Our protein vaccine represents one successful example of using the translocation domains of a bacterial toxin for development of cancer vaccine and immunotherapy. Previous study has employed the translocation domain of an exotoxin linked to a targeted protein to facilitate the entry of protein and DNA into the cytosol, whereas the truncated forms of this chimeric protein that lacks the translocation domain failed to facilitate efficient protein or DNA transfer (23). This indicates that the bacterial domain may serve as a useful tool for introduction of exogenous protein into the cytosol, although the precise mechanism of such translocation remains unclear. The domain II of exotoxin without the KDEL signaling transducer is enough to link to a DNA-binding protein and facilitate DNA entry into the cytosol (23, 24). In our study, however, PE(ΔIII)-E7 protein without the KDEL signaling transducer did not significantly enhance antigen-specific immunologic response (Fig. 3). Naked DNA vaccine functions without the KDEL signaling transducer (11). Thus, it seems reasonable to suggest that protein-format vaccine needs the KDEL signaling transducer to facilitate the transfer of DNA into the cytosol.
processing and presentation of E7 antigen protein in vivo. The KDEL signal enhances protein retention in the endoplasmic reticulum (32) and/or controls glycosylation of the therapeutic proteins for antitumor effect (33). These may account for the fact that KDEL signaling is required for the protein vaccine to enhance the processes of antigen processing and presentation.

The PE(DIII)-E7-KDEL protein enhances MHC class I presentation of E7 in cells expressing this fusion protein to enhance E7-specific CD8+ T-cell activity in vivo. One of the potential explanations is that linkage of PE(DIII) to E7 may lead to enhanced stimulation of E7-specific CD8+ T cells in vivo via the so-called cross-priming mechanism. The PE(DIII)-E7-KDEL protein mixed with ISA206 adjuvant may facilitate antigen uptake, transport, and presentation by antigen-presenting cells (APCs; ref. 34) and lead to antigen uptake and processing by these APCs via the MHC class I–restricted pathway (35). Indeed, in our in vitro assays, we found that BMDCs pulsed with PE(DIII)-E7-KDEL fusion protein presented E7 through the MHC class I pathway more efficiently than BMDCs pulsed with the E7 protein (Fig. 2). These PE(DIII)-E7-KDEL protein-phagocytosed APCs may directly enhance E7 presentation through the MHC class I pathway to CD8+ T cells and contribute to the generation of E7-specific CD8+ T-cell precursors in vivo.

Another important factor for the enhancement of antigen-specific CD8+ T-cell activity and the antitumor effect generated by PE(DIII)-E7-KDEL3 protein may be CD4+ T-cell–mediated immunity. It is clear that CD4+ T cells play a major facilitative role in B-cell antibody production. More recently, it has become evident that CD4+ T-helper cells also control induction and maintenance of CD8+ T cells (36) and help other CD4+ T cells by a process of linked
T-cell help (37). The pivotal position of CD4+ T-helper cells has stimulated our focus on their importance in protein vaccination and motivated design of fusion proteins for induction of activating Th responses (38). In addition to the helper effect of CD4+ T cells for CD8+ CTLs, the former can also induce direct killing of tumor cells (39, 40). Thus, the role of E7-specific CD4+ T cells for CD8+ CTLs, the former can also induce direct killing of tumor cells (39, 40). Thus, the role of E7-specific CD4+ T cells for CD8+ CTLs, the former can also induce direct killing of tumor cells (39, 40).

The success of the PE(ΔIII)-E7-KDEL3 fusion protein vaccine, as shown by the exotoxin retrograde-delivery domains component, suggests that consideration of strategies that use the domains of other bacterial toxins to enhance vaccine potency is warranted. Several studies have investigated the retrograde-delivery domains for several bacterial toxins, including diphtheria toxin (41), clostridial neurotoxins (42), anthrax toxin lethal factor (43), Shiga toxin (44), E. coli heat-labile toxin (45), Yersinia cytotoxins (46), Listeria toxin (47), and pertussis adenylate cyclase toxin (48). It will be interesting to discover whether these bacterial toxins can also generate comparatively potent vaccine effects when linked with the HPV 16 E7 antigen.

The mechanisms underlying the antitumor effects of the available vaccine formats differ. We tested previously the bacterial toxin PE(ΔIII) strategy in a mammalian expression DNA vector (pDNA; ref. 11). We found that naked DNA or protein vaccine enhanced E7-specific CD8+ T-cell-mediated immune responses and antitumor effects. Our studies have shown that CD8+ T cells are important for both naked DNA and protein vaccines. By contrast, CD4+ T cells are essential only for the antitumor effect generated by the protein vaccine. Depletion of CD4+ T or NK cells did not lead to any significant loss in antitumor effect in mice vaccinated with naked DNA vaccine (11). However, depletion of CD4+ T cells led to a complete loss of antitumor effect in mice vaccinated with the

Figure 5. Immunologic profiles and antitumor effects for mice vaccinated with PE(ΔIII)-E7-KDEL3 fusion protein. Mice were immunized with one, two, or three injections of PE(ΔIII)-E7-KDEL3 fusion protein and splenocytes were prepared as described in Materials and Methods. A, number of IFN-γ-secreting CD4+ T cells in the presence (white columns) or absence (black columns) of the corresponding E7 peptide (amino acids 30-67). The numbers of IFN-γ-secreting CD4+ T-cell precursors increased significantly in mice with increasing numbers of vaccinations with PE(ΔIII)-E7-KDEL3 protein (22.5 ± 4.2, 102.0 ± 8.5, and 232.5 ± 17.7 for one, two, and three injections, respectively; P < 0.001, ANOVA). B, number of IFN-γ-secreting CD8+ T-cell precursors in the presence (white columns) or absence (black columns) of the corresponding E7 peptide (amino acids 49-57). The number of IFN-γ-secreting CD8+ T-cell precursors also increased in mice vaccinated with PE(ΔIII)-E7-KDEL3 protein once to thrice (38.5 ± 7.8, 96.0 ± 8.5, and 547.5 ± 21.5 for one, two, or three injections, respectively; P < 0.001, one-way ANOVA). C, ELISA shows E7-specific antibodies in mice vaccinated with one, two, or three injections of PE(ΔIII)-E7-KDEL3 fusion protein. The titers of anti-E7 antibodies in the sera of the vaccinated mice also increased as the number of immunizations increased (1:100 dilution: 0.917 ± 0.045, 1.912 ± 0.061, and 3.593 ± 0.45 for one, two, and three injections, respectively; P < 0.01, one-way ANOVA). D, in vivo tumor protection experiments using mice vaccinated with one, two, or three injections of PE(ΔIII)-E7-KDEL3 fusion protein. Mice were immunized with PE(ΔIII)-KDEL3 fusion protein once, twice, or thrice and challenged with TC-1 tumor cells as described in Materials and Methods. All of the naive mice and those immunized only once with PE(ΔIII)-KDEL3 fusion protein vaccine grew tumors within 14 days of the tumor challenge. However, 60% and 100% of the animals immunized twice and thrice, respectively, with the PE(ΔIII)-KDEL3 fusion protein vaccine were tumor-free 60 days after tumor challenge. E, in vivo tumor treatment experiments using mice vaccinated with one, two, or three injections of PE(ΔIII)-E7-KDEL3 protein. Mice were challenged with TC-1 tumor cells and subsequently treated with PE(ΔIII)-KDEL3 fusion protein once, twice, or thrice as described in Materials and Methods. The number of pulmonary tumor nodules decreased significantly comparing subgroups treated with PE(ΔIII)-KDEL3 fusion protein vaccine (103.0 ± 8.8, 28.8 ± 6.1, and 0.6 ± 0.4 for one, two, or three injections, respectively; P < 0.001, one-way ANOVA).
PE(ΔIII)-E7-KDEL3 fusion protein. Thus, the CD4+ T cells were important for the antitumor effect generated by the PE(ΔIII)-E7-KDEL3 fusion protein because the vaccine actively induced E7-specific CD4+ T cells (Fig. 4), suggesting that CD4+ T cells contribute to the antitumor effect via an antigen-specific mechanism. Further, we discovered that NK cells also play a role in the antitumor effect generated by the PE(ΔIII)-E7-KDEL3 fusion protein but not the naked DNA vaccine. It seems reasonable to suggest, therefore, that different types of vaccines encoding the same construct may activate different subsets of effector cells in the vaccinated host and have specific immunologic or antitumor mechanisms.

We observed that E7-specific antibody titers and the number of E7-specific CD4+ T-helper cells were significantly enhanced by PE(ΔIII)-E7-KDEL protein vaccination. We propose that the mechanism for enhancement of antibody responses operates via the pathway of CD4+ T-helper cells. Although it has not been shown that antibody-mediated responses play an important role in controlling HPV-associated malignancies, antigen-specific antibodies are significant in other tumor models, such as breast cancer, with the HER-2/neu antigen. It has also been shown that HER-2/neu-specific antibodies induce growth arrest where high levels of HER-2/neu are expressed on the surface of breast and ovarian cancer cells (49, 50). The fusion protein vaccine strategy by linked with the domains of P. aeruginosa exotoxin A may also be used to generate HER-2/neu antibodies for treatment of other cancers.

Protein vaccines can eliminate the concern associated with DNA vaccines of DNA integration. Our previous studies have shown that many chimeric E7 DNA vaccines linked to E7 hold promise for mass immunization in the murine model (11–13, 29, 51). Certain safety issues still need to be resolved, however.

Figure 6. Kinetic immunologic responses and in vivo tumor protection experiments using mice immunized with the PE(ΔIII)-E7-KDEL3 fusion protein vaccine. Mice were immunized with PE(ΔIII)-E7-KDEL3 fusion protein thrice, and the splenocytes and sera of the vaccinated mice were then collected 7, 14, 21, 30, or 60 days after the last vaccination to detect the immunologic profiles as described in Materials and Methods. A, number of E7-specific CD4+ T-cell precursors. The numbers of E7-specific CD4+ T-cell precursors decreased gradually from day 7 to day 60 after immunization (264.5 ± 7.5, 233.5 ± 19.5, 198.5 ± 12.5, 169.5 ± 6.5, and 19.0 ± 2.0 on days 7, 14, 21, 30, and 60, respectively). B, number of E7-specific CD8+ T-cell precursors. The numbers of E7-specific CD8+ T-cell precursors dropped dramatically 14 days after immunization (524.0 ± 26.5, 94.0 ± 12.0, 73.0 ± 10.5, 54.0 ± 6.0, and 12.5 ± 1.5 on days 7, 14, 21, 30, and 60, respectively). C, titers of E7-specific antibodies from ELISA. The E7-specific antibodies of the PE(ΔIII)-E7-KDEL3 fusion protein vaccinated mice were significantly enhanced from 14 days after immunization, with the effect persisting as far out as 60 days (1:100 dilution: 1.040 ± 0.045, 3.451 ± 0.027, 3.621 ± 0.014, 3.505 ± 0.020, and 3.070 ± 0.058 on days 7, 14, 21, 30, and 60, respectively). D, in vivo tumor protection experiments. Mice were immunized with PE(ΔIII)-E7-KDEL3 fusion protein thrice and challenged with TC-1 tumor cells 7, 30, or 60 days after the last immunization as described in Materials and Methods. All of the mice receiving the PE(ΔIII)-E7-KDEL3 fusion protein 7 or 30 days later, when challenged with TC-1, remained tumor-free 60 days after the TC-1 challenge. In the group receiving the PE(ΔIII)-E7-KDEL3 fusion protein 60 days later, when challenged with TC-1, 60% remained tumor-free 60 days after the TC-1 challenge.
Notably, the concern that DNA may integrate into the host genome, although it is estimated that the frequency of such integration is much lower than that of spontaneous mutation and should not, therefore, pose a significant risk (52). The concern with respect to DNA integration is avoided with protein vaccines. The PE(ΔIII)-E7-KDEL3 fusion protein vaccine may be used for future human clinical trials into prevention of HPV 16 infection and therapy for HPV 16–related cervical cancer. The PE(ΔIII)-E7-KDEL3 fusion protein could induce long-term protective antitumor effects as well as antigen-specific T-cell and antibody memory responses. Immunologic memory, the most important consequence of adaptive immunity, is the capacity of the immune system to respond more rapidly and effectively to antigens that have been encountered previously. It reflects the preexistence of a clonally expanded population of antigen-specific lymphocytes.

Memory T cells are increased in frequency and have distinct activation requirements and cell-surface proteins that distinguish them from effector T cells (53, 54). Secondary and subsequent responses to the antigens are mediated solely by memory lymphocytes and not by naive lymphocytes (55). In terms of future clinical utilization of the PE(ΔIII)-E7-KDEL3 fusion protein vaccine, knowledge of the capacity of vaccines to induce these memory responses and the frequency and phenotype of the generated effector and central memory lymphocytes is important.

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References

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