CTL Responses to a DNA Vaccine Encoding E7 Gene of Human Papillomavirus Type 16 from an Iranian Isolate

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Abstract

Background: Cervical cancer is the most prevalent tumor in developing countries and the second most frequent cancer among female population worldwide. Specific human papillomaviruses and, most notably, HPV types 16 and 18 are recognized as being causally associated with cervical carcinomas. The early HPV type 16 genes, E6 and E7, directly participate in the in vitro transformation of primary human keratinocytes and represent an excellent target for immune therapy of HPV related disease. Objective: The aim of this study was the evaluation of the efficacy of a DNA vaccine containing human papillomavirus type 16 E7 gene (Iranian isolate) in induction of CTL responses in an animal model. Methods: In this study, the expression vector containing HPV type 16 E7 gene was constructed and chosen as a model antigen in the development of a therapeutic DNA vaccine in an animal model. CTL responses, cytokine assay, lymphocyte stimulation test, CD4 and CD8 staining and flow cytometry were done for evaluating the immune responses. Results: Our findings indicate that the target DNA vaccine can induce an E7-specific CTL response, which is important in the lysis of infected tumor cells, compared to negative control (p<0.005) after in vivo immunization in the mouse system. Conclusion: The developed vaccine may be promising as an anti-cancer vaccine.

Keywords: Human papillomavirus type 16, E7 gene, Immune responses, DNA vaccine

INTRODUCTION

Papillomaviruses are small DNA viruses with a double stranded circular genome of about 8 kbp (1).
HPV infection is currently the most common sexually transmitted disease worldwide. HPV infection and HPV-associated cervical and other anogenital cancers are significant public health problems. The clinical spectrum of disease ranges from asymptomatic infection to benign warts (primarily low-risk types 6 and 11 (2, 3), to invasive malignancy (4, 5, 6). Over 70% of cervical cancers are associated with the high-risk genotypes 16 and 18 (3). The HPV E7 and E6 oncogenes are constitutively expressed in HPV infected cells and interact with different cellular proteins known to be involved in the control of the cell cycle and DNA repair, most notably the tumor suppressor proteins p53 and pRb (7, 8). Recent efforts have focused on targeting of E7 and/or E6 proteins that may provide an opportunity to prevent and treat HPV-associated cervical malignancies. The continued expression of the E7 ORF in malignant carcinomas makes E7 a potential vaccine candidate for HPV-associated cervical disease (6). The first prophylactic HPV virus-like particle (VLP) vaccine against HPV types 6/11/16/18 was licensed in 2006 for girls and women aged 9-26 years (8). These vaccines are almost 100% effective in preventing infection and in high-grade pre-cancer associated disorders with the HPV, which are included in the vaccine. The currently developed prophylactic HPV vaccines are formulations of the major capsid protein, L1, of the natural HPV particle. Vaccination with L1 in a number of animal models neutralizes species-specific papillomaviruses (8). Vaccination with naked DNA might be attractive because of simple production protocols, greater efficiency than some other approaches, stability and overall safety (9, 10). In fact, human trials are underway to test DNA vaccines in the fight against well-known infectious diseases like HIV, HBV and malaria (11, 12, and 13). Investigation into the immune response mounted against E7 and other viral proteins is necessary before using a DNA vaccine in clinical trials.

In this study, we engineered HPV16 E7 coding sequence (wild type) as a candidate therapeutic vaccine for targeting already infected individuals. A cloning vector containing HPV16 E7 gene (Iranian Isolate) was designed and constructed as described previously (14). In the present study, expression vector containing the HPV16 E7 gene was constructed and confirmed using MAb in Western blot analysis. Mice were immunized and induction of immune responses was investigated.

**MATERIALS AND METHODS**

**Construction of Recombinant Expression Vector.** The HPV16-E7 gene was isolated by PCR, cloned in pTZ57R/T-E7, confirmed by sequencing and submitted to Gene Bank (DQ 323401) as described earlier (14). The target gene was obtained from pTZ57R-E7, and sub-cloned into the unique EcoRI and XbaI cloning sites of the pcDNA3 expression vector (Invitrogen, Canada), down-stream of the cytomegalovirus promotor.

**Confirming the Accuracy of Constructed Plasmid.** Colonies containing the constructed plasmid were obtained by transformation of E. coli DH5α as a host. The colonies containing the target gene were selected by ampicillin resistance and the accuracy of constructed plasmid was confirmed by colony-PCR and restriction enzyme analysis. The colonies were used directly as template in colony-PCR using E7 forward and reverse primers. PCR mixture consisted of 50 pmol of each primer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 1 U of Taq polymerase (Cinagen, Iran) in a total reaction volume of 15 µl. Amplification was carried out for 35 cycles (94°C for 30s, 66.5°C for 45s,
72°C for 45s) after an initial denaturation step at 94°C for 5 min, on a Techne Thermal Cycler. The cycles were followed by a 5 min extension at 72°C and the PCR product was visualized on a 1.5% agarose gel by ethidium bromide staining.

Restriction enzyme analysis was used for further confirmation. The colonies that were positive in colony-PCR were selected, propagated and purified. The restriction enzymes EcoRI and XbaI were used for enzyme analysis. The confirmed construct was prepared in a large scale and used in a eukaryotic transfection system.

Cell Line. Wehi-164 cells (as negative control) and Wehi-164 transfected cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml and 1 mM sodium pyruvate. Then, the cells were grown at 37°C in a 5% CO2 atmosphere.

Generation of Stable Eukaryotic Cells Expressing E7 Gene. To monitor the expression of E7 recombinant protein, 10⁶ Wehi-164 cells were seeded into a 6-well microplate and incubated overnight in complete medium without antibiotics. The Wehi-164 cells were transfected with pcDNA3/E7 using Lipofectamin 2000 (Invitrogen) in 70% confluency. To produce stable cells expressing HPV16 E7 gene generation, 48h after transfection, the cells were cultured in medium containing G418 antibiotic at the appropriate concentration and G418-resistant foci were picked up and expanded in 48-well plates. The accuracy of stable Wehi-164 cells expressing E7 (Wehi-164/E7) was determined by Western blotting using E7-specific monoclonal antibody (Abcam, UK). In brief, total cell lysate was extracted and 1 mM of phenylmethylsulfonyl fluoride (PMSF), was added to the cell lysate to inactivate proteinases. The cell lysate was subjected to 5 times freezing and thawing and then sonicating (60 HZ, 0.5 amplitude). The samples were loaded on a 12% SDS-PAGE gel after boiling for 10 min and transferred to a PVDF Western blotting membrane (Roche, Germany) by electrophoresis for 1 h at 90 V. Membranes were blocked in 3% BSA in PBS for 1 hour. E7 protein was detected using anti-E7 monoclonal antibody. The specific band was visualized with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G antibody (TEBSUN, Iran) and staining with diaminobenzidine (DAB) substrate (Biogene, Iran).

Antigen Preparation. Forty eight hours following the induction with pcDNA3/ E7, cell extracts were prepared from both the un-transfected and transfected Wehi-164 cells. Total cell lysates were subjected to sonication (60 HZ, 0.5 Amplitude) after 5 times freezing and thawing. PMSF (1 mM) was added to the cell lysates to inactivate proteinases and the protein concentration was determined by Bradford method.

Mice. Six to eight weeks old inbred female BALB/c mice were obtained from Pasture Institute of Iran. Given free access to food and water, the mice were housed for one week and maintained under standard conditions prior to experimentation. All the experiments were done according to the Animal Care and Use Protocol of the Tarbiat Modares University (Tehran, Iran).

DNA Vaccination. Three groups of mice were vaccinated with PBS (negative control), pcDNA3 (negative control), and pcDNA3/E7. Negative control mice groups were used for elimination of NK cell responses and other environmental interferences. The mice were injected with 50 µl of 10 µM cardiotoxin into each tibialis anterior muscle 5-6 days prior to DNA injection. For vaccination, 90µl of plasmid DNA (1 µg/µl in PBS) was injected into each pretreated muscle. After ten days, the mice were sacrificed and their splenocytes were isolated.

CTL Assay. The mice were immunized as described above. Ten days after vaccination, the spleens were removed and the splenocytes from the immunized mice were harvested.
The Wehi-164/E7 target cells were incubated with splenocytes at effector-to-target cell ratio of 40:1 in duplicate Falcon centrifuge tubes and centrifuged at 350g at 4°C for 5 minute. Then they were incubated at 37°C for 3.5 hours. After incubation, IQ Products Phosphatidyl Serine Detection Kit (IQ Product, Netherlands) was used for staining of apoptotic and necrotic cells. After annexin staining, the cells were resuspended in 300 µl of 100 µg/ml propidium iodide at least 10 minutes before analysis. Some 10,000 cells were counted on the Fascalibur flowcytometer (Becton Dickinson, USA), and the data were analyzed by WIN MDI software. The results were expressed as percent of apoptosis or necrosis, separately, as obtained by the following formula:

% Apoptosis = percent of experimental apoptosis - (percent of spontaneous spleen cell apoptosis + percent of spontaneous Wehi-164/E7 cell apoptosis).

% Necrosis = percent of experimental necrosis - (percent of spontaneous spleen cell necrosis + percent of spontaneous Wehi-164/E7 cell necrosis).

**Cytokine Assay.** The splenocytes (5 × 10^5 viable cells/well) were harvested and cultured in 96-well flat-bottom tissue culture plates with 100 µl of medium (RPMI 1640, 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 1 mM sodium pyruvate) alone or the medium containing crude E7 expressing cell lysate (500 µg/ml) or PHA (5µg/well, Gibco, UK) for 48 h. The supernatants were harvested and assayed for the presence of IFN-γ and IL-4 using ELISA kits (R&D Systems, UK) according to the manufacturer's instructions.

**CD4 and CD8 Staining and Flowcytometry Assay.** To determine the number of CD4 or CD8 positive cells by flowcytometry, the splenocytes from vaccinated mice were harvested and stained with FITC/RPE -conjugated monoclonal rat anti- mouse CD8 or CD4 antibody (Serotec, UK).

**Lymphocyte Stimulation Test.** The Splenocytes from the vaccinated and negative control mice were cultured under the same conditions. The cell viability ascertained with trypan blue dye exclusion method was usually greater than 98%. The cell concentration was adjusted to 3.50 ×10^5 viable cells/100 µl. Splenocyte suspensions (100 µl) from each animal were added to nine wells in a 96-well flat-bottom tissue culture plate. A total of 100 µl of medium alone or medium containing crude E7 expressing cell lysate (250 µg/ml) or PHA (5µg/well) were added to triplicate wells containing the cells from each animal. The plates were incubated at 37°C for 72 hours in a humidified atmosphere containing 5% CO2. A 100 µl aliquot of the supernatant was removed from each well and 20 µl of MTT (5 mg/ml) solution in sterile PBS was added to each well. The plates were incubated for an additional four hours, and then 100 µl of dimethyl sulfoxide was added to each well. Formasan crystals formed upon metabolism of MTT by proliferating cells were dissolved by vigorous pipetting and the plates were incubated for 15 minutes at 37°C. Optical density was recorded at 540 nm in an ELISA reader and stimulation index was calculated as follows:

\[
SI = \frac{OD \text{ of stimulated cultures}}{OD \text{ of un-stimulated cultures}}
\]

Where, SI= stimulation index by MTT assay and OD= optical density.

**Statistical Analysis.** Mann-Whitney U analysis was used for the statistical analysis of CTL, cytokine assays and MTT.
RESULTS

The authenticity of the desired plasmids was confirmed by colony-PCR using E7 forward and reverse primers. The colonies containing the desired plasmid were positive and the size of their DNA fragment was about 350bp. The positive colonies on the colony-PCR were propagated and their plasmids were purified. For further confirmation, restriction enzyme analysis using EcoRI and XbaI enzymes was performed. The confirmed construct was prepared in a large scale, purified and used for transfection studies.

To evaluate the expression of E7 in the Wehi-164 transfected cells, Western blot analysis using E7-specific monoclonal antibody was used. Non-transfected Wehi-164 Cell lysate was used as a negative control. The Wehi-164 stable cell lysate showed a protein band at about 11 KDa for E7 in the Western blot (Figure 1).

![Western Blot](image)

**Figure 1.** Detection of E7 protein in stable Wehi164 cells by Western blot analysis. Western blot analysis using E7-monoclonal antibody showed negative results in pcDNA3 transfected cells (lane 1) and a protein band with a size of approximately 11 KDa in pcDNA3/E7 stable cells (lane 2).

**Determination of the Specific and Non Specific Cytotoxicity.** To determine the induction of apoptosis or necrosis using the designed construct, CTL assay was performed using Annexin/PI kit. The target cells were stable Wehi-164 cells transfected with HPV16 E7 gene using G418 antibiotic-resistant selection. The splenocytes were re-stimulated in vitro by the E7 expressing Wehi-164 target cells and 3.5 hours after re-stimulation, Annexin/PI staining was performed. Apoptotic and necrotic cells were counted by BD flowcytometer. Data for statistical analysis are shown in Table 1. As shown in Figure 2, vaccination with pcDNA3/E7 induced both apoptosis and necrosis in the vaccinated mice compared to the mice vaccinated with pcDNA3 or PBS (p=0.0001).

**Determination of IFN-γ and IL-4 Levels Following Vaccination.** Since many of the tumoricidal effects of CD8+ T cells in vivo are mediated through the secretion of the inflammatory cytokines like IFN-γ, the IFN-γ and IL-4 cytokine profiles of in vitro
re-stimulated splenocytes from the immunized mice were examined using ELISA. In cytokine assay, IFN-γ was significantly increased in the pcDNA3/E7 vaccinated mice compared to negative control groups (Figure 3A) (p<0.005), but IL-4 assay did not show any statistically significant differences among tested groups (Figure 3B).

Table 1. E7-specific CTL responses (apoptosis and necrosis) data.

<table>
<thead>
<tr>
<th>Different vaccinated mice groups</th>
<th>Apoptosis</th>
<th>Necrosis</th>
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<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
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<tr>
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<tr>
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Figure 2. E7-specific CTL responses (apoptosis A, necrosis B) from BALB/c mice immunized with pcDNA3/E7DNA vaccine (E7 compared with pcDNA3 and PBS vaccinated groups). Mice (6 per group) were immunized with the above plasmids via intramuscular injection. Splenocytes were obtained from immunized mice ten days after the vaccination. CTL assay was performed using Annexin/PI kit and stable transfected Wehi-164 cells expressing E7 gene were used as target cells. The calculated results of apoptosis and necrosis from each group are presented.

Figure 3. IFN-γ (A) and IL4 (B) assays were performed using ELISA kit. Splenocytes were obtained from immunized mice ten days after vaccination with pcDNA3 (negative control), and pcDNA3/E7 plasmids via intramuscular injection. Splenocytes were re-stimulated with lysate of Wehi-164 expressing E7 protein. IFN-γ and IL-4 concentration was determined 48 h after specific induction by commercial ELISA kit. As seen, IFN-γ increased significantly in pcDNA3/E7 and vaccinated mice compared to negative controls, but IL-4 did not increase significantly.
**Determination of the CD4/CD8 Ratio Following Vaccination.** The splenocytes stained with anti-CD4 and anti-CD8 monoclonal antibodies conjugated with FITC/RPE were used for flowcytometric analysis. The CD8\(^+\) cells (but not CD4\(^+\) cells) showed significant increases (p=0.0001) in the test group compared to the negative control groups (Figure 4).

![Figure 4](image)

**Figure 4.** Splenocytes were harvested from immunized mice similar to CTL assay and anti-CD8 and anti-CD4 monoclonal antibodies conjugated with FITC and RPE, respectively were CD4\(^+\) or CD8\(^+\) cells were counted by a BD flowcytometer. The differences observed for E7 vaccinated mice group for CD8 (A) and CD4 (B) were statistically significant (p=0.0001).

**Determination of the T Cell Proliferation Following Vaccination.** The lymphocyte proliferation in response to the specific antigen increased in the pcDNA3/E7 vaccinated mice using MTT cell proliferation assay, but not in the mice injected with pcDNA3 or PBS (p=0.004) (Figure 5, Table 1).

![Figure 5](image)

**Figure 5.** Lymphocyte proliferation shown as the stimulation Index (SI) for vaccinated mice. Splenocytes from immunized mice were obtained and re-stimulated with cell lysate of target cells, 72 hours after induction. Cell proliferation assay was performed using MTT solution. Formasan crystals were dissolved in dimethyl sulfoxide by vigorous pipeting. Their optical densities were measured at 540 nm. The stimulation index was calculated and the results shows that the SI of E7 vaccinated mice increased significantly compared to the negative controls.

**DISCUSSION**

More than 200 types of human papillomaviruses have been recognized and human papillomavirus type 16 (HPV16) is the most common one associated with severe cervical displasia and cancers (14). The essential factors necessary for HPV-induced malig-
The HPV16-E7 protein has been shown to be continuously expressed in cervical carcinoma cells, therefore it is a logical target in HPV16 vaccination of patients with cervix dysplasia and cancer (1,2). Since the E7 protein represents the ideal target for the development of HPV therapeutic vaccines, DNA vaccines targeting E7 may generate a more potent immune response (15).

DNA vaccines have several potential advantages compared to protein or peptide-based vaccines. The elimination of time-consuming purification of recombinant proteins, the ability to stimulate both cell-mediated and humoral immunity, and the perceived safety advantages of subunit vaccines over live vaccines makes it of potential interest in anticancer studies (16, 17). In DNA vaccination, the immune responses are induced upon injection of gene expression cassettes directly into a host target tissue. This delivery of non-replicating transcript units drives the synthesis of specific foreign proteins within the inoculated host. These adapted synthetic foreign proteins then become the subject of immune surveillance resulting in Ag-specific cellular and humoral immune responses without the associated risk of viral pathogenesis (18, 19).

In this study, HPV16 genotype was chosen because this genotype is commonly present in more than 50% of cervical cancers. Experimental studies with HPV-transformed cells in vivo have demonstrated that E7-specific T lymphocytes can prevent the growth of these cells. Expression can be aided by enhanced distribution of the plasmid DNA which has been accomplished by pre-injection of muscles with appropriate volumes of hypertonic solutions or myotoxic agents such as cardiotoxin (11). In the present study we used cardiotoxin because it has the advantage of selectively destroying myofibers without harming myoblasts or the vascular endothelial cells, thus enabling complete recovery of the muscle (11). Uptake of naked DNA in muscle cells depends on muscle swelling which may mimic the tissue conditions associated with natural infections.

As mentioned by Hung et al, understanding the molecular mechanisms that delay immune attack in the tumor microenvironment will lead to the identification of novel molecular targets that can be blocked in order to enhance the therapeutic effect of HPV DNA vaccines. With continued attempts in the development of HPV therapeutic vaccines, HPV therapeutic DNA vaccines will appear as a significant approach that can be combined with existing forms of therapy such as chemotherapy and radiation leading to effective translation from bench to bedside for the control of HPV-associated malignancies (15).

In this study E7-specific immune responses in intramuscularly vaccinated mice were evaluated using pcDNA3-E7 construct. Activated CTLs function directly as effector cells, providing anti-tumor immunity through lysis of the tumor cells or through the release of the cytokines capable of interfering with the propagation of tumors. Furthermore, depletion of CD8$^+$ CTL has led to the loss of anti-tumor effects in several cancer vaccines (11,15, 19). Therefore, the enhancement of antigen presentation through MHC class I pathway to CD8$^+$ T cells has been a primary focus of cancer immunotherapy (17, 20).

Our findings indicated that the DNA vaccine encoding E7 protein (Iranian isolate) can induce an E7-specific CTL response. DNA vaccine with high immunopotentiating action can be a useful vehicle for immunological phenomena. Although the HPV16 E7 DNA vaccine is an attractive DNA vaccine, safety issues need to be resolved to prevent the integration of DNA into the host genome which may result in subsequent inactivation of the tumor suppressor genes or the activation of oncogenes and may lead to a malignant transformation of the host cells (17, 21, 22).
nately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation, and integration should not pose any real risk (17). Such efforts are likely to lead to development of improved methods, enabling greater expression efficiencies in vivo and having greater clinical utility.

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