Original Article

Further Stimulation of Cellular Immune Responses through Association of HPV-16 E6, E7 and L1 Genes in order to produce more Effective Therapeutic DNA Vaccines in Cervical Cancer Model

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Abstract

Background: Cervical cancer has been shown to be highly associated with human papillomavirus (HPV) infection. The viral oncogenes E6 and E7 are constantly expressed by the tumor cells and are therefore potent targets for therapeutic genetic vaccination. In the present study, it was investigated the potential effect of HPV-16 E6, E7 and L1 co-administration to activate specific cytotoxic T lymphocytes in tumor mice models.

Methods: The HPV-16 E6, E7 and L1 genes from Iranian isolate were separately inserted into the mammalian expression vector, pcDNA3, to construct the DNA vaccine candidates. Tumor-bearing Animals (C57BL/6 mice) were immunized with the vaccine candidate; then, Lymphocyte Proliferation Assay (LPA) and relative tumor volume measurements were carried out in order to examine the immunological effects of the vaccine.

Results: Obtained results showed that co-administration of the HPV-16 E6, E7 and L1 DNA induced HPV-16 specific cellular immune responses and also protected against TC-1-induced tumor in vivo compared with negative controls.

Conclusion: The results showed that mixed delivery systems might be valuable to improve the magnitude of the induced immune responses and confirmed therapeutic effects of HPV-16 E6, E7 through cytotoxic T lymphocyte induction and illustrate the new promising role for HPV-16 L1 CTL epitopes as a suitable CTL inducer.

Keywords: pcDNA3/E6; pcDNA3/E7; pcDNA3/L1; immunocellular responses

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Introduction

Worldwide, cervical cancer, with approximately 470,000 new cases and 233,000 deaths each year is the second most common cancer in women [1]. Approximately 70% of cervical cancers occur in developing countries [2, 3]. Today, the causal role of high-risk strains of HPV (especially types 16 and 18) in cervical cancer has been documented [4, 5].

Since, some of the HPV early proteins, such as E6 and E7 are constantly expressed in cervical cancer cells as oncogenes that promote tumor growth and malignant transformation [3], they represent ideal targets for immunotherapy of cervical cancer [6]. E6 can bind to a C-terminal region of p53 and degrade it [7]. Also, E7 proteins is a small nuclear proteins that binds to hypo-phosphorylated form of the retinoblastoma protein (Rb), causing that to be disabled [8]. In this situation, cell cycle proceeds to S phase and ultimately cellular DNA synthesis and cell proliferation occur [3, 9]. In some studies, this has been reported that memory T cells specific for an E7-derived cytotoxic T cell (CTL) epitope were generated in Cervical Intraepithelial Neoplasia (CIN) and cervical cancer patients [9, 10]. So, E6, E7 can be considered the logical targets for therapeutic HPV vaccines.

In order to reduce the number of deaths from cervical cancer, prophylactic vaccines which protect against primary HPV infection in naïve individuals would be of unquestionable value. Anti L1 and L2-directed antigens are efficient in this role [11]. Prophylactic vaccines containing L1 as DNA or VLP have shown significantly effective in clinic [11]. Also today, some studies have shown the therapeutic potential of L1-specific CD4(+) and CD8(+) T lymphocytes responses in cervical cancer patients [12, 13]. A study indicated that HPV-16 L1 DNA vaccine stimulates the vaginal CD8(+) T lymphocytes which are essential to eliminate virus-infected cells [14]. It seems to be required more studies about the ability of L1 gene to stimulate immune cells.

In the current study, DNA-based immunization was examined for induction of cell-mediated immune responses of L1. Also, the effect of co-administration of HPV-16 E6, E7 and L1 genes on the protection and cellular immune system was evaluated and discussed in C57BL/6 mice model.

Materials and Methods

The TC-1 cells were purchased from Pasteur Institute, Tehran, Iran. They were derived from the primary lung epithelial cells of C57BL/6 mice and were immortalized with the amphotropic retrovirus vector, containing E6 and E7 genes and subsequently transformed with the plasmid expressing the activated human c-Ha-ras oncogene [15]. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 cell-culture medium (Gibco Invitrogen; Paisley, Scotland, UK), supplemented with 10% fetal calf serum (FCS) (Gibco BRL), 100 IU/ml penicillin,100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, at 37°C with 5% CO2.

Female C57BL/6 mice (3–4 weeks old) were purchased from the Pasteur Institute, Tehran, Iran (n=63). Given free access to food and water, the mice were housed for one week before the experiment, and maintained in a good standard condition. All experiments were done according to the guidelines for the care and use of laboratory animals by the Ethical Commission of the Tarbiat Modares University.

Tumor Monitoring

For the therapeutic experiments, C57BL/6 mice were challenged by a subcutaneous (s.c) injection of a suspension of 100 µl PBS containing 10^6 TC-1 cells/mouse in the left flank and then, were grouped into nine cages (seven for each group).

After two weeks, the resulting tumors were obvious and palpable in mice. The neoplastic masses were measured with calipers every other day and tumor volume was estimated according to Carlsson's formula [16, 17]. The average tumor size in millimeter was reported as the average of all dimensions measured. Smallest diameter (a) and biggest diameter (b) were measured and tumor volume was calculated using the following formula: V= (a2b)/2 (data not shown).

Vectors Preparation

The HPV-16 E7 gene was isolated by PCR, cloned in pTZ57R/T, confirmed by sequencing and subcloned into the unique *EcoRI* and *XbaI* cloning sites of the pcDNA3 expression vector (Invitrogen, Burlington, Canada), down-stream of the cytomegalovirus promoter as described previously by Meshkat et al., [18]. Also, in the study that was conducted by Mirshahabi et al., the HPV-16 E6 gene was isolated by PCR, and cloned in the pTZ57R/T. Cloning was confirmed by sequencing. Then, this gene was subcloned into the unique *BamHI* and *HindIII* cloning sites of the pcDNA3 expression vector (Invitrogen, Burlington, Canada), down-stream of the cytomegalovirus promoter [19].

Also, pET/L1 that was produced by Teimoori et al. previously [20] and subcloned into the unique site of *XhoI* and *NheI* cloning sites of the pcDNA3 expression vector (Invitrogen, Burlington, Canada), down-stream of the cytomegalovirus promoter.

Competent cells of DH5α strain of *E. coli* were transformed with confirmed recombinant pcDNA3/E6, pcDNA3/E7 and pcDNA3/L1 vectors in Luria-Bertani medium. The presence of the HPV-16 E6, E7 and L1 genes in the constructed vectors was determined using restriction enzyme analysis. Large-scale preparation of the plasmids were performed according to standard polyethylene glycol (PEG) precipitation method [21].

Mice immunization and *in vivo* tumor rejection assay

Two weeks after TC-1 cells injection, mice were immunized intramuscularly (IM) twice at a two weeks interval with 100 µl phosphate buffered saline (PBS; negative control), 100 µg naked DNA vaccine

encoding pcDNA3 (negative plasmid control), pcDNA3/L1, pcDNA3/E6, pcDNA3/E7, pcDNA3/E6 & pcDNA3/E7, pcDNA3/E6 & pcDNA3/L1, pcDNA3/E7 & pcDNA3/L1 and pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1 in PBS. Negative control mice groups were used for elimination of none specific responses and other environmental interferences. Finally, three animals per group were sacrificed randomly on day 14 after the final immunization and their spleens were isolated aseptically for in vitro splenocytes culture. Remained mice followed up to 60 days for tumor appearance and size of tumors. The tumor volume was estimated according to Carlsson's formula as described in previous [16].

Lymphocyte Proliferation Assay (LPA)

The CTL assay was performed as described earlier [22]. Briefly, the suspension of isolated spleen cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, 1% HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)], 0.1 mM minimal essential medium with nonessential amino acids, 0.1% penicillin/streptomycin and incubated in the presence of 4×10^5 inactivated treated TC-1 cells per well. A total of 100 µl of medium alone or 5 µg/well phytohemoagglutinin (PHA) (Sigma Chemical Co, St Louis, MO, USA) was added in triplicate wells as negative and positive control respectively. All of the plates were incubated at 37°C for 72 hours in a humidified atmosphere containing 5% CO2. A 100 µl aliquot of supernatant was removed and 20 µl of MTT (3-(4, 5-dimethyl tetrazolyl-2) 2, 5 diphenyl) tetrazolium bromide (Sigma Chemical Co, St Louis, MO, USA) in concentration of 5 µg/ml was added per well and incubated for additional 5 h at 37°C in 5% CO_2. DMSO (dimethyl sulfoxide) (100 µl) was added to dissolve produced formazan crystals by proliferating cells.

Plates were incubated for 15 minutes at 37°C and read at 540 nm. The results were expressed as stimulation index (SI). The SI was calculated as follows: OD values of stimulated cells (Cs) minus relative cell numbers of unstimulated cells (Cu) by relative OD values of unstimulated cells [23].

SI = (Cs _ Cu)/Cu

All tests were performed in triplicate for each mouse.

Statistical Analysis

The SPSS version 21 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis .The significance of statistical comparisons was calculated using one way ANOVA. The p-values less than 0.05 were considered statistically significant. All values were expressed as means±SD.

Results

In vivo Tumor Challenge Assays

The TC-1 cells were used as a tumor cell model in this study (Figure 1). As shown in figure 2, tumor was appeared rapidly in mice after s.c inoculation of 10^6 TC-1 cells. To determine whether produced plasmids can induce regression of E7 & E6-related tumors, mice were immunized intramuscularly with pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1 after TC-1 inoculation and were followed for tumor development.

Determination of the T Cells Proliferation Following Vaccination

Lymphocyte proliferation was measured as the Stimulation Index (SI) for vaccinated mice. The results showed a significantly enhanced Ag-specific splenocytes proliferation in the pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1 vaccinated mice group compared with other groups using MTT cell proliferation assay ($p<0.005$).

In vivo Tumor Rejection Assays

In this study, we evaluated the effect of DNA vaccination on the progression of tumor in the TC-1 tumor-bearing mice. The inhibition rate of tumor growth was significantly different ($p<0.05$) in the vaccinated group with all test groups in compare to the control groups. The efficacy of DNA vaccination appeared to be in order of pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1>pcDNA3/E6 & pcDNA3/E7> pcDNA3/E6 & pcDNA3/L1> pcDNA3/E7 & pcDNA3/L1> pcDNA3/E6> pcDNA3/E7> pcDNA3/L1. The enhanced inhibition of tumor growth was shown in pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1.

Discussion

In this study, we provides evidences that E6, E7 and L1 genes of HPV-16 cloned in mammalian expression vector are able to produce immune response against cervical cancer model in term of reduction in tumor size and increased cell proliferation in comparison to control groups.

Cervical cancer that is the second-leading cause of cancer death in women usually seems to occur by exposure to the high risk papillomaviruses. So far, two types of preventive vaccines against

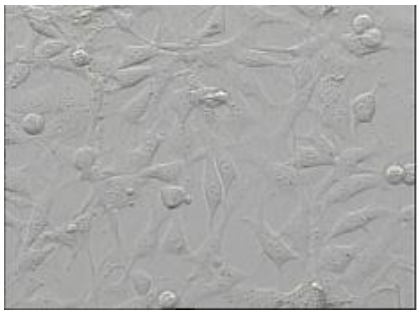

Figure 1. TC-1 cell line in media culture before counting, and injecting into the left flank of C57BL/6.

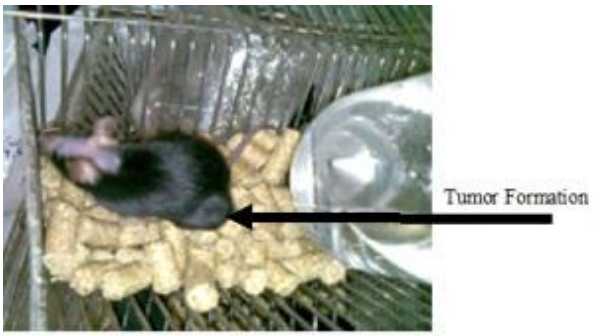

Figure 2. Observation of tumor in mice that were inoculated with 10^6 TC-1 cells.

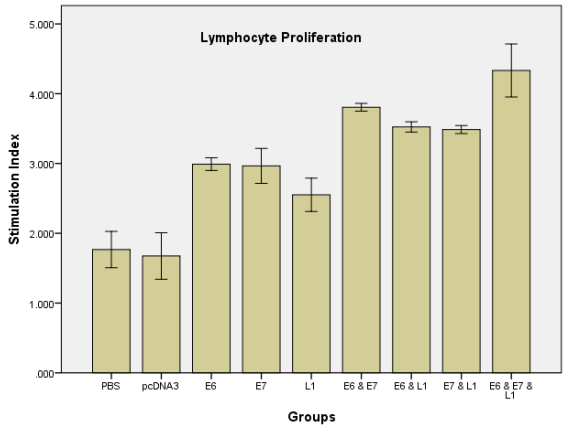

Figure 3. The stimulation calculated indexes for different vaccinated groups; C57BL/6 mice were injected subcutaneously with TC-1 cells. After two week, the mice were immunized intramuscularly (IM) twice at a two weeks interval with 100 µl phosphate buffered saline (PBS; negative control), 100 µg naked DNA vaccine encoding pcDNA3 (negative plasmid control), pcDNA3/L1, pcDNA3/E6, pcDNA3/E7, pcDNA3/E6 & pcDNA3/E7, pcDNA3/E6 & pcDNA3/L1, pcDNA3/E7 & pcDNA3/L1 and pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1 in PBS. Two weeks after final immunization, spleen of individual mice (three/group) was removed and lymphocyte proliferation was evaluated using the MTT method. Formazan crystal formation after incubation with MTT was determined by solving the crystals in DMSO, and the OD was read at 540 nm. Lymphocyte proliferation in the pcDNA3/E6 & pcDNA3/E7 & pcDNA3/L1 group was significantly higher than in the other groups especially negative control group ($p<0.05$).

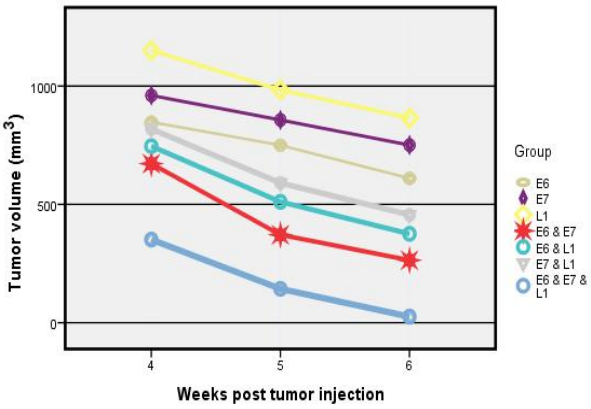

Figure 4. Assessment of therapeutic vaccine on tumor size between the 4th and 6th weeks after the tumor inoculation

cervical cancer have been approved by the Food and Drug Administration (FDA). However, nowadays, due to the cost of these vaccines in developing countries, they are considered as inappropriate strategies. Therapeutic vaccines may be effective in inducing cellular immunity against cells expressing viral antigens and improvement of the HPV lesions. Also today, researches demonstrated DNA vaccines have significant potential, compared to traditional protein vaccines, in terms of priming CTL responses and generating memory CD8+ T-cell responses [11, 24, 25].

The HPV-16 E6 and E7 proteins have been shown to be continuously expressed in cervical carcinoma cells [26]; therefore they are logical targets for therapeutic HPV-16 vaccination in patients with cervical dysplasia and cancer. Studies showed that endothelial cells can take up the purified E7 oncoprotein added to the culture medium [27]. Also, the HPV-16 E6/E7+ tumor cells could be genetically modified with DNA coding for

immunostimulatory cytokines or co-stimulatory molecules and used for vaccination. The genetically modified tumor cells were found to be substantially more effective than the parental tumor cell vaccines. Immunological examination had revealed an important role for CD8+ lymphocytes in eradication of an established and actively growing carcinoma [28].

Recent studies have shown that HPV-16 L1 gene has an effect on cellular immunity, so it can be used in the production of therapeutic vaccines [13]. Cheung et al. (2004) engineered L1E7hpSCA1 plasmid encoding the L1 and E7 genes with the codon usage optimized for mammalian cell expression. After injection into a muscle, they showed that this plasmid had efficiently induced T cell cytotoxicity and also can lyse the TC-1 cells expressing E7; also, tumor growth was stopped in vaccinated mice [29]. The protection against TC-1 tumor challenge was also observed after vaccination with chimeric papillomavirus-like particles consisting of the HPV-16 L1/L2 viral capsid proteins plus the entire E7 non-structural protein [30].

Since CD8+ T cells exert a major immunological role in tumor rejection, we compared *in vitro* cytotoxic function of the splenic cells from the various vaccinated animals. Our results indicated an increase CTL activity of the mice treated with E6, E7 and L1 as a DNA vaccine. In the interpretation of this experiment, we considered that enhancement of E6 and E7 effects as stimulators of cellular immunity in the presence of L1 is probably due to specific epitopes on it. Bellone et al. (2009) showed that the HPV-16 L1 DNA vaccine strongly promotes the stimulation of vaginal CD8+ T cells, which are essential for the elimination of virus-infected cells [13].

In our setting, the amount of CTL response was lower than previous studies [13, 14] which may be due to several reasons such as the stage of tumor, the method of CTL assay, the mouse model and cell line type. On the other hand, pro-inflammatory cytokines can activate the natural killer (NK) cell, and the NK cells have important role in rejecting tumors [31]. Although, we did not assay NK cell infiltration in our established tumor model, but the role of NK cells in tumor resistance and development cannot be ignored. Since modulation of T helper subsets as effector cell populations is responsible for protective immunity, therefore, directing immune responses is needed for considerable protection [32].

Conclusion

We have studied HPV16 DNA vaccines containing HPV-16 E6, E7 and L1 genes as a therapeutic vaccine. Based on the obtained data, they are ideal targets for HPV vaccine development and tumor rejection. Since, whole genomes of E6 and E7 are toxic for human cells and given the strong antitumoric effects of them, it seems that further examinations of these approaches are acquired.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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