Strong Immune Responses Induced by a DNA Vaccine Containing HPV16 Truncated E7 C-terminal Linked to HSP70 Gene

Zahra Meshkat¹, Hoorieh Soleimanjahi¹*, Hessam Mirshahabi¹, Mojtaba Meshkat², Maryam Kheirandish³, Zuhair Mohamad Hassan¹

¹Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, ²School of Medicine, Islamic Azad University, Mashhad, ³Blood Transfusion Center, Tehran, Iran

ABSTRACT

Background: Vaccines capable of controlling tumor virus based infections are found difficult to develop due to the consistence latent infection in the host. DNA vaccines are attractive tools for the development of HPV vaccines and inducing antigen-specific immunity owing to the stability, simplicity of delivery, safety and cost effectiveness. However, there is a need to increase their potency by procedures such as using HSP70 gene as an adjuvant. Objective: To evaluate a DNA vaccine containing HPV16 truncated E7 C-terminal cytotoxic T-lymphocyte epitopes linked to HSP70 gene (HSP70-tE7) in an animal model. Methods: Mice were immunized with the plasmid DNA after pre-treatment with cardiotoxin. The splenocytes of immunized mice were then tested for CTL activity by detecting the apoptosis and necrosis in target cells, cytokine production by ELISA, CD4 and CD8 frequencies by flow cytometry, and lymphocyte stimulation by MTT assay. Results: The recombinant expression vector was able to elicit immune responses close to that of full length E7 complete gene. Although the use of a small part of a target antigen can induce immune responses equivalent to the full length antigen, it fails to elicit statistically significant stronger immune responses when fused with HSP70 compared to the complete E7 gene alone. Conclusion: The potent immunogenicity of HPV16 E7 was preserved in the HSP70-tE7 vaccine and may represent a target of choice for the therapeutic vaccination strategies. However, to improve the immunogenicity polytope DNA vaccines which elicit multiple effector and memory CTL responses should be considered in future studies of DNA-based cancer vaccines.

Keywords: CTL Epitope, DNA Vaccine, E7, HSP70, Human Papillomavirus Type 16
INTRODUCTION

DNA vaccination is a potential means of expressing desired genes in vivo for the generation of immune responses especially cell-mediated immunity. The plasmid DNA has low immunogenicity; therefore it can be administered for several times. DNA vaccines can be prepared in high purity. They are more stable than proteins, peptides and other biological polymers (1-8). The important concern in the use of DNA vaccine is its limited potency. For increasing the potency of DNA vaccines, different methods such as fusing antigens to chemokines or to a pathogen sequence such as fragment C of tetanus toxin, targeting antigens for rapid intracellular degradation, directing antigens to APCs by fusion to ligands of APC receptors, co-injecting of cytokines, co-stimulatory molecules and co-administration with CpG oligonucleotides have been applied (9-16).

Linkage of different genes to HSP represents a potential approach for increasing the potency of DNA vaccines. Recent studies have shown that immunization with HSP complexes isolated from tumor or virus infected cells are able to induce potent anti-tumor or antiviral immunity (17-19). Papillomaviruses form a heterogeneous group of non-enveloped viruses that target the epithelium. These viruses contain a double stranded circular DNA genome of approximately 8000 bp. A subgroup of the human papillomavirus (HPV) targets the mucosal epithelium and is considered as the causative agent of anogenital cancers (20). The prominent HPV type in this respect is HPV-16 which can be detected in about half of all invasive cervical cancers. Worldwide, about half a million women develop this type of tumors every year. Being a major health hazard, great efforts are currently undertaken to develop means for the treatment and prevention of HPV infections. A number of strategies have been presented for immunotherapy of HPV-associated diseases. Many of these approaches are aimed at targeting the immune system toward the viral tumor antigen E7. In a number of studies, it has been demonstrated that the E7 protein is expressed in all tumor tissues and there are in vitro data indicating that E7 protein is required for the growth of tumor cells. Therefore, DNA vaccines targeting HPV E7 gene may provide useful means for preventing and treatment of HPV-associated cancers.

In this study, DNA was selected from a region proximal to the C-terminal end of the HPV 16 E7 protein (codons 83-93). The truncated E7 (tE7) was fused to human HSP70 gene by a three step PCR. The chimeric gene was inserted into the expression vector and then sequenced. The constructed DNA vaccine was administered to mice and the induction of immune responses was evaluated.

MATERIALS AND METHODS

Linkage of HPV 16 tE7 to Human HSP70 Gene. The expression vector containing human HSP70 was kindly provided by Dr P.J. McLean (Department of Neurology, Massachusetts General Institute for Neurodegenerative Disease, Massachusetts General Hospital, USA). In order to Link the tE7 to HSP70 gene, a three steps PCR was performed. One forward (F) and three reverse (R1, R2, R3) primers were designed for the insertion of Kozak sequence, generation of a mutation for the deletion of HSP70 stop codon, fusion of a linker and the linkage of tE7 to HSP70 (Figure 1).
The F and the R3 primers also allowed insertion of flanking BamHI and XbaI restriction enzyme sites, respectively. The sequences of the designed primers are showed in Table 1. PCR mixture consisted of 50 pmol of F and R1 primers, 2 µl of DNA sample, 1.5 mM MgCl2, and 0.2 mM each dNTP, 1 U of Taq DNA polymerase (Cinagen, Iran). Amplification was carried out for 30 cycles (94°C for 45s, 67°C for 35s, 72°C for 1min) after an initial denaturation step at 94°C for 5min on a Techne Thermal Cycler. The cycles were followed by a 10min extension at 72°C and the PCR product was visualized on a 1.5% agarose gel by ethidium bromide staining.

The HPV 16 tE7 fragment which contained 33 nucleotides and two reverse primers i.e., R2 and R3, were designed for the insertion of E7 sequence in to HSP70 (Figure 1 and Table 1). PCR mixture was prepared in the same manner as before using F and R2 primers and amplification was performed for 30 cycles (94°C for 45s, 67.5°C for 35s, 72°C for 1min) with the initiation and extension steps under the same conditions as F-R1 PCR.

In the third step of PCR, amplification was carried out for 30 cycles (94°C for 45s, 69°C for 35s, 72°C for 1min) using F and R3 primers (Figure 1 and Table 1). PCR mixture, initiation and extension steps were the same as the first step PCR.

The final PCR product (HSP70-tE7) was excised from the agarose gel, purified by Bioneer extraction kit (Korea) and used for cloning in to the vector upon by digestion with BamHI and XbaI enzymes.

**Construction of Chimeric HSP70-tE7 Plasmid.** The chimeric HSP70-tE7 fragment was inserted into pcDNA3 plasmid after digestion with the above mentioned enzymes. Colonies containing the plasmid were obtained by transformation of E. coli DH5α and the presence of the desired plasmid was confirmed by colony-PCR using F/R3 primers, restriction enzyme analysis with BamHI and XbaI enzymes and sequencing. For the large scale preparation of the plasmid DNA, the transformed E. coli DH5α was grown in LB medium in the presence of ampicillin, and the plasmids were extracted using MN commercial Kit. DNA concentrations were determined by measuring the optical density at 260 nm and the absence of contaminating E. coli DNA or RNA was checked by agarose gel electrophoresis.
Table 1. Primers used in the three steps of PCR method for the construction of HSP70-tE7.

<table>
<thead>
<tr>
<th>primer</th>
<th>Primer Sequence (5' to 3')</th>
<th>Primer properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>TACAGAggatccGGAACACCATGGgCCAA</td>
<td>Kozak sequences are shown by underlined letters. BamHI restriction enzyme site is written in small letters.</td>
</tr>
<tr>
<td>Reverse primer 1</td>
<td>GCCGCCCCCATCTACCTCCTCAATG</td>
<td>HSP70 stop codon was mutated and three glycine codons (underlined letters) were inserted as a linker between HSP70 and tE7.</td>
</tr>
<tr>
<td>Reverse primer 2</td>
<td>ACAATTCCTAGTGTGCCCATTAA GCCGCCCCCARCAAACACACGCCGTATA</td>
<td>Italic letters are showed first part of tE7. Bold letters are the overlapped sequences between the first part and the second part of tE7.</td>
</tr>
<tr>
<td>Reverse primer 3</td>
<td>TATCATcttagaTTAGATGGGCACACAATTCCATG</td>
<td>Italic letters are shown as the second part of tE7. Bold letters are the overlapped sequences between the two parts of tE7. Stop codon is marked as highlighted letters and XbaI restriction enzyme site is written in small letters.</td>
</tr>
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**Cell line.** Wehi-164 cells (as negative control) and Wehi-164/HSP70-tE7 transfected cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, USA), 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 1 mM sodium pyruvate. The cells were grown at 37°C and 5% CO₂.

**Analysis of Eukaryotic Protein Expression.** In order to control the expression of HSP70-tE7 recombinant protein, Wehi-164 cells were transfected with pcDNA3/HSP70-tE7 using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Forty eighth hour after the transfection, the cells were used for RT-PCR and Western blot analysis.

**Generation of Stable Target Cells Expressing HPV 16 E7 Gene.** Wehi-164 cells were used for generation of stable cells expressing HPV 16 E7 gene as target cells in CTL assay as described previously (22).

**Western Blot Analysis.** Cell extract was prepared from both the un-transfected and transfected Wehi-164 cells 48 h following transfection and Western blotting was performed using a monoclonal mouse anti-HSP70 antibody (Sertec, UK) (22).

**RT-PCR for Confirming Expression of HSP70-tE7.** To determine the presence of HSP70-tE7 mRNA in the Wehi-164 transfected cells, RT-PCR was performed. Total cellular RNA was purified from the cells using RNX Plus kit (Cinagen, Iran). The contaminating DNA was removed by DNase treatment and the extracted RNA was used in RT-PCR. In brief, cDNA synthesis was performed on 1 µg total RNA with Oligo-dT primers at 37°C. PCR was performed on cDNA by the F and R3 primers as described above.

**Mice.** Six to eight weeks old inbred female BALB/c mice were obtained from Pasture
Institute of Tehran (Karaj, IR. Iran). One week before the experiments, the mice were housed and maintained in a good standard condition. All the experiments were done according to the Animal Care and Use Protocol of the Tarbiat Modares University (Tehran, Iran).

**DNA Vaccination.** The mice were injected with 50 µl of 10 µM cardiotoxin into each tibialis anterior muscle 5-6 days prior to DNA injection. For DNA vaccination, 90µl of plasmid DNA (1 µg/µl in PBS) was injected into each pretreated muscle. Ten days later, the mice were sacrificed and their splenocytes were isolated (20, 23, 24).

**CTL Assay.** The splenocytes from the immunized mice were harvested and used for measuring their CTL activity by detecting the apoptosis and necrosis in target cells as described previously (22).

**ELISA for Cytokines.** The splenocytes obtained from vaccinated mice were used for the measurement of IFN-γ and IL-4 production using ELISA commercial kits (R&D Systems, UK) according to the manufacturer's instructions (22).

**CD4 and CD8 Staining and Flowcytometry Assay.** For analysis of the number of CD4 or CD8 positive cells by flowcytometry, the splenocytes from the vaccinated animals 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 obtained and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), L-glutamine, 2 mM; penicillin, 100 U/ml; streptomycin, 100 µg/ml; and sodium pyruvate, 1 mM. The cell viability was determined and stimulation index was calculated by MTT assay as described previously (22).

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

**RESULTS**

**Generation of HSP70-tE7 Fusion Construct.** Full length HPV 16 E7 is an oncogenic protein which cannot be used in DNA vaccination. Therefore, in the present study, a part (83-93 codons) of E7 without any oncogenicity or transforming activity was chosen. Our hypothesis was that the fusion of HSP70 to HPV 16 tE7 would introduce the protein after DNA vaccination in antigen presenting cells (APCs). The HSP70-tE7 fusion fragment was generated by an overlapping PCR. Chimeric HSP70-tE7 was formed by linking the full length HSP70 (without stop codon) to truncated HPV 16 E7 (aa 83-93, containing stop codon for proper protein production). The construct was inserted into an expression vector under the control of cytomegalovirus immediate early promoter (pCMV).

The presence of desired plasmid in bacteria was confirmed by colony-PCR using F/R3 primers. The colonies containing the desired plasmid were positive and their segment was about 2000 bp using F/R3 primers. Restriction enzyme analysis using BamHI and XbaI restriction enzymes was performed on positive colonies and the plasmids containing the desired fragment were confirmed by sequencing.

**Expression and Characterization of HSP70-tE7 Fusion Protein in Vitro.** To analyze the expression of the encoded fusion protein, the DNA was introduced into the Wehi-164 cells by transient transfection method as described under Materials and Methods.
About 48 hours after transfection, the cells were harvested and the extracts were analyzed by SDS-PAGE and Western blotting. Cells transfected with either HSP70-tE7 or HSP70 (alone) showed a protein band of approximately 70 KDa corresponding to HSP70 gene (lanes 1 and 3 in Figure 2 for HSP70 and HSP70-tE7, respectively).

**Figure 2.** Detection of HSP70-tE7 protein in the transfected cells by Western blot analysis. Wehi-164 cells were transfected with the HSP70-tE7 or HSP70 expression construct. HSP70 protein was detected in the lysates by Western blot analysis using HSP70-specific monoclonal antibody. Western blot analysis showed a protein band with a size of approximately 70 KDa corresponding to HSP70 in the HSP70 or HSP70-tE7 transfected cells (lanes 1 and 3 for HSP70 and HSP70-tE7, respectively, but not in the pcDNA3 (alone)-transfected cells (lane 2). The protein was not detected in the culture medium of pcDNA3 (empty vector) transfected cells (lane 2). The results indicated correct expression of the protein as it migrated according to its corresponding size.

In addition, RT-PCR was performed for confirming the expression of HSP70-tE7 in the Wehi-164 transfected cells using the F and R3 primers. The experiment showed the desired fragment in HSP70-tE7 transfected cells but not in HSP70- or pcDNA3-transfected Wehi-164 cells.

**Vaccination with HSP70-tE7 Fusion DNA can Induce E7-Specific CTL Responses.** CTL responses to E6 and E7 genes were demonstrated to correlate with an effective immunotherapy of HPV immortalized tumors (12). To determine if fusion to HSP70 enhances the ability of tE7 to induce cytotoxic T-cells in vivo, four groups of mice were considered and vaccinated with pcDNA3 (negative control 1), pcDNA3/HSP70 (negative control 2), pcDNA3/E7 (positive control), and pcDNA3/HSP70-tE7 (test). Two negative control mice groups were used for the elimination of NK cell responses and other environmental interferences. Ten days after intramuscular injection of 90µg of vector DNA, the mice were sacrificed and the splenocytes were prepared as described under Materials and Methods.

**Determination of Specific and non Specific Cytotoxicity.** The splenocytes of vaccinated animals 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. Vaccination with pcDNA3/E7
and pcDNA3/HSP70-tE7 induced both apoptosis and necrosis in the vaccinated mice compared with the mice vaccinated with pcDNA3 or pcDNA3/HSP70 (p<0.05, Figure 3). The pcDNA3/E7 and pcDNA3/HSP70-tE7 vaccinated mice did not show any significant differences (p<0.05).

**Figure 3.** E7-specific CTL responses (apoptosis A, necrosis B) from the BALB/c mice immunized with pcDNA3/E7 and pcDNA3/HSP70-tE7 DNA vaccines compared with the pcDNA3 alone or pcDNA3/HSP70 plasmids. The mice (6 per group) were immunized with the above mentioned plasmids via intramuscular injection. Splenocytes were obtained from the immunized mice 10-days after vaccination. CTL assay was performed using Annexin/PI kit and the 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 4.** IFN-γ (A) and IL-4 (B) assays were performed using ELISA kit. Splenocytes were obtained from the immunized mice 10 days after the vaccination with pcDNA3 (negative control 1), pcDNA3/HSP70 (negative control 2), pcDNA3/E7 (positive control), and pcDNA3/HSP70-tE7 (test) plasmids via intramuscular injection. The splenocytes were re-stimulated with the lysate of Wehi-164 expressing E7 protein. IFN-γ and IL-4 concentrations were determined by commercial ELISA kit 48 hours after specific induction. As seen in this Figure, IFN-γ increased in the pcDNA3/E7 and pcDNA3/HSP70-tE7 vaccinated mice compared to the negative controls. The differences observed for E7 and HSP70-tE7 IFN-γ but not for IL-4, were statistically significant.
**Determination of IFN-γ and IL-4 Levels Following Vaccination.** Since most of the antitumor activity of CD8+ T cells are mediated through secretion of the inflammatory cytokines such as IFN-γ, the cytokine profiles of IFN-γ and IL-4 were examined by ELISA. In cytokine assay, IFN-γ was significantly increased in the pcDNA3/E7 and pcDNA3/HSP70-tE7 vaccinated mice compared to the two negative control groups (Figure 4A), but IL-4 assay did not show any statistically significant differences in all groups (Figure 4B). The differences observed for E7 and HSP70-tE7 were not statistically significant.

**Determination of CD4/CD8 Ratio Following Vaccination.** The splenocytes of vaccinated mice were used for stained by anti-CD4 and anti-CD8 monoclonal antibodies conjugated with FITC/RPE, and used for analysis by a flowcytometry. The CD8+ cells showed significant increases (p<0.05) in the positive control and the test groups compared with the two negative control groups (Figure 5).

![Figure 5](image)

**Figure 5.** The splenocytes were harvested from the immunized mice similar to the CTL assay and anti-CD8 and anti-CD4 monoclonal antibodies conjugated with FITC and RPE were used for staining and analyses were done using a BD flowcytometer. This Figure shows that the differences observed for CD8 (A) and CD4 (B) the E7 and HSP70-tE7 vaccinated groups were statistically significant (p<0.05).

Elevations in CD8/CD4 ratios were also demonstrated. Therefore, the responses of both the E7 and HSP70-tE7 vaccinated groups shifted to CD8 response which is necessary for tumor cell elimination. The difference between E7 and HSP70-tE7 vaccinated mice were not statistically significant (p<0.05).

**Determination of the T cell Proliferation Following Vaccination.** MTT cell proliferation assay which measures the cell proliferation in response to specific antigen was increased in the pcDNA3/E7 and pcDNA3/HSP70-tE7 vaccinated mice but not in the mice vaccinated with pcDNA3 or pcDNA3/HSP70 (Figure 6).
Figure 6. Stimulation Index (SI) of vaccinated mice. The splenocytes from the immunized mice were obtained and re-stimulated with the cell lysate of the target cells. Seventy two hours after induction, cell proliferation assay was performed using MTT solution. Formazan crystals were dissolved in dimethyl sulfoxide by vigorous pipetting. Optical densities were obtained at 540 nm. The results show that the Stimulation Index values of the E7 and the HSP70-tE7 vaccinated mice increases significantly compared to the negative controls.

DISCUSSION

Certain types of human papillomaviruses have been recognized as major etiological factors for the development of cervical cancer (25). The most prominent HPV type in this respect is HPV 16, which can be detected in about half of all invasive cervical cancers. E7 protein is expressed in all tumor tissues and there are in vitro data indicating that E7 is required for the growth of tumor cells (20).

Different studies using HPV-transformed cells have shown that the E7- specific T cells can prevent the growth of transformed cells and reject the established tumors (26). Activated CTLs function directly as effector cells, providing anti-tumor immunity through lysing the tumor cells or releasing the cytokines capable of interfering with the propagation of tumors. In several cancer vaccine studies, depletion of CD8$^+$ CTL causes the loss of anti-tumor effects (27-28). Therefore, increasing of MHC class I antigen presentation pathway has been a primary concern in cancer immunotherapy (12).

Our previous study indicated that the DNA vaccine encoding wild type E7 protein could induce E7-specific CTL responses and E7-expressing cells were killed by the CTLs which were induced by construct inoculation (22).

In this study, we have demonstrated that linkage of tE7 to HSP70 gene can preferentially induce CD8$^+$ T cell responses. One possible mechanism for the enhancement of CD8$^+$ T cell responses is the adjuvant effects of HSP70 and its ability to enter into professional APCs via receptor-mediated endocytosis. Lin et al. (28) showed that DNA vaccines with HSP70 fused to mutant HPV16E7 elicited strong E7-specific cellular immunity and generated significant CD8+ T cell-dependent therapeutic
responses’ but in our study, E7 CTL epitopes were linked to HSP70 and did not enhance immune responses when compared to E7 wild type.

Further aspects of such fusion proteins have been studied by several researchers. Recently, TLR family of cell surface receptors and CD91 were demonstrated to be involved molecules in HSP-receptor complex formation (29). Following the attachment of these receptors to HSP70, the complex was endocytosed by APCs. In the intracellular compartment, they will gain access to the endoplasmic reticulum and the peptides are represented by MHC class I and class II molecules on the surface of APCs.

We compared the E7-specific immune responses of the mice immunized with the expression vectors containing either E7 alone or tE7 fused to HSP70 gene. The E7-specific immune responses were significantly increased in the E7 and HSP70-tE7 vaccinated mice compared to the negative control groups, but no significant differences between the E7 and HSP70-tE7 immune responses were observed. Previous studies had shown that the HSP70 gene can represent an adjuvant activity by linkage to the genes in different DNA vaccines, but our data shows that although the DNA vaccine encoding tE7 linked to the HSP70 gene can induce immune responses, the induced responses do not show any significant differences compared with the E7 gene alone. Therefore linkage of HSP70 gene to complete genes may induce strong immune responses compared to the desired gene alone as shown by previous studies, but it can not probably show a significant increase in the immune response in DNA vaccines encoding a small section of the gene. Based on our results, DNA vaccines representing either E7 alone or immunogenic epitope of E7 gene with HSP70 also did not show any statistically significant differences in the ability to induce CTL responses in vaccinated mice. One limitation of full length E7 DNA vaccine is its oncogenicity and there are potential risks associated with the presence of HPV 16 E7 protein in the host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRb protein in the nuclei. Thus, the presence of E7 in the host cells may lead to malignant transformation of the host cells. Considering this point that the full length E7 protein is known to have transforming activity and oncogenic potential, a known CTL epitope of HPV16 E7 protein linked to C-terminal of HSP70 without any transforming activity and oncogenic function may represent a safer candidate for DNA vaccination, although the necessary steps should be taken to ensure the safety of the vaccine.

In conclusion, although E7-specific immune responses were significantly increased in the E7 and HSP70-tE7 vaccinated mice compared to the negative control groups, it was not strong enough to be used as a therapeutic vaccine. In future, we should use multi-epitope design of polytopes for vaccine development which will be able to deliver multiple B and T epitopes as immunogens to the MHC class I and class II pathways, creating strong immune responses.

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