HSP70 modified response against HPV based tumor

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Abstract. – BACKGROUND AND OBJECTIVES: DNA immunization is quite inventive vaccination strategies that engage the direct introduction of plasmid DNA encoding the desired antigen into the host. DNA vaccines expand strong protective responses against tumors. The desired target E7 oncogene products represent a target of choice for the therapeutic vaccination. The efficacy of vaccination is limited and it is often necessary to enhance the immune response by using adjuvant in order to achieve the desired responses. Numerous approaches have been applied to boost the effectiveness, such as the fusion or co-administration of cytokine and co-stimulatory molecules gene. Heat-shock protein 70 a family of chaperone proteins makes possible delivery of non-covalently bound peptide to MHC I molecules and influences peptide-specific CTL responses and cure treated individuals. HSP70 have been proposed as the affective adjuvant and expected to act as an appropriate substitute of both cytokine and co-stimulatory genes.

MATERIALS AND METHODS: In the current study, the impact of HSP70 co-delivery and HPV-E7 boosting on cellular immune responses and protection has been investigated by intramuscular injection of mixed DNA constructs.

RESULTS: Our results reveal that the target DNA vaccine can influence an E7-specific CTL response, which is imperative in the lysis of infected tumor cells, compared to negative control ($p < 0.05$). Additionally, treatment of tumor-bearing mice with pcDNA/E7 + HSP70 plasmid generates stronger immune responses and decreased significantly tumor sizes. Immunization with HSP-based vaccine with special target immunogen can induce potent and specific anti-tumor or anti-viral immune responses.

CONCLUSIONS: Co-administration of pcDNA/E7 + HSP70 plasmid was immunologically more effective than pcDNA/E7 alone. It was concluded that all the characteristics observed during our investigation demonstrate the potent adjuvant activities of HSP70 and could be an efficient approach to persuade dramatically E7-specific immune responses as future cervical cancer vaccine.

Key Words: HPV16-E7, DNA vaccine, Enhancement, HSP70.

Introduction

DNA based immunization works well in animal models for the prevention and induction of both humoral and cell-mediated immune responses.

Human papillomavirus type16 (HPV-16) infection in humans is coupled with most squamous cell carcinoma are common all over the world. Expression of the early oncogenic proteins E6 and E7 are necessary to preserve the transformed state of the tumor cells. Therefore, an efficient vaccination is highly required to control these infections.

Most experimental evidence points the notion that tumor rejection is mainly based, if not completely, on T cell reactivity. Indeed, the combinations of CTL and antibody response have been shown to be necessary for efficient tumor rejection. Also, several lines of evidence suggest that cell-mediated immunity is important in controlling both HPV infection and HPV-associated neoplasm’s. Therefore, immunotherapies targeting E7 protein may provide an opportunity to prevent and treat HPV-associated cervical malignancies.

Many studies have shown that the route of DNA vaccine administration plays an important role in the induction of protective immune responses. Administration of DNA vaccine via muscle, inducing a long-term cellular immunity, may be a promising approach to other kinds of vaccine administration. DNA vaccine strategies based on the use of full-length or truncated or
modified HPV16-E7 gene and route of administration induce protective immunity or therapeutics effect against tumors in animal models\(^1,10\). E7 is a potential target antigen for cytotoxic T-cell responses and an ideal antigen for incorporation into a therapeutic vaccine\(^11\). Some studies have benefited the effect of cytokines, stimulatory molecules, or heat shock proteins (HSPs) to enhance the antitumor potency of DNA vaccines\(^8,11,12\).

HSPs have been used as a potent adjuvant in immunotherapy of cancers and infectious disease\(^12,13\). They are families of highly conserved and abundant intracellular proteins involved in chaperoning, cross-presentation of class I-restricted epitopes, and activation of antigen presenting cells\(^13\).

The purpose of this study was to evaluate and discuss the effect of co-administration of HSP70 and HPV16-E7 DNA vaccine in animal models and the findings showed encouraging result as a therapeutic vaccine strategy against HPV-associated tumors.

### Material and Methods

#### Cell Line

TC-1 cell line was purchased from Pasteur Institute, Tehran, Iran. 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% CO\(_2\).

#### Mice

Female C57BL/6 mice (6-8 weeks old) were purchased from the Pasteur Institute (Tehran, Iran). The mice were housed for one week before the experiment, given free access to food and water, 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.

The cages were coded, and the suspension of 100 µl containing 5×10\(^6\) TC-1 cells/mouse was injected in the left flank subcutaneously in order to create tumor in mice.

#### Tumor Monitoring

For the *in vivo* experiments, C57BL/6 mice were challenged subcutaneously and after a week, tumors were noticeable and palpable in treated mice. The neoplastic masses were measured with calipers every other day and tumor volume was estimated according to Carlsson’s formula\(^14\). The average tumor size in millimeter is reported as the average of all dimensions measured. Smallest diameter (1) and biggest diameter (2) were measured and tumor volume was calculated using the following formula:

\[
V = \frac{(a \times b)}{2}
\]

#### Vectors Preparation

Previously, the HPV16/E7 gene was isolated by PCR, cloned in pTZ57R/T-E7, 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\(^15\). The expression vector containing human HSP70 was kindly provided by Dr McLean P.J. (Department of Neurology, Mass General Institute for Neurodegenerative Disease, Massachusetts General Hospital, Boston, MA, USA) and the presence of the HSP gene in the constructed vector (pcDNA3/HSP70) was determined using restriction enzyme analysis. A confirmed HPV16-E7 DNA plasmid and pcDNA3/Hsp70 were amplified using DH5\(\alpha\) strain of E.coli in Luria-Bertani medium and the plasmids were extracted and purified using Bioneer commercial Kit (Daejon, Korea). Large-scale purification of plasmid DNA samples (pcDNA/E7 and pcDNA/Hsp70) was conducted by ion-exchange chromatography QI-AGEN kit (Hilden, Germany) according to the manufacturer’s instructions.

#### Mice Immunization

For the therapeutic experiments, C57BL/6 mice (7 per group) were challenged by subcutaneous injection in the right flank with 5×10\(^3\) TC-1 cells and were grouped into 5 cages and each group was intramuscularly vaccinated 2 times by two week intervals with phosphate buffered saline (PBS) (negative control), pcDNA3 (negative plasmid control), pcDNA3/Hsp70, pcDNA3/E7, and pcDNA3/Hsp70 and pcDNA3/E7. Negative control mice groups were used for elimination of none specific responses and other environmental interferences. Mice were vaccinated at 7 and 14 days post-tumor cell inoculation and monitored.
up to 60 days (Table I). The tumor volume was estimated according to Carlsson’s formula as described in previous section. Two weeks after the last immunization 3 mice/group were sacrificed and their splenocytes were removed.

**Lymphocyte Proliferation Assay (LPA)**

Two weeks after the last immunization, single cell suspension of mononuclear cells were harvested from immunized and negative control groups of mice and used for lymphocyte proliferation assay. 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 treated TC-1 cells per well. A total of 100 µl of medium alone or 5 µg/well phytohemagglutinin (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 thiazol-2-yl) 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% CO₂. 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.

\[
SI = \frac{(Cs - Cu)}{Cu}
\]

All tests were performed in triplicate for each mouse.

**Cytotoxic T Lymphocyte (CTL) Assay**

The CTL activity of the spleen mononuclear cells (MNCs) (used as the effector cells) from the variously treated mice was assayed immediately by lactate dehydrogenase release (LDH) assay, according to manufacturer’s instructions (Takara Biotechnology Co., Ltd., Dalian, China). Various concentrations of spleen mononuclear with 1×10⁴ stimulated target EL4 cells (2 × 10⁴ cells/well) in a 100 µl volume at various 100:1, 50:1, and 25:1 effector/ target ratios for 4 h in phenol red-free RPMI 1640 containing 3% FCS. After centrifugation at 250 g for 10 minutes, the supernatants (50 µl/well) were transferred to the 96-well round-bottom plates, and lyses of target cells were determined by measuring LDH release. Several controls were used for the cytotoxicity assay. “High control” was the total LDH released from the target cells, and all EL4 cells were lysed by medium containing 1% Triton X-100. “Low control” was the natural release of LDH from the target cells, which was obtained by adding EL4 cells only in the assay medium. The assay for all samples, including the controls, was performed in triplicates. The percentage of specific lysis was determined by the following formula: the specific lysis (%) = (optical density [OD] of experimental LDH release – OD of spontaneous LDH release of effector cells – OD of spontaneous LDH release of target cells)/(maximum LDH release of target cells – OD of spontaneous LDH release of target cells) × 100%. All determinations were performed in triplicate.

**Statistical Analysis**

The SPSS version 13 (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 significant. All values were expressed as means ± SD.

Table I. The results of one way ANOVA test. Comparison of cytolytic activity in different vaccinated groups.

<table>
<thead>
<tr>
<th>Hsp70 &amp; E7</th>
<th>E7</th>
<th>Hsp70</th>
<th>pcDNA3</th>
<th>PBS</th>
<th>One-way anova test</th>
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<td>0.00</td>
<td>0.00</td>
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<td>−</td>
<td>0.24*</td>
<td>pcDNA3</td>
</tr>
<tr>
<td>0.00</td>
<td>0.8*</td>
<td>−</td>
<td>0.00</td>
<td>0.00</td>
<td>HSP70</td>
</tr>
<tr>
<td>0.00</td>
<td>−</td>
<td>0.8*</td>
<td>0.00</td>
<td>0.00</td>
<td>E7</td>
</tr>
<tr>
<td>−</td>
<td>0.00</td>
<td>0.00</td>
<td>0.006</td>
<td>0.02</td>
<td>Hsp70 &amp; E7</td>
</tr>
</tbody>
</table>

*Non-significant.
Results

Effect of DNA Vaccines on Tumor Therapy Assay (Immunotherapy)

In this study, we evaluated the effect of DNA vaccination on the progression of tumor in the TC-1 tumor-bearing mice. The protocol of the study from the beginning of subcutaneous tumor establishment and inoculation of DNA vaccine was performed according to standard protocols.

The inhibition rate of tumor growth was significantly different ($p < 0.05$) in the group vaccinated with all test groups in comparison with the control groups. The tumor-bearing mice were monitored every other day and the numbers and the inhibition rate of tumor growth from the beginning of formation until 6 weeks after the last vaccination was shown in Figure 1.

The efficacy of DNA vaccination appeared to be in order of pcDNA3/Hsp70 & pcDNA3/E7 > pcDNA3/E7 > pcDNA3/Hsp70 > pcDNA3 > PBS. The enhanced inhibition of tumor growth was shown in pcDNA3/Hsp70 and pcDNA3/E7.

Evaluating the Percentage of Protection In Vivo

The immunized and control mice were challenged by intramuscular injection of TC-1 cell line and observed every other day for possible signs and symptoms of tumor and survival rate. As shown in Figure 2, the mice inoculated with pcDNA3/Hsp70 and pcDNA3/E7 products were protected mice from death significantly vs. other test groups ($p = 0.04$ and $p = 0.03$ for pcDNA3/E7 and pcDNA3/Hsp70 respectively). All control negative mice died on the 40th day, whereas 75% of pcDNA3/Hsp70 & pcDNA3/E7 (main test group) mice survived.

![Figure 1](image1.png)

**Figure 1.** The inhibition rate of tumor growth in vaccinated group of tumor-bearing mice.

![Figure 2](image2.png)

**Figure 2.** Protection of mice against TC-1 cell line challenge. The effect of pcDNA3/Hsp70 & pcDNA3/E7 immunization on the survival of tumor-bearing mice was significant vs. other test groups ($p = 0.04$ and $p = 0.03$ for pcDNA3/E7 and pcDNA3/Hsp70 respectively). All challenged groups with $5 \times 10^5$ TC-1 cells received two intramuscular injections.
**Co-administration of HSP70 and HPV-16 E7 Strategy Leads to Enhanced CTL Response**

The enhancement of the specific cytolytic activity in immunized mice was evaluated in this study by lactate dehydrogenase (LDH) release assay as described in Materials and Methods. As shown in Figure 3 lymphocytes in vaccinated mice with pcDNA3/Hsp70 & pcDNA3/E7 (52.86±4.1) increased specific cytolytic activity at an E/T ratio of 50:1 significantly as compared to that of pcDNA3/E7 (30.67±3.2), pcDNA3/Hsp70 (36.17±2.4), pcDNA3 (negative plasmid control) and PBS (negative control) (18.49±1.9 and 16.01±1.6% respectively) (*p* < 0.05) (Table I). However, no statistically significant difference in cytolytic activity was found between negative control groups. Based on the results, the CTL activities were much stronger in Co-administration of HSP70 and HPV-16 E7 strategy as compared with those in positive control ones.

**Determination of the T cell Proliferation Following Vaccination**

Lymphocyte proliferation was measured 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 formed and suspended in dimethylsulfoxide by vigorous pipeting. Their optical densities were measured at 540 nm.¹⁵ The stimulation index was calculated and the results shows that the SI of pcDNA3/Hsp70 and pcDNA3/E7 group improved obviously compared to the other groups especially negative controls (3.89±0.11) (Figure 3). All of the test groups (pcDNA3/Hsp70, pcDNA3/E7 and pcDNA3/Hsp70 and pcDNA3/E7) showed significant difference with negative controls (*p* ≤ 0.05). E7-specific CD8 T-cell in immunized C57BL/6 mice with E7 or Hsp70 itself had less effect than pcDNA3/Hsp70 and pcDNA3/E7 group on the stimulation Index (SI) (Table II).

**Discussion**

DNA vectors have been extensively used for cancer immunotherapy and vaccines development against various pathogens. It has been reported that in the absence of appropriate adjuvants, only weak immune responses were observed in mice vaccinated by DNA vaccines.¹⁶ Innovative strategies are necessary to improve a strong T cell-dependent antitumor immunity

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**Table II.** The results of one way ANOVA test. Comparison of stimulation calculated indexes for different vaccinated groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.00</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>0.00</td>
</tr>
<tr>
<td>Hsp70</td>
<td>0.00</td>
</tr>
<tr>
<td>pcDNA3/Hsp70 &amp; E7</td>
<td>52.86±4.1</td>
</tr>
</tbody>
</table>

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*Not-significant.*
which are important for the anti-tumor effect. Genetic adjuvants and HSPs defined generally as any substance that promote the immunogenicity of antigen, have been extensively used in vaccine technology. They were originally thought of as agents mixed with an efficient antigen for use in animal models\textsuperscript{3,4,19}.

HSPs are among the most important groups of ubiquitously expressed protein chaperones involved in protein folding and in assisting refolding of recombinant foreign proteins under normal and stress conditions\textsuperscript{14,20}.

Inducible HSP70 is a member of HSPs with similar biological characteristics to other chaperones. It may function as a sort of link between innate and adaptive immune response\textsuperscript{21} and stimulate cellular immunity.

It was revealed that tumor cells can be recognized by CTL following presentation of Tumor Associated Antigens (TAA) when physically connected with MHC molecules\textsuperscript{22}.

Therefore, sufficient CTL responses are often needed for controlling viral based tumor and rate of disease progression.

The immunized mice were significantly protected against lethal tumor challenge. They exhibited significant cell mediated immunity specific for the tumor cells, as measured by immunization significantly enhanced survival ($p = 0.00$) compared to control groups.

In conclusion our results raised the possibility that HSPs could regulate cell differentiation and proliferation and accelerate the CTL response comparing to DNA vaccination alone. In those studies co-delivery of HSP genes were able to expand the immune response and influence the immune pathway. However, it has also been provided new insights into the regulation of immune system and its activation against a pathogen may be generally beneficial in management and treatment of human cancers.

The type of immunity clarified in those studies in animal models, the origin of tumor derived cells, the concentration, formulation, guidelines and the route of vaccine administration should also be implemented. As mentioned by Khanna\textsuperscript{22} "our understanding of immune escape mechanisms employed by tumor cells will be helpful in designing more effective strategies to control human cancers".

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References


