Antitumor Immunity Induced by Genetic Immunization with Chitosan Nanoparticle Formulated Adjuvanted for HPV-16 E7 DNA Vaccine

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ABSTRACT

Background: In recent years attention has been paid to develop effective adjuvant systems for DNA vaccines. Co-formulation of a gene delivery vector with an immunostimulator can enhance therapeutic efficiency of DNA vaccine. **Objective:** To investigate the efficacy of chitosan as a nanodelivery system to enhance antitumor effects of human papilloma virus (HPV)-16 DNA vaccine with IL-12 gene for protection against TC-1 tumor using an animal model. **Methods:** The mice were challenged by subcutaneous injection of TC-1 cells and immunized intramuscularly with DNA vaccine thrice at seven-day intervals. One week after the last immunization, mice were sacrificed and antitumor effects were assessed through measuring lymphocyte proliferation, cytotoxicity, cytokines production, and tumor regression. **Results:** We found that co-formulation and co-administration of chitosan nanoparticles and IL-12 with HPV-16 E7 DNA vaccine induced higher antitumor effects compared with chitosan or IL-12 alone. E7-specific lymphocyte proliferation index and CTL activity were found to be significantly higher in combination group in comparison to single vaccination with either chitosan or IL-12. Co-formulation of chitosan and IL-12 resulted in higher IFN-γ and IL-4, and decreased IL-10 production. Furthermore, combined vaccination highly inhibited the tumor progression compared with chitosan or IL-12 alone. **Conclusion:** Chitosan nanoparticle is a promising delivery system for DNA vaccine and IL-12 is an effective genetic adjuvant for the induction of strong antitumor immune response.

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Keywords: Cellular immunity, Chitosan nanoparticles, DNA vaccine, IL-12, Papillomavirus, Tumor

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INTRODUCTION

DNA vaccines offer valid approaches to the generation of antigen-specific immunotherapy (1). DNA vaccines produce antigen-specific immunity in vivo by expression foreign antigens that can become the subject of immune surveillance in the context of both major histocompatibility complex (MHC) class I and II molecules of antigen-presenting cells (APCs) in the vaccinated host (2). However, while DNA vaccines have shown safety and potential utility for cancer therapy, the approach suffers from low immunogenicity and has restricted specificity for APCs (1). The strength of DNA vaccines encoding weak immunogenic tumor-associated antigens can be enhanced through the use of an adjuvant injected together with the gene delivery vector (3). Gene delivery vector can protect DNA vaccine from degradation by nucleases and facilitate cell uptake and intracellular delivery (4); adjuvant induces the molecular signals for enhancing the immune response against tumor (5). In this regard, co-formulation of an effective gene delivery vector and a potent adjuvant with DNA vaccine can enhance the therapeutic benefits.

Nanoparticle-based gene delivery has been the subject of several studies over the past few decades because they are widely employed biomaterials with an established safety profile in humans (6,7). The most effective nanoparticle-based gene delivery vectors are cationic polymers, especially chitosan, which create condensed complexes with negatively charged DNA to protect and improve cell entry (8). Our previous studies have demonstrated that chitosan-based nanoparticles are able to deliver the therapeutic gene to specific cells in vivo (9,10). The study also showed that intramuscular injections of chitosan nanoparticles with DNA vaccine induces significant antitumor effects (10).Interestingly, it has been reported that chitosan nanoparticles have immunostimulatory activity, and chitosan-based formulations for the delivery of genetic adjuvants might enhance the adjuvancity of cytokine co-administered with antigens (11-14). Recently, co-formulations and co-administration of cytokines with chitosan have been proved to increase local cytokine retention and bioactivity (15).

Gene-based immunostimulators (known as genetic adjuvants) have gained a lot of attention owing to their ability to stimulate protective antitumor immune responses (16,17). Among these genetic adjuvants, cytokine-based adjuvants such as interleukin (IL)-12 have been suggested as the most effective for enhancing immune responses (18). The IL-12 provides the molecular signals required for the stimulation of antitumor responses such as T cells and natural killer (NK) cells (19). Using exogenous IL-12 to treat malignant neoplasms has been well studied and heavily pursued (20). According to previous reports, cytokine-based immunotherapies would be more effective and less toxic if delivered locally and via an effective gene delivery vector (21,22). In the present study, the objective was to assess the gene delivery potential of chitosan nanoparticles and immunostimulatory ability of IL-12 in the co-formulation of chitosan and IL-12 to enhance the antitumor efficacy of human papilloma virus (HPV)-16 DNA vaccine in tumor mice model. The results presented here provide evidence as to the fact that chitosan/IL-12 formulation induces strong antitumor immune response.

MATERIALS AND METHODS

Cell line and mice. The TC-1 cell line (C57BL/6 primary lung epithelial cells transduced with retroviral vectors expressing HPV-16 E6/E7 and an activated H-ras gene) was provided from Pasteur Institute, Tehran, Iran. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco-BRL, UK) containing 10% FBS (Gibco), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.4 mg/ml G418. The cells were maintained in a humidified incubator at 37ºC and 5% CO2. Six- to eight-week-old female C57BL/6 mice were obtained from Pasteur Institute, Karaj, Iran, and maintained in individual cages with food/water ad libitum and in a controlled environment (temperature and humidity) in the animal house of Golestan University of Medical Sciences (Gorgan, Iran). This experi¬ment was approved by the animal ethics committee of the Golestan University of Medical Sciences.

Plasmids and adjuvant. The construction, amplification and purification of pcDNA3.1 plasmid expressing HPV-16 E7 has been previously described (23). The constructed pcDNA3.1 plasmid expressing IL-12 was kindly provided by Dr T. Sakai from University of Tokushima (24).

Preparation and characterization of low molecular weight chitosan nanoparticles as well as in vitro HPV-16 E7 transfection studies have been previously elucidated by our group (9, 10). The particle size was between 40 and 150 nm with a mean diameter of ~70 nm and positive zeta potential ~20 mV. The complex formation between chitosan nanoparticles and pcDNA at different N/P ratios was analyzed by agarose gel electrophoresis. Moreover, to evaluate the stability of the complex, the release behavior of pcDNA from nanoparticles in 100 h time duration at different time points was studied and measured via UV spectrophotometry as was previously described (9).

Tumor protection responses. Using the TC-1 tumor model, in vivo evaluations were carried out in the C57BL/6 mice. Mice were primarily challenged with subcutaneous injection of 2×10^5 TC-1 tumor cell lines in 100 µl PBS in the right flank. After one week, each group of mice (10 mice/group) were immunized intramuscularly three times at one-week intervals with 90 µg of E7 DNA, IL-12, E7 DNA+IL-12, chitosan-E7 DNA, chitosan-IL-12, chitosan-E7 DNA+IL-12 (test groups) and chitosan alone, pcDNA alone, and PBS (control groups). The control groups received a protocol similar to the test groups. In combination groups, mice received 90 µg of each vaccine.

Seven days after the final administration, five mice per group (5/10) were sacrificed and the splenocytes were obtained aseptically; further examined were immunological responses such as lymphocyte proliferation, cytotoxic responses, and cytokines production. For up to 6 weeks, the sizes of tumors in these mice were measured twice a week according to Carlsson's formula: (longest diameter) × (shortest diameter) 2) × 0.5

Lymphocyte proliferative response. In order to characterize the splenocytes obtained from the immunized mice, we performed the in vitro lymphocyte proliferation. For this purpose, lymphocytes were harvested from obtained splenocytes and prepared as effector cells by removing the erythrocytes with ammonium chloride-potassium lysing buffer. Next, a 50 µl aliquot containing 2×10^5 cells was added to each well of 96-well flat-bottom culture plates (Nunc, Denmark). The TC-1 cell lines (4×10^5) previously treated with mitomycin C (Sigma, St. Louis, Mo) (30 µg/ml for 3 h) as antigen, were added to wells in triplicate and incubated at 37ºC in 5% CO2 for 3 days. After that, 5 µg/ml MTT (3-(4,5-dimethyl tetrazolyl-2) 2,5 diphenyl) tetrazolyum bromide (Sigma

chemicals) was added to each well and incubated for 5 h at 37ºC in 5% CO2. DMSO (dimethyl sulfoxide) at a concentration of 100 µl was added to dissolve the produced formazan crystals. Plates were read at 540 nm, and the stimulation index (SI) was determined by the following formula: [(OD values of stimulated cells - relative OD values of unstimulated cells) / (relative OD values of unstimulated cells)].

Lymphocyte cytotoxicity assay. So as to evaluate the cytotoxic responses from the obtained lymphocytes, we performed the in vitro lymphocyte cytotoxic response. Briefly, the lymphocytes (as effector cells) were cultured in phenol red-free RPMI containing 3% FCS with EL-4 cells previously stimulated with antigen (4×10^5 mitomycin-treated (30 µg/ml for 3 h) TC-1 cells) as target cells (at 50:1 effector to target cell ratio). After 4 h of incubation, the culture plates were centrifuged and the supernatants (50 µl/well) were transferred to the 96-well flat-bottom plates. The CTL activity was measured based on the measurement of LDH release from lysed target cells using Cytotoxicity Detection Kit (Takara, Japan) according to the manufacturer's instructions. Plates were read at 490 nm after a 30 min incubation at room temperature, and cytotoxicity was determined by:

% Cytotoxicity= (Experimental value - Low control / High control - Low control) × 100

In the 'high control', all EL-4 cells were lysed with Triton X-100 and in the 'low control', EL-4 cells were treated only with assay medium.

Cytokine secretion assay. To assess cytokines production responses, lymphocytes were harvested from the obtained splenocytes. An aliquot of 5×105cells/100 µl was added to the wells of 96-well plates. Then, 4×10^5 TC-1 cells, previously treated with mitomycin C (30 µg/ml for 3 h), were added to each well. Three days later, cell supernatants were collected and used to detect the levels of IL-4, IFN γ and IL-10, using ELISA commercial cytokine detection kits (eBioscience, Inc. San Diego, CA) according to the manufacturer's recommendation.

Statistical analysis. Statistical analysis was performed with one-way ANOVA test. Differences were considered significant for p values< 0.05.All tests were performed in triplicate and all data are expressed as mean ± SD.

RESULTS

Co-formulation of chitosan and IL-12 enhances E7-specific T cell proliferation. The lymphocyte proliferative response was assayed seven days following the last vaccination. As shown in Figure 1, chitosan and IL-12 alone significantly enhanced the proliferative response of E7 DNA vaccines compared with E7 DNA vaccine group (chitosan-E7 DNA vs. E7 DNA vaccine: $p< 0.01$, and E7 DNA+IL-12 vs. E7 DNA: $p< 0.05$). However, chitosan-E7 DNA+IL-12 group induced the strongest antigen-specific proliferation of lymphocytes in response to E7 protein stimulation. The response was significantly higher in the chitosan-E7 DNA+IL-12 compared with the E7 DNA vaccine group ($p< 0.001$). More importantly, the proliferative response of chitosan-E7 DNA+IL-12 group was significantly higher than mice immunized with E7 DNA+IL-12 ($p< 0.01$), and chitosan-E7 DNA ($p< 0.05$). In this experiment, no statistically significant differences were found between the control groups (IL-12, chitosan, pcDNA, and PBS). The results indicated that chitosan, as a delivery system for DNA vaccine, has the potential to enhance the lymphocyte proliferative responses against E7 antigen, which could be augmented by boosting the antitumor immune response with IL-12 adjuvant.

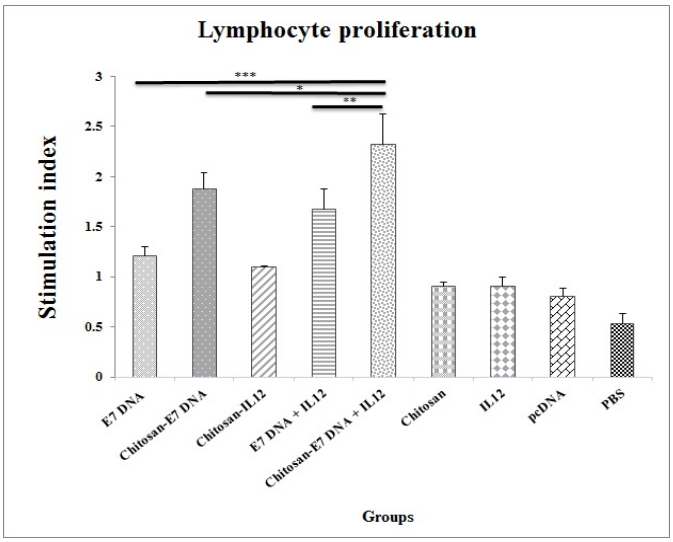

Figure 1. Chitosan/IL-12 adjuvant system induces E7-specific T cell proliferation. Each group of mice was challenged subcutaneously with 2×10^5 TC-1 cells, and immunized intramuscularly three times at one-week intervals. One week after the last immunization, mice were sacrificed and splenocytes were obtained, and lymphocyte proliferation was performed with MTT method. Results represent the mean ± SD of 5 animals in each group. (*p< 0.05, **p< 0.01, *** p< 0.001). Co-formulation of chitosan and IL-12 promotes E7-specific CTL response.

To determine the ability of chitosan and IL-12 co-formulation to enhance HPV DNA vaccine cytotoxicity, we assessed cytolytic activity of lymphocyte from immunized mice with LDH assay. As shown in Figure 2, chitosan and IL-12 alone significantly enhanced the cytotoxic response of E7 DNA (chitosan-E7 DNA (mean: ~46%)vs. E7 DNA vaccine (mean: ~28 %): p< 0.05, and E7 DNA+IL-12 (mean: ~47%) vs. E7 DNA (mean: ~28%): p< 0.05). However, mice immunized with chitosan-E7 DNA+IL-12 induced higher cytotoxic response (~65%) against E7 antigen compared to the E7 DNA vaccine group (p< 0.001). More importantly, the cytotoxic response in chitosan-E7 DNA+IL-12 group was higher than mice immunized with either chitosan (chitosan-E7 DNA) (p< 0.05) or IL-12 alone (E7 DNA+IL-12) (p< 0.05). In this experiment, the cytotoxic response in mice immunized with chitosan-IL-12 (~20%), IL-12 (~17%) and chitosan (~16%) had a higher cytotoxic response compared with the PBS group. Taken together, the combination of chitosan as delivery vector with IL-12 genetic adjuvant has the potential to ameliorate the CTL response of HPV DNA vaccine.

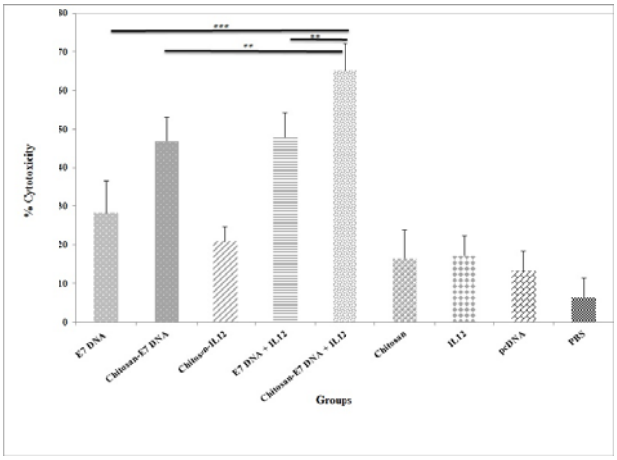

Figure 2. Chitosan/IL-12 adjuvant system induces E7-specific CTL response. Each group of mice was challenged subcutaneously with 2×10^5 TC-1 cells and immunized intramuscularly three times at one-week intervals. One week after the final immunization, mice were sacrificed and splenocytes were obtained, and CTL responses were performed with LDH assay. Results represent the mean ±SD of 5 animals in each group. (**p< 0.01, *** p< 0.001).Co-formulation of chitosan and IL-12 induces IFN-γ and IL-4, and decreases IL-10 production.

Via ELISA method, the levels of IL-4, IFN-γ and IL-10 in the lymphocytes culture supernatants were measured seven days following the last vaccination (Figure. 3). The data showed that mice immunized with chitosan-E7 DNA+IL12 more significantly produced IFN-γ and IL-4 comparisons with either chitosan (chitosan-E7 DNA) or IL-12 alone (E7 DNA+IL-12) ($p<0.001$). Furthermore, mice immunized with chitosan-E7 DNA+IL12 significantly decreased the production of immunosuppressive cytokine IL-10 compared with chitosan-E7 DNA and E7 DNA+IL-12 ($p< 0.001$). The control pcDNA, PBS, chitosan, and IL-12 groups induced no substantial increase in E7-specific cytokine secretion. The data indicate that the co-formulation of chitosan and IL-12 enhances E7-specific IFN-γ and IL-4, while reducingE7-specific IL-10 levels to HPV DNA vaccine in comparison with chitosan-E7 DNA and E7 DNA+IL-12 groups.

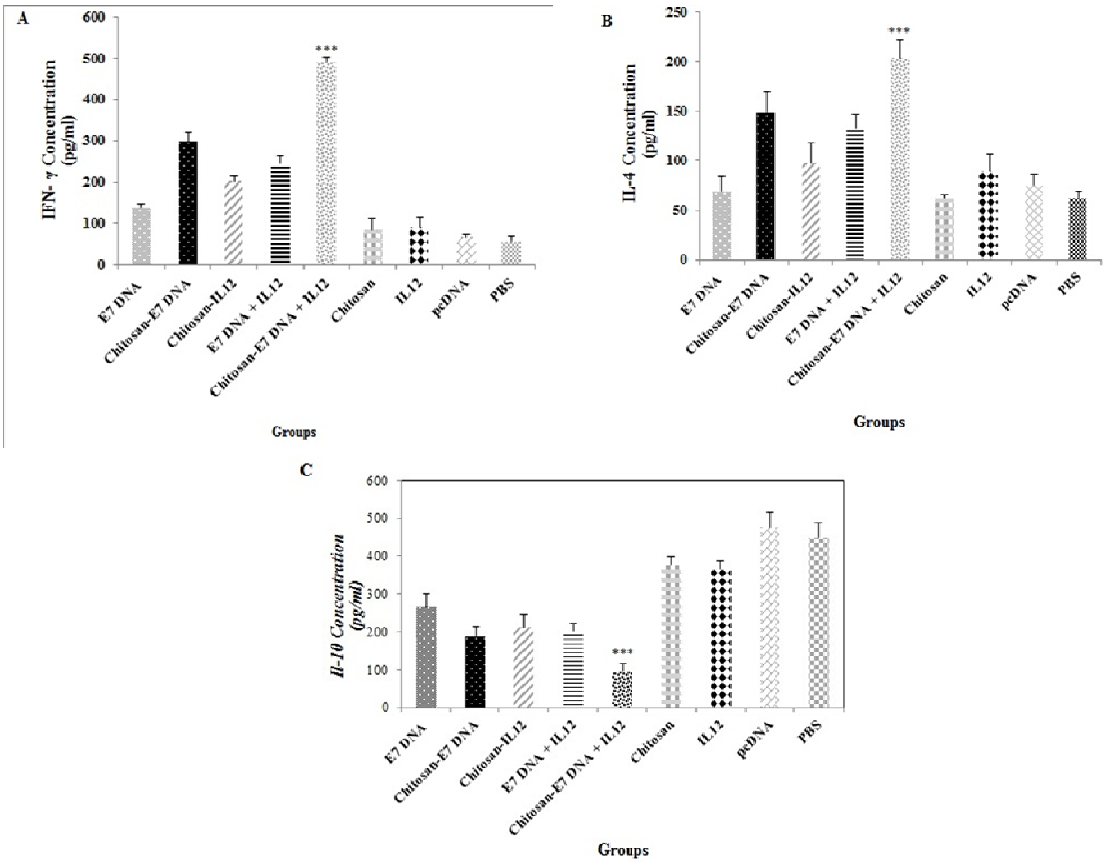

Figure 3. Chitosan/IL-12 adjuvant system induces IFN-γ and IL-4, while reducing IL-10 production. Quantitative ELISA analysis of IFN-γ (A), IL-4 (B) and IL-10 (C) secreted by lymphocytes upon re-stimulation with antigen. The production levels of cytokines were assessed from the supernatant of splenocytes culture using ELISA method. Each bar represents the mean optical density values ±SD from three replicate wells from five individual mice. (*** $p< 0.001$).

Co-formulation of chitosan and IL-12 induces tumor regression. Following the increase in the E7-specific immunity through the use of co-formulation system, we

assessed the reduction of tumor volume by therapeutic immunization. In this regard, mice challenged with 2×10^5 TC-1 tumor cell lines were monitored twice a week following immunization for up to 6 weeks. As shown in Figure 4, in agreement with the increase in the E7-specific immunity by novel adjuvant system, chitosan-E7 DNA+IL12 more significantly reduced tumor size compared with control groups (PBS, pcDNA, chitosan, IL-12, and chitosan-IL-12). A similar but not statistically significant decrease was observed in comparison with chitosan-E7 DNA, E7 DNA, and E7 DNA+IL12 groups. Additionally, the average tumor volume in the chitosan-E7 DNA, E7 DNA, and E7 DNA+IL12 groups was significantly lower than that in the control groups. Animals in control groups exhibited a completely stabilized tumor growth. No differences were found among the various control groups. Taken together, chitosan as a delivery system for DNA vaccine, has the potential to significantly reduce tumor growth, which could be maximized by boosting antitumor immune response with IL-12 adjuvant.

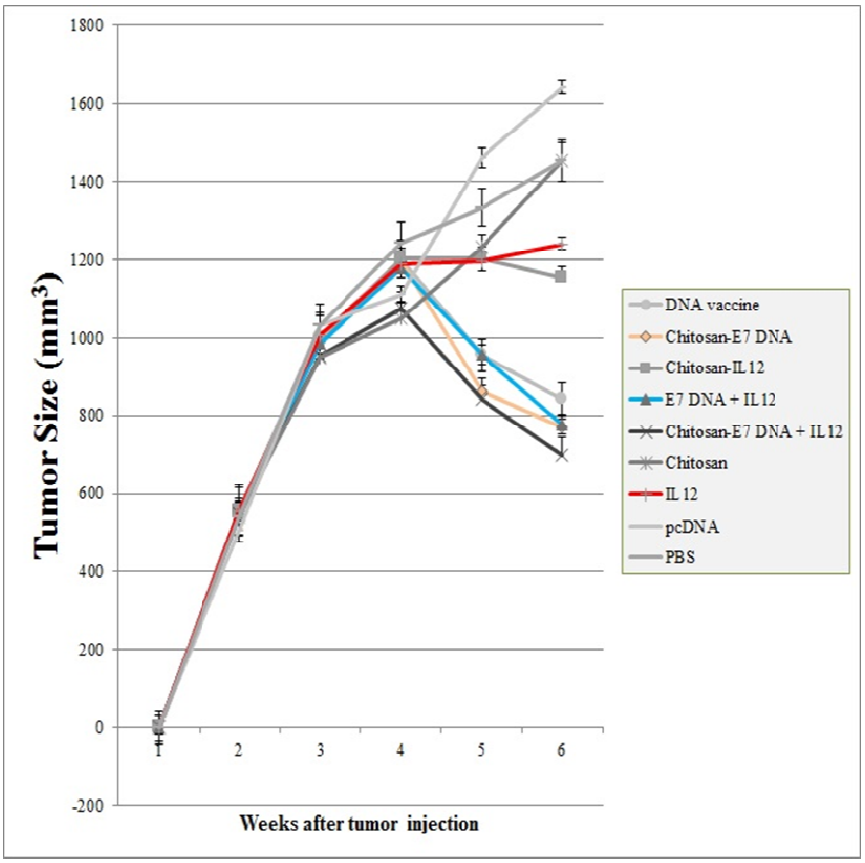

Figure 4. Chitosan/IL-12 adjuvant system induces tumor regression. The tumor size of the immunized mice was evaluated for up to 6 weeks. Tumor sizes represent the mean ± SD of 10 mice for 4 weeks and 5 mice after week 4 in each group. Line and scatter plot graphs depicting the tumor volume (in mm3) are also presented. The data presented are a representation of three independent experiments.

DISCUSSION

Papillomaviruses, accounting for 10% of worldwide cancer incidence, are the primary cause of cervical cancer and detected in 95% of such cancers. Infection with more than 10 high-risk genotypes of HPVs, especially HPV-16 and HPV-17, has been casually linked to the incidence of cervical cancer. These viruses are also associated with 50% of vulvar, vaginal, penile, anal and peri-anal cancers and up to 20% of oral, laryngeal and nasal cancers (25).Cervical cancer is the second most common malignancy in women worldwide, for which no effective therapy has been developed yet (26). More and more attention has been paid to cancer immunotherapy using DNA vaccines over the past few years (27). However, the limited delivery of therapeutic genes into APCs results in poor immunogenicity of DNA vaccines. Thus, enhancing the efficiency of DNA vaccine delivery and protecting DNA from degradation are important for the increase in DNA vaccine potency (2). Given the ability of chitosan in gene delivery, and the immunostimulatory activity of IL-12, we hypothesized that co-formulation and co-administration of chitosan nanoparticles with IL-12 can enhance antitumor effects of HPV-16 DNA vaccine encoding E7 gene. It has been reported that the oncogenic activity of the tumor cell line expressing HPV-E7 protein plays a crucial role in tumor establishment and development (28). To verify this hypothesis, we designed an in vivo tumor therapy experiment using an E7-expressing murine tumor cell line, TC-1, as a model of cervical carcinoma.

Chitosan-based gene delivery has been the subject of myriad investigations, as it has a rapid degradation in lysosomal compartment after cellular uptake, allowing for enhanced MHC-I restricted antigen presentation (28). Our previous findings have demonstrated that chitosan, as a non-toxic biodegradable and biocompatible cationic polymer, can bind and protect the entrapped DNA from degradation by nuclease and increasing the efficiency of cellular DNA uptake in vitro (9).More importantly, several studies have investigated the immunostimulatory activity of chitosan nanoparticles such as activation of APCs, cytokine and cytotoxic CTL response induction (11, 12, 29). Mori et al. has reported that chitosan is a suitable alternative to alum as a vaccine adjuvant where a potent cell-mediated immunity is required, because chitosan can enhanceTh1 and Th17 responses (30). According to Xie et al., Helicobacter pylori vaccine with chitosan as an adjuvant can protect against infection and induce both Th1 and Th2 type immune response (31). It has been demonstrated that intramuscular or intranasal administration of the chitosan-DNA nanoparticle complex in BALB/c mice induces significant antibody titers compared to those of naked DNA vaccine (32). Furthermore, mice immunized with the chitosan containing the swine influenza DNA vaccine stimulated higher immune responses and sustained the release of the plasmid DNA compared to the DNA vaccine alone (33).

IL-12-based therapies potentiate immunologic memory and have demonstrated remarkable antitumor effects for the induction of cell-mediated immunity in numerous animal tumor models (34). In this regard, several studies have investigated the immunostimulatory activity of IL-12 in the field of HPV DNA vaccine. Jin et al. demonstrated that immunization with adenoviral vectors carrying recombinant IL-12 and HPV-16 E7 resulted in the effective suppression of tumor growth in mice injected with TC-1 tumor cells (35). In Li et al., intranasal immunization with recombinant Lactococci carrying HPV-16 E7 protein combined with the genetic adjuvant of IL-12 DNA induced strong antitumor responses in TC-1 tumor model (18). The utility of IL-

12 gene as an adjuvant for a Hepatitis C virus (HCV) DNA vaccine in a mouse model has also been demonstrated (5).Furthermore, Park et al. showed that photodynamic therapy with recombinant adenovirus carrying IL-12,enhanced therapeutic immunity through improved expansion of the CTL subset mediated by CD8+ T cells (36).

In the present study, we used the co-formulation of chitosan/IL-12 as a new adjuvant system to enhance antitumor effects of HPV-16 DNA vaccine. Our results clearly indicate that co-formulation and co-administration of chitosan/IL-12 with HPV-16 E7 DNA vaccine in comparison with either chitosan or IL-12 alone induces: (a) higher E7-specific lymphocytes proliferation, (b) strong E7-specific cytotoxic response, (c) greater IFN-γ and IL-4 production, (d) lower IL-10 level, and (e) reduction in tumor size. These observations are in agreement with the potential benefit of chitosan/IL-12 combination in tumor therapy reported by others (37-41). It has been reported that the formulation of chitosan nanoparticles with IL-12 prevents colorectal cancer because liver resident antitumor immunity is triggered (38). Kim et al. showed that the intratumoral injection of chitosan, as IL-12 delivery system, into BALB/c mice bearing CT-26 carcinoma cells clearly suppresses tumor growth and angiogenesis, and significantly induces cell cycle arrest and apoptosis (16). In Zaharoff et al. study, the intravesical administration of chitosan/IL-12 induced a potent immunity against bladder cancer (39). Moreover, it has been shown that chitosan/IL-12 co-adjuvant increases IL-12 retention in the tumor microenvironment, eradicates murine tumors, and produces systemic tumor-specific protective immunity (15). According to Heffernan et al., the combination of chitosan and IL-12 promotes the generation of ovalbumin-specific CD4+ and CD8+ T-cell responses, as demonstrated by CD4+ splenocytes proliferation, Th1 cytokine release and CD8+ T cellresponse (20). Yang and Zaharoff reported that chitosan increases the IL-12-mediated leukocytic expansion in tumors and tumor-draining lymph nodes (40). Furthermore, Smith et al. showed that intravesical administration of chitosan/IL-12 induces tumor-specific systemic immunity against murine bladder cancer (41).

In addition to enhancing the tumor-specific CD4 T cells proliferation and the cytolytic activity of CD8 T lymphocyte, this chitosan/IL-12 co-adjuvant promotes cellular immunity through the down-regulation of the immunosuppressive cytokine IL-10. This is in line with the previous result where IL-12, as a genetic adjuvant, enhanced IFN-γ secretion, but inhibited IL-10 production. This implies that IL-12 gene delivery was capable of activating T cells that generate Th1-type cytokines (IFN-γ) but reduce immunosuppressive cytokines (42). IFN-γ is a cytokine that plays a pivotal role in antitumor host immunity, which elicits potent antitumor immunity by inducing Th1 polarization, CTL activation, and dendritic cell tumoricidal activity (43).Furthermore, elevated production of a Th2 cytokine IL-4 was observed in the current study. The increase in both IFN-γ and IL-4 cytokines is indicative of a mixed Th1-Th2 response. The higher the increase in IFN-γ is, the more dominant Th1 will be. In the initial murine experiments, IL-4 exhibited potent antitumor ability through inducing effective immune responses (44). Our cytokine results showed that the combination treatment has the potential to induce a wide range protective immunity, for which a balanced and potent stimulation of both the cellular and humoral responses is required. Corroborating the immunosuppressive action of IL-10 and antitumor activity of IFN-γ/IL-4, the decrease in IL-10 and the increase in IFN-γ/IL-4 level lead to the stimulation of antitumor response and tumor regression.

It is concluded that IL-12 may improve vaccine efficiency when included in delivery systems and as a gene-based adjuvant of the DNA vaccine. Taken together, the high

anti-cancer activity of HPV-16 DNA vaccine with chitosan/IL-12 co-adjuvant is a powerful tool against cervical cancer and is a promising subject for further investigation. Furthermore, the simplicity, versatility, and biocompatibility of chitosan/IL-12 immunotherapy have made it a suitable candidate for clinical translation. Although this study introduces a novel combination in order to enhance HPV DNA vaccine efficacy, further comprehensive investigations are required to explore the antitumor effect and other underlying mechanisms in tumor microenvironment. Using novel adjuvants and molecules may be effective in the field of HPV DNA vaccine for enhancing the immune responses (45-48).

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