

RESEARCH ARTICLE

Cytotoxic T Lymphocytes Elicited by Dendritic Cell-Targeted Delivery of Human Papillomavirus Type-16 E6/E7 Fusion Gene Exert Lethal Effects on CaSki Cells

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Abstract

Human papillomavirus (HPV) is the primary etiologic agent of cervical cancer. Consideration of safety and non human leukocyte antigen restriction, protein vaccine has become the most likely form of HPV therapeutic vaccine, although none have so far been reported as effective. Since tumor cells consistently express the two proteins E6 and E7, most therapeutic vaccines target one or both of them. In this study, we fabricated DC vaccines by transducing replication-defective recombinant adenoviruses expressing E6/E7 fusion gene of HPV-16, to investigate the lethal effects of specific cytotoxic T lymphocytes (CTL) against CaSki cells *in vitro*. Mouse immature dendritic cells (DC) were generated from bone marrow, and transfected with pAd-E6/E7 to prepare a DC vaccine and to induce specific CTL. The surface expression of CD40, CD68, MHC II and CD11c was assessed by flow cytometry (FCM), and the lethal effects of CTL against CaSki cells were determined by DAPI, FCM and CCK-8 methods. Immature mouse DC was successfully transfected by pAd-E6/E7 *in vitro*, and the transfecting efficiency was 40%-50%. A DC vaccine was successfully prepared and was used to induce specific CTL. Experimental results showed that the percentage of apoptosis and killing rate of CaSki cells were significantly increased by coculturing with the specific CTL ($p < 0.05$). These results illustrated that a DC vaccine modified by HPV-16 E6/E7 gene can induce apoptosis of CaSki cells by inducing CTL, which may be used as a new strategy for biological treatment of cervical cancer.

Keywords: Cervical cancer - Human papillomavirus - E6/E7 protein - CaSki cell

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Introduction

Cervical cancer is the second most common cancer and leading cause of cancer-related death to women in worldwide (Zeng et al., 2011). Human papillomavirus (HPV) which presents in 99% of cervical cancer cases is one of the causative agents of cervical cancer (Walboomers et al., 1999). This small, non-enveloped DNA virus that infects epithelial cells of skin and mucosa, which only replicates in differentiating keratinocytes. Infection of the mucosa with high-risk HPV types is considered as the single most important etiological factor in cervical carcinogenesis. Particularly HPV-16, is found in over 50% of squamous cell carcinomas of the uterine cervix (Milde-Langosch et al., 2000; zur Hausen, 2000). Despite the recent development of various therapeutic strategies, there is no specific therapy available for invasive squamous cell carcinoma of the cervix and its precursor lesions. Millions of patients suffer from HPV-associated morbidity or mortality (Parkin et al., 2006). An estimated 5 million

cervical cancer deaths will occur in the next 20 years due to existing HPV infections (Frazer et al., 2004). Thus, more efforts are needed to develop an effective therapy to achieve cure.

Some inspired results appeared to the prophylactic randomized HPV vaccination trials using virus-like particle vaccines against HPV-16 and -18 or HPV-6, -11, -16, and -18 (Koutsky et al., 2006). With the implementation of HPV vaccination programs, the preventive HPV vaccine is being used more and more widely for cervical cancer therapy in the Asian Pacific (Young, 2010). The preventive vaccine of HPV-16 and HPV-18 has been registered to fully protective against persistent infection and the associated with the development of high-grade genital lesions, but it is poorly benefit to the individual which has been infected (Wheeler et al., 2007). Therefore, there is an urgent need to develop therapeutic HPV vaccines. Peptide vaccination strategy was improved to be an appealing immunotherapeutic approach for the treatment of established viral-induced tumors (Barrios et

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al., 2012). Protein vaccine has become the most popular form of HPV therapeutic vaccines because it is safe and has no human leukocyte antigen (HLA) restriction. To date, there has been no report on using cytotoxic T lymphocytes elicited by dendritic cell-targeted delivery of human papillomavirus type-16 E6/E7 fusion gene for gene therapy of papillomavirus infections.

DC are cells that consist of heterogeneous subsets with different lineages and maturity. They not only initiate immunity, but also involve in the induction of tolerance *in vivo* (Banchereau et al., 2000; Steinman et al., 2002; Morelli et al., 2003). From their original application to the cancer, DC have been used in the more areas at present, for example, infectious diseases, immunoregulatory diseases, allergic diseases and allograft rejection. Genetic modification of DC with genes encoding immunoregulatory molecules provides a potential approach for Ag-specific regulation of T cell-mediated immunity by selectively targeting antigen-specific T cells (Matsue et al., 1999; Lu et al., 1999; Morita et al., 2001; Liu et al., 2003; Watanabe et al., 2003).

Here we used E6/E7 fusion protein for gene therapy of papillomavirus infections. Cloned HPV16 E6/E7 fusion gene from human cervical cancer cells, assayed the ability of CTL to produce lethal effect against CaSki cells *in vitro*. Our results showed the increased apoptosis and significantly killing rate of CaSki cells, suggesting genetically modified DC vaccine is a new way to develop therapeutic HPV vaccines.

Materials and Methods

Female C57BL/6 mice, 4-5 weeks of age, were purchased from the Experimental Animal Center of Chongqing Medical University (China). Animals were housed under pathogen-free conditions. All experimental procedures were carried out following approval of the Institutional Animal Care Committee. CaSki cell line was purchased from the Cell Bank of the Chinese Academy of Science (China). Recombinant adenovirus vector carrying E6/E7 fusion gene was constructed successfully by our study group. RmGM-CSF and rmIL-4 were purchased from Peprotech (USA). RPMI 1640 were purchased from Gibco (USA). CD40, CD86, MHCII and Hamster Mab anti-mouse CD11c were purchased from EBioscience (USA). Penicillin and streptomycin were purchased from Hyclone (USA). PVDF membrane was purchased from Immobilon (USA). Mouse polyclonal antibodies against E6 and E7 were purchased from Santa Cruz (USA). Peroxidase conjugated IgG antibodies were purchased from MultiSciences Biotech (China). ECL detection kit was purchased from keygen (China). Annexin V and propidium iodide (PI) were purchased from Sigma (USA). DAPI staining kit was purchased from Roche (USA). Cell Counting Kit-8 kit (CCK-8) was purchased from Dojindo Molecular Technologies (Japan)

Generation, adenoviral infection and characterization of murine DC

DC were generated from bone marrow as described previously [16]. In brief, bone marrow was flushed from

the tibias and femurs of C57BL/6 mice and depleted of erythrocytes with Tris-NH₄Cl. The cells were washed twice in RPMI-1640 medium and cultured in a six-well plate at 5×10⁶ cells per well with RPMI-1640 medium containing 10 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) and recombinant murine interleukin-4 (rmIL-4). ImDC were collected after 6 days. Transduction of imDC with Ad vector was conducted in six-well plates with 1×10⁶ DC per well in a 3 ml volume of RPMI-1640 medium containing 10 % FBS. Cells were then infected with pAd-mock as control and with adenovirus at 200 multiplicity of infection (MOI) for 2h. Subsequently, the adenovirus containing medium was removed and DC culture medium was added back. The infected cells were left to express the transgenes of interest for another 36h before further use. The imDC and infected cells (pAd-E6/E7-DCs) were harvested, incubated with rat-anti-mouse CD40, CD86, MHC II (IA/IE) and Hamster Mab anti-mouse CD11c, and analyzed by FCM, respectively.

Western blot analysis

The imDC, pAd-E6/E7-DC and pAd-DC were harvested, and total protein was isolated and quantified. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto a PVDF membrane. After saturation, the membranes were incubated at room temperature for 2h in TBS with 0.1 % Tween-20 (TBS-T) containing 5 % nonfat dry milk, and subsequently incubated with primary antibodies against E6 and E7 (1:200 dilution) overnight at 4°C. Peroxidase conjugated IgG antibodies were used as secondary antibodies. The protein was detected using the ECL detection kit following the manufacturer's protocol. β-actin immunoblotting was used as protein loading control.

Induction of specific CTL in mice

Spleen was moved from female BALB/c mice and soaked in PBS, then was homogenized. Suspensions of splenocytes were filtered by mesh screens. The lymphocytes were purified by lymphocytes separating solution and suspended in RPMI 1640 supplemented with 10 % FBS. The pAd-E6/E7-DC and pAd-DC co-cultured with lymphocytes respectively for 24h at the ratio of 1:10 to induce specific CTL.

Detection of apoptosis of CaSki cells induced by CTL

CaSki cells were divided into pAd-E6/E7-DC group, pAd-DC group and blank control group (untreated CaSki cells). After co-culturing the CTL with CaSki cells for 24h, CTL were washing-out softly by PBS. CaSki cells were taken as target cells at the ratio of effector to target of 1:1. Apoptosis was detected by FCM and DAPI staining. CaSki cells were collected by trypsinization and washed with cold phosphate-buffered saline and incubated for 10 min with Annexin V for 15 min, then stained with propidium iodide (PI). The analysis was performed with a FAC Scan flow cytometer using the CellQuest software. Cells that are in early apoptosis are Annexin V positive and PI negative. DAPI staining for the detection of apoptotic cells was performed according to the manufacturer's instructions.

Detection of *in vitro* killing efficiency of CaSki cells

The killing efficiency of CaSki cells were evaluated *in vitro* with a Cell Counting Kit-8 kit (CCK-8). Cells were divided into four groups: pAd-E6/E7 infected DC vaccine group, loaded-CaSki cells lysates DC vaccine group, untreated CaSki cells (control group) and RMPI 1640 (blank control). Cells (1×10^6 per well) were seeded in 96-well plates and incubated for 24h in a humidified atmosphere containing 5% CO₂. CaSki cells were taken as target cells at the ratio of effector to target of 10:1 and 20:1. Tests were performed in triplicate. After 24h incubation in a humidified atmosphere containing 5% CO₂, 20 μ l CCK-8 was added to each well and the incubation to more 1h. The absorbance of each well was determined with an ELISA reader (Wellsan MK3; Labsystems Dragon, Finland), with a measurement wavelength of 570 nm. Killing efficiency (%) was calculated as the following equation: $(1 - (\text{experimental group} - \text{blank control}) / (\text{control group} - \text{blank control})) \times 100\%$.

Statistical analysis

All experiments were performed at least 3 times. Statistical tests with package SPSS13.0 (SPSS incorporated, Chicago, USA) were carried out, all values were expressed as mean \pm SD. Statistical significance of differences between two groups was determined using the Student's t-test or Mann-Whitney's U test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Culture of bone marrow derived DC

DC became adherent and grew in a clonal manner after 3 days. At 5 days, more colonies were observed, some cells became suspended in round or irregular shape and increased in size, showing a burred or pseudopod-like projections on their surfaces. At 7 days, examined by FCM, the relative specificity surface of immature dendritic cell (imDC) CD11c was 71.19%, is high level expression. While other markers including CD40, CD86 and MHC II were 34.5%, 39.53% and 55.35% respectively, lower than the expression of CD11c, which were consistent with the characteristic surface expression of imDC. FCM results were shown in Table 1.

Construction of E6/E7 specific DC vaccine with recombinant adenovirus transfecting

After being infected with pAd-E6/E7 for 24 h, As shown in table 1, the expression of CD11c, CD40, CD86 and MHCII in DC was 62.87%, 55.5%, 78.51% and 95.95% respectively, consistent with the characteristic surface expression of mature dendritic cell (mDC). To determine whether the foreign genes could be expressed or not, imDC were transfected with recombinant adenovirus and the fluorescence were detected, because the cloned genes E6/E7 was co-expressed as fusions with the N-terminus of EGFP. Different sizes of dendritic and burr-like protrusions DC can be observed. some green fluorescence and some dendritic processes appeared as well (as shown in Figure 1), indicating that foreign genes E6/E7 could be exactly expressed in mammalian cells.

Table 1. Surface Molecules of DC Detected by Flow Cytometry

	CD11c (%)	CD40 (%)	CD86 (%)	MHCII (%)
imDC	71.19	34.5	39.53	55.35
pAd-E6/E7-DC	62.87	55.5	78.51	95.95
pAd-DC	52.09	52.21	74.02	89.84

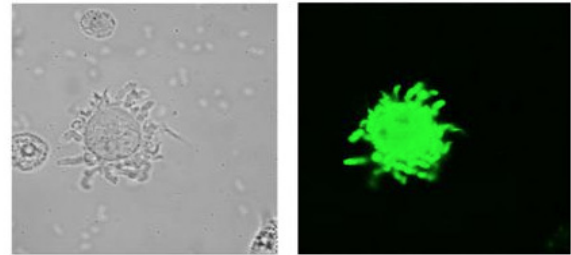


Figure 1. Expression of GFP in Dendritic Cells (DC) 24h After Transfection with pAd-E6/E7 by Laser Scanning Confocal Microscope. (original magnification $\times 2000$). Dendritic green fluorescence can be observed in DC

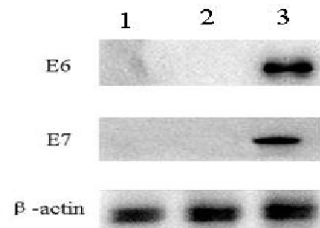


Figure 2. Western Blot Analysis of E6 and E7 Protein Expression 36h After Transfection with Adenovirus. E6 and E7 were expressed in lysates of pAd-E6/E7 transfected cells, (1) untreated DC were used as control; (2) DC were transfected with pAd-mock, which named pAd-mock-DC; (3) DC were transfected with pAd-E6/E7, which named pAd-E6/E7-DC

Moreover, the transfected cells were collected and also verified by Western blotting. As shown in Figure 2, two bands were respectively recognized by E6 and E7 antibody in lysates of pAd-E6/E7 transfected cells, while in the control groups (untreated control and the DC group which infected with pAd-mock (pAd-mock-DC) the expression of E6 and E7 protein is too poor to test, proving that the foreign genes were indeed expressed in DC vaccine.

DC vaccine inducing specific CTL to induce apoptosis of CaSki cells

DC vaccine was used to induce specific CTL. After co-culturing the CTL with CaSki cells, the apoptosis of CaSki cells were significantly increased (Figure 3). Approximately 72.82% of apoptotic cells were checked by FCM, while no significant changes in the control groups (Figure 4).

In vitro killing of CaSki cells with CTL induced by pAd-E6/E7 infected DC vaccine

At the effector/target ratio of 10:1 and 20:1, the killing rates of pAd-E6/E7 infected DC vaccine group were $(60.4 \pm 2.6)\%$ and $(71.2 \pm 3.7)\%$ respectively. Those of loaded-CaSki cells lysates DC vaccine group were

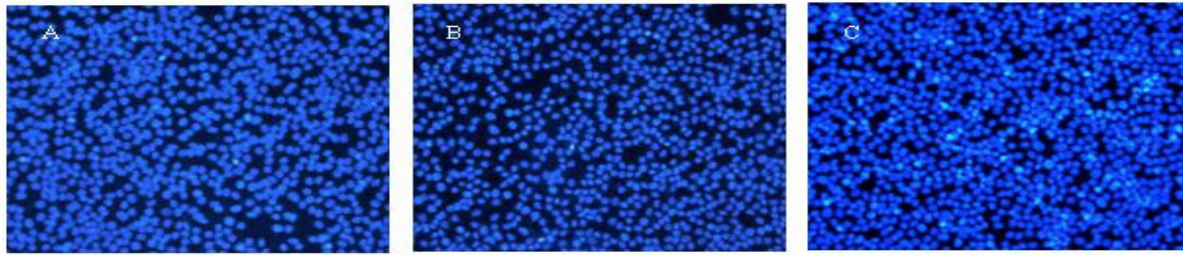


Figure 3. Induction of CaSki Cells Apoptosis by Treatment of pAd-E6/E7 DC Vaccine (DAPI, Original Magnification×200). (A) CaSki cells were used as blank control. (B) CaSki cells were treated with pAd-mock. (C) CaSki cells were treated with pAd-E6/E7 DC vaccine

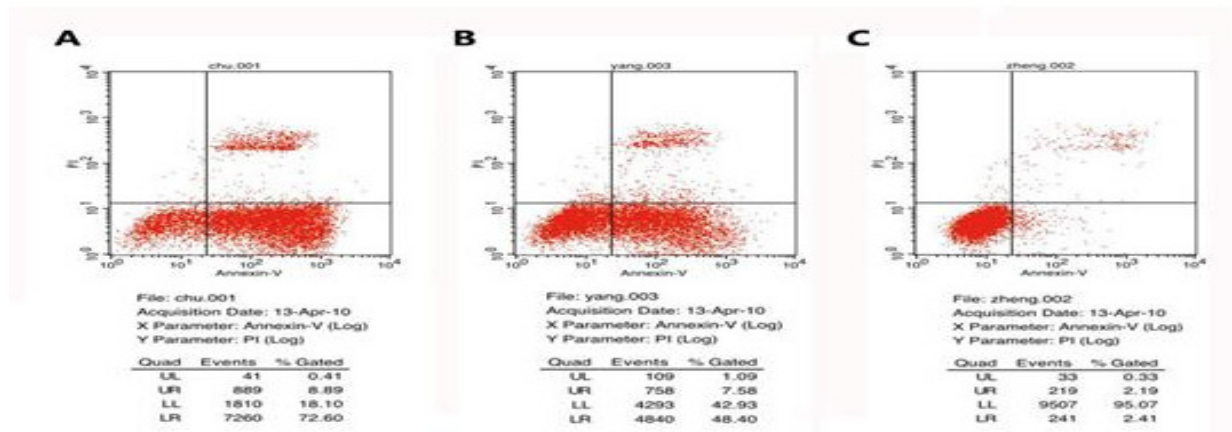


Figure 4. Induction of CaSki Cells Apoptosis by Treatment of pAd-E6/E7 DC Vaccine (FCM). (A) CaSki Cells were Treated with pAd-E6/E7 DC Vaccine. (B) CaSki cells were treated with pAd-mock. (C) CaSki cells were used as blank control

Table 2. The Killing Efficiency of CaSki Cells Induced by CTLs in Three Groups Detected by CCK-8. (*, Δp<0.05, $\bar{x} \pm s$)

	10:1 ratio (%)	20:1 ratio (%)
pAd-E6/E7 infected DC vaccine group	60.4±2.6*	71.2±3.7Δ
loaded-CaSki cells lysates DC vaccine group	30.1±2.3*	38.4±1.6Δ
untreated CaSki cells	5.7±2.5*	7.2±3.4Δ

(30.1±2.3)% and (38.4±1.6)% respectively. Those of blank control group were (5.7±2.5)% and (7.2±3.4)% respectively. The rates were significantly higher in pAd-E6/E7 infected DC vaccine group than in loaded-CaSki cells lysates DC vaccine and blank control group in a statistically significant manner ($p<0.05$) (Table 2), suggesting that transduced DC vaccine recombinant adenoviral vector-mediated E6/E7 gene fragment induced a more significant CTL cytotoxic effect on CaSki cells than cells lysates-loaded counterpart.

Discussion

Gene therapy of human cancers is most effective way when tumor specific expression of therapeutic gene is achieved. The human papillomavirus is presented in most cervical cancers, and is one of the presumed etiological agents. Thus specific targeting of the virus would provide an ideal strategy for the treatment of cervical cancer (Chen et al., 1996). E6 and E7 are the major oncogenic proteins produced by the cervical cancer associated HPVs (Zur Hausen, 1996). Association of the E6 can result in

ubiquitin-dependent degradation of p53 (Werness et al., 1990). E7 can interact with the pRb tumor suppressor protein which results in release of transcription factor E2F. The release of E2F can increase cell cycle progression (Munger et al., 2001). Frazer and colleagues declared that the HPV-16 Immunotherapeutic comprising HPV-16 E6/E7 fusion protein can induce vaccine antigen specific cell mediated immunity in patients (Frazer et al., 2004). Continued expression of the E6 and E7 genes is necessary for the maintenance of the malignant phenotype (Wu et al., 2006). Thus, the E6 and E7 gene products are important oncoproteins and feasible targets for anticancer therapies. However, the mechanism is far away to clear.

Deregulation of cell death pathways is an important feature of carcinogenesis (Evan et al., 1998). The tumor suppressor gene p53 is crucial to the control of apoptosis. One of the important functions of p53 is to recognize DNA damage and arrest cell growth in the G1 phase of the cell cycle for DNA repair and, if repair is not possible, to promote apoptosis (Levine, 1997). E6 and E7 of HPV-16 induce malignance by degradation of the cellular antitumor proteins, p53 and Rb respectively in cervical cells. Thus, interference HPV E6/E7 would be expected to affect cell cycle and apoptosis. In this study, we used genetically modified DC vaccine expressing HPV-16 E6/E7 fusion gene to induce specific CTLs, to investigate the lethal effects of specific cytotoxic T lymphocytes (CTL) against CaSki cells *in vitro*. We found that pAd-E6/E7 infected DC vaccine affected the viability and morphology of the cervical carcinoma CaSki cells and induced apoptosis. It is likely that the pAd-E6/E7 infected

DC vaccine induced apoptosis by reducing expression of viral E6 and E7 proteins, increasing expression of p53 protein. Our results demonstrated that immunization with full-length HPV-16 E6/E7 protein might be capable of eliciting specific protective immunity against cervical cancer cells growth.

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