Vaccination of full-length HPV16 E6 or E7 protein inhibits the growth of HPV16 associated tumors

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Abstract. Cervical cancer is the second most common cancer in women worldwide. Human papillomavirus (HPV) is the primary etiologic agent of cervical cancer. Two HPV16 proteins, E6 and E7, are consistently expressed in tumor cells. Most therapeutic vaccines target one or both of these proteins. Taking the advantages of safety and no human leukocyte antigen restriction, protein vaccine has become the most popular form of HPV therapeutic vaccines. Here we demonstrate that immunization with full-length HPV16 E6 or E7 protein elicited specific immunological effect and inhibition of TC-1 cell growth using TC-1 mouse model. HPV16 E6 and E7 genes were cloned into pET-28a(+) and introduced into *E. coli* Rosetta. Expression of the genes was induced by IPTG. Proteins were purified by Ni-NTA agarose and they were detected by SDS-PAGE and Western blotting. C57BL/6 mice were vaccinated with 1.5 nmol HPV16 E6 or E7 protein. Then they were implanted with $1x10^5$ TC-1 cells. No tumor was detected in any mouse vaccinated with E7 protein. Forty days later, the tumor-free mice and control mice were challenged with $2x10^5$ TC-1 cells. All control mice developed tumors 6 days later, but E7 immunized mice were tumor free until 90 days. Tumor growth was slow in the E6 immunized mice, but 83% of the mice developed tumors and the survival percentage was not significantly different from the control. An adoptive immune model was used to demonstrate the therapeutic effect. Results showed that the development of TC-1 cells was obviously reduced by transfusion of T-cells but not serum from mice immunized with E7 protein. T-cells from E7 immunized mice also induced the lysis of TC-1 cells in the cytotoxic T lymphocyte assay. These findings show that immunization with HPV16 E6 or E7 protein was able to elicit specific protective immunity against TC-1 tumor growth.

Introduction

Cervical cancer (CC) is the second most common cancer in women worldwide (1). In developing countries, CC is often the most common cancer in women. Human papillomavirus (HPV) is the primary etiologic agent of CC. Persistent infection with high-risk HPV is required for the development and maintenance of CC (2,3). Thus, CC might be prevented or treated by HPV related vaccines.

Currently, a preventive vaccine of HPV16 and HPV18 has been registered, which is fully protective against persistent infection and the associated development of high-grade genital lesions. However, the prophylactic vaccine showed no benefit in women who were already infected with the HPV types covered by this vaccine (4). Millions of patients currently suffer from HPV-associated morbidity or mortality (5). An estimated 5 million CC deaths will occur in the next 20 years due to existing HPV infections (6). Thus, there is an urgent need to develop therapeutic HPV vaccines.

HPV16 is by far the most common carcinogenic type, and 54.6% of CC was caused by HPV16 (7-9). Two HPV16 oncogenic proteins, E6 and E7, are critical to the induction and maintenance of cellular transformation. They are expressed in the majority of HPV16-induced CC cells (7,10). Thus, E6 and E7 proteins represent good targets for developing therapeutic vaccines of CC (11). Various forms of HPV16 therapeutic vaccines have been described targeting E6 and/or E7 proteins in animal models and/or clinical trials including viral or bacterial vectors expressing E6 and/or E7 proteins (12-19), plasmid DNA (20-23), dendritic or tumor cell-based vaccines (24-26), RNA interfering (RNAi) E7 expression (27-29), peptides (30-32) and recombinant proteins (33-35).

Protein vaccine has become the most popular form of HPV therapeutic vaccines because it is safe and has no human leukocyte antigen (HLA) restriction. In this study, we cloned HPV16 E6 and E7 genes from human CC cells, expressed and purified full-length E6 and E7 proteins in *Escherichia coli* (*E. coli*) system. Then we investigated the inhibition of TC-1 cell growth by using the TC-1 mouse model (36). In this model, mice were immunized with E6 or E7 protein. Our results demonstrated that immunization with full-length HPV16 E6 or E7 protein was capable of eliciting specific protective immunity against TC-1 tumor growth.

Materials and methods

Mice and tumor cell lines. Female C57BL/6 mice aged 6-8 weeks were purchased from Shanghai Laboratory Animal Center. All animals were maintained under specific-pathogen-free conditions, and all procedures in animal experiments were approved by the Animal Study Committee at the Institute of

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Table I. The primer sequences of E6 and E7 genes used for amplification.

Gene	Primer sequences	Restriction site
HPV16 E6	Sense: 5'-CGGGATCCATGCACCAAAAGAGAACTG-3'	*Bam*HI
	Antisense: 5'-CCGCTCGAGTTACAGCTGGGTTTCTCTAC-3'	*Xho*I
HPV16 E7	Sense: 5'-CGGAATTCATGCATGGAGATACACCTAC-3'	*Eco*RI
	Antisense: 5'-CCGCTCGAGTTATGGTTTCTGGGAACAG-3'	*Xho*I

Molecular Medicine, Nanjing University. Human CC cell line CaSki was obtained from China Center for type culture collection (Wuhan). CaSki cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated newborn calf serum (NCS) (Gibco), 2 mM L-glutamine (Hyclone, Logan, UT), 1.5 mg/ml sodium bicarbonate (Amersco, Cleveland, OH), 10 mM HEPES (Promega, Madison, WI), 1 mM sodium pyruvate (Amersco), 100 U/ml penicillin (North China Pharmaceutical, Group, Shijiazhuang, China) and 100 μg/ml streptomycin (Lu-Kang Pharmaceuticals, Jining, China). Human CC cell line SiHa was obtained from Chinese Academy of Sciences Committee type culture collection cell bank (Shanghai). SiHa cells were maintained in MEM (Gibco) supplemented with 10% NCS, 2 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 0.1 mM non-essential amino acid (Gibco), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. TC-1 tumor cells were provided by Dr T.C. Wu from Johns Hopkins University. TC-1 cells were derived from primary epithelial cells of C57BL/6 mice transformed with HPV16 E6, E7 and c-Ha-ras oncogenes. TC-1 cells were maintained in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) (Gibco), 400 μg/ml of G418 (Gibco), 2 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin. Freshly isolated T cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 50 U/ml recombinant human interleukin (IL)-2 (Four Rings Biopharmaceuticals, Beijing, China), 5 μg/ml concanavalin A (Promega), 2 mM L-glutamine, 2 mg/ml sodium bicarbonate, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37˚C in 5% CO_2.

Plasmid construction. The HPV16 E6 and E7 genes (gene sequence of Genebank: E6, AAD33252; E7, AAD33253) were amplified by reverse transcriptional-polymerase chain reaction (RT-PCR) from human CC cell line SiHa and CaSKi using a set of primers (Table I), then cloned into a prokaryotic expression vector pET-28a(+) (Novagen).

The PCR-amplified HPV16 E6 or E7 gene was directly inserted into a pMD 18T vector (Takara Biotechnology Co., Dalian, China). The clone was transformed into *E. coli* strain DH5α (Invitrogen) by heat shock and selected for ampicillin resistance. Colonies were picked and plasmid DNA in each colony was amplified by PCR using E6 and E7 primers. Plasmids containing inserts of the correct size were purified using a Plas/mini Isolation Spin-kit (Sigma). E6 and E7 coding regions were verified by sequencing (Shanghai Sangon Biological Engineering Technology and Service Co., Shanghai, China). All synthetic oligonucleotides were purchased from Shanghai, Sangon.

Then E6 and E7 genes were transcloned into pET-28a(+). Plasmids of pET-28a(+)-E6 and pET-28a(+)-E7 were verified by PCR and two restriction enzyme digestion. They were then transformed into *E. coli* strain Rosetta (Invitrogen) by heat shock and selected for kanamycin resistance.

Expression of E6 and E7 proteins. The *E. coli* strain Rosetta containing the pET-28a(+)-E6 or pET-28a(+)-E7 plasmid was grown at 37˚C in Luria-Bertani (LB) medium supplemented with 50 μg/ml kanamycin, until the culture reached an absorbance of 0.6-0.8 at 600 nm. Protein synthesis was induced by the addition of isopropyl-ß-D-thiogalactoside (IPTG, final concentration, 1 mM) to the medium and the culture was allowed to continue for up to 20 h. Cells were harvested by centrifugation [7000 rpm, at 4˚C for 6 min) and the cell pellet was resuspended in bacterium lysate [0.5 M NaCl, 20 mM Tris, 10 mM ß-mercaptoethanol (ß-ME), 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9]. The cells were then sonicated (amplitude of vibration 70-75, 20 min, square pulse 2.0s 2.0s) and the lysate was centrifuged at 4˚C, 8000 rpm for 20 min. Inclusion body and supernatant were analyzed by SDS-PAGE in a 12% gel under denaturing conditions.

Purification and characterization of E6 and E7 proteins. The inclusion bodies that contain the E6 or E7 protein were dissolved at 37˚C for 2 h in a solution I [0.5 M NaCl, 20 mM Tris, 6 M guanidine hydrochloride (GHCl), pH 7.9], then centrifuged at 16000 rpm for 20 min at 4˚C to obtain supernatant (soluble protein). The supernatants were loaded onto a Ni-NTA agarose (Qiagen) column that was balanced with a solution I. The columns were washed extensively with 10 times colume volum of solution II, III and IV separately (solution II, 0.5 M NaCl, 20 mM Tris, 5 mM ß-ME, 5 mM imidazol, 6 M GHCl, pH 7.9; solution III, 0.5 M NaCl, 20 mM Tris, 5 mM ß-ME, 5 mM imidazol, 8 M urea, pH 7.9; solution IV, 0.5 M NaCl, 20 mM Tris, 5 mM ß-ME, 20 mM imidazol, 8 M urea, pH 7.9). Then the columns were eluted with solution V (0.5 M NaCl, 20 mM Tris, 5 mM ß-ME, 200 mM imidazol, 8 M urea, pH 7.9) and the protein solution was collected. Next the protein solution was dialysised extensively with PBS at 4˚C and centrifuged at 16000 rpm for 20 min at 4˚C to obtain supernatant (pure protein solution).

SDS-PAGE was used to determine the identity and purity of the recombinant proteins. Concentrations of the recombinant proteins were measured by the Bradford assay (37). To confirm the identity of the recombinant proteins, all purified proteins were verified by Western blot analysis against the human HPV16 E6 or E7 antibody (Santa Cruz).

Prevention of TC-1 cell growth by immunization with E6 or E7 protein. Female C57BL/6 mice were immunized with 1.5 nmol E6 or E7 protein with Complete Freund's Adjuvant (CFA, Sigma) subcutaneously (s.c.). Negative control mice received PBS with CFA. A second equivalent dose of protein with Incomplete Freund's Adjuvant (IFA, Sigma) was given by intraperitoneal (i.p.) injection two weeks later. Mice were injected s.c. with $1x10^5$ TC-1 cells in the right flank 7 days after the second immunization and tumor growth was monitored every 3 days until control mice began to die. Percentage of tumor-free mice and survival rate was recorded. Tumor growth was determined by measuring maximal and minimal diameters with a vernier caliper, and tumor volumes were calculated according to: volume = (length x width^2) x 0.52.

The tumor-free mice immunized with E7 protein were challenged with $2x10^5$ TC-1 cells 40 days after the first tumor planting. Control group mice were injected with $2x10^5$ TC-1 cells at the same time. Percentage of tumor-free mice was recorded.

The cytotoxic T lymphocyte (CTL) assay. Female C57BL/6 mice were immunized s.c. with 1.5 nmol E7 or PBS with CFA, and the same protein dose with IFA was given by i.p. injection two weeks later. Seven days later, the T lymphocytes (T cells) were harvested from immunized mouse spleens by nylon wool and plated into 24-well plates with 50 μg/ml mitomycin C (Union Pharmaceuticals, Beijing, China) pre-treated TC-1 cells for 72 h at a ratio of 5:1. T cells were then harvested and co-cultured with the target TC-1 cells for 6 h in a 96-well U-bottom plate at different ratios. The supernatant was measured for lactate dehydrogenase (LDH) released from lysed cells using the CytoTox 96 cytotoxicity assay kit (Promega). The percentage of specific release of LDH was determined by the following equation: percent specific release = (experimental release - spontaneous T cell release - spontaneous TC-1 cell release)/(maximal TC-1 cell release - spontaneous TC-1 cell release) x 100.

Passive immune transfusion. Donor female C57BL/6 mice were immunized s.c. with 1.5 nmol E7 protein with CFA. A second equivalent dose with IFA was given by i.p. injection two weeks later. T-cells and serum of the immunized mice were harvested 7 days after the second immunization. Ten days after the subcutaneous injection of $1x10^5$ TC-1 cells (all mice have tumor growth), $4x10^6$ T-cells or 100 μl serum were given intravenously (i.v.) by tail vein per mouse, respectively. Control mice received PBS. Each recipient mouse was transfused 4 times at 3-day intervals. The size of the tumor was monitored every 3-6 days until mice in the control group began to die.

Statistical analysis. The data are expressed as means ± SEM. Comparisons of tumor volume and oganic weight between individual data points were made using a Student's t-test. Data for survival percentage were evaluated by long-rank test. Differences were considered statistically significant for $p<0.05$.

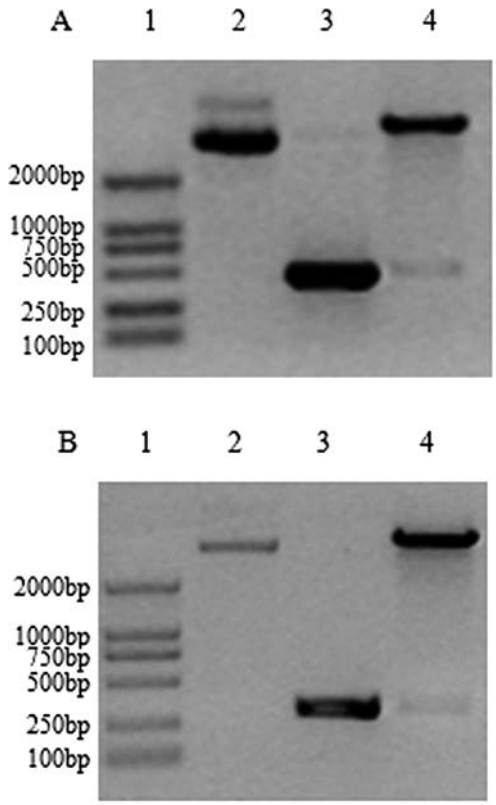

Figure 1. Construction of procaroytic recombinant expression vector of pET28a(+)-E6 (A) and pET28a(+)-E7 (B). (A) 1, DNA marker; 2, pET-28a(+)-E6; 3, PCR product; 4, enzyme digestion (*Bam*HI/*Xho*I). (B) 1, DNA marker; 2, pET-28a(+)-E7; 3, PCR product; 4, enzyme digestion (*Eco*RI/*Xho*I).

Results

Construction of prokaryotic recombinant expression vector of pET28a(+)-E6 and pET28a(+)-E7. HPV16 E6 gene was cloned into pMD18-T by RT-PCR from human CC cell line SiHa. HPV16 E7 gene was from human CC cell line CaSki. The positive clone was detected by enzyme digestion and PCR, then sequenced by Shanghai Sangon. Next the E6 and E7 genes were transformed into pET28a(+). The recombinant pET28a(+)-E6 and pET28a(+)-E7 were confirmed by PCR and enzyme digestion (Fig. 1).

Protein preparation and characterization. The E6 and E7 proteins were expressed efficiently in *E. coli* Rosetta after 4 h induction by IPTG. These proteins were expressed as inclusion body (Fig. 2A and B). E6 and E7 proteins with histidine tag (his-tag) were purified by a Ni-NTA Agarose column. The purification of E6 and E7 proteins was confirmed by SDS-PAGE (Fig. 2C). HPV16 E6 or E7 protein was recognized by human E6 or E7 antibody (Fig. 2D). Concentration of the purified proteins were measured by Bradford assay.

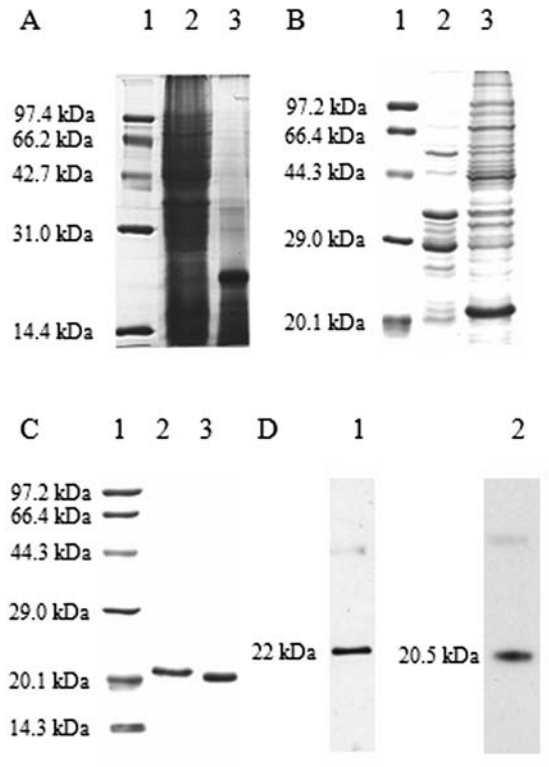

Figure 2. Expression and characterization of E6 and E7 proteins. (A) Suspension and inclusion body of E6 protein with IPTG induction for 4 h. (B) Suspension and inclusion body of E7 protein with IPTG induction for 4 h. Protein molecular weight marker (lane 1), suspension protein (lane 2), inclusion body (lane 3). (C) SDS-PAGE analysis of E6 protein (lane 2) and E7 protein (lane 3). Protein molecular weight marker (lane 1). (D) Western blot analysis of purified E6 protein (lane 1) and E7 protein (lane 2).

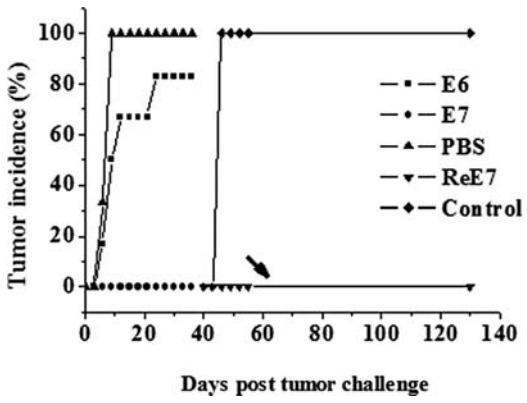

Figure 3. Immunization with HPV16 E6 or E7 protein elicited protective immunity against TC-1 cells. Female C57BL/6 mice were immunized twice with HPV 16 E6 protein (■), E7 protein (●), PBS (▲) with CFA or IFA respectively, followed by challenged with $1x10^5$ TC-1 cells 7 days after the second immunization. The tumor-free mice immunized with E7 protein were challenged with $2x10^5$ TC-1 cells 40 days after the first tumor planting (ReE7, ♦), using female C57BL/6 mice as control (▼). The tumor incidence was monitored for 130 days. Each group comprised 6 mice.

Prevention of TC-1 growth by immunization with HPV16 E6 or E7 protein. The TC-1 mouse model was used to test the anti-tumor immunity of HPV16 E6 or E7 protein. Successful immune responses should inhibit the development of TC-1 tumors. Female C57BL/6 mice were immunized twice with

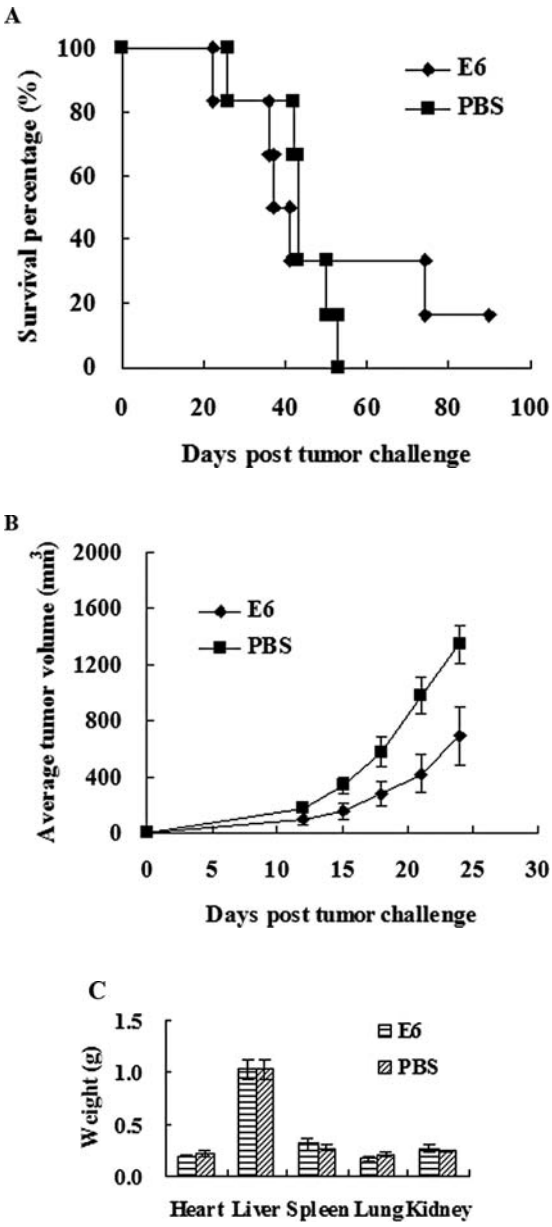

Figure 4. Immunization with HPV 16 E6 protein inhibited the TC-1 tumor growth. Female C57BL/6 mice were immunized twice with HPV 16 E6 protein or PBS with CFA or IFA respectively, followed by challenged with $1x10^5$ TC-1 cells 7 days after the second immunization. Survival was monitored for 90 days (A). The volume of the tumors was monitored with calipers every 3 days until control mice began to die (B). Organs were weighed immediately after the death of TC-1 tumor-bearing mice (C). Each group comprised 6 mice.

HPV16 E6 or E7. After being challenged with $1x10^5$ TC-1 cells, all mice in the control group developed tumors and died within 53 days. Only 17% of mice immunized with E6 protein were tumor free, whereas the E7 protein immunized groups demonstrated that no tumors had developed at all. Next the tumor-free mice of E7 protein vaccination were challenged with $2x10^5$ TC-1 cells 40 days after the first tumor planting. All control mice developed tumors 6 days later, but E7 immunized mice were tumor free until 90 days (Fig. 3). There was no significant difference in the survival rate of the mice immunized with E6 and the control group (Fig. 4A). However, the growth rate of the tumors in the mice immunized

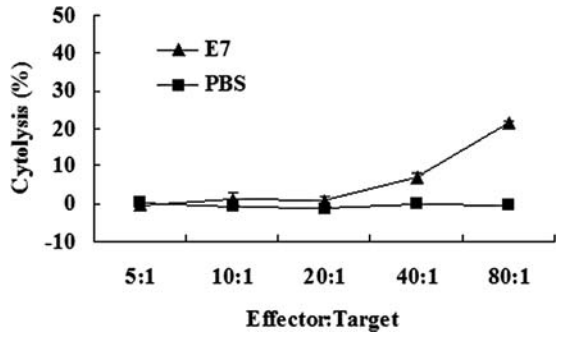

Figure 5. CTL analysis. After activation *in vitro*, T cells from mice immunized with E7 protein (▲) and PBS (■) were incubated with target TC-1 cells at indicated ratios. LDH released from lysed TC-1 cells was measured. The data are expressed as the mean ± SEM of 3 replicates.

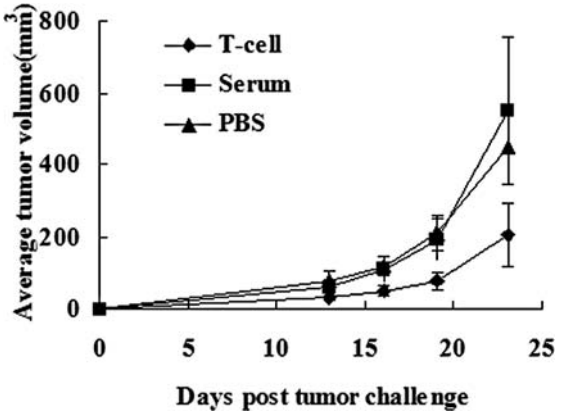

Figure 6. Passive immune transfusion. PBS (▲), T-cell (♦) and serum (■) from C57BL/6 mice vaccinated with HPV 16 E7 protein were injected i.v., respectively to recipient mice challenged s.c. in the right flank with $1x10^5$ TC-1 cells. Tumor growth was monitored until the control mice began to die. The results are expressed as the mean ± SEM of 5 replicates.

with E6 was much slower than that observed in the control group (Fig. 4B). On day 18, 21 and 24, average tumor volumes in HPV16 E6 and PBS-treated mice were 277±89 and 576±100, 421±139 and 981±129, 694±209 and 1349±136 mm^3 ($p<0.05$), respectively. Organs were observed and weighed immediately after the death of TC-1 tumor-bearing mice. No tumor metastasis was found and the weight of the organs in E6 immunized and the control group showed no significant difference (Fig. 4C).

Elucidation of cellular immunity in immunized mice. T cells were isolated from E7 immunized mice spleens by nylon wool. After incubation with mitomycin C-treated TC-1 cells, T cells were co-cultured with the target TC-1 cells. Specific lysis of TC-1 cells was successfully induced by the T cells from E7 immunized mice. However, the T cells from the control mice were not able to induce the lysis of TC-1 cells (Fig. 5). The extent of specific lysis was correlated with the ratios of effector cells over target cells.

To further study the mechanism of the anti-tumor immunity, a treatment model of passive immunization was employed. T-cells ($4x10^6$) or 100 μl serum from immunized allogeneic mice were transfused to C57BL/6 mice. It was found that the TC-1 cell growth was inhibited by the transfusion of T-cells but not serum (Fig. 6). These findings indicated that immunized T lymphocytes contributed to the inhibitory effect of the vaccination in tumor growth.

Discussion

Taking advantage of safety and no HLA restriction, protein vaccine has become the most popular form of HPV16 therapeutic vaccines. In this study, we constructed a procaryotic expression system of full-length HPV16 E6 and E7 proteins and purified these two proteins. Then we demonstrated that immunization with full-length E6 or E7 protein could elicit protective immunity against TC-1 cell growth by using TC-1 mouse model. E7 protein elicited a long-term immunological effect. Our data further demonstrated that immunized T lymphocytes predominantly contributed to the inhibitory effect of the vaccination in TC-1 tumor growth.

HPV16 E6 and E7 genes were cloned into pMD18-T by RT-PCR from CC cells SiHa and CaSki. Two genes were then transformed into pET28a(+). Two recombinant expression plasmids pET28a(+)-E6 and pET28a(+)-E7 were introduced into *E. coli* Rosetta. E6 and E7 proteins were expressed efficiently after 4 h induction by IPTG. Proteins were purified by Ni-NTA Agarose and detected by SDS-PAGE. The molecular weight (MW) of E6 protein was 22 kDa which is close to the calculated MW, but E7 protein was 20.5 kDa, larger than the theoretical MW 15 kDa. The reason for MW variation was that the electrophoretic migration of acidic E7 protein was changed by the positive charged basal amino acids of his-tag (38). HPV16 E6 or E7 protein was recognized by human E6 or E7 antibody.

Protein-based vaccines have become an attractive approach for generating antigen-specific immunotherapy because there was no HLA restriction and they are safe. Most of the HPV16 protein vaccines are according to the specific antigen site (30-32). We used the full-length protein sequence which contains all the antigen sites. The reason the full-length sequence was used is because it elicits stronger immunological effect against the protein expressed tumor cells. Based on our data, E7 protein could elicit stronger immunological protection than E6 protein and has a very long-term immunological effect protection of TC-1 cell. Thus, E7 protein should be used in clinical trials for the patient after tumor incision to inhibit tumor regrowth and metastasis. Our data further demonstrated that T lymphocytes from the E7 protein immunized mice spleen inhibited the growth of TC-1 cells *in vivo*, while serum from the immunized mice showed only slight inhibition of TC-1 tumor growth. Specific lysis of TC-1 cells was induced by the CTLs *in vitro*. We concluded that cellular immunity is primarily responsible for the inhibition of TC-1 tumor growth.

TC-1 mouse model is the most widely used CC mouse model. TC-1 cells were derived from primary epithelial cells of C57BL/6 mice transformed with HPV16 E6, E7 and c-Ha-ras oncogenes (36). Because HPV can not infect other animals, this transgenic mouse model is a very good model

for detecting the immunological effect of different HPV16 E6 and/or E7 related vaccine. In our study, in order to investigate the immunic effect of E6 protein on the metastasis of TC-1 tumors, organs were observed and weighed immediately after the death of TC-1 tumor-bearing mice. We found that no tumor had metastasized to organs and there was no difference in the weight of the organs between the E6 immunized and the control group. From these data we concluded that this TC-1 tumor model was not a suitable model for spontaneous metastasis from subcutaneous tumor to organs.

In conclusion, our study showed that HPV16 full-length E6 and E7 protein vaccination can induce protective immunity against TC-1 cells. Moreover, we showed that E7 protein could elicit 100% inhibition with a prolonged immunological protection.

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