Immunological Protection against HPV16 E7-Expressing Human Esophageal Cancer Cell Challenge by a Novel HPV16-E6/E7 Fusion Protein Based-Vaccine in a Hu-PBL-SCID Mouse Model

Yuanzhi LU, Zhixin ZHANG, Qiao LIU, Bo LIU, Xinxin SONG, Mingrong WANG, Xinhua ZHAO, and Qingzheng ZHAO*

Laboratory of Cellular and Molecular Biology, State Key Laboratory of Molecular Oncology, Cancer Institute & Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences; PO. Box 2258, Beijing 100021, P.R. China; and Beijing Blood Center; No.37 Beisanhuan Zhonglu, Haidian District, Beijing 100088, P.R. China.

Received July 19, 2006; accepted October 16, 2006

Increasing evidence has suggested that infection with high-risk human papillomavirus (HPVs) is closely associated with esophageal squamous cell carcinoma (ESCC) in China. The E6 and E7 oncoproteins expressed in ESCC are considered as attractive tumor-specific antigen targets for immunotherapy. We have reported that the HPV16 mE6Δ/mE7/TBhsp70Δ fusion protein vaccination induced powerful anti-tumor immunity against TC-1 tumor cells in a C57BL/6 mouse model. In the present study, we further evaluate the protective efficacy of this fusion protein vaccine using an HPV E7-expressing human ESCC cell line (EC9706) and a Hu-PBL-SCID mouse model. We demonstrated that immunization with the fusion protein vaccine caused significant inhibition of tumor growth with the delay time to tumor detection (tests vs. controls, 16 d vs. 9 d, p<0.01) and much smaller tumor size (p<0.01) in vivo. The inhibitory rate was ca. 69.6%, and 25% of the fusion protein vaccinated-mice remained tumor free by the end of the experiment (42 d). Furthermore, the activated lymphocytes (CD8+ T) were capable of infiltrating into the tumor site, and much more apoptotic cells along with activation of caspase-3 were observed in the tumors from vaccinated-mice. Also, high expression levels of human IFN-γ, TNF-α, granzyme B and perforin were detected in the tumors from vaccinated-mice. Therefore, we concluded that the HPV16 mE6Δ/mE7/TBhsp70Δ fusion protein vaccine is able to stimulate cellular-mediated immune response against E7-containing ESCC cells through CD8+ T-dependent CTL-induced apoptosis in Hu-PBL-SCID mice. These findings provide a scientific basis for HPV E7-expressing ESCC active immunotherapy.

Key words esophageal squamous cell carcinoma; human papillomavirus 16(HPV16); E6 protein; E7 protein; vaccine; Hu-PBL-SCID mouse

Esophageal carcinoma is one of the most common and highly aggressive malignant neoplasms in the world, particularly in China. More than 90% of esophageal cancers are ESCC.1,2 Despite advances made in surgical techniques and chemo-radiotherapy during the past several decades for the treatment of ESCC, the prognosis of ESCC remains poor with the 5-year survival rate of approximately 15%.2,3 Moreover, unresectable and relapsed esophageal cancers are still resistant to the currently available chemotherapy or radiotherapy regimens, and there is no substantial change in overall survival. The poor prognosis of esophageal cancer is due to the lack of early screening strategies, and most of patients are in advanced stage at diagnosis. As a result, its mortality rate is almost equal to its incidence in some countries.2 Consequently, there is an urgent need to develop a novel therapeutic strategy for this disease, and immunotherapy may be a promising approach for improvement of ESCC treatment. However, the tumor associated antigens that can be used as vaccine for successful ESCC immunotherapy have not been well identified, and it has been suggested that the HPV16 E7 protein could be a potential target for ESCC vaccine.4

Epidemiological studies indicate that many factors may be associated with esophageal cancer. However, the molecular mechanisms of ESCC still remain unclear.3 Accumulating evidence has also suggested that infection with high-risk HPVs, particularly HPV type 16, has been reported as a potential risk factor for ESCC, especially at high incidence areas in China.11-13 The E6 and E7 oncoproteins of the high-risk HPV types can efficiently destroyed the cell cycle regulatory machinery and the apoptotic pathways by binding to a number of host-cell proteins.8 Thus, the ability of HPV oncoproteins to alter control of the cell cycle imparts growth advantage on the cells and leaves them vulnerable to other genetic changes that ultimately result in malignant transformation. Therefore, the consistent expression of E6 and E7 oncoproteins with strong immunogenicity in HPV-containing precancerous lesions and carcinomas suggests that these proteins represent attractive targets for immunotherapeutic strategies against HPV-associated malignancies.3 Additionally, circulating HPV E7 specific effector T cells were detectable in patients with squamous cell carcinoma of the oropharynx (SCCO) and in squamous cell carcinoma of the head and neck (SCCHN) patients.9,10 Indeed, the malignant transformation of human esophageal epithelial cells was also induced in vitro by high-risk E6/E7 in synergy with TPA or human telomerase reverse transcriptase (hTERT), and these cells showed many features similar to those of ESCC.11,12 These observations supported the role of HPV in esophageal carcinogenesis. Therefore, the immunogenicity of HPV 16 E7-encoded antigen might be critical factors to developing vaccine-based strategies for enhancing antitumor immunity in patients with HPV16 E7-expressing ESCC. However, there were few reports on successfully experimental vaccination using the HPV 16 E7-encoded antigen as cancer-specific immunotherapy for this disease.

So far, the immunogenicity of anti-HPV vaccines and their antitumor efficacy have been evaluated in vivo by using TC-1 cell line and C57BL/6 mouse model which mainly generated...
immune responses to the HPV16-E6/E7 H-2Db-restricted epitope.13,14 Yet, the conclusions drawn from these animal models are incomplete in reflection of the interaction between human immune system and HPVs vaccines. Consequently, it is indispensable to develop a small animal model in which human lymphocytes, particularly human CD4+/CD8+ T cells, can be engrafted in an in vivo environment to mimic the human immune system and induce specific antitumor immunity. The Hu-PBL-SCID mouse model, produced by engrafting the immunocompetent human peripheral blood lymphocytes (HuPBLs) into SCID mice, is an ideal animal system designed to study the human immune responses to vaccination15,16 and cancer immunotherapy.17 Since this animal model was originally reported by Mosier et al. in 1988,18 numerous reports have demonstrated that the coengraftment of tumor cells and HuPBLs donated by healthy adult volunteers into SCID mice provides the opportunity to evaluate anti-tumor immune response of human lymphocytes that is more clinically relevant. This animal model has been successfully applied to evaluate the BCG-based vaccine breast cancer vaccine,19 the DC-based vaccine against human hepatocarcinoma cells20, and the anti-tumor efficacy against a wide spectrum of human tumors including lymphoma, glioblastoma, head and neck squamous cell carcinoma, lung and ovarian cancer using cytokine-based immunotherapies. In the majority of these studies, the HuPBLs were allogeneic with respect to the tumor and the allo-anti-tumor response was found to be mediated primarily by CD8+ T- and CD56+ NK-cells without HLA typing. These observations suggest that utilizing the Hu-PBL-SCID mouse model for evaluation of cancer vaccine is quite feasible.

We have successfully fused the truncated Mycobacterium tuberculosis heat shock protein 70 (TBHp70) with HPV-16 mE6Δ/mE7 to generate a recombinant fusion protein as an adjuvant-free vaccine, HPV16 mE6Δ/mE7/TBHp70Δ, and demonstrated that the fusion protein vaccine or the mE6Δ/mE7 protein plus IFA induced anti-tumor immunity against TC-1 tumor cells in C57Bl/6 mouse model. In the present study, we further evaluated the protective activity of this vaccine against HPV16 E7-expressing human ESCC cell challenge in a Hu-PBL-SCID mouse model. We showed that modulation of the tumor microenvironment by HPV16 mE6Δ/mE7/TBHp70Δ fusion protein vaccine can induce tumor cells to undergo apoptosis, and result in the inhibition of tumor growth in Hu-PBL-SCID mice.

MATERIALS AND METHODS

Cell Lines and Animals The ESCC cell line EC9706 and an HPV 16-negative cell line, EC/CUHK1, were described previously, respectively. The HPV16-E7 positive CaSki cervical carcinoma cell line was from American Type Culture Collection (ATCC). The ESCC and CaSki cells were maintained in RPMI 1640 medium (Gibco-BRL), and the EC/CUHK1 cells were grown in DMEM medium (Gibco-BRL). Both media were supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and 10% (v/v) heat inactivated fetal calf serum (FCS) (HyClone). Cells were cultured at 37 °C in 5% CO2 humidified incubator. SCID mice were obtained from the Experimental Animal Center of Peking Union Medical College (PUMC) and kept under specific pathogen-free (SPF) conditions. All experiments were undertaken in accordance with ethical guidelines for care and use of laboratory animals at PUMC.

Reconstitution of Hu-PBL-SCID Mice, Immunization and Tumor Cell Challenge Details of the reconstitution of Hu-PBL-SCID mice were as described by others. The vaccine and control proteins used in this study included HPV16 mE6Δ/mE7/TBHp70Δ, HPV16 mE6Δ/mE7, TBHp70Δ (control) and PBS (sham vaccine as negative control) were prepared as previously described. Fourteen days after lymphocyte reconstitution, proteins of 2.0 nm were diluted in a total volume of 200 μl of PBS or PBS alone were injected s.c. into the Hu-PBL-SCID mice in the groin of the hind leg (n=8), respectively. All the mice were boosted 10 d later from the first immunization with the same dose. Then, one week after the last vaccination, 3×106 EC9706 cells or 1×106 CaSki cells (E7-positive control) or 3×106 EC/CUHK1 cells (E7-negative control) were injected s.c into the right flank of mice. Following the tumor cell challenge, tumor development in mice was monitored twice weekly. Tumor growth was monitored by measuring maximal and minimal diameters by a caliper, and tumor volumes were calculated according to: (length×width2)/2.

Histological Analysis and Immunohistochemistry Tumor-bearing mice were sacrificed by a deep anesthesia when the control mice became moribund. Tumor tissues recovered from each group were fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections 4 μm thick were cut, and then were carried out standard H&E staining or immunohistochemical staining with mAb specific for human CD8 using a standard Non-Biotin HRP Detection system (Zymed Corporation, San Francisco, California, U.S.A.) following the manufacturer's instructions.

Detection of Tumor Apoptosis To detect apoptotic cells in tumor section from tumor-bearing SCID mice, TUNEL assay was performed using the DeadEnd Colorimetric Apoptosis Detection System Kit (Promega, Madison, WI, U.S.A.) follow by the manufacturer's instructions and as described previously. Apoptotic cells were identified using light microscopy. Cells exhibiting nuclear staining without cytoplasmic background were regarded as undergoing apoptosis. Apoptotic index (AI) was randomly estimated in 10 high-power fields (1000 total cells) and the percentage of apoptotic cells were determined as follows: apoptotic cells/total number of cells×100.

Antibodies and Western Blotting Cancer cells and fresh tumor tissues recovered from the animal model were rinsed three times with cold PBS and lysed with lysis buffer, then Western blot was performed using standard immunoblotting protocols and as described elsewhere. The following antibodies were used in the experiments: HPV 16 E7 (Cat #: sc-1597), Actin (Cat #: sc-7210), γ-Tubulin (Cat #: sc-17788), Granzyme B (Cat #: sc-1968), Perforin (Cat #: sc-9105), INF-γ (Cat #: sc-1377), TNF-α (Cat #: sc-8301), Caspase-3 (Cat #: sc-7272) and PARP (Cat #: sc-8007). These antibodies were commercially provided from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All experiments were repeated in triplicate.

Cytotoxic Assay Hu-PBL-SCID mice were immunized and boosted as above described. Then, mouse spleens from
each experimental groups (three per group) were disrupted, connective tissue and debris were allowed to settle, and the single cell suspensions were washed twice in RPMI 1640 medium. Human lymphocytes were enriched by Ficoll density gradient centrifugation and used as effector cells in a non-radioactive cytolytic analysis using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions and as described previously.\(^{26,27}\) EC9706, EC/CUHK1 and Caski tumor cells were used as target cells, respectively. The assay was repeated twice with similar results.

**Statistical Analysis** Tumor volume and results of CTL assay were expressed as mean ± S.E. of each independent experiment. One-way ANOVA was used for the comparison among three or more groups at individual time points. Tukey's HSD was used for the post-test of ANOVA using SPSS 12.0 software. Differences were considered statistically significant when the \(p\) value was <0.05.

**RESULTS**

**Expression of the HPV16 E7 Protein in Human ESCC Cell Lines** In order to determine the expression levels of the HPV16 E7 protein in the cell lines for this study. ESCC cell lines and the cervical cancer cell line, CaSki, were examined for HPV16 E7 protein expression using Western blot. As shown in Fig. 1, high level of HPV16-E7 protein is expressed in EC9706 and CaSki cells, whereas E7 protein was undetectable in the EC/CUHK1 cells. The in vivo growth kinetics and the concentration of these three cancer cell lines were determined in a primary experiment before embarking on the vaccine experiments, and the suitable concentration of tumor cells was \(3 \times 10^5\) (EC9706), \(1 \times 10^6\) (CaSki) and \(3 \times 10^5\) (EC/CUHK1), respectively. Thus, EC9706, CaSki and EC/CUHK1 tumor cells were chosen for this study as test, positive and negative control cells, respectively.

**The HPV16 mE6A/mE7/TBhsp70Δ Fusion Protein Vaccine Induced Anti-tumor Response against the Challenge of HPV16 E7-Expressing Human ESCC Cells in Hu-PBL-SCID Mice** To confirm whether HPV16 mE6A/mE7/TBhsp70Δ fusion protein vaccine could induce anti-tumor immune response against HPV16 E7-expressing human ESCC in Hu-PBL-SCID mice, the fusion protein vaccine immunized-mice (\(n=8\)) were challenged s.c. in the right flank with \(3 \times 10^5\) EC9706 tumor cells. As indicated in Fig. 2A, all mice immunized with the HPV16 mE6A/mE7 or PBS developed a palpable tumor with a median time to tumor detection of 9 d after the inoculation of the tumor cells. Whereas 25% mice immunized with the HPV16 mE6A/mE7/TBhsp70Δ fusion protein vaccine were still remained tumor-free by the end of the observation period (42 d). The median time for gross tumor detection in vaccinated-mice was 16 d (\(p<0.01\)). In addition, tumor xenografts in the control groups (HPV16 mE6A/mE7, PBS) showed a continual increase in size with time. The tumor growth curves for these two groups were essentially identical (Fig. 2A).

However, the growth rate of tumors in the animals immunized with HPV16 mE6A/mE7/TBhsp70Δ was much slower than that observed in the control groups. On day 42, average tumor volumes in HPV16 mE6A/mE7/TBhsp70Δ, HPV16 mE6A/mE7 and PBS-treated mice were 261.58 ± 75.71, 856.73 ± 153.51 and 858.5 ± 113.91 mm\(^3\) (\(p<0.01\)), respectively. These results suggest that the adjuvant-free vaccine, HPV16 mE6A/mE7/TBhsp70Δ, is capable of protecting against HPV16 E7-expressing ESCC cell challenge. However, TBhsp70Δ and mE6A/mE7 protein mixture or TBhsp70Δ alone have no prophylactic effect on the E7-expressing ESCC cell challenge (data not shown), these results were also supported by our and other studies.\(^{26,31,33}\)

The HPV16 E7-expressing CaSki and E7-negative tumor cell line, EC/CUHK1, were employed as positive and negative controls to evaluate the antitumor activity of the fusion protein vaccine.

**Immunobotting** revealed that the EC9706 cell line exhibit high level HPV16-E7 expression compared with the HPV16 E7-expressing cell line, CaSki, but the expression of E7 protein was undetectable in EC/CUHK1 cells. \(\beta\)-Actin was used as a loading control.

---

**Fig. 1.** Western Blot Analysis of the HPV 16-E7 Expression Levels in Different ESCC Cell Lines and CaSki Cell Line

**Fig. 2.** Recombinant HPV16 mE6A/mE7/TBhsp70Δ Fusion Protein Protects Hu-PBL-SCID Mice against the Growth of HPV16E7-Containing Esophageal Tumors (A) and Cervical Tumors (B), But Unable to Confer Protection against the Growth of HPV16E7-Negative Esophageal Tumors (C)

For in vivo tumor studies, Hu-PBL-SCID mice (\(n=8\)) were first immunized s.c. in the groin of the hind leg with 2.0 mg HPV16 mE6A/mE7/TBhsp70Δ (\(\bullet\)), HPV16 mE6A/mE7 (\(\bigcirc\)) and PBS (\(\Delta\)) without adjuvant, and then boosted 10 d later. Seven days after the boost, mice were challenged s.c. in the right flank with \(3 \times 10^5\) EC9706 (A), \(1 \times 10^6\) CaSki (B) and \(3 \times 10^5\) EC/CUHK1 (C) tumor cells, respectively. The volume of the tumors was monitored with calipers every 3 d for 6 weeks. Data are presented as average tumor volume (mean ± S.E.).
tive control, respectively. The Hu-PBL-SCID mice were challenged with $1 \times 10^6$ CaSki cancer cells or $3 \times 10^5$ EC/CUHK1 cells. As expected, mice immunized with the fusion protein vaccine showed significantly protective effect against the CaSki cancer cell challenge (Fig. 2B, $p<0.01$), but no noticeable effect was observed for the EC/CUHK1, an HPV16 E7-negative tumor cells (Fig. 2C), indicating that in EC/CUHK1 cells there is no suitable antigen which could prime the allogeneic HuPBLs in the SCID mice, and could not elicit sufficiently immune responses against EC/CUHK1 tumors, since the tumor growth rate in the three negative-control groups are similar. Therefore, These results demonstrated that immunization with the HPV16 mE6/mE7/TBhsp70Δ, an adjuvant-free protein vaccine, is able to induce anti-HPV16 E7-positive tumor immunity.

**Infiltration of CD8$^+$ T Lymphocytes into the Tumor Site** In order to further investigate the mechanisms underlying the anti-tumor effects of the vaccine, we analyzed the infiltration of CD8$^+$ T-cells in the tumor microenvironment using H&E stain and immunohistochemical analysis, respectively. As illustrated in Fig. 3, a greater number of infiltrating lymphocytes and human CD8$^+$ lymphocytes were observed in the tumors obtained from animals treated with HPV16 mE6Δ/mE7/TBhsp70Δ (Fig. 4a, d) compared with control-treated mice (Fig. 3b, e, and 3c, f).

**Enhancement of Apoptosis in Tumor Xenograft in Animals Immunized with the HPV16 mE6Δ/mE7/TBhsp70Δ Fusion Protein Vaccine** To test whether the fusion protein vaccine treatment could enhance CD8$^+$ T-cell anti-tumor function by inducing apoptosis in the tumor site, tumor tissues from experimental animals were subject to TUNEL assay. As shown in Fig. 3, much more apoptotic tumor cells were observed in the tumor sections from vaccinated-animals (Fig. 3g) compared with the tumors from control groups (Fig. 3h, i). The AI in the experimental group was higher than that in the two control counterparts ($p<0.001$) (Fig. 3). To confirm the results, immunoblotting analysis revealed that the activation of caspase-3 and the product of a $Mr$ 85000 fragment (cleaved fragment) of poly (ADP-ribose) polymerase (PARP, $Mr$ 116000), were markedly increased in the lysate of tumor tissues from the fusion protein vaccine treated mice (Fig. 4A).

**Up-Regulation of IFN-$\gamma$, TNF-$\alpha$, Granzyme B and Perforin Protein Levels in the Tumors Derived from Mice Immunized with the HPV16 mE6Δ/mE7/TBhsp70Δ Fusion Protein Vaccine** Granzyme B and perforin are two downstream factors critical for CD8$^+$ T-cell function, while IFN-$\gamma$ and TNF-$\alpha$ are important cytokines involved in tumor cell apoptotic pathway, and also these cytokines are the markers for T-cell activation. To investigate whether these cytokines are involved in the anti-tumor effects in the vaccinated-mice, immunoblotting analysis was performed to evaluate the expression levels of IFN-$\gamma$, TNF-$\alpha$, granzyme B and perforin in fresh tumor tissues from mice vaccinated by either the fusion protein vaccine or the HPV16 mE6Δ/mE7 or

![Fig. 3. Detection and Characterization of Tumor-Infiltrating Lymphocytes and Apoptosis in Tumors](Image)

Tumor tissues obtained from mice immunized with HPV16 mE6Δ/mE7/TBhsp70Δ, HPV16 mE6/mE7 and PBS were stained for the presence of CD8$^+$ tumor-infiltrating lymphocytes. Tumor-infiltrating CD8$^+$ T cells were mainly detected in tumors obtained from animals vaccinated with HPV16 mE6Δ/mE7/TBhsp70Δ (a, d). TUNEL assay revealed that most tumor cells in the tumor sections from mice immunized with the fusion protein vaccine (g) were undergone apoptosis compared with control groups (h, i). The AI in the experimental group was higher than that in the two control counterparts ($p<0.001$).

![Fig. 4. Up-Regulation of Granzyme B, Perforin, IFN-$\gamma$ and TNF-$\alpha$ as well as Activation of Caspase-3 and PARP in Tumors Derived from Hu-PBL-SCID Mice Immunized with HPV16 mE6Δ/mE7/TBhsp70Δ Fusion Protein Vaccine](Image)

Fresh tumor tissues recovered from mice were lysed and analyzed by Western blotting using IFN-$\gamma$, granzyme B, perforin, TNF-$\alpha$, caspase-3 and PARP specific antibody. Activated caspase-3 and the cleaved PARP were markedly increased in the lysates of tumors from the fusion protein vaccine treated mice (A), and high expression levels of granzyme B, perforin, IFN-$\gamma$ and TNF-$\alpha$ were also detected in these tumors (B). (a) HPV16 mE6Δ/mE7/TBhsp70Δ vaccine group, (b) HPV16 mE6Δ/mE7 fusion protein group; (c) PBS γ-Tubulin was used as a loading control. Experiments were performed on five individual tumors from each group and repeated in triplicate with the similar results. Data shown here were one set representative result of the experiments.
PBS. As expected, significantly high levels of granzyme B (32 kDa) and TNF-α (17 kDa) were detected in the tumor lysates from mice vaccinated by the fusion protein vaccine (Fig. 4B), although slight signals of the two cytokines were also observed in the tumor lysates obtained from the control groups. Observably enhanced levels of perforin (70 kDa) and IFN-γ (25 kDa) were detected only in the tumor tissues from vaccinated mice relative to the control groups.

Specific CTL Response Induced by Immunization with the HPV16 mE6Δ/mE7/TBhsp70Δ Protein Vaccine To determine whether HPV16 mE6Δ/mE7/TBhsp70Δ fusion protein vaccine may elicit specific cytotoxic activity in Hu-PBL-SCID mice, spleen cells isolated from animals of each experimental groups (three per group) were collected as effector cells for CTL assay. As shown in Fig. 5, splenocytes from mice treated with HPV16mE6Δ/mE7/TBhsp70Δ protein exhibited a significant increase in tumor lystate of EC9706 and CaSki cancer cells compared with HPV16 mE6Δ/mE7 or PBS-treated counterparts (p<0.01; Fig. 5). Most importantly, this cytolytic activity seems to be more specific for HPV16 E7-expressing target cells (EC9706, CaSki, as positive control, but not found in animals inoculated with HPV16 mE6Δ/mE7 or PBS developed a highly aggressive tumor with a continual increase in size with time. Notably, 25% mice immunized with the fusion protein vaccine were still remained tumor-free by the end of the experiment (42 d). Similar results were also observed using an HPV16 E7- expressing cervical cancer cell line, CaSki, as positive control, but not found in animals inoculated by HPV16 E7- negative cells, EC/CUHK1. These findings suggested that unprimed HuPBLs in SCID mice exhibited no significantly inhibitory effect on the growth of the ESCC tumor xenograft. However, the adjuvant-free vaccine, HPV16mE6Δ/mE7/TBhsp70Δ, is able to induce antigen-specific immune response resulting in the selective elimination of E7-positive ESCC and the cervical cancer cells in Hu-PBL-SCID mice. In accordance with these findings, it has been recently reported by Chung and colleagues that immunization with a BCG-MUC1-based breast cancer vaccine can induce anti-tumor immune response and cause inhibition of breast tumor xenograft in Hu-PBL-SCID mice which reconstituted by HuPBLs from normal donors. This allo-antitumor response was primarily mediated by CD8+ T cells regardless of HLA restriction. Our findings also supported by other reports that vaccination with a DC-based vaccine has an inhibitory effects on the growth of human hepatocarcinoma in SCID mice. Thereby, together with other reports, our data shown here support the notion that hu-PBL-SCID mouse model is an in vivo animal system prior to...
evaluate human immune responses to a range of prophylactic or therapeutic vaccine candidates for cancer immunotherapy. This study, to the best of our knowledge, is the first to demonstrate that the HPV16 mE6Δ/mE7/TBhsp70Δ fusion protein-based vaccine is capable of inducing strong immune responses to human esophageal carcinomas in Hu-PBL-SCID mouse model.

Consistent with the inhibition of tumor growth in vivo, the presence of CD8+ T-cells were mainly detected in the tumors from animals immunized with the fusion protein vaccine. Also, comparison of the specific-CTL responses from splenocytes of vaccinated mice and the control groups demonstrates that vaccination with the HPV16 mE6Δ/mE7/TBhsp70Δ protein results in far better responses (Fig. 5). Furthermore, much more tumor cells in these tumors were undergone apoptosis (Fig. 3g) compared with the tumors from control groups (Fig. 3h, 3i). With these findings, our data further demonstrated that the activated form of caspase-3 and the production of cleaved PARP were markedly increased in the lysates of tumors from the fusion protein vaccinated mice (Fig. 4A). These results suggested that the apoptotic cascade was triggered and the downstream effector caspases were activated when the activated CD8+ T-cells infiltrated into the tumor site, and these observations are also supported by recent findings that the protective or therapeutic effect of HPV16 E7/TBhsp70 fusion protein or DNA vaccine using E7 gene linked to TBhsp70 gene against E7 expression of HPV16 or therapeutic vaccine candidates for cancer immunotherapy. This study, to the best of our knowledge, is the first to demonstrate that a recombinant adjuvant-free fusion protein vaccine against HPV16 E7-expressing human esophageal carcinoma using this model. Our study demonstrated that a recombinant adjuvant-free fusion protein vaccine is superior to inducing specific cellular immune responses, resulting in the inhibition of HPV16 E7-expressing ESCC tumor growth in the in vivo model. These data provide a scientific basis for esophageal carcinoma active immunotherapy and offer the evidence of therapeutic vaccines for HPV-related cancer in the future study.

Acknowledgements We thank Jietao Song and Baochun Duan for help with animal care. This work was supported by a grant from the “863” High-Tech Projects of the Chinese Government (No.2002AA216041) and the Beijing City Natural Science Foundation (No. 7052047)