In vitro antigen-specific cytotoxic T cell response against esophageal carcinoma cells induced by HPV18E7-transfected dendritic cells

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Summary. Human Papillomavirus (HPV)-associated esophageal carcinoma (EC) is a high incidence tumor worldwide. Dendritic cell (DC)-based tumor vaccine is considered an alternative therapy to treat EC. Here we developed a DC-based vaccine by transfecting cord blood CD34^{+} stem cell-derived DC with HPV18E7 gene, observed its biological characteristics and the antigen-specific T-cell cytotoxicity on EC cells induced by HPV18E7-DC in vitro. Our results showed that 1) HPV18E7 gene transfer did not change the typical morphology of mature DC, 2) the representative phenotypes of mature DC (CD80, CD86, and CD83) were highly expressed in HPV18E7- DC (81.6%, 80.5%, and 86.6%, respectively), 3) the expression level of 18E7 protein in HPV18E7-DC was 47.5%, and 4) the specific cytotoxicity against EC cells was significantly higher than that in controls (p<0.01). This study indicates the possibility of a DC-based immunotherapy in HPV-associated EC.

Key words: Dendritic cell (DC), Esophageal carcinoma (EC), Human Papillomavirus (HPV), Cord blood stem cell, Immunotherapy

Introduction

Esophageal carcinoma (EC) is one of the most common and highly aggressive malignancies worldwide, and it is especially prevalent in China (Li et al., 2001; Zemin et al., 2006). Despite the advances in early diagnosis and treatment, the prognosis (the 5-year survival rate) is still very poor (McCabe and Dlamini, 2005; Tew et al., 2005). As several studies have shown promising results with immunotherapy in other human neoplasms (Joseph-Pietras et al., 2007; Hu et al., 2008), immunotherapy is considered an alternative approach to treat EC.

Currently, one of the most attractive strategies for cancer immunotherapy is inhibition of tumor growth by generating tumor-specific Cytotoxic T Lymphocyte (CTL) response via immunization with tumor antigens. Although the tumor-specific antigens or tumor-associated antigens in most tumors are unknown, viral antigens expressed in tumor cells are potential targets for immunotherapeutic vaccines against virus-associated malignancies.

Human Papilloma viruses (HPV) are commonly associated with various malignancies including esophageal carcinoma (EC) (Li et al., 2005; Lu et al., 2007). Epidemiologic studies have divided these viruses into low-risk types (HPV 6, 11, 40, 42, 54, and 57) and high-risk types (HPV 16, 18, 31, 33, 45, and 58) for cervical cancer (zur-Hausen, 1999; Wolf and Ramirez, 2001). HPV E6 and E7 oncoproteins expressed in the tumors are necessary for the maintenance of the malignant phenotype (Hudson et al., 1990), and they, especially E7 (Bosch et al., 2002), interfere with cell cycle regulation in cervical cancer (zur-Hausen, 2002). These oncoproteins are therefore considered potential tumor-specific target antigens for immunotherapy of HPV-associated neoplasms (Nonn et al., 2003; Bellone et al., 2007).

Dendritic cells (DCs) are the most efficient professional antigen-presenting cells (APCs), and are able to initiate primary immune responses in vivo and in vitro. DCs can be prepared in vitro from peripheral blood, bone marrow, or cord blood, and DC-based

immunotherapy protocols have been developed to elicit immunity against cancer, transplantation, and autoallergic diseases (Lim et al., 2006; Santin et al., 2006; Wenink et al., 2006).

Various gene transfer methods, such as electroporation, lipofection, and viral vectors, have been used to introduce into, and then express, antigenic peptides in DCs. Several studies have shown successful lipofection-mediated gene transfers into human DCs with resultant induction of T-cell response against tumor cells (Muderspach et al., 2000; Ceppi et al., 2005).

In this study, we investigated DC-mediated, tumor antigen-specific CTL response against EC cells by first developing a DC vaccine using human cord blood CD34^{+} stem cell-derived DC transfected with an EC-related virus antigen gene, HPV18E7, by liposome-mediated transfection, and then examining HPV18E7-specific CTL response against EC cells.

Materials and methods

This study was preapproved by the Ethical Committee of ShanTou University Medical College, and conducted in conformity with the guidelines and with informed consent from participants.

Esophageal carcinoma cell culture

Esophageal carcinoma cell line (EC-109) expressing HPV18E7 protein was obtained from the Department of Oncopathology of ShanTou University Medical College. EC-109 cells were grown in DMEM (Gibco, USA) containing 10% fetal calf serum (FCS; Hyclone, USA), 100 u/ml penicillin, and 100ug /ml streptomycin.

Preparation of DCs

Human cord blood mononuclear cells (CBMCs) were isolated from umbilical cord blood of 52 term deliveries collected in the Department of Obstetrics, the Second Affiliated Hospital of ShanTou University Medical College, by density-gradient centrifugation with Ficoll- Hypaque (d=1.077g/ml). CD34^{+} stem cells in CBMCs were labeled with a Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Germany) and separated from CBMCs by Mini magnetic activated cell sorter (Mini-MACS; Miltenyi Biotec, Germany) according to the manufacturer's instructions. DCs were prepared from cord blood CD34^{+} stem cells as previously described (Caux et al., 1996). Briefly, CD34^{+} stem cells were plated in a 6-well tissue-culture plate (Costar, USA) at 1.0×10^5 cells/well, and cultured in DMEM/F12 (Gibco, USA) containing 10% FCS, 100 u/ml penicillin, 100ug/ml streptomycin, 200ng/ml recombined human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Peprotech, USA), and 100u/ml recombined human tumor necrosis factor-α (rhTNF-α; Peprotech, USA) in a humidified 37°C incubator with 5% CO_2. Every 2 days, half of the medium was replaced with fresh complete medium supplemented with 200 ng/ml rhGM-CSF and 100 u/ml rhTNF-α for a total period of 2 weeks. Mature DCs were then collected and analyzed for typical DC phenotypes by fluorescence-activated cell sorter (FACS) analysis or co-cultured with T cells for sensitization assays.

Preparation of HPV18E7-DCs

A recombinant plasmid, pcDNA3.1-HPV18E7, was constructed by our team in 2004. The plasmid DNA was isolated with Qiagen Plasmid Mini Kit (Qiagen, GERMANY) and its concentration and purity was determined by spectrophotometry. Sequence identity of HPV18E7 DNA was confirmed at BioSia Co., Ltd. (Shanghai, China). The plasmid was stored at -20°C

CD34^{+} stem cells that had been cultured in complete medium with 200 ng/ml rhGM-CSF and 100 U/ml TNF-α for 12 days, as described before, were harvested as immature DCs for subsequent treatment with liposome-DNA complexes. On one hand, HPV18E7 DNA was mixed with cationic liposome DMRIE-C (Invitrogen, USA; 2.5 µl/µg of DNA) in 1 ml of serum-free OPTI-MEMI (Gibco, USA), and incubated for 30 minutes with gentle shaking at room temperature to form liposome-DNA complexes. On the other hand, the immature DCs in OPTI-MEMI were placed in a 6-well tissue-culture plate (2×10^6 cells/well, 200 µl/well). The liposome-DNA complexes were added dropwise to each well, followed by incubation at 37°C in 5%CO_2 for 7 h. Then, fresh complete medium with 200 ng/ml rhGM-CSF and 100 u/ml rhTNF-α was added to bring the cultures to 2 ml per well. After further incubation for 48 h, the mature HPV18E7-DCs were harvested. The expression of 18E7 protein in HPV18E7-DCs was analyzed by indirect immunofluorescence and FACS assays.

Preparation of T lymphocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors by density-gradient centrifugation with Ficoll-Hypaque (d=1.077g/ml) and suspended in complete medium. After incubation for 2 h at 37°C in 5% CO_2, the nonadherent cells were harvested. T lymphocytes were isolated from the nonadherent cells by sterile nylon-wool isolation column (Wako, Japan) as previously described (Pan et al., 2006). T lymphocytes were plated in a 6-well tissue-culture plate at 1.0×10^6 cells/well in complete medium supplemented with 80 u/ml recombinant human interleukin-2 (rhIL-2; Peprotech, USA). Purity of about 85-95% was obtained with this method.

DC phenotyping and HVP18E7 expression analysis by flow cytometry

To examine the expression of co-stimulatory molecules (CD80 and CD86) and DC activation marker

(CD83), mature untransfected DCs and HVP18E7-DCs were collected and resuspended in cold FACS buffer (phosphate-buffered saline with 0.2% BSA and 0.09% sodium azide). Cells were immunostained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD83 antibody (PeproTech, USA) and, phycoerythrin (PE)-conjugated mouse anti-human CD80 and CD86 antibodies (PeproTech, USA). Corresponding FITC- and PE-conjugated immunoglobulin G (IgG) isotype control antibodies (PeproTech, USA) were included in staining. A total of 1×10^6 cells were incubated overnight at 4°C with the antibodies. The cells were then washed twice with FACS buffer, resuspended, and phenotyped using a FACScan (Beckman Coulter, USA).

The detection of 18E7 proteins in HPV18E7-DCs was performed by an intracellular staining method followed by FACScan analysis. Mature HPV18E7-DCs were collected and fixed in 2% paraformaldehyde. Cell membranes were permeated in 0.2% Triton X-100 (Amresco, USA), then incubated with anti-HPV18E7 rabbit monoclonal antibody (Peprotech, USA) at 4°C overnight. After washing with PBS twice, the cells were immunostained with PE-conjugated goat anti-rabbit IgG (Sigma, USA) for 30 min at 37°C. The cells were then washed twice with FACS buffer, resuspended, and analyzed on a FACScan (Beckman Coulter, USA).

Lymphocyte proliferation assays

Methyl thiazolyl tetrazolium (MTT, 5 mg/ml; Sigma, USA) assay was used to detect lymphocyte proliferation. HPV18E7-DCs, untransfected DCs, and EC-109 were used as stimulator cells and T lymphocytes were used as responder cells. Stimulator cells were incubated with Mitomycin C (MMC; Sigma, USA) at 25 ug/ml at 37ºC in 5% CO_2 for 30 min, and then co-cultured with T lymphocytes isolated from PBMCs at ratios of 1:5, 1:10, 1:20, 1:50, 1:100, and 1:200 in a 96-well tissue-culture plate (Costar, USA) for 96 h at 37°C in 5%CO_2. Then, 20 ul freshly prepared and filtered MTT was added to each well, incubated for 4 h, and the supernatant was removed. Subsequently, 150 µl dimethyl sulfoxide (DMSO; Amresco, USA) was added to each well, followed by agitation for 10 min or until the crystals were fully dissolved. Absorbance was measured at 570 nm on an automatic ELISA reader (3550-UV, BIO-RAD, USA). T cells incubated in medium alone were used as control. All determinations were carried out in triplicate and repeated four times. The extent of induced T lymphocyte proliferation as represented by stimulation index (SI) was calculated as follows: SI = (experimental - blank) / (control - blank).

Induction of CTLs by HPV18E7-DCs

HPV18E7-DCs were added to T cells at a stimulator-to-responder ratio of 1:20 in DMEM/F12 containing 20% FCS and 40 u/ml rhIL-2. They were co-cultured in a 24-well tissue-culture plate at 37°C in 5% CO_2 for a total of 14 days with re-stimulation with HPV18E7-DCs on day 7. CTLs thus obtained were used as the effector cells in CTL assays against EC-109.

Cytotoxicity assays

EC-109 cells were seeded into a 96-well tissue-culture plate at a concentration of $1 \times 10^4 / 200$ µl per well. After 24h incubation the culture medium was removed and fresh medium containing HPV18E7-DC-primed CTLs was added at an effector-to-target ratio of 5:1, 10:1, and 20:1. They were co-cultured for 48 h, and cytotoxic activities were determined by MTT assay as described before. All determinations were carried out in triplicate and repeated four times. The percentage of specific cytotoxicity was calculated as [(experimental-minimal) / (maximal-minimal)]x100%. Target cells incubated in medium alone or in medium containing 1% Triton X -100 were used to determine minimal and maximal cytotoxicity, respectively. T cells isolated from the PBMCs and the untransfected DC-primed T cells were used as controls.

Statistical analysis

Data were analyzed with SPSS 11.0 statistical software. The significant difference between groups was determined by one-way ANOVA test, and p values less than 0.05 were considered statistically significant.

Results

Morphological features of cord blood DCs and HPV18E7-DCs

Under the stimulation of rhGM-CSF and rhTNF-α· for 2 weeks, cord blood CD34$^+$ stem cells were amplified about 10-20-fold, and the purity of DCs was above 90%. During the proliferation process, DCs increased in the number and formed cell clones, achieving the largest colonies from 8 to 9 days. Mature DCs with typical long dendritic processes shed from the clones in the later period of culture. HPV18E7-DCs were grown well and had a more irregular shape and
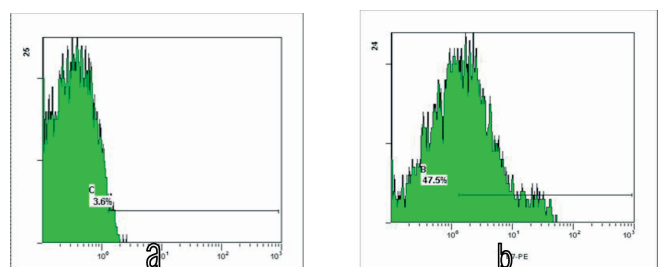

Fig. 1. Flow cytometric analysis showing the expression level of 18E7-protein in untransfected DCs (3.6%) **(a)** and HPV18E7- DCs (47.5%) **(b)**.
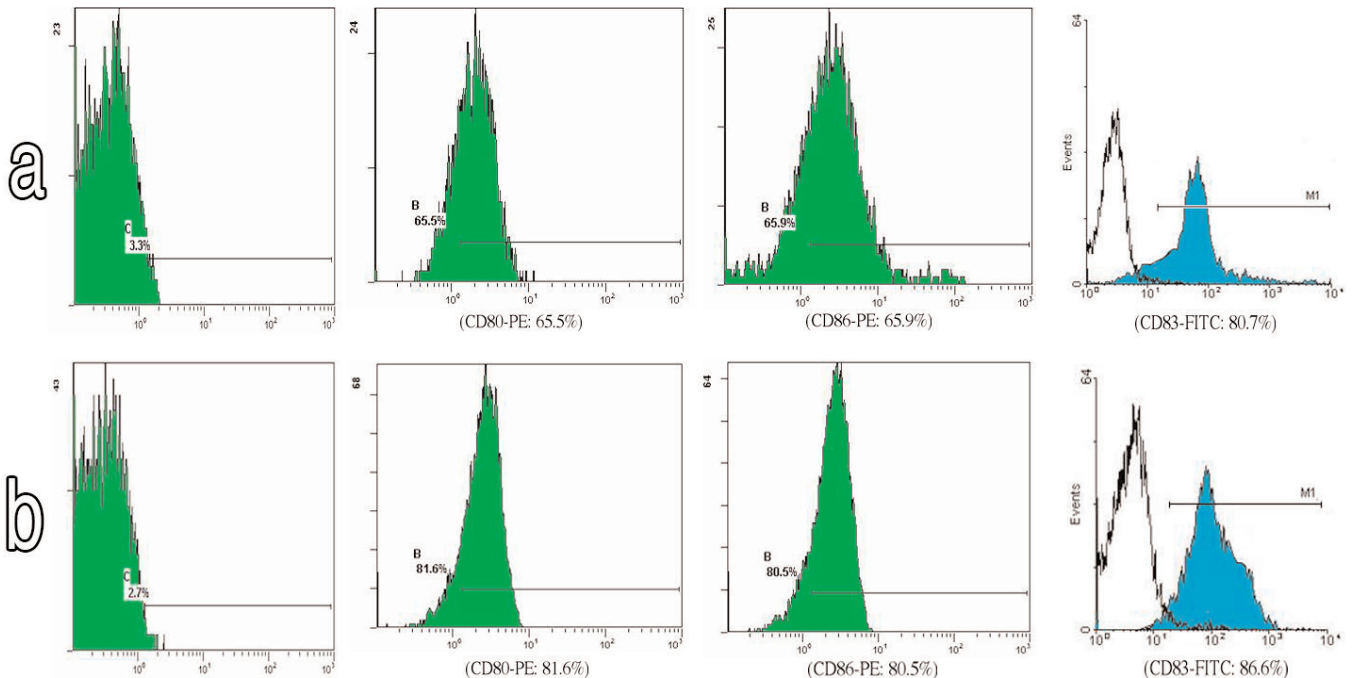
Fig. 2. Flow cytometric analysis showing the expression of CD80, CD86, and CD83 detected with PE conjugated mouse anti-human CD80 and CD86 antibodies and FITC conjugated mouse anti-human CD83 antibody. The expression levels of CD80 and CD86 in HPV18E7-DCs **(b)** were significantly higher than that in untransfected DCs **(a)** (81.6% vs. 65.5%; 80.5% vs. 65.9%; ps<0.01), and CD83 expression was also higher in HVP18E7-DCs **(b)** than that in untransfected DCs **(a)** (86.6% vs 80.7%; p<0.05).
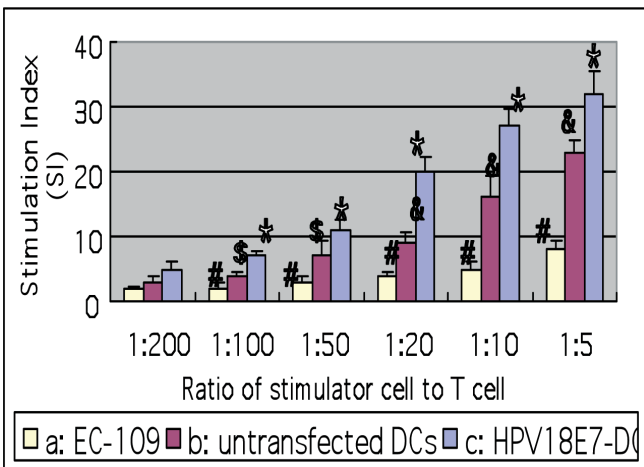
Fig. 3. T cell proliferation, as determined by methyl thiazolyl tetrazolium (MTT) assay and represented by stimulation index (SI), after induction with HPV18E7-DCs **(a)**, untransfected DCs **(b)**, and EC-109 **(c)**. The results are expressed as means ± SD of three replicates. At stimulator-responder ratios of up to 1:100, T cell stimulation by HPV18E7-DCs was significantly higher than that by untransfected DCs and EC-109 (p = <0.01 ~ <0.05); at a ratio of 1:200, significant difference was observed only between HPV18E7-DCs and EC-109 (p<0.05). (★vs #, p<0.01; ★vs &, p<0.01; ★vs $, p<0.05)
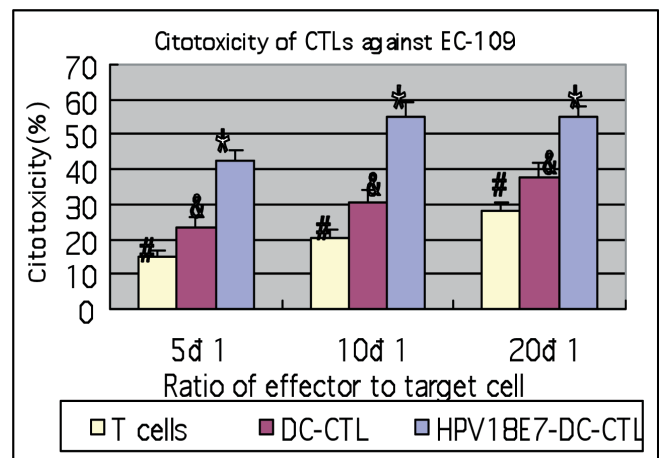
Fig. 4. Cytotoxicity of CTLs induced by HPV18E7-DCs, untransfected DCs, and T cells against EC-109. Percent cytotoxicity (mean ± SD of three replicates) was determined by methyl thiazolyl tetrazolium (MTT) assay. The cytotoxic activity with HPV18E7-DCs was significantly higher than that with untransfected DCs and T cells (p<0.01). And the cytotoxic activity with untransfected DCs was significantly higher than that with T cells (p<0.05). (★vs &, p<0.01; ★vs #, p<0.01; & vs #, p<0.05)

longer dendritic processes.

Expression of 18E7 protein in HPV18E7-DCs

By flow cytometric analysis, the percentage of cells expressing 18E7 protein for transfected DCs and untransfected DCs was found to be 47.5%, and 3.6%, respectively (Fig. 1).

Phenotype of HPV18E7-DCs

Both untransfected DCs and HPV18E7-DCs expressed co-stimulatory molecules CD80 and CD86 and DC activation marker CD83. The expression levels of CD80, CD86, and CD83 were 65.5%, 65.9%, and 80.7% for untransfected DCs and 81.6%, 80.5%, and 86.6% for HPV18E7-DCs, respectively. The expression level of CD80 and CD86 was significantly higher in HVP18E7-DCs than untransfected DCs ($p < 0.01$), and CD83 expression was also higher in HVP18E7-DCs than untransfected DCs ($p < 0.05$) (Fig. 2)

Stimulation of T lymphocytes by HPV18E7-DCs

T cell proliferation, as represented by stimulation index, was observed after a single treatment with stimulator cells. The stimulation of T lymphocytes by HPV18E7-DCs was significantly stronger than that by untransfected DCs ($p < 0.01$) and EC-109 ($p < 0.01$) at stimulator-to-responder ratios of 1:5, 1:10, and 1:20 (Fig. 3).

Induction of anti-EC-109 cytotoxicity

MTT assay showed that HPV18E7-specific anti-EC-109 cytotoxicity was induced after stimulation twice with HPV18E7-DCs.Cytotoxicity induced by HPV18E7-DC-primed CTLs was significantly higher than that by untransfected DC-primed CTLs and T cells ($ps < 0.01$). The cytotoxic activity of untransfected DC-primed CTLs was also higher than that of T cells ($p < 0.05$). The cytotoxic activity was significantly enhanced with higher ratios (10:1 and 20:1) of effector-to-target cells ($p < 0.05$) (Fig. 4).

Discussion

Dendritic cells (DCs) are the most potent professional APCs and are able to stimulate naive and quiescent CD4$^+$ and CD8$^+$ T-cell responses in vivo and *in vitro* (Vieweg and Jackson, 2005; Rasaiyaah et al., 2007). However, DCs from patients with cancer are deficient in number and functional activity, leading to poor induction of T-cell antitumor responses and thus inadequate tumor immunosurveillance. Loaded dendritic cell therapy is a vaccination strategy aimed at eliciting tumor antigen-specific, T-cell immune responses (Brossart et al., 2000; Dorothee et al., 2001).

In this study, we applied a DC-based vaccination strategy to induce an effective therapeutic anti-tumor immunity, by using DCs derived from CBMCs expressing the tumor-associated virus antigen. This approach has apparent advantages over protein- or peptide-based immunization (Diebold et al., 2001), because tumor-associated antigen gene transfected into DCs could induce endogenous processing and presentation of multiple defined and undefined antigenic peptides independent of MHC alleles, and induce specific T cell-mediated immunity without prior knowledge of relevant MHC class I- or class II-restricted peptide epitopes (Pan et al., 2006).

Morphological features of cord blood DCs and HPV18E7-DCs

Compared with bone marrow, cord blood contains a lower proportion of CD34$^+$/CD38$^+$ cells but a higher proportion of CD34$^+$/CD38$^-$ cells, which are early stem cells and thus more sensitive to the stimulation of cytokines (Gigant et al., 2001; Ueda et al., 2001). HPV18E7 gene transfer did not change the typical morphology of mature DC.

In our study, we found that cord blood-derived DCs grew well and displayed typical long dendritic processes. Moreover, HPV18E7-DCs showed a more irregular shape and longer dendritic processes. These results showed that HPV18E7 gene transfer did not change the typical morphology of mature DC.

Phenotype of HPV18E7-DCs

The co-stimulatory molecules identified on DCs with their respective T-cell receptors are CD80, CD86 and CD83. In order to activate T-cells, high levels of co-stimulatory molecules are necessary on the surface of mature DCs. In this study, the co-stimulatory molecules CD80, CD86 and CD83 increased significantly after HPV18E7-DNA modification of DCs compared with untransfected DCs, demonstrating that HPV18E7- DNA transfection had a notable effect on DC maturation and antigen-presenting function.

Expression of 18E7 protein in HPV18E7-DCs

We used cationic lipid-DMRIE-C to transfer HPV18E7-DNA into cord blood-derived DCs, and found that the expression rate of 18E7 protein was about 47.5%, indicating that DMRIE-C is an appropriate medium for introducing foreign genes into DCs, and that transfected HPV18E7-DNA in DCs can successfully induce endogenous processing and antigen presentation.

Stimulation of T lymphocytes by HPV18E7-DCs

DCs are well known for their ability to induce antigen-specific T cell responses with extremely high efficiency, partly attributed to high expression of T cell stimulatory cytokines (IL-1, IL-6, and IL-12) (Kanangat

et al., 1995). Dendritic cells have been loaded with tumor-derived material in a number of ways, each of which had some ability to stimulate T cell proliferation and induced some level of anti-EC immunity in vitro and in vivo (Yamana, 2002; Babatz et al., 2003; Milano et al., 2007). The finding in our study of significantly strong stimulation of T lymphocytes by HPV18E7-DCs indicates that DCs loaded with HPV18E7-DNA can effectively induce HPV18E7-specific CTLs.

Induction of anti-EC-109 cytotoxicity

Recent studies have shown that DCs loaded with autologous tumor-derived antigens are able to induce tumor-specific CTL response against cervical cancer, melanoma, and hepatocellular cancer (Xu et al., 2007; Dillman et al., 2006; Butterfield et al., 2007). Likewise, we also found that DCs loaded with HPV18E7 antigen can elicit the potent specific CTL activity against EC cells.

In conclusion, we demonstrated that DCs transfected with HPV18E7-DNA were able to stimulate T-cell proliferation and HPV18E7- specific cytotoxic T-cell responses in vitro. This study denotes a potential DC-based immunotherapeutic strategy for HPV-associated EC.

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