

RESEARCH ARTICLE

HPV16 CTL Epitope Peptide-activated Dendritic Cell and Natural Killer Co-culture for Therapy of Cervical Cancer in an Animal Model

Yan-Xia Hu¹, Min Li², Xiao-Hui Jia¹, Qu-Xiao Du¹, Feng-Tai Miao¹, Li Yao¹, Ji-Duo Shen^{3*}

Abstract

There is increasing evidence that natural killer (NK) cells play an important role in antitumor immunity following dendritic cell (DC) vaccination. Little is known, however, about the optimal stimulation of DCs by epitopes and NK interactions for cytotoxicity in tumors. In this study, DC cells activated by the HPV16E7.49-57 epitope and LPS were co-cultured with NK cells *in vitro*, and then used to immunize mice to study CTL activity of TC-1, which constitutively expresses HPV16E6E7, with an LDH release assay. Cytotoxicity in mice immunized with DC loaded with epitope HPV16E7.49-57 vaccine co-cultured with NK was enhanced significantly ($p < 0.01$). In conclusion, talk-across between DC and NK cells enhances their functions, also improving cytotoxicity against tumor cells, suggesting that activated DC-NK by epitopes has potential application for cancer-specific immuno-cellular therapy.

Keywords: Epitope stimulation - dendritic cells - natural killer cells - interaction - cervical cancer therapy

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Introduction

Cervical cancer is currently the only one gynecologic malignancy with definite cause, and reported to be related with persistent infection due to high-risk human papillomavirus (HPV). Current treatments include surgery, radiotherapy and chemotherapy. All of these treatments have certain effect on cervical cancer but accompanying side effects, recurrence and metastasis. The current available commercialized cervical cancer vaccine has been proved to have no therapeutic effect on patients infected with HPV or cervical cancer patients. Thus it is still necessary to explore new treatment for HPV infection or cervical cancer, among which the immune cell therapy that has been proved to be functional for cancer treatment in recent years has been gaining more attention.

It is now accepted that dendritic cells (DCs) vaccine can activate antigen-specific T cells response, which has been confirmed in preclinical animal tumor models and clinical trials (Gilboa, 2007; Ilett et al., 2010; Nakai et al., 2010). DC-CTL immune response has an important role in the immune response to tumor, but it cannot be ruled out that other immune cells are involved. In fact, recent clinical trials have shown that the clinical results are more related with NK (Natural killer) response after DCs or DC derived-exosomes inoculation than T-cell responses (Osada et al., 2006; Di Nicola et al., 2009; Viaud et al., 2009).

NK cell is very important in maintaining homeostasis and preventing tumor formation and virus infection (Gasser et al., 2006). Despite NK cells can clear transformed cells without stimulation, the previous studies using several mouse models showed that NK cells require additional activation to provide protection against lethal tumor invasion (van den Broeke et al., 2003; Grzywacz et al., 2008; Karimi et al., 2008). The cross talk between DC and NK cells has been increasingly concerned, and it has been reported that DC vaccine has the potential to activate NK cells (van den Broeke et al., 2003; Degli-Esposti et al., 2005; Grzywacz et al., 2008; Boudreau et al., 2009). In this study, we used DC cells activated by HPV16E7.49-57 epitope to investigate the interaction between DC and NK cells, and the cytotoxicity against TC-1 cells expressing HPV16E7 in mice that were immunized with co-cultured DC-NK. This will provide new ideas and experimental basis for cervical cancer immunotherapy.

Materials and Methods

Synthesis of HPV16E7.49-57 peptide

E7.49-57 (RAHYNIVTF) and control HBV surface antigen R187 (aa183-191: FLLTRILTI) were synthesized by Zhongtai Biotechnology Co., Ltd, Hangzhou, China. Purity is greater than 95%. Dissolved in DMSO at a concentration of 20mg/ml, storing at -20 °C.

¹Department of Gynecology and Obstetrics, ²Department of Oncology, People's Hospital of Zhengzhou, ³College of Pharmacy, Henan University of Traditional Chinese Medicine, Zhengzhou, Henan, China *For correspondence: jiduoshen@163.com

DC cell culture and activation

Mice bone marrow-derived DCs (BMDCs) were induced following protocol from Shen's lab (Shen et al., 2004). In brief, cells from bone marrow were flushed out from hind legs with a syringe, passed through a nylon mesh and collected. Red blood cells were lysed with lysis buffer containing ammonium chloride. Then cells were incubated in complete culture medium (RPMI1640 containing 10% FCS) at 37 °C and 5% CO₂ for 4 h, the suspension cells were discarded, and attached cells were rinsed for several times with RPMI-1640 and cultured in complete culture medium containing rmGM-CSF (20 ng/ml, PeproTech, Rocky Hill, New Jersey, USA) and rmIL-4 (20 ng/ml, PeproTech, Rocky Hill, New Jersey, USA). The medium with rmGM-CSF and rmIL-4 was changed every other day. Unattached granulocytes were removed after 48 hours. DC cultured for 6 days were infected with different doses of adenovirus for 8-12 h, and then stimulated with LPS (0.5 µg/ml) for 24 h. The cell suspensions pipetted gently and collected on the next day were *in vitro* amplified bone marrow-derived dendritic cells. Cell growth and morphological changes were observed daily with phase contrast microscope. DCs were seeded as 10⁵ cells in 400 µl RPMI-1640 culture medium per well into 12-well plates. HPV16E7.49-57 epitope peptide (1 µl/ml) was added to activate DCs cells, accompanying with LPS (5 ng/ml) for DC maturation. 24 hours later, DCs were used to co-culture with NK cells. HBsAg R187 polypeptide was used as a control.

NK Cells *in vitro* expansion

Splenic mononuclear cells isolated by immune-magnetic beads (MiltenyiBiotec, Bergisch Gladbach, Germany) were irradiated with 25 Gy to prevent massive proliferation and used as feeder cells. NK cells (1×10⁵ cells/mL) purified by immuno-magnetic beads were cultured in AIM-V medium containing 5 % fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) and 500IU/mL recombinant mouse IL-2 (rmIL-2) in 24-well tissue culture plates. Anti-CD3 monoclonal antibody (10 ng/mL) was added into culture medium for 5 days but then depleted. Cells further expanded in fresh culture medium containing IL-2 for 4 days and were examined by flow cytometry. Trypan blue dye was used to detect cell total number.

TC-1 or MCF-7 cells culture

TC-1 cells, which integrated HPV16E6E7 gene and were often used in mouse model to study cervical cancer treatment, and MCF-7 breast cancer cell lines were cultured following conventional cell culture method.

Co-culture of DC cells and NK cells

NK cells (2×10⁵ cells/well) and DC (1×10⁵ cells/well) were mixed and cultured in 24-well cell culture plate for 12 hours for subsequent experiments.

DC-NK mice immunization

20 C57BL/6(H-2 b) female mice were purchased from Animal Center, Henan Province, and housed in a pathogen-free filter top cages. All animal experiments

were performed in compliance with institutional guidelines and were approved by local animal ethical committees. 5×10⁶ cell/ml DC-NK cells suspended in 100 µL Hank 's buffered salt solution (HBSS) (Hyclone, Logan, Utah, USA) were injected subcutaneously into C57BL/6 mice (10 per group) for immunization experiment. One week later mice were re-immunized and sacrificed one more week later to detect CTL response.

CTL experiments

Spleen cells (3×10⁷ cells) were re-stimulated in an upright T25 tissue culture flasks containing 1 µ M E7.49-57 CTL medium for 7 days and collected for cytotoxicity experiments. Spleen cells were cultured in CTL responses medium which was RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% FCS, 2 mM L- glutamine, 1 mM sodium pyruvate (Gibco, Grand Island, NY, USA), 50 µ M 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA), and 50µ g/ml gentamicin sulfate neomycin (Gibco, Grand Island, NY, USA). Cytotoxicity experiment was carried in 96 well plates as triplicates. Cultured effector cells and TC-1 target cells expressing HPV16E6E7 were co-cultured in 96 well plates at aratios of 20:1 (effector cell/target cell, E/T) at 37 °C, 5% CO₂ for 4h, centrifuged for 5 min at 200 g using Beckman G5-6R centrifuge (Beckman-Coulter, Miami, FL, USA). 100uL supernatant from each well was transferred to Beckman ready caps to determine LDH activity released by the cells. Target cells that were cultured in control medium or Triton X-100 (1% wt/vol) without effector cells were used to determine spontaneous release of LDH or general release. The results were shown as percentage of specific lysis form, according to the following formula: [(LDHtest-LDHspont)/(LDHtotal-LDHspont)] * 100%.

Statistical analysis

SPSS 17.0 statistical software (SPSS Inc, Chicago, IL, USA) was used for data processing. Two independent samples were compared using t-test statistics and considered to have statistically significant difference when the data *P* <0.05.

Results

Identification of DC and NK cells

Cultured DC and NK cells were analyzed by ordinary optical microscopy and FACS (Figure 1, 2). DC cells were stimulated with LPS in the late stage of culture, and

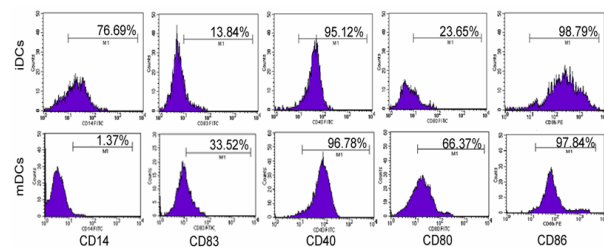


Figure 1. Phenotype Analysis of Immature and Mature DC (The Phenotypic Analysis of iDCs and mDCs). The results showed that CD83 and CD80 increased significantly on the surface of matured DC while CD14 was significantly reduced

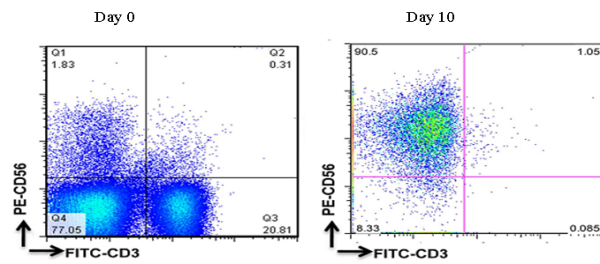


Figure 2. Flow Cytometry Analysis of NK Cells on Day 0 and Day 10 (The Identification of NK Cells on Day 0 and Day 10.) The results showed that NK cells were massively amplified in vitro, and the purity of NK cells was improved from 1.83% to 90.5% which can meet the needs of the experiment

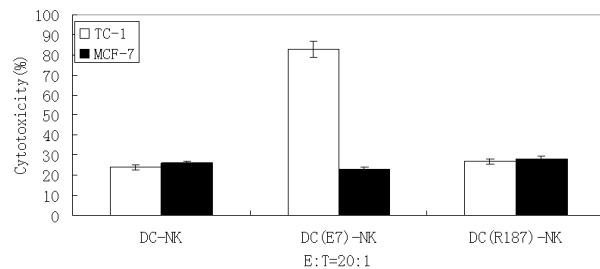


Figure 3. CTL Assay. Mice were immunized with co-culture of stimulated-DC and NK cells to detect CTL responses. The cytotoxicity analysis after mice was immunized by DC-NK. Experiment was repeated for three times

analyzed of DC surface markers using flow cytometry. Results showed that LPS promoted the maturation of DC, expressing levels CD83 and CD80 on matured DC were significantly increased ($p < 0.01$), CD14 expressing level was significantly decreased ($p < 0.01$), while CD40 and CD86 did not change significantly. We used irradiated spleen cells as stimulator cells and IL-2 and anti-CD3 antibody to amplify NK cells in vitro, and observed that CD3 + CD56 + cells increased significantly, demonstrating that our method is feasible for NK amplification in vitro.

Cytotoxicity against tumor cells in vitro

Mice were immunized with co-cultured DC-NK cells or CTL detection. At the ratio of effector: target cells = 20:1, HPV16E7.49-57 stimulated DC had much stronger cytotoxicity against TC-1 cells, as compared with other DC groups ($p < 0.01$), while the cytotoxicity against MCF-7 was not significantly different ($p > 0.05$). DC stimulated with R187 cell had no significant difference with DC-NK on the aspect of cytotoxicity against either TC-1 cells or MCF-7 cells (Figure 3). These data showed that mice immunized with antigen-stimulated DC-NK cells behaved specific anti-tumor effect.

Discussion

The transformation protein of high-risk cervical cancer includes E5, E6 and E7. These proteins are often focused as target for vaccine design and drug therapy, in particular E7 protein. In this study, we used HPV16E7.49-57 epitope peptide as an antigen, but polypeptide is poorly immunogenic and hard to invoke a strong CTL response, and thus is not used alone.

DC cells interact with a number of effector cells such as T cells, macrophages and NK cell in vivo after vaccination (Degli-Esposti et al., 2005; Shanker et al., 2007; Bellora et al., 2010; Eissmann et al., 2010; Shanker, 2010; Shanker et al., 2010). Recent studies showed that NK cells could remove the antigen-loaded DC and facilitate adaptive T cell response (Petersen et al., 2011). It was also found that NK cells selectively kill immature DC through TNF-related apoptosis-ligand-mediated pathway, since immature DC had reduced expression of MHC-I molecules while mature DC had no change (Degli-Esposti et al., 2005).

DC-originate signal through cytokines and cell-cell contact is important for the activation of NK. DC produced IL-12 plays an important role in IFN- γ secretion by NK cells (Degli-Esposti et al., 2005). In this study, we used irradiated spleen cells as stimulator cells which were capable of amplifying NK cells (purity >90%) in vitro as a basis for subsequent research. DC sensitized with peptide and stimulated with LPS was induced to mature DC with distinguishing mark including elevated expression of CD83 and CD80, reduced expression of CD14 and no significant change of the expression of CD40 and CD86. In the co-culture of stimulated-DC and NK cells, while NK cells killed immature DC cells and retained fully mature DC cells, the mature DC cells activated the cytotoxicity of NK cells on the other hand. Cytokines secreted by interaction of DC-NK co-culture, such as IL-2, IL-12, TNF- α and IFN- γ , etc. can inhibit the effect of prostaglandin E on DC-NK interaction was imprinted during DC maturation (Van Elssen et al., 2011). Our results indicated mice immunized with antigen epitope peptide stimulated-DC-NK co-culture had specific cytotoxicity against antigen expressing target cells. Recent studies showed that DC vaccine from antigen-stimulated DC-NK co-culture could elicit strong CTL responses (Morandi et al., 2012), consisting with our results, but exact mechanism needs to be further studied.

In conclusion, DC-NK cross-talk benefits respective function of DC and NK cells, which means NK cells eliminate immature DC cells and help DC vaccine activate CTL responses in vivo. This has potential clinical application in immunotherapy including cervical cancer treatment.

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