HPV16E7 silencing enhances susceptibility of CaSki cells to natural killer cells

HUIMIN GUO, RUILI HU, XINLEI GUAN, FANG GUO, SHUZHEN ZHAO and XUEYING ZHANG

Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan 453100, P.R. China

Received July 25, 2013; Accepted February 7, 2014

DOI: 10.3892/mmr.2014.1975

Abstract. The aim of the present study was to investigate the cytotoxicity of natural killer (NK) cells to CaSki cells following knockdown of the E7 protein of the human papillomavirus type 16 (HPV16E7). Recombinant adenovirus-short hairpin-E7 protein of the human panillomavirus type 16 (Ad-sh-HPV16E7) was constructed and used to infect CaSki cells. The expression of HPV16E7 in CaSki cells was assessed using western blot analysis. The expression of cell surface molecule major histocompatibility complex-I (MHC-I) in CaSki cells infected with Ad-sh-HPV16E7 was examined using flow cytometry. The cytotoxicity of NK cells isolated and expanded from healthy volunteers on Ad-sh-HPV16E7-infected CaSki cells was assessed using the lactate dehydrogenase (LDH) release assay. Ad-sh-HPV16E7 was successfully constructed and able to inhibit HPV16E7 the expression in CaSki cells. The expression of major histocompatibility complex I (MHC-I), a surface molecule, in CaSki cells was increased after infection with Ad-sh-HPV16E7. Compared with the controls, the cytotoxicity of NK cells on CaSki cells, which were infected with Ad-sh-HPV16E7, was decreased ($p<0.05$). In conclusion, HPV16E7 suppresses the expression of MHC-I on CaSki cells to evade cytotoxic T-cell (CTL) response. However, it was possible to enhance the cytotoxicity of expanded NK cells to cervical cancer cells or HPV16-infected cells *in vitro*, indicating that NK cells may be used for immunotherapy of cervical cancer.

Introduction

Persistent infection with high-risk human papillomavirus (HPV) is the major cause of cervical cancer. The transforming proteins E6 and E7 encoded by high-risk HPV are able to interact with the intracellular tumor suppressor protein p53 and the retinoblastoma protein (pRb), respectively, leading to cell immortalization (1). Surface molecules of virus-infected cells have frequently been observed to transform in order to evade recognition by immune cells. Major histocompatibility complex-I (MHC-I) is one of these molecules and has an important role in tumor immune responses. MHC-I expression levels have been observed to decrease in cervical cancer cells and the functional components such as transporter associated with antigen processing 1 (TAP1) are frequently deactivated, enabling tumor cells to evade immune surveillance (1). Natural killer (NK) cells are highly significant effector cells of the innate immune response and are able to kill virus-infected or transformed cells. NK cells are derived from cluster of differentiation molecule 34 $(CD34)^+$ progenitor cells which account for 1-32% of human peripheral blood lymphocytes (2-4). These large granular lymphocytes express the cluster of differentiation molecule 56 (CD56); however, not the cluster of differentiation molecule 3 (CD3) or T-cell receptor molecule (TCR). Their functions are regulated through balanced activity of inhibitory receptors, including killer-cell immunoglobulin-like receptors (KIR) and activation receptors, including the natural killer cell-related proteins 30, 44 and 46 (NKp30, NKp44 and NKp46). NK cells have prospective applications in adoptive immunotherapy of tumors as they are able to lyse target cells directly without sensitization (3,4).

The present study aimed to investigate MHC-I expression in CaSki cells infected with the recombinant adenovirus Ad-sh-HPV16E7 as well as to assess the cytotoxicity of NK cells isolated from healthy human peripheral blood to cervical cancer cells.

Materials and methods

Construction and infection of adenovirus vector. Short helix RNA (shRNA)-HPV16E7 was constructed by Shanghai Gene Pharma Ltd., Shanghai, China. Its sequence was as follows: sense, 5'-*GATCC*GCATGGAGATACACCTACAttcaagaga TGTAGGTGTATCTCCATGCTTTTTTG-3' and antisense, 5'-*AATTC*AAAAAAGCATGGAGATACACCTACAtctcttgaa TGTAGGTGTATCTCCATGC*G*-3' (*Bam*HI and *Eco*RI, restriction sites). shRNA-HPV16E7 and the negative control were cloned into the pShuttle vector (BD Biosciences, Franklin Lakes, NJ, USA), then they were inserted into the

Correspondence to: Dr Ruili Hu, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xinxiang Medical University, No. 88 Jiankang Road, Weihui, Henan 453100, P.R. China
E-mail: ruilihu@126.com

Key words: HPV16, natural killer, cervical cancer, adenovirus

replication-defective adenoviral vector pAdeno-X (BD Biosciences) according to the manufacturer's instructions.

Cell culture, adenovirus preparation and titration. 293T cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Recombinant adenovirus plasmids and blank vectors were transfected when the confluence rate reached 50-70% with stable cell growth. Following transfection for 8-12 h (37˚C, 5% CO_2), the mixture was replaced with complete medium. The virus was collected when 1/3 to 1/2 of the cells were floating, ~3-5 days post-infection. The recombinant adenovirus vector was examined using polymerase chain reaction (PCR) and sequencing. The primers were designed using the BD Adeno-XTM PCR Screening Primer set (BD Biosciences). Only a 287 bp DNA fragment was obtained by amplification. The PCR reaction consisted of the following steps: 94˚C for 2 min, 94˚C for 15 sec, 72˚C for 2 min, 72˚C for 3 min for 30 cycles. Titration was performed using Adeno-X Rapid Titer kits with the verified adenovirus vector. The human CaSki cell line was purchased from Typical Species Preservation Center of Wuhan University, China. CaSki cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The CaSki cells were infected with the recombinant adenovirus at a multiple of infection (MOI) of 10 following titration. The expression of MHI-I was assessed using flow cytometry with ad-mock adenovirus as a control.

Expression of HPV16E7. The expression of HPV16E7 was assessed using western blot analysis. A phosphate-buffered saline (PBS)-treated group and adenovirus-enhanced green fluorescence protein (Ad-EGFP)-infected group were used as negative controls. A HPV16E7-siRNA transfected with a liposome group was used as the positive control and the Ad-shRNA-HPV16E7 group was the experimental group. HPV16E7-siRNA was designed by our laboratory and has been proven to inhibit HPV16E7 expression effectively. CaSki cells were collected 72 h post-infection in all the groups, and subsequently lysed with radio-immunoprecipitation assay (RIPA) reagent [0.3% NP40, 1 mM EDTA, 50 mM Tris-Cl (pH 7.4), 2 mM EGTA, 1% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na_3VO_3, 10 μg/ml benzene methylation sulfonic acid oriented fluorine] on ice for 30 min. Cells were then centrifuged at 4˚C at 12,000 x g for 15 min. The supernatant was used for western blot analysis. The first antibody was anti-HPV16E7 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), while the secondary antibody was sheep anti-mouse immune globulin (Chemicon, Temecula, CA, USA).

MHC-I expression in CaSki cells. Three days following infection, five tubes of CaSki cells were harvested with 10^5 cells/tube. Mouse anti-human monoclonal antibody labeled with fluorescein isothiocyanate (FITC) was added into every tube. The isotype control was mouse immunoglobulin G (IgG). Cells were incubated for 30 min, washed once with PBS and added into 500 μl PBS prior to analysis by flow cytometry.

NK cell isolation. Cells collected from peripheral blood mononuclear cells (PBMCs) using immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were irradiated with 25 Gy in order to prevent them from overgrowth. The NK cells ($1x10^5$/ml) purified by immunomagnetic beads were cultured in a 24-well plate at a dilution of 1:20. AIM-V medium containing 5% human serum (Sigma-Aldrich, St. Louis, MO, USA) and 500 IU/ml recombinant human interleukin 2 (rhIL-2) in it. During the first five days, anti-cluster of differentiation 3 (CD3) monoclonal antibody (10 ng/ml) was added into the medium. The medium with anti-CD3 was replaced with IL-2 in fresh medium. The amplified cells were analyzed using flow cytometry. The number of cells was directly calculated following trypan blue staining.

In vitro cytotoxicity assays. Cytotoxicity of the NK cells to CaSki cells was assessed using the lactate dehydrogenase (LDH) release assay. Amplified NK and CaSki cells were co-cultured at 37˚C for 4 h at ratios of 1:10, 1:30, 1:50. Culture medium (100 μl) excluding effector cells was added via the natural release hole, followed by the addition of 100 μl 1% nonyl phenoxypolyethoxyethanol (NP40) via the maximum release hole. Each experiment was performed in three replicates. The specific cytotoxicity was calculated as: Cytotoxicity (%) = [(OD experimental group-OD total spontaneous release)/(OD maximum release group-OD total spontaneous release)] x100%, with OD being the optical density.

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Two independent samples were compared using the t-test. Differences were considered statistically significant for $P<0.05$.

Results

Construction of the adenovirus vector. The PCR results for the virus were collected at 72 h following transfection with Ad-shRNA-HPV16E7 (Fig. 1). PCR bands were significant on the 3rd and 4th lanes at 287 bp, indicating that pAd-shRNA-HPV16E7 and pAd-EGFP were constructed successfully. The CaSki cells were infected with Ad-EGFP and the infection was confirmed following 48 h of incubation.

Assessment of HPV16E7 protein suppression using western blot analysis. The expression of HPV16E7 was inhibited in the HPV16E7-siRNA and Ad-shRNA-HPV16E7 groups compared with the PBS control group. There was significant statistical difference between the Ad-shRNA-HPV16E7 and PBS groups ($P<0.01$) (Fig. 2A). The expression levels of HPV16E7 were statistically increased in the HPV16E7-siRNA group compared with those in the Ad-shRNA-HPV16E7 group ($P<0.05$) (Fig. 2B).

MHC-I on CaSki cells assessed using flow cytometry. The amount of MHC-I cell-surface molecules was increased by almost 55% in CaSki cells infected with Ad-sh-HPV16E7 compared with isotype antibody-infected cells, while there was almost no effect in the PBS-treated controls. The number of MHC-I molecules was significantly increased following HPV16E7 knockdown by Ad-sh-HPV16E7 ($P<0.01$). This indicates that HPV16 infection may lead to decreased expression of MHC-I (Fig. 3).

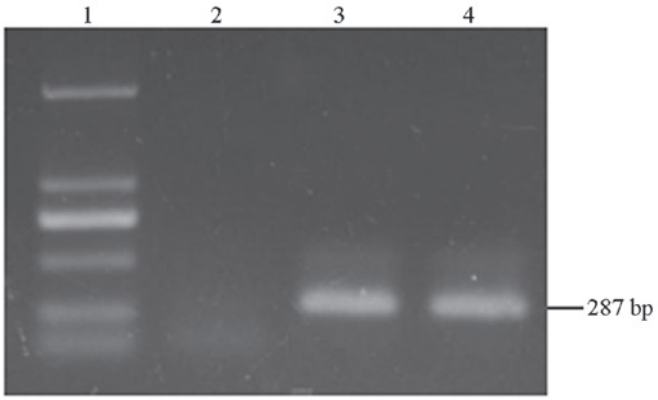

Figure 1. Recombinant adenovirus vector was identified by PCR. Lane 1, DL 2000 ladder; lane 2, control; lanes 3 and 4, positive clones. PCR, polymerase chain reaction.

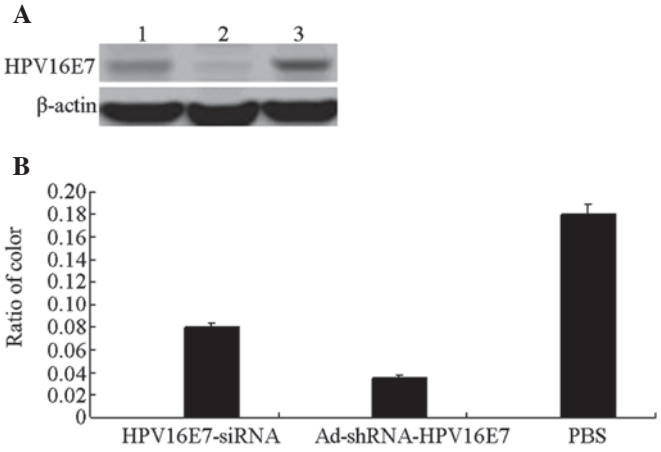

Figure 2. (A) HPV16E7 expression in CaSki cells assessed by western blot analysis. Lane 1, HPV16E7-siRNA; lane 2, Ad-shRNA-HPV16E7; lane 3, PBS. (B) Grayscale ratio of HPV16E7 and β-actin. HPV16E7, E7 protein of the human papillomavirus type 16. siRNA, small interfering ribonucleic acid; Ad-shRNA; recombinant adenovirus short helix ribonucleic acid; PBS, phosphate-buffered saline.

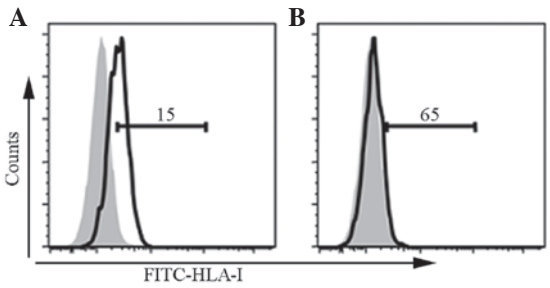

Figure 3. MHC-I on CaSki cells quantified by flow cytometry. (A) Ad-sh-HPV16E7; (B) PBS. FITC, fluorescein isothiocyanate; HLA-1, HLA class 1 histocompatibility antigen 1; MHC-I, major histocompatibility complex I; Ad-sh-HPV16E7, recombinant adenovirus-short hairpin-E7 protein of the human panillomavirus type 16; PBS, phosphate-buffered saline.

Amplification of NK cells. Immunomagnetic separation was used to purify $CD56^+$ lymphocytes without the effect of $CD3^+$ T cells. NK cell amplification was stimulated by recombinant IL-2 and anti-CD3 monoclonal antibodies. The final purity of the NK cells was 95.3% according to flow cytometric analysis,

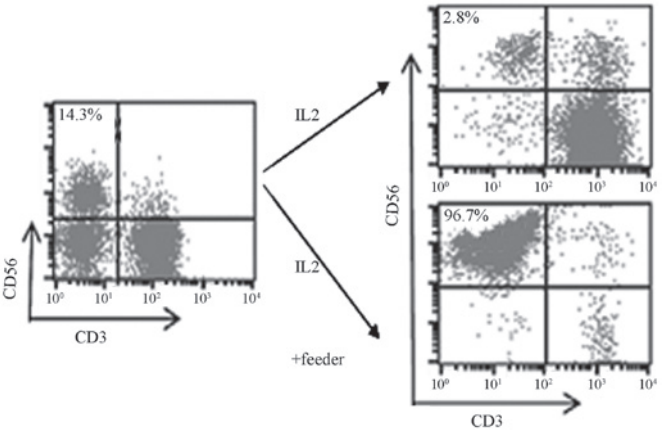

Figure 4. Peripheral blood lymphocytes were amplified for two weeks *in vitro*. The NK markers CD56 and CD3 were assessed by flow cytometry. The number of NK cells increased from 14.3 to 96.7% following amplification, while $CD56^+$ cells decreased if there was only IL-2 stimulation. NK, natural killer; CD56, cluster of differentiation molecule 56; CD3, cluster of differentiation molecule 3; IL-2; interleukin 2.

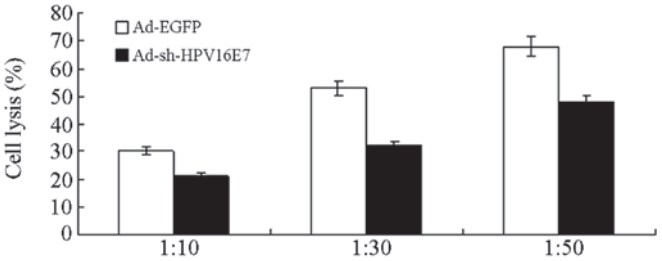

Figure 5. Cytotoxicity of the NK cells to CaSki cells was tested by LDH release assay. Ad-sh-HPV16E7 represents the CaSki cells infected by Ad-sh-HPV16E7; Ad-EGFP represents the CaSki cells infected by Ad-EGPF. Differences were statistically significant ($P<0.05$). NK, natural killer; LDH, lactate dehydrogenase; Ad-sh-HPV16E7, recombinant adenovirus short helix of E7 protein of the human papillomavirus type 16; Ad-EGFP; Ad-EGFP, adenovirus-enhanced green fluorescence protein.

indicating that relatively high levels of purity of NK cells are able to be achieved by this method; however, a small number of T cells may still be present (Fig. 4).

Cytotoxicity of the NK cells to CaSki cells. Infection of CaSki cells with the recombinant adenovirus Ad-sh-HPV16E7 resulted in increases in the amounts of MHC-I which may increase the susceptibility to NK cells. The cytotoxicity in this group was significantly higher than that in the group infected with adenovirus Ad-EGFP at 1:50, 1:10 and 1:30. The effect was was more significant at higher ratios (Fig. 5).

Discussion

Infection with high-risk HPV16 accounts for 50-70% of all incidences of cervical cancer, with HPV16E7 being among the main pathogenic genes. Therefore, the HPV16E7 gene has an important role in the pathogenesis of cervical cancer and other diseases associated with HPV infection (1). In the present study, MHC-I expression in CaSki cells increased following HPV16E7 silencing by recombinant Ad-sh-HPV16E7, which indicated that the HPV16E7 protein reduced MHC-I

expression in HPV-infected CaSki cells. A decrease in the number of MHC-I molecules reduces T-cell recognition. However, inhibitory receptors are present on NK cells which are able to bind to MHC-I, including KIR. Thus, the decrease of MHC-I expression reduces the inhibitory signaling, which activates NK cells (2-4). Accordingly, the decrease in MHC-I levels induced by HPV16E7 results in an increase of the cell susceptibility to NK cells. A decrease in HPV16E7 levels and an increase in MHC-I levels reduce recognition by cytotoxic NK cells.

In the present study, lymphocyte demixing fluid was used to separate PBMCs and immunomagnetic beads were used to isolate NK cells while removing $CD3^+$ cells in order to enhance the purity of NK cells in the subsequent culture (5). The content of NK cells in peripheral blood was relatively low, accounting for 1-20% of PBMCs. Furthermore, aging and reduction in telomeric length were present in NK cells *in vitro*. Achieving a high amplification number of NK cells *in vitro* to meet clinical requirements has become the major bottleneck in clinical use. Several methods are applied to increase the amplification number of NK cells *in vitro*, including the use of different cytokines (IL-2, IL-15 and IL-21) (6,7) and stimulation with feeder cells (wild-type or modified K562) (8). In the present study, PBMCs without NK cells, of which the T cells were considered as feeder cells, were treated with radiation to stimulate the amplification of NK cells. The results showed that a high amplification of NK cells was achieved by this method and $CD56^+$ NK cells accounted for 95.3% of the cultured cells. A number of T cells and other cells may have remained since the NK cells were isolated by immunomagnetic beads; however, the purity of NK cells did not affect the subsequent experiments. Furthermore, the use of IL-2 without feeder cells failed to effectively amplify NK cells or any other immune cells; thus, the relative proportion of NK cells was reduced. The LDH release assay was used to demonstrate that the cytotoxicity of NK cells to CaSki cells was lower than to Ad-sh-HPV16E7-infected CaSki cells, indicating that NK cells are able to recognize the changes in MHC-I expression in the target cells. Inhibition of the expression of E7 increased MHC-I levels in CaSki cells by 55%, similar to a study reporting the increase of MHC-I levels by 30% induced by adenovirus early region 1A (AdE1A) suppression with an antisense oligonucleotide (9). Another study reported that reduced levels of MHC-I on an adenovirus type 12 (Ad12)-transformed cell surface were related to the susceptibility to NK cells (3). These results suggested that the expression of MHC-I was inhibited by HPV to evade host-immune cell recognition; however, this also increased the cytotoxic activity of NK cells to CaSki cells.

Besides MHC-I, NK cells are regulated by several molecules expressed on the target cells. The activity of NK cells is able to be inhibited by the non-classical MHC-I molecule HLA class 1 histocompatibility antigen, α chain E (HLA-E) through interactions of the heterodimer CD94/NK gene 2A (NKG2A) (10). However, the expression levels of HLA-E in CaSki cells are extremely low (11,12). Whether the HLA-E expression is modified by siRNA-mediated E7 knockdown requires further study.

In conclusion, the inhibition of antigen presentation and decrease in the amounts of MHC-I are mediated by E7 through a variety of mechanisms, enabling viruses to escape immune responses in the replication process (13-15). This indicates that amplification of NK cells *in vitro* may be used for immunotherapy of cervical cancer.

References

1. Vaccarella S, Lortet-Tieulent J, Plummer M, Franceschi S and Bray F: Worldwide trends in cervical cancer incidence: Impact of screening against changes in disease risk factors. Eur J Cancer 49: 3262-3273, 2013.
2. Cheng M, Chen Y, Xiao W, Sun R and Tian Z: NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol 10: 230-252, 2013.
3. Jost S and Altfeld M: Control of human viral infections by natural killer cells. Annu Rev Immunol 31: 163-194, 2013.
4. Cooper MA, Colonna M and Yokoyama WM: Hidden talents of natural killers: NK cells in innate and adaptive immunity. EMBO Rep 10: 1103-1110, 2009.
5. Bottley G, Cook GP and Blair GE: A flow cytometric assay for analysis of natural-killer cell-mediated cytolysis of adenovirus transformed cells. Methods Mol Med 131: 221-230, 2007.
6. Lim O, Lee Y, Chung H, *et al*: GMP-compliant, large-scale expanded allogeneic natural killer cells have potent cytolytic activity against cancer cells in vitro and in vivo. PLoS one 8: e53611, 2013.
7. Denman CJ, Senyukov VV, Somanchi SS, *et al*: Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS one 7: e30264, 2012.
8. Shook DR and Campana D: Natural killer cell engineering for cellular therapy of cancer. Tissue Antigens 78: 409-415, 2011.
9. Zhao B and Ricciardi RP: E1A is the component of the MHC class I enhancer complex that mediates HDAC chromatin repression in adenovirus-12 tumorigenic cells. Virology 352: 338-344, 2006.
10. Lee N, Llano M, Carretero M, Ishitani A, Navarro F, López-Botet M and Geraghty DE: HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci USA 95: 5199-5204, 1998.
11. Kren L, Valkovsky I, Dolezel J, *et al*: HLA-G and HLA-E specific mRNAs connote opposite prognostic significance in renal cell carcinoma. Diagn Pathol 7: 58, 2012.
12. Kren L, Fabian P, Slaby O, *et al*: Multifunctional immune-modulatory protein HLA-E identified in classical Hodgkin lymphoma: possible implications. Pathol Res Pract 208: 45-49, 2012.
13. McCoy WH IV, Wang X, Yokoyama WM, Hansen TH and Fremont DH: Cowpox virus employs a two-pronged strategy to outflank MHCI antigen presentation. Mol Immunol 55: 156-158, 2013.
14. Qiu T, Wang L, Liu XH, *et al*: Over-expressing transporters associated with antigen processing increases antitumor immunity response in prostate cancer. Cell Immunol 279: 167-173, 2012.
15. Oliveira CC and van Hall T: Importance of TAP-independent processing pathways. Mol Immunol 55: 113-116, 2013.