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Human papillomavirus 16E6 suppresses major histocompatibility complex class I by upregulating lymphotoxin expression in human cervical cancer cells

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ABSTRACT

Major histocompatibility complex (MHC) class I is a major host defense mechanism against viral infections such as type 16 and type 18 of the human papillomavirus (HPV). Here, we found that the E6 oncogene from HPV16, but not HPV18, suppressed MHC I expression. Ectopic expression of HPV16E6 in HeLa cells, which are infected with HPV18, suppressed MHC I expression, and that knockdown by antisense or siRNA of the HPV16E6 strongly enhanced MHC I expression in Caski cells, which are infected with HPV18, but not HPV16. The expression of HPV16E6 strongly enhanced cellular resistance to cytotoxic T lymphocytes (CTLs)-mediated lytic activity, and knockdown of HPV16E6 by antisense had the opposite effect. The regulation of HPV16E6-mediated MHC I suppression might be through the regulation of lymphotoxin (LT) and its receptor, LTβR. In addition, cells from the spleen and liver of LTα- or LTβR-deficient mice showed increased MHC I expression. Overall, these results demonstrated that the E6 oncogene of HPV16 might play an important role in cell transformation and cancer development through LT-mediated MHC I downregulation in humans.

1. Introduction

Persistent infection with high-risk types of human papillomavirus (HPV) causes oncogenic transformation of the cervical epithelium [1,2]. Although several mechanisms have elucidated the relationship between tumor growth and the host immune defense, including the downregulation of major histocompatibility complex class I (MHC I), the secretion of cytokines and a lack of Fas and FasL, detailed mechanisms directly involved in the suppression of MHC I expression are not clearly understood.

Previous studies have indicated that HPV E6 and E7 oncoproteins disrupt cell cycle control through the inactivation of the tumor suppressors p53 and pRB via the ubiquitin-dependent proteolytic pathway [3] by binding to several PSD95/Dlg/ZO-1 (PDZ) domains in the protein [4]. Recent publications suggest that membrane-associated RING-CH (MARCH) 4 and 9 proteins, which possess PDZ motifs, might play a role in the downregulation of MHC I and CD4 by acting as E3 ubiquitin ligases; this effect occurs because MARCH1 facilitates the ubiquitination of MHC II, B7.2 and Fas [5]. However, there is no evidence as to whether HPV16E6 proteins are directly involved in the degradation of MHC I.

Lymphotoxin (LT) is composed of two molecular forms: a secreted form consisting of an $LT\alpha_3$ homotrimer that binds to tumor necrosis factor (TNF) receptors and a membrane-associated heterotrimeric form comprising $LT\alpha_1\beta_2$ that binds to LTβR [6]. The genes encoding LT and TNF-α are located in tandem within the MHC [7]. LT and TNF-α increase MHC I expression and the subsequent lysis of sarcomas by cytotoxic T lymphocytes (CTLs) [6]. Although previous studies have reported that high-risk HPV-positive cervical carcinoma tissues exhibit reduced MHC I expression [8,9], the exact mechanism by which HPV16E6 regulates MHC I expression and whether LT can regulate MHC I expression have not been elucidated.

Here, we identify a novel molecular mechanism that LT regulated by the expression of HPV16E6 downregulates MHC I in human cervical cancer cells axis might play an important role in cancer development in cervical cancer through the suppression of immunity and cancer cell evasion from the host defense immune system.

2. Materials and methods

2.1. Mice, cell lines, and reagents

C57BL/6J mice were obtained from the Damul Science (Daejeon, Korea). $LT\alpha^{-/-}$ and $LT\beta R^{-/-}$ mice were kindly provided by Dr. Y.X. Fu of the University of Chicago. HeLa (HPV18-positive) and Caski (HPV16-positive) cells were cultured in α-MEM supplemented

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with 10% FBS at 37 °C, 5% CO_2. HT3 (HPV-free) cells were cultured in McCoy's 5A medium (Gibco, Carlsbad, CA, USA). Recombinant human LT$\alpha_1\beta_2$ (rLT), human Ig (hIg) and LTβR-Ig were obtained from R&D Systems (Abingdon, UK).

2.2. Antibodies and flow cytometric analysis

The antibodies for Western blot and flow cytometric analysis were purchased from Santa Cruz Biotechnology (CA, USA) and BD Biosciences Pharmingen (San Diego, CA), respectively. Flow cytometric analysis was performed on a FACS Calibur® using CellQuest software (BD Bioscience Pharmingen) as described previously [10].

2.3. DNA constructs

HPV16E6, LTα_1 and LTβ_2 were amplified by the reverse transcription-polymerase chain reaction (RT-PCR) using the total RNA from HeLa, Caski and HT3 cells with specific primers. The cDNAs were then introduced into pcDNA3.1, pLNCX2 and pIRES vectors, respectively, and were confirmed by DNA sequencing and restriction endonuclease mapping.

2.4. Small interfering RNA (siRNA)

siRNA (5 μg) was synthesized by Samchully Pharmaceuticals (Seoul, Korea) using oligonucleotides specific for HPV16E6, HPV18E6 or LTβR (Supplementary Table 1) and transfected into cells using Lipofectamine reagent. At 4 h post-transfection, the cells were replaced with fresh complete cell culture medium and cultured for 48 h. The knockdown effects of each target mRNA were measured by RT-PCR or real-time RT-PCR.

2.5. RT-PCR and real-time RT-PCR

The cDNAs were reverse transcribed with 5 μg of total RNA, 2.5 U of Moloney murine leukemia virus (MMLV) reverse transcriptase and 10 pmol of oligo(dT) primers at 42 °C for 1 h. The PCR was performed using the indicated primers (Supplementary Table 1), as described previously [10]. For real-time RT-PCR, total cytoplasmic RNA was digested with DNase I (Life Technologies, Grand Island, NY) to remove chromosomal DNA, and the remaining DNase I was inactivated at 75 °C for 20 min. After cDNA synthesis, 20 μL of the PCR mixture [iQ® SYBR Green PCR buffer (Bio-Rad, Hercules, CA, USA) and 10 pmol of each specific primer] was added to 1 μL of the cDNA template, and real-time RT-PCR was performed in the Bio-Rad sequence detection system using the indicated primers (Supplementary Table 1).

2.6. Cytotoxic T lymphocyte (CTL) assay

CTL activity was measured by the lactate dehydrogenase (LDH) release assay according to the manufacturer's instructions (Promega, Madison, WI). Briefly, CTLs were purified by immunomagnetic depletion of non-$CD8^+$ T cells using anti-CD8-conjugated beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The CTLs were utilized as effector cells, and cervical cancer cells were used as target cells for measuring cytolytic activity. The effector cells were stimulated with target cell lysate (10 μg/mL) for 24 h and then mixed with target cells (1×10^4) at different effector-to-target (E:T) ratios ranging from 10:1 to 1:1. After 4 h, 50 μL of culture supernatants was collected from each well and was tested for LDH activity using a chromogenic substrate. Absorbance was measured at 495 nm using an ELISA reader (Bio-Rad), and the percentage of specific lysis was determined by the following formula: (experimental effector spontaneous release − experimental target spontaneous release)/(target maximum release − target spontaneous release) × 100.

2.7. Injection of LT plasmid DNA into mice

Six-week-old C57BL/6J mice were injected intramuscularly on days 0, 7 and 14 with PBS, 50 μg of pIRES expression vector (Mock) or pIRES-LT$\alpha_1\beta_2$ (LT plasmid DNA). Twenty-one days after injection, RT-PCR was performed to measure gene expression of MHC I in the spleens of the mice.

2.8. Statistical analysis

For the statistical analyses of the data, *P* values were analyzed using a paired Student's *t* test software program (Startview 5.1; Abacus Concepts, Berkeley, CA). The results were considered statistically significant when *P* values were <0.05.

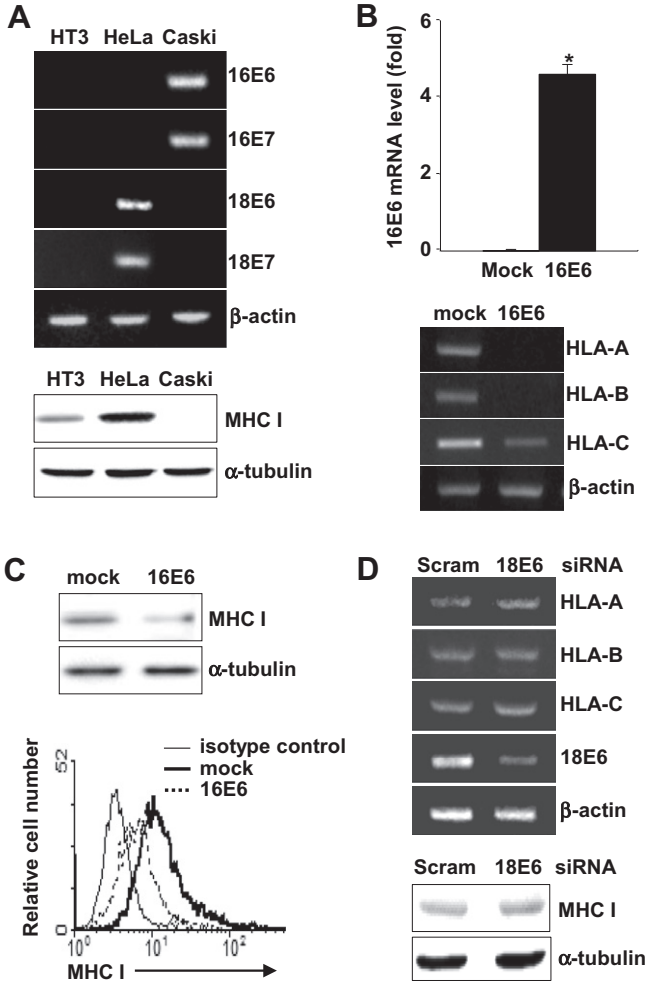

Fig. 1. The E6 oncoprotein of HPV16, but not HPV18, downregulates MHC I expression. (A) Confirmation of the E6 and E7 oncoproteins in HT3, HeLa and Caski cells. The E6 and E7 gene expression of HPV16 (16E6 and 16E7) or HPV18 (18E6 and 18E7) were assessed by RT-PCR (top) and MHC I protein expression were visualized by Western blot (bottom). (B) Ectopic expression of HPV16E6 suppresses MHC I gene expression. The HT3 cells were transfected with pcDNA3.1–HPV16E6, and ectopic expression of HPV16E6 was confirmed by real-time RT-PCR in HT3 cells (graph; $^*P < 0.05$). Expression of MHC I components, HLA-A, HLA-B and HLA-C, was analyzed by RT-PCR (bottom). (C) MHC I protein expression was evaluated by Western blot (top) and cell surface expression of MHC I was analyzed by flow cytometric analysis (bottom). (D) HPV18E6 does not affect MHC I expression. HeLa cells transfected with siRNA for HPV18E6 were used for confirmation of the expression of the MHC I components by RT-PCR (top) and Western blot analysis (bottom). (A–D) β-Actin and α-tubulin were used as internal controls and the data are representative of at five independent experiments.