COOPERATIVE EFFECT OF HUMAN PAPILLOMAVIRUS TYPE 18 E5, E6 E7 ONCOPROTEINS IN PROMOTING CELL PROLIFERATION, MIGRATION, INVASION AND IN MODULATING CELLULAR REDOX STATE

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BACKGROUND

High-risk human papillomaviruses (HR-HPVs) are the etiological agents of cervical cancer. Among them, types 16 and 18 are the most prevalent worldwide. The HPV genome encodes three oncoproteins (E5, E6 and E7) which have a high transformation potential when transduced together. Reactive oxygen species (ROS) constitute a potentially harmful by-product of many cellular reactions, hence an efficient clearance is required. HR-HPVs can adapt to oxidative stress (OS) conditions by upregulating the formation of endogenous antioxidants such as catalase, glutathione (GSH) and peroxiredoxin (PRX). The main aim of this work was to study how these oncoproteins cooperate to boost key cancer cell features such as uncontrolled cell proliferation, invasion potential, and oxidative stress, which is known to aid the carcinogenic process. We generated three different HaCaT cell lines, expressing the following combination of oncogenes: E5-18, E6/E7-18 and E5/E6/E7-18. Our results showed a statistically significant increment in cell viability, proliferation in the cell line containing the three oncogenes. The highest invasion potential, and expression of two proangiogenic proteins (u-PA, CapG) was observed in HaCaT E5/E6/E7-18 cells. Additionally, we assessed the levels of three systems involved in ROS detoxification and noticed that cells carrying HPV-18 E5/E6/E7 presented a decrease in catalase activity and a significant augmentation of GSH, PRX1 and HO-1/HMOX1 levels relative to E5, E6/E7, and HaCaT cells. In conclusion, this study demonstrates that the three HPV-18 E5, E6 and E7 oncoproteins can cooperate to enhance the malignant transformation of HaCaT cells.

MATERIAL AND METHODS

Transduction of HPV-18 E5,E6 and E7 in spontaneously immortalized human keratinocytes (HaCaT). HaCaT E5/E6/E7 cells were obtained by co-infection with a lentiviral vector carrying the MSCV-N-puro-18E5 plasmid, and with a pLXSN retroviral vector that contained cloned HPV-18 E6/E7 genes. 15 µg of each plasmid were used to transfect the packaging ecotropic Bosc23 cells using the FuGENE® 6 Transfection Reagent (Promega, Wisconsin, USA). After 48 h, cell supernatants were used to transduce amphotropic Am-12 cells in order to obtain high-titer retroviral supernatants in the presence of 10 mg mL⁻¹ polybrene. At 48 h post infection, HaCaT cells infected with retroviral vector containing E6/E7 HPV-18 were selected with 0.5 mg mL⁻¹ G418 (Gibco, Massachusetts, USA), and cells infected with retroviral vector containing E5 HPV-18 were selected with 0.5 µg mL⁻¹ of Puromycin (Santa Cruz Biotechnology, Texas, USA). Viral stocks were titrated by a NIH3T3 cells G418-resistant colony assay. Equal amounts of virus were used to infect HaCaT cells (at MOI=10). Total cellular RNA was extracted using TRIzol® (Life Technologies, California, USA). cDNAs were generated by reverse transcription from 2.000 ng of total RNA in a total volume of 20 µL using the High Capacity RNA to cDNA kit (Life Technologies, California, USA). cDNAs (100 ng µL⁻¹), cDNA samples of cells transduced with HPV-18E5, HPV-18E6/E7, and HPV-18 E5/E6/E7, were subjected to PCR amplification with specific primers for HPV-18 E5, and E7 amplicons.

Viability and proliferation assays. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). In this assay, cells were stained with a 5 mg mL⁻¹ solution of MTT (Sigma-Aldrich, Missouri, USA). After 4 hours, 100 µL of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Finally, the absorbance was measured at 570 nm in a Varioskan FLASH equipment (Thermo Fischer Scientific, Massachusetts, USA). A cell proliferation curve was performed by counting viable cells in a Neubauer chamber for four days. For this, 5.0 × 10⁴ cells (day 0) of the stable HaCaT lines were plated; HaCaT E5-18; HaCaT E6/E7-18 and HaCaT E5/E6/E7-18 in 6-well plates, each plate was numbered corresponding to a different day of the experiment (day 1, day 2, day 3 and day 4).

Invasion assay: The invasiveness potential of HaCaT, HaCaT E5, HaCaT E6/E7, HaCaT E5/E6/E7 was measured using the QCM™ Collagen Cell Invasion Assay (Merck Millipore, Darmstadt, Germany) following manufacturer's instructions.

ROS quantification: Intracellular oxidant levels were measured in the four cell lines (HaCaT, HaCaT E5, HaCaT E6/E7, HaCaT E5/E6/E7) using the fluorescent probe 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Thermo Fischer Scientific, Massachusetts, USA). CM-H2DCFDA (10 µM) in DMEM without phenol red (Sigma-Aldrich, Missouri, USA) was added to a 6-well plate containing 1×10⁴ cells per well. Plates were incubated for 30 min at 37 °C and cells were analyzed by flow cytometry, in a BD FACS Calibur cytometer.

Catalase activity and GSH quantification: Catalase activity was assayed spectrophotometrically, following the decomposition of 10 mM H2O2 by catalase contained in the samples at 240 nm (ε (240 nm) = 39.4 M⁻¹cm⁻¹). Dilutions of cell lysates were used with concentrations ranging from 0.75 µg µL⁻¹ to 1.0 µg µL⁻¹ of total protein in Hank's Balanced Salt Solution (HBSS). Absorbance measurements were conducted at room temperature in a UV-Vis Varian Cary 50 spectrophotometer for 2 minutes or until the absorbance value decreased by 10% with respect to the initial rate conditions. Total glutathione in cell lysates was quantified by HPLC.

PRX1 expression level: HaCaT cell pellets from the four cell lines (HaCaT, HaCaT E5, HaCaT E6/E7, HaCaT E5/E6/E7) were washed with ice cold phosphate buffered saline (PBS), centrifuged, and protein lysates were extracted by incubation on ice for 30 min with RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) containing complete protease inhibitor cocktail (Roche, Basel, Switzerland). Membranes were blocked with TBS (1X), 5% BSA, 0.6% Tween 20 for one hour, and then incubated with the primary antibody against peroxiredoxin 1 (Anti-PRX1) diluted 1/1000 in TBS (1X), 5% BSA, 0.1% Tween 20 (AB Frontier, LF PA0086, Seoul, Korea).

Proteome profiling of keratinocytes transduced with HPV18 oncogenes: Relative intensity of 84 different proteins involved in different signaling mediated pathways related to cancer were evaluated using the Human XL Oncology Array Kit (R&D Systems, MN, USA, ARY026)

RESULTS

Figure 1. Characterization of stable HaCaT cell lines transduced with viral HPV-18 E5, E6 and E7. (A) Morphology of the four cell lines (HaCaT, HaCaT E5, HaCaT E6/E7, HaCaT E5/E6/E7), was analyzed with a Zeiss Primovert microscope (Zeiss, Jena, Germany). Magnification 10x. (B) Left: RT-PCR for E5 oncogene in four HaCaT cell lines. Right: RT-PCR for E7 in four HaCaT cell lines. Human keratinocytes with HPV-18 entire genome (HF) cells DNA was employed as positive control for RT-PCR assays

Figure 2. Effect of E5, E6 and E7 oncogenes on cell viability, cell proliferation and modulation of E5, E6 and E7 oncogenes of HPV-18 in the production of reactive oxygen species (ROS) in HaCaT cells. (A) A total of 8.0×10³ HaCaT parental cells or HPV-18 transduced cells were seeded in 96-well plates for 48 hours at 37°C, 5% CO₂, and absorbance at 570nm was measured spectrophotometrically. Three independent experiments were performed in triplicate. Mean ± SD is shown for each cell line. A one-way ANOVA test was conducted to assess statistical significance among groups along with Tukey's HSD test for intra group comparison. (n.s.) not significant, (*) P < 0.05, (**) P < 0.01, (***) P < 0.001. (B) Cell proliferation was evaluated by counting viable cells in the Neubauer Chamber. We plated 5.0 × 10⁴ cells (day 0) of the stable HaCaT cell lines: HaCaT E5-18; HaCaT E6/E7-18 and HaCaT E5/E6/E7-18 in 6-well plates, each plate was numbered corresponding to a different day of the experiment (day 1, day 2, day 3 and day 4). (C) The expression profile of proteins involved in cell proliferation and glycolytic metabolism. (D) Reactive oxygen species were determined measuring CM-DCF fluorescence by flow cytometry in a BD FACSCalibur cytometer. Geometrical mean fluorescence intensity of the cell population was obtained and expressed relative to control values (n = 3 culture dishes per group). Results are the mean ± S.D. A one-way ANOVA test was conducted to assess statistical significance among groups along with Tukey's HSD test for intra group comparison. (n.s.) not significant (*) P < 0.05, (**) P < 0.01, (***) P < 0.001. (E) Expression profile of two proteins involved in angiogenesis, cell invasion (u-PA, and CapG), and one protein involved in oxidative stress responses (HO-1/HMOX1) were shown in the figure.

Figure 3. Effect of HPV-18 E5, E6 and E7 oncogenes on invasion in HaCaT cells. Invasiveness potential was assessed by means of a QCM™ Collagen Cell Invasion Assay. (A) A total of 9.0×10⁵ starved cells were seeded in each chamber containing serum-free DMEM Low Glucose medium. The bottom chamber was filled with DMEM Low Glucose 10% FBS which acted as a chemoattractant and plates were incubated at 37°C, 5% CO₂ for 48 hours. (B). Quantification of results obtained in (A), mean ± SD is showed. A one-way ANOVA test was conducted to assess statistical significance among groups along with Tukey's HSD test for intra group comparison. (n.s.) not significant (*) P < 0.05, (**) P < 0.01, (***) P < 0.001.

Figure 4. Evaluation of the effect of HPV-18 E5 E6 and E7 oncogenes on the expression levels of PRX-1, and in the enzymatic activity of catalase and GSH. (A) Total protein extracts from four cell lines (HaCaT, HaCaT E5, HaCaT E6/E7, HaCaT E5/E6/E7) were subjected to SDS-PAGE followed by immunoblotting using an antibody against the Peroxiredoxin 1 (PRX1). (B) Densitometric analysis of PRX1 levels in the four cell lines as mentioned above. Values were normalized to Coomassie Gel, and averages ±SDs of three independent experiments are shown. (C) GSH levels were studied in four HaCaT cell lines. (D) Catalase enzymatic activity in parental and HPV-18 transduced HaCaT cells

CONCLUSIONS

- Cells containing HPV-18 E5,E6 and E7 increased cell viability, proliferation and invasion potential compared to control cells HaCaT
- HPV-18 E5,E6 and E7 oncoproteins increases the production of moderate intracellular reactive oxygen species (ROS)
- It is hypothethized that the increment in the intracellular levels of ROS in cell expressing E5, E6 and E7 oncogenes could trigger the activation of different cellular processes, such as cell proliferation and invasion
- ROS might in turn augment PRX1, HO-1/HMOX1 expression, and GSH levels likely through Nrf2 activation