The role of gC1qR in regulating survival of human papillomavirus 16 oncogene-transfected cervical cancer cells

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Abstract. Human papillomavirus 16 (HPV-16) is strongly associated with the development of 50% of cervical cancers. The E6 and E7 proteins encoded by high-risk HPV types are associated with the immune evasion of cervical cancer cells, but the mechanism is poorly understood. The purpose of this study was to investigate whether cells transfected with E6 and E7 expression constructs reduce the expression of the globular heads of the C1q receptor (gC1qR), a mitochondrial surface protein overexpressed in certain cancer cells. First, C-33A cells were transiently transfected with the HPV-16 E6 and E7 oncogenes which resulted in gC1qR inhibition and a reduction in apoptosis. Second, gC1qR overexpression in cells showed that caspase-3 activation and mitochondrial dysfunction were involved in gC1qR-induced apoptosis. Cells transfected with a GFP-gC1qR vector resulted in upregulated gC1qR protein and a gradual increase in the generation of reactive oxygen species (ROS). Additionally, ROS generation and increased Ca2+ influx in mitochondria resulted in the loss of the mitochondrial transmembrane potential. Interestingly, when gC1qR was overexpressed in C-33A cells, apoptosis was significantly inhibited when cells were treated with metformin, which may protect mitochondrial function. These data suggest that gC1qR could play an important role in HPV-16-induced cervical cancer immune evasion depending on its level of expression and subcellular localization.

Introduction

High risk human papillomavirus 16 (HPV-16) is thought to be responsible for over 500,000 malignancies per year, including carcinomas of the vagina, anus, vulva, penis and oropharynx (1,2). Moreover, HPV-16 has now been strongly associated with the development of >50% of cervical carcinoma cases (3). The E6 and E7 proteins encoded by HPV-16 have been associated with the cellular tumour gene, which has been suggested as an immune evasion mechanism (4,5).

Most of the HPV infections are eliminated through immune responses. Moreover, only a percentage of the HPV-infected women show persistent infection that leads to malignant disease (6,7). HPV displays several mechanisms for evading the host's immune system including, but not limited to, maintenance of low protein levels in the cell, changes of viral protein expression and inhibition of host's immune responses (8,9).

The complement system has been characterised extensively, both biochemically and functionally. The receptor for the globular heads of C1q, gC1qR, is a ubiquitous and highly anionic cellular protein of 33 kDa that was initially identified as a protein of the mitochondrial matrix (10). Evidence that gC1qR induced T-cell dysfunction involves the induction of suppressor of cytokine signaling (SOCS), a powerful inhibitor of cytokine signaling, which represents a novel mechanism (11). Indeed, the gC1qR mediates many biologic responses, including inflammation, infection and immune regulation (12). Examples of such responses include phagocytosis and the uptake of apoptotic cells (13). In the present study, we provide evidence that the gC1qR in E6 and E7 proteins encoded by HPV-16 play an important role in cervical carcinoma cells evading the host's immune system.

Materials and methods

Reagents. The C-33A cervical cancer cell lines were obtained from Boster Technology (Wuhan, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Pyrrolidine dithiocarbamate (PDTC) and ethyleneglycoltetraacetic acid (EGTA) were obtained from Sigma-Aldrich (St. Louis, MO). A Phototope-HRP Western blot detection system, including anti-mouse IgG, HRP-linked antibody, biotinylated protein ladder, 20X LumiGLO reagent and 20X peroxide, was purchased from Cell Signaling Technology (Beverly, MA, USA). H2DCFDA was obtained from Molecular Probes. Propidium iodide flow cytometry assay kit was purchased from Invitrogen. Antibodies

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directed toward gC1qR, caspase-3, procaspase-3, calnexin, histone H1, mtSSB and COXII were products of Santa Cruz (Santa Cruz, CA, USA) and Cell Signaling Technology. Cell culture supplies were purchased from Life Technologies (Gaithersburg, MD). Unless otherwise specified, all other reagents were of analytical grade.

The C-33A cell line culture and DNA transfection. C-33A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 10% foetal bovine serum and 5 μg/ml insulin in a 37°C incubator with 5% CO2. Complementary DNA (cDNA) to gC1qR was constructed in frame within the BamHI/EcoRI sites of the pEGFP-C1 vector (BD Biosciences). Then, the GFP-gC1qR vector was transfected into C-33A cells. According to the vendor's protocol, 500 pmol of GFP-gC1qR vector and Lipofectamine 2000 (10 μl) were diluted in 750 μl of OptiMEM in one well (Life Technologies, USA). After preincubation for 45 min at 37°C, both solutions were mixed and incubated for an additional 15 min at room temperature. The Lipofectamine 2000/GFP-gC1qR vector mixture was subsequently overlaid onto the cells and incubated for 2 h. Finally, 1 ml of growth medium (20% FCS) per well was added for further cultivation of the cells.

Western blot analysis. The C-33A cells were collected and placed in sample buffer after specific treatments, and then these cells were incubated in lysis buffer containing (150 mM NaCl, 1 mM Na2VO4, 50 mM NaF, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10% glycerol, 20 mM Tris-HCl pH 7.5) and protease inhibitor mixtures for 30 min on ice. The supernatants were collected by centrifugation at 13,000 x g at 4°C for 15 min. An equal amount of protein was separated by SDS-PAGE on a 10-15% polyacrylamide gel and then transferred onto a PVDF membrane. The transferred membranes were blocked for 1 h in 5% non-fat milk in PBST (PBS containing 0.05% Tween-20) and incubated with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualised using the enhanced chemiluminescence (ECL) Western detection system.

Assay of intracellular ROS in C-33A Cells. C-33A cells were incubated with H2DCFDA (10 μM) under various conditions for 10 min in the dark and lysed with RIPA buffer in ice-cold conditions (14). Assays were detected by fluorescence microscopy at an excitation wavelength of 488 nm and emission at 530 nm. A spectrofluorometer with a slit width of 5 nm was used to quantify the fluorescence in the supernatant. The experiments were repeated at least ten times. Results were shown as increase in fluorescence intensity with respect to normoxic untreated control by subtracting basal fluorescence.

Measurement of intracellular Ca2+ concentration ([Ca2+]i). Fluorescence with Fluo-4 AM was used to quantify the intracellular Ca2+ levels. C-33A cells were treated with various conditions for indicated times and then washed with ice-cold PBS. The cells were resuspended in 1 ml of PBS and incubated with 5 ml of Fluo-4 AM 1 mM for 1 h. The fluorescence intensity of intracellular Ca2+ concentration was detected by Beckman Coulter Paradigm™ detection platform at an excitation wavelength of 485 nm and an emission wavelength at 530 nm. Fluorometric measurements were performed in ten different sets and expressed as the fold increase in fluorescence per microgram of protein compared with the control group.

Measurement of mitochondrial membrane potential (Δψm). JC-1 is a cationic mitochondria-specific fluorescent dye, which was used to detect the loss of mitochondrial membrane potential (Δψm) (15). At an excitation wavelength of 485 nm and emission at 530 nm, the dye accumulates in mitochondria with increasing Δψm in monomeric conditions. C-33A cells that had undergone various treatments after 60 h of growth were washed with serum-free medium and incubated with 10 μM JC-1 at 37°C. Then, C-33A cells were resuspended with medium containing 10% serum and measured at the two different wavelengths. The data are representative of ten individual experiments.

Detection of apoptotic cells. Propidium iodide staining was utilised to detect apoptotic C-33A cells. After different treatments at indicated times, C-33A cells were washed and resuspended with binding buffer (2.5 mM CaCl2, 10 mM HEPES pH 7.4, and 140 mM NaCl) before being transferred to a 5-ml tube. The cells were incubated in the dark with propidium iodide for 15 min. Binding buffer was then added to each tube, and the samples were analysed by a Beckman Coulter Epics XL flow cytometer.

Statistical analysis. Most results are presented as means ± SD. Differences between various data sets were tested for significance using Student's t-test, and p<0.05 was considered significant (*p<0.01; **p<0.01; ***p<0.05).

Results

The expression and subcellular localisation of gC1qR protein induced by HPV-16-transfected C-33A cells. To explore the role of gC1qR on HPV-16-transfected C-33A cell survival, we have established a cell culture system based on the expression of E6 and E7 from HPV-16. In this experiment, the gC1qR protein level was assessed in C-33A cells transfected with or without HPV-16 E6 and E7 oncoproteins. The expression of gC1qR protein was measured by using Western blotting (Fig. 1A). Western blot analysis results showed that the expression of gC1qR protein was significantly decreased in C-33A cells treated with HPV-16 E6 and E7 when compared with controls (C-33A cells transfected without HPV-16 E6 and E7 oncoproteins).

The subcellular localisation of gC1qR was examined by Western blotting. Calnexin, histone H1 and mtSSB were used as markers for endoplasmic reticulum, nuclei and mitochondria, respectively. As shown in Fig. 1B, the expression of gC1qR protein was localised to the mitochondrial fraction.

Accumulated gC1qR induces the apoptotic death of C-33A cells. To determine if accumulated gC1qR could trigger the apoptotic pathway, the apoptotic death of C-33A cells was assessed in this experiment. After the treatment with GFP-gC1qR vector for 72 h, C-33A cells were subjected to flow cytometric analysis. Fig. 2 shows that accumulated gC1qR increased the number of C-33A cells in the subG1 region in HPV + GFP-gC1qR vector group and GFP-gC1qR vector group. However, the number of subG1 cells in HPV E6- and E7-transfected C-33A cells showed
a slight decrease when compared with C-33A cells transfected without HPV E6 and E7 oncogenes. Additionally, we evaluated the activation of caspase-3. The presence of the caspase-3 fragment was examined in C-33A cells by treating cells with HPV, HPV + GFP-gC1qR vector, HPV + empty vector, GFP-gC1qR vector and plain medium. Cells were lysed and examined for the procaspase-3 and the active form fragment by Western blotting. As shown in Fig. 2B, the expression of procaspase-3 decreased and the active form of caspase-3 significantly increased in the GFP-gC1qR vector group when compared with the HPV + GFP-gC1qR vector group. Additionally, in HPV + GFP-gC1qR vector group, the expression of procaspase-3 decreased and the active form of caspase-3 increased when compared with HPV + empty vector group.

The effect of gC1qR on ROS generation and [Ca\textsuperscript{2+}]i concentration in C-33A cells. In subsequent experiments, ROS production was quantified in GFP-gC1qR vector-mediated gC1qR overexpressing cells and shown to be increased compared to controls. After treatment with HPV, HPV + GFP-gC1qR vector, HPV + empty vector, GFP-gC1qR vector or plain medium for the indicated time periods, ROS generation was determined by H\textsubscript{2}DCFDA fluorescence and quantified by flow cytometric analysis. The data show that ROS generation reached the maximal level at 60 h after the initial manipulation. ROS levels in the GFP-gC1qR vector group were increased by ~1.7-fold compared to HPV + GFP-gC1qR vector-treated C-33A cells (Fig. 3A). Cytosolic Ca\textsuperscript{2+} was determined using a fluorescent ELISA reader and revealed a notable elevation at 72 h after the initial manipulation (Fig. 3B). At this time period, the [Ca\textsuperscript{2+}]i concentration of the GFP-gC1qR vector group was 2.1-fold that of HPV + GFP-gC1qR vector-treated C-33A cells. The HPV + GFP-gC1qR vector group was increased by 2.7-fold over that of HPV + empty-treated C-33A cells (Fig. 3C).

To investigate whether the effect of gC1qR on intracellular Ca\textsuperscript{2+} influx and ROS generation was interlinked, GFP-gC1qR vector-mediated gC1qR overexpressing cells were treated either with the antioxidant, PDTC (25 µM), or with EGTA, a Ca\textsuperscript{2+} ion chelator (30 µM). As shown in Fig. 3D, there was a 3.5-fold decrease in ROS generation in presence of antioxidant or Ca\textsuperscript{2+} ion chelator. Furthermore, the intracellular Ca\textsuperscript{2+} levels significantly diminished after treatment with PDTC and EGTA (Fig. 3E). The data indicate that blocking the excess ROS generation by PDTC diminished the intracellular Ca\textsuperscript{2+} accumulation. Similar results were also demonstrated with the inhibition of Ca\textsuperscript{2+} accumulation by EGTA. Time-dependent changes in relative Δψm values in gC1qR overexpressing C-33A cells were explored. We used the JC-1 dye to monitor Δψm estimated as the 590:527 nm emission ratio at a specific time period from 0 to 72 h of growth. The value of Δψm in the GFP-gC1qR vector alone treatment group resulted in a loss of ~43% as compared to HPV + GFP-gC1qR vector group at 72 h. The HPV + GFP-gC1qR vector group showed a 56% decrease in relative Δψm, as compared with the HPV + empty vector group. There was an apparent increase in Δψm in C-33A cells exposed to HPV alone for 72 h when compared to plain medium after the initial manipulation (Fig. 3F).

Loss in respiratory chain complex II protein synthesis in C-33A cells. Next, we verified the effects of gC1qR on the inhibition of respiratory chain complex II protein synthesis in GFP-gC1qR vector treated cells. C-33A cells were transfected with GFP-gC1qR vector and harvested every 24 h for 96 h. gC1qR increased after the GFP-gC1qR vector transfection, whereas mtSSB was not altered for up to 96 h. This finding suggests that the expression of mtSSB may be internally controlled in the mitochondria. Consistent with the strong decrease in COX II expression, the COX II protein level was decreased at 24 h after the GFP-gC1qR vector transfection (Fig. 4).

Protection of mitochondrial function reverses the gC1qR-induced C-33A cells apoptosis. Metformin can promote mitochondrial biosynthesis. Therefore, to evaluate whether...
the protection of mitochondrial function could reverse the gClqR-induced C-33A cell apoptosis. C-33A cells were treated with HPV + GFP-gClqR vector, HPV + empty vector and metformin + HPV + GFP-gClqR vector for the indicated time periods. Fig. 5A shows that the HPV + GFP-gClqR vector resulted in an increase in the number of cells at the subG1 region when compared with HPV + empty vector in the C-33A cell line. However, the number of subG1 cells in C-33A cells showed an apparent decrease when treated with metformin + HPV + GFP-gClqR vector. Next, we evaluated the activation of caspase-3. The presence of the caspase-3 fragment was examined in C-33A cells by treating cells with the HPV
Figure 3. The biological change of mitochondria in C-33A cells. (A) ROS generation was quantified by the fluorescence of H$_2$DCFDA for 30 min and subjected to flow cytometric analysis. The data represent the mean ± SD. These data are representative of three independent experiments. (B) Constitutive expression of gC1qR induced the cellular calcium ion influx in C-33A cells (C-33A cells transfected with or without HPV16 E6 and E7 oncogenes for 7 days). Quantitative estimation of intracellular Ca$^{2+}$ was monitored using the fluorescence probe, fluo-4 AM, in five cell lines at different time points from 0 to 72 h. All data are representative of five independent experiments in which the data were calculated by averaging the value as the mean ± SD. (C) The elevated levels of mitochondrial Ca$^{2+}$ in GFP-gC1qR vector cell line was measured by fluo-4 AM fluorescence with respect to the parent cell line. (D) Oxidant generation at 60 h of growth was detected by H$_2$DCFDA fluorescence in GFP-gC1qR vector cell line, with or without treatment with PDTC (an antioxidant) and EGTA (a Ca$^{2+}$ chelator). *p<0.01 uses a t-test. (E) In the GFP-gC1qR vector cell line, intracellular Ca$^{2+}$ levels at 60 h of growth were detected in the presence or absence of PDTC (an antioxidant) and EGTA (a Ca$^{2+}$ chelator) by fluo-4 AM, respectively. In both cases, the p-value was <0.001. (F) The mitochondrial membrane potential was observed. Time-dependent changes in the relative Δψm value were observed, as detected by fluorescence of JC-1 (590:527 nm). These data are representative of three separate experiments.
+ GFP-gC1qR vector, HPV + empty vector or metformin + HPV + GFP-gC1qR vector for the indicated times. As shown in Fig. 5B, the presence of procaspase-3 decreased and the active form of caspase-3 significantly increased in HPV + GFP-gC1qR vector when compared with HPV + empty vector treated cells. In the metformin + HPV + GFP-gC1qR vector group, the levels of procaspase-3 and caspase-3 showed no apparent change when compared with the HPV + empty vector group.

**Discussion**

The complicated balance between cell proliferation and differentiation is crucial for maintaining homeostasis and normal development within the cell, and disruption of either of these processes may result in oncogenesis (16). HPVs are small DNA tumour viruses that infect, persist in and cause proliferative lesions in the epithelial cells of the skin, ectoderm-derived mucosae and their adnexa (17,18). HPV-16 E6/E7 oncoproteins have been demonstrated to cause immortalisation of primary human keratinocytes and are expressed in malignant cancers (19,20). Many studies have previously reported the ability of HPV-16 E6/E7 oncoproteins to disrupt the normal process of differentiation of human foreskin keratinocytes (21) by targeting key tumour suppressors, such as p53 (5) and pRb (2), resulting in increased levels of cell survival proteins, such as Akt (22), and disruption of the cell cycle (23). Moreover, HPV-16 E6/E7 oncoproteins acted via mitochondrial-dependent pathways to control cellular apoptosis and fate (24). Among mitochondrial matrix proteins, gC1qR has been described in controlling diverse cellular processes, such as cell growth, differentiation and apoptosis (25). The present study has provided an essential framework for assessing the role of gC1qR protein in HPV 16-transfected C-33A cell survival. HPV-16 E6/E7 oncoprotein suppression is non-specific; therefore, the expression of gC1qR is not completely suppressed by the HPV-16 E6/E7 oncoproteins. However, our
study noted that the expression of gC1qR was mostly confined to mitochondria. The gC1qR is a multi-compartmental and multi-functional cellular protein expressed in a wide range of tissues and cell types, including lymphocytes, endothelial cells, dendritic cells and platelets (26,27). Our data demonstrate that transfecting C-33A cells with HPV-16 E6 and E7 oncoproteins inhibited expression of gC1qR. Additionally, gC1qR may not only be involved in the regulation of innate immunity and adaptive immunity (28), but also it is the underlying molecular mechanisms, especially in virus infection. Xu et al (29) provided evidence that viruses use the gC1qR host protein to inhibit host antiviral responses and promote viral proliferation by activating a suppressive pathway to negatively regulate antiviral signalling. In the present study, we report that gC1qR is a physiological inhibitor in HPV-16-induced C-33A cell survival. A role for gC1qR in HPV-16 E6 and E7 oncoproteins-mediated apoptosis was demonstrated as well. As shown in Fig. 2A, the flow cytometry analysis revealed that the number of cells at the subG1 region increased after treatment with the GFP-gC1qR vector. Under this condition, gC1qR over-expression remarkably increased the number of subG1 cells. The inhibition of C-33A cell growth was further characterised by examining caspase-3 expression. These data indicate that for immune evasion, HPV-16 E6 and E7 oncoproteins might inhibit gC1qR protein expression in C-33A cells through the involvement of mitochondrial dysfunction.

Increasing evidence suggests that mitochondrial dysfunction is linked to apoptosis initiated by cytotoxic factor, such as ROS, which are generated in excess in defective mitochondria. Our study demonstrated that the generation of ROS, which leads to mitochondrial dysfunction, resulted in apoptosis in GFP-gC1qR-treated C-33A cells expressing gC1qR, a unique amino acid hypusine, following its accumulation in the mitochondria. Oxidant generation correlated with intracellular Ca2+ accumulation. Thus, the continuation of ROS generation in gC1qR overexpressed cells was associated with intracellular Ca2+ accumulation, which may lead to mitochondrial dysfunction. Indeed, a synergistic interaction was shown in intracellular Ca2+ influx and ROS generation. It was expected that interference with electron transport by ROS and intracellular Ca2+ would influence mitochondrial membrane potential. Loss in Δψm also occurred in GFP-gC1qR-treated C-33A cells. These observations of gC1qR augment our present observations that mitochondrial Ca2+ overload occurs in gC1qR overexpressing cells, suggesting its role in mitochondrial-dependent apoptosis. This observation was also supported by the treatment with metformin because metformin can promote mitochondrial biosynthesis (30). These findings indicate that promoting the mitochondrial biosynthesis may reverse the gC1qR-induced C-33A cell apoptosis.

In summary, the present work demonstrates, for the first time, important findings related to HPV-16 infection. First, HPV-16 induces C-33A cell immune evasion via suppressing gC1qR expression. Second, our data demonstrate that gC1qR-induced C-33A cell death is mitochondrial-dependent apoptosis. Overexpression of gC1qR attenuates the HPV-16 effect on C-33A survival, suggesting that gC1qR plays an important role in controlling HPV-16-transfected cervical cancer immune evasion. Promotion of gC1qR translocation to mitochondria could be a therapeutic approach to enhance the antiviral response of the host and may be an alternative means to treat viral and associated diseases.

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References


