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Recombinant viral capsid protein VP1 suppresses migration and invasion of human cervical cancer by modulating phosphorylated prohibitin in lipid rafts

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ABSTRACT

Recombinant capsid protein VP1 (rVP1) of foot-and-mouth disease virus inhibits invasion/metastasis of cancer cells. Here we studied its mechanism of action on human cervical cancer cells. The inhibition of cell invasion by rVP1 was accompanied with reduction in phosphatidylinositol (3,4,5)-triphosphate (PIP3), phospho-Akt S473, phosphorylated prohibitin (phospho-PHB) T258 in lipid rafts, dissociation of phospho-PHB T258 with Raf-1 and the inactivation of Raf-1/ERK. Addition of PIP3 or overexpression of constitutively active Akt and raft-anchored PHB T258 but not PHB T258I mutant protein reversed the inhibitory effects of rVP1. rVP1 inhibited cervical tumor growth and metastasis, and prolonged survival in xenograft mouse models. These results suggest that rVP1 inhibits cancer metastasis *via* de-phosphorylation of Akt and PHB T258 in lipid rafts to downregulate Raf/ERK signaling.

1. Introduction

Cervical cancer is one of the most common cancers in women. There are an estimated half million new cases worldwide per year and, in 2008, cervical cancer was responsible for approximately 275,000 deaths [1,2]. More than 85% of cervical cancer related deaths occur in developing countries where it is the second leading cause of cancer related mortality among women [1,2]. Cancer metastasis leads to around 90% of human cancer deaths [3,4]. Even though some progress has been made in the treatment of metastatic cervical cancer that has recurred outside the pelvis, nearly all patients still eventually succumb to their disease [5]. Due to the shortage of effective therapy for advanced metastatic cervical cancer, continued investigation of new therapeutic modalities and treatment strategies is clearly needed.

Foot-and-mouth disease virus (FMDV) is a member of the *Aphthovirus* genus in the *Picornaviridae* family. It is composed of 60 copies of each of four capsid proteins termed VP1, VP2, VP3, and VP4 [6,7]. Among these, VP1 contains the Arg-Gly-Asp (RGD) tripeptide motif that binds to integrins to mediate FMDV infection [8]. Recombinant DNA-derived VP1 (rVP1) of FMDV has previously been found to induce apoptosis of human cancer cell lines MCF-7, PC-3, and 22Rv1 *via* modulation of the integrin/Akt signaling pathway [9]. Recently, rVP1 has also been shown to suppress progression of hepatocellular carcinoma [10] and invasion of SKOV3 ovarian adenocarcinoma cells [11] by decreasing MMP-2. However, how rVP1 suppresses MMP-2 and cancer metastasis remains largely unclear.

Activation of Ras, an important proto-oncogene, may upregulate cellular migration and invasion *via* several important downstream effector signaling pathways, notably Raf-1/MAPK(ERK) and the PI3K/Akt cascade [12,13]. Elevation of PI3K activity has been correlated with clinicopathological significance in cervical cancer [14] and Raf/ERK has been proposed as a target for cancer treatment [15]. Although Ras may bind directly to Raf-1, full activation of Raf-1 requires prohibitin (PHB, also known as PHB1) [16–19] which localizes to the mitochondria, cytosol, nucleus, lipid rafts, and cell plasma membrane [20–23]. PHB contains multiple phosphorylation sites and has been reported to be phosphorylated

Abbreviations: Akt, serine/threonine-specific protein kinase; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinases; FAK, focal adhesion kinase; FBS, fetal bovine serum; FMDV, foot-and-mouth disease virus; H&E, hematoxylin and eosin; IC_{50}, half maximal inhibitory concentration; IP, immunoprecipitation; MEK, mitogen-activated protein kinase/ERK kinase; MMPs, matrix metalloproteinases; PBS, phosphate-buffered saline; PHB, prohibitin; PI3K, phosphatidylinositol 3-kinases; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homologue; RIPA, radioimmunoprecipitation assay; rVP1, recombinant viral capsid protein VP1; SCID, severe combined immunodeficiency; TBS, tris-buffered saline; WST-1, water soluble tetrazolium salt.

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by Akt at T258 and by insulin at both Y114 and Y259 [24,25]. Since rVP1 has been shown to inhibit Akt signaling [9] which might lead to a decrease in phosphorylation of PHB at T258, we hypothesized that de-phosphorylation of PHB T258 might play an important role in the rVP1-mediated suppression of cancer metastasis.

In this study we investigated whether rVP1 can suppress the migration/invasion and metastasis of human cervical cancer cell *in vitro* and *in vivo*. We evaluated the mechanism of rVP1 action and found that it involves modulation of PI3K, decrease in PIP3 and phospho-PHB T258 in the lipid rafts as well as dissociation of Raf-1 from PHB, resulting in a reduction in Raf-1/ERK signaling and MMP-2 activity, and a decrease in epithelial mesenchymal transition (EMT). By using an orthotopic xenograft mouse model of cervical cancer and analyzing phospho-PHB T258 and Raf-1 in the tumor, we further verified that the rVP1-mediated decrease in phospho-PHB T258 in the lipid raft domain is important for inhibition of tumor growth and metastasis of cervical cancer.

2. Materials and methods

2.1. Materials

The following cell lines were obtained from American Type Culture Collection (Rockville, MD): human cervical carcinoma SiHa and CaSki cell lines and human cervical adenocarcinoma HeLa cell lines. HeLa and SiHa cells were maintained in DMEM medium and CaSki cells in RPMI-1640 medium at 37 °C under 5% CO_2. All media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Rabbit anti-PHB (H-80), rabbit anti-E-cadherin (H-108) and mouse anti-β-actin (C-4) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-integrin β1 (MAB1965) antibody was obtained from Millipore (Bedford, MA). Rabbit anti-FAK and mouse anti-phospho-FAK^{Y397} antibodies were obtained from BD Biosciences (Bedford, MA). Mouse anti-vimentin antibody was obtained from Sigma–Aldrich (St. Louis, MO). Rabbit anti-phospho-PI3Kp85, rabbit anti-phospho-PI3K-p85^{Y458}, rabbit anti-PTEN, rabbit anti-phospho-PTEN^{S385}, rabbit anti-Akt and rabbit anti-phospho-Akt^{S473} antibodies were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-Raf-1, mouse anti-phospho-Raf-1^{S338}, rabbit anti-ERK1/2, rabbit anti-phospho-ERK1/2$^{T185/Y187}$ and rabbit anti-caveolin-1 antibodies were obtained from Upstate Biotechnology (Charlottesville, VA). Rabbit anti-phospho-PHB^{T258} antibody was generated by Abnova Corporation, Taiwan. Phosphatidylinositol (3,4,5)-trisphosphate diC16 (PI(3,4,5)P3 diC16) was purchased from Echelon Biosciences Inc. (Salt Lake City, UT).

2.2. Purification of recombinant VP1 protein

Purification of recombinant VP1 protein was carried out as described previously [26]. In brief, the VP1 gene in the expression vector pET24a(+) (Novagen, Madison, WI) was expressed in *Escherichia coli*. After breaking up the bacteria with a microfluidizer in TEN buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl), the pellet was washed with 0.5% deoxycholate in TEN buffer, followed by rinsing with TEN buffer and resuspended in binding buffer (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 8 M urea). The solution was then applied to a metal-chelating affinity column and eluted with a gradient of 0–0.2 M imidazole. SDS was then added to the protein solution to a final concentration of 1%. The protein solution was subsequently applied to a Superdex 200 column (GE Healthcare, Piscataway, NJ) and eluted with a buffer solution containing 25 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl and 0.05% SDS. The fractions containing rVP1 protein were pooled, concentrated and dialyzed against PBS before use.

2.3. Construction of plasmids

Human PHB cDNA was amplified by PCR from the pOTB7-PHB plasmid (Open Biosystems, Huntsville, AL) and subcloned into the pDisplay™ vector by Gateway cloning technology (Invitrogen, Carlsbad, CA) to generate plasmid pD-PHB, which fused the C-terminus of the PHB gene to the platelet-derived growth factor receptor (PDGFR) transmembrane domain and tagged it with the hemagglutinin (HA) epitope at the N-terminus. The fusion of expressed PHB with the platelet-derived growth factor receptor (PDGFR) transmembrane domain resulted in anchoring PHB to the plasma membrane and lipid rafts. PHB cDNA was also cloned into the pcDNA6/BioEase-DEST vector (Invitrogen) to generate the plasmid pBio-PHB which expressed biotin-tagged PHB. Human PHB cDNA was subcloned into the pEGFP-N1 vector (Clontech, Mountain View, CA) to generate the plasmid pPHB-GFP, with the GFP tagged to the C-terminus of PHB. The PHB-GFP or GFP gene was subcloned into the pDisplay™ vector to obtain the pD-PHB-GFP and pD-GFP plasmids. The PHB mutants were produced using the QuikChange Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The following primers were used to generate the PHB mutants: T258I: forward-5′ CTCTCGGAACATCATCTACCTGCCAGCGG 3′ and reverse-5′ CCGCTGGCAGGTAGATGATGTTCCGAGAG 3′. All constructs were verified by DNA sequencing. The pUSEamp-*Akt1* (wild type) and pUSEamp-myr-*Akt1* (activated) were purchased from Upstate Biotechnology. Transfections were performed using FuGENE HD Transfection Reagent (Roche, Nutley, NJ) according to the manufacturer's protocol.

2.4. Cell viability assay

Cell viability was measured by water soluble tetrazolium salt (WST-1) assay according to the manufacturer's instructions (Roche). In brief, 2×10^4 cells were added to 100 μl media per well on a 96-well plate and incubated at 37 °C in 5% CO_2 overnight in a humidified incubator. The cells attached to the wells were incubated in medium supplemented with 0.5% FBS (0.5% FBS-medium) and treated with serial dilutions of rVP1. After incubation at 37 °C in 5% CO_2 for 24 h to allow the drug to take effect, 10 μl WST-1 reagent was added to each well, and the plate was placed on a shaking table. After shaking at 150 rpm for 1 min, the cells were incubated at 37 °C in 5% CO_2 for another 2 h to allow the WST-1 reagent to be metabolized, and the proportion of surviving cells were determined by optical density (450 nm test wavelength, 690 nm reference wavelength). The half maximal inhibitory concentration (IC_{50}) is the concentration at which the reagent yields 50% inhibition of the cellular viability.

2.5. Clonogenic assay

Human cervical cancer cells were seeded at 2000 cells/well in a 6-well plate. One day after seeding, the cells were treated with 0, 0.2 or 0.4 μM of rVP1 in 0.5% FBS-medium at 37 °C in 5% CO_2 for 24 h. After washing, the cells were cultured in 0.5% FBS-medium for 11 days. The cells were subsequently rinsed with PBS, fixed in methanol and stained with 0.5% crystal violet. Colonies of >50 cells were counted. All the experiments were conducted in triplicate.

2.6. Boyden chamber assay

Cell migration/invasion ability was determined by using the Boyden chamber assay [10]. Briefly, the upper membrane (8 μm pore size, Corning, Corning, NY) within a Boyden chamber was coated with 20 μg/ml fibronectin (Millipore) for cell migration, or 500 μg/ml Matrigel (BD Biosciences) for cell invasion.

2.7. Gelatin zymographic analysis

Gelatinolytic activities of the gelatinases MMP-2 and MMP-9 were evaluated as previously described [11].

2.8. Immunoprecipitation, pull-down assay, and western blot

Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) containing proteinase and phosphatase inhibitor cocktail (Roche). The cell lysates (500 μg) were incubated with anti-PHB antibody and protein A/G agarose (Santa Cruz Biotechnology) for immunoprecipitation or streptavidin agarose (Sigma–Aldrich) for biotin-PHB pull-down on a rotating device at 4 °C overnight. Subsequently, pellets were washed sequentially with ice-cold RIPA and TBS buffer. Immunoprecipitated or pull-down proteins were separated by 12% SDS–PAGE and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% skim milk for 30 min and incubated with primary antibody (5–10 μg/ml) at 4 °C overnight. After washing with TBST buffer, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The presence of antibody-protein complexes was detected by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and exposure to Hyperfilm ECL film (GE Healthcare).

2.9. In vivo experimental metastasis assay

Human cervical cancer cells were pretreated with or without 0.4 μM rVP1 in 0.5% FBS-medium at 37 °C for 24 h. The cells (1×10^6 cells/100 μl PBS/mouse) were injected into five female SCID mice *per* group *via* the tail vein. After 4 weeks, the mice were sacrificed and their lungs were collected, processed for hematoxylin and eosin (H&E) staining and the number of tumor colonies in each lung was counted.

2.10. Establishment of an orthotopic xenograft murine model of human cervical cancer

An orthotopic human cervical cancer xenograft model was established in SCID mice. Briefly, 11 female SCID mice *per* group aged 8–10 weeks were anesthetized by 0.4% isofluorane inhalation. Following exposure of the uterus by an abdominal midline incision, the top and bottom sites of the cervix were stitched using