Oncotropic H-1 parvovirus infection degrades HIF-1α protein in human pancreatic cancer cells independently of VHL and RACK1

IL-RAE CHO1, SIRICHAT KAOWINN1, JEONG MOON1, JIWON SOH2, HO YOUNG KANG2, CHO-ROK JUNG3, SANGTAEK OH4, HAYNE SON5, SANG SEOK KOH5 and YOUNG-HWA CHUNG1

1BK21+, Department of Cogno-Mechatronics Engineering and 2Department of Microbiology, Pusan National University, Busan 609-736; 3Gene Therapy Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333; 4Department of Bio and Fermentation Convergence Technology, Kookmin University, Seoul 136-702; 5Department of Biological Sciences, Dong-A University, Busan 604-714, Republic of Korea

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Abstract. Overexpression of HIF-1α, a transcription factor responsive to hypoxia, is frequently observed in malignant tumors, which sometimes show resistance to chemotherapy and radiation therapy. Consequently, decrease of HIF-1α through virotherapy offers a logical strategy for the treatment of aggressive tumors. In this study, we found that infection with the oncolytic H-1 parvovirus decreased HIF-1α protein levels in pancreatic cancer cells under CoCl2 or hypoxia. The H-1 virus-induced decrease of HIF-1α was regulated by a proteasome-mediated pathway. Suppression of VHL, an E3 ligase and a critical regulator of HIF-1α, or enforced expression of UCP, an E2 ubiquitin-conjugating enzyme, failed to inhibit the H-1 virus-induced decrease of HIF-1α. Furthermore, siRNA-mediated suppression of RACK1, another regulator of HIF-1α, did not prevent H-1 viral infection from lowering HIF-1α protein levels. Although decrease of HIF-1α was observed after H-1 viral infection, constitutive expression of HIF-1α limited H-1 viral replication. After combined treatment with H-1 parvovirus and YC-1, an inhibitor of HIF-1α, the apoptosis of pancreatic cancer cells was greater than after treatment with H-1 virus alone or YC-1 alone. Accordingly, we propose that H-1 parvovirus could be used with YC-1 as a potential therapeutic agent against aggressive tumors exhibiting hypoxia and increased levels of HIF-1α.

Introduction

Hypoxia is a prevalent feature of solid tumors. Normal tissues have an oxygen partial pressure of 50-60 mmHg, compared with 10 mmHg or less in most solid tumors (1,2). The hypoxic environment induces adaptive changes in tumor cell metabolism, which can distort the local microenvironment. These changes are clinically important because hypoxia enhances resistance to chemotherapy and radiation therapy and it is predictive of metastasis and malignancy (1).

Members of the HIF family of transcription factors are crucial regulators of adaptive cellular responses to hypoxia. Overexpression of HIF-1α is a hallmark of diverse tumors and its constitutive activation is frequently observed in aggressive tumor phenotypes (3). HIF-1α is degraded via a ubiquitination-mediated, proteasome-dependent pathway under normoxic conditions (4). The oxygen-dependent turnover of HIF-1α is regulated by prolyl HIF hydroxylase (PHD) enzymes that hydroxylate two conserved proline residues located in the oxygen-dependent degradation domain of HIF-1α. During normoxia, hydroxylation of HIF-1α allows it to bind to the von Hippel-Lindau (VHL) protein, a recognition component of the E3 ubiquitin ligase complex. The interaction promotes the ubiquitination of HIF-1α, which is mediated by a complex that includes VHL, Elongin-B, Elongin-C, Cullin-2 and Rbx1, and leads to the degradation of HIF-1α (5,6).

Pancreatic cancer has an extremely poor prognosis, with a 5-year survival rate of <5% (7,8). The only potential curative treatment for pancreatic cancer is surgery, but only 10-20% of patients are candidates for surgery at the time of presentation. Because most patients are diagnosed with malignant pancreatic cancers of advanced metastatic stages, the therapeutic options are very limited (9). Moreover, the efficacy of current treatments, such as monoclonal antibodies (10,11) or small molecule tyrosine kinase inhibitors (12), is low. The use of viral vectors derived from adenovirus, vaccinia virus, herpesvirus, or H-1 parvovirus has been suggested as a promising way to expand the current options for pancreatic cancer therapy (13-15).

The autonomous H-1 parvovirus comprises a small, non-enveloped icosahedral particle with a single-stranded DNA genome of ~5 kb (16). H-1 parvovirus has received attention because of its oncotropic and oncotoxic properties (17). Its lytic cycle leads to tumor cell death via an apoptotic or a lysosomal pathway (18). It was shown that administration of H-1 virus prolongs the survival of rats with transplanted glioma cells in the brain without having cytotoxic effects on other tissues (19).
Furthermore, clinical phase I/IIa trials have been performed in patients with progressive primary or recurrent glioblastoma multiforme, the most malignant type of glial tumor (20). In pancreatic tumors, another aggressive cancer model, IFN-γ released from immune cells accelerates the efficacy of H-1 virus-mediated oncolysis in pancreatic cancer cells (21). H-1 virus uses cellular SMAD4, a transcription factor that can bind to the viral P4 promoter, for efficient replication in pancreatic cancer cells (22).

In this study, we investigated whether H-1 parvovirus could downregulate HIF-1α, a malignant tumor marker, to trigger apoptosis in pancreatic cancer cells. We found that H-1 parvovirus reduces HIF-1α protein levels independently of VHL and RACK1. Furthermore, combined treatment with H-1 parvovirus and YC-1 accelerated the apoptosis of pancreatic cancer cells constitutively expressing HIF-1α, suggesting that H-1 parvovirus could be used with YC-1 as a therapeutic agent against aggressive pancreatic tumors.

Materials and methods

Cell culture and virus amplification. Normal rat kidney (NRK) cells and Mia PaCa-2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Mia PaCa-2 cells with stable knockdown of VHL (MiaPaCa-shVHL cells) and the corresponding control cells (MiaPaCa-shGFP cells) were additionally supplemented with puromycin (1 μg/ml). H-1 parvovirus was purchased from American Type Culture Collection (Manassas, VA, USA) and was propagated in NRK cells. The virus was purified as described elsewhere (23) and the viral titer was determined as TCID₅₀/ml.

Reagents and antibodies. Cycloheximide and YC-1 were purchased from Sigma (St. Louis, MO, USA) and MG132 was obtained from Calbiochem (San Diego, CA, USA). For immunoblotting, anti-β-tubulin and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the anti-HIF-1α antibody was obtained from BD Biosciences (San Jose, CA, USA). The anti-caspase-8 antibody was purchased from Cell Signaling (Danvers, MA, USA). Polyclonal anti-H-1 parvovirus antibodies were prepared after immunization of rabbit 3 times with purified H-1 virus. For construction of stable pancreatic cancer cells in which VHL protein expression was stably suppressed, the pRS-shVHL vector was purchased from OriGene (Rockville, MD, USA).

Western blot assay. Cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl, pH 7.5) containing 0.1 mM Na₂VO₃, 1 mM NaF and protease inhibitors (Sigma). For immunoblotting, proteins from whole cell lysates were resolved by 10 or 12% SDS-PAGE and then transferred to nitrocellulose membranes. Primary antibodies were used at 1:1,000 or 1:2,000 dilutions and secondary antibodies conjugated with horseradish peroxidase were used at a dilution of 1:2,000 in 5% non-fat dry milk. After the final washing steps, nitrocellulose membranes were exposed to enhanced chemiluminescence reagent and imaged using LAS 4000-mini (Fuji, Tokyo, Japan).

RT-PCR analysis. Total RNA was extracted from cells using an RNeasy Protect Cell Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer’s instructions. Three micrograms of total RNA was converted to cDNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and PCR was performed using specific primers described elsewhere (24). The cDNA in each sample was diluted and PCR was run for the optimized number of cycles. β-actin mRNA was measured as an internal standard. After amplification, the products were subjected to electrophoresis on 1.5% agarose and detected with ethidium bromide staining.

Statistical analysis. Data are presented as the mean ± standard error of the mean (SEM). Student’s t-test was used for statistical analysis, with P values <0.05 defined as statistically significant.

Results

H-1 parvovirus infection induces rapid degradation of the HIF-1α. Because many solid tumors display features of hypoxia (1,2), we wondered how tumor cells growing under hypoxic conditions would respond to infection with the oncolytic H-1 virus. To test this, we grew Mia PaCa-2 pancreatic cancer cells under CoCl₂ (which mimics hypoxic conditions), infected them with H-1 virus and measured the levels of HIF-1α protein at 12 h post-infection. We found that H-1 virus infection significantly reduced the levels of HIF-1α protein, which had been increased by CoCl₂ (Fig. 1A). Furthermore, when cells under hypoxia (1% O₂) were treated with H-1 virus, HIF-1α levels were again markedly reduced at 12 h post-infection (Fig. 1B). Thus, H-1 viral infection significantly reduces the abundance of HIF-1α protein, which is otherwise increased by hypoxia. To test whether the H-1 virus-induced decrease in HIF-1α abundance occurred at the transcriptional level, we analyzed HIF-1α mRNA levels in cells infected with H-1 virus during hypoxia. We detected no significant alteration in the abundance of HIF-1α transcript after H-1 virus treatment under normoxia (Fig. 1C), even though HIF-1α protein levels changed dramatically (Fig. 1A and B). These results suggest that H-1 virus induces a rapid decrease in HIF-1α protein abundance at the post-transcriptional level.

The H-1 virus-induced decrease in HIF-1α is independent of VHL but requires proteasomes. HIF-1α is degraded via its binding to VHL protein, which functions as a recognition component of the E3 ubiquitin ligase complex under normoxia (5,6). Therefore, we asked whether VHL protein was involved in the H-1 virus-induced decrease in HIF-1α abundance. To answer this question, we suppressed VHL protein levels using siRNA, which resulted in stable expression of HIF-1α in Mia PaCa-2 cells even under normoxic conditions. When the VHL-suppressed Mia PaCa-2 cells were treated with H-1 virus, HIF-1α protein levels decreased at 12 h post-infection (Fig. 2A). In addition, we used a vector encoding ubiquitin carrier protein (UCP), an E2-EPF ubiquitin-conjugating enzyme, which promotes the ubiquitination and degradation of VHL (25). Introduction of UCP stabilized the expression of HIF-1α in Mia PaCa-2 cells even under normoxic conditions. When the Mia PaCa-2 cells with enforced expression of UCP were treated with H-1 virus, HIF-1α protein levels decreased
The H-1 virus-induced degradation in HIF-1α is independent of RACK1 and HAF. Because the receptor of activated protein kinase C (RACK1) interacts with the PAS-A domain of HIF-1α and promotes HIF-1α degradation by recruiting Elongin-C/B ubiquitin ligase (26) in an O2/PHD/VHL-independent manner, we next asked whether RACK1 was involved in the H-1 virus-induced degradation of HIF-1α. We suppressed RACK1 levels with siRNA, which resulted in stable expression of HIF-1α even under normoxic conditions. When RACK1-suppressed MIA PaCa-2 cells were treated with H-1 virus, HIF-1α protein levels decreased at 12 h post-infection (Fig. 3A). This result indicates that H-1 virus-induced HIF-1α degradation is independent of RACK1. Furthermore, we treated MIA PaCa-2 cells with 17-AAG, an HSP90 inhibitor, under CoCl2 or hypoxia because RACK1 competes with HSP90 for binding to the PAS-A domain of HIF-1α (26). Treatment with 17-AAG enhanced the degradation of HIF-1α protein (Fig. 3B), as seen in a previous study (26). In addition, because hypoxia-associated factor (HAF) promotes HIF-1α degradation in an O2/VHL-independent manner (27), we wondered whether HAF played a role in the H-1 virus-induced degradation of HIF-1α. We repressed HAF levels with siRNA, but did not observe a significant increase in HIF-1α protein levels under normoxic conditions, in contrast to the effects of VHL or RACK1 knockdown (Fig. 3C). Nonetheless, H-1 viral infec-
H-1 VIRAL INFECTION DEGRADES HIF-1α PROTEIN

Figure 2. H-1 virus-mediated HIF-1α degradation is independent of VHL but requires proteasomes. (A) MIA PaCa-2 cells were transfected with VHL siRNA (200 µM) and then infected with H-1 virus (MOI = 10) at 24 h post-treatment. The cells were harvested at 12 h post-infection and the expression of HIF-1α and VHL was examined by immunoblotting with anti-HIF-1α and anti-VHL antibodies, respectively. (B) MIA PaCa-2 cells were transfected with different concentrations of a Flag-tagged UCP expression vector (0.5, 1 and 2 µg). The cells were harvested at 48 h post-treatment and the expression of HIF-1α, VHL and UCP was examined by immunoblotting with the corresponding antibodies. MIA PaCa-2 cells were transfected with different concentrations of a Flag-tagged UCP expression vector and then infected with H-1 virus (MOI = 10) 24 h post-treatment. The cells were harvested at 12 h post-infection and the expression of HIF-1α, VHL and UCP was examined by immunoblotting with the corresponding antibodies. (C and D) MIA PaCa-2 cells were transfected with VHL siRNA or the UCP vector and then infected with H-1 virus (MOI = 10) at 24 h post-treatment. The cells were then treated with MG132 (10 µM) or cycloheximide (CHX; 10 µM) for 8 h before the cells were harvested. At 12 h post-infection, the cell lysates were separated on 10% SDS-PAGE gels and the expression of HIF-1α, VHL and UCP was examined by immunoblotting with the corresponding antibodies.

Figure 3. H-1 virus-mediated HIF-1α degradation is independent of RACK1 and HAF. (A and C) MIA PaCa-2 cells were transfected with RACK1 or HAF siRNA (200 µM) and then infected with H-1 virus (MOI = 10) at 24 h post-treatment. The cells were harvested at 12 h post-infection and the expression of HIF-1α, RACK1 and HAF was examined by immunoblotting with the corresponding antibodies. (B) MIA PaCa-2 cells were treated with CoCl₂ or cultured under hypoxia and then treated with 17-AAG (0.5 µM) for 12 h. The cells were harvested and the expression of HIF-1α, Hsp90 and RACK1 was examined by immunoblotting with the corresponding antibodies.

Constitutive expression of HIF-1α limits H-1 virus-induced apoptosis. To investigate the biological consequence of the constitutive expression of HIF-1α in cells infected with H-1 virus, we first established MIA PaCa-2 cells in which VHL was stably suppressed (Miapaca-shVHL cells) and prepared corresponding control (Miapaca-shGFP cells). We then evaluated H-1 virus-mediated cytotoxicity in Miapaca-shVHL cells and Miapaca-shGFP cells. Interestingly, Miapaca-shGFP
cells were more sensitive than Miapaca-shVHL cells to H-1 virus-induced cell death (Fig. 4A). When live cells lines were counted at 48 h post-infection using the trypan blue exclusion assay, the number of live Miapaca-shVHL cells was ~2-fold

Figure 4. Constitutive expression of HIF-1α limits H-1 viral replication. (A) Miapaca-shVHL and Miapaca-shGFP cells were infected with H-1 virus (MOI = 10) for 48 h and cell viability was assessed under a light microscope. (B) Miapaca-shVHL and Miapaca-shGFP cells were infected with H-1 virus for 48 h and the number of viable cells was counted using the trypan blue exclusion assay. A white bar indicates the initial cell number and a black bar indicates the cell number at 48 h post-infection. *P<0.05 for Miapaca-shVHL cells vs Miapaca-shGFP cells. (C) Miapaca-shVHL and Miapaca-shGFP cells were infected with H-1 virus (MOI = 10) for 48 h and harvested for immunoblot detection of H-1 replication with a rabbit polyclonal anti-H-1 antibody and apoptosis with anti-caspase-8 and anti-PARP antibodies.

Figure 5. Combined treatment with H-1 virus and YC-1 efficiently induces apoptotic cell death in MIA PaCa-2 cells constitutively expressing HIF-1α. (A) Miapaca-shVHL cells were treated with YC-1 at 1, 5, 10, 30 and 50 µM for 12 h and harvested for immunoblotting with an anti-HIF-1α antibody. (B and C) Miapaca-shVHL cells were treated with YC-1 (30 µM), H-1 (MOI = 10), or YC-1 (30 µM) and H-1 virus (MOI = 10) for 48 h. The cells were observed under a light microscope at 12, 24 and 48 h post-treatment to assess cell viability and the viable cell number was determined using the trypan blue exclusion assay. Open circle, YC-1; closed rectangle, H-1; closed diamond, H-1 virus plus YC-1; *P<0.05 for co-treatment with YC-1 and H-1 virus vs treatment with H-1 virus alone. The cell lysates were separated on 10% SDS-PAGE gels and the expression of HIF-1α, caspase 8 and PARP was examined by immunoblotting with the corresponding antibodies.
higher than the number of live Miapaca-shGFP cells (Fig. 4B). Next, we investigated what caused the greater sensitivity of Miapaca-shGFP cells to cell death during H-1 virus infection. We examined H-1 capsid protein levels in whole cell lysates from H-1 virus-infected Miapaca-shVHL and Miapaca-shGFP cells. We found that the levels of H-1 capsid protein VP2 were higher in Miapaca-shGFP cells than in Miapaca-shVHL cells (Fig. 4C). The result suggests that greater viral replication is associated with the sensitization of Miapaca-shGFP cells to cell death. Consistent with this result, Miapaca-shGFP cells exhibited higher levels of cleaved PARP and active caspase-8 than did Miapaca-shVHL cells (Fig. 4C). Taken together, our results suggest that the constitutive expression of HIF-1α in Miapaca-shVHL cells limits replication of the H-1 virus and inhibits cellular apoptosis to some extent, although we cannot presently rule out the possibility that the suppression of VHL protein also modulates apoptotic responses through other pathways.

YC-1, a drug targeting HIF-1α, promotes H-1 virus-induced apoptosis. Because the constitutive expression of HIF-1α appears to restrict H-1 viral replication and subsequent cell death (Fig. 4), we attempted to overcome this hurdle using YC-1, a compound that targets HIF-1α. First, we identified the minimal concentration of YC-1 necessary to degrade HIF-1α in Miapaca-shVHL cells in order to avoid the cytotoxic effects of YC-1. Although YC-1 at 50 µM efficiently reduced HIF-1α levels (Fig. 5A), the concentration induced apoptosis in ~70% of the cells at 12 h post-treatment (data not shown). We therefore used YC-1 at 30 µM during treatment with H-1 virus and confirmed that degradation of HIF-1α was greater after co-administration of YC-1 and H-1 virus than after treatment with H-1 virus or YC-1 alone. We then examined the efficiency of the co-administration killing pancreatic carcinoma Miapaca-shVHL cells. As shown in Fig. 5B, YC-1 treatment alone did not induce cell death during the treatment period. Pancreatic carcinoma Miapaca-shVHL cells showed ~30% cell death at 24 h after H-1 viral infection alone and 50% cell death at 48 h post-infection (Fig. 5B). On the other hand, combined treatment with YC-1 and H-1 virus induced more rapid cell death: ~60% at 24 h and 85% at 48 h post-treatment (Fig. 5B). We confirmed that Miapaca-shVHL cell death induced by H-1 virus alone or by combined treatment occurred through apoptosis, with activation of caspase 8 leading to PARP cleavage (Fig. 5C). Thus, because of its ability to overcome the inhibitory effects of HIF-1α, co-administration of YC-1 and H-1 virus might be an efficient strategy for the treatment of solid tumors.

Discussion

HIF-1α is activated not only by hypoxia but also by a number of stimulants, including cytokines, LPS and certain bacterial infections (28). In addition, HIF-1α is upregulated upon infection with hepatitis B virus through its interaction with hepatitis B virus X protein (29). LMP-1, a primary oncoprotein of Epstein-Barr virus, has been reported to induce HIF-1α synthesis (30). Recent studies have shown that infection with respiratory syncytial virus (RSV) stabilizes HIF-1α protein via the release of nitric oxide (31) and that stabilization of HIF-1α by RSV infection does not require hypoxia (32). In contrast to studies showing that some viruses can activate HIF-1α, we show here that HIF-1α, which is often elevated in malignant tumors under hypoxic or stress conditions, is degraded following oncotropic H-1 viral infection, with down-regulation achieved at the post-transcriptional level, as seen in reviral infection (24). We found that the downregulation of HIF-1α induced by H-1 viral infection was independent of oxygen levels and VHL. Because recent studies have reported that both RACK1 and HAF are able to trigger HIF-1α degradation in a manner that is independent of oxygen, PHD and VHL (26,27), we suppressed the expression of these proteins with siRNA. We found that H-1 viral infection still reduced HIF-1α protein levels under these conditions. However, the mechanism underlying H-1 virus-mediated HIF-1α degradation is still under investigation.

Many lines of evidence have shown that the constitutive expression of HIF-1α functions as a hurdle to the treatment of chemotherapy- or radiotherapy-resistant cancer cells. Reovirus infection induces the degradation of HIF-1α but the constitutive expression of HIF-1α restricts reovirus replication to some extent (24). Other studies have shown that the renal carcinoma 786-O cell line with constitutive expression of HIF-1α inhibits the replication of vesicular stomatitis virus through the upregulation of proteins involved in the immune defense response, such as IFN-β, OAS and interferon-stimulated genes (ISGs) (33). Therefore, we examined IFN-β and OAS transcripts in our study. Surprisingly, we found no significant difference in the levels of IFN-β or OAS transcripts in Miapaca-shVHL and Miapaca-shGFP cells (data not shown). This might be attributable to the different backgrounds of the cell lines and viruses.

Strategies to target HIF-1α, such as screening small molecules from chemical libraries, have been developed in an effort to improve the treatment of solid tumors. Several small molecule inhibitors of HIF-1α activity have been shown to have antitumor and anti-angiogenic activity in addition to other known roles. These include the microtubule depolymerizing agent 2-methoxyestradiol (34); inhibitors of the redox protein thioredoxin-1 (35); YC-1, an agent developed for circulatory disorders (36); and the HSP90 inhibitor geldanamycin (37). Herein, we have described the potential utility of YC-1. Some lines of evidence indicate that the introduction of Mdm2, a HIF-1α binding partner, reverses YC-1-mediated HIF-1α decrease, suggesting Mdm2 has an effect opposite that of YC-1 in the regulation of HIF-1α (38). Recent studies have also shown that the Akt/NF-κB signaling pathway contributes to YC-1-mediated HIF-1α downregulation (39). Although we have yet to describe the detailed mechanism of the synergic effect of combined YC-1 and H-1 virus treatment in Mia PaCa-2 pancreatic cancer cells constitutively expressing HIF-1α, we suggest that combined treatment with these two agents offers a novel strategy for the treatment of solid tumors constitutively expressing HIF-1α.

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References


