HIV-1 viral protein R (Vpr) induction of apoptosis and cell cycle arrest in multidrug-resistant colorectal cancer cells

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Abstract. Colorectal cancer is a significant health problem, and the advanced stages of the disease have a low response rate to chemotherapy and easily acquire chemoresistance. HIV-1 viral protein R (Vpr) has been shown to possess inhibitory effects on various malignant cells in vivo and in vitro. In this study, an Ad-Vpr construct was used to infect the multidrug-resistant human colorectal cancer HCT-8/5-FU(MDR) cell line in vitro for cell viability, apoptosis, gene expression and gene activity using the MTT, flow cytometry, immunoblotting and gel shift assays, respectively. The data showed that Ad-Vpr significantly reduced HCT-8/5-FU(MDR) cell viability in a dose- and time-dependent manner. Ad-Vpr infection promoted HCT-8/5-FU(MDR) cells to undergo apoptosis and to arrest at the G2 phase of the cell cycle. The G2 cell cycle protein Cyclin B1 accumulated in the cells after Ad-Vpr infection. Furthermore, Ad-Vpr induced activation of caspase-3 and -9, but not caspase-8, in HCT-8/5-FU(MDR) cells. Ad-Vpr suppressed expression of the Bcl-xl protein, but upregulated Bax expression and cytochrome c release from the mitochondria in HCT-8/5-FU(MDR) cells. Ad-Vpr infection also resulted in a time-dependent decrease in nuclear translocation of NF-kB/p65 protein and p65 DNA-binding activity in HCT-8/5-FU(MDR) cells. The data from the current study provide mechanistic insights into understanding the molecular basis and utility of Ad-Vpr as a novel anticancer agent for multidrug resistance in human colorectal cancer.

Introduction

Colorectal cancer is a significant health problem in the world, and is the third most common cancer in women and the fourth most common in men. More than half a million patients with colorectal cancer die of the disease annually worldwide (1,2). To date, surgical intervention is the most effective tool to cure colorectal cancer, whereas chemotherapy is another important means to treat colorectal cancer, especially for advanced staged disease. In those patients, chemotherapy appears to be the only therapeutic modality and offers some benefit. For chemotherapy, 5-fluorouracil (5-FU)-based regimens are the most common choice for patients. The combination of 5-FU with irinotecan and oxaliplatin has significantly improved the response rate of colorectal cancer patients. Additionally, combination regimens of 5-FU with irinotecan have been evaluated as the first-line therapeutic selection for advanced colorectal cancer with a response rate of 30-50% and an overall survival of 14-20 months (3,4). However, the response rate for these advanced targeted therapies and third-generation chemotherapies remains low (5). The reason may be due to innate and acquired chemoresistance (6) of colorectal cancer, which often leads to relapse and poor patient prognosis. Thus, a more aggressive and effective therapy to control colorectal cancer is imperative.

To this end, our research focuses on the viral protein R (Vpr), which is an accessory protein and plays an important role in the regulation of nuclear import of the HIV-1 pre-integration complex, and is required for virus replication in non-dividing cells. Expression of Vpr protein was able to induce cell cycle arrest and apoptosis. Therefore, many studies have analyzed the potential antitumor activity of this molecule both in vitro and in vivo (7-15). Previous studies have shown that the Vpr protein appears to preferentially inhibit the growth of rapidly dividing tumor cells, but does not affect the normal cells regardless of changes in p53 expression (7-9,15). Most importantly, the Vpr protein can easily penetrate the cell membrane without the help of a receptor or carrier (16-18). Therefore, these characteristics of the Vpr protein potentiate...
its possibility as an effective anticancer agent. Indeed, a number of previous studies have pursued this protein as a therapeutic tool in control of different types of human cancer (7-15). However, the anticancer effects of the Vpr protein in colorectal cancer have not been elucidated. Therefore, in this study, we explored the possible effects of the Vpr protein on regulating colorectal cancer cell viability and apoptosis in HCT-8/5-FU(MDR) cells. Our data provide novel insight into the underlying mechanisms by which Vpr overrides drug resistance in HCT-8/5-FU(MDR) cells.

Materials and methods

Adenoviral vector construction. An adenoviral vector containing Vpr cDNA was constructed according to a previous report (19). Briefly, the HIV-1 Vpr cDNA sequence was synthesized and cloned into a plasmid vector pUC57. The Vpr expression cassette was then excised from the plasmid pUC57-Vpr endonuclease Xhol and Nhel, and ligated into a linearized adenoviral shuttle vector, pGStrack-CMV, to construct pGStrack-CMV-Vpr. After DNA sequence confirmation, the pGStrack-CMV-Vpr was co-transfected with the pAd vector backbone into DH5α bacteria for the recombinant generation of Ad-Vpr, which was further amplified in HEK293 cells. Viral particles were purified by cesium chloride density gradient centrifugation. Multiplicity of infection (MOI) was assessed as plaque forming units (pfu)/cell number.

Cell culture and adenoviral transduction. The multidrug-resistant human colorectal cancer cell line HCT-8/5-FU(MDR) was developed previously in our laboratory by incubating parental HCT-8 cells (CCTCC, Wuhan, Hubei, China) in increasing concentrations of 5-FU (Tianjin Amino Acid Pharmaceutical Company, Tianjin, China) over a period of seven months according to the method described by Han et al (20). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St Louis, MO, USA). 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

HCT-8/5-FU(MDR) cells in serum-free RPMI-1640 medium were infected with the Ad-Vpr at the indicated MOI. Infection with an adenovirus designed to express green fluorescent protein (Ad-GFP) served as a mock control. The virus-containing medium was removed 8 h later and replaced with fresh RPMI-1640 medium containing 10% FBS.

MTT assay. Cell viability was measured by an MTT assay. Briefly, cells were grown, trypsinized and centrifuged at 1000 rpm for 5 min to remove the culture medium. Next, the cells were resuspended and diluted to 50,000 cells/ml with complete culture medium. Then, 100 µl of cell solution (5,000 cells in total) was seeded into each well of a 96-well plate and incubated overnight at 37°C. The next day, the cells were infected with Ad-Vpr or Ad-GFP (the latter served as the infection efficiency control and mock control). After that, the culture medium was removed and the cells were washed with phosphate-buffered saline (PBS) and 150 µl of dimethyl sulfoxide (DMSO) was added to each well. The plates were then gently agitated for 15 min and the absorbance at 570 nm was measured using a Synergy2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA). For each infection, three duplicate wells were set up and repeated three times. The data were then summarized as mean ± SD.

DNA content analysis. The cells were harvested at 48 h and 72 h after viral infection for cell cycle and apoptosis analysis. Briefly, after washing cells twice with 2 ml of 5 mM EDTA-PBS and centrifugation at 1,500 rpm, the cells were resuspended in 1 ml of 5 mM EDTA-PBS and fixed with 2.5 ml of cold 70% ethanol (-20°C) containing 10% FBS for 30 min. The fixed cells were then stored overnight at 4°C. The following day, the cells were centrifuged again and washed twice with 2 ml of 5 mM EDTA-PBS through centrifugation. Next, the cells were resuspended in 0.5 ml PBS and then incubated with RNase A (10 µg/ml) at 37°C for 30 min and then at 4°C with propidium iodide (PI, 10 µg/ml) for 1 h. The cells were then filtered and the DNA content of the cells was analyzed using a FACSscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Nuclei to the left of the G1 peak containing hypodiploid DNA were considered apoptotic (Fig. 2C).

Protein extraction and western blotting. HCT-8/5-FU(MDR) cells were infected with Ad-Vpr or Ad-GFP at an MOI of 100 or RPMI-1640 medium (control) for a specific period of time. Total cell lysates were then prepared using an M-PER extraction reagent (Thermo Scientific Co., Ltd., Covington, KY, USA). The protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). After that, the proteins were stored at -80°C until use. For western blot analysis, protein samples (40 µg/lane) were separated using 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto PVDF membranes. Membranes were then incubated with a 5% fat-free milk in Tris-buffered saline containing Tween 20 (TBST) for 1 h at room temperature. Primary antibodies were then applied to the membrane and incubated overnight at 4°C. The next day, after washing three times in TBST, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (at a dilution of 1:5000) for 1 h at room temperature. Membranes were then washed again and an electrochemiluminescence (ECL) kit was used for detection (Thermo Scientific Co., Ltd.).

Electrophoretic mobility shift assay (EMSA). Nuclear protein was first prepared from the cells after viral infection using an NE-PER extraction reagent (Thermo Scientific Co., Ltd.). EMSA was performed using a commercial kit (Viogene Biotech, Inc. Ningbo, China) by incubating 20 µg of nuclear protein with Gel Shift Binding buffer and 2 µl poly (dI-dC) at 4°C for 30 min. After adding the biotin-labeled NF-αB oligonucleotides (5’-AGTTGAGGGGACTTTCCCAGGC-3’), the incubation was continued for 20 min at room temperature. The mixture was separated from free oligonucleotide on 4% polyacrylamide gels containing a 0.25X Tris-borate-EDTA (TBE) buffer. The dried gels were visualized with FluorChem TMIS-8900 (Alpha Innotech, San Leandro, CA, USA), and radioactive bands were quantified with AlphaEase FC software 6.0.2 (Alpha Innotech).
Hoechst staining. The cells were washed twice with PBS and fixed for 20 min at room temperature in 3.7% paraformaldehyde/PBS 72 h after viral infection. Subsequently, the cells were washed twice with PBS and stained for 30 min at 37°C in Hoechst 33258 (1 µg/ml; Sigma Chemical). The nuclear morphology of resuspended cells was observed under a fluorescence microscope (Olympus, Tokyo, Japan). The cells with condensed or fragmented nuclei were considered apoptotic.

Statistical analyses. Data are presented as means ± SD. The differences between the two groups were analyzed by the Student’s unpaired t-test. Multiple comparisons were performed by one-way or two-way analysis of variance (ANOVA), followed by an appropriate post hoc test. Statistical significance was set at p<0.05.

Results

Ad-Vpr infection inhibits viability of HCT-8/5-FU(MDR) cells. In this study, we first determined the effects of Ad-Vpr on the regulation of HCT-8/5-FU(MDR) cell viability. After the cells were infected with Ad-Vpr, the data showed that Ad-Vpr significantly reduced HCT-8/5-FU(MDR) cell viability in a dose- and time-dependent manner (Fig. 1). To investigate whether Vpr preferentially inhibited the growth of rapidly dividing HCT-8/5-FU(MDR) cells, we cultivated these cells in
RPMI-1640 medium supplemented with different concentrations of FBS and infected them with Ad-Vpr or Ad-GFP, and found that Vpr reduced HCT-8/5-FU(MDR) cell viability in a FBS concentration-dependent manner (Fig. 1B) (p<0.01 vs. the control). This suggested that Vpr preferentially inhibited the viability of the rapidly dividing HCT-8/5-FU(MDR) cells. Ad-GFP showed minimal cytotoxicity to HCT-8/5-FU(MDR) cells (Fig. 1A), and the viability of the cells after infection with different MOIs for 5 days was over 95% (Fig. 1A).

Ad-Vpr infection induces cell cycle arrest at G2/M and apoptosis of HCT-8/5-FU(MDR) cells. Next, we determined the cause of the reduced tumor cell viability using a flow cytometer. The tumor cells displayed cytotoxicity by morphology and Hoechst 33258 staining 72 h after infection with Ad-Vpr (Fig. 2A and B). Ad-Vpr induced a marked increase in the percentage of hypodiploid DNA (an indicator of apoptosis) in tumor cells (36.8±4.36%) compared to the control cells (3.32±2.54%, p<0.01) or Ad-GFP infected cells (3.10±2.73%, p<0.01) (Fig. 2C and D).

We also assessed cell cycle distribution in these cells because Vpr-mediated cell cycle arrest appears to be dependent on the cell type (11). We found that 48 h after infection, Ad-Vpr led to a significant increase in the proportion of hyperploid (>4n) cells (39.97±1.88%) compared to the control (10.0±3.24%, p<0.05) or Ad-GFP infected cells (9.66±3.51%, p<0.05) (Fig. 3A and B). To confirm this result, we analyzed the expression of Cyclin B1 protein in these Ad-Vpr-infected cells using western blot analysis (Cyclin B1 is the protein that controls the G2 phase of a cell cycle). There was a significant increase of Cyclin B1 protein levels in the cells after Ad-Vpr infection (Fig. 3C), suggesting that Vpr protein plays a role in G2 phase cell cycle arrest of HCT-8/5-FU(MDR) cells.

Ad-Vpr infection regulates expression of apoptosis-related genes in HCT-8/5-FU(MDR) cells. Subsequently, we confirmed Vpr-induced apoptosis in HCT-8/5-FU(MDR) cells by analyzing the expression of Bcl-2 family proteins and release of mitochondrial cytochrome c in HCT-8/5-FU(MDR) cells. The cells were grown and infected with Ad-Vpr or Ad-GFP (100 MOI each) for 48 h and subjected to western blot analysis for Bcl-2, Bcl-xl, Bax, and cytochrome c. β-actin or VDAC were used as a loading control.

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by investigating the activation of various caspases in Ad-Vpr infected cells. We discovered that Ad-Vpr induced the activation of caspase-3 and -9, but not of caspase-8, as revealed by western blot analysis (Fig. 4). Ad-Vpr infection also induced proteolytic cleavage of PARP, a known substrate of caspase-3. Changes in caspase-3 and -9 activation and PARP cleavage in control or Ad-GFP infected cell lines were not detected (Fig. 4).

Next, we determined the underlying mechanisms responsible for caspase-3 and -9 activation. It is evident that the Bcl-2 family of proteins includes the best characterized regulators of apoptosis- anti-apoptotic members, such as Bcl-2 and Bcl-xl, and pro-apoptotic members, including Bax and various BH3-only proteins (21). We assessed the effects of Ad-Vpr on the protein levels of Bcl-xl and Bcl-2 in HCT-8/5-FU(MDR) cells. Ad-Vpr suppressed the expression of Bcl-xl but did not alter the expression of Bcl-2 compared to the controls (Fig. 5). By contrast, Ad-Vpr infection induced a time-dependent increase in Bax protein expression and cytochrome c release from the mitochondria into the cell cytoplasm (Fig. 5). The data indicate that Ad-Vpr-induced apoptosis occurred by an activation of the mitochondrial pathway via Bax translocation and mitochondrial release of cytochrome c in HCT-8/5-FU(MDR) cells.

**Ad-Vpr inhibits NF-κB activation in HCT-8/5-FU(MDR) cells.** NF-κB signaling plays a role in colorectal cancer development and progression, and its suppression may represent a potential therapeutic strategy. We therefore investigated whether Ad-Vpr is able to modulate NF-κB signaling. As shown in Fig. 6, HCT-8/5-FU(MDR) cells infected with Ad-Vpr resulted in a time-dependent decrease in nuclear translocation of NF-κB/p65 protein and p65 DNA-binding activity (p<0.05 vs. the mock control), with a concomitant increase in IκBα in the cell cytoplasm.

**Discussion**

Acquired and de novo resistance of cancer cells to chemotherapy remains a central problem in colorectal cancer treatment. Consequently, this restricts the successful control of advanced disease for a potential cure or survival of patients. The dose escalation necessary to overcome such a drug resistance will significantly increase severe toxicity to normal cells. Exploration of novel agents with great efficacy for refractory colorectal cancer is urgently required. In this study, we demonstrated the usefulness of Ad-Vpr in inhibition of colorectal cancer cell viability, further validating previous in vivo and in vitro studies on different human cancer (6-9).

The advantage of Ad-Vpr is that Vpr is a 96 amino acid 14-kDa protein and induces G2 cell cycle arrest and apoptosis in proliferating cells (6-9). Our current study also confirmed the effects of Vpr in multidrug-resistant colorectal cancer cells.

Apoptosis is a homeostatic process utilized by multicellular organisms to eliminate cells without disturbing the corresponding architecture or function. Two major apoptotic pathways exploited by cells were discovered as an extrinsic or intrinsic pathway (31) by apical activation of caspase-8 or caspase-9, respectively. Early observations in various cell types showed that Vpr induced apoptosis either via the extrinsic pathway or intrinsic pathway (32-35). Cell type-specific variations and context may account for these discrepancies (36). In the current study, Ad-Vpr infection induced mitochondrial release of cytochrome c and a time-dependent proteolytic cleavage of PARP.
caspase-3 and -9, but not of caspase-8, in HCT-8/5-FU(MDR) cells. Vpr also induced a time-dependent proteolytic cleavage of PARP, a substrate of caspase-3. Collectively, these findings indicate that Vpr induced apoptosis in HCT-8/5-FU(MDR) cells through the intrinsic apoptosis pathway. The intrinsic apoptosis pathway is regulated by the Bcl-2 family proteins, which comprise both pro-apoptotic and anti-apoptotic proteins (21). Vpr perturbed the balance of pro- and anti-apoptotic Bcl-2 family proteins by repressing the expression of Bcl-xl, by upregulating expression and promoting translocation of Bax in HCT-8/5-FU(MDR) cells.

Nevertheless, many agents and genes are able to mediate cell apoptosis. The NF-κB protein complex is considered an anti-apoptotic protein. Activated NF-κB protein will undergo nuclear translocation, and in turn enhance its DNA-binding activity and transcription of a variety of downstream genes for cell proliferation, invasion, angiogenesis, metastasis, and suppression of apoptosis and chemoresistance in multiple tumors (28-30). Thus, NF-κB is increasingly considered as a target for anti-cancer drug treatment (23-27). Many studies have demonstrated that Vpr was able to block NF-κB activation (37,38), whereas others reported that Vpr may also activate NF-κB and protect cells from apoptosis (18,39). However, in the current study, we further confirmed that Ad-Vpr was able to inhibit NF-κB activation in a time-dependent manner through the upregulation of IκBα in the cytoplasm, and inhibition of nuclear translocation of p65 and DNA-binding activity of p65. We believe that suppression of NF-κB mediated by Ad-Vpr correlated with its ability to regulate cell cycle G2/M arrest in HCT-8/5-FU(MDR) cells. Blockage of NF-κB signaling can delay mitotic entry and keep cells at the G2 phase (40). Based on previous studies, protein phosphatase 2A (PP2A), a serine/threonine phosphatase, could play an essential role in G2 cell cycle arrest induced by Vpr (15,41). PP2A can dephosphorylate and modulate the activity of IκKβ (42), one of two catalytic subunits of IKK. Dephosphorylated IκKβ protein can reduce the degradation of IκBα and eventually inhibit the nuclear translocation and activation of NF-κB (42). The data suggests that the Vpr-inhibited NF-κB activity may occur in part through a PP2A-mediated mechanism and eventually promote progression of cell cycle G2/M arrest. The elucidation of the exact underlying signaling mechanisms is certainly required.

Collectively, this study demonstrated that Ad-Vpr was able to significantly inhibit proliferation of refractory colorectal cancer HCT-8/5-FU(MDR) cells and that the reduced tumor cell viability was due to the induction of apoptosis and G2 cell cycle arrest. Mechanistically, Ad-Vpr suppressed NF-κB activity and inhibited Cyclin B1 and Bcl-xl expression, but up-regulated Bax expression in HCT-8/5-FU(MDR) cells. According to the data presented herein, Ad-Vpr could become a particularly useful anticancer agent against colorectal cancer, especially refractory colorectal cancer.

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