Reovirus Oncolysis - Role of cyclin-dependent kinase inhibitor 1

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Received: March 24, 2015
Published online: May 13, 2015

New therapeutic interventions are essential for improved cancer management. Over the past decade, reovirus, a naturally-occurring oncolytic double-stranded RNA virus harboring an intrinsic preference to destroy mutant KRAS driven tumors, has shown novel promissory potential. The fact that more than 30-40% of human tumors harbor KRAS mutations had previously guided us to investigate the efficacy of reovirus in KRAS mutant colon cancer cells (CRC), and also to examine the cellular consequences of this biologic when used in combination with the chemotherapeutic drug, irinotecan, a Topoisomerase I inhibitor. Although much research is being undertaken to improve the drug efficacy by using in combination with other chemotherapeutic drugs, the plausible contribution of underlying signaling mechanisms which includes p21, a cyclin-dependent kinase inhibitor 1, in virus recognition and dissemination remains largely unexplored. This research highlight provides an insight into our current work to elucidate the precise role of p21 in favoring viral propagation in KRAS mutated CRC cells and in the process identify unique molecular players that can be effectively harnessed for improvement of the therapeutic efficacy of reovirus.

cells still remains elusive.

The Dearing strain of reovirus serotype 3 (ReoT3D) is a non-engineered wild type reovirus strain with innate ability to kill KRAS transformed cells [6]. Several studies have attempted to elucidate the precise molecular mechanism of reoviral oncolysis with little success. It has been reported that reoviral cytotoxicity is beta interferon independent and is enhanced by interferon regulatory factor 3 and NF-kB-dependent expression of Noxa, a protein that promotes activation of caspases and apoptosis [7]. Activation of caspase 3 has also been reported to be necessary for development of reovirus induced encephalitis [8]. Our own study conducted with KRAS activated colorectal carcinoma cell lines clearly indicated enhanced cytotoxicity upon reovirus infection as compared to KRAS wild type isogenic cells [9]. Studies involving TUNEL staining and flow cytometry, revealed that reovirus preferentially induced apoptosis in KRAS mutant HCT116 CRC cells compared to its isogenic derivative Hke3. At the molecular level, reovirus showed a greater degree of Caspase 3 activation with PARP1 cleavage and preferential inhibition of p21 protein expression in HCT116 cells when compared to Hke3 cells [9].

Reovirus engage cells by binding to cell-surface carbohydrates along with the immunoglobulin superfamily member, junctional adhesion molecule-A (JAM-A). Following attachment, reovirus internalization is promoted by β1 integrins, most likely via clathrin endocytosis [10]. Although the mechanism of reovirus entry to the host cells has been scientifically elucidated, its recognition and downstream interactions with cell cycle regulators is poorly understood.

Thorough analysis showed that activated RAS signaling plays an integral role in reovirus infectivity [6, 11]. Cell lines expressing constitutively active KRAS were found to be permissive to reovirus replication [12]. Specifically, when activated KRAS was placed under a Zinc-inducible promoter by transformation [12], a productive replication by reovirus occurred rapidly following induction of expression of activated RAS suggesting the activation of RAS signaling itself rather that the secondary consequences was essential to permit reovirus replication [11]. Furthermore, the association of double stranded RNA dependent protein kinase (PKR) and effective reoviral replication has been well established [13]. PKR dimerization, autophosphorylation and activation upon binding to dsRNA are the critical steps towards prohibiting viral translation initiation. Specific chemical inhibitors of PKR phosphorylation allows the usurpation of reovirus translation [13].

In our previous studies, we demonstrate preferential reoviral cytopathy in KRAS mutant colorectal cancer cell lines. Our study is consistent with several previous reports [12-14] that demonstrate that reovirus preferentially induces cell lysis in KRAS mutant cells. The increased sensitivity of KRAS mutant HCT116 cells to reovirus was associated with a pronounced decrease in the percentage of cells in S phase, a significant G2M arrest, an increased release of LDH and induction of apoptosis with activation of Caspase 3 followed by a robust suppression of p21 expression [9].

KRAS being a cell membrane bound signaling molecule with intrinsic GTPase activity can generate aberrant cell signals when mutated, a property that is efficiently utilized by reovirus for the preferential induction of oncolysis [11]. When studied in nonmalignant mouse intestinal epithelial cells (IEC-iRAS) in which mutant KRAS can be selectively expressed by IPTG, we found that reovirus preferentially induces cell lysis even in non-malignant environment. Interestingly, this effect was only evident under serum free conditions indicating the presence of an exogenous factor or factors dampen the influence of KRAS mutation. Evidently, the identification of this factor or factors that can compensate for mutant KRAS may provide valuable insight into how response to reovirus is determined [9].

It has been reported that Caspase-3 activation is required for reovirus induced encephalitis in vivo. In the study, caspase-3 (-/-) mice showed lesser degree of tissue damage with better survival [8] Our findings clearly demonstrate that reovirus activates procaspase-3 in KRAS mutant HCT116 cells. One of the primary functions of Caspase-3 is the cleavage of DNA repair enzyme PARP1. Inactivation of PARP1 inhibits the cells ability to repair the damaged DNA, committing the cells towards programmed cell death/apoptotic pathway.

Human proliferating cell nuclear antigen (PCNA) is a protein involved in several DNA processes [15]. PCNA is a ring shaped homo-trimeric protein that functions on DNA as a clamping platform to recruit proteins involved in DNA metabolism. Interaction between PARP1 and PCNA has been demonstrated by in vitro co-immunoprecipitation assays [15]. It has been speculated from this study that the association of PCNA with PARP1 occurs at the same region which is involved in the interaction of PCNA with polymerase δ. Interestingly, the region of the inter domain connector loop of PCNA that is involved in the association with polymerase δ is identical to the sequence interacting with the C-terminus of p21[16]. This feature enables p21 to compete with DNA polymerase δ to regulate the differential inhibition of DNA replication vs. DNA repair. During DNA repair, the multiple interactions established by PCNA could be regulated by p21 in a disassembly and or recycling process of PCNA.
molecules at the repair sites \cite{17}.

The finding that PARP1 associates with both PCNA and p21 suggested a possible cooperation of PARP1 and p21 in regulating the functions of PCNA during DNA replication/repair \cite{15}. Our findings revealed cleavage of PARP-1 by Caspase-3 which is then followed by down regulation of the expression of p21 in reovirus treated \textit{KRAS} mutant CRC cells \cite{9}. We hypothesize that upon activation of Caspase-3 and subsequent cleavage of full length PARP-1, the PCNA mediated DNA repair complex involving PARP-1 and p21 and other associated protein complex is disrupted leading to the rapid turnover and down regulation of p21 expression as observed in our study\cite{9}.

It is well established that p21 can protect cells from stress-induced apoptosis \cite{18}. Hence, down regulation of the expression of p21 would thus further promote apoptosis. Cell cycle arrest is performed by p21 not only through CDK inhibition, but also by direct binding to PCNA, thereby interfering with PCNA-dependent DNA synthesis \cite{19}. The dual effect on cell cycle regulatory proteins is mediated in the p21 sequence via distinct interaction sites for cyclin-Cdk complexes (n-terminal residue 45-60) and for PCNA (c-terminal 142-163) \cite{19}.

Although a major effect of p21 is considered to be exerted during G1 phase of the cell cycle, p21 gene knock-out studies suggested its involvement in G2/M checkpoint as well \cite{20}. Our flow cytometry analysis showed a clear G2/M arrest with prominent S-phase ablation upon virus treatment, the effect being significantly pronounced in cells with mutant \textit{KRAS} \cite{9}.

Furthermore, it has been reported that PARP1 interacts with several proteins involved in DNA metabolism, including DNA topoisomerase I \cite{21}. In this aspect, it has been shown that poly(ADP-ribosylation) by PARP1 regulates the catalytic activity of topoisomerase I \cite{22} \cite{23}. We showed that irinotecan, a topoisomerase 1 inhibitor, synergizes with reovirus. The cleavage of PARP1 through reovirus treatment and inhibition of topoisomerase I by irinotecan irreversibly affects the cellular DNA metabolism causing an enhanced cytotoxicity. In the double drug combination scenario, p21 cannot be induced to the extent as seen by single agent treatment as the whole PCNA complex is disrupted and destroyed by reovirus leaving no scope for irinotecan to induce p21 rather only inhibit topoisomerase 1 and thus making the cells more vulnerable to cell death.

The cyclin-dependent kinase (CDK) inhibitor p21 can mediate both p53-dependent and independent cell cycle arrest \cite{24}. Cell cycle arrest permits cells to pause, repair damage and then to continue cell division. The function of p21 to inhibit cell proliferation may contribute to its ability to act as tumor suppressor. On the other hand, the capacity of p21 to induce cell cycle arrest following cell stress may protect cells from stress-induced apoptosis \cite{18}. As a proliferation inhibitor, p21 is therefore poised to play an important role in preventing tumor development \cite{18}. Our experiments demonstrated marked repression of p21 following viral infection selectively in \textit{KRAS} mutant colon cancer cells. Furthermore, the p21 protein can also interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair \cite{15}. Absence of p21 protein would evidently affect the S-phase, a phenomenon clearly seen by flow cytometry analysis of HCT116 as compared to Hke3. It has also been reported that p21 does not induce cell death on its own \cite{25}. The protein is specifically cleaved by Caspase-3-like Caspases, and may be instrumental in the execution of apoptosis following Caspase activation \cite{18}. Our current study shows that viral oncolysis is achieved by Caspase 3 mediated apoptosis under \textit{KRAS} mutated conditions.

Apoptosis is a crucial mechanism by which multi-cellular organisms control cell numbers and ensure the removal of damaged or potentially harmful cells \cite{26}. Our flow cytometry analysis also supported by TUNEL staining demonstrated that reovirus stimulates apoptosis more prominently in \textit{KRAS} mutant cells. The exact mechanism by which the \textit{KRAS} mutation favors enhanced virus induced apoptosis is yet to be determined. What is clear however, is that an early onset of apoptosis along with processing of procaspase 3 and significant suppression of p21 expression in HCT116 cells as compared to Hke3. The cell cycle analysis showed marked ablation of the S phase upon viral treatment in the \textit{KRAS} mutated HCT116. This observation along with simultaneous processing of procaspase 3 detected by Western blotting provides evidence that the virus infected cells are triggered for apoptotic dissemination. This phenomenon can be further harnessed as a therapeutic approach towards \textit{KRAS} mutated CRC patients.

Drugs that produce overtly similar effects are often given in combination \cite{27}. These include drugs for treating many disease conditions and certainly almost all cancers where chemotherapy is used with radiation or other antimicrobial therapy for better patient outcome. There is an increasing awareness that the use of synergistically acting drug combinations for treating cancer can lower the doses of individual drugs and consequently lower adverse effects \cite{28}. This principle prompted us to determine whether reovirus therapy could act synergistically with a viable FDA approved chemotherapy. Irinotecan is a topoisomerase I inhibitor
which eventually leads to inhibition of both DNA replication and transcription [29]. It is often the only regimen available to patients with KRAS mutant tumors once they progress on frontline 5FU/platinum based therapy. We intended to find a synergistic correlation between inhibition of p21 expression and enhanced apoptosis by the action of irinotecan in inhibiting DNA replication. To understand better, we investigated the effect of combination treatment of reovirus and irinotecan in 13 CRC cell lines with both KRAS mutated and KRAS WT background. Synergism was observed in all but one cell line. Importantly, we observed a greater synergy as per the combination index at ED50 and ED75 in the KRAS mutated HCT116 cells as compared to the KRAS WT Hke3 cells.

The fact that we are unable to observe increased levels of cleaved caspase 3 and PARP1 by Western blotting in samples treated with irinotecan single agent helps us to conclude that this topoisomerase I inhibitor does not directly activate the Caspase 3 mediated extrinsic apoptotic pathway but disrupts the DNA repair complex formation and thus renders the cells more vulnerable to the apoptosis inducing agents like reovirus. Consequently, the combination group reveals significantly increased apoptosis by flow cytometry analysis although no activation of apoptotic markers is observed by Western blotting method. We justify the observation by speculating that the combinatorial treatment renders the cellular nucleic acids so extensively damaged that the apoptotic markers are completely turned over and although apoptosis was followed by propidium iodide staining of the damaged DNA the associated proteins cannot be detected by specific antibodies in Western blot analysis.

Our current research endeavor focuses on identifying the molecular players and critical virus mediated signals that might play a crucial role in the suppression of p21 towards favoring unabated viral propagation in KRAS mutated environment. Such investigation of the molecular mechanism of selective viral oncolysis in KRAS tumor cells and the cross talk between mutant KRAS and cyclin-dependent kinase inhibitor / DNA repair complexes will help unravel the intricate cellular pathways that co-operatively contribute to cell transformation. This in turn will not only help in identifying novel targets for cancer therapy but also shed light on the particular cancer backgrounds that might be compatible with viral oncolysis strategies. Reovirus thus has immense potential not only as a therapy but also a tool to unravel the aberrant cellular pathway leading to carcinogenicity.

**Conflicting interests**

The authors have declared that no competing interests exist.

**References**


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