Parainfluenza Virus Infection Sensitizes Cancer Cells to DNA-Damaging Agents: Implications for Oncolytic Virus Therapy

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ABSTRACT A parainfluenza virus 5 (PIV5) with mutations in the P/V gene (P/V-CPI⁻) is restricted for spread in normal cells but not in cancer cells *in vitro* and is effective at reducing tumor burdens in mouse model systems. Here we show that P/V-CPI⁻ infection of HEp-2 human laryngeal cancer cells results in the majority of the cells dying, but unexpectedly, over time, there is an emergence of a population of cells that survive as P/V-CPI⁻ persistently infected (PI) cells. P/V-CPI⁻ PI cells had elevated levels of basal caspase activation, and viability was highly dependent on the activity of cellular inhibitor-of-apoptosis proteins (IAPs) such as Survivin and XIAP. In challenge experiments with external inducers of apoptosis, PI cells were more sensitive to cisplatin-induced DNA damage and cell death. This increased cisplatin sensitivity correlated with defects in DNA damage signaling pathways such as phosphorylation of Chk1 and translocation of damage-specific DNA binding protein 1 (DDB1) to the nucleus. Cisplatin-induced killing of PI cells was sensitive to the inhibition of wild-type (WT) p53-inducible protein 1 (WIP1), a phosphatase which acts to terminate DNA damage signaling pathways. A similar sensitivity to cisplatin was seen with cells during acute infection with P/V-CPI⁻ as well as during acute infections with WT PIV5 and the related virus human parainfluenza virus type 2 (hPIV2). Our results have general implications for the design of safer paramyxovirus-based vectors that cannot establish PI as well as the potential for combining chemotherapy with oncolytic RNA virus vectors.

IMPORTANCE There is intense interest in developing oncolytic viral vectors with increased potency against cancer cells, particularly those cancer cells that have gained resistance to chemotherapies. We have found that infection with cytoplasmically replicating parainfluenza virus can result in increases in the killing of cancer cells by agents that induce DNA damage, and this is linked to alterations to DNA damage signaling pathways that balance cell survival versus death. Our results have general implications for the design of safer paramyxovirus-based vectors that cannot establish persistent infection, the repurposing of drugs that target cellular IAPs as antivirals, and the combined use of DNA-damaging chemotherapy agents in conjunction with oncolytic RNA virus vectors.

KEYWORDS DNA damage, oncolytic viruses, parainfluenza virus

There is intense interest in developing oncolytic viral vectors with increased potency against cancer cells. As examples, a modified herpes simplex virus 1 strain designated talimogene laherparepvec (T-VEC) (Imlygic; Amgen) is the first oncolytic virus approved for human use by the Food and Drug Administration (1), and there has been a large increase in the number of clinical trials for new viruses for tumor therapy (2, 3). A number of paramyxoviruses have shown promise as oncolytic vectors based in part on their inherent cytopathic properties, including measles virus (MV), Newcastle disease virus (NDV), Sendai virus, and mumps virus (MuV) (4–10). The goal of the work described here was to understand how cancer cells infected with a cytopathic parain-

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fluenza virus oncolytic vector respond to external inducers of apoptosis such as cisplatin.

Wild-type (WT) parainfluenza virus 5 (PIV5) has an unusual property among paramyxoviruses of being a poor inducer of host cell responses in most human epithelial and fibroblast cell types (11, 12). This property is related in part to the active evasion of host cell responses. One such evasion mechanism is through the viral V protein hijacking the host protein damage-specific DNA binding protein 1 (DDB1). In virus-infected cells, DDB1 becomes part of a cytoplasmic "V degradation complex," which induces the degradation of signal transducer and activator of transcription 1 (STAT1) or STAT2, resulting in the inhibition of type I interferon (IFN) signaling (13–15). The PIV5 V protein targets the degradation of STAT1, whereas the related virus human parainfluenza virus type 2 (hPIV2) targets STAT2 degradation (14–17).

For reasons that are not fully understood, WT PIV5 is also largely noncytopathic to most cell types *in vitro*. Interestingly, alterations to the PIV5 P/V gene can convert the noncytopathic WT virus into a highly cytopathic mutant (P/V-CPI[−]) (12, 18–22). The PIV5 P/V gene encodes phosphoprotein P and accessory protein V, which share an identical 164-residue amino-terminal domain (the shared P/V region) but have unique C-terminal domains. The P protein is an essential subunit of the viral RNA-dependent RNA polymerase (23). The V protein is thought to function in the regulation of viral RNA synthesis (24) but also has additional roles in blocking IFN signaling (14, 15), disrupting apoptosis pathways (12), and inhibiting IFN- β gene expression through binding to mda-5 (25). In the case of the P/V-CPI[−] mutant, the introduction of six amino acid changes in the shared P/V region (listed in Materials and Methods) results in a mutant with properties very different from those of WT PIV5, including the activation of IFN and proinflammatory cytokine responses (18, 26), the overexpression of viral RNA and proteins (18, 27), and the induction of massively cytopathic effects in most cancer cell lines tested so far (18, 21, 22).

The PIV5 P/V-CPI[−] mutant is currently being developed as a novel oncolytic vector. This mutant is restricted for growth in normal cells but is fully capable of growth and spread through a population of tumor cells *in vitro* (19, 28). These differences in cell type restriction have been proposed to be due in part to effective IFN responses following P/V-CPI[−] infection of normal cells but not cancer cells (28). During the transformation process, tumor cells can accumulate specific defects in IFN pathways that contribute to resistance to the antiproliferative effects of IFN (e.g., see references 29 and 30). It has been proposed that these alterations can also confer increased susceptibility to viral infection (3, 31), particularly in the case of mutants such as P/V-CPI[−], which is defective in blocking IFN.

The mechanism of cell killing by the P/V-CPI[−] mutant is not completely understood at this time; however, it could be tied mechanistically to the high-level induction of double-stranded RNA (dsRNA) during replication and the shutoff of host and viral protein synthesis through protein kinase R (PKR) pathways (22). Similarly, cell killing by the P/V-CPI[−] mutant involves the activation of caspase-dependent death pathways (32, 33). Importantly, we have also shown that the P/V-CPI[−] mutant is very effective at reducing prostate cancer tumor burdens *in vivo* in a mouse model system (28).

Here we show that P/V-CPI[−] infection of HEp-2 human laryngeal cancer cells results in the majority of the cells dying, but unexpectedly, over time, there is an emergence of a population of cells that survive as persistently infected (PI) cells. While testing the hypothesis that PI cells have altered apoptotic pathways, we found that PI cells and cells acutely infected with the P/V-CPI[−] virus show enhanced DNA damage and cell death induced by chemotherapy agents such as cisplatin. Our results have general implications for the design of safer paramyxovirus-based vectors that cannot establish persistent infection as well as the potential for combining chemotherapy with oncolytic RNA virus vectors.

FIG 1 Persistent infection with a cytopathic oncolytic virus. (A) Naive HEp-2 cells were mock infected or infected with rPIV5-WT or the P/V-CPI⁻ mutant at an MOI of 10 PFU/cell. Cell viability was determined by an MTT assay at the indicated hours postinfection. Values are the means for three samples, with *** indicating a *P* value of <0.001 comparing WT and P/V-CPI⁻ infections. (B) Cells that survived P/V mutant infection were examined by fluorescence microscopy for GFP expression. (C) Lysates from naive, PI, and acutely P/V-CPI⁻-infected cells (24 hpi) were analyzed by Western blotting for the indicated viral proteins. (D) Titers of virus released from PI HEp-2 cells or after 24 h of acute infection with P/V-CPI⁻ were determined. *** indicates a *P* value of <0.001.

RESULTS

The cytopathic PIV5 P/V-CPI⁻ mutant is capable of establishing persistent infection. The PIV5 P/V-CPI⁻ mutant is highly cytopathic to a number of cancer cell lines (18, 21, 28). This is illustrated in Fig. 1A, where HEp-2 human laryngeal cancer cells were mock infected or infected with WT PIV5 or the P/V-CPI⁻ mutant. Cell viability was monitored over 72 h postinfection (hpi) by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays. In contrast to WT PIV5, the P/V-CPI⁻ mutant induced ~70% cell death at 48 and 72 hpi. Unexpectedly, at these later time points, a substantial percentage of viability remained. Cells that survived this persistent infection could be passaged and retained green fluorescent protein (GFP) expression (Fig. 1B). Western blotting confirmed that PI cells expressed all viral proteins examined so far (Fig. 1C). PI cells produced infectious virions (Fig. 1D) albeit with a ~3-log-lower yield in PFU per milliliter than with acute P/V-CPI⁻ infection. These data indicate that in some cell types, the cytopathic P/V-CPI⁻ mutant can establish a PI cell line that still expresses viral proteins and produces virus.

PI cells have elevated basal levels of apoptotic markers and are highly dependent on inhibitors of apoptosis for survival. The above-described findings raised the question of whether PI cells had downregulated apoptotic pathways in order to survive P/V-CPI⁻ infection. To determine the levels of steady-state stress, naive and PI HEp-2 cells were examined for cell viability and apoptotic effector proteins. As shown in Fig. 2A (top), ~10 and ~20% of PI cells stained positive for annexin V and propidium iodide under steady-state conditions, respectively, compared to 2 to 3% of naive HEp-2 cells. The levels of steady-state propidium iodide staining of PI cells varied between experiments but were always in the range of ~10 to 20%, a variability which may reflect differences in cell passage number, confluence, or time after plating or other differences in cell culture conditions. In addition, a higher fraction of PI cells showed cleaved caspase 3 and active caspase 3/7 than in naive cells (Fig. 2A, bottom, and B). PI cells and

FIG 2 PI cells have elevated basal levels of apoptotic markers and are highly dependent on inhibitors of apoptosis for survival. (A) Naive HEP-2 and PI HEP-2 cells were stained with annexin V and propidium iodide or with antibodies to cleaved caspase 3 or active caspase 3/7 before analysis by flow cytometry. Values are the means for three samples, with *** indicating a *P* value of <0.001. (B and C) Lysates from naive HEP-2 cells, PI cells, or acutely P/V-CPI⁻-infected cells were analyzed by Western blotting for the indicated apoptotic proteins (B) or for cellular inhibitors of apoptosis (C). (D and E) Naive and PI HEP-2 cells were treated with the Survivin inhibitor YM155 (D) or with the XIAP inhibitor Embelin (E) for 24 h. Cells were stained with propidium iodide before analysis by flow cytometry. Values are the means for three samples, with ** and *** indicating *P* values of <0.01 and <0.001, respectively.

acutely P/V-CPI⁻-infected cells showed similar levels of cleaved caspase 3 (Fig. 2B). In contrast, PI cells did not contain substantially higher levels of downstream apoptotic markers such as cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 2B). Together, these data suggest that PI cells have high basal levels of cell stress, indicated by caspase activation; however, this stress does not lead to substantial levels of cell death.

Cellular inhibitor-of-apoptosis proteins (IAPs) have been found to have distinct mechanisms of action to block apoptosis. For example, XIAP can directly bind and inhibit cleaved caspases 3, 7, and 9 from activating their substrates, whereas Survivin binds to and inhibits cleaved caspases 3 and 7 (34, 35). Our finding of high levels of caspase activation and low levels of cell death in PI cells suggested that IAPs may be important for survival of P/V-CPI⁻ infection. To test this hypothesis, levels of IAPs were determined by Western blotting. As shown in Fig. 2C, levels of c-IAP1, Survivin, and XIAP expression in PI cells were very similar to those in naive HEP-2 cells. In contrast, acute infection with P/V-CPI⁻ led to reduced levels of all three of these IAPs, a result which may be due to PKR activation and a global reduction of protein synthesis (22).

To determine the functional importance of IAPs in PI cell survival, naive and PI cells were treated with increasing concentrations of a Survivin inhibitor (Fig. 2D) or an XIAP inhibitor (Fig. 2E) for 24 h before determining viability by propidium iodide staining.

FIG 3 PI cells have increased sensitivity to DNA-damaging agents. (A and B) Naive and PI HEP-2 cells were treated with the indicated concentrations of cisplatin for 24 h (A) or with 100 μ M cisplatin for the indicated times (B). The remaining cell viability was determined by annexin V and propidium iodide staining followed by flow cytometry. Values are the means for three samples, with *, **, and *** indicating *P* values of <0.05, <0.01, and <0.001, respectively. (C) Naive and PI HEP-2 cells were treated with 75 μ M cisplatin for 24 h, and lysates were analyzed by Western blotting for levels of caspase 3 and PARP.

The viability of PI cells was sensitive to IAP inhibition compared to naive HEP-2 cells, suggesting that PI cells are highly dependent on IAPs in order to survive the high levels of cell stress under steady-state conditions.

PI cells have increased sensitivity to DNA-damaging agents. Given the survival of PI cells harboring the cytopathic P/V-CPI⁻ mutant and the increased sensitivity of PI cells to IAP inhibitors, we hypothesized that PI cells have increased resistance to inducers of cell death. To test this, naive and PI cells were challenged with the DNA-damaging agent cisplatin, and cell viability was measured by annexin V and propidium iodide staining. As shown in Fig. 3, annexin V staining of PI cells showed enhanced sensitivity to cisplatin compared to that of naive cells, and this sensitivity was both dose dependent (Fig. 3A) and time dependent (Fig. 3B). This was also observed with propidium iodide staining, whereby 24 h of cisplatin treatment resulted in about 60% propidium iodide-positive PI cells, compared to ~10% of naive cells (Fig. 3B). Although P/V-CPI⁻ is defective at blocking IFN signaling, IFN treatment of naive Hep2 cells did not alter sensitivity to cisplatin (data not shown).

Western blotting was carried out to confirm that cisplatin activated apoptotic pathways in PI cells. As shown in Fig. 3C, cisplatin treatment of PI cells resulted in higher levels of cleaved caspase 3 and cleaved PARP than those detected in untreated PI cells. Higher levels of cleaved PARP were also detected in cisplatin-treated PI cells than in naive cisplatin-treated cells (Fig. 3C). Levels of cleaved caspase and PARP were similar in PI and naive cells. Taken together, these data indicate that PI cells are more sensitive to cisplatin-induced cell death than are naive cells.

To test whether cisplatin treatment resulted in more DNA damage in PI cells, naive and PI cells were treated with increasing concentrations of cisplatin, and *in situ* terminal

FIG 4 PI cells have enhanced DNA damage following cisplatin treatment. Naïve (A) and PI (B) HEp-2 cells were treated with the indicated concentrations of cisplatin for 18 h. Alternatively, cells were treated with DNase as a positive control (bottom). All samples were also treated with DAPI to visualize DNA. Cells were analyzed by an *in situ* TUNEL assay to determine levels of DNA fragmentation.

deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were used to visualize DNA fragmentation by microscopy. As shown in Fig. 4A (left), naïve cells did not show substantial DNA damage until treatment with 10 μ M cisplatin. In contrast, treatment of PI cells with the lowest tested cisplatin concentration (2 μ M) resulted in the majority of cells staining TUNEL positive (Fig. 4B). These data indicate that PI cells have enhanced sensitivity to cisplatin-induced cell death and DNA damage.

PI cells have altered DNA damage repair pathways. DNA adducts induced by cisplatin are mainly recognized by the nucleotide excision repair (NER) pathway (36), which can lead to cell cycle arrest to efficiently repair the DNA or can lead to cell death based on the extent of DNA damage. To examine DNA damage repair pathways, naïve and PI cells were treated with and without cisplatin and examined by Western blotting for the phosphorylation of upstream sensors of damaged DNA, such as the ataxia telangiectasia- and Rad3-related (ATR) protein and repair mediator protein gamma-histone H2A.X. As shown in Fig. 5A, both naïve and PI cells responded to cisplatin treatment in similar fashions, as evidenced by similar levels of phosphorylation of ATR. The level of phosphorylation of H2A.X was slightly lower in PI cells treated with cisplatin than in naïve cells treated with cisplatin.

Downstream of H2A.X, checkpoint kinase 1 (Chk1) and Chk2 are two DNA repair effector proteins, which, when activated by phosphorylation, can lead to cell cycle arrest and the repair of damaged DNA (37). As shown in Fig. 5B, phosphorylation of Chk1 at Ser317 and Ser345 was detected in naïve cells treated with cisplatin, whereas phosphorylation at these sites was not detected in PI cells treated with cisplatin. Similarly, phosphorylation of Chk2 at Ser516 was detected in naïve cisplatin-treated cells but was greatly diminished in PI cells (Fig. 5C). Taken together, these data indicate that DNA damage is sensed in PI cells, as evidenced by the phosphorylation of H2A.X; however, PI cells are altered in downstream Chk1 and Chk2 signaling, resulting in a defect in DNA repair.

Wild-type p53-inducible protein 1 (WIP1) is a stress-responsive phosphatase that acts as a negative-feedback loop to terminate the DNA repair pathways (38) through dephosphorylation of signaling proteins, including Chk1 and Chk2 (39–42). As shown in

FIG 5 PI cells have altered DNA repair pathways. (A to D) Naïve and PI HEP-2 cells were treated with 75 μ M cisplatin for 24 h, and lysates were analyzed by Western blotting for the DNA damage repair sensors ATR and H2A.X (A), the effector proteins Chk1 (B) and Chk2 (C), or p53 and WIP1 (D). (E) PI HEP-2 cells were treated with or without a WIP1 inhibitor for 24 h, followed by an additional 24 h of cisplatin treatment with or without the WIP1 inhibitor before analysis by flow cytometry. Values are the means for three samples, with ** and *** indicating *P* values of <0.01 and 0.001, respectively. (F) PI HEP-2 cells were treated with or without a WIP1 inhibitor for 24 h, followed by an additional 24 h of cisplatin treatment with or without the WIP1 inhibitor, and lysates were analyzed by Western blotting for the DNA damage repair effector protein Chk1.

the Western blot in Fig. 5D, levels of p53 and WIP1 were elevated in cisplatin-treated PI cells compared to those in naïve cells. This finding raised the hypothesis that WIP1 activity against DNA repair proteins contributed to the enhanced sensitivity of PI cells to cisplatin. A prediction of this hypothesis is that WIP1 inhibition should result in decreased cell killing by cisplatin treatment. To test this, PI cells were pretreated with a WIP1 inhibitor, followed by treatment with increasing concentrations of cisplatin and cell viability assays. As shown in Fig. 5E, treatment of PI cells with 125 μ M cisplatin resulted in ~45% of the population being positive for propidium iodide staining. In the presence of the WIP1 inhibitor, cisplatin treatment resulted in ~25% of cells staining positive for propidium iodide. Consistent with a decrease in phosphatase activity, WIP1 inhibition of PI cells followed by cisplatin treatment restored the phosphorylation of Chk1 (Fig. 5F). These data are consistent with the hypothesis that an elevated expression level of WIP1 in PI cells results in alterations in DNA repair pathway effector proteins, which are unable to effectively repair damaged DNA.

Acute infection with the PIV5 P/V-CPI⁻ mutant sensitizes cancer cells to DNA damage-induced death. In the above-described studies, we sought to understand how PI cells were sensitized to stress pathways leading to cell death. Our results raised the question of whether the phenotype of enhanced sensitivity to cisplatin-induced death was limited to P/V-CPI⁻-derived PI cells or was also seen during acute infections with the P/V-CPI⁻ virus. To test this, naïve cells were mock infected or infected with the P/V-CPI⁻ mutant, followed by treatment for 24 h with increasing doses of cisplatin and cell viability assays. As shown in Fig. 6A, treatment of mock-infected cells with 75 μ M cisplatin resulted in ~12% annexin V-positive cells and ~18% propidium iodide-positive cells. In contrast, acute infection with the P/V-CPI⁻ mutant followed by treatment with 75 μ M cisplatin resulted in >50% annexin V-positive cells and ~40%

FIG 6 Acute infection by the P/V mutant sensitizes cells to a DNA-damaging agent and results in deficient DNA repair signaling. (A) Naive HEp-2 cells were mock infected or infected with the P/V-CPI⁻ mutant for 18 h. Cells were treated with the indicated concentrations of cisplatin for 24 h before analysis for annexin V and propidium iodide staining by flow cytometry. Values are means for three samples, with *** indicating *P* values of >0.001. (B) Naive cells, PI HEp-2 cells, or acutely P/V-CPI⁻-infected cells were treated with 75 μ M cisplatin for 24 h. Cell lysates were analyzed by Western blotting for the indicated DNA repair proteins.

propidium iodide-positive cells. Under similar conditions, the DNA damage repair pathway proteins Chk1 and Chk2 were phosphorylated in mock-infected cisplatin-treated cells, as expected, but not in the case of cells acutely infected with P/V-CPI⁻ or in PI cells (Fig. 6B). These data indicate that acute infection with the P/V-CPI⁻ mutant can sensitize cancer cells to cisplatin-induced death and alter DNA damage response pathways similarly to what is seen with PI cells.

PI cells show a difference in the distribution of the DDB1 protein following treatment with a DNA-damaging agent. Upon DNA damage, DDB1 binds to DDB2, forming the DNA damage binding (DDB) complex that can then translocate to the nucleus. DDB1 is also known to function in a cytoplasmic complex with the rubulavirus V protein to induce STAT degradation (14–17). As such, we hypothesized that the DDB1 protein in PI cells would be localized to the cytoplasm and altered in its translocation to the nucleus in response to DNA damage. To test this hypothesis, naive and PI cells were mock treated or treated with 100 μ M cisplatin before immunostaining for DDB1. As shown in Fig. 7A for naive cells, DDB1 appeared in both the nucleus and cytoplasm but had exclusively nuclear staining after cisplatin treatment. While untreated PI cells had staining similar to that of untreated naive cells, cisplatin treatment of PI cells resulted in a perinuclear localization with very little nuclear accumulation. Thus, these data suggest that DDB1 is unable to enter the nucleus when PI cells are treated with cisplatin.

FIG 7 PI cells show a difference in the distribution of the DDB1 protein following treatment with a DNA-damaging agent. (A) Naïve and PI HEP-2 cells were treated with 150 μ M cisplatin for 18 h and stained with antibody to DDB1 and with DAPI. Representative pictures are shown. (B) Naïve and persistently P/V mutant-infected HEP2 cells were treated with 50 μ M cisplatin for 18 h prior to cell lysis and separation into nuclear (Nuc) and cytoplasmic (Cyto) fractions. Samples were analyzed for the indicated proteins by Western blotting.

As an alternative approach to localize DDB1 after DNA damage, naïve and PI cells were treated with 50 μ M cisplatin, and cell extracts were fractionated into nuclear and cytoplasmic fractions, followed by Western blotting for a nuclear control protein, PARP; a cytoplasmic control protein, cofilin; and DDB1. As shown in Fig. 7B, PARP localized to the nucleus as a high-molecular-weight protein in untreated cells and as a cleaved smaller form in cisplatin-treated cells. Cofilin largely localized to the cytoplasm, although PI cells had some of this protein detected in the nucleus. Most importantly, DDB1 was detected in the nuclear extract of cisplatin-treated naïve cells but was not detected in the nuclear extract of cisplatin-treated PI cells. Taken together, these data support the proposal that DDB1 is defective in translocating to the nucleus upon DNA damage in PI cells.

Acute infections with WT PIV5 and hPIV2 sensitize cancer cells to DNA damage-induced death. We tested the hypothesis that WT viruses in the *Rubulavirus* family could also sensitize cancer cells to DNA-damaging agents. Naïve HEP-2 cells were mock infected or infected with WT PIV5 or hPIV2, followed by 24 h of treatment with doses of cisplatin and cell viability assays. Treatment of WT PIV5-infected cells with 100 μ M cisplatin resulted in about 90% annexin V-positive cells and 65% propidium iodide-positive cells, whereas about 20% of the mock-infected cells were annexin V positive (Fig. 8A). As shown in Fig. 8B, treatment of cells with 50 μ M cisplatin after acute hPIV2 infection resulted in ~80% annexin V-positive and 75% propidium iodide-positive cells, values much higher than those seen for cisplatin-treated mock-infected cells or infected cells that did not receive cisplatin treatment. Taken together, these data indicate that acute infections with at least two WT rubulaviruses can sensitize cells to DNA damage-induced cell killing.

FIG 8 Acute WT PIV5 and hPIV2 infections sensitize cells to a DNA-damaging agent. Naive HEp-2 cells were mock infected or infected with WT PIV5 (A) or hPIV2 (B) at an MOI of 10 for 18 h. Cells were treated with the indicated concentrations of cisplatin for 24 h, followed by analysis for annexin V (left) and propidium iodide (right) staining by flow cytometry. Values are the means for three samples, with *** indicating a *P* value of <0.001.

DISCUSSION

During our analysis of the capacity of the P/V-CPI⁻ mutant to kill a range of cancer cell lines, we discovered that infection results in the majority of the cells dying, but in some cases, there is an emergence of a population of cells that survive as P/V-CPI⁻ PI cells. The goal of the work described here was to understand how cells are altered to survive harboring a cytopathic virus. Our most striking finding, which emerged from challenge experiments aimed at testing resistance to apoptosis, is that cells either acutely or persistently infected with P/V-CPI⁻ have an enhanced sensitivity to DNA-damaging agents. This was evidenced in PI cells by increased cisplatin-induced DNA damage and death relative to those of naive cells. This was also reflected in alterations in PI cell DNA damage response signaling pathways in the nucleus (e.g., Chk1) as well as in alterations of the translocation of key DNA damage response factors from the cytoplasm to the nucleus (e.g., DDB1). Based on these findings, we present a model below for how cytoplasmically replicating RNA viruses such as PIV5 and hPIV2 alter cellular responses to DNA damage. These results with PI cells and acute infections have implications for the design of oncolytic RNA virus-based vectors and the possible use of combinatorial virus and chemical approaches to cancer therapy.

The mechanisms that lead to the transition from a cytopathic acute infection to a noncytopathic persistent infection are not completely understood (reviewed in reference 43). This can involve apparently unrelated changes in cell morphology, such as with a persistent infection with foot-and-mouth disease virus (FMDV), where there are alterations in cell shape and increased growth, as well as acquired resistance to acute FMDV infection (44). In the case of MuV, previous studies have shown that PI cells *in vitro* continually express MuV antigen and are resistant to the cytopathic effects of parental MuV (45). Host cell type can also be key in developing a persistent paramyxovirus infection (46). Consistent with this, a persistent NDV infection was successfully established in one ovarian cancer cell line, OVCAR3, while other cell lines, such as OAW28, CAL27, FaDu, and PE/CA PJ15, were unable to establish a persistent infection

(47). PI cells can have an altered antiviral state induced by low levels of IFN production and the presence of IFN-stimulated genes, as seen in the case of NDV infection (47, 48). The P/V-CPI⁻ virus is a potent inducer of IFN (18), and work is in progress to understand the role that this cytokine plays in the differential ability to establish P/V-CPI⁻ PI cells in different cancer cell lines.

IFN induction could also play a role in the lower level of virus production from PI cells than that during acute infection (Fig. 1D), but other possibilities include lower cell growth rates, elevated cell stress responses (e.g., translation arrest), or the accumulation of mutations in the virus population. Consistent with this, virus derived from persistently MV-infected cells leading to subacute sclerosing panencephalitis (SSPE) had mutations in the M, H, and F genes due to polymerase errors and hypermutation events (49). Since the level of virus released from P/V-CPI⁻ PI cells is lower than that with acute P/V-CPI⁻ infection (Fig. 1D), we hypothesize that the persistently infecting virus acquired mutations that impair virus production and enhance persistence. Future studies will examine mutations in the virus genome derived from P/V-CPI⁻ PI cells.

Under standard cell culture conditions, P/V-CPI⁻ PI cells survive with elevated levels of stress markers (e.g., annexin V) and cleaved caspases compared to those in naive cells. This survival was at least partially dependent on the activities of IAPs, since the inhibition of Survivin and XIAP dramatically increased cell death of PI cells relative to naive cells. These findings support a model where the continuous production of inducers of cell death during low-level P/V-CPI⁻ replication (e.g., dsRNA) is countered by IAP activity, and once IAP activity is inhibited, PI cells are induced to die. This raises the attractive hypothesis that screening tumors for IAP expression could provide a tool to determine their susceptibility to the establishment of a persistent infection by an oncolytic virus. In addition, the findings that PI cells are sensitized to IAP inhibitors, which are currently in various phases of clinical trials (50), raise the possibility of their repurposing as antivirals and as combined chemotherapy agents in conjunction with oncolytic viruses.

Since P/V-CPI⁻ PI cells survive while harboring a cytopathic virus, we anticipated that they would be more resistant to cell killing after challenge with external inducers of apoptosis. Unexpectedly, cisplatin-treated PI cells had more DNA damage than did naive cells and showed dose- and time-dependent enhancement of cisplatin-induced cell death. While the phosphorylation of "upstream" sensor molecules such as H2A.X appeared normal, PI cells were defective in the phosphorylation of "downstream" molecules such as Chk1 and -2. The phosphatase WIP1 was upregulated in PI cells by cisplatin treatment, and WIP1 inhibitors decrease cisplatin-induced cell death. WIP1 has also been reported to be upregulated and to have enhanced activity by the human T cell leukemia virus type 1 Tax protein. Here WIP1 upregulation results in suppressed DNA repair capabilities and cell cycle progression in the presence of DNA damage, although the exact mechanism has yet to be elucidated (51). We have found that the combination of P/V-CPI⁻ and cisplatin is effective in HEp-2 (Fig. 3) and A549 human non-small-cell lung carcinoma cells (data not shown), both of which have wild-type p53 (52, 53). Future studies will determine the roles of wild-type versus mutated p53 and WIP1 in P/V-CPI⁻-enhanced sensitivity to cisplatin.

How does an RNA virus that replicates in the cytoplasm sensitize cells to DNA-damaging agents and enhanced cell killing? The cellular protein DDB1 normally shuttles from the cytoplasm to the nucleus in response to DNA damage and acts to assemble sensors and effectors to efficiently repair DNA damage (54). This includes DNA repair signaling proteins such as Chk1 and -2, which are activated by phosphorylation (reviewed in reference 55). Once DNA repair is completed, WIP1 can be induced to deactivate the DNA damage signaling pathways by dephosphorylation for continued cell cycle progression and return the cell to homeostasis (38). PIV5 (and other rubulaviruses) hijacks DDB1 as part of the "V degradation complex," which targets the cytoplasmic degradation of STAT proteins to inhibit IFN signaling pathways (14–17). Our microscopy and cell fractionation data on P/V-CPI⁻ PI cells show that DDB1 is localized largely in the cytoplasm in structures distinct from those in naive cells, and it

is altered in accumulating in the nucleus in response to cisplatin treatment. We propose a model whereby the treatment of PI cells with cisplatin induces a typical DNA damage response, including the phosphorylation of ATR and H2A.X (Fig. 5A). The retention of DDB1 in the cytoplasm of PI cells results in the reduced sensing of the extent of DNA damage, in effect telling the cell that DNA damage is not extensive. The finding that WIP1 is induced in cisplatin-treated PI cells is consistent with our finding of unphosphorylated Chk1 and Chk2 and with a model where PI cells shut down signaling pathways due to the lack of the DDB1-mediated ability to sense the extent of DNA damage. In our model, ultimately, the cell is unable to recognize the extent of DNA damage, which is then not repaired, and cells undergo apoptosis.

Our studies raise the possibility of using combination therapies with oncolytic paramyxoviruses and chemotherapies, as were studied previously with other viruses. For example, a phase II clinical trial has shown that the combination of the oncolytic adenovirus ONYX-015, cisplatin, and 5-fluorouracil was more effective than these therapies alone in patients with recurrent head and neck cancers (56). A phase I/II study investigated the combination of the oncolytic virus T-VEC, radiotherapy, and cisplatin for the treatment of patients with head and neck cancers. These clinical trials have found that combination therapy with an oncolytic virus and chemotherapy decreases disease progression and the rate of relapse and improves overall survival (57).

The finding that acute infection with the oncolytic virus P/V-CPI⁻ sensitizes cells to DNA-damaging agents raises a practical application for our work in terms of the resistance of some cancer cells to DNA-damaging agents. Although cisplatin is the gold standard for numerous cancers, chemoresistance is a major concern (58). For example, cisplatin treatment of ovarian cancer patients is initially very effective, but tumor recurrence occurs in up to 75% of cases, resulting in chemotherapy-resistant tumors (59). Previous studies have shown that cisplatin resistance can result when cells increase DNA repair capabilities (36, 60–63). Conversely, cisplatin sensitivity has been associated with a lower capacity of DNA repair pathways in cells (64–67). Thus, an attractive property of an oncolytic virus would be its ability to impair DNA damage repair pathways and increase cisplatin sensitivity, especially in the case of drug-resistant tumors that have upregulated DNA repair pathways. This is in line with what we report here for the ability of P/V-CPI⁻ to reduce DNA repair capacities.

A number of paramyxoviruses and rhabdoviruses are being developed as oncolytic vectors for tumor therapy, including MV, mumps virus, Sendai virus, and NDV (4–10). Our finding that the highly cytopathic PIV5 P/V mutant can establish persistent infection in some cells raises concern about the potential use of RNA viruses for oncolytic therapy. Future work will focus on the extent to which the establishment of PI cells occurs during treatment of tumors in animal model systems as well as the potential for combinations of cisplatin and P/V-CPI⁻ to reduce tumor burdens.

MATERIALS AND METHODS

Cells, viruses, and plaque assays. Cultures of HEp-2, MDBK, Vero, and CV-1 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI FBS; HyClone, Logan, UT). WT recombinant PIV5 (rPIV5)-GFP was recovered as described previously from a cDNA plasmid (33) kindly provided by Robert Lamb (Northwestern University) and Biao He (University of Georgia) and was grown in MDBK cells. The P/V mutant rPIV5-P/V-CPI⁻ (P/V-CPI⁻) expressing GFP was generated and grown in Vero cells as described previously (18). PIV5 P/V-CPI⁻ encodes 6 naturally occurring mutations in the amino-terminal region of the P/V gene, resulting in amino acid changes of Y26H, V32I, T33I, L50P, L102P, and S157F (21). Human parainfluenza virus type 2 was grown in CV-1 cells. Viral titers were determined on CV-1 cells as described previously (18).

To obtain PI cell lines, HEp-2 cells were infected with the P/V-CPI⁻ mutant at a multiplicity of infection (MOI) of 10, and the medium was replaced every 3 days postinfection (p.i.) for 2 weeks. P/V mutant-infected cells were sorted for high GFP expression levels by using a FACSCalibur flow cytometer (BD Bioscience, San Diego, CA) and were designated PI HEp-2 cells.

Fluorescence microscopy. Media were removed from cell monolayers and replaced with phosphate-buffered saline (PBS) before analysis by microscopy using a Zeiss Axiovert fluorescence microscope with a 10× objective lens. Exposure times were 46 ms for bright field and 500 ms for fluorescence. Slides were imaged on a Zeiss 710 confocal microscope with a 40× objective lens.

Cell viability assays. MTT cell viability assays were performed in 96-well dishes using Cell Titer 96 Aqueous One solution reagent (Promega) according to the manufacturer's instructions. Data are expressed as a percentage of the value for mock-infected cells analyzed in parallel.

Alternatively, PI or naive cells were treated as indicated in each figure legend (concentration of drug and time). Media and trypsinized adherent cells were centrifuged and analyzed for annexin V binding (BD Bioscience) and propidium iodide staining (BD Bioscience) as described by the manufacturer. Cells were analyzed by flow cytometry using the CytoFLEX system (Beckman Coulter), and 10,000 independent events were recorded and analyzed by using CytExpert software.

Western blotting. Dishes (60-mm diameter) of cells were treated as described in the figure legends, followed by lysis in 1× protein lysis buffer (Cell Signaling Technology). The cell lysate was resolved on 10 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes. Samples were probed with the antibodies indicated in the figure legends (Cell Signaling) or with anti-β-actin antibody (catalog number A5316; Sigma) and rabbit polyclonal antisera to the PIV5 NP, hemagglutinin-neuraminidase (HN), and P proteins (18). Blots were visualized with horseradish peroxidase-conjugated antibodies and chemiluminescence (Pierce ThermoScientific).

Chemical challenge experiments. YM155 was purchased from EMD Millipore and reconstituted in sterile dimethyl sulfoxide (DMSO) at a stock concentration of 3 mM. Embelin was purchased from Tocris and reconstituted in sterile DMSO at a stock concentration of 5 mM. Cisplatin was purchased from Sigma-Aldrich and reconstituted in sterile water at a stock concentration of 5 mM. The WIP1 inhibitor (GSK 2830371) was purchased from Tocris and reconstituted in sterile DMSO at a stock concentration of 5 mM. PI and naive cells cultured in 24-well plates (diameter of 2 cm) were challenged as indicated in the figure legends and treated with drugs that were diluted in DMEM containing 10% HI FBS.

Immunostaining and TUNEL staining. Cells grown on 8-chamber slides (ThermoFisher) were treated with cisplatin as indicated in the figure legends and analyzed by staining with anti-DDB1 (1:250 dilution; Zymed, Invitrogen) followed by goat anti-rabbit Alexa Fluor 568 (1:2,000 dilution; Invitrogen). 4',6-Diamidino-2-phenylindole (DAPI) was included to stain for nuclei. To visualize DNA damage, cells were treated with cisplatin as indicated in the figure legends and analyzed by using the Click-iT TUNEL Alexa Fluor 647 assay kit according to the manufacturer's instructions (Invitrogen).

Nuclear extraction. Cells were grown in 6-well dishes and treated with cisplatin as indicated in the figure legends. Nuclear and cytoplasmic extracts were obtained by using a kit according to manufacturer's guidelines (Active Motif). Ten micrograms of extracts was lysed in 1× protein lysis buffer (Cell Signaling Technology) and analyzed by Western blotting.

Statistical analyses. Statistical analysis was performed by using GraphPad Student's *t* test.

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