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Malignant Peripheral Nerve Sheath Tumors with High and Low Ras-GTP are Permissive for Oncolytic Herpes Simplex Virus Mutants

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Background. Malignant peripheral nerve sheath tumors (MPNSTs) occur most frequently in patients with neurofibromatosis type 1 and are often fatal. Current therapy relies upon radical surgical resection, which often fails to completely remove the tumor. To address the need for novel treatment approaches for this disease, we sought to determine if human MPNST-derived cell lines are sensitive to oncolytic Herpes simplex virus (oHSV) infection. Activation of the Ras pathway and its inhibitory effects on protein kinase R (PKR) activation have been shown to dictate cellular permissivity to oHSV mutants. Because NF-1-associated MPNSTs possess inherent hyperactive Ras, we hypothesized these tumors would be ideal therapeutic targets for oHSVs. ***Procedure.*** Human MPNST-derived cell lines were examined for sensitivity to oHSV-mediated gene transduction, virus replication, cytotoxicity, and apoptosis. These parameters were correlated with PKR activation following oHSV infection and compared with normal human Schwann cells (NHSCs) without hyperactive Ras. ***Results.*** MPNST-derived cell lines were efficiently transduced, supported virus replication and were killed by the oncolytic HSV mutants, including sporadic MPNSTs without hyperactive Ras. In contrast to the highly sensitive MPNST cell lines, NHSCs did not support mutant virus replication. ***Conclusions.*** MPNSTs are susceptible to lysis by oncolytic HSV mutants, regardless of Ras status. Tumor-selective virus replication in MPNST cells appears to be mediated by both cellular expression of ribonucleotide reductase and prevention of eIF2α phosphorylation. Virus-induced cytotoxicity of MPNST cell lines was caused by both direct lysis and apoptosis. Our data suggest the use of oncolytic HSV mutants may represent a novel treatment approach for patients with MPNSTs. Pediatr Blood Cancer 2006;46:745–754.
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Key words: malignant peripheral nerve sheath tumor; neurofibromatosis type 1 (NF1); oncolytic Herpes simplex virus; protein kinase R; Ras

INTRODUCTION

Neurofibromatosis type 1 (NF1) occurs at a prevalence of approximately 1 in 5,000 in the general population [1]. Patients with NF1 develop neurofibromas, which are benign peripheral nerve tumors composed of Schwann cells, fibroblasts, neurons, perineural cells, and mast cells embedded in a collagen-rich extracellular matrix [2]. Neurofibromas present as both discrete dermal lesions and, in approximately 30% of patients, large plexiform lesions that occasionally undergo malignant conversion, becoming highly invasive and often metastatic [3–5]. These so-called malignant peripheral nerve sheath tumors (MPNSTs) arise in NF1 patients at a frequency of 10%–13% but occur in the general population at a frequency of only ~0.0001% [6,7]. MPNSTs account for 4%–10% of all soft tissue sarcomas in childhood, and over half of MPNSTs are associated with NF1 [8]. MPNSTs are believed to be derived from abnormal Schwann cells within neurofibromas [9]. Supporting the notion that benign neurofibromas are an ideal environment for malignant progression, Schwann cells isolated from these lesions exhibit increased proliferation, invasiveness, and secretion of a number of pro-angiogenic cytokines [10,11].

NF1 is caused by mutation of neurofibromin, a GTP-activating protein (GAP) that functions to limit cellular Ras activity through acceleration of Ras GTPase activity [12,13]. Loss of neurofibromin in Schwann cells causes dysregulation of Ras activity and enhanced tumorigenic potential [14]. MPNSTs may also occur in non-NF1 patients through alternative mutations and these tumors often retain normal neurofibromin activity. The mainstay of therapy for patients

Abbreviations: NF1, neurofibromatosis type-1; MPNST, malignant peripheral nerve sheath tumor; oHSV, oncolytic Herpes Simplex Virus; NHSC, normal human Schwann cell; PKR, double stranded RNA-inducible protein kinase; RR, ribonucleotide reductase; PFU, plaque forming units; MOI, multiplicity of infection (# of PFU/cell); hpi, hours post infection.

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with MPNSTs is surgery, but obtaining wide surgical margins is difficult because MPNSTs often grow adjacent to vital structures [15]. MPNSTs exhibit a high rate of recurrence with frequent metastases to the lung, liver, and brain [16]. The results of alternatives to surgery such as chemotherapy and irradiation for MPNSTs have remained discouraging. In addition, these treatments hold increased risk since NF1 patients are hypersensitive to therapy-induced malignancies [17,18]. New therapies for patients with MPNSTs are therefore warranted.

Oncolytic viruses are being investigated as a possible new cancer selective alternative for patients who have failed traditional therapy. Rationale for utilizing a selectively replication competent virus for cancer therapy is virus replication and amplification with lysis of cells within tumor tissues, and sparing of cells in adjacent normal tissues [19]. Oncolytic Herpes simplex viruses (oHSVs) have shown anti-tumor efficacy against a number of cancer types including brain, prostate, colon, neuroblastoma, rhabdomyosarcoma, breast, and others [20–26]. The oHSVs G207, 1716, and NV1020 have shown safety and efficacy in phase I and II clinical trials [27–29]. It has previously been shown that, tumor cell selective oHSV-mediated cell killing and replication (termed oncolysis) are dictated by cellular Ras activity [30] and metabolic state [31]. oHSVs deleted for viral ICP6, the large subunit of ribonucleotide reductase (RR), show restricted viral replication in quiescent cells but retain robust virus production in rapidly dividing cancer cells. Cellular Ras activity also dictates permissivity to oHSV infection through inhibition of the double stranded RNA-dependent protein kinase (PKR) pathway [30]. PKR is a well described kinase, activated upon viral infection and exposure to interferon [32]. As a countermeasure to the effects of PKR, wild type HSV-1 expresses the ICP34.5 protein to restore protein translation by recruitment of protein phosphatase 1α to dephosphorylate eIF2α [33,34]. ICP34.5 has also been shown to play additional roles in supporting efficient virus replication [35,36]. As an additional measure of safety, a portion of the HSV-1 latency genes are deleted in ICP34.5$^{-/-}$ oHSVs [20]. Therefore these mutants are not able to replicate in cells with intact PKR defense pathways and are unable to establish latency.

Because abnormal Ras activity has been demonstrated in ~30% of all human malignancies including ~50% of colorectal, 70%–90% of pancreatic, and 30% of non-small cell lung cancers [37], oHSVs may have a large number of potential cancer targets. Based on the apparent oHSV selectivity for cells possessing hyperactive Ras activity, we predicted that MPNSTs would be permissive for oncolysis by oHSVs. We also hypothesized that due to low basal Ras signaling and intact PKR defense pathways, normal human Schwann cells (NHSCs) would not support oHSV replication. To assess the utility of oHSVs as a treatment for MPNSTs we evaluated two different oHSV vectors of different strain and genetic background. G207, derived from HSV-1 strain F, is deleted for both ICP6 and ICP34.5 [38]. hrR3, derived from HSV-1 strain KOS, is an ICP6 deletion mutant that retains ICP34.5 and thus has a higher replicative potency [31].

Here we show that all MPNST cell lines tested were efficiently killed by the ICP34.5$^{-/-}$ oHSV, G207, and by the ICP34.5$^{+/+}$ oHSV, hrR3, although to varying degrees. MPNST cell lines showed higher replication of hrR3 than G207, as predicted. Because the sporadic MPNST derived cell line STS26T supported replication of both oHSVs, and because hrR3 was able to replicate to higher titer than G207, even in MPNST lines with high basal Ras, our data suggest that alternate signaling pathways may play a role in influencing cellular susceptibility to oHSV. As a measure of oHSV attenuation in normal cells, NHSCs did not support replication of either HSV mutant. Following oHSV infection, PKR was activated in NHSCs, but to a lesser degree in MPNST cell lines, partially accounting for oHSV tumor selective virus replication. MPNST cell lines showed evidence of HSV-induced apoptosis, suggesting that multiple mechanisms are responsible for virus-associated cytotoxicity in MPNST cell lines.

MATERIALS AND METHODS

Cell Culture and Viral Description

Cell line Vero was obtained from the ATCC (Rockville, MD). Cell lines ST8814, S462, T265p21, STS26T, and 90-8 (human MPNSTs) were kindly provided by Jeff DeClue (National Cancer Institute, Fredrick, MD). Cell lines, except 90-8, were maintained in DMEM, 10% FBS (Hyclone, Logan, UT) and 25 mM HEPES. 90-8 was cultured in RPMI 1640, 15% FBS, bovine pituitary extract (Invitrogen, Carlsbad, CA), and mito+ serum extender (Invitrogen, Carlsbad, CA). NHSCs were provided by the University of Miami organ procurement team [39] and their use was approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. In brief, NHSCs were collected from the cauda equina of trauma patients. Tissue was dissociated and expanded for 2–7 passages on 50 µg/ml poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and 10 µg/ml laminin (BD Biosciences, San Jose, CA) coated plates. NHSCs were cultured in a 1:1 mix of RPMI 1640 and F12 media with N2 supplement (Invitrogen). Rabbit skin cells (RSCs), kindly provided by Nancy Sawtell (CCHMC), were grown in MEM, with 10% FBS. All cells were grown in penicillin/streptomycin and incubated in an atmosphere at 37°C and 5% CO_2.

Viruses were kindly provided by MediGene, Inc. (San Diego, CA), and Sandra Weller (University of Connecticut Health Center, Farmington, CT). G207, derived from strain F, is an ICP6 and ICP34.5 double deleted virus. hrR3, derived form strain KOS, is an ICP6 single deletion virus. Both these viruses contain the reporter gene β-galactosidase driven by

the early HSV-1 promoter, ICP6. Wild type HSV-1 strain KOS was a kind gift from Nancy Sawtell.

Ras Activation Assay

Cells were grown to 90% confluency and serum starved for 2 hr. Basal levels of activated Ras were determined using a Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's specifications. Data are representative of two independent experiments.

Gene Transfer Efficiency

Cells were infected with G207 or hrR3. Protein lysates were collected, ultra centrifuged, and assayed for protein concentration by micro BCA protein assay kit (Pierce, Rockford, IL). β-galactosidase levels were assayed in triplicate using the Galacto-Star kit (Tropix, Bedford, MA). Relative light units from each sample were normalized to μg protein per sample.

Virus Replication Assay

Cells were infected with G207 or hrR3 at a MOI of 0.1. After a 3 hr infection period, media in each well was replaced. Cells were harvested with cell scraping. Harvested cells and media were freeze-thawed twice and assayed for infectious viruses particles via standard plaque assays. In brief, viral titering was done by infecting RSC monolayers with serially diluted samples, addition of a carboxymethylcellulose overlay, and staining with crystal violet (Fisher Chemicals, Fairlawn, NJ). Data shown is one representative of at least three independent experiments.

Viral Cytotoxicity Assay

Cells were infected with G207 or hrR3 at a range of MOI. Cells were incubated in an atmosphere at 37°C with 5% CO_2 and assessed for viable cells compared to uninfected controls. Remaining viable cells were quantified via modified MTT assay (Promega, Madison, WI).

Apoptosis Assays

Cells were infected with G207 or hrR3 at a MOI of 1, or treated with camptothecin at 100 μM to induce apoptosis. Cells were collected via scraping and centrifugation. Supernatant media was removed and the cell pellet was lysed in 50 μl lysis buffer. Cells were chilled on ice for 30 min, followed by high speed centrifugation. Protein concentration was measured by micro BCA protein assay kit (Pierce). Activated caspase 3 was detected using the ApoTarget kit (BioSource International, Camarillo, CA). Measured activated caspase 3 values were normalized to μg protein per sample. For cell staining, cells were infected at MOI of 0.1 for 20 hr, fixed and stained with X-gal (1 mg/ml) for 12 hr, and Hoechst dye (2 μg/ml) for 15 min.

Denaturing Electrophoresis and Immunoblotting

Cells were infected with G207 or hrR3 at a MOI = 1. At 16 hpi, cells were washed twice with cold PBS and cell lysates were collected using RIPA buffer (10 mM Tris pH 7.4, 160 mM NaCl, 5 mM EDTA, 1% Deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4) plus 1× protease inhibitor cocktail (BD pharmingen, San Diego, CA). Lysates were chilled on ice followed by ultra centrifugation. Samples were assayed for protein concentration by micro BCA protein assay kit (Pierce) and subjected to denaturing electrophoresis, followed by electro-transfer to PVDF membranes (Biorad, Hercules, CA). Primary antibodies incubated with blots overnight at 4°C included: anti-HSV-1 protein (Dako-Cytomation, Carpinteria, CA), or anti-actin (a kind gift from James Lessard, CCHMC), or anti-RR M1 subunit (US Biological, Swampscott, MA), or anti-P^{T451}-PKR (BioSource, Camarillo, CA), or anti-PKR (Santa Cruz Biotechnology), or anti-P-eIF2α (Cell Signaling Tech., Beverly, MA), or anti- eIF2α (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, secondary, anti-rabbit IgG or anti-mouse IgG, HRP conjugated antibodies (Amersham Biosciences, Piscataway, NJ) were incubated on a rocking shaker for 30 min. Western lightning ECL reagent plus (Perkin Elmer, Boston, MA) was incubated with blots for 1 min and shaken by hand. Blots were exposed to Blue Lite film (ISCBioExpress, Kaysville, UT) and imaged at various exposure times.

RESULTS

Basal Ras-GTP and RR Levels are Elevated in NF1-Associated MPNSTs

Because sensitivity to lytic infection by oHSVs has previously been shown to be dictated by levels of Ras and levels of cellular deoxynucleotide (dNTP) pools, we tested MPNST cell lines for basal Ras-GTP and as an indirect indicator of dNTP pools, cellular RR. Our panel consisted of five MPNST cell lines derived from NF1 patients and two MPNST cell lines derived from tumors arising spontaneously in non-NF1 patients. Because Schwann cells, or their precursors, are the likely cell of origin of MPNSTs, NHSCs were chosen as a normal control. Neurofibromin deficient cells displayed hyperactive basal Ras activation in comparison to NHSCs, while the sporadically arising MPNSTs had lower basal levels (Fig. 1A). To compare basal cell growth between MPNST cell lines and the highly HSV permissive positive control cell line, Vero, we measured cellular division time. Each of the cell lines grew with similar kinetics, with cell doubling times ranging between 40 and 50 hr (Fig. 1B). Because some oHSV vectors currently in clinical trials are

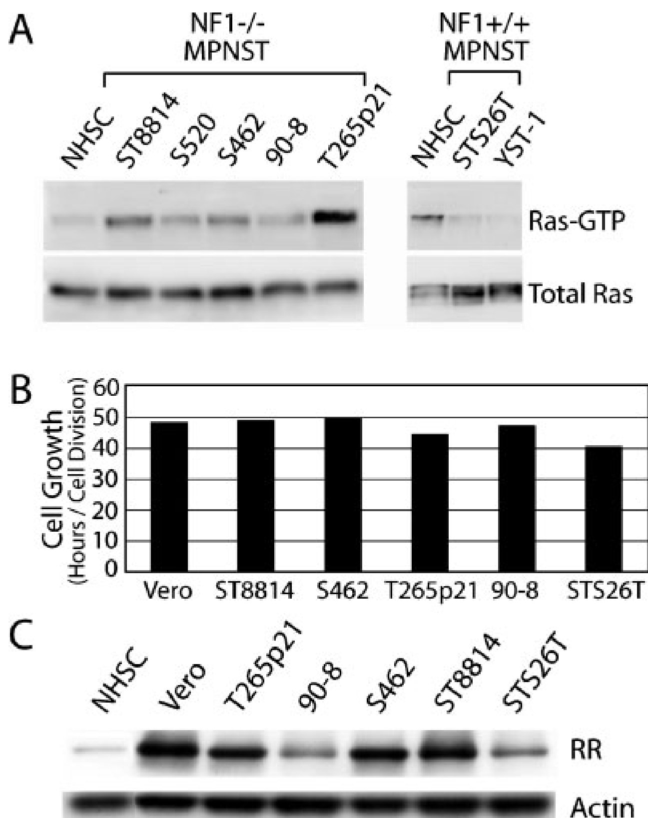

Fig. 1. Basal Ras, growth kinetcs, and Ribonucleotide Reductase (RR, M1 subunit) levels in MPNSTs. Levels of activated Ras (Ras-GTP) and total Ras are shown for NF1-associated ($NF^{-/-}$) and sporadic ($NF1^{+/+}$) MPNSTs (**A**). The positive control cell lines Vero and MPNST cell lines were grown in culture and examined for number of cell divisions over time. Shown is the number of hours required for one cell division, based upon cell growth for 8 days in culture (**B**). Immunoblot showing endogenous levels of RR (M1 subunit) in NHSCs, Vero, and MPNST cell lines. Actin is shown a loading control (**C**).

ICP6 mutants, that require high cellular DNA precursor pools for viral DNA replication, we evaluated the panel of MPNST cell lines, Vero, and NHSCs, for cellular RR activity as an indirect indicator of cellular nucleotide pools. Each of the transformed cell lines showed expression of RR above levels seen in NHSCs (Fig. 1C). The dramatic difference in cellular RR levels between NHSC and MPNST cell lines is an attractive target for future tumor selective based anti-cancer strategies. Overall, these results suggest that NF-1-associated MPNSTs are ideal targets for ICP6 deleted oHSV based therapies.

HSV Mutants Infect and Replicate in MPNST Cell Lines

To investigate if MPNST cell lines were indeed permissive for oHSV infection, we measured oHSV mediated β-galactosidase gene transduction, HSV-1 protein expression, and virus replication. Upon infection of MPNST cell lines by G207 or hrR3 at a MOI of 1, all of the cell lines showed >95% transduction efficiency by X-gal staining (data not shown). Because the β-galactosidase gene is driven by an early HSV promoter (ICP6), levels of gene expression seen in MPNST cell lines reflect the cells' ability to activate such a viral promoter. MPNST cell lines infected at various MOIs, showed a strong dose dependent increase in β-galactosidase gene transduction (Fig. 2A). At high MOIs, both G207 and hrR3 transduced MPNST cell lines to levels on par with the positive control, Vero. However, at the lower MOI of 0.01, MPNST cell lines infected with either virus showed significant variation (Fig. 2A). S462 showed transduction similar to Vero, while ST8814, STS26T, and 90-8 showed lower β-galactosidase gene transduction by 5–9-fold. T265p21 showed the lowest levels of β-galactosidase expression upon infection with G207, ~37-fold, or hrR3, ~116-fold reduction compared to Vero. To further assess oHSV gene expression in infected MPSNT cell lines, we examined total HSV-1 protein synthesis using a polyclonal antibody generated against HSV-1 proteins. MPNST cell lines showed strong expression of HSV proteins (Fig. 2B).

To determine if MPNST cell lines would support replication of oHSV, we infected the panel of cell lines and measured viral titer at 48 and 72 hpi, by plaque assay. MPNST cell lines showed robust oHSV replication (Fig. 2C) roughly correlating with levels of HSV gene expression seen previously (Fig. 2A and B). While S462 showed strong replication of G207, to levels almost reaching that of Vero, T265p21 did not support replication of this virus. Overall, G207 replicated in MPNST cell lines as predicted from our gene transduction data in that cell lines showing lower gene transduction showed poorer replication kinetics. All MPNST cell lines showed strong replication of hrR3 (Fig. 2C). Because T265p21 supported replication of hrR3 but not G207, high Ras activity may not be enough to dictate overall permissivity to oHSV. As predicted, MPNST cell lines infected with the $ICP34.5^{+/+}$ oHSV, hrR3, replicated to higher titer than cells infected by the $ICP34.5^{-/-}$ oHSV, G207 (Fig. 2C).

MPNST Cell Lines are Sensitive to Oncolysis by oHSV

MPNST cell lines examined for oHSV mediated cytotoxicity were permissive for lytic infection by oHSVs G207 and hrR3 (Fig. 3). G207 showed stronger cytotoxicity for cell lines S462 and 90-8, with day 6 IC_{50} values of <0.001 and 0.001 respectively (Fig. 3A). Less efficient cell killing was seen in cell lines STS26T, ST8814, and T265p21, with observed day 6 IC_{50} values between 0.01 and 0.1. Cell lines that showed the highest cytotoxicity upon infection by G207 were only moderately improved for oncolysis by infection with the $ICP34.5^{+/+}$ oHSV, hrR3. Alternatively, MPNST cell lines that showed less effective cytotoxicity upon infection

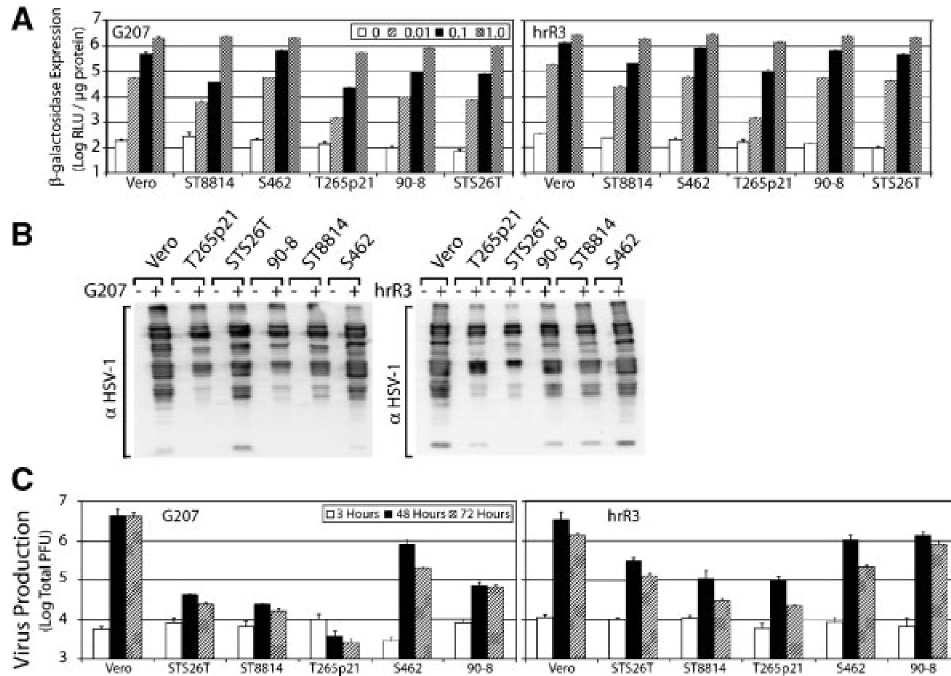

Fig. 2. oHSV gene transduction, protein expression and replication in MPNST cell lines. MPNST cell lines and Vero were infected with G207 or hrR3 and analyzed for β-galactosidase expression. Data are presented as the Log base 10 of Relative Light Units (RLU) per μg of protein per sample (**A**). Infected MPNST cell lines were immunoblotted for expression of HSV-1 proteins (**B**). MPNST cell lines infected with G207 or hrR3 were examined for virus replication at 48 and 72 hpi. Data are presented as the Log base 10 of the total plaque forming units (PFU) as titered by plaque assay (**C**).

with G207 (STS26T, ST8814, and T265p21) showed up to 10-fold enhancement in day 6 IC_{50} when infected by hrR3 (Fig. 3B). Overall, MPNST cell lines were uniformly highly sensitive to oncolysis by oHSVs, with some being equal in sensitivity to Vero.

The PKR Pathway is Upregulated and Activated in Response to Infection by oHSV

MPNST cell lines were infected with either G207 or hrR3, lysed and submitted to denaturing electrophoresis. Cellular

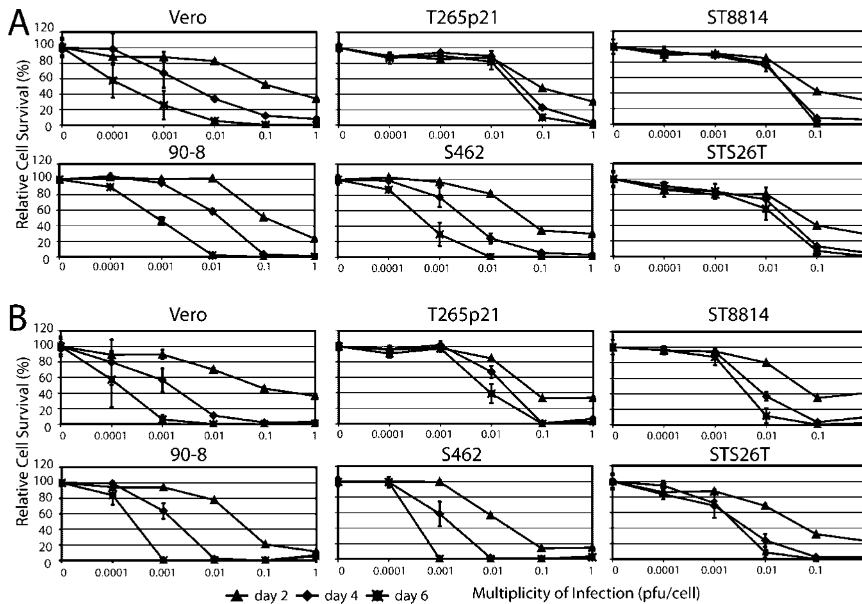

Fig. 3. MPNST cell lines are sensitive to oHSVs at low multiplicity of infection (MOI). MPNST cell lines and Vero infected with G207 (**A**) or hrR3 (**B**) over a range of MOI from 0.0001 to 1, and examined for remaining viable cells at 2, 4, and 6 days post infection. Data are presented as the percentage of viable cells compared to mock infected cultures.

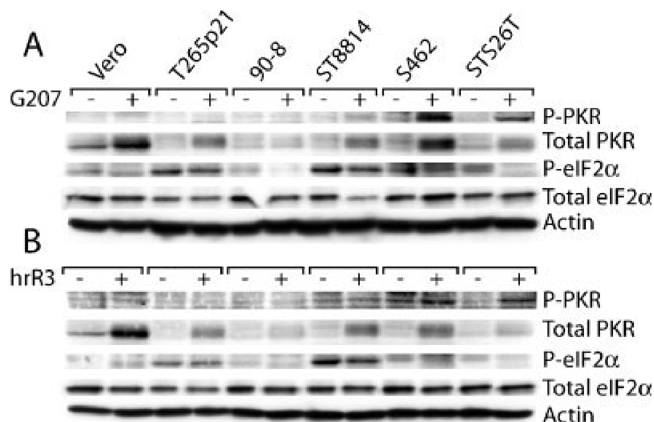

Fig. 4. Functional response of the of the PKR pathway to infection, in MPNST cell lines. MPNST cell lines were infected with G207 (**A**) or hrR3 (**B**) at an MOI of 1.0 for 16 hr, lysed, and submitted to SDS–PAGE. Immunoblots show levels of active PKR (P-PKR), total PKR, phosphorylated eIF2α (P-eIF2α), total eIF2α, and actin.

lysates were immunoblotted to determine both the level of PKR activation (P-PKR) and the phosphorylation status of cellular eIF2α. Infection of nearly all of the tested cell lines, MPNST and Vero, by either oHSV induced a large upregulation of total PKR levels with activation of the PKR pathway (P-PKR, Fig. 4A and B). In response to infection by either oHSV, total levels of eIF2α remained constant and levels of phosphorylated eIF2α remained low possibly due to a Ras-mediated effect. As predicted, the sporadically arising MPNST cell line, STS26T, showed robust PKR activation. Even though this cell line showed activation of the PKR pathway, phosphorylation of eIF2α remained low upon infection with the ICP34.5$^{-/-}$ oHSV, G207. This result suggests that STS26T is sensitive to lytic oHSV infection by a mechanism other than Ras inhibition of PKR activation. Overall, our data suggest that MPNST cell lines maintain low levels of eIF2α phosphorylation in response to oHSV infection, suggesting perhaps that viral/cellular phosphatases are able to counterbalance PKR mediated attempts to shut down protein translation. This result explains a possible mechanism for the high sensitivity of MPNST cell lines to oHSV.

oHSV Replication and cytotoxicity are Attenuated in Normal Human Schwann Cells

As is the case for any potential therapeutic, safety, and toxicity of oHSVs is of high concern. To evaluate the safety of oHSV infection for non-tumor derived tissues, we examined oHSV infection of NHSCs isolated from the cauda equina of trauma patients. To examine the relative attenuation of oHSV we compared infection of NHSCs by wild type HSV-1 KOS, to infection by either G207(strain F) or hrR3 (strain KOS). All three tested viruses were able to efficiently transduce and express viral proteins in NHSCs (Fig. 5A). Infection of NHSCs by G207 as well as hrR3, resulted in high levels of PKR activation and upregulation (Fig. 5B). Infection of NHSCs with wild type HSV-1, KOS, showed upregulation of total PKR levels, however PKR was not activated (Fig. 5B). Thus, it appears that unlike the wild type HSV-1, KOS, G207 and hrR3 are not able to block activation of PKR.

Because NHSCs exhibited low basal levels of RR (Fig. 1C) and robust PKR activation (Fig. 5B), we predicted that in these cells oHSVs would be attenuated in both replication and cytotoxicity. Wild type HSV-1 KOS showed robust replication within NHSCs and reached a high titer; ~3 logs above baseline (Fig. 5C). Unlike the wild type virus, both G207 and hrR3 were unable to replicate in NHSCs. In comparison to levels of replication seen by KOS, G207 was attenuated 602-fold at 48 hr and 229-fold at 72 hpi, respectively; hrR3 attenuated 214-fold at 48 hr and 390-fold at 72 hpi respectively. These results provide dramatic evidence of the selective nature of oHSVs to replicate

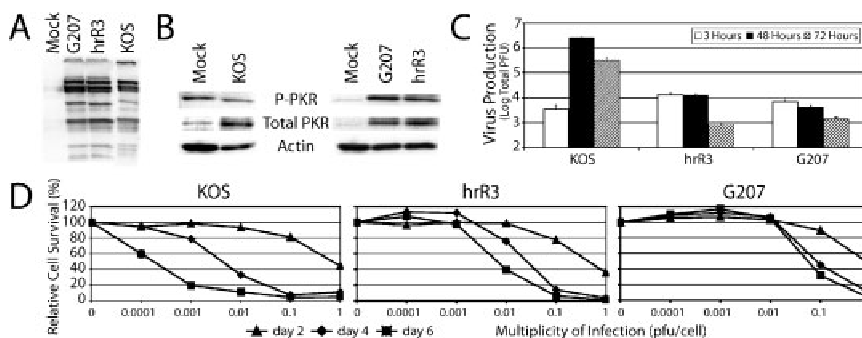

Fig. 5. oHSVs are attenuated for replication and cytotoxicity in normal human Schwann cells (NHSCs). NHSCs were mock infected or infected with G207, hrR3, or wild type KOS followed by lysis and SDS–PAGE followed by immunoblotting for HSV-1 proteins (**A**). NHSCs infected as in panel A were examined for activated PKR (P-PKR), total PKR and actin (**B**). NHSCs infected with wild type KOS, hrR3 or G207 examined for production of infectious progeny at 48 and 72 hpi. Data are presented as the Log base 10 of the total plaque forming units (PFU) (**C**). NHSCs infected with wild type KOS, hrR3 or G207, and examined for remaining viable cells at 2, 4, and 6 days post infection. Data are presented as the percentage of viable cells compared to mock infected cultures (**D**).

selectively within tumor derived cells. Finally, we examined the cytotoxic effect of HSV on NHSCs upon infection with either wild type KOS, G207, or hrR3. NHSCs were highly permissive to infection by wild type KOS, with even the lowest tested MOI showing significant cytotoxicity by day 6 post infection (Fig. 5D). Comparison of the day 6 IC_{50} values for NHSCs infected with wild type KOS against NHSCs infected with either hrR3 or G207, indicates that these viruses are attenuated by ~1.5 logs and >2 logs respectively. These results suggest that oHSVs are attenuated in normal cells.

Apoptosis Plays a Role in oHSV-Induced Cytotoxicity of MPNST Cell Lines

To further explore the mechanism of oHSV mediated cytotoxicity in MPNSTs we sought to determine if oHSV infection could induce apoptosis. Upon infection with either hrR3 or G207, cell lines STS26T (Fig. 6A) and ST8814 (Fig. 6B and C) showed a high degree of transduction by X-gal staining. oHSV infected cells were stained with Hoechst dye to view infected cell nuclei. hrR3 infected STS26T (Fig. 6D) and G207 infected ST8814 (Fig. 6E and F) cells displayed sporadic fragmented nuclei, indicative of apoptosis. When the X-gal and Hoechst images were overlaid it was apparent that some apoptotic cells are infected while others appear to be neighboring uninfected cells (Fig. 6G,H,I). To examine apoptosis induction by oHSV on a molecular level, we assayed the panel of MPNSTs for activation of the caspase pathway. Caspase 3 activation in MPNST cell lines was compared to levels in mock infected cells, or to camptothecin treated cells as a positive control. Upon oHSV infection, a few MPNST cell lines showed activation of caspase 3 (Fig. 6J). Interestingly, cell lines S462 and ST8814 showed no caspase 3 activation following oHSV infection in similar fashion to the highly HSV sensitive cell line, Vero.

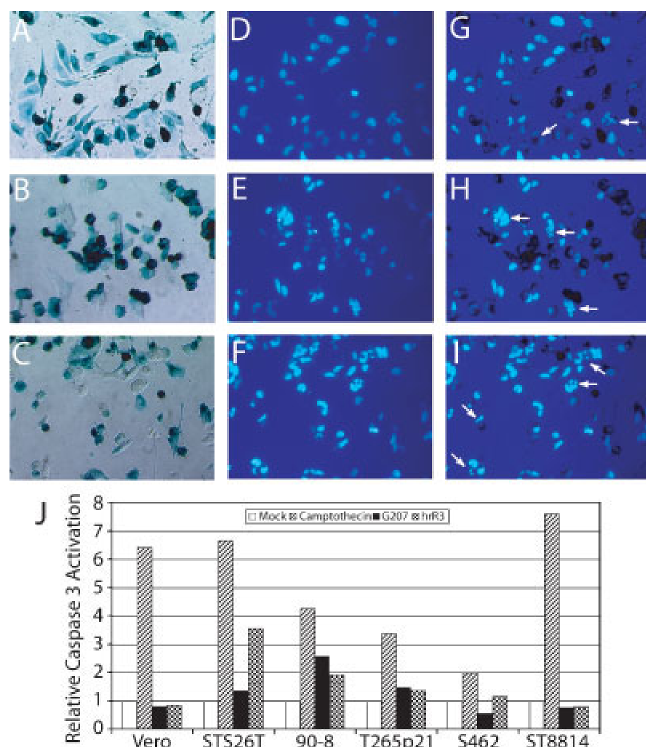

Fig. 6. oHSV induced apoptosis in MPNST cell lines. MPNST cell lines were infected with either G207 or hrR3 for 14 hr, followed by fixation and staining for X-gal (**A**–**C**). oHSV infected cultures were also stained with Hoechst dye and viewed by fluorescent microscopy (**D**–**F**). X-gal and Hoechst staining images were overlaid to examine nuclear morphology of oHSV infected and uninfected cells. White arrows indicate cells with nuclei that appear apoptotic (**G**–**I**). MPNST cell lines were infected with G207 or hrR3 for 20 hr, following by harvesting and lysis. Lysates were measured for caspase 3 activation relative to mock infected cultures. Camptothecin treatment was used as a positive control for induction of apoptosis (**J**).

DISCUSSION

The tumor suppressor protein neurofibromin plays an important role in the regulation of cellular Ras activity [12,13]. Mutation of both alleles of this gene, in Schwann cells of patients with NF1, leads to the development of benign neurofibromas. In 10%–13% of NF1 patients, these lesions undergo a malignant conversion, likely due to the acquisition of additional genetic alterations [6,7]. Effective treatment for MPNSTs has remained a significant therapeutic problem and to address this need we sought to investigate the potential therapeutic utility of oHSV. We show that human MPNST-derived cell lines possess inherent hyperactive Ras activity and high levels of cellular RR (indicative of dNTP pools), making this tumor type potentially an ideal target for oHSV-mediated therapies. Overall, MPNSTs supported efficient HSV gene transduction and replication and were highly sensitive to lytic infection by oHSV.

Our data clearly show that MPNST cell lines support efficient oHSV replication and are highly sensitive to oHSV. The high sensitivity of MPNST cell lines to oHSV is best illustrated by the extremely low IC_{50} values for some cell lines. Because hrR3 replicated to higher levels than G207, we conclude that that ICP34.5 is able to provide features which enhance the efficiency of HSV replication, even in cells with hyperactive Ras signaling. Many oHSV-infected MPNST cell lines showed a robust upregulation of total PKR levels and a modest activation of the PKR pathway in response to viral infection. Even though oHSV infection caused PKR activation, MPNST cell lines maintained low levels of phosphorylated eIF2α. We were surprised to note that in our experimental system levels of phosphorylated eIF2α did not differ between G207 and hrR3 infected MPNST cell lines. Although data in the literature support the assertion that ICP34.5 containing oHSV are more potent than ICP34.5 mutants, our results could possibly be explained by HSV-1 strain variance. The ability of MPNST cell lines to maintain low levels of phosphorylated eIF2α,

even upon infection, could be explained by a number of possible mechanisms. Cellular or viral phosphatases could counteract effects of PKR mediated phosphorylation of eIF2α, active PKR could be physically separated from eIF2α, or hyperactive basal Ras activity in MPNST cell lines could serve as a blockade to effects of active PKR on eIF2α.

In comparison to the highly sensitive MPNST cell lines, we predicted that oHSVs would be minimally toxic to NHSCs. Our prediction was based upon the fact that NHSCs possess low basal Ras activity and low levels of cellular RR or dNTP pools. We found that in NHSCs infected by oHSVs total PKR was upregulated and activated. Correspondingly, both oHSV vectors were unable to replicate within NHSCs. In contrast, wild type HSV-1 infected NHSCs showed no PKR activation above mock, and replicated to high titer. Since only wild type HSV-1 replicated in these normal cells, we believe the selective nature of oHSV most likely relies heavily upon the requirement for cellular nucleotide pools as well as inhibition of PKR activation. These results suggest that PKR activation in NHSCs is important to protect these cells from productive HSV-1 infection. Although highly attenuated, oHSVs showed some cytotoxic virus induced effect on NHSCs, such toxicity may be the result of cellular anti-viral mechanisms such as virus-induced interferon.

Although MPNST cell lines were sensitive to oHSV as predicted based upon Ras and RR status, it is interesting to note two exceptions. First, T265p21, a high Ras-GTP cell line, was not permissive for G207. Although hyperactive Ras may confer sensitivity of some cell lines to HSV infection, there may be alternative signaling pathways necessary for effective oHSV replication. Additionally, the lack of G207 replication in T265p21 may be due to the low activity of HSV promoters in that cell line. Second, STS26T, a low Ras-GTP cell line, was permissive for both oHSVs. It is plausible that this cell line supported oHSV replication due to hyperactive signaling in a common or overlapping pathway with Ras, such as the epidermal growth factor (EGF) receptor signaling pathway. Supporting this notion, overexpression of the EGF receptor has been documented in primary human MPNST tissue sections, human MPNST derived cell lines and transformed $NF1^{-/-}$ murine Schwann cell lines, suggesting that the EGF receptor triggered pathway is key in the malignant conversion of benign neurofibromas to MPNSTs [40–42]. Ras and EGF receptor pathways have been reported to interact and it is possible that both pathways are acting to confer sensitivity of MPNSTs to oHSV [43]. Activation of the EGF receptor pathway may also play a role in the activation of angiogenic and invasive tumor promoting genes.

It is well accepted that normal cells may become apoptotic upon viral infection in order to prevent viral replication and spread; however the role of apoptosis in the context of a cancer cell infected by an oncolytic virus is still debatable. For instance, it may be of benefit to kill tumor cells by both apoptosis and oncolysis, though, it may be detrimental to oHSV replication and spread if cells that could potentially produce more virus are killed by apoptosis before undergoing a full cycle of virus replication. Wild type HSV-1 has been shown to induce apoptosis upon infection, an effect blocked by viral proteins such as ICP27 and others [44]. Infection of human gastric cancer cells with the oHSV NV1066 caused induction of apoptosis in cells neighboring virus infected cells [45]. Because it has been suggested that such apoptotic events may inhibit productive virus infection, we felt it was important to assess the induction of apoptosis in MPNST cell lines. In a few MPNST cell lines, oHSVs induced signs of nuclear fragmentation and activation of caspase 3 and thus, apoptosis may play a role in the levels of virus replication attainable by MPNST cell lines. It will be important to further define the affect of apoptosis on oHSV replication and intratumoral spread of virus.

Because MPNSTs remain a significant clinical challenge, it is important to discover novel treatment approaches. Our results support the idea of using attenuated HSV mutants as therapy for these cancers. While our data suggest MPNSTs may be suitable targets for oHSV therapy, the susceptibility of these tumors to virus infection in vivo may differ from cells in culture. Unfortunately, a reliable in vivo MPNST model that mimics human disease is lacking. One group has been able to show that neuroblastoma cells grow upon injection into murine nerve sheaths, though they were unable to create such a tumor model using human MPNST derived cells [46]. Authentic in vivo models will be essential to further evaluate the potential clinical utility of novel therapeutics such as oHSVs to treat MPNSTs.

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