**ORIGINAL ARTICLE**

\( \gamma_1 34.5 \)-deleted HSV-1-expressing human cytomegalovirus \( IRS1 \) gene kills human glioblastoma cells as efficiently as wild-type HSV-1 in normoxia or hypoxia

This article has been corrected since advance online publication and an erratum is also printed in this issue

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Pathophysiological hypoxia, which fosters the glioma stem-like cell (GSC) phenotype, is present in high-grade gliomas and has been linked to tumor development, invasiveness and resistance to chemotherapy and radiation. Oncolytic virotherapy with engineered herpes simplex virus-1 (HSV-1) is a promising therapy for glioblastoma; however, the efficacy of \( \gamma_1 34.5 \)-deleted HSVs, which have been used in clinical trials, was diminished in hypoxia. We investigated the ability of a chimeric human cytomegalovirus (HCMV)/HSV-1 virus, which expresses the human CMV protein kinase R evasion gene \( IRS1 \) and is in preparation for clinical trials, to infect and kill adult and pediatric patient-derived glioblastoma xenographs in hypoxia and normoxia. Infectivity, cytotoxicity and viral recovery were significantly greater with the chimeric virus compared with the \( \gamma_1 34.5 \)-deleted virus, regardless of oxygen tension. The chimeric virus infected and killed CD133+ GSCs similarly to wild-type HSV-1. Increased activation of mitogen-activated protein kinase p38 and its substrate heat-shock protein 27 (Hsp27) was seen after viral infection in normoxia compared with hypoxia. Hsp27 knockdown or p38 inhibition reduced virus recovery, indicating that the p38 pathway has a role in the reduced efficacy of the \( \gamma_1 34.5 \)-deleted virus in hypoxia. Taken together, these findings demonstrate that chimeric HCMV/HSV-1 efficiently targets both CD133+ GSCs and glioma cells in hypoxia.

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**INTRODUCTION**

High-grade gliomas are the most common brain malignancy in adults and represent \( \sim 10\% \) of childhood brain tumors.1 Outcomes for adult and pediatric glioblastoma multiforme (GBM) are poor with 2-year survival rates \(< 20\% \) despite multimodality therapy including surgery, chemotherapy and radiotherapy.2,3 Novel therapies are desperately needed, and oncolytically engineered herpes simplex virotherapy (oHSV) is one such therapy that offers a promising approach by targeting glioma cells while sparing normal cells. Normal cells are unharmed through deletions of various dispensable genes such as the neurovirulence gene \( \gamma_1 34.5 \), which enables the wild-type virus to overcome a host cell’s defense mechanism to prevent virus replication.4 Oncolytic HSVs with \( \gamma_1 34.5 \) deleted cannot evade protein kinase R (PKR)-mediated translational arrest, which occurs in normal cells in response to HSV double-stranded RNA and prevents viral replication, rendering the virus safe for normal cells. Mutant \( \gamma_1 34.5 \)-deleted (ICP34.5−) viruses may replicate in tumor cells with defective signaling pathways that result in an attenuated PKR response. Non-essential genes can be replaced with therapeutic foreign genes that augment the oncolytic effect, such as the human cytomegalovirus (HCMV) \( IRS1 \) gene. The \( IRS1 \) gene product restores a critical function that is lost with the \( \gamma_1 34.5 \) deletion by facilitating late viral protein synthesis and viral replication without contributing to virulence for normal cells.5,6 First-generation ICP34.5−viruses have been used safely in phase I human adult high-grade glioma trials with evidence of virus replication in tumors and several radiologic responses seen; however, many responses were modest.7,8 One recently discovered limitation of the first-generation ICP34.5−HSV-1, which may explain some of the moderate clinical responses, is the reduced efficacy of the virus under hypoxic conditions.9 Pathophysiological hypoxia is a hallmark of high-grade gliomas with hypoxic gradients varying from 10–2.5% (mild to moderate) to as low as 0.1% (severe), which can be found in necrotic areas of tumors.10,11 This hypoxia has been associated with tumor initiation, invasiveness, angiogenesis, loss of apoptotic potential and resistance to radiation and chemotherapy, all characteristics that have been ascribed to glioma stem-like cells (GSCs).12 Furthermore, hypoxia has been shown to maintain and drive the GSC phenotype with the number of GSCs, as measured by the most commonly used GSC marker CD133, increasing markedly in hypoxia.13,14 Accordingly, the reduced efficacy of the

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ICP34.5− virus in hypoxia has important implications for clinical application of oHSV because hypoxia has a critical role in shaping tumor behavior and therapeutic resistance. An effective oHSV must be able to replicate well under hypoxic conditions to eradicate the resistant GSC population.

Therefore, we sought to determine if the second-generation, chimeric HCMV/HSV-1 oncolytic virus C134, which has proven safe in normal brain cells and prolonged survival in two in vivo experimental murine brain tumor models, has an improved ability to infect, replicate in and kill pediatric and adult GBM xenograft lines (xenolines) in hypoxia compared with the first-generation ICP34.5− virus and to establish a mechanism for the reduced efficacy of the ICP34.5− virus in hypoxia.17 C154 is a green fluorescence protein (GFP)-expressing version of C134, which is being prepared under current GMP procedures and qualified by the NExT Program (RAID) for clinical application. We explored the role of the p38 mitogen-activated protein kinase (MAPK) signaling pathway, including its known substrate heat-shock protein 27 (Hsp27), which together enhance the expression of late viral genes, in the moderation of the ICP34.5− virus in hypoxia.15,16 We hypothesized that reduced activation of p38 MAPK and Hsp27 in hypoxia would result in reduced efficacy of the ICP34.5− virus. As the C154 chimeric virus contains the HCMV IRs1 gene, which facilitates late viral protein synthesis, we anticipated that C154 would be less affected by hypoxia. Importantly, our data demonstrate that the chimeric HCMV/HSV-1 is superior to the ICP34.5− virus in hypoxia and can target both CD133+ GSCs and glioma cells similar to wild-type HSV-1. Furthermore, the p38 pathway has an important role in the reduced efficacy of ICP34.5− viruses in hypoxia.

RESULTS

Viral infectivity

We previously showed that the ability of ICP34.5− viruses to infect and kill GBM xenolines was diminished in hypoxia.9 To determine if the chimeric virus had improved infectability, GBM-XD456, GBM-X12 and GBM-X6 cells that were maintained in Neurobasal medium either in normoxia or hypoxia (1% oxygen) were infected with the recombinant GFP-tagged C154 virus at 1, 3.3 and 10 multiplicities of infection (MOI) for 30 h. One percent oxygen was chosen to recapitulate the severe hypoxia observed in high-grade gliomas by Evans et al.11 Using this assay, we previously demonstrated that the ICP34.5− virus infected a similar percentage of cells between 1 and 5% oxygen levels.17 Thirty hours was established as the optimal time point in our previous study, which examined multiple time points, because it allowed the infection to be well underway but was before cells lysis.7 The proportion of cells infected was determined by fluorescence-activated cell sorting (FACS) analysis. Similar to our previous observations about the sensitivity of these GBM xenolines to the ICP34.5− virus, GBM-X12 was the most sensitive in both normoxia and hypoxia, while GBM-X6 was the most resistant to infection with the chimeric virus. The chimeric virus infected a significantly greater number of cells than the ICP34.5− virus in both hypoxia and normoxia in all three xenolines at 10 MOI (Table 1). GBM-X6 cells experienced the greatest increase in infection with C154 infecting 2.7- and 5.9-fold more cells than the ICP34.5− virus in normoxia and hypoxia, respectively. Although the chimeric virus infected significantly fewer GBM-XD456 cells (P = 0.008) and GBM-X12 cells (P = 0.001) in hypoxia compared with normoxia, the relative decrease in infection (25% for GBM-XD456 and 15% for GBM-X12) was less than that observed for the ICP34.5− virus, which infected 36% fewer GBM-XD456 cells and 26% fewer GBM-X12 cells in hypoxia. Unlike the ICP34.5− virus, the chimeric virus infected more GBM-X6 cells in hypoxia (58.8 ± 0.4%) than in normoxia (54.3 ± 1.4%; P = 0.006) after 30 h. These data demonstrate that the chimeric HCMV/HSV-1 is superior at infecting adult and pediatric GBM xenolines in both normoxia and hypoxia and is not limited to the same degree in hypoxia as the ICP34.5− virus.

We next compared the abilities of the chimeric virus, the ICP34.5− virus and a wild-type HSV-1 (M2001) to infect CD133+ GSCs from GBM-X6, the xenoline that was most resistant to the ICP34.5− virus, in normoxia and hypoxia. By 30 h postinfection, the C154 virus infected a significantly greater number of CD133+ cells in both hypoxia and normoxia at each MOI (1, 3.3 and 10) compared with the ICP34.5− virus (Figure 1). At an MOI of 3.3, the chimeric virus infected fourfold more CD133+ cells than the ICP34.5− virus in normoxia and sevenfold more CD133+ cells in hypoxia. Despite GBM-X6 being the most resistant xenoline, C154 was able to infect over half (55.9 ± 4.6%) of the CD133+ cells in hypoxia at 10 MOI within 30 h compared with only 8.2 ± 0.9% for the ICP34.5− virus. At a low MOI of 1, the chimeric virus infected fewer cells than wild-type HSV-1; however, at an MOI of 3.3 and 10, the chimeric virus was as effective at infecting cells as wild-type virus. At 10 MOI, C154 infected 46.9 ± 4.6% of CD133+ cells in normoxia, and the wild-type virus infected 44.8 ± 5.1% (P = 0.62); in hypoxia, C154 infected 55.9 ± 4.6% of CD133+ cells versus 48.9 ± 3.4% for the wild-type virus (P = 0.10). Importantly, in all three xenolines, the chimeric virus was able to infect as many CD133+ cells in hypoxia as in normoxia at various MOI (Figure 2). Taken together, these data show that the chimeric HCMV/HSV-1 infects CD133+ GSCs similar to wild-type HSV-1 except at low MOI. Furthermore, the chimeric virus is not limited to the same degree as the ICP34.5− virus in hypoxia, and its ability to infect GSCs is not limited by hypoxia.

Cytotoxicity and viral recovery

To confirm the infections were productive, we next determined the cytotoxic ability of the chimeric virus in normoxia and 1% hypoxia 3 days after infection, and we measured virus recovery 48 h postinfection. Consistent with the infectivity data, GBM-X12 was the most sensitive to killing with the lowest dose (plaque-forming units (PFU) per cell) required to kill 50% of the cells (LD50) followed by GBM-XD456, and GBM-X6 was the most resistant to killing with the chimeric virus. The virus recovered from the infected xenoline at levels similar to those from normoxia (17–21% of the input virus) and hypoxia and showed no significant differences in the viral titers recovered from hypoxia or normoxia (17–19% of the input virus).

The proportion of cells infected was determined by fluorescence-activated cell sorting (FACS) analysis. Similar to our previous observations about the sensitivity of these GBM xenolines to the ICP34.5− virus, GBM-X12 was the most sensitive in both normoxia and hypoxia, while GBM-X6 was the most resistant to infection with the chimeric virus. The chimeric virus infected a significantly greater number of cells than the ICP34.5− virus in both hypoxia and normoxia in all three xenolines at 10 MOI (Table 1). GBM-X6 cells experienced the greatest increase in infection with C154 infecting 2.7- and 5.9-fold more cells than the ICP34.5− virus in normoxia and hypoxia, respectively. Although the chimeric virus infected significantly fewer GBM-XD456 cells (P = 0.008) and GBM-X12 cells (P = 0.001) in hypoxia compared with normoxia, the relative decrease in infection (25% for GBM-XD456 and 15% for GBM-X12) was less than that observed for the ICP34.5− virus, which infected 36% fewer GBM-XD456 cells and 26% fewer GBM-X12 cells in hypoxia. Unlike the ICP34.5− virus, the chimeric virus infected more GBM-X6 cells in hypoxia (58.8 ± 0.4%) than in normoxia (54.3 ± 1.4%; P = 0.006) after 30 h. These data demonstrate that the chimeric HCMV/HSV-1 is superior at infecting adult and pediatric GBM xenolines in both normoxia and hypoxia and is not limited to the same degree in hypoxia as the ICP34.5− virus.

Table 1. Percentage (mean ± s.d.) of GBM xenoline cells infected with ICP34.5− virus and chimeric C154 virus at 10 MOI 30 h after infection in normoxia or 1% hypoxia as measured by GFP expression

| Virus         | GBM-XD456 |          | GBM-X6 |          | GBM-X12 |          |
|---------------|-----------|----------|--------|----------|---------|----------|
|               | Normoxia  | Hypoxia  | Normoxia| Hypoxia  | Normoxia| Hypoxia  |
| ICP34.5−      | 41.5 ± 0.9| 26.4 ± 0.9| 20.4 ± 1.3| 10.0 ± 0.2| 65.5 ± 2.0| 48.2 ± 0.8|
| C154          | 65.4 ± 3.4| 48.9 ± 1.2| 54.3 ± 1.4| 58.8 ± 0.4| 86.3 ± 1.6| 73.6 ± 2.1|
| P-value       | 0.0003    | < 0.0001 | < 0.0001| < 0.0001| 0.0003  | 0.0006  |

Abbreviations: GBM, glioblastoma multiforme; GFP, green fluorescent protein; ICP34.5−, γ34.5-deleted virus; MOI, multiplicity of infection.
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Table 2. Virus dose (PFU per cell) (mean ± s.d.) required to kill 50% of cells (LDso) in normoxia or 1% hypoxia as measured by the alamarBlue assay after a 3-day incubation period

| Virus  | GBM-XD456 (Normoxia) | GBM-XD456 (Hypoxia) | GBM-X6 (Normoxia) | GBM-X6 (Hypoxia) | GBM-X12 (Normoxia) | GBM-X12 (Hypoxia) |
|--------|----------------------|---------------------|-------------------|-----------------|-------------------|-------------------|
| ICP34.5– | 7.0 ± 2.9            | 10.7 ± 0.8          | 44.8 ± 3.0        | 47.1 ± 2.9      | 1.9 ± 0.1         | 3.2 ± 0.2         |
| C154   | 2.1 ± 0.6            | 3.9 ± 0.4           | 18.1 ± 2.7        | 15.8 ± 4.4      | 0.4 ± 0.1         | 1.0 ± 0.2         |
| P-value| 0.015                | 0.0002              | 0.0003            | 0.0005          | 0.009             | 0.004             |

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demonstrate that activation of p38 MAPK and Hsp27 was inhibited greatest inhibition at 24 h (52% of the control). The results of Hsp27 were inhibited at all time points with greatest decrease at 6 h (61% of the control (total p38 MAPK); p-Hsp27 was seen in GBM-X12-infected cells in normoxia with hypoxia (14-fold greater signal intensity in normoxia compared with hypoxia (2.1-fold greater in ICP34.5 compared with hypoxia for ICP34.5−Vero cells.

As p38 MAPK signaling was greater in normoxia, we next sought to determine if inhibiting p38 MAPK activation in normoxia could decrease virus yields. GBM-XD456 cells were treated with a single 10 μM dose of p38 MAPK inhibitor (SB 203580). At 2, 4, 6 or 24 h after the inhibitor was added, cells were infected with ICP34.5− virus for 24 h and p-p38 MAPK and p-Hsp27 were assessed by western blotting. Adding the inhibitor 2, 4 or 6 h before infection resulted in decreased p-p38 MAPK signal with the greatest decrease at 6 h (61% of the control (total p38 MAPK); Figure 7a). By 24 h the signal intensity of p-p38 MAPK was back to 81% of the control. Hsp27 was inhibited at all time points with greatest inhibition at 24 h (52% of the control). The results demonstrate that activation of p38 MAPK and Hsp27 was inhibited by a single dose of SB 203580.

Next, we determined virus recovery in GBM-XD456 cells 24 and 48 h postinfection with ICP34.5− or C154 virus in normoxia with and without a single 10 μM dose of p38 MAPK inhibitor 24 h before infection. The single dose of the inhibitor significantly reduced virus yields in both the ICP34.5− and C154 virus at both 24 and 48 h (Figure 7b). To confirm that inhibition of Hsp27 results in decreased virus recovery, GBM-XD456 cells transfected with small interfering RNA (siRNA) specific for Hsp27 (Figure 7c) or nonspecific, control siRNA were infected for 24 h with ICP34.5− virus and the virus recovery was determined. Knockdown of Hsp27 resulted in a 38.7% (P = 0.002) and 40.9% (P = 0.0021) decrease in virus yield at 24 and 48 h, respectively. Taken together, these data show that inhibition of p38 signaling or knockdown of its substrate Hsp27 reduces virus yields, and based on the increased activation of p38 MAPK in normoxia compared with hypoxia, these data indicate that the p38 pathway may have a role in the reduced efficacy of the ICP34.5− virus in hypoxia.

To establish if p38 MAPK inhibition reduced virus recovery by affecting virus entry, the expression of nectin-1 (CD111), an adhesion molecule that is the most efficient mediator of HSV-1 entry, was compared in GBM-XD456 cells treated or untreated with a single 10 μM dose of p38 MAPK inhibitor for 6, 12 or 24 h. At each time point, CD111 expression was not significantly different (P = 0.9) in untreated and treated cells. Next, cells treated or untreated with a 10 μM dose of p38 MAPK inhibitor for 24 h were infected with ICP34.5− virus at 10 MOI for 6 h, and the percentage of cells infected was determined by FACS for GFP expression. A similar percentage of cells that received the p38 MAPK inhibitor were infected as compared with the control (25.4 ± 6.9% versus 25.5 ± 3.4%, respectively; P = 1.0). As GFP is produced with IE gene expression kinetics, the infectivity data combined with the entry receptor expression data suggest that effects of the p38 MAPK inhibitor on virus recovery occur postvirus entry.

**DISCUSSION**

Increasing evidence has implicated hypoxia in mediating phenotypic changes that result in tumorigenesis; increased invasiveness, metastatic potential, induction of angiogenesis, resistance to chemotherapy and radiation; and decreased patient survival. Many of these resultant changes appear to be due to hypoxia’s regulation of GSCs; hypoxia increases the CD133+ GSC fraction and promotes the self-renewal, proliferation and survival capability of GSCs. Thus, for an oHSV to have a maximum therapeutic effect, it must be able to infect, replicate in and kill GSCs in hypoxia. Although previous phase I adult recurrent GBM trials using ICP34.5− viruses demonstrated safety and some evidence of efficacy, the responses were more modest compared with the preclinical studies, and one explanation may be the differential sensitivity of GBM cells under normoxia, the oxygen...
tension traditionally used in in vitro experiments, compared with hypoxia.\textsuperscript{7,8}

We previously showed that CD133+ GSCs express the primary HSV-1 entry molecule CD111 in similar amounts to CD133\textsuperscript{−} tumor cells and that the GSCs that expressed CD111 were sensitive to ICP34.5\textsuperscript{−} viruses in normoxic conditions.\textsuperscript{17} We subsequently showed that CD111 increased significantly in hypoxia; however, just having more HSV entry molecules per cell did not improve efficacy.\textsuperscript{9} Our data showed that the first-generation ICP34.5\textsuperscript{−} virus had decreased infectivity, replication and cytotoxicity in GBM xenolines under hypoxia. In this study, we demonstrate that the chimeric HCMV/HSV-1 oncolytic virus outperformed the ICP34.5\textsuperscript{−} virus in infecting, replicating in and killing pediatric and adult GBM xenoline cells including CD133+ GSCs in hypoxia and normoxia. Significantly, the chimeric virus infected and killed a GBM xenoline, which is resistant to ICP34.5\textsuperscript{−} virus oncolysis, in a manner similar to that of wild-type HSV-1. These compelling data suggest that the chimeric virus, which has been produced in clinical grade, will have improved therapeutic effect and, combined with earlier data showing safety and efficacy of the chimeric virus in vivo, strongly support the use of the chimeric virus for upcoming brain tumor trials.\textsuperscript{6}

Figure 5. In vivo infectivity, as measured by GFP expression (green), 24 h postinoculation of 1 × 10\textsuperscript{7} PFU of ICP34.5\textsuperscript{−} or C154 virus in GBM-XD456MG cells (DAPI (4',6-diamidino-2-phenylindole); blue) grown in the right cerebral hemisphere of nude mice. Areas of hypoxia are marked by carbonic anhydrase 9 (CA9; red). Controls were injected with saline. Rabbit IgG was used as a negative control. Photos were taken microscopically with a x10 objective and representative of 10 sections.

Figure 6. Total P38 MAPK (T-p38 MAPK), activated P38 MAPK (p-P38 MAPK) and activated Hsp27 (p-Hsp27) in normoxia or hypoxia at baseline (control), 4–6 h or 24 h after infection at 10 MOI with ICP34.5\textsuperscript{−} virus (34.5\textsuperscript{−}) or chimeric C154 virus in GBM-D456MG and GBM-X12 cells. Thirty-five micrograms of whole-cell lysate was used for western blot analysis.
In an animal model, hypoxia upregulated p38 activated in normal tissue by oxidative and environment stresses c-Jun N-terminal kinases. P38 is known to be phosphorylated and includes the extracellular signal-regulated protein kinases and the pathways driven by members of the MAPK superfamily, which oncolytic HSVs. The p38 MAPK pathway is one of three signaling previously examined in tumor cells infected with attenuated oncolytic HSVs. The 38 MAPK pathway is one of three signaling pathways driven by members of the MAPK superfamily, which includes the extracellular signal-regulated protein kinases and the c-Jun N-terminal kinases. P38 is known to be phosphorylated and activated in normal tissue by oxidative and environment stresses such as hypoxia and has an important role in apoptosis regulation. In an animal model, hypoxia upregulated p38 MAPK phosphorylation in cortical and hippocampal neurons. Although there are limited studies on the effects of hypoxia on p38 MAPK in gliomas and none in GBM xenografts or xenolines, activation of p38 MAPK in established glioma cell lines under normoxic conditions resulted in increased levels of vascular endothelial growth factor secretion. In the GBM xenolines, we found that total p38 MAPK levels were similar in hypoxia and normoxia without infection, which is consistent with the findings by Levin et al. in established glioma cell lines, and postinfection. A unique finding is that hypoxia did not increase the activation of p38 MAPK in the glioma xenoline cells without infection. This may be owing to the fact that the cells were allowed to equilibrate in the hypoxic environment for 4–7 days before any experiments were performed.

Importantly, although p38 MAPK activation increased after infection with either the ICP34.5 – virus or the chimeric virus, the relative increases were less in hypoxia and p38 activation had returned almost to baseline 24 h postinfection, whereas activation of p38 MAPK remained elevated at 24 h after infection in normoxia. Under hypoxic conditions, the stresses on the cell to maintain levels of ATP needed for regulatory states of multiple kinase-driven pathways likely govern the continued activation processes, and protective mechanisms are likely evoked by these stresses to limit the expenditure of energy resources. Evanescent activation of mechanisms used by the virus for its replication likely limited the amount of virus produced under hypoxia. Furthermore, we showed that inhibiting p38 MAPK or Hsp27 knockdown significantly decreased viral recovery in the ICP34.5 – virus and the chimeric virus. The effect of p38 MAPK inhibition on virus replication is likely postentry as inhibition did not affect the expression of the entry molecule CD111 or alter early infectivity.

To gain a better understanding of the differential sensitivity of GBM xenolines to the ICP34.5 – virus in normoxia compared with hypoxia, we explored the p38 MAPK signaling pathway and its known substrate, Hsp27, which to our knowledge has not been previously examined in tumor cells infected with attenuated oncolytic HSVs. The 38 MAPK pathway is one of three signaling pathways driven by members of the MAPK superfamily, which includes the extracellular signal-regulated protein kinases and the c-Jun N-terminal kinases. P38 is known to be phosphorylated and activated in normal tissue by oxidative and environment stresses such as hypoxia and has an important role in apoptosis regulation. In an animal model, hypoxia upregulated p38 MAPK phosphorylation in cortical and hippocampal neurons. Although there are limited studies on the effects of hypoxia on p38 MAPK in gliomas and none in GBM xenografts or xenolines, activation of p38 MAPK in established glioma cell lines under normoxic conditions resulted in increased levels of vascular endothelial growth factor secretion. In the GBM xenolines, we found that total p38 MAPK levels were similar in hypoxia and normoxia without infection, which is consistent with the findings by Levin et al. in established glioma cell lines, and postinfection. A unique finding is that hypoxia did not increase the activation of p38 MAPK in the glioma xenoline cells without infection. This may be owing to the fact that the cells were allowed to equilibrate in the hypoxic environment for 4–7 days before any experiments were performed.

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The IE HSV-1 protein ICP27 induces activation of p38 MAPK. Zachos et al. demonstrated that wild-type HSV-1 infection stimulates activation of the p38 pathway to enhance transcription of specific viral gene promoters and to improve viral replication; however, a more recent study found that inhibition of p38 did not affect the transcriptional programs of wild-type HSV-1. Mezhir et al. showed that in pancreatic tumors treated with cHSV, ionizing radiation activates late HSV-1 promoters through the activation of the p38 MAPK pathway, although the mechanism by which p38 MAPK acts to enhance expression of late viral genes has yet to be elucidated. Mathew et al. found that Hsp27 is rapidly phosphorylated in a p38-dependent manner, and in HeLa cells depleted of all forms of Hsp27, virus replication was significantly reduced suggesting that the chaperone protein is necessary for productive viral replication. In this study, we show that similar to p38 activation, Hsp27 activation was increased in normoxia postinfection. Thus, the increased signaling of p38 MAPK, as measured by phosphorylated p38 MAPK and Hsp27, in normoxia in contrast to hypoxia coupled with the reduced viral yields after p38 inhibition and Hsp27 knockdown suggest that the p38 pathway has an important role in the reduced efficacy of the ICP34.5 – virus in hypoxia.

Interestingly, the chimeric virus was not limited to the same extent as the ICP34.5 – virus in hypoxia and was able to infect CD133+ GSCs equally in normoxia and hypoxia. Unlike ICP34.5 – mutants, the chimeric virus expresses the CMV PKR-evasion gene IRS1 and has improved late viral protein synthesis, which likely provides the virus with a replication advantage over the ICP34.5 – virus in hypoxia despite decreased p38 MAPK activation. It is not known if other genetic modifications to improve replication of ICP34.5 – HSV would be as effective in hypoxia. For example, T-VEC (talimogene laherparepvec) has a deletion of the α7 gene that juxtaposes its promoter to the Us11 coding sequences causing Us11 to be expressed with α-gene kinetics rather than in its native late expression. Us11, expressed early, is able to block shut-off of host protein synthesis by a mechanism different from that of γ34.55 This improves virus replication but whether it overcomes the inhibitory effects of hypoxia or has improved infectivity of GSCs is unknown. The greater activation of p38 MAPK seen after infection with the chimeric virus relative to the ICP34.5 – virus may at least partially contribute to the improved efficacy in hypoxia. What role the IRS1 gene has in the ability of chimeric virus to replicate in hypoxia requires further investigation. In conclusion, the chimeric HCMV/HSV-1 oncolytic virus was
superior to the ICP34.5− virus at infecting and killing pediatric and adult GBM xenolines in hypoxia, and our data suggest that the chimeric virus is better equipped to target resistant cells in the hypoxic microenvironment.

MATERIALS AND METHODS

Human glioblastoma xenolines

All xenograft lines or ‘xenolines’ were established as tumor cell lines by implanting freshly resected human GBM tissues directly into the flanks of athymic nude (nu/nu) mice. The xenolines were then maintained by serial transplantation in athymic nude mice. GBM-X12 and GBM-X6 were established from adult patients and were provided by C David James, PhD and Jann Sarkaria, MD (Mayo Clinic, Rochester, MN, USA). GBM-XD456 was from a pediatric patient and was provided by Darell D Bigner, MD, PhD (Duke University Medical Center, Durham, NC, USA). The University of Alabama at Birmingham Institutional Animal Care and Use Committee approved the uses of all animal subjects (APN1300008972, APN090108642 and APN 130509395).

Genetically engineered herpes simplex viruses

C101 is a ICP34.5− recombinant virus that has been described previously.5 C154 was constructed from the C134 chimeric HSV, which has been described previously.6 C154 was derived from the ΔICP34.5 recombinant C101 and contains the HCMV IES1 gene and the gene encoding enhanced green fluorescent protein under the control of the HCMV IE promoter in the UL3–UL4 domain. M2001 has previously been described as an HSV-1 wild-type strain F virus with the gene encoding enhanced green fluorescent protein under the control of the CMV IE promoter.8 GFP expression from the viruses was used to determine the percentage of infected and uninfected cells after a specified time period by FACS analysis.

Tumor disaggregation and tissue culture

Xenoline tumors were aseptically harvested from the flank of mice and disaggregated as described previously9,14 or via a gentleMacs Dissociator (Miltenyi Biotec, Auburn, CA, USA) per the standard protocol. Collected cells were washed twice with serumless Dulbecco’s modified Eagle’s medium/ F12 (200 µg/ml, room temperature) and added to the Neurobasal medium (Invitrogen, Grand Island, NY, USA) prepared with fibroblast growth factor-β (Invitrogen) and epidermal growth factor (Invitrogen) at 10 ng/ml, 2% B-27 (Invitrogen, Grand Island, NY, USA) and gentamicin (50 µg/ml). This medium was used to promote growth and slow differentiation of GSCs. The medium was exchanged every 3–4 days as needed, and cells grew non-adherently as neurospheres. Cells were maintained in either normoxia (20.8% O2) or hypoxia (1% O2) in a hypoxia chamber (BioSpherix, New York, USA), prepared for FACS analysis as described previously.9,17 Single cells were replated at 5 × 105 cells per well, dissociated with Accutase-DNAse I and prepared for FACS analysis as described previously.9 To determine the survival. These controls normalized each experimental oxygen condition.

Viral recovery assay

Xenoline cells (3 × 103) grown in normoxia or hypoxia were infected in parallel with C101, C154 or M2001 virus at an MOI of 0.1. 48 h postinfection, the cultures were frozen (−80°C) and virus recovery was measured by limiting plaque dilution on Vero cells as described previously.3 For p38 MAPK inhibitor studies, 2.5×103 cells were grown in normoxia with and without a single 10 µM dose of SB 203580 (Sigma-Alrich) and infected with C101 or C154 for 24 or 48 h and then viral recovery was determined as above.

Immunoblotting

Cells were seeded at 1 × 106 cells per well in 1600 µl of the medium in 6-well plates in normoxia or hypoxia. The next day, cells were infected at 10 PFU per well with C101 or C154 or 24 h. Uninfected cells served as controls. An equal amount of protein (35 µg) from each lysate sample was loaded in a well of 4–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini gels (Bio-Rad, Hercules, CA, USA), electrophoretically separated, transferred to PVG membranes and immunoblotting was performed as described previously.5 Antibodies used were p38 total (H147; Santa Cruz, Dallas, TX, USA), p-p38 (Thr180/Tyr182; Cell Signaling Technology, Danvers, MA, USA), p-Hsp27 (ser82; Cell Signaling Technology) and β-actin (Sigma-Alrich), which was used to confirm equal protein loading.

In vivo infectivity

GBM-XD456 cells (3 × 106) were injected in the right cerebral hemisphere of athymic nude mice as described previously.16 The tumor bed was injected 10 days later with 1 × 107 PFU of C101, C154 or saline. Mice were killed 24 h postinjection and the tumors were harvested, fixed in formalin and paraffin embedded for immunohistochemistry and fluorescence microscopy. Tumor sections were deparaffinized, treated in a citrate buffer (Dako, Carpenteria, CA, USA) to retrieve antigens, incubated in Power Block (BioGenex, Fremont, CA, USA) to block non-specific binding. Sections were then exposed to carbonic anhydrase 9 (Abcam, Cambridge, MA, USA) primary antibody at 1:500 or normal rabbit IgG as a negative control. Cy3-conjugated AffinityPure Fab fragment donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories, Suffolk, UK) was used for a secondary antibody. DNA dye DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen) was used to counter- stain sections. Slides were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Photos were taken microscopically with Olympus IX70 connected to a DP1 digital camera (Olympus, Center Valley, PA, USA) and analyzed with the software provided by the manufacturer.

Transfection with siRNA

GBM-XD456 cells were transfected with siRNA designed to interfere with hsp-27 (5′-GUCACUGAAUUUUGAGCGC-3′; Dharmacon, Lafayette, CO, USA) or scrambled siRNA (5′-CAGGCGUGGACAGAAGUUCU-AU-3′; D-001206; Dharmacon) as a control at a concentration of 200 nM per well using Oligofectamine (Invitrogen) transfection reagent. Cells were infected 48 h posttransfection with C101 and virus recovery was determined after 24 and 48 h.
Statistical analyses

Student’s t-test analyses for significance of mean differences were performed using Microsoft Excel (Microsoft Corp, Redmond, WA, USA). All experiments were performed at a minimum in triplicate. A P-value of ≤0.5 was considered significant.

CONFLICT OF INTEREST

Dr Markert and Dr Gillespie are founders of and own stock and stock options (≤0.5% interest) in Cathexer Inc. and in Aetts Inc., biotechnology companies that are developing other oncolytic HSV that are not the subject of this current investigation. They serve as consultants for Cathexer Inc. as well. Dr Gillespie currently serves as one of five unpaid members of the Board of Directors for Cathexer Inc. The virus used in this study is not licensed by either company or the subject of any company plans for clinical use. Dr Gillespie has served as a paid advisor to the Program Project at the Ohio State University that seeks to find improved methods for the application of oncolytic HSV to treat localized and metastatic cancers. This is generally, but not specifically, related to the subject matter of this investigation.

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