Preferential Replication of Systemically Delivered Oncolytic Vaccinia Virus in Focally Irradiated Glioma Xenografts

Sunil J. Advani1, Lisa Buckel2, Nanhai G. Chen1,2, Daniel J. Scanderbeg1, Ulrike Geissinger2, Qian Zhang1,2, Yong A. Yu1,2, Richard J. Aguilar2, Arno J. Mundt1, and Aladar A. Szalay1,2,3

Abstract

Purpose: Radiotherapy is part of the standard of care in high-grade gliomas but its outcomes remain poor. Integrating oncolytic viruses with standard anticancer therapies is an area of active investigation. The aim of this study was to determine how tumor-targeted ionizing radiation (IR) could be combined with systemically delivered oncolytic vaccinia virus.

Experimental Design: U-87 glioma xenografts were grown subcutaneously or orthotopically. Oncolytic vaccinia viruses GLV-1h68 and LIVP 1.1.1 were injected systemically and IR was given focally to glioma xenografts. In a bilateral tumor model, glioma xenografts were grown in both flanks, oncolytic vaccinia was injected systemically and radiation was delivered specifically to the right flank tumor, whereas the left flank tumor was shielded. Viral replication and tumor regression, after systemic injection, was analyzed and compared in irradiated and nonirradiated glioma xenografts.

Results: Systemically administered oncolytic vaccinia virus replicated to higher titers in preirradiated U-87 xenografts than in nonirradiated glioma xenografts. This increased oncolytic viral replication correlated with increased tumor xenograft regression and mouse survival in subcutaneous and orthotopic U-87 glioma models compared with monotherapies. The ability of focal IR to mediate selective replication of oncolytic vaccinia was shown in a bilateral glioma model in which systemically administered oncolytic vaccinia replicated preferentially in the irradiated tumor compared with the nonirradiated tumor in the same mouse.

Conclusion: These findings show a potential clinical role of focal IR in sensitizing irradiated tumor sites for preferential vaccinia virus–mediated oncolysis. Clin Cancer Res; 18(9); 2579–90. ©2012 AACR.

Introduction

High-grade gliomas remain a therapeutic challenge with a median survival of 12 to 14 months with conventional therapies of surgical resection followed by chemoradiotherapy (1). The resistance of high-grade gliomas to standard therapies has resulted in testing oncolytic viruses to improve tumor control. Oncolytic viruses are replication-competent viruses that selectively infect and destroy tumor cells (2, 3) while sparing surrounding normal tissue. Multiple viruses that selectively replicate in cancer cells and achieve an acceptable safety profile have been identified, and initial clinical trials have been completed in high-grade gliomas showing the feasibility and safety of oncolytic viral therapy (4–6).

However, a major issue in the development of clinically safe viral oncolytic is the therapeutic trade-off of efficacy for safety. Preferential tumor replication of oncolytic viruses has primarily been achieved by deletion of viral genes that hampers their replication efficiency (3, 7). One mechanism to augment the replication of oncolytic viruses is to combine them with standard anticancer therapies such as chemotherapy and radiotherapy (7–11). The cellular response to ionizing radiation (IR) provides a conducive environment for viral replication by upregulation of genes and activation of stress pathways. Because IR is given focally to targeted tumor tissue, studies published to date have primarily evaluated the combination of IR and intratumoral oncolytic viral delivery. It was shown that IR can be combined with various intratumoral delivered oncolytic viruses to increase viral replication and enhance tumor xenograft regression (12–17). However, intratumoral injections of
replication-competent viruses have limited viral distribution within tumor xenografts. To improve viral spread within tumors, oncolytic viruses have been delivered intra-vascularly (18, 19).

Recently, vaccinia viruses (VACV) have been evaluated for their oncolytic potential (20, 21). VACV is large double-stranded DNA virus in the poxvirus family. Oncolytic VACV has several advantages over other oncolytic viruses. It is unique among DNA viruses in that it replicates exclusively in the cytoplasm to minimize the risk of integrating within the host genome. From a safety issue, VACV has an excellent track record in humans as an agent for immunization against smallpox. VACV also have a large cloning capacity. Oncolytic VACV have shown antitumor efficacy in multiple preclinical animal tumor models and have entered clinical trials (22–25). GLV-1h68 was constructed by inserting 3 expression cassettes encoding for β-galactosidase, Renilla luciferase, and Aequorea GFP fusion protein, β-galactosidase, and β-glucuronidase were inserted into the F14.5L, J2R, and A56R loci, respectively, of the viral genome of the LIVP strain. LIVP 1.1.1 is a less virulent wild-type isolate of the LIVP strain (Chen and colleagues; unpublished data).

Viral proliferation assay
Standard viral plaque assays were used to quantify viral replication (23). Infected cells were harvested in triplicate at 24, 48, and 72 hours postinfection (hpi). Viral titers were determined in duplicates by plaque assay using serial dilutions on CV-1 monolayers.

Subcutaneous U-87 glioma xenografts
Mice were cared for in accordance with approved protocols by the Institutional Animal Care and Use Committee of LAB Research International, Inc. and Explora Biolabs (San Diego Science Center, San Diego, CA). Five- to 6-week-old male Hsd: athymic Nude-Foxn1nu mice (Harlan) were implanted s.c. with 5 × 10⁶ U-87 cells (in 100 μL PBS) into the right or bilateral flanks. Treatment started when tumors were 200 to 300 mm³. GLV-1h68 or LIVP 1.1.1 was administered systemically by retro-orbital inoculation of 2 × 10⁶ plaque-forming units (pfu) in 100 μL PBS at day 0.

For irradiation, mice were anesthetized by intraperitoneal (i.p.) ketamine (3 mg/mouse) and xylazine (0.2 mg/mouse). The body was shielded with lead except for the tumor-bearing hind limb, blocking 95% of the given dose as determined by RadCal device. IR was delivered with a RadSource RS 2000 (RadSource Technologies, Inc.). Tumor growth was measured twice a week, and tumor volume was calculated as 0.5 × (length − 5) × width × height. Fractional tumor volume (FTV) was defined by

**Translational Relevance**

Glioblastoma remains a therapeutic challenge and with concurrent chemoradiotherapy, the median survival is 12 to 14 months. Oncolytic viruses are being evaluated for their efficacy in glioblastoma and systemically delivered oncolytic vaccinia viruses have currently entered clinical trials. Because focal radiotherapy is a mainstay of glioblastoma therapy, it is of clinical relevance how systemically administered oncolytic vaccinia virus could be combined with targeted ionizing radiation for therapeutic gain. Focal irradiation of glioma xenografts resulted in preferential replication of systemically delivered vaccinia virus in the irradiated tumor site compared with nonirradiated tumors. These data provide a rationale for future clinical trials combining systemically injected oncolytic virus with radiotherapy. In this therapeutic paradigm, focal radiotherapy can sensitize irradiated tumor sites for viral oncolysis resulting in enhanced tumor control.

We show for the first time that systemically delivered virus had increased levels of viral replication in focally irradiated tumor xenografts compared with nonirradiated glioma xenografts. In a bilateral animal tumor model, where oncolytic VACV were delivered systemically to infect both tumors equally and focal IR was delivered to one tumor 1 day before virus administration, the irradiated glioma xenograft had higher levels of viral gene expression and viral replication. These data establish a novel use of focal tumor irradiation with systemically delivered oncolytic viruses that takes advantage of the physical delivery properties of radiotherapy. In such a therapeutic paradigm, IR can serve as a means to ‘radiopaint’ tumor areas for targeted enhancement of viral replication and tumor cell kill of systemically administered oncolytic viruses.

**Materials and Methods**

**Cell culture and VACV constructs**

African green monkey kidney fibroblasts (CV-1) and human U-87 glioma cells were obtained from American Type Culture Collection. CV-1 and U-87 cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. The recombinant, triple mutant VACV GLV-1h68 has been previously described (23). Briefly, 3 expression cassettes encoding for Renilla luciferase, Aequorea GFP fusion protein, β-galactosidase, and β-glucuronidase were inserted into the F14.5L, J2R, and A56R loci, respectively, of the viral genome of the LIVP strain. LIVP 1.1.1 is a less virulent wild-type isolate of the LIVP strain (Chen and colleagues; unpublished data).

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tumor volume at each time point divided by initial tumor volume. Body weight was measured as net body weight (Body weight – tumor volume/1,000) to exclude tumor mass.

**Orthotopic U-87 glioma xenografts**

For the intracranial glioma xenografts, athymic nude mice were stereotactically implanted with 1 × 10⁸ U-87 cells. Mice were anesthetized and fixed with a small animal stereotactic frame (David Kopf Instruments). Cells were inoculated into the right frontal lobe. Mice were reversed with Antisedan (Altipamezole; 0.5 mg/kg) and analgesia (Buprenophine; 0.1 mg/kg) was administered. Five days after cell implantation, GLV-1h68 or LIVP 1.1.1 was delivered systemically by retro-orbital injection of 2 × 10⁶ pfu in 100 μL PBS. In irradiated mice, the irradiated target volume was the entire cranium.

**Expression of virus-encoded marker genes GFP and luciferase in tumors**

GLV-1h68 GFP expression within tumors was monitored under UV light by a stereo fluorescence macroimaging system (LighTools Research). GFP expression was scored with a 4 point system: 0, no GFP signal; 1, one spot; 2, 2 or 3 local spots; 3, more than 3 spots; and 4, diffuse signal. For GLV-1h68 luciferase expression, 5 μL coelenterazine (0.5 μg/μL) in 95 μL PBS was given by tail vein injection. Photon emission was recorded for 1 minute by the Argus100 Low Light Imaging System (Hamamatsu).

**Tumor vessel permeability in nonirradiated and irradiated glioma xenografts**

Tumor vessel permeability was analyzed by an Evans Blue Dye assay. U-87 glioma xenografts were grown s.c. as described above. Irradiated tumor xenografts received a focal dose of 6 gray (Gy) and 24 hours after irradiation, mice were injected with 0.1 mL of 1.5% Evans Blue Dye (Sigma) via retro-orbital inoculation. The dye was allowed to circulate for 45 minutes followed by perfusion with 20 mL PBS. Tumors were harvested and placed in 1 mL of N,N,-dimethylformamide (Sigma) per 0.1 g tissue. Dye extraction was conducted at 55°C for 72 hours, and absorbance was quantified at 620 nm. Values were normalized to dye extracted from the spleen of the same mouse.

**Vaccinia viral titers in tumor xenografts and immune-related profiling**

Tumors were excised and placed in 2 volumes of homogenization buffer (50 mmol/L Tris-HCl and 2 mmol/L EDTA) supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics). Tumors were homogenized with a MagNA Lyser (Roche Diagnostics). After 3 freeze and thaw cycles, the supernatants were collected by centrifugation. Viral titers were measured by standard plaque assay on CV-1 cells.

In addition, samples (n = 2 per group) were analyzed for immune-related protein antigen profiling by Multi-Analyte Profiles (Rules Based Medicine) using antibody-linked beads. Results were normalized on the basis of total protein concentration and cutoff value was set at minimum 1.5-fold increase in treated samples compared with control.

**Histology**

Tumors were fixed with 10% neutral-buffered formalin overnight, dehydrated, and then embedded in paraffin. Five-μm sections were stained for VACV as described previously (26). Sections were examined with low magnification (× 2) on a Nikon Eclipse 6600 microscope. Images were taken by the Diagnostics Instruments model 24.4 camera and MetaMorph v. 7.7 software.

**Statistical analysis**

Statistical analysis was conducted with SPSS, version 11 (SPSS, Inc.). One-way ANOVA was used to compare the tumor volumes among different treatment groups at each time point. The differences between the groups were analyzed with Bonferroni tests when the ANOVA showed an overall significance at a time point. To determine significance between only 2 treatment groups, a 2-tailed unpaired t test was used (Excel 2007 for Windows). Differences in survival were analyzed using log-rank method.

**Results**

**Combining GLV-1h68 and IR increases U-87 glioma xenograft growth delay**

We assessed the efficacy of combining GLV-1h68 and IR in a subcutaneous U-87 xenograft model. U-87 glioma xenografts were grown in the right flank of athymic nude mice. GLV-1h68 was delivered systemically on day 0. In irradiated groups, 6 Gy of focal IR was given to the tumor-bearing hind limb. We used a dose of IR that was therapeutically suboptimal and would result in tumor growth delay without tumor regression. IR was delivered either 1 day before or 1 day after systemic viral administration to determine whether there was an effect on sequencing IR and oncolytic VACV. Tumor volumes were measured biweekly and plotted as mean FTV (Fig. 1A). The response of individual glioma xenografts in each experimental group is shown in Supplementary Fig. S1. Mice were weighed once a week to monitor general well being (Supplementary Fig. S2A). Untreated control glioma xenografts grew exponentially and all mice were sacrificed by day 23, secondary to tumor burden. Systemic GLV-1h68 injection alone had minimal effect on tumor growth delay and all animals were sacrificed by day 27, secondary to tumor burden. Delivery of a 6 Gy fraction resulted in an initial tumor growth delay, but then irradiated glioma xenografts grew exponentially. Combining 6 Gy with GLV-1h68 resulted in tumor xenograft growth delay compared with either GLV-1h68 or IR alone. Tumors of mice treated with GLV-1h68 and 6 Gy were significantly smaller (P < 0.05) than in all other treatment groups by day 27. Sequencing of IR, either before or after systemic viral injection, had a similar effect on U-87 xenograft growth delay. To further quantitate the effects of...
GLV-1h68 and IR on glioma growth delay, we calculated the mean time to reach 10 times the initial starting volume, $FTV = 10V(0)$, for each of the groups (Table 1A). Control U-87 xenografts grew to $FTV = 10V(0)$ by 17.8 days. Systemic GLV-1h68 caused minimal tumor growth delay increasing $FTV = 10V(0)$ by 2 days. Focal 6 Gy caused a 10-day increase in $FTV = 10V(0)$ over control tumors. The combination of systemic GLV-1h68 delivery and focal IR, given either 1 day before or after GLV-1h68 injection, resulted in a 22-day increase of $FTV = 10V(0)$.

Combining GLV-1h68 and IR increases survival in mice implanted intracranially with U-87 glioma xenografts

We then verified the efficacy of combining GLV-1h68 and IR in an intracranial U-87 xenograft model. U-87 glioma cells were implanted intracranially, and GLV-1h68 was delivered systemically on day 5. In irradiated groups, 2 fractions of 6 Gy were given to the entire cranium on days 4 and 6. Mice were followed for survival (Fig. 1B, Table 1B). Median survival for control mice was 25 days after implantation. GLV-1h68 did not increase survival. IR increased median survival by 7 days. Mice treated with combination of IR and GLV-1h68 survived significantly longer ($P < 0.01$) than all other groups, showed by an increase of median survival by 21 days. The effects of combining GLV-1h68 and IR in the intracranial glioma model corroborated our results in the s.c. glioma model. To further characterize the interaction of GLV-1h68 and IR, we then used the s.c. glioma model.

Irradiation increases in situ expression of GLV-1h68-encoded GFP and luciferase in glioma xenografts

To investigate whether focal IR and systemic viral delivery interacted, we monitored in situ real-time GLV-1h68 reporter gene expression within tumor xenografts from groups treated with systemic injection of GLV-1h68 with or without focal tumor irradiation. GLV-1h68 encodes a Renilla luciferase–GFP fusion protein. Viral GFP expression was monitored within the tumor xenografts and scored on a 4-point scale on days 7 and 14 after systemic viral delivery. Tumor xenografts from control mice did not have any detectable GFP expression. At 1 week after systemic injection of GLV-1h68 alone, U-87 xenografts had focal spots of GFP expression (Fig. 2A, Supplementary Fig. S3). In contrast, U-87 xenografts treated with 6 Gy focal to the tumor 1 day before systemic GLV-1h68 injection had more diffused GFP expression within the tumor xenografts (Fig. 2A, Supplementary Fig. S4). Interestingly, when 6 Gy of focal IR was given 1 day after systemic GLV-1h68 injection, GFP expression within the tumor was again focal at day 7 similar to nonirradiated GLV-1h68–injected alone group (Fig. 2A).

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**Table 1. Growth inhibition of subcutaneously (A) or intracranially (B) implanted U-87 glioma xenografts**

<table>
<thead>
<tr>
<th>Time to reach $FTV = 10$, d</th>
<th>Tumor growth delay over control tumors, d</th>
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<td>Pre 6 Gy + GLV-1h68</td>
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<tr>
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<tr>
<td>GLV-1h68 + Post 6 Gy</td>
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<table>
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<th>Median survival (days postimplantation)</th>
<th>Survival increase over control mice, d</th>
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<td>25</td>
</tr>
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<tr>
<td>2 x 6 Gy</td>
<td>32</td>
</tr>
<tr>
<td>2 x 6 Gy + GLV-1h68</td>
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<tr>
<td>GLV-1h68</td>
<td>21</td>
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**Figure 1. Inhibition of glioma xenograft growth by combination of GLV-1h68 and IR.**

A, subcutaneous U-87 xenografts were grown in flanks of athymic nude mice. Mice were treated with systemic GLV-1h68 and focal 6 Gy IR either 1 day before or after virus injection. Glioma xenografts were measured twice a week and plotted as mean FTV. B, Kaplan–Meier survival curves of IC U-87 glioma xenografts. U-87 glioma cells were orthotopically implanted into the brains of nude mice. GLV-1h68 was injected systemically and IR was given in two 6 Gy fractions 1 day before and after GLV-1h68 injection.
Supplementary Fig. S5). The mean GFP expression is shown in Fig. 2B. While preirradiation initially had more diffuse viral GFP expression, by day 14 viral GFP expression was equivalent if IR was given before or after viral injection. Importantly, irradiated glioma xenografts had higher viral GFP expression than nonirradiated glioma xenografts. To verify higher viral GFP expression in irradiated tumor xenografts, we quantitated viral luciferase activity. Ten days after viral injection, photon counts from GLV-1h68 luciferase activity in 8 nonirradiated and 8 preirradiated U-87 glioma xenografts were measured. As with GLV-1h68 GFP expression, IR-treated U-87 xenografts had higher luciferase activity than nonirradiated glioma xenografts (Fig. 2C). Mean and median photon counts from GLV-1h68–encoded luciferase increased 2.6-fold and 5.2-fold, respectively, in preirradiated U-87 xenografts compared with nonirradiated U-87 glioma xenografts ($P < 0.05$). These data show that IR interacted with oncolytic GLV-1h68 to increase oncolytic VACV gene expression.

**Focal IR does not alter tumor vessel permeability of U-87 glioma xenografts**

Our studies showed an increase in viral marker gene expression in tumors that received a dose of 6 Gy 1 day before GLV-1h68 administration. To determine whether the focal dose of 6 Gy increases permeability of the tumor vasculature at the time of systemic virus delivery, we conducted an Evans Blue Dye assay. One and a half percent Evans Blue Dye in PBS was injected systemically into U-87 tumor-bearing mice 1 day after focal irradiation of tumors, similar to the time of oncolytic VACV injection. Nonirradiated and irradiated tumors along with spleens were analyzed for vessel permeability. We found that there was no significant difference ($P = 0.134$) in dye extravasation in nonirradiated versus irradiated tumors. The mean normalized Evans Blue extravasation of control and irradiated tumors is shown in Supplementary Fig. S6. These data suggest, that increased viral replication in irradiated tumors is not due to a higher viral dose that reaches the irradiated tumor initially because of increased permeability of the vessels.

**Combining IR with the less attenuated oncolytic vaccinia LIVP 1.1.1 enhances U-87 xenograft delay**

We next determined whether the interaction of IR with oncolytic vaccinia was limited to attenuated GLV-1h68, or if IR could interact with a less attenuated oncolytic VACV, LIVP 1.1.1. We first compared the replication of GLV-1h68...
and LIVP 1.1.1 in U-87 cells grown in culture. U-87 cells were infected at a multiplicity of infection (MOI) of 0.01 with either GLV-1h68 or LIVP 1.1.1 and harvested at 24, 48, and 72 hpi. LIVP 1.1.1 replicated to higher titers in U-87 cells than in GLV-1h68 (Fig. 3A). U-87 cells infected at an MOI of 0.01 had 65-fold more infectious particles produced by LIVP 1.1.1 than in GLV-1h68 by 24 hpi. We then assessed the efficacy of LIVP 1.1.1 and IR in U-87 glioma xenografts. LIVP 1.1.1 was injected on day 0 systemically and 6 Gy of IR was given as a single 6 Gy fraction 1 day before LIVP 1.1.1, or in two 3.5 Gy fractions 1 day before and 1 day after LIVP 1.1.1 injection. Glioma xenografts were measured twice a week and normalized to the volume at initiation of treatment.

Figure 3. LIVP 1.1.1 replicates more efficiently in glioma cells and increases tumor growth delay with IR. A, increased replication of LIVP 1.1.1 versus GLV-1h68 in U-87 glioma cells. U-87 glioma cells were infected with either GLV-1h68 or LIVP 1.1.1 and harvested 24, 48, and 72 hpi. Viral titers were determined on CV-1 monolayer and normalized to the input virus. B, U-87 glioma xenograft growth delays with LIVP 1.1.1 and IR. U-87 glioma xenografts were grown in nude mice and systemically injected with LIVP 1.1.1 on day 0. IR was given as a single 6 Gy fraction 1 day before LIVP 1.1.1, or in two 3.5 Gy fractions 1 day before and 1 day after LIVP 1.1.1 injection. Glioma xenografts were measured twice a week and normalized to the volume at initiation of treatment.

Fractionated IR in combination with oncolytic vaccinia virus achieves similar glioma xenograft regression

Clinical radiotherapy is primarily delivered in a multifractionated scheme for gliomas, and in above intracranial models we tested the efficacy of IR given in 2 fractions before and after VACV. To be able to fairly compare the different options, we then validated our results in a U-87 orthotopic model as above. LIVP 1.1.1 was injected systemically on day 5 and IR delivered in 2 fractions of 6 Gy, given on day 4 and 6. LIVP 1.1.1 injection increased median survival by 3 days, and irradiation alone increased survival by 7 days over control mice. Mice treated with combination showed a 21-day increase over control mice (Supplementary Fig. S7). These results indicate that the interaction of IR with oncolytic VACV is not restricted to the triple deleted GLV-1h68 but is also seen with the less attenuated isolate of LIVP, LIVP 1.1.1.
radiation fractionation schedules, we tested similar biologic effective doses (BED) than in the flank glioma model. Assuming an α/β ratio of 10 for tumor cell sensitivity, 6 Gy given as a single fraction is equivalent to 3.5 Gy given in 2 fractions (total dose of 7 Gy). For the 2 fractions IR schedule, 3.5 Gy was given 1 day before and after LIVP 1.1.1 injection. IR alone, given as a single fraction of 6 Gy or 2 fractions of 3.5 Gy, produced equivalent growth delays of approximately 7 days, which is as expected as both IR schedules have an equivalent BED for tumor cell kill (Fig. 3B). Splitting IR in two 3.5 Gy fractions resulted in similar tumor volume regression as seen with 6 Gy and LIVP 1.1.1 and was statistically significant compared with single treatment groups (P < 0.05). With the split fraction regimen, 6 of 7 tumors had a FTV ≤ 1 at day 41. These data indicate IR can be delivered as a large single fraction or as 2 smaller fractions in combination with LIVP 1.1.1.

IR increases oncolytic vaccinia viral replication and distribution in U-87 xenografts

We next determined whether IR increased the replication and spread of oncolytic VACV in glioma xenografts. Because LIVP 1.1.1 does not encode reporter genes, we checked viral spread within glioma xenografts by immunohistochemistry for antibody to VACV protein A27L at 7 days after LIVP 1.1.1 injection. Glioma xenografts from control mice showed no staining for VACV protein A27L (Fig. 4A). LIVP 1.1.1 injection alone showed focal areas positive for VACV (Fig. 4B). Irradiation alone showed no staining for VACV. Irradiation in combination with LIVP 1.1.1 resulted in a much more diffused staining pattern for VACV protein A27L (Figs. 4C and D). The staining pattern for VACV protein was similar when 6 Gy was given before LIVP 1.1.1 or if IR was given as 2 fractions of 3.5 Gy, 1 day before and after LIVP 1.1.1 systemic injection. Because IR doses used were therapeutically suboptimal resulting in tumor xenograft growth delay, no necrosis was evident on hematoxylin and eosin staining in irradiated xenografts compared with control xenografts at day 7. We then quantitated the number of infectious LIVP 1.1.1 viral particles in nonirradiated and irradiated glioma xenografts. IR was given as a 6 Gy fraction 1 day before LIVP 1.1.1 systemic injection. By day 7, glioma xenografts injected with LIVP 1.1.1 had a mean of $1.8 \times 10^7$ pfu per gram tumor and irradiated glioma xenografts had a mean of $12 \times 10^7$ pfu per gram tumor. Irradiation resulted in a statistically significant 6-fold increase in infectious viral particle production ($P = 0.03$; Fig. 4E).

**IR in combination with oncolytic vaccinia virus induces a strong proinflammatory tissue response in U-87 xenografts**

Because oncolytic VACV replication was enhanced with U-87 tumor xenografts, we then determined how the combination of IR and oncolytic VACV influenced the inflammatory cytokine profile within tumors. U-87 glioma xenografts from control mice and mice treated with LIVP 1.1.1, 6 Gy, or the combination of 6 Gy followed by LIVP 1.1.1, were harvested 7 days after infection and analyzed for the expression of murine cytokines and proteins regulating inflammation (Table 2). Radiation alone had minimal effects on the expression of murine inflammatory cytokines. As expected, LIVP 1.1.1 replication in the tumor resulted in increase in inflammatory cytokines, in particular RANTES, granulocyte macrophage colonystimulating factor (GM-CSF), IP-10, lymphotoxin, and macrophage inflammatory protein (MIP)-1β. Interestingly, the combination of 6 Gy followed by LIVP 1.1.1 injection resulted in a robust proinflammatory reaction within the tumor. Four cytokines were expressed greater than 10-fold (MCP-1, IL-18, MCP-3, and IP-10). In addition, the majority of the remaining cytokines

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**Figure 4.** Combination of LIVP 1.1.1 with IR results in increase of VACV in tumors. A–D, distribution of LIVP 1.1.1 in nonirradiated and irradiated U-87 glioma xenografts. Immunohistochemistry (IHC) of VACV in U-87 glioma xenografts harvested 7 days after LIVP 1.1.1 systemic injection. All pictures were taken at 2 × magnification. A, control U-87 xenografts from noninfected mice. B, LIVP 1.1.1 treatment alone. C, 6 Gy 1 day before LIVP 1.1.1 injection. D, 3.5 Gy 1 day before and 1 day after LIVP 1.1.1 injection. E, infectious LIVP 1.1.1 particles within nonirradiated and irradiated U-87 glioma xenografts. IR was given as a single 6 Gy fraction 1 day before systemic LIVP 1.1.1 injection. Glioma xenografts were harvested 7 days after LIVP 1.1.1 injection and infectious viral particles were quantitated on CV-1 monolayer.
Table 2. Mouse immune-related protein antigen profiling of s.c. U-87 xenografts

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<th>Antigen name</th>
<th>LIVP 1.1.1/ control ratio</th>
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<td>4.9</td>
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Fold upregulation: > 1
Fold downregulation: > 1, 2–5, 5–10, >10
profiled were higher in the combined treatment group than in LIVP 1.1.1 or 6 Gy alone. Interleukin (IL)-1β was interesting in that it was the only cytokine strongly downregulated by the combination of 6 Gy and LIVP 1.1.1.

In a bilateral glioma tumor model, systemically delivered oncolytic vaccinia preferentially replicates in focally irradiated glioma xenografts

To further pursue whether focal IR could serve to target systemically delivered virus to replicate preferentially within irradiated tumors, U-87 xenografts were grown bilaterally in the flanks of athymic nude mice. GLV-1h68 was injected systemically, and 6 Gy was given focally to the exposed right flank tumor. The rest of the mouse including the left flank tumor was shielded with lead. A, serial measurements of FTV of the left and right flank glioma xenografts. B, GLV-1h68 reporter gene expression in a representative mouse with bilateral glioma xenografts. 6 Gy was given focally to the right flank tumor (upper tumor) with the left flank (lower tumor) shielded. Top left, bright field; top right, GFP expression; bottom left, luciferase activity (photons/minute); bottom right, overlay of luciferase activity photon count with mouse contour. C, IHC of VACV 9 days after GLV-1h68 systemic injection in shielded left flank tumor (left) and irradiated right tumor (right). Pictures were taken at 2 x magnification.

Figure 5. Preferential replication of systemically delivered GLV-1h68 within the focally irradiated right tumor as opposed to the nonirradiated left tumor in a bilateral flank tumor model. U-87 glioma xenografts were grown in the bilateral flanks of mice and GLV-1h68 was injected systemically. IR was given focally as single 6 Gy fraction 1 day before viral injection to the right flank tumor. The remainder of the mouse and the left flank tumor were shielded with lead. A, serial measurements of FTV of the left and right flank glioma xenografts. B, GLV-1h68 reporter gene expression in a representative mouse with bilateral glioma xenografts. 6 Gy was given focally to the right flank tumor (upper tumor) with the left flank (lower tumor) shielded. Top left, bright field; top right, GFP expression; bottom left, luciferase activity (photons/minute); bottom right, overlay of luciferase activity photon count with mouse contour. C, IHC of VACV 9 days after GLV-1h68 systemic injection in shielded left flank tumor (left) and irradiated right tumor (right). Pictures were taken at 2 x magnification.

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In the mice with bilateral flank glioma xenografts, we determined how well systemically delivered GLV-1h68 replicated in the shielded left flank tumor compared with the right flank tumor exposed to 6 Gy. Of the 6 mice treated with systemic GLV-1h68 and focal IR to the right flank, all 6 mice had higher GFP expression in the irradiated exposed right flank tumor than in the shielded left flank tumor. A representative mouse is shown (Fig. 5B). GLV-1h68 GFP expression was higher in the irradiated right flank tumor (top flank) than in the shielded left flank tumor. GLV-1h68 luciferase expression was 2-fold higher in the irradiated flank tumor than in the contralateral-shielded flank tumor. Finally, bilateral xenografts were harvested on day 9 and tumor sections were stained for VACV protein. Similar to viral GFP and luciferase expression, immunohistochemical staining revealed a greater distribution of VACV protein in
the irradiated right flank tumor than in the shielded left flank tumor (Fig. 5C).

Discussion

In this series of studies, we have shown that how focal IR can be incorporated with systemically delivered oncolytic VACV to increase viral replication and enhance tumor xenograft regression. Focal IR increased replication of systemically injected oncolytic VACV GLV-1h68 and LIVP 1.1.1 in the targeted irradiated tumors compared with nonirradiated glioma xenografts. More importantly, we showed for the first time that focal IR can result in preferential replication of systemically delivered oncolytic viruses in a preirradiated tumor target compared with nonirradiated tumors using a bilateral murine tumor model system. Interestingly, there was qualitatively a more diffuse GLV-1h68 GFP signal and quantitatively increased GLV-1h68 luciferase activity in the preirradiated right flank glioma xenografts compared with the shielded left flank U-87 xenografts. To our knowledge, this is the first demonstration that focal IR resulted in preferential oncolytic viral replication in an irradiated tumor xenograft compared with nonirradiated tumor xenograft in the same animal. In this treatment paradigm, systemically delivered oncolytic viruses are not sensitizers for radiotherapy as is the case with systemically delivered chemotherapy. Instead, IR functions as an oncolytic viral sensitizer to promote viral replication within targeted irradiated tumors.

The safety concerns in the use of oncolytic viruses have prompted the generation of oncolytic viruses genetically engineered with multiple mutations (3, 7). We and others have shown that increased viral attenuation for safety comes at the cost of antitumor efficacy. Our data reported here, with LIVP 1.1.1 and GLV-1h68, are further proof of this concept. The less attenuated LIVP 1.1.1 replicated more efficiently in glioma cells and translated in a more profound U-87 xenograft delay than in GLV-1h68. One approach to improve the therapeutic efficacy of attenuated oncolytic viruses is to create a more favorable environment for viral replication. Immunomodulation with cyclophosphamide or rapamycin has previously been shown to enhance the efficacy of systemically delivered oncolytic VACV in experimental glioma models (27). Our results showed that the combination of IR and LIVP 1.1.1 resulted in a robust proinflammatory response. In particular, MCP-1, MCP-3, IL-18, and IP-10 were found within higher levels in the tumor than seen with IR or LIVP 1.1.1 alone. In a pancreatic tumor model, these 4 cytokines were also observed to be upregulated by GLV-1h68 infection at day 21 and 42 postinfection (28). Immunohistochemical staining of infected tumors in previous studies showed an intense peri- and intratumoral infiltration of mononuclear cells, indicating the activation of innate immune mechanisms (23). We proposed that tumor regression induced by GLV-1h68 was, at least partially, mediated through activation of innate immune mechanisms. Our results here show the combination of IR to LIVP 1.1.1 resulted in high expression of these cytokines by as early as 7 days postinfection. These results suggest that the combination of IR and oncolytic VACV can activate a proinflammatory tumor response. Further studies are currently underway to determine whether cytokine expression mediates an inflammatory tumor response or is secondary to enhanced oncolytic VACV replication mediated by IR.

Data presented here indicate that IR has an ability to enhance the replication of oncolytic VACV that is not restricted to a specific viral mutant. Both GLV-1h68 and wild-type LIVP 1.1.1 replicated to higher titers in irradiated U-87 xenografts. One explanation for how focal tumor IR can enhance systemically delivered VACV replication in tumor xenografts is that IR increases the vascular permeability in tumors allowing increased extravasation of oncolytic VACV into irradiated tumor xenografts. However, in our experimental tumor model system, we were not able to detect any significant difference in dye extravasation 24 hours following IR. Thus, focal IR does not appear to result in enhanced oncolytic VACV as a result of IR altering the tumor vasculature.

The ability of IR to enhance oncolytic VACV replication also appeared to be specific to tumor cells and not normal tissue. Following focal irradiation of tumor xenografts and LIVP 1.1.1 delivered systemically, there was no increase in viral particles recovered from nonirradiated normal tissue (spleen, liver, lungs, and brain; data not shown). Moreover, when normal tissue was focally irradiated in non–tumor-bearing mice and GLV-1h68 systemically injected, the irradiated normal tissue had no viral particles detected by viral titration (data not shown). This suggests that IR-enhanced VACV replication maintains the tumor selectivity of oncolytic VACV.

To characterize the interaction of oncolytic VACV with IR, we investigated how IR delivery sequence and IR dose influenced oncolytic VACV replication. To determine whether a temporal relationship existed with IR and oncolytic VACV, a single 6 Gy fraction of IR was delivered either 1 day before or after systemic viral injection. Both temporal sequences of oncolytic virus and IR enhanced viral replication and tumor xenograft regression. While IR delivered 1 day before GLV-1h68 infection had an earlier peak (day 7) of VACV GFP expression within tumors, by day 14 postinfection VACV GFP tumor expression was similar if IR was given 1 day before or after GLV-1h68 infection. One explanation for these results is that IR delivered 1 day after VACV replication may prime cells for infection by progeny virus released by the first wave of tumor infecting VACV.

Clinical radiotherapy is delivered in a fractionated scheme for gliomas. When the BED of IR was held constant, 2 fractions of 3.5 Gy produced a similar glioma xenograft growth delay as a single 6 Gy fraction alone. When both fraction schemes were combined with LIVP 1.1.1, they both resulted in increased glioma xenografts regression and more diffuse LIVP 1.1.1 spread in U-87 xenografts. Thus, it appears that oncolytic vaccinia virus could be incorporated into either larger hypofraction or more conventionally...
fractionated IR. The choice of radiotherapy fractionation scheme would be dictated by clinical relevance and surrounding normal tissue constraints.

Here, we have shown a rationale and use of combining focal IR with systemic oncolytic virus administration. Currently, IR is routinely combined with systemically delivered cytotoxic chemotherapies such as cisplatin and temozolomide or targeted agents such as erlotinib and cetuximab. Here, we showed that IR could act as a sensitizer for tumor oncolysis by VACV. Our preclinical results have implications in how focal radiotherapy can be combined with systemic oncolytic viral administration for locally advanced tumors. Focal IR to tumors can provide a spatial target for systemically delivered oncolytic virus. Such “radio-painting” of tumors may result in preferential and enhanced oncolysis within the irradiated tumor target while sparing surrounding normal tissue. Furthermore, clinically this may lead to reduction of both viral and radiation dose resulting in safe and efficient tumor regression.

Disclosure of Potential Conflicts of Interest

N.G. Chen, U. Geissinger, Q. Zhang, Y.A. Yu, R.J. Aguilar, and A.A. Szalay are salaried employees of Genelux Corporation and have personal financial interest in Genelux Corporation. No potential conflicts of interest were disclosed by the other authors.

References


Preferential Replication of Systemically Delivered Oncolytic Vaccinia Virus in Focally Irradiated Glioma Xenografts
