Ionizing Radiation Improves Survival in Mice Bearing Intracranial High-Grade Gliomas Injected with Genetically Modified Herpes Simplex Virus

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ABSTRACT

Malignant gliomas remain incurable with current interventions. Encouraging investigational approaches include the use of genetically modified herpes simplex-1 (HSV-1) viruses as direct cytotoxic agents. Combining attenuated HSV-1 with standard therapy, human U-87 malignant glioma xenografts grown in the hind limb or intracranially in athymic nude mice were exposed to ionizing radiation, inoculated with genetically modified HSV R3616, or received both virus and radiation. The combination of virus with fractionated ionizing radiation suggests a synergistic action and results in reduced tumor volumes and longer survivals when compared with treatment with either modality alone.

INTRODUCTION

The current treatment for malignant astrocytoma, the most common primary tumor of the central nervous system, consists of surgery followed by radiation therapy. The median survival for the most malignant astrocytoma, GBM,1 (1) remains approximately 1 year (2). Astrocytomas recur in more than 75% of treated patients within a 2-cm margin of the primary tumor (3). These observations suggest that extending effective therapy beyond the margins of resection will improve survival. Experimental therapies in the treatment of gliomas include the use of retroviruses as shuttle vectors and viruses as direct tumoricidal agents. Retroviruses used in the treatment of GBM encode enzymes such as the HSV thymidine kinase, which converts the prodrug gancyclovir to toxic metabolites in retrovirally infected cells. This strategy relies heavily on a bystander effect because only a small proportion of tumor cells are infected with retrovirus (4–6). A second strategy uses genetically modified HSV that has been engineered to replicate preferentially within dividing tumor cells, compared with neurons. Because of the risk of encephalitis, HSV has been modified by either deletions in viral genes encoding enzymes for viral DNA synthesis (thymidine kinase and ribonucleotide reductase) and/or deletions in the γ1 34.5 gene (7–11). The γ1 34.5 gene product allows viral replication in neurons and inhibits host shutoff of protein synthesis following HSV-1 infection. R3616, with both copies of γ1 34.5 deleted, has been the prototype for various antiglioma strategies. This virus, compared with wild-type HSV, has a replicative advantage in cycling glioma cells when compared with quiescent neurons and has a limited distribution of tissue damage along the injection site (7, 8, 11–15).

Studies on human intracranial gliomas in murine models treated with the R3616 recombinant virus show more modest antitumor effects than other more virulent mutants because the tumor cell kill is limited by decreased viral proliferation and distribution within the tumor (16). Ionizing radiation, in addition to its own cytoidal properties, interacts with R3616, resulting in enhanced viral replication, improved distribution, and tumor volume reduction (16). These studies involved large doses of 20 and 25 Gy in a hindlimb glioma model. Here we report on the effects of R3616 administration combined with clinically relevant fraction sizes of radiation in both hindlimb and orthotopic intracranial glioma models.

MATERIALS AND METHODS

Cells and Viruses. The U87-MG cell line was originally obtained from a patient with GBM and was obtained from American Type Culture Collection (Rockville, MD). The genetically engineered R3616 was derived from wild-type HSV-1(F). As described previously, it has both copies of the γ1 34.5 gene deleted (7).

Hindlimb Glioma Experiment. All of the animal work was carried out under protocols approved by the University of Chicago Animal Research Committee. U87-MG cells (1 × 107) suspended in a volume of 10 μl of sterile PBS were injected s.c. into the right hind limb of athymic nude mice. When the tumor volume reached 200 mm3, the mice were randomized to the following treatment arms: R3616 alone, radiation alone, and R3616 + radiation. Viral doses were 1 × 107 PFU per tumor. The fractional tumor volume means were calculated for each treatment arm and compared. The fractional tumor volume was...
defined as the tumor volume at the measurement time point divided by the initial tumor volume.

For irradiation, the mice were immobilized in Lucite chambers. Their whole body was shielded with lead except for the tumor-bearing right hind limb. Radiation was delivered with a Maxitron 250 X-ray generator (General Electric, Milwaukee, WI) using 150 kVp at a dose rate of 1.91 Gy/min. Fractionated irradiation consisted of 5-Gy fractions on Monday, Tuesday, Thursday, and Friday over 2 weeks to a total dose of 40 Gy.

**Intracranial Glioma Experiment.** Surgery was performed using sterile technique. The animals were anesthetized by i.p. administration of ketamine (20 mg/ml) and xylazine (0.3 mg/ml) in saline at 0.07 ml/10 g of body weight. A midline scalp incision was made, and a 0.5-mm burr hole was drilled 1.5–2.0 mm to the right of midline and 1 mm posterior to the coronal suture. The tumor cells were injected stereotactically, using a 250-μl Hamilton syringe with a prepared 30 gauge needle mounted in a Stoelting stereotaxic apparatus. A plastic sleeve surrounding the needle allowed reproducible injections of tumor cells or virus to a depth of 2.5 mm. Tumor cells (5 \( \times \) \( 10^5 \)) suspended in 5 ml of modified Eagle medium (MEM) and 5% methylcellulose were injected with a Hamilton repeatable dispenser. The needles were left in place for 2 min to minimize reflux of the inoculum along the needle track. The incision was closed with 9-mm Michel wound clips. The mice were placed on a heating pad in sterile microisolator polycarbonate cages and allowed to awaken from anesthesia. During recovery, the animals were randomly assigned to treatment groups. The day of tumor inoculation was assigned as day 0.

On day 5, the animals assigned to receive R3616 virus were inoculated with 1 \( \times \) \( 10^7 \) PFU in 5 μl of MEM. The same sterile surgical materials and procedures were used. Animals not assigned to viral groups received sham intracranial injections consisting of 5 μl of MEM. The scalp wounds were closed with nexabond glue to avoid scatter dose from metallic wound clips.

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**Table 1** Median survival times for experiments 1 and 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Estimate (days)</th>
<th>Difference (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>XRT</td>
<td>10</td>
<td>53</td>
<td>13</td>
</tr>
<tr>
<td>R3616</td>
<td>10</td>
<td>56</td>
<td>16</td>
</tr>
<tr>
<td>XRT + R3616</td>
<td>10</td>
<td>69</td>
<td>29</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>38.5</td>
<td>0</td>
</tr>
<tr>
<td>XRT</td>
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<td>2</td>
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<tr>
<td>R3616</td>
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<td>42</td>
<td>3.5</td>
</tr>
<tr>
<td>XRT + R3616</td>
<td>10</td>
<td>55</td>
<td>16.5</td>
</tr>
</tbody>
</table>

*Statistical differences across arms are \( P = 0.002 \) by the Log-rank method and 0.006 by the Wilcoxon method.

*Statistical differences across arms are \( P = 0.05 \) by both Log-rank and Wilcoxon methods.
during radiation treatment. Animals in the second experiment received a second R3616 viral or sham injection on day 12. Irradiation consisted of 30 Gy delivered in 5-Gy fractions to the whole brain, delivered on Monday, Wednesday, and Friday for 2 weeks. This dose was not intended as a curative dose. The radiation energy was 150 kVp as described above for the hindlimb experiment. Irradiation started 6 h after viral injection on day 5. This regimen has been shown to increase viral replication and spread within the tumor mass.4 Irradiated mice were immobilized in Lucite chambers. Their bodies were shielded with 3 mm of lead placed over the chamber, extending from behind the ears to the tail. The mice in this treatment regimen received their radiation fractions on Monday, Wednesday, and Friday for 2 weeks. The mice were followed until death or until they became moribund from progressive tumor growth and were euthanized. Mice euthanized for progressive tumor growth had uncontrolled tumor growing beyond the scalp. The date of death for euthanized mice was marked as the date of euthanasia.

Immunohistochemical Staining. To demonstrate tumor growth and viral replication, a separate set of animals was inoculated intracranially with 5 × 10⁵ of U87-MG cells. The mice were inoculated with 1 × 10⁷ PFU of R3616 on day 5, and half of these underwent whole brain irradiation to 30 Gy in 5-Gy fractions as above. To demonstrate viral proliferation in vivo, two mice in each group were sacrificed on days 1, 3, and 12 postinfection. Tumor harvest was performed after intracardial formalin infusion. The brains were fixed an additional 24 h at 4°C and sectioned coronally through the injection site. The tumors were then embedded in paraffin, sectioned, and stained. Immunohistochemistry studies used rabbit polyclonal antibody to HSV (PU086-UP; Biogenex, San Ramon, CA) at a dilution of 1:80.

Statistical Analysis. For the hindlimb model, a Kaplan-Meier analysis was performed comparing the three groups with respect to end points ≤ 2.5 and ≥ 2.5 times the original tumor volume. For the intracranial model, tests for homogeneity were performed for the two groups by both the Wilcoxon and Log-rank methods. A pairwise comparison was then performed for both groups using Bonferroni’s method. Survival curves were plotted for all arms in experiments 1 and 2, using absolute survivals. To test for interaction between irradiation and R3616, the statistical method of Machado and Bailey (17) was performed for both groups tested.

RESULTS AND DISCUSSION

Hindlimb Glioma Experiment. To test the therapeutic effects of R3616 and fractionated radiation on U87-MG x-
nografts, an experiment was carried out in the hind limbs of nude mice. U87-MG cells were injected s.c. into the right hind limb of 5- to 6-week-old female nude mice and allowed to grow to 200–300 mm³. The mice were then randomly allocated to three groups: (a) 2 × 10⁷ PFU of R3616 injected directly into tumor, (b) tumors irradiated locally with daily doses of 5 Gy, and (c) R3616 as described above plus local irradiation. The results for the hindlimb experiments are shown in Fig. 1. The mean times to reach a fractional tumor volume of 2.5 were 14, 42, and 59 days for the R3616, radiation, and R3616 + radiation groups, respectively (P < 0.0001; 95% confidence intervals, 10–14, 42–45, and 56–64 days, respectively). Mice receiving R3616 + radiation had longer growth delays than either the R3616 or radiation alone groups (P < 0.0001 and 0.0001, respectively).

Intracranial Glioma Experiments. To study whether fractionated radiation and R3616 is effective in an orthotopic model, we stereotactically injected U87-MG tumor cells into the right caudate/putamen of nude mice. After tumor inoculation, the mice were randomly allocated into groups of 10 mice each and treated with buffer, R3616 alone, radiation alone, or R3616 + radiation. The viral dose was 1 × 10⁷ PFU. The radiation dose was 5 Gy on Monday, Wednesday, and Friday, to a total dose of 30 Gy. A second experiment was conducted with the same number of animals. This second experiment differed from the first only in that the R3616 alone and the R3616 + XRT animals received a second injection of virus during the radiation course. Control and radiotherapy alone animals received a second sham injection at this time. The median survival times and survival differences from the control group for each U87-MG intracranial arm in experiments 1 and 2 are shown in Table 1.

There is a significant difference of treatment effect across arms for both experiments 1 and 2 (group 1, P = 0.002 by the Log-rank method and 0.006 by the Wilcoxon method; group 2, P = 0.05 by both Log-rank and Wilcoxon methods). The survival curves for group 1 are shown in Fig. 2. The data demonstrate that the significance of the differences between arms in experiment 1 by pairwise comparisons was P = 0.002, P = 0.05, and P = 0.08 for the combined treatment versus the control, XRT, and R3616 arms, respectively. Survival curves for the two-injection experiment are shown in Fig. 3, with similar results. Two mice in group 1 required euthanasia for gross progressive tumor growth. All other mice in both intracranial experiments died of tumor progression as identified by visual inspection. No mice in either study demonstrated signs of encephalitis.

Because the effect of the combination of R3616 and irradiation at 5 Gy/fraction was greater than either treatment alone, we conducted an analysis to determine whether the interaction was additive or synergistic. When statistical methods reported
by Machado and Bailey (17), based on a proportional hazards model, were used, the interaction of virus and ionizing radiation appears synergistic for both groups 1 and 2 ($P = 0.0005$ and $P = 0.0053$, respectively).

Animals in experiment 2 experienced shorter median survivals in each of the treated groups when compared with the animals in experiment 1. The reason for the shorter survivals in the second experiment is unclear. Our observation is that the third intracranial injection within a 12-day period was poorly tolerated, resulting in longer recovery times and shorter median survivals. Regardless, the results of both experiments suggest that the addition of ionizing radiation to cells containing R3616 results in a synergistic cell kill. Further study is needed to confirm these findings.

Immunohistochemistry staining for R3616 was performed to show enhanced viral proliferation in irradiated tumors. Brains containing tumor were harvested on days 1, 3, and 12 post-viral injection and stained for virus in both the R3616 and the XRT + R3616 groups. Viral staining shows infection by R3616 in both irradiated and unirradiated tumors 1 day following injection. The unirradiated tumors did not stain for virus after 3 days. However, the irradiated tumors remained positive after 12 days (Fig. 4). Viral counts were 15/hpf and 13/hpf in the irradiated group at 3 and 12 days following R3616 inoculation compared with 0/hpf in the unirradiated mice at 3 and 12 days. Thus, fractionated doses produce a 2- to 5-fold enhancement in viral proliferation similar to that seen previously with 20- and 25-Gy doses (16).

The inhibitory effects of HSV mutants on intracranial glioma models have been reported by several investigators. Martuza et al. (8) used an HSV mutant lacking the thymidine kinase gene in U87 gliomas and demonstrated improved survivals in mice infected intratumorally with HSV. Mice receiving $1 \times 10^5$ PFU of virus had longer survivals than those given $1 \times 10^3$. Mineta et al. (11) tested HSV G207 in mice harboring both s.c. and intracranial U87-MG tumors. HSV G207 caused decreased tumor growth and prolonged survivals. Chambers et al. (12) injected MT539MG glioma tumors with R3616, R4009, or saline buffer and found prolonged survivals in mice receiving either type of modified HSV versus controls.

The interaction of R3616 with ionizing radiation was initially reported by Advani et. al. (16) in hindlimb U87-MG xenografts. This study used large doses (20 + 25 Gy) of...
radiation and reported a 2- to 5-fold enhancement of viral replication and improved viral distribution in irradiated versus unirradiated tumors. Complete regression of the tumor was seen in 22 of 33 mice in the combined group versus 4 of 33 in the group receiving R3616 alone. The mechanism of enhanced HSV proliferation with ionizing radiation remains unknown. Our hypothesis is that tumor cells produce some gene product in response to irradiation that stimulates viral proliferation. Experiments are under way to delineate this mechanism.

The combination of R3616 and fractionated ionizing radiation significantly extended the growth delay versus either virus or radiation alone in hindlimb xenografts (Fig. 1). The growth delay seen in the hindlimb model correlates with the longer survivals seen in the intracranial model (Figs. 2 and 3). The advantage of using hindlimb tumors in addition to intracranial tumors is that these tumors can be measured directly for differences in volume and correlated to tumor control within the brain. Better tumor control results in longer survival. This benefit is consistent in both intracranial studies.

REFERENCES
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