Journal of Neuro-Oncology **57:** 9–18, 2002.
© 2002 *Kluwer Academic Publishers. Printed in the Netherlands.*

Laboratory Investigation

The combination of X-ray-mediated radiosurgery and gene-mediated immunoprophylaxis for advanced intracerebral gliosarcomas in rats

H.M. Smilowitz[1], J.A. Coderre[2,4], M.M. Nawrocky[2], W. Tu[1], A. Pinkerton[3], G.H. Jahng[3,5], N. Gebbers[1] and D.N. Slatkin[1,2]
[1]*Department of Pharmacology, University of Connecticut Health Center, Farmington, CT;*
[2]*Medical Department, Brookhaven National Laboratory, Upton, NY;*
[3]*Department of Diagnostic Imaging and Therapeutics, University of Connecticut Health Center, Farmington, CT;*
[4]*Department of Nuclear Engineering, Massachusetts Institute of Technology, Cambridge, MA;*
[5]*MR Unit, University of California, San Francisco-VA Medical Center, San Francisco, CA, USA*

Key words: combination therapy, gene-mediated immunoprophylaxis, gliosarcomas, radiosurgery

Summary

Rats with advanced, imminently lethal, ∼4 mm diameter, left-sided intracerebral 9L gliosarcoma (9LGS), a well characterized malignant tumor with some similarities to human high-grade astrocytomas, were used as a therapy model 14 days post-implantation of 10^4 cells. Such tumor-bearing rats die within two weeks (median, 6 days) thereafter if untreated. However, if these tumors are exposed on day 14 to 12–25 Gy of an electron-equilibrated 6 MV photon beam (radiosurgery), survival is extended about 5–6 fold to a median of 34 days, but long-term survival (>1 year) is increased only to ∼18%. Multiple subcutaneous inoculations of radiation-disabled 9LGS cells post-radiosurgery (immunoprophylaxis) extended lifespan and long-term (>1 year) survival minimally (median, 37 days; 25%, respectively). In sharp contrast, radiosurgery followed by multiple subcutaneous inoculations of radiation-disabled 9LGS cells that had been transfected with granulocyte macrophage colony stimulating factor (GMCSF), a cytokine with demonstrated immune-enhancing properties (i.e. gene-mediated immunoprophylaxis, GMIMPR) increased long-term survival to ∼67%. To our knowledge, these results are the first to show that the combination of photon radiosurgery and GMIMPR is effective for an advanced, imminently lethal brain tumor in a mammal. These data raise the possibility that GMIMPR following radiation therapy might prove effective for the treatment of some human malignant gliomas.

Abbreviations: 9LGS – 9L gliosarcoma; IMPR – immunoprophylaxis; GMIMPR – gene-mediated immunoprophylaxis; FWHM – full width at half maximum; MRI – magnetic resonance imaging; GBM – glioblastoma multiforme; MRT – microbeam radiation therapy; BNCT – boron neutron-capture therapy; GMCSF – granulocyte macrophage colony stimulating factor; Gy – gray; sc – subcutaneous; ic – intracerebral; im – intramuscular; UCHC – University of Connecticut Health Center.

Introduction

Despite improvements in radiotherapy and chemotherapy, the long-term prognosis for patients with glioblastoma multiforme (GBM), which disables, and then kills about 7000 Americans per year, remains dismal. Those diagnosed with that neoplasm usually succumb to it within two years after the onset of neurological symptoms. At the time of diagnosis, which is often several days after the first serious neurological symptoms, a GBM tumor is usually large, that is, ∼3 cm in greatest diameter. Soon thereafter, as much as possible of the tumor is removed surgically, which alleviates the immediate danger of death. Postoperative fractionated photon-based radiotherapy (usually megavoltage photons generated by an

electron linear accelerator) in doses up to the limit of tolerance of normal brain structures (in effect, of normal brain endothelial and oligodendroglial cells) usually extends life. But GBM, most often a unilateral, unifocal neoplasm of the cerebrum, usually recurs within 3 cm of its original margin, presumably from microscopic nests of cells that infiltrate the surrounding edematous brain tissue [1]. Therefore adjunct therapies are needed that can be combined with postoperative radiotherapy to destroy microscopic remnants of GBM. Tumor-directed chemotherapy, inhibition of tumor angiogenesis, immunotherapy, and gene therapy are being studied. Alternative methods of postoperative irradiation, including boron neutron-capture therapy (BNCT) [2] and microbeam radiation therapy (MRT) [3], to allow more effective tumor suppression while minimizing radiotoxicity in normal brain structures, are also under investigation.

We have been using rats with advanced, clinically relevant-sized, imminently lethal malignant brain tumors as a model for human malignant gliomas. We have been testing the efficacy of immunoprophylaxis (IMPR), a form of immunotherapy, in combination with minimally palliative radiosurgery using 6 MV photons generated from an electron linear accelerator, a kind of radiation generator widely available in radiation oncology clinics. We define IMPR to be active immunization to avert or delay tumor regrowth from microscopic, clinically occult nests of tumor cells remaining after macroscopic tumor-debulking procedures such as surgical excision and/or irradiation, in contrast to the term immunotherapy, which usually refers to active immunization to treat a clinically evident tumor. 'Clinically relevant' or 'imminently lethal' refers to a tumor so advanced that the median residual life span of the concomitantly untreated control animals will be no more than one third of the total time between tumor inoculation and death from tumor overgrowth. The untreated 9L gliosarcoma (9LGS), a well established, demonstrably immunogenic, malignant rat brain tumor cell line, causes death 21 ± 3 (mean $\pm$ SD) days after the intracerebral implantation in isogeneic rats of about 10^4 cultured 9LGS cells in 1 μl of culture medium. Clinically relevant therapeutic intervention is not begun until 14 days after tumor inoculation, at which time the tumor volume relative to the brain volume approximates that of human GBM at diagnosis. We have shown [4,5] that while immunization with a series of subcutaneous (SC) injections of radiation-killed unmodified 9LGS cells initiated on day 14 after tumor inoculation is therapeutically ineffective, the same regimen becomes highly effective if preceded by deliberately suboptimal boron neutron-capture therapy (BNCT, a binary treatment modality that can selectively irradiate tumor tissue [2]). To our knowledge, the use of active IMPR to successfully treat any large, clinically relevant-sized experimental brain tumor is novel [5]. Since BNCT is an experimental therapy available to only a small number of glioblastoma patients in Japan and Europe at this time, and since most radiotherapy of human GBM is mediated by megavoltage photons, we have tested the combination of IMPR with megavoltage photon irradiation of relatively large, clinically relevant-sized, imminently lethal, intracerebral rat 9LGS brain tumors. In this paper we show that, unlike the combination of BNCT and IMPR with unmodified radiation-disabled 9LGS cells, which is decidedly effective, the combination of single-fraction photon irradiation and IMPR is only marginally effective. However, in sharp contrast, the combination of photon irradiation and granulocyte macrophage colony stimulating factor (GMCSF) gene-mediated IMPR (GMIMPR) is highly effective, presumably due to the immune-enhancing properties of GMCSF [6–8]; of a large number of immunomodulators tested, GMCSF was the most potent inducer of anti-tumor immunity. To our knowledge, these results are the first to demonstrate the therapeutic efficacy of the combination of photon-based radiosurgery and GMIMPR for an advanced, imminently lethal malignant glioma in any species. Thus, these data raise the possibility that GMIMPR following radiation therapy may prove useful in the treatment of some human malignant gliomas.

Materials and methods

Cell lines, animals, anesthesia, cell culture, intracranial implantation of 9LGS cells

All protocols used were approved by the UCHC animal care committee, protocol # 98-002 and were described previously [4,5].

GMCSF transfectant

A rat GMCSF expression vector, in which rat GMCSF PCR product was cloned into the eukaryotic expression vector pCR3 (Invitrogen), was generously provided by Dr. Martin Oaks, University of Wisconsin Medical School, Milwaukee, WI [9]. Transfections were performed using a Eukaryotic Transfection Kit

(Stratagene Corp., La Jolla, CA). Supernatants from 9LGS-GMCSF transfected and 9LGS control cultures were collected after 24 h of post-confluent culture and were compared with known quantities of recombinant rat GMCSF for both: (a) their ability to support the growth of FDC-P1 cells (American Type Culture Collection), which require GMCSF for their growth and viability [9] and (b) the presence of GMCSF by capture ELISA (R & D Corp.) Robust transfectants were subcloned. The subclone 9LGMCSF 27-1 ((a) ~150 ng GMCSF by the FDC-P1 test and (b) ~375 ng GMCSF by capture ELISA/10^6 cells/24 h) was used for these experiments. GMCSF was undetectable in supernatants from control cultures using both assays.

Magnetic resonance imaging (MRI)

MRI was performed in a 1.5 T whole-body clinical scanner (Siemens Vision) with a human extremity (knee) coil. High-resolution turbo spin-echo sagittal imaging was used for selecting slice position with imaging parameters as follows: repetition time (TR) = 3000 msec; echo time (TE) = 15 msec; field of view (FOV) = 100 mm; matrix size = 256 × 256; slice thickness (TH) = 2 mm; total number of slices = 9. For collecting tumor images, conventional spin-echo T1-weighted axial images were collected at various times after a single ip injection of 0.2 ml of the undiluted contrast agent gadodiamide (287 mg gadodiamide/ml; Omniscan, Nycomed, Princeton, NJ) with imaging parameters as follows: TR = 660 msec; TE = 15 msec; TH = 2 mm; FOV = 80 mm; matrix size = 256 × 256; imaging times = 3 min/scan; resolution = 0.313 mm.

Subcutaneous (sc) injection of gamma-irradiated 9LGS cells

9LGS cells or GMCSF-transfected 9LGS cells were removed from culture. Cell clumps were disrupted by gentle hydrodynamic shearing through a Pasteur pipet. A suspension of 5×10^7 cells per ml was exposed to 50 Gy (0.765 Gy/min) in a cesium-137 Gamma Cell 40, (Nordion). Five million irradiated cells in 0.1 ml of medium were injected sc through a 25-gauge needle into the left flank of an anesthetized rat within 1 h after radiosurgery. The injections were repeated after 7 days and every two weeks thereafter as described.

Megavoltage photon irradiation

Therapy was initiated 14 days after intracranial implantation of 9LGS tumor cells. The irradiation field, which straddled the site of tumor injection, contained much peritumor brain tissue as well as intracranial, extracerebral tissue. Irradiations were performed using 6 MV photons from a Varian 2100C linear electron accelerator (LINAC) at the Department of Radiation Oncology, UCHC. Since the accelerator is used exclusively for patients on weekdays, irradiations were performed on Saturdays. The anesthetized rats were positioned on a mobile rat cradle (Figure 1A) within a clear Lexan™ plastic box during irradiation. Anesthesia was by im ketamine/xylazine (50/5 mg/kg, respectively). The beam was geometrically collimated to be approximately 1.2 cm × 1.2 cm square in cross-section. The FWHM boundary represented by the two vertical dashed lines in Figure 1F was also approximately square, about 0.8 cm × 0.8 cm in cross-section. The average whole-body radiation dose of ~1 Gy was readily tolerated. Irradiation times of ~10 min, reflected a maximum head-entrance dose rate of ~2.5 Gy/min in the scalp around the central axis. Because of the relatively shallow depth of the tumor <1 cm deep, the Lexan™ box was fitted with a 9-mm-thick Lexan™ lid which was used as a beam spoiler [10] to achieve electron equilibration and, thereby, approximate uniformity of physical absorbed dose with increasing depth in each vertically irradiated tissue column. The maximum head-entrance radiation doses were determined from measurements with a miniature Markus tissue-equivalent ionization chamber. In a preliminary study, three normal rats each received maximum head-entrance absorbed doses of 15, 20 or 25 Gy, respectively as described. All were alive and apparently well one year later. Near the margins of the full width at half maximum (FWHM) boundary, 4 mm from the field's central axis, absorbed head-entrance doses were by definition half-maximum. Since we wished to deliver a proven maximally tolerable radiation dose, 25 Gy_{max} was used in the LINAC/IMPR studies reported here. The prone rat's body was rotated 45 degrees (i.e. roll angle, 45 degrees counterclockwise around its long anatomical body axis) in the rat cradle (Figure 1A–F). The radiation field was centered about 1 mm to the right of the midsagittal plane (Figure 1F) and about 9 mm caudal to the interocular plane (Figure 1B). The interocular plane is defined here as the anatomic coronal plane that bisects both eyeballs. Just before irradiation, the rat's head was manually

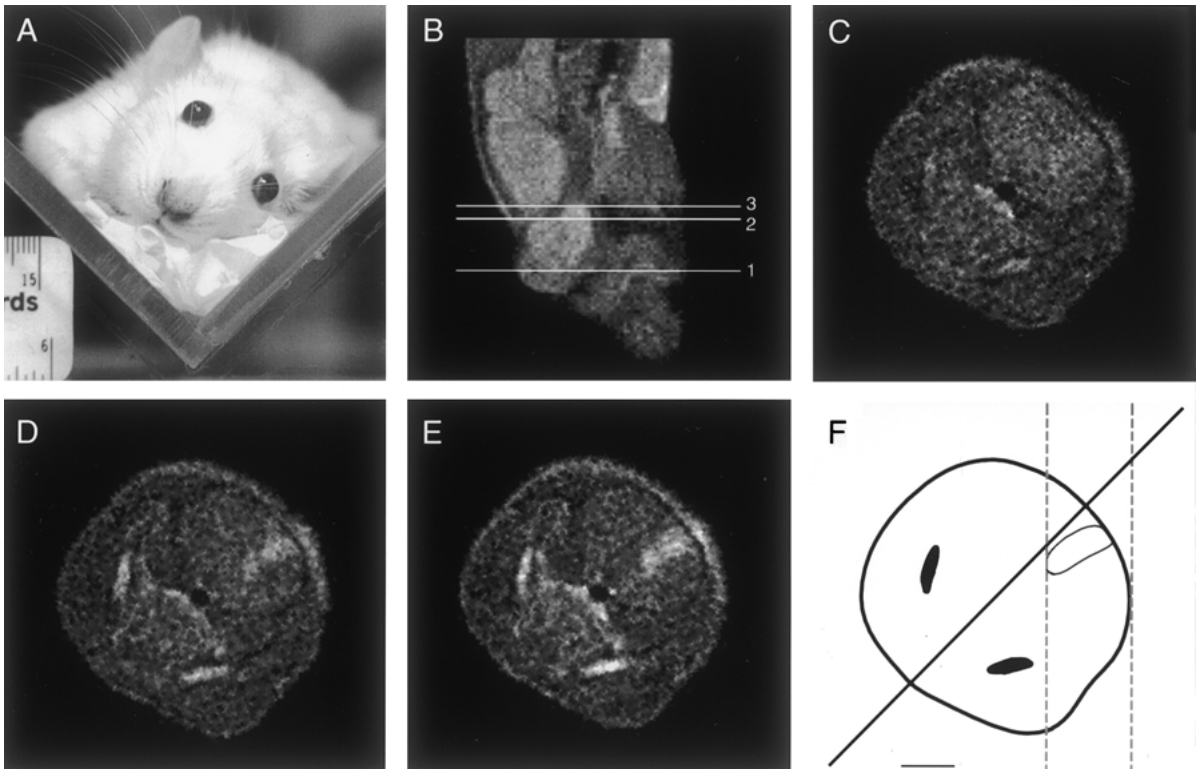

Figure 1. MRI (T1 images) and irradiation of a rat with an advanced intracerebral 9LGS. Intracerebral 9LGS tumors were initiated as described in the section Methods. Imaging is at 13.5 days post-cell implantation. Ketamine/xylaxine anesthesia was used (see section Methods). (A) An antero-posterior view of a rat's head in the movable rat cradle is shown. The rat head, bolstered by crumpled tissue paper, is cradled between two planes of the holder, each plane of which is angled 45 degrees from the horizontal. The head is positioned so that the line joining the apparent centers of the eyeballs is positioned approximately parallel to the plane of the holder upon which the rats jaw primarily rests. Since the interocular plane (see section Methods) is positioned perpendicular to the rat's body axis, all the parasagittal planes of the rat are approximately perpendicular to that same plane of the holder during irradiation. A millimeter scale is seen below the right forefoot of the rat, which is magnified ~50% less in frame A than are the linear scales of frames C–F. (B) Parasagittal slice through the tumor centered ~4 mm to the left of the midsagittal plane. The position of the 2-mm-thick coronal slice of the rat's head imaged in frames C–E is shown here between lines 2 and 3. In the rat, line 3 is 1.0 cm posterior to line 1, the mid-ocular plane. The tumor, located ~7–10 mm posterior to the mid-ocular plane, is seen as a dark zone extending as a band deep into the striatum from the surface of the brain. (C) Coronal head slice (2-mm-thick) image centered 8 mm behind the mid-ocular plane, acquired immediately after the ip injection of 0.20 ml of a 287 mg/ml solution of gadodiamide (Nicomed ™) into the ~190 g rat. (D) ~8 min after ip gadodiamide. Note the enhancement in contrast of the left striatal tumor. (E) ~16 min after ip gadodiamide. Note the increased contrast-enhancement of the left striatal tumor. The tumor measures about 2 mm × 6 mm in this ~2-mm-thick coronal slice of the rat's head. The resultant swelling of the left striatum is indicated by its displacement of the right striatum farther to the right. (F) A line drawing showing the apparent path of the high-energy photons (dotted lines) within the 8-mm-wide (FWHM) irradiation field after manual positioning as described above. The horizontal line represents 5 mm in the rat here as well as in frames C–E.

positioned and supported by crumpled tissue paper in the movable cradle so that its interocular plane was perpendicular to the long axis of its body. Weight loss was slight (median, 3% and maximum, 8% of the body weight on the day of irradiation) and transient (3–5 days after irradiation). Subcutaneous fluid supplementation was neither required nor given.

Combination of LINAC-based megavoltage photon irradiation and either IMPR or GMIMPR

Half of the rats received sc injections of 5×10^6 irradiated unmodified 9LGS cells (IMPR) or 5×10^6 irradiated GMCSF-transfected 9LGS cells (GMIMPR) immediately after 25 Gy_{max} LINAC therapy, one

week later, and every two weeks thereafter, as indicated.

Measurements and statistics

The rats were randomly assigned to each treatment Group following ic implantation of 9LGS cells. The difference between Kaplan–Meier survival plots was analyzed for statistical significance by the log-rank test [11].

Results

Magnetic resonance imaging of an advanced intracerebral 9LGS in a rat

Intracerebral 9LGS tumors were initiated as described. Imaging of the tumor (Figure 1B–E) was at 13.5 days post-cell implantation. Figure 1B is the image of a 2-mm-wide parasagittal slice, 3–5 mm to the left of the midsagittal plane. The tumor is centered ∼8 mm posterior to the mid-ocular coronal plane (line 1, Figure 1B). This image was taken about 5 min after ketamine/xylazine anesthesia (see section Methods). The dark zone 8–10 mm posterior to the mid-ocular slice (between lines 2 and 3, Figure 1B) evidently contains non-CNS soft tissue (presumably, tumor tissue) extending in a band deep into the striatum from the surface of the brain. The distance between lines 2 and 3 represents 2 mm in the rat. Half an hour later, 0.20 ml of a 287 mg/ml solution of gadodiamide was injected ip into the anesthetized, 190 g rat. MRI scans are shown immediately after administration of the ip gadodiamide (Figure 1C), then 8 min and 16 min after ip gadodiamide (Figure 1D,E, respectively) in the same 2-mm-thick coronal slice. Highest signal intensity was seen 16 min after ip gadodiamide (Figure 1E). The tumor in that slice measured ∼2 mm × ∼6 mm. The coronal MRI images (Figure 1C–E) are rotated 45 degree counterclockwise just as the rats were rotated during irradiation (Figure 1A). The middle of the skin-entrance zone of the collimated photon beam was placed approximately 1 mm to the right of the midsagittal plane of the rat's head (Figure 1F). From these images, it is seen that the most superficial tumor tissue is centered about 4 mm to the left of the midsagittal plane, and that the 8-mm-wide zone within the FWHM boundary would encompass such a tumor macroscopically in its entirety (Figure 1F). However, the absorbed dose at the anatomic depth of the tumor was about half of the maximum dose because there it grazed the FWHM boundary. Some deviations from nominal tumor dosimetry, therefore, would be expected due to minor variations in the positioning of rats and/or variations in the macroscopic growth pattern of the tumor from one rat to another.

LINAC-based megavoltage photon palliation for advanced intracerebral 9LGS in rats without IMPR

10^4 9LGS cells were injected ic (12–14 rats per group) into the left striatum in 1 μl of culture medium as described. Fourteen days later, when the resultant brain tumors were expected to be about 4 mm in diameter (∼40 mg) (12; D.D. Joel, personal communication), the rats were anesthetized and the tumors were irradiated with megavoltage photons to a physical absorbed head-entrance dose of 25 Gy_{max}. Seventy-six rats have been treated this way over the course of six experiments (N = 12, 12, 12, 13, 13, 14). In each experiment, another group of rats harboring brain tumors (5–6 control rats) was not irradiated. The results, Figure 2, show that, in the absence of radiation treatment, 97% of the rats die ∼2 weeks (median survival: 6 days) after the day of therapy, that is, ∼3 weeks after tumor cell implantation. Irradiation alone extends life

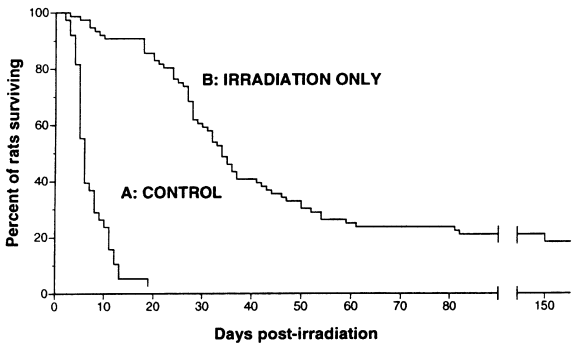

Figure 2. Linear accelerator-based megavoltage photon irradiation for advanced rat brain tumors. Kaplan–Meier plot showing the fraction of surviving rats vs. days after therapy. Fischer 344 rats received an ∼1 μl ic injection of 10^4 9LGS cells 14 days before treatment (see section Methods). Fourteen days later, day 0: (A) Untreated control group, n = 38; (B) Radiation-only group, n = 76, 25 Gy_{max} irradiation given as a single (radiosurgical) fraction. The data of both groups A and B represent the combined results of six similar trials implemented at different times.

over fivefold (median: 34 days); long-term survival is $18 \pm 12\%$ (mean$\pm$SD). Approximately 8% of the irradiated rats died within the same time frame as that of the control rats.

The combination of LINAC-based megavoltage photon palliation with IMPR for advanced intracerebral (ic) 9LGS in rats

Two experiments were performed combining megavoltage photon therapy followed by IMPR for advanced intracerebral 9LGS. Of the ~30 rats in each experiment, 24 were anesthetized and their brains were irradiated with megavoltage photons at a head-entrance dose of 25 Gy_{max}. Twelve of these rats also received sc injections of 5×10^6 irradiated (50 Gy) 9LGS (IMPR). Figure 3 shows the combined results of both experiments. The day of death for each of the rats after the date of irradiation, day 0, and the number of rats that died on that day are shown on a Kaplan–Meier survival plot. A survival analysis using the log-rank test reveals that, of the rats that survived no longer than six weeks, those that received irradiation plus IMPR died 30% later than those that received only LINAC irradiation ($p < 0.045$). Although there are twice the number of long-term survivors in the irradiation plus IMPR group as in the irradiation-only group (6 vs. 3), statistical significance of the difference at the >90% confidence level was not quite achieved. One of these irradiation plus IMPR long-term survivors died on day 236. No tumor recurrence was seen, but radiation damage resulted in shrinkage of brain tissue, behavioral abnormalities and weight loss. Long-term survivors in both groups survived for >12 months. If the radiation plus IMPR survival curve (Figure 3) is superimposed on the aggregate radiation-only curve (Figure 2), the two curves merge; there is no statistically significant difference between them.

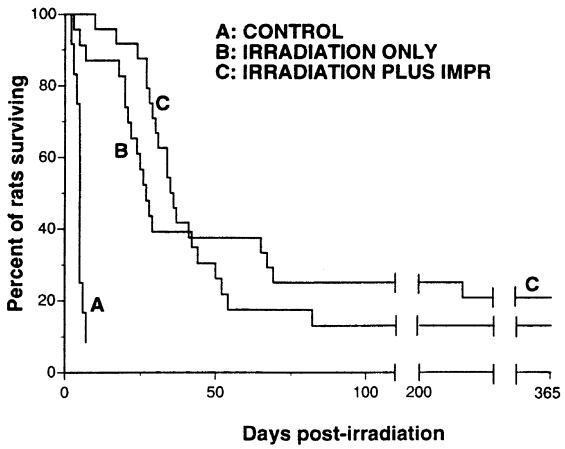

Figure 3. Combination of megavoltage photon irradiation and IMPR for advanced rat brain tumors. Kaplan–Meier plot showing the fraction of surviving rats vs. days after therapy. Fischer 344 rats received an ~1 μl ic injection of 10^4 9LGS cells 14 days before treatment (see section Methods). Fourteen days later, day 0: (A) Untreated controls, $n = 13$; (B) Radiation-only given as a single fraction, 25 Gy_{max}, $n = 24$; (C) Radiation plus multiple injections of 5×10^6 irradiated (50 Gy) cells on post-irradiation days 0, 7, 21 and every two weeks thereafter for one year, $n = 24$. The data represent the combined results of two similar trials implemented at different times. A survival analysis using the log-rank test reveals that the rats that received LINAC irradiation plus IMPR died significantly later than the rats that received LINAC irradiation ($p < 0.045$), but that difference was observed only within six weeks after LINAC therapy.

The combination of LINAC-based megavoltage photon palliation with gene-mediated (GMCSF-transfected) IMPR for advanced intracerebral 9LGS in rats

Two experiments were performed combining megavoltage photon therapy with GMIMPR for advanced intracerebral 9LGS in rats. The combined results of both experiments (Figure 4) show that, as before, irradiation alone significantly prolongs median survival (6–33 days); there are approximately 15% long-term survivors. In sharp contrast with the results using irradiated 9LGS cells (Figure 3), immunization of irradiated rats with radiation-disabled GMCSF-transfected 9LGS cells resulted in about 67% long-term survivors of all such rats in both experiments as of May 1, 2001 (day 233) at which time long-term, apparently healthy survivors in experiment 1 had survived twelve months; those in experiment 2 had survived about seven months. As of day 365 in both experiments, there were 5 rats alive of the original 27 in the radiation-only group and 16 rats alive of the original 27 in the radiation plus GMIMPR group. One of the irradiation plus GMIMPR long-term survivors (experiment 1) died on day 275. There was no tumor recurrence but shrinkage of the left striatum with anterior lateral ventricle enlargement, anorexia and behavioral abnormalities were seen. Immunization in experiments 1 and 2 continued every two weeks for nine and four months, respectively. Although the median post-irradiation lifespan of rats in the immunized group that did die is about the same as that in the radiation-only group, that is, 34 days,

the fraction of long-term survivors is about four-fold greater, a highly significant increase as determined by the log-rank test ($p < 0.0007$). If the radiation-plus-GMIMPR survival curve (Figure 4) is superimposed on the aggregate radiation-only curve (Figure 2), the two curves are distinctly different. If rats bearing advanced (14 days) 9LGS tumors are immunized with irradiated GMCSF-transfected 9LGS cells without prior irradiation ($n = 10$), they die on the same schedule (median, 6 days) as do untreated controls (median, 6 days) ($n = 9$).

Discussion

Radiotherapy for human brain tumors is generally delivered by a LINAC in thirty daily fractions of 1.8–2.0 Gy each over a six-week interval. However, such fractionated megavoltage photon irradiation of experimental, advanced, large imminently lethal (~7 days to death if untreated) intracerebral tumors in rats would be prohibitively time-consuming, labor-intensive and costly. Therefore, we adopted a radiosurgical paradigm in which a single irradiation is performed on day 14 after intrastriatal implantation of 9LGS cells, when the tumor weighed ~20–40 mg [12]; MRI on one of the rats performed on day 13.5 revealed a tumor with dimensions about 3 mm × 2 mm × 6 mm (~36 mm^3) which occupied ~2% of the ~1800 mm^3 volume of the brain of this ~190 g rat [13]. Such a single-fraction radiosurgical treatment regimen is analogous to our previous work with BNCT [5], which was also delivered in a single fraction 14 days after ic (intrastriatal) inoculation of 10^4 9LGS cells.

A 25 Gy_{max}, 8-mm^2 (FWHM), 6 MV photon irradiation (see Figure 1F) of an advanced 9LGS in the rat brain on day 14 after cell inoculation can provide significant extension of life when compared to no treatment at all (median survival of 34 days vs. 6 days post-radiation therapy, respectively) but only modest long-term survival (~18% vs. 2%, respectively). Median survival times afforded by 40–80 Gy cesium-137 whole-brain irradiations administered on day 10 post-9LGS cell inoculation administered either as ten daily fractions over two weeks or 10 twice-daily fractions administered in 5 days were shorter [14]. Immunization with either unmodified 9LGS cells [5] or GMCSF-transfected 9LGS cells, initiated on day 14 of advanced tumor growth, was ineffectual without prior radiation therapy. This is consistent with reports that GMCSF gene-mediated immunotherapy, when applied to gliomas 10 days post ic implantation, is without therapeutic benefit [15]. All of the immunotherapy studies of which we are aware that utilize tumor cells implanted in the brain employ either pre-immunization strategies using cytokines or cytokine-transfected tumor cells or inoculation regimens that are started only a few days (usually within 3 days) after tumor cell implantation ([15–19] for recent references; [5] for expanded list). For the study reported here, therapy was started on day 14 after tumor cell implantation, when the tumor was well vascularized, growing robustly, and had already occupied a significant fraction of the intracranial volume. IMPR using irradiated unmodified 9LGS cells, initiated immediately after radiation therapy (Figure 3) may have provided a statistically significant, but modest, extension of life within the first six weeks after therapy (median 29.5 vs. 20.5 days) but little or no significant increase in long-term survival (increase from 18% to 25%). However GMIMPR using GMCSF-transfected 9LGS cells (Figure 4) increased the number

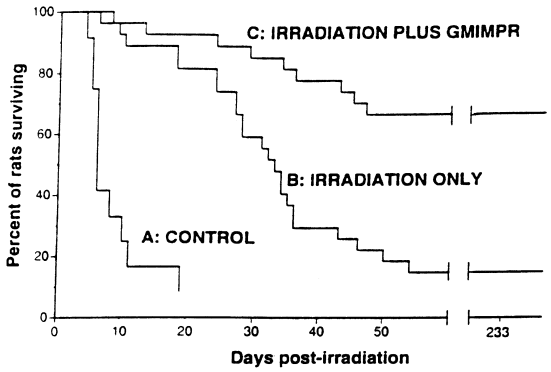

Figure 4. Combination of LINAC-based megavoltage photon irradiation and IMPR for advanced rat brain tumors. Kaplan–Meier plot showing the fraction of surviving rats vs. days after therapy. Fischer 344 rats received an ~1 μl ic injection of 10^4 9LGS cells 14 days before treatment (see section Methods). Fourteen days later, day 0: (A) Untreated controls, $n = 13$; (B) Radiation-only given as a single fraction, 25 Gy_{max}, $n = 27$; (C) Radiation plus multiple injections of 5×10^6 irradiated (50 Gy) GMCSF-transfected 9LGS cells on post-irradiation days 0, 7, 21 and every two weeks thereafter for nine months (trial 1, $n = 13$) and four months (trial 2, $n = 14$). The data represent the combined results of the two similar trials (1 and 2) implemented at different times. A survival analysis using the log-rank test shows that the number of long-term surviving rats that received LINAC irradiation plus GMIMPR is significantly greater than the number of long-term surviving rats that received LINAC irradiaton only ($p < 0.0007$) and significantly greater than the number of long-term surviving rats that received the combination of LINAC irradiation plus IMPR with unmodified, irradiated 9LGS cells ($p < 0.001$).

of rats that survived long-term from 25% to 67%. This represents the first demonstration that immunization with GMCSF-transfected autologous tumor cells in combination with megavoltage photon-mediated radiosurgery can rescue most rats that present with large (∼2% of the rat brain), otherwise imminently lethal brain tumors. Although the immunogenicity of the 9LGS cell line is readily demonstrable [4,5,20,21], its innate level of immunogenicity is evidently insufficient to rescue rats with advanced 9LGS tumors after radiation therapy. Only after *in vitro* genetic transfection with the GMCSF gene does the immunogenicity of the 9LGS tumor cell become sufficiently enhanced to enable rescue of the majority of such rats. Immunization was initiated on the day of radiation therapy, repeated one week later and every two weeks thereafter for nine months in experiment 1 and four months in experiment 2. There was one radiation-damage related death (day 275) in experiment 1 after immunization was stopped and there have been no further deaths in experiment 2 (as of July 1, 2001 or day 294). The question of how long it would be necessary to immunize patients is of great importance. Our rat data suggest that GMIMPR for four months after radiotherapy may be sufficient for maximal benefit.

There are many data from various experimental tumor systems suggesting that tumor cells engineered to secrete various cytokines, such as GMCSF [6,7] or interleukin-4 [22,23] may evoke enhanced anti-tumor immune responses ([24], for recent review), possibly by promoting dendritic cell differentiation [25] and antigen presentation to T cells by dendritic cells [8]. We are currently determining the level of the rat's immune response that is associated with efficacy in our combined radiation plus GMIMPR system. A number of other parameters of this system also merit investigation to identify parameters critical to efficacy. These include: (1) the level of GMCSF production. (Would transfectants that produce higher levels of GMCSF offer even greater therapeutic efficacy?) [26], (2) the effectiveness of exogenous GMCSF [27] and (3) different IMPR regimens such as the number of irradiated cells used, the timing of their administration and their route of administration.

The results of photon radiosurgery plus IMPR using unmodified 9LGS cells (Figure 3) can be contrasted with the combination of BNCT plus IMPR, which rescued about half the rats that would probably have died had they received BNCT alone [5, Table 1]. It is difficult to compare these two treatments directly because the radiation doses used were different and the kinds of radiation used in the two techniques are largely different. The BNCT physical dose (absorbed dose from BNCT in the boronated animal) used without IMPR, 40 Gy, resulted in 60% long-term (>1 year) survivors whereas single-fraction LINAC (25-Gy-maximum) without IMPR resulted in only ∼10–15% long-term survivors. We postulate that the superior debulking provided by the 40 Gy BNCT treatment (∼4 logs kill) compared with 25 Gy_{max} LINAC (∼3 logs kill) [28] resulted in many fewer remaining viable cells to be confronted by the immune system. Our data suggest, therefore, that, for IMPR with irradiated unmodified 9LGS cells to be as useful with photons as it was with BNCT, 9LGS cytoreduction should be about tenfold greater than was apparently achieved by our 25 Gy_{max} photon radiosurgery. Alternative scenarios include (1) the combination of photon therapy plus IMPR could become more effective if access of the tumor-reactive immune cells to the brain were substantially increased and (2) the rapidity and steepness of the cytoreduction afforded by BNCT could be a factor in the superiority of BNCT plus IMPR over LINAC plus IMPR. BNCT radiation is a complex mixture of both high and low linear energy transfer (LET) components [2]. The high-LET radiation dose delivered during BNCT may produce more rapid clonogenic cell killing and possibly a more effective immune response; to our knowledge there are few or no data on the mechanisms of cell death following BNCT compared to photon therapy or on the relative enhancement by IMPR of BNCT vs. photon therapy.

In conclusion, our studies of an advanced experimental 9LGS show that, while IMPR has the ability to significantly augment the prolongation of life afforded by either BNCT and photon radiotherapy delivered in a single fraction, the synergy of IMPR with the latter is much more effective using GMCSF-transfected tumor cells than using untransfected tumor cells. This shows that in at least one rat model of advanced malignant brain tumor, most of the animals can be treated successfully by the combination of radiation therapy and GMIMPR and lends credence to the postulate that GMIMPR following radiotherapy may prove useful in the treatment of human gliomas.

Acknowledgements

We wish to acknowledge the generous support by the Robert Leet and Clara Guthrie Patterson Trust, The Elsa U. Pardee Foundation and by the Institute

of Pathology, University of Bern, Switzerland of Henry M. Smilowitz, Ph.D., University of Connecticut Health Center, for his contributions to this work. This report is dedicated to the remembrance of William Herbert Sweet, February 13, 1910 – January 22, 2001.

Abstract presented at the AACR-NCI-EORTC International Conference of the American Association for Cancer Research (Clinical Cancer Research 7S: 3751S Nov. 2001; #484).

References

1. Hartmann M, Jansen O, Egelhof T, Forsting M, Albert FK, Sartor K: Eifluss des hirnodems auf das rezidivwachstum maligner gliome. Radiologe 38: 948–953, 1998
2. Coderre JA, Morris GM: The radiation biology of boron neutron capture therapy. Radiat Res 151: 1–18, 1999
3. Laissue JA, Geiser G, Spanne PO, Dilmanian FA, Gebbers J-O, Geiser M, Wu X-Y, Makar MS, Micca PL, Nawrocky MM, Joel DD, Slatkin DN: Neuropathology of ablation of rat gliosarcomas and contiguous brain tissues using a microplanar beam of synchrotron-wiggler-generated x-rays. Int J Cancer 78: 654–660, 1998
4. Smilowitz HM, Joel DD, Slatkin DN, Micca PL, Nawrocky MM, Youngs K, Coderre JA: Long-term immunological memory in the resistance of rats to transplanted intracerebral 9L gliosarcoma (9LGS) following subcutaneous immunization with 9LGS Cells. J Neuro-Oncol 43: 193–203, 2000
5. Smilowitz HM, Micca PL, Nawrocky MM, Slatkin DN, Coderre JA: The combination of boron neutron-capture therapy and immunoprophylaxis for advanced intracerebral gliosarcomas in rats. J Neuro-Oncol 43: 231–240, 2000
6. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC: Vaccination with irradaited tumor cells engineered to secrete murine GMCSF stimulates potent, specific, and long-lasting anti tumor immunity. Proc Nat Acad Sci USA 90: 3539–3543, 1993
7. Hodi, SF, Dranoff G: Genetically modified tumor cell vaccines. Cancer Gene Ther 7: 471–485, 1998
8. Pardoll D: Paracrine cytokine adjuvants in cancer immunotherapy. Ann Rev Immunol 13: 399–415, 1995
9. Oaks MK, Penwell T, Suh C-H, Tector AJ: Polymerase chain reaction and expression of the rat granulocyte-macrophage colony-stimulating factor. J Interferon Cytokine Res 15: 1095–1102, 1995
10. Lee PC, Thomason C, Glasgow GP: Med Phys 20: 717–721, 1993
11. Hosmer D, Lemeshow S: Applied Survival Analysis: Regression Modeling of Time to Event Data. John Wiley & Sons Inc., New York, 1999
12. Joel DD, Fairchild RG, Laissue JA, Saraf SK, Kalef-Ezra JA, Slatkin DN: Boron neutron capture therapy of intracerebral rat gliosacomas. Proc Natl Acad Sci USA 87: 9808–9812, 1990
13. Zeman W, Innes JRM: Craigie's Neuroanatomy of the Rat. Academic Press, New York, 1963
14. Kimler BF, Martin DF, Evans RG, Morantz RA, Vats TS: Combination of radiation therapy and intracranial bleomycin in the 9L rat brain tumor model. Int J Radiat Oncol Biol Phys 18: 1115–1121, 1990
15. Herrlinger U, Kramm CM, Johnston KM, Louis DN, Finkelstein D, Reznikoff G, Dranoff G, Breakfield XO, Yu JS: Vaccination for experimental gliomas using GM-CSF-transduced glioma cells. Cancer Gene Ther 4: 345–352, 1997
16. Jean WC, Spellman SR, Wallenfreidman MA, Hall WA, Low WC: Interleukin-12-based immunotherapy against rat 9L glioma. Neurosurgery 42: 850–857, 1998
17. Wallenfriedman MA, Conrad JA, DelaBarre L, Graupman PCX, Lee G, Garwood M, Gregerson DS, Jean WC, Hall WA, Low WC: Effects of continuous localized infusion of granulocyte-macrophage colony-stimulating factor and inoculations of irradiated glioma cells on tumor regression. J Neurosurg 90: 1064–1071, 1999
18. Liau LM, Black KL, Prins RM, Sykes SN, DiPatre P-L, Cloughesy TF, Becker DP, Bronstein JM: Treatment of intracranial gliomas with bone marrow-derived dentritic cells pulsed with tumor antigens. J Neurosurg 90: 1115–1124, 1999
19. Heimberger AB, Crotty LE, Archer GE, McLendon RE, Friedman A, Dranoff G, Bigner DD, Sampson JH: Bone marrow-derived dendritic cells pulsed with tumor homogenate induce immunity against syngeneic intracerebral glioma. J Neuroimmunol 103: 16–25, 2000
20. Blume MR, Wilson CB, Vasques DA: Immune response to a transplantable intracerebral glioma in rats. Recent Prog Neurol Surg 5: 129–132, 1974
21. Denlinger RH, Axler DA, Koestner A, Liss L: Tumor-specific transplantation immunity to to intracerebral challenge with cells from a methylnitrosourea-induced brain tumor. J Med 6: 249–259, 1975
22. Yu JS, Wei MX, Chiocca EA, Martuza RL, Tepper RL: Treatment of glioma by engineered interleukin-4-secreting cells. Cancer Res 53: 3125–3128, 1993
23. Giezeman-Smits KM, Okada H, Brissette-Storkus CS, Villa LA, Attanucci J, Lotze MT, Pollack IF, Bozik ME, Chambers WH: Cytokine gene therapy of gliomas: Induction of reactive CD4+ T cells by IL-4-transfected 9L gliosarcoma is essential for protective immunity. Cancer Res 60: 2449–2457, 2000
24. Mach N, Dranoff G: Cytokine-secreting tumor cell vaccines. Curr Opin Immunol 12: 571–575, 2000
25. Inaba I, Inaba M, Romani N, Aya H, Dequchi M, Ikehara S, Muramatsa S, Steinman RM: Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176: 1693–1702, 1992
26. Chang EY, Chen C-H, Ji H, Wang, T-L, Hung W, Lee BP, Huang AYC, Kurman RJ, Pardoll DM, Wu T-C: Antigen-specific cancer immunotherapy using a GM-CSF secreting allogeneic tumor cell-based vaccine. Int J Cancer 86: 725–730, 2000

27. Shi F-S, Weber S, Gan J, Rakhmilevich A, Mahvi DM: Granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by cDNA-transfected tumor cells induces a more potent antitumor response than exogenous GM-CSF. Cancer Gene Ther 6: 81–88, 1999
28. Coderre JA, Makar MS, Micca PL, Nawrocky MM, Liu HB, Joel DD, Slatkin DN, Amols, HI: Derivations of relative biological effectiveness for the high-LET radiations produced during boron neutron-capture irradiations of the 9L rat gliosarcoma *in vitro* and *in vivo*. Int J Radiat Oncol Biol Phys 27: 1121–1129, 1993

Address for offprints: Henry M. Smilowitz, Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06030, USA; Tel.: (860)679-2710; Fax: (860)679-3693; E-mail: smilowitz@nso1.uchc.edu