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Laboratory Investigation

Synergy of gene-mediated immunoprophylaxis and microbeam radiation therapy for advanced intracerebral rat 9L gliosarcomas

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Summary

Purpose: Microbeam radiation therapy (MRT), a novel experimental radiosurgery that largely spares the developing CNS and other normal tissues, is tolerated well by developing animals and palliates advanced 9LGS tumors. This report, to our knowledge, is the first demonstration that gene-mediated immunotherapy (GMIMPR) enhances the efficacy of MRT for advanced 9LGS tumors.

Methods: Seventy-six male Fischer 344 rats were implanted ic with 10^4 9LGS cells on d0. By d14, the cells had generated ~40 mm^3 ic 9LGS tumours, experimental models for therapy of moderately aggressive human malignant astrocytomas. Each of the 14 untreated (control) rats died from a large (> 100 mg) ic tumor before d29 (median, d21). On d14, the remaining 62 rats were given deliberately suboptimal microbeam radiation therapy (MRT) by a single lateral exposure of the tumor-bearing zone of the head to a 10.1 mm-wide, ~11 mm-high array of 20–39 μm-wide, nearly parallel beams of synchrotron wiggler-generated radiation (mainly ≈ 50–150 keV X-rays) that delivered 625 Gy peak skin doses at ~211 μm ctc intervals in ~300 ms either without additional treatments (MRT-only, 25 rats), with post-MRT GMIMPR (MRT + GMIMPR, 23 rats: multiple sc injections of irradiated (clonogenically-disabled) GM-CSF gene-transfected 9LGS cells), or with post-MRT IMPR (MRT + IMPR, 14 rats: multiple sc injections of irradiated (clonogenically-disabled) 9LGS cells.

Results: The median post-implantation survivals of rats in the MRT-only, MRT + GMIMPR and MRT + IMPR groups were over twice that of controls; further, ~20% of rats in MRT-only and MRT + IMPR groups survived > 1 yr with no obvious disabilities. Moreover, over 40% of MRT + GMIMPR rats survived > 1 yr with no obvious disabilities, a significant ($P < 0.04$) increase over the MRT-only and MRT + IMPR groups.

Significance: These data suggest that the combination of MRT + GMIMPR might be better than MRT only for unifocal CNS tumors, particularly in infants and young children.

Abbreviations: 9LGS – 9L gliosarcoma; BNCT – boron neutron-capture therapy; cc – cubic centimeter; CNS – central nervous system; ctc – center-to-center; d – day; ELISA – enzyme-linked immunosorbant assay; ELISPOT – enzyme-linked immunospot; ESRF – European Synchrotron Radiation Facility; GMCSF – granulocyte-macrophage colony-stimulating factor; GMIMPR – gene-mediated immunoprophylaxis; Gy – gray; ic – intracerebral(ly); IMPR – immunoprophylaxis; mm – millimeter; MRT – microbeam radiation therapy; s – second; sc – subcutaneous(ly); UCHC – University of Connecticut Health Center; w – week; yr – year

Introduction

Better therapies are needed, especially for children [1], to improve the palliation of malignant tumors with little or no added toxicity. Novel radiation therapies based on diverse physical principles, including boron neutron-capture therapy (BNCT) [2], microbeam radiation therapy (MRT) [3], and metal-enhanced radiation therapy [4], are among those under investigation. MRT, a novel form of radiosurgery [5, 6], uses high-intensity, spatially fractionated, microscopically wide X-ray beams generated with negligible divergence by wigglers in electron synchrotron storage rings [7, see below: 'Materials and methods-Irradiation']. It spares the developing CNS [8–10] and other normal tissues (J.-O. Gebbers and J.A. Laissue, unpublished observations) remarkably well. The brains of 4-day pre-hatch duck fetuses *in ovo* [8], the cerebella of suckling rats [9] and of weanling piglets [10], the chorio-amniotic membranes of chicken embryos *ex ovo* [11], and the cerebra of rats [12] and mice [13] have been irradiated from one direction by a parallel array of microbeams using target-entrance absorbed doses of 50–600 Gy. The growth and behavior of weaning piglets having cerebella irradiated

Table 1. Summary of rats used in Experiment 1 and 2 & results

	Experiment 1	Experiment 2	Total	Post-irrad. survival (median, d)	% Long-term survival
Untreated control	7	7	14	na	0
MRT only	11	14	25	25	20
MRT plus IMPR	–	14	14	25	21
MRT plus GMIMPR	10	13	23	32	44

Experiment 1 and 2 were performed 8 months apart with different lots of animals.

with relatively high entrance doses and with various combinations of microplanar widths and beam spacings could not be distinguished from those of sham-irradiated controls by veterinary specialists [10]. The microplanar beam's normal-CNS-sparing effect has been ascribed to rapid repair of microscopic lesions by minimally irradiated adjacent endothelial cells and, in the brain, possibly by adjacent oligodendroglial stem cells [12]. MRT implemented by a single array of nearly parallel microplanar beams, i.e., unidirectional MRT by itself will cure only a small fraction of rats with a moderately aggressive malignant brain tumor [14, 15]. Therefore more aggressive MRT protocols, such as biaxial MRT, may need to be developed [16–18] possibly in synergy with immunotherapy and/or other modes of cancer therapy. In this paper, we explore the synergy of combining unidirectional MRT with a form of immunotherapy termed GMIMPR.

Of immunomodulators screened in mice, GMCSF ranked among the best inducers of anti-tumor immunity [19]. When single-fraction, spatially uninterrupted (i.e., 'seamless') 6 MeV photon radiosurgery of otherwise imminently lethal 9LGS brain tumors in rats was followed by immunization with multiple sc injections of GMCSF-transfected, radiation-disabled 9LGS cells (gene-mediated immunoprophylaxis; GMIMPR), most rats survived > 1yr [20]. Post-irradiation injections of otherwise identical non-transfected cells were ineffective in prolonging survival. That was the first *in vivo* demonstration of the therapeutic efficacy of the combination of photon-based radiosurgery and GMIMPR for such an advanced, imminently lethal glioma in a mammal.

We performed the experiments described below to determine if similar GMIMPR might also enhance unidirectional MRT of 9LGS brain tumors. An abstract of this study has been published [21].

Materials and methods

Cell lines, animal anaesthesia, cell culture, intracranial implantation of 9LGS cells

Published protocols were used for these experiments [20, 22]. The transplantable rat 9LGS cell line was originally induced in a Fischer 344 rat by weekly iv injections of *N*-nitrosomethylurea [23], characterized histopathologically at the Massachusetts General Hospital, Boston [24] and subsequently serially passaged *in vitro* at the Department of Medicine, Montefiore Medical Center by Dr Victor Hatcher before transfer to the late Dr Ralph G. Fairchild at the Medical Department, BNL and subsequently to Dr H.M. Smilowitz at the UCHC [25]. Cells were maintained in DMEM medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Gemini Bio-Products), glutamine, Pen-Strep, fungizone (GIBCO BRL), removed from tissue culture flasks with trypsin/EDTA solution (GIBCO BRL) and used between passages 12 and 25. Animal use protocols sanctioned by the UCHC animal care committee, protocol #98-002, were implemented. For each experiment, on d0, after surgical reflection of the scalp, 10^4 cultured 9LGS cells in 1 μl of DMEM medium were injected ic 5 mm deep into the left striatum of anaesthetized, prone, ~175–200 g male, pathogen-free Fischer rats purchased from Taconic Farms (Germantown, NY) and housed in the UCHC animal care facility and maintained on Harlan Teklad (Madison, WI) maintenance diet #2918. On d12, the rats were transported to the ESRF from the UCHC. On d14, when the tumors were ~40 mm^3 [26, 27; D.D. Joel, personal communication] the rats were either untreated or treated with MRT-only, MRT + IMPR or MRT + GMIMPR as described in Table 1. The rats were returned to the UCHC on d16.

Irradiation

MRT and methods for implementing MRT have been described [3, 5, 6, 11, 14–18, 28, 29]. Briefly, MRT is an experimental, pre-clinical form of X-ray (mainly ≈50–150 keV) radiosurgery that delivers very large doses (~200–800 Gy) to a target using a negligibly divergent nearly parallel array of synchrotron-generated X-ray microbeams.

Such synchrotron-generated photon beams are 10^4–10^5 times more intense than those used at present for any kind of clinical radiotherapy. They are derived from compact bunches of electrons, several billion electrons per bunch, that circulate several dozen millions times per second around the 'electron-storage' ring of the ESRF, virtually at the speed of light, with energies of 6 billion electron volts per electron. A 'wiggler' i.e., a linear array of powerful magnets inserted in a straight section of the synchrotron ring produces magnetic fields of successively opposite polarities. As the electrons in the ring traverse the wiggler, they vibrate horizontally and emit a flat, extremely intense minimally divergent fan beam of synchrotron radiation tangential to the ring – hence the term 'wiggler-generated' synchrotron radiation.

In this study, rats were anaesthetized and placed prone on a computer-guided movable platform with their long axis transverse to the microbeams. Irradiation was implemented by moving the rat vertically (at an average rate of ~1.8 mm/s) through the synchrotron radiation beam produced at the ID 17 wiggler beamline of the ESRF. For this experiment, the fan beam was

collimated by the Archer multislit microcollimator [28]. The latter produced a 10.1 mm-wide, 0.5 mm-high array of nearly parallel, horizontally propagated, vertically oriented microplanar X-ray beams with widths ranging from ~20 to 39 μm; median ~27 μm [30]. There was little variation of on center intervals (~211 μm) between adjacent microbeams impinging on the rat. For the irradiations, a 100 μm-thick depleted uranium filter, sandwiched between two 3.9-mm aluminum foils, was placed upstream from the Archer collimator. The resultant beam spectra are shown in Figure 1. There was about a 2-fold reduction in beam intensity as it traversed the depleted uranium filter. The beam sustained another 2-fold reduction in intensity as it traversed the 16 mm-deep radiolucent aluminum built into the Archer multislit collimator. Concomitantly, the beam's median energy and penetrativeness increased.

The microbeams irradiated the rat's head from the anatomic left lateral direction while the rat was elevated past the array of microbeams at ~1.8 mm/s. The array was centered anteroposteriorly 9 mm posterior to the mid-interocular coronal plane. The prone rat was tilted slightly upward so that the plane tangent to the scalp over the slightly convex calvarium was horizontal and approximately parallel to the plane of the fan beam; the entire field irradiated was bounded by a pair of horizontal planes parallel to that tangent, the upper ~3 mm above it and the lower ~11 mm below it. Each element of irradiated tissue was exposed to the 0.5 mm – high X-ray beam for an average of 283 milliseconds. This rate of upward displacement of the rat during the exposure was reduced slightly in successively irradiated rats to compensate for the slow decline in the synchrotron ring current (over ~5 h: experiment 1, 172 to 167 mA; experiment 2, 188–176 mA) to keep the imparted peak surface dose ('skin-entrance dose') invariant at 625 Gy. The effect of this technique was to transect the ~4 mm-wide rat brain tumor and an ~3 mm-wide swath of adjacent cerebrum on each side of it by an array of 49 quasi-parallel, 'unidirectional' ~11 mm-high, ~25 μm-wide microbeams at ~211 μm intervals on center.

Immunoprophylaxis

A rat GMCSF expression vector, in which the rat GMCSF PCR product was cloned into the eukaryotic expression vector pCR3 (Invitrogen) [31] was generously provided by Dr Martin Oaks, University of Wisconsin, Madison, Wisconsin. Transfections were performed using the Eukaryotic Transfection Kit (Stratagene Corp., La Jolla, CA). After 24 h, supernatants from cultures of post-confluent GMCSF-transfected and of non-transfected 9LGS cell cultures were assayed for GMCSF by capture-ELISA (R&D Corp., Minneapolis, MN) and compared with known quantities of recombinant rat GMCSF for their ability to support GMCSF-dependent cells [20]. Neither of those assays could detect GMCSF in supernatants from cultures of the non-transfected cells. Subclones of our robust 9LGS cell transfectant #27-1 (~150 ng {FDC-P1 test}/~150–375 ng {capture-ELISA test} GMCSF/10^6 cells/24 h) or of the non-transfected 9LGS cells were grown *in vitro* and used for rat immunoprophylaxis. Clumps of cells were disrupted and suspended (5×10^7 cells/ml) by gentle shearing through a Pasteur pipette and then clonogenically-disabled by exposure to 50 Gy (0.013 Gy/s) in a ^{137}Cesium irradiator (Nordion Gamma Cell 40, Kanata, Ontario). 10^6 irradiated cells in 0.1 ml of medium were injected sc through a 25G needle into the left flank of the anaesthetized rats 3 days after MRT. Injections were repeated after 7 d and every 2 w thereafter for 16 w.

Combination of MRT with either IMPR or GMIMPR

Post-irradiation immunization with multiple sc injections of radiation-disabled 9LGS cells is termed immunoprophylaxis or IMPR; post-irradiation immunization with multiple sc injections of radiation-disabled GMCSF-transfected, radiation-disabled 9LGS cells is termed gene-mediated IMPR or GMIMPR. The MRT-treated rats received the first of a series of sc injections of 5×10^6 transfected or non-transfected, irradiated 9LGS cells in their left thigh on d3 post-irradiation (Table 1). Injections were repeated after 7 d and every 2 w thereafter for 16 w. There were 7 sham-irradiated, uninjected controls in each

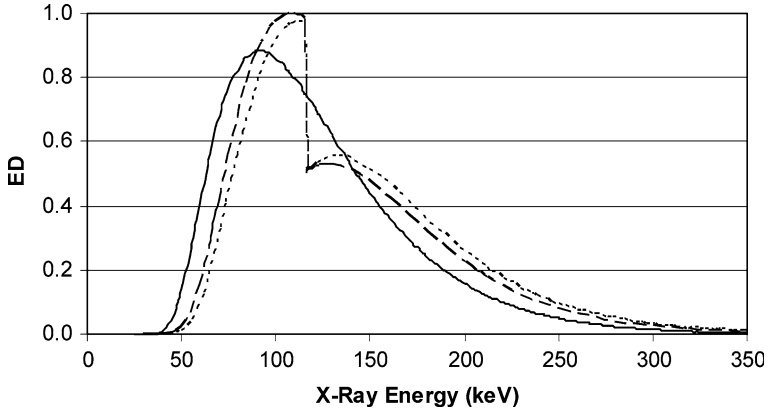

Figure 1. The ESRF ID17 spectrum weighted with the probability of energy deposition (ED) in a truncated 3–13 mm-diameter right cone of water (a rough surrogate for the tumor-bearing quarter of the rat's head) from a beam normal to its axis. The solid line results from the ESRF ID17 beam with its intrinsic filtering, the dashed line results from additional filtration through 0.1 mm depleted uranium sandwiched between two 3.9 mm Al foils, and the dotted line results from final filtration through 16 mm of aluminum in the Archer collimator. The three curves were normalized to the same total ionization energy deposited in the cone, i.e., the areas under the curves were equalized.

of the two experiments (Table 1). The rats were randomly assigned to each treatment group 1 w after the ic implantation of 9LGS cells.

Lymphocyte-enriched leukocyte preparation and ELISPOT assay

The blood was collected by cardiac puncture and spleens were removed from anaesthetized rats. Blood: 5 cc rat blood was diluted with 15 cc Dulbecco's Minimal Essential Medium (DMEM), layered on 10 cc Ficoll-Paque (Pharmacia) and centrifuged at $400\times g$ for 40 min at room temperature. The buffy coat was removed, diluted and washed twice in DMEM. Approximately 10^7 blood leukocytes were so isolated. Spleen: The spleen was minced and sifted through a 200 μm-mesh metal screen. The cells were diluted in DMEM and washed. The pelleted cells were resuspended in red blood cell (RBC)-lysing buffer (0.15 M Ammonium chloride, 1.0 mM Potassium bicarbonate, 0.1 mM Sodium ethylenediamine tetraacetic acid, pH 7.2–7.4) and incubated at room temperature for 5 min. After washing in DMEM, the cell suspension was applied to Ficoll-Paque to remove lysed cells. The buffy coat was removed and washed. Approximately 10^8 spleen leukocytes were so isolated.

Using a monoclonal (mAb) anti-rat Pan T Cell IgG1 as first antibody at 1:100 (anti-alpha, beta T cell receptor (TCR), Accurate, Inc.) and a FITC-labeled rat anti-mouse IgG1 as second antibody at 1:500 (Zymed) we determined that T cells comprised 31% of all such spleen cells and 49.5% of all such blood cells. ELISPOT assay kits for Interferon gamma (IFNγ) were purchased from U-Cy Tech, University of Utrecht, The Netherlands. Assays to detect lymphocytes secreting IFNγ were performed according to kit instructions. Briefly, 96 well ELISPOT plates were coated with 1:100 dilution of anti-rat IFNγ (capture) mAb overnight. The wells were then washed six times with Phosphate Buffered Saline (PBS)-tween-20 (0.05%) (PBST) and blocked with 1% Bovine Serum Albumin (BSA) for 1 h at 37 °C. After BSA removal, leukocytes (20,000) from blood or spleen of untreated, IMPR-treated, or GMIMPR-treated rats were added to the wells of 24-well tissue culture plates with or without 9LGS cells (40,000) and incubated in cell culture medium in a CO_2 incubator for $\leq$ 18 h. The wells were then washed with ice-cold double-distilled water for 10 min, washed 10 times with PBST, then incubated with a 1:100 dilution of biotin-labeled anti-rat IFNγ, diluted ϕ-labeled anti-biotin antibodies and finally a 1:1 mixture of activator solutions for spot development, all according to company specifications.

Table 2. Interferon gamma production by leukocytes derived from spleen and blood of 9LGS immunized (IMPR) and GM-CSF-transfected 9LGS immunized (GM-IMPR) rats measured by the ELISPOT assay

Control rats (9LGS added – 9LGS not added)	IMPR immunized rats (9LGS added – 9LGS not added)	GMIMPR immunized rats (9LGS added – 9LGS not added)
74(81)	90(38)	74(126)
89(49)	60(41)	122(70)
44(68)	592(246)	767(20)
49(49)	97(83)	55(13)
68(45)	39(39)	110(28)
47(31)	121(43)	128(20)
108(32)	202(126)	
120 (113)	98 (122)	
1(0)	65(59)	
56(76)	345(343)	

Shown are the number of spots (interferon gamma producing leukocyte) counted in wells seeded with an enriched lymphocyte preparation derived from either spleen or (blood, in parenthesis) removed from either an untreated (control, $n=10$) or 9LGS immunized (IMPR immunization protocol, $n=10$) or GM-CSF transfected 9LGS (GMIMPR immunization protocol, $n=6$) rat. Each table entry represents the difference in the total number of spots between wells that had 9LGS cells added (the sum of two duplicate wells) and wells that did not have 9LGS cells added (the sum of two duplicate wells). Leukocytes from the spleens of both IMPR treated rats (90% confidence) and GMIMPR treated rats (95% confidence) are different from control rats (Wilcoxon Non-parametric Statistics).

Histopathology

The brains of 32 rats (MRT-only, 9 rats; MRT + IMPR, 2 rats; MRT + GMIMPR, 14 rats; 7 unirradiated, tumor-bearing rats) were processed for histopathology (Table 3) as described previously [32]. Briefly, the brains were fixed in sodium phosphate-buffered formalin, embedded in 2% agar, then sliced [28] into five 2-mm-thick horizontal slabs for each rat. Each slab was embedded in a block of paraffin wax, and several horizontal sections approx. five micrometers thick cut from each block were stained by hematoxylin and eosin (HE), Luxol fast blue-PAS (LFB-PAS), Elastica Van Gieson (EvG) and silver stains (Ag) where indicated. Sections from these brains were also stained immunohistochemically for vimentin and glial fibrillary acidic protein.

Statistics

Differences between various treatment groups, displayed as Kaplan–Meier survival plots were assessed for statistical significance by the log-rank and Chi-square tests [33].

Results

The advanced 9LGS tumors were palliated despite the use of deliberately suboptimal MRT, i.e., the doses used were below those maximally tolerated by the surrounding normal CNS tissues and the beams were only unidirectional. The combined results of experiments 1 and 2, shown as Kaplan–Meier survival plots (Figure 2), indicate that unidirectional microbeam irradiation using 625 Gy peak skin-entrance absorbed doses and 211 μm ctc intervals between intensely irradiated tissue slices significantly prolongs median survival (from 7 days to 32 days); there were ~20% 1-yr survivors. However, the combination of MRT + GMIMPR provided better palliation than did MRT or GMIMPR alone. Repeated inoculation of similar rats with radiation-disabled GMCSF-transfected 9LGS cells for 4 months after identical irradiation resulted in 44% 1-yr survival. By 300 days after irradiation, 5/25 rats in the MRT-only

Table 3. Summary of rats used for histopathology data

Exposure	Experiment	Death, days	Tumor, number of nodule(s)	No tumor (Nt)
MRT only	1	28E	1	
	2	29E	1	
	1	31E	1	
	2	33D	1	
	1	57E	1	
	1	57E	1	
	1	365E		Nt
	1	365E		Nt
	1	365E		Nt
MRT plus	1	21E	1	
GM-IMPR	1	22E	2	
	1	24E	1	
	2	32E	1	
	2	38E	1	
	1	322D		Nt
	1	365E		Nt
	1	365E		Nt
	1	365E		Nt
	1	365E		Nt
	1	365E		Nt
	2	309D		Nt
	2	365E		Nt
	2	365E		Nt
MRT + IMPR	2	26E	1	
untreated control	2	365E		Nt
	2	8E	1	
	1	8E	1	
	1	8E	1	
	1	8E	1	
	2	10E	2	
	2	11E	1	
	2	12E	1	

E, euthanasia; D, died.

group and 10/23 rats in the MRT + GMIMPR group were alive. Although the median post-irradiation lifespans of rats in those two groups were about the same, 25–32 days, the fraction of long-term survivors in the latter group was > 2-fold greater. By applying the log-rank and Chi-square tests [33] to the data displayed in Figure 2, the proportion of long-term survivors that received MRT + GMIMPR was shown to be significantly greater than the proportion of long-term survivors of the MRT-only group ($P < 0.04$). Such rats, if immunized with irradiated GMCSF-transfected 9LGS cells without prior irradiation, died on the same schedule as did untreated tumor-bearing control rats [20].

Another 14 rats with similarly advanced 9LGS tumors received identical unidirectional MRT and post-MRT injections of non-transfected, irradiated 9LGS cells (IMPR) on the schedule described above (Table 1). Whereas 1 of 25 rats in the MRT-only group and 1 of 23 rats in the MRT + GMIMPR group died early (as if they had not received any treatment), 3 of the 14 MRT + IMPR rats died early. This shifted the survival curve to the left. Perhaps these rats had especially large tumors at the time of therapy. One yr after irradiation, 3/14 rats (21%) were still alive (Table 1). Long-term survivorship was thus not significantly different ($P < 0.09$) from that of the MRT-only group, i.e., 5/25 rats (20%).

ELISPOT assays for IFNγ production were performed on leukocytes isolated from blood and spleens of control rats ($n = 10$) and rats treated with either IMPR ($n = 10$) or GMIMPR ($n = 6$). Results are shown in Table 2. Seven out of ten IMPR-treated rats had greater numbers of IFNγ-producing spleen-derived leukocytes than had the corresponding control rats ($P < 0.1$); five of those seven also had increased numbers of IFNγ-producing blood-derived leukocytes. Four of the six GMIMPR-treated rats had greater numbers of IFNγ-producing spleen-derived leukocytes than had the corresponding control rats and were different from all the control rats when rank-ordered ($P < 0.05$). Three of the six GMIMPR-treated rats had greater numbers of spleen-derived leukocytes than had their paired IMPR-treated and control rats.

Histopathologically, transverse microbeam radiation 'stripes', characterized by a partial loss of eosinophilia in the neuropil in HE-stained sections [12] were seen in all irradiated rats, aligned in a parallel, equidistant array in the tissue section, reflecting the array of beams from the microcollimator that generated them. Stripes extended anteroposteriorly in both hemispheres from proximal parts of the olfactory bulbs to the thalami, including the frontoparietal lobes and the striatum; they contained fewer cell nuclei and fewer glial fibrillary acidic protein-positive fibers than did the 'valley' tissue between the stripes. Conversely, no stripes were seen in sham-irradiated rats (Table 3). All of the latter displayed large tumors, as did 6/9 rats of the MRT-only group that survived for 28–57 days, one of two rats in the MRT + IMPR group, and 5/14 in the MRT + GMIMPR group.

17/19 tumors were uninodular (Table 3), with maximal diameters ranging from 4 to 13 mm, involving the left or both hemispheres. A correlation between tumor size and post-irradiation survival time was not apparent. The vimentin-positive, glial fibrillary acidic protein-negative tumors grew expansively, but also frequently infiltrated surrounding brain parenchyma, leptomeninges, perivascular Virchow-Robin spaces and the subependymal layer, sometimes breaking into the lateral ventricle(s). The infiltration was rarely by groups of just a few tumor cells. Marked focal infiltrative growth patterns were seen in 25% of both unirradiated and irradiated rats. The 9LGS elicited peritumoral reactions, particularly in rats surviving for several weeks: Astrocytosis and gliosis were evident in glial fibrillary acidic protein- and vimentin-stained sections; increased numbers of small blood vessels, focal hemorrhage and round-cell infiltrates were often noted in the tumor periphery. Those peritumoral changes were modest in the unirradiated rats, moderate in rats treated with MRT alone, and slightly more marked in rats treated with MRT + GMIMPR.

The hemispheres of the brains of long-term surviving rats that were euthanized at day 365 and of two others surviving for 309 or 331 days (Table 3) displayed some atrophy of the left frontal cortex and the left striatum. No 9GLS tissue was detected in any of these rats, but, typically, scars were found in 8 rats, mostly located in the left striatum, near the lateral ventricle. In sections, those scars measured $\sim 3 \times 1$ mm at most; they consisted

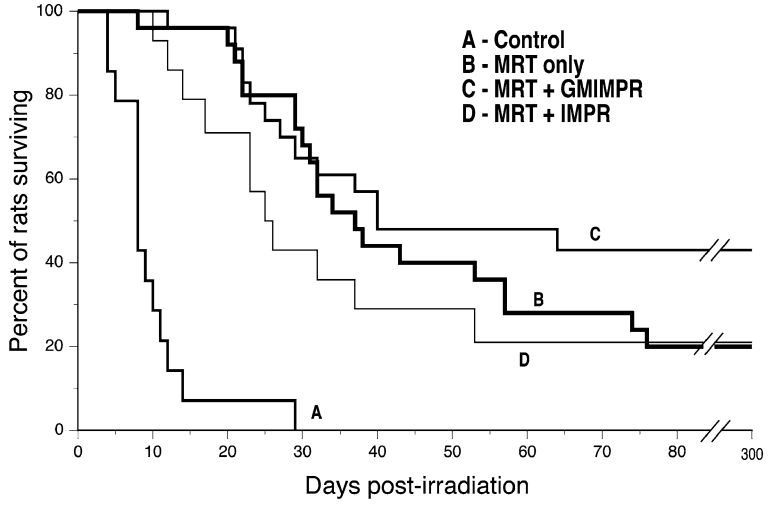

Figure 2. Combinations of MRT, IMPR and GMIMPR for advanced rat brain tumors. Kaplan–Meier plot showing the fraction of surviving rats vs. days after therapy. (A) Untreated controls, $n=14$; (B) unidirectional MRT-only given as a single-fraction ($n=25$); (C) unidirectional MRT plus multiple injections of 5×10^6 irradiated (50 Gy) GMCSF-transfected 9LGS cells (MRT + GMIMPR) on post-irradiation days 0, 7, 21 and every 2 weeks thereafter for 4 months ($n=23$); (D) unidirectional MRT plus multiple injections of 5×10^6 irradiated (50 Gy) 9LGS cells (MRT + IMPR) on post-irradiation days 0, 7, 21 and every 2 weeks thereafter for 4 months ($n=14$) The data represent the combined results of the two similar trials (experiments 1 and 2) implemented at different times (see Table 1).

of bands of hyaline collagen, stained red by EVG- and purple by LFB-PAS, often with some adjacent microcalcifications and sparse leukocytosis.

In one of the three long-term survivors studied in the MRT-only group, rather than a scar there was a 3 mm-diameter cyst in the left frontal cortex and striatum, lined by a loose network of glial tissue with many astrocytes, macrophages, the latter often PAS-positive, some containing granular haemosiderin. Two out of the 9 surviving rats of the MRT + GMIMPR group showed similar cysts, but they were in the right hemisphere, adjacent to and possibly communicating with the right lateral ventricle. Possibly, those cysts originated after irradiation as a consequence of loss of tumor and brain substance and/or gradual compensatory dilation of the right lateral ventricle. The ventricle showed a partial interruption of the ependymal layer with macrophages (some with haemosiderin) as well as unidentified small round nuclei, possibly of lymphocytes or neuroglial stem cells mainly in the subependymal zones.

Microcalcifications were frequently (92%) found in striatum and thalamus of long-term surviving rats, often bilaterally. They were seen as focal accumulations (typically about 2×1 mm overall) of spherical, intensely basophilic, PAS-positive structures, mostly with diameters somewhat larger than the bodies of large cortical neurons. The cerebral architecture of long-term survivors, as seen in LFB/PAS-stained sections, remained intact, even in microcalcified areas, but displayed stripes and, in some rats, microangiectasias – less distinctively on the right than on the left.

One of the MRT + GMIMPR-treated rats from experiment #1 died on day 331. In the right frontoparietal cerebrum, there was a superficial, thrombosed arterial aneurysm, extra-aneurysmal scarring, and perifocal small (older) hemorrhages containing some granulation tissue with foam cells. A florid fibrinoid necrosis was not present. Another of the MRT + GMIMPR-treated rats from experiment #2 died on day 309 with a dark, hemorrhagic, possibly neoplastic lesion at the base of the brain. This mostly extracerebral lesion consisted of small and medium-sized round cells closely packed and surrounded by silver-positive fibers, resembling a lymphoma histologically. There was no 9GLS tissue detected. Spontaneous lymphomas are not uncommon in aged Fischer and SD rats [34].

Discussion

We show for the first time that gene-mediated immunoprophylaxis (GMIMPR) significantly increases the fraction of long-term surviving rats (from ~20% to 44%, $P<0.04$) when administered after unidirectional, deliberately suboptimal MRT of advanced ic 9GLS tumors. Unirradiated rats bearing such advanced (14 days) tumors died on the same schedule as did untreated controls despite immunization with irradiated GMCSF-transfected 9LGS cells [20].

Previously, we showed that GMIMPR was even more palliative and even curative (long-term survivors from ~20% to 67%) when administered after LINAC-based megavoltage photon radiosurgery delivered in a seamless radiation field [20]. MRT and LINAC-based radiosurgery share the attribute of not acting synergistically with multiple injections of untransfected, irradiated 9LGS cells (IMPR). However, such a synergy can be achieved by multiple injections of GMCSF-transfected 9LGS cells for both MRT and LINAC radiosurgery [20]. The combination of BNCT and IMPR was effective [25]. Perhaps the cytoreduction effected by BNCT is sufficiently great to act in synergy with the immune response induced by multiple injections of irradiated unmodified 9LGS cells (IMPR) [22, 25] whereas the lesser cytoreduction effected by LINAC and MRT requires a greater immune response such as that induced by multiple injections of irradiated

GMCSF-transfected tumor cells. Similarly, it is possible that megavoltage photon radiosurgery kills a greater proportion of clonogenic tumor cells than does MRT and thus allows a greater degree of synergy with GMIMPR.

Consistent with such a model are experiments [27] that have compared a relatively low-blood-boron BNCT [35; *p*-boronophenylalanine (BPA)-mediated BNCT] thought to mainly affect the clonogenicity of tumor parenchymal cells with a relatively high-blood-boron BNCT [27, 36; Sodium Borocaptate-Disulfide (BSSB)-mediated BNCT] believed to affect tumor angiogenesis as well as tumor cell clonogenicity. Immediately after irradiation the clonogenicities of 9LGS cells, derived from aliquots of fresh tumor tissues removed at necropsy, were assayed *in vitro*. The data suggested that MRT [29] and BSSB-mediated BNCT eliminated a roughly 10-fold smaller proportion of clonogenic tumor cells than did BPA-mediated BNCT, for comparable percentages of tumors controlled long-term *in vivo* (Darrel D. Joel, Personal Communication; March 13, 2005). Conceivably, MRT and BSSB-mediated BNCT may exert their tumor-palliating actions not only by disabling clonogenic tumor cells but, in large part, by inhibiting the tumor vasculature. It is also formally possible that post-megavoltage photon radiosurgery GMIMPR was more effective than post-MRT GMIMPR because the former was initiated immediately following irradiation whereas the latter was initiated 3 days after irradiation. We speculate that GMIMPR implemented before radiosurgery might be even more effective in this model.

These data suggest that multiple injections of GMCSF-transfected cells tend to promote long-term survival in those rats that would otherwise die relatively late (>1 month) after MRT since the median day of death is virtually unchanged with or without GMIMPR; it is only the number of long-term survivors that increases significantly after adding GMIMPR to LINAC-based radiosurgery [20]. Thus more effective MRT, implemented either by bi-directional irradiation [18] and/or by more effective beam spacing and dosing [16], may further enhance synergy with GMIMPR by further decreasing the pool of rats that die within the first couple of months. For example, after unidirectional MRT with skin entrance doses of 625 Gy and 100 μm beam spacing, more than half of such rats died within 2 months after MRT; the remaining 35% of the rats died over the next 9 months [14]. A greater degree of short-term palliation of the 9LGS was achieved after a more aggressive form of MRT that utilized closer (75–100 μm) beam spacing and skin entrance doses of 250–500 Gy; half of such 9LGS-bearing rats survived 6 months but there were no 1-yr survivors [15].

The mechanisms underlying this observation are not yet understood. Another observation may be relevant. A preliminary safety/efficacy trial of BPA-mediated BNCT for human adult glioblastoma multiforme (1994–1999) [37] resulted in $\sim$10% $\geq$5-yr survival (Patricia A. Edwards, MSN; Upton, NY 11973, USA; personal communication), yet the median survival was similar to that expected from standard glioblastoma therapy (primary post-neurosurgery temporally fractionated megavoltage photon-based (LINAC) radiotherapy).

Although the study was not designed to systematically study the radiopathology of brains bearing 9LGS treated by MRT + GMIMPR, the opportunity was taken to study especially the brains of those rats that survived 1 yr after MRT and MRT + GMIMPR, mainly to gain some qualitative sense of the neuropathology of some brains that bore those tumors. The results confirm that the irradiation was correctly performed and that the examined unirradiated control rats all bore tumors. 9GLS tissue was eradicated in all 12 long-term survivors examined. In most long-term survivors, the less intensively, but equally extensively irradiated right hemisphere displayed minor alterations, mainly stripes and microcalcifications. Scars, little doubt the residua of 9LGS ablation, were confined to the left hemisphere, the site of tumor implantation and the entrance of the microbeam array into the brain, with some exceptions. The aneurysm of a right leptomeningeal artery in one rat was most likely due to radiation damage; vascular necrosis can be observed when longer segments of blood vessels, perhaps >100 μm-long, run parallel to the direction of propagation of the beam [12]. The cystic lesions seen in the right hemisphere of two rats are consistent with residual changes after eradication of 9GLS that had invaded the right hemisphere, although radiation damage to normal tissue cannot be entirely ruled out as their cause.

Our ELISPOT data confirm that the number of tumor-stimulated, IFNγ-producing splenic leukocytes isolated from 9LGS-immunized rats vs. non-immunized rats was increased [38]. The further increase in the number of tumor-stimulated IFNγ-producing splenocytes following immunization with GMCSF-transfected 9L cells may be analogous to the increase in IFNγ production by splenocytes immunized with Interleukin-4 (IL4)-transfected 9L cells [38] and may be related to the increased efficacy of GMIMPR compared to IMPR. Experiments are now needed to determine the mechanism for the increased efficacy of GMIMPR, and particularly to identify the species of splenocytes that secrete IFNγ after immunization with either 9L or 9LGMCSF cells. Preliminary measurements showed that about twice as many CD8+ lymphocytes isolated from the spleen of an IMPR-treated rat secreted IFNγ after incubation with 9LGS cells in culture for $\sim$18 h compared to CD8+ lymphocytes isolated from the spleen of a control rat. However, nearly 3-fold more CD8+ lymphocytes isolated from a GMIMPR-treated rat secreted IFNγ under the same conditions. Further, a low level of IFNγ-secreting CD4+ lymphocytes [38] was also detectable among splenocytes isolated from a GMIMPR-treated rat but not from a reference IMPR-treated rat. Studies of the tissue kinetics of tumor-specific vs. non-specific lymphocytes after MRT might prove useful. In contrast to tumor vessels preferentially damaged by MRT, normal blood vessels adjacent to the tumor are likely to be spared by MRT [12, 14], thus remaining available for transportation of immune effector cells to the margins of the tumor.

In this study, we filtered the broad-spectrum synchrotron-generated X-rays with a 100 μm depleted-uranium foil so as to minimize the proportion of MRT photons in the 115.6 keV [K-edge of uranium] to ~150 keV energy range. It was intended that the uranium foil sharpen the margins of tissue microslice dose profiles by reducing the median range of Compton-scattered electrons. Thus it might have enhanced the normal-CNS-sparing effect. A disadvantage of the foil may have been its reduction of the dose rate, which might have allowed extra blurring of these profiles by occult micromotion of the head during the correspondingly lengthened exposure times. To date, no advantage of such filtration has been demonstrated experimentally.

The use of the Fischer 344 rat strain may have been fortuitous, since these rats are particularly unresponsive to some antigenic and pro-inflammatory stimuli. Those rats, with their more robust hypothalamic-pituitary-adrenal axis and higher levels of endogenous corticosteroids, should be inherently more refractory than Lewis rats to immunoprophylaxis [39]. It would be instructive to study the effect of increased steroid levels on the effectiveness of post-irradiation GMIMPR for advanced brain tumors since dexamethasone is widely used to control peritumor brain edema clinically. Additional forms of anti-tumor therapy, such as anti-angiogenesis chemotherapy might complement the tumor-suppressive effects of a combination of MRT + GMIMPR.

MRT represents the third modality of radiation therapy (BNCT [25], LINAC [20], MRT [21]) that we have shown to be synergistic with GMIMPR to significantly increase long-term survival of rats with advanced 9LGS brain tumors. The ELISPOT data support the existence of immunologically based mechanisms for this synergy. The advanced 9LGS we have treated represent malignant brain tumors that have traversed the complex processes of peri- and intra-tumoral angiogenesis, peritumoral edema, microscopic centrifugal tissue invasion, and have reached a size (~4 mm-diameter) that bears nearly the same volume ratio to the normal rat brain as do neurologically eloquent human brain tumors to the human brain volume when initially diagnosed. However, advanced 9LGS tumors are only moderately aggressive and have a number of important differences from high-grade human astrocytomas. The latter are gliomas, generally more invasive, putatively less immunogenic and possibly less radiation-sensitive than is the 9LGS. For these reasons, it would be informative to extend these studies to tumor models that bear greater similarity to human glioblastoma multiforme; one such model is possibly the rat F98 glioma [40, 41].

Conclusion

Although the unidirectional MRT, used alone, palliated these advanced 9LGS tumors, 80% of those rats died within 1 yr after therapy. However, 44% of rats that received the combination of MRT + GMIMPR survived for a year after therapy. These data suggest that the combination of MRT + GMIMPR may prove superior to MRT alone. Experiments in developing animals [7–10] suggest that MRT may be useful for radiosurgery of infantile brain tumors, particularly in children under 3-years-old for whom standard radiation therapy might be contraindicated or excessively toxic [1], Therefore the combination of MRT and GMIMPR may prove superior to any broad-beam radiotherapy for unifocal CNS tumors, particularly in infants and young children.

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