Abstract

Purpose: High-grade glioma treatment includes ionizing radiation therapy. The high invasiveness of glioma cells precludes their eradication and is responsible for the dismal prognosis. Recently, we reported the down-regulation of MHC class I (MHC-I) products in invading tumor cells in human and mouse GL261 gliomas. Here, we tested the hypothesis that whole-brain radiotherapy (WBRT) up-regulates MHC-I expression on GL261 tumors and enhances the effectiveness of immunotherapy.

Experimental Design: MHC-I molecule expression on GL261 cells was analyzed in vitro and in vivo by flow cytometry and immunohistochemistry, respectively. To test the response of established GL261 gliomas to treatment, mice with measurable (at CT imaging) brain tumors were randomly assigned to four groups receiving (a) no treatment, (b) WBRT in two fractions of 4 Gy, (c) vaccination with irradiated GL261 cells secreting granulocyte-macrophage colony-stimulating factor, or (d) WBRT and vaccination. Endpoints were tumor response and survival.

Results: An ionizing radiation dose of 4 Gy maximally up-regulated MHC-I molecules on GL261 cells in vitro. In vivo, WBRT induced the expression of the β2-microglobulin light chain subunit of the MHC class I complex on glioma cells invading normal brain and increased CD4+ and CD8+ T cell infiltration. However, the survival advantage obtained with WBRT or vaccination alone was minimal. In contrast, WBRT in combination with vaccination increased long-term survival to 40% to 80%, compared with 0% to 10% in the other groups (P < 0.002). Surviving animals showed antitumor immunity by rejecting challenge tumors.

Conclusion: Ionizing radiation can be successfully combined with peripheral vaccination for the treatment of established high-grade gliomas.

Radiation therapy remains the principal approach for the treatment of adult patients with high-grade glioma and pediatric patients with brainstem gliomas, generally involving fractionated radiotherapy with total doses up to 60 Gy (1–4). The addition of concurrent temozolomide to radiotherapy has been shown to significantly prolong the life of patients with glioblastoma, but long-term survivors of this disease remains extremely rare (3).

Because of the pressing need for adjunctive therapies to enhance the effectiveness of radiation therapy for glioma, we have developed an experimental murine GL261 model that mimics the aggressive and invasive growth observed in human brain tumors (6–8) and whose growth can be monitored noninvasively by CT imaging. Using this model, we showed significant down-regulation of MHC genes in vivo in tumor cells invading normal brain tissue in comparison with the glioma cells in the core of the tumor, similar to what we had observed in human glioma (9). We hypothesized that one of the mechanisms for invading glioma cells to defeat the host immune surveillance system is down-regulating expression of MHC molecules at the growing edge of the tumor. The general hypothesis of tumor immune surveillance was originally formulated by Burnet in 1970 (10). It is now clear that there is a complex relationship between the host’s immunosurveillance system and the ability of transformed cells to escape immune recognition and destruction (reviewed in refs. 11–14). The ability of tumor cells to modulate the host’s immune response has important implications for the development of immunotherapies, including the design of effective cancer vaccines (15, 16).
Recently, there has been growing interest in the interactions between ionizing radiation (IR) and the immune system. Several studies have shown that irradiation of human and murine tumor cell lines up-regulates the expression of numerous immunologically relevant molecules, including Fas, ICAM-1, MHC class I, and the human carcinoma-associated antigens, CEA and MUC-1 (17–21), which may make them better targets for immune recognition. In the present study, we tested the hypothesis that a low dose of whole-brain radiotherapy (WBRT) of GL261 intracranial (i.c.) tumors may up-regulate MHC class I expression, as measured by the surrogate marker, β2-microglobulin, thus providing a target for a T cell–mediated antitumor immune response elicited by vaccination. We show significant increases in the long-term survival of mice with well-established i.c. GL261 gliomas treated with WBRT and vaccination, concomitant with the development of antitumor immunity. The growing awareness that IR can make tumors more amenable to immune recognition has important implications for the development of novel immunotherapies. Importantly, the more insidious invading tumor cells that invariably lead to fatal relapse may be particularly targeted as a result of this strategy.

Materials and Methods

GL261 glioma cell line and reagents. The murine GL261 glioma cell line was obtained from the National Cancer Institute-Frederick Cancer Research Tumor Repository (Frederick, MD). The cells were transfected with the plasmid encoding the gene for green fluorescent protein (GFP) according to the manufacturer’s protocol (Clontech, Palo Alto, CA) and cultured in 5% CO2 and 95% humidified air atmosphere at 37°C in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). 0.25% gentamicin (Life Technologies) and 1% l-glutamine (Life Technologies) as described (7). The GL261 cell line retrovirally transduced to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) was a kind gift from Dr. X.O. Breakefield (22) and was cultured as described for the nontransduced GL261 cells. Both GL261 glioma cell lines were split every 3 days to ensure logarithmic growth.

Flow cytometric analysis of the GL261 cell line. GL261 cells were plated (5 × 10^5/75 cm² flask) and after 48 hours, the flask were either irradiated (0, 2, 4, and 6 Gy) using a 100Gy Co source (Theratron 780-C, AECL Medical, Canada) or treated with IFN-γ (150 pg/mL). Cells were harvested 72 hours after irradiation in cold PBS containing 0.04% EDTA and processed in parallel for MHC expression by fluorescence-activated cell sorting (FACS) analysis or for mRNA gene expression by RT-PCR analysis as described below. For FACS analysis, cells were washed and incubated with phycoerythrin-conjugated anti-H2-Kb (clone AF6-88.5), phycoerythrin anti-H2-D^b (clone KH95), and phycoerythrin anti-Fas/CD95 or isotype controls (all antibodies from BD PharMingen, San Jose, CA) and cultured in 5% CO2 and 95% humidified air atmosphere at 37°C in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 0.25% gentamicin (Life Technologies) and 1% l-glutamine (Life Technologies) as described (7). The GL261 cell line retrovirally transduced to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) was a kind gift from Dr. X.O. Breakfield (22) and was cultured as described for the nontransduced GL261 cells. Both GL261 glioma cell lines were split every 3 days to ensure logarithmic growth.

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To obtain the total number of lymphocytes, we took the total number of viable cells isolated from the two pooled brains (control, 3.7 × 10^6; WBRT, 8.0 × 10^6) and multiplied by the percentage of cells in the lymphocyte gate (Fig. 3A; control, 44.5% gated represents 1.65 × 10^6; WBRT, 60.3% gated represents 4.82 × 10^6). This number of total lymphocytes was then multiplied by the percentage of cells in the lymphocyte gate positive for CD4+, CD8+, or NK1.1 to obtain the total number of lymphocytes in each subset depicted as a bar graph (Fig. 3B).

RNA isolation and reverse transcription-PCR. Total RNA was isolated from suspension cultures of GL261 cells (see above for FACS analysis) using the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s directions. RNA was quantitated by absorbance at 260 nm. Two micrograms of total RNA was reverse-transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers (Invitrogen) at 25°C for 10 minutes and at 42°C for 1 hour for CDNA synthesis. Two microliters of the reverse transcription product was used as a template for PCR amplification. PCR was done under standard conditions in a 50 μL reaction mixture containing 1 μL PCR buffer, 1 unit of Platinum Taq polymerase (Invitrogen), 200 μmol/L of deoxynucleotide triphosphate mix, 1.5 mmol/L of MgCl₂, and 150 μmol/L of H-2Kβ primers (5'-GAAAAAGAGGGGACTATGC-3', sense; 5'-TCCATAAGGCTCACAGGGAAC-3', antisense), or 25 μmol/L of β-actin primers (5'-CACAGGGGAGTGGATACATGC-3', sense; 5'-TCGAAGTGCGCACAAAG-3', antisense). The PCR conditions consisted of 3 minutes of an initial denaturation step (95°C) followed by 25 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 30 seconds), and extension (72°C, 50 seconds) followed by a final elongation step of 7 minutes at 72°C. Twenty microliters of PCR product was analyzed on 3% agarose gels stained with ethidium bromide. Quantitation of bands was done with the Bio-Rad Fluor-S apparatus (Bio-Rad, Hercules, CA) with Quantity One (version 4.2.1) software.

Immunohistochemistry. Single and double label immunohistochemistry was done using the NexES automated immunostainer and detection systems (Ventana Medical Systems, Tucson, AZ). Formalin-fixed, paraffin-embedded, 6 μm sections were deparaffinized in xylene (three changes), rehydrated through a graded series of alcohols, and rinsed in distilled water. All incubations were carried out at 37°C, unless noted otherwise.

To detect MHC class I expression in mouse tissue, sections were immunostained with rabbit anti-human/mouse β2-microglobulin antibody (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). This antibody reacts with the light chain component of MHC class I molecules (9). Antigen retrieval was done by proteolytic enzyme digestion (alkaline endopeptidase, 0.5 units/mL; Ventana Medical Systems) for 4 minutes. Endogenous peroxidase was blocked by the application of hydrogen peroxide for 4 minutes at 37°C. Anti-β2-microglobulin was diluted 1:1,000 and β2-microglobulin antibody was detected by the application of a secondary biotinylated goat anti-mouse (Ventana Medical Systems) for 8 minutes, followed by the application of streptavidin-horseradish peroxidase for 8 minutes. The chromogen, 3,3′-diaminobenzidine/hydrogen peroxide mix was
GL261 glioma cells expressing GFP were used to detect tumor cells invading the brain adjacent to the tumor. Tissue sections were immunostained with mouse anti-GFP (clone GFP01; Neomarkers, Fremont, CA). Antigen retrieval was done by microwaving tissue sections in 0.01 mol/L (pH 6.0) citrate buffer for 20 minutes in a 1,200 W microwave oven. Slides were allowed to cool to room temperature, then washed in 0.05 mol/L Tris-HCl (pH 7.6) containing 0.3 mol/L NaCl. Endogenous peroxidase was blocked by the application of hydrogen peroxide for 4 minutes at 37°C. For double label immunohistochemistry, tumor sections were first stained with β2-microglobulin. Heat-induced antigen retrieval was done as described above. Next, slides were incubated with anti-GFP antibody diluted 1:300 and incubated overnight at room temperature. GFP antibody was detected as described above using alkaline phosphatase fast red as chromogen.

To evaluate lymphocyte populations in brain tissue, tissue sections were immunostained with mouse anti-human CD3 (clone PS2, Novocastra Laboratories). Antigen retrieval was done by microwaving tissue sections in 0.01 mol/L (pH 6.0) citrate buffer for 10 minutes as described above. Endogenous peroxidase was blocked by the application of hydrogen peroxide for 4 minutes at 37°C. Anti-CD3 was diluted 1:200, incubated for 30 minutes at room temperature, and then anti-CD3 antibody was detected by the application of a secondary biotinylated goat anti-mouse (Ventana Medical Systems) for 8 minutes, followed by the application of streptavidin-horseradish peroxidase for 8 minutes. The chromogen, 3,3'-diaminobenzidine/hydrogen peroxide mix was applied for 3 minutes.

Animals and i.c. tumor implantation. Female C57BL/6 mice were obtained from Taconic (Germantown, NY) and maintained under aseptic conditions in microisolator cages in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, The Department of Health and Human Services, and the NIH. Animal studies were done under an approved protocol by the Institutional Animal Care and Use Committee. The animals were allowed food and water ad libitum. GL261 cells were cultured to subconfluence, trypsinized, washed twice in DMEM without serum, and resuspended in DMEM for inoculation into animals.

To establish i.c. tumors, GL261 glioma cells were implanted in the brains of 10- to 12-week-old female C57BL/6 mice (20 g) as described (24). Briefly, animals were anesthetized with an i.p. injection of xylazine (80 mg/kg) and ketamine (10 mg/kg) and a burr hole was drilled in the skull 0.1 mm posterior to the bregma and 2.3 mm lateral to the midline. GL261 cells (5 × 10^6/mL) in 2 μL of medium were inoculated stereotactically using a head frame (David Kopf Instruments, Tujunga, CA) in the defined location of the caudate/putamen (0.1 mm posterior to the bregma, 2.3 mm lateral to the midline) using a 10 μL Hamilton syringe (Reno, NV) with a 1-in. 30-gauge needle attached and inserted into a Kopf microinjection unit (Model 5000 with Model 5001 Hamilton syringe holder). The needle was advanced to a depth of 2.3 mm from the brain surface and the cell suspension was delivered slowly over the course of 3 to 4 minutes. Following injection, the needle was left in place for 2 minutes, after which time, it was raised to a depth of 1.5 mm below the brain surface and left in place for an additional 1 minute. Upon withdrawal of the needle, the burr hole was immediately sealed with bone wax and the incision sutured. Animals were randomly assigned into control and treatment groups (n = 5/group). Animals were observed weekly and when they showed signs of neurologic deficit (lethargy, failure to ambulate, or lack of feeding resulting in loss of >20% body weight) they were sacrificed. Prior to sacrifice, the animals were anesthetized and then perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were removed and placed in cold 4% paraformaldehyde overnight, then sliced into 2 mm coronal sections prior to processing and embedding in paraffin. Tumors were measured grossly in three orthogonal axes to determine tumor volumes. H&E-stained coronal sections were used as a check of tumor volumes and for overall tumor histopathology.

CT imaging. Animals were anesthetized with xylazine and ketamine, as described above, prior to imaging the brain using a CT scanner (GE LightSpeed QX/i). Animals received an intraorbital injection of contrast agent (100 μL; Conray, Mallinckrodt, Inc., St. Louis, MO) and within 10 minutes of contrast injection, axial images of the brain were acquired using the CT scanner to detect the presence of brain tumors. Contrasted tumors appear as regions of brighter intensity (similar to that of the bone tissue, in the animal skull) in the CT scan compared with surrounding brain tissue which appears dark due to lower intensity.

Preparation of the GL261 vaccine. To analyze the secretion of GM-CSF into the medium, cells (5 × 10^5) were plated in a 60 mm dish in 2.5 mL of medium. After 24 hours, the supernatant was harvested and either stored at −80°C or tested directly by ELISA for GM-CSF using a commercial ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer’s instructions. The GM-CSF transduced cells that we obtained secreted 100 ng/10^6 cells/24 hours GM-CSF, similar to the original report (22). For vaccine preparation, GL261 cells were irradiated with 25 Gy in 75 cm^2 flasks, using a 60Co source (Theratron 780-C, AECL Medical), trypsinized, washed, and inoculated s.c. into the left and right flanks of mice (2.5 × 10^7/100 μL/injection in the inguinal regions).

WBRT combined with GL261 peripheral vaccine treatment. In the first experiment, on day 12 post-i.c. implantation, animals were randomly assigned to four treatment groups (n = 5/group): (a) control with sham irradiation, (b) WBRT given in two fractions of 4 Gy, 48 hours apart on days 12 and 14, (c) vaccination treatment given on both flanks with irradiated (25 Gy) GL261 tumor cells secreting GM-CSF on day 14 after i.c. tumor cell implantation, (d) WBRT + vaccination treatment, where peripheral vaccination was given a few hours after the animals had received their second fraction of WBRT on day 14. WBRT was delivered to the head of the mouse centered in a 5 cm radiation field using a 60Co radiation source (Theratron 780-C, AECL Medical) on days 12 and 14 postimplantation.

In the second experiment, CT on day 15 post-i.c. implantation, when tumors were large enough to be reliably measured, was used to compute tumor volumes. Images were digitally transferred to a Varian Eclipse radiation planning system workstation and tumor volumes were calculated using the formula for ellipsoid volume. Two dimensions (in frontal plane) were measured directly and the third dimension was measured using three-dimensional reconstructing feature of the planning system. Animals were assigned to four treatment groups (n = 5/group) so that the mean tumor volumes were 3.5 mm^3 (range, 2.1-4.8) in all groups. The groups consisted of (a) control with sham irradiation, (b) WBRT given in two fractions of 4 Gy, 48 hours apart on days 15 and 17, (c) vaccination as described above on day 17, and (d) WBRT + vaccination, where peripheral vaccination was given a few hours after the animals had received their second fraction of WBRT on day 17. On day 28, tumor volume was assessed by CT. On day 60, control “naïve” 6- to 8-week-old female C57BL/6 mice or surviving animals were challenged by s.c. injection in the hind limb of 2 × 10^6 cells in 0.05 mL of serum-free media. We chose to evaluate antitumor immunity in the long-term survivors using s.c. rather than i.c. tumor rechallenge in order to assess their systemic state of immunity. Tumor growth was measured twice weekly using calipers and tumor volumes were calculated using the formula (length × width^2)/2, where the length represents the longest axis and the width was measured at right angles to the length. Animals were observed for an additional 60 days and/or sacrificed when tumor volumes reached 900 to 1,200 mm^3. Brains from “cured” animals were removed and processed for histopathology.

Statistics. Kaplan-Meier survival curves with a log-rank test were used to compare the survival proportions of the four treatment groups (control, WBRT, vaccination, and WBRT + vaccination). Differences were considered significant at P < 0.05.
Results

IR up-regulates MHC class I expression on GL261 glioma cells in vitro. We recently reported that invading GL261 glioma tumor cells in the brain down-regulate MHC class I expression, a molecule required for recognition by CTLs (9). We investigated whether irradiation could reverse this low expression, as has been reported for other cell lines in vitro (17). GL261 cells were irradiated with different doses of IR (0, 2, 4, or 6 Gy) or treated with IFN-γ (150 pg/mL) as a positive control (25, 26).

Irradiation of GL261 cells increased surface MHC class I H-2Kb expression 72 hours after irradiation (Fig. 1A). Treatment with 4 or 6 Gy induced similar increases in H-2Kb expression, whereas 2 Gy was less effective (data not shown). Expression of H-2Dβ on GL261 was undetectable on untreated cells and was not induced by radiation. Similarly, there was no induction of Fas/CD95 molecules after irradiation (data not shown). As expected, IFN-γ markedly increased the surface expression of both H-2Kb and H-2Dβ MHC class I molecules. Up-regulation of the H-2Kb MHC protein complex at the cell surface by IR or IFN-γ treatment was accompanied by increased levels of H-2Kb mRNA gene expression as assessed by RT-PCR (Fig. 1B).

IR up-regulates β2-microglobulin expression on GL261 glioma cells in vivo. GL261-GFP tumor cells were used to identify glioma cells in the brain and to test whether irradiation would increase the expression of MHC in vivo. WBRT was delivered to the brains of mice on days 12 and 14 after implantation, when tumors were ~4 ± 1 mm³ in volume (24). On the basis of our in vitro MHC expression data, we selected two 4-Gy doses 48 hours apart for the in vivo treatments. Brains were harvested 48 hours following the second fraction of WBRT and processed for immunohistochemistry as described previously (9). We used double-labeled immunohistochemistry to visualize GFP-positive tumor cells invading the brain adjacent to tumor (Fig. 2A) and expression of the light chain of the β2-microglobulin complex to assess MHC class I expression. Brains from one representative control and one WBRT animal of a group of four animals are shown (Fig. 2). In untreated mice, there was no detectable expression of β2-microglobulin by the invading tumor cells (Fig. 2B). In contrast, following WBRT, there was strong surface staining of the invading tumor cells, consistent with up-regulation of MHC class I complexes (Fig. 2C).

IR increases lymphocytes infiltrating GL261 tumors. A possible consequence of the administration of IR is the induction of an inflammatory response (27–30). To test this, mice were randomly assigned to receive mock treatment or WBRT given in two doses of 4 Gy on days 15 and 17 post-i.c. tumor implantation. Brains were harvested 7 days later and single-cell suspensions were prepared and partially purified on Percoll gradients for the analysis of lymphocyte subsets by flow cytometry (Fig. 3). Representative data from one of two groups of control or WBRT animals are shown. The contamination of control or WBRT samples by the inflammatory response (27–30) was <1% in all samples (data not shown). The numbers of CD4+, CD8+, and natural killer cells recovered from brain tumors of control and irradiated mice are shown in the bar graph (Fig. 3B). There was a 4-fold increase in the number of CD4+ and a 4.5-fold increase in CD8+ T cells in the brain tumors of irradiated mice, whereas natural killer cells showed a smaller increase compared with brain tumors of control mice.
Increased tumor-free survival of animals bearing i.c. GL261 tumors treated with WBRT combined with peripheral vaccination. Vaccination with irradiated autologous tumor cells transduced to secrete GM-CSF has been shown to induce a T cell–mediated antitumor response against GL261 glioma (31). The elicited immune response, which is mediated, at least in part, by CD8+ T cells can inhibit the growth of early brain tumors (31). To test whether WBRT can increase the susceptibility of established invasive GL261 glioma to the antitumor immune response elicited by vaccination, we used the treatment protocol shown in the schema (Fig. 4A). At day 12 after i.c. implantation, animals were randomly assigned to four treatment groups: (a) control, (b) WBRT given in two fractions of 4 Gy, (c) vaccination with irradiated GL261 cells transduced to secrete the cytokine GM-CSF, and (d) WBRT + vaccination. The mean survival time was 33 ± 7 days for control animals, 55 ± 24 days for animals treated with fractionated WBRT alone, and 45 ± 21 days for animals treated with vaccination only (Fig. 4B). In the group treated with WBRT + vaccination, 80% of the animals had long-term survival times of >75 days compared with only 10% in the other treatment groups. This difference in overall survival was statistically significant (P < 0.002).

To test whether the long-term survivors had developed effective systemic antitumor immunity, all surviving animals were rechallenged with viable GL261 cells in the hind limb and observed for tumor development for an additional 8 weeks (Fig. 4C). All five of the naive animals developed tumors by day 28. In contrast, none of the long-term survivors from the different treatment groups developed tumors, indicating that these animals had developed immunologic memory capable of rejecting GL261 tumor.

Radioresponse of established i.c. GL261 tumors treated with WBRT combined with peripheral vaccination. In a subsequent experiment, CT scanning was used to image the i.c. tumors and follow the kinetics of tumor response to treatment. CT scanning was first done on day 15 after implantation, at which time, tumors were usually large enough to be reliably visualized. Animals were then assigned to four treatment groups (control, WBRT, vaccination, or WBRT + vaccination), such that the tumor volumes were comparable between groups. Treatment was administered as described above (Fig. 4A) except that WBRT was given on days 15 and 17, and vaccination on day 17. Representative CT scans from animals prior to treatment on day 15 and after treatment on day 28 are shown (Fig. 5A, arrows indicate tumors) together with the Kaplan-Meier survival curves (Fig. 5B). The mean survival was 33 ± 3 days for control animals, 37 ± 7 days for animals treated with fractionated WBRT alone, and 34 ± 6 days for animals treated with vaccination only. The survival advantage observed following WBRT or vaccination alone was less than in the previous experiment (Fig. 4B), suggesting that a delay of only a few days in treatment and correspondingly larger tumors dramatically reduces the effectiveness of vaccination and radiation as single modalities. Importantly, although the group treated with WBRT + vaccination had an overall reduced survival advantage as compared with the previous experiment (Fig. 4B), 40% of

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Fig. 3. IR increases tumor-infiltrating lymphocytes into GL261 tumors. GL261 cells were implanted i.c. on day 0, and mice were either left untreated (Control) or given WBRT of 4 Gy on days 15 and 17 (WBRT). On day 23, brains were harvested and the lymphocyte-enriched fractions were collected as described in Materials and Methods and stained with phycoerythrin-Cy5 anti-CD8, and phycoerythrin anti-CD4 or anti-NK1.1 monoclonal antibodies, followed by flow cytometry analysis. A, the lymphocyte gate was set based on the scatter plots of spleen cells. Representative data from one of two groups of mice. B, the total number of CD4, CD8, and natural killer cells were calculated based on the total viable cells recovered from each group, and multiplied by the percentage of gated lymphocytes as described in Materials and Methods; columns, number of cells in each subset.
The animals experienced long-term survival as compared with none of the animals in the other treatment groups. This difference in overall survival was statistically significant ($P < 0.002$). Again, the long-term survivors failed to develop tumors upon rechallenge with GL261 glioma cells in the hind leg after 60 days compared with the controls.

**Discussion**

Our previous work has shown that the murine GL261 cell line can be used to develop a valid transplantable orthotopic model of human glioma. Traditionally, mouse models of glioma have primarily used xenografts of human glioma cell lines implanted i.c. into immunoincompetent mice (32). As a result, they have been unable to test immunotherapeutic strategies (33). Furthermore, such tumors do not show the invasive phenotype characteristic of human gliomas that is often associated with treatment failure (33). In contrast to the xenograft models, GL261 tumors show extensive invasion into the brain parenchyma adjacent to the tumor with visible single or multiple tumor cells advancing into the brain along vascular channels (7, 9). The derivative of GL261 expressing GFP was used to readily identify invading tumor cells (7). Although GFP can be immunogenic, our data show that it does not change the tumorigenicity of this poorly immunogenic glioma (7). Moreover, using neuroimaging techniques, including perfusion MRI, we have documented a correlation between tumor growth, relative cerebral blood volume, microvascular density, and histologic features associated with the highly angiogenic nature of the GL261 tumors (34).

Contrary to the earlier belief that the brain was refractory to infiltration with immune cells, there is now ample evidence that immune cells can traffic to the brain and affect the growth of experimental gliomas (35–37). More than a decade ago, it was shown that peripheral vaccination with cytokine-secreting autologous tumor cells could induce an immune response against the intracerebral tumor in experimental animals (38).

In the current study, we used GL261 glioma cells transduced to secrete the cytokine GM-CSF as a vaccine. Vaccination with tumor cells modified to secrete GM-CSF has been shown to be effective in several preclinical cancer models, as well as in clinical trials in generating a systemic and durable antitumor response.
immune response (reviewed in ref. 39). In an experimental setting of well-established, radiologically detectable i.c. tumors, the combination of the vaccine with WBRT was superior to either treatment alone. Similar data were obtained by Lumnizky et al. (31). However, in their study, treatment was given 3 days following i.c. implantation of GL261, at a time when the tumor was unlikely to be well established. To better mimic the clinical setting, we elected to study GL261 glioma tumors when they were firmly established, as documented by CT imaging.

Time after implantation and tumor size are critical factors affecting the therapeutic response to vaccination, as shown by the fact that 40%, 10%, and 0% of mice treated with vaccination alone achieve long-term survival when vaccine is given on days 3, 14, and 17, respectively (ref. 31; Figs. 4 and 5). Although we do not know whether delayed vaccination elicits an antitumor immune response of the same magnitude as vaccination given earlier in the course of tumor growth, the response is sufficient to obtain a therapeutic effect when combined with WBRT. GL261 gliomas are relatively radioresistant tumors (TCD$_{50}$ = 65 Gy; ref. 8). Consequently, the modest dose of radiation used in our experiments is unlikely to have resulted in sufficient cell death to explain the clinical response observed (Fig. 5B), suggesting that its major effect may indeed have been to facilitate infiltration and/or recognition of tumor cells by antitumor T cells. Our studies identify one possible mechanism whereby this could occur in that radiation treatment up-regulated the expression of MHC class I H2-K$^b$ and β2-microglobulin molecules on GL261 glioma cells in vitro and in vivo, respectively. In vitro, treatment with 4 Gy was sufficient to up-regulate the expression of H2-K$^b$ but not H2-D$^b$ molecules on GL261 cells. Differential regulation of H2-K and H2-D locus products by radiation has been previously reported in the B16 melanoma model (40). Other studies using sublethal doses of radiation on a wide variety of human cancer cell lines also showed up-regulation of one or more surface molecules involved in T cell–mediated immune responses, including MHC class I, Fas, ICAM-1, CEA, and MUC-1 (17–21). Increase of MHC class I expression on GL261 cells has been shown to confer greater sensitivity to tumor-specific CTLs in vitro, as shown by using T cells specific for the melanoma-associated antigen gp100 expressed by GL261 cells (41).

Significantly, irradiation induced the expression of β2-microglobulin on invading GL261 glioma cells in vivo (Fig. 2C), a surrogate marker used in this study for MHC class I expression. The finding of very low MHC class I expression on glioma cells invading the normal brain tissue (9) of nonirradiated mice could, at least in part, be responsible for the resistance of established tumors to antitumor T cells elicited by vaccination (Fig. 5B). Therefore, it is possible that the significant tumor response, as determined by CT imaging and increased survival, among the mice treated with WBRT and vaccination is due to the increased susceptibility of irradiated GL261 to antitumor T cells (Fig. 5B). Nevertheless, other radiation-induced changes in brain tumor microenvironment are likely to contribute to the therapeutic benefit of the combination treatment. For instance, the observed influx of inflammatory cells, including CD4+ and CD8+ T cells in response to local radiation (Fig. 3) may be triggered by local production of cytokines and other inflammatory signals in addition to the up-regulation of vascular adhesion molecules (reviewed in ref. 30). Moreover, we cannot exclude the possibility that the effects of radiation not only on the tumor but also on normal brain tissue, which was included in the radiation field, contribute to the observed results. MHC class I antigens are usually not expressed in the central nervous system, but they might be induced by radiation. However, by immunohistochemistry, we did not detect the expression of β2-microglobulin in the normal brain adjacent to the irradiated tumor, suggesting that an autoimmune response is unlikely to be generated and a true therapeutic benefit might result from the combination of radiation treatment with a vaccination strategy.

Immunotherapy of brain tumors is rapidly emerging as a viable clinical option (42), but its use as a first-line approach remains controversial (43). At least two “proof of principle” studies have been reported using vaccines prepared from the patients’ autologous irradiated glioma tumor cells admixed with GM-CSF or IL-4-secreting fibroblasts (44, 45). In a phase I clinical trial using tumor vaccination plus adoptive T cell immunotherapy for patients with newly diagnosed malignant gliomas, several objective clinical responses were observed without any long-term toxicities (44). More recently, several groups have developed dendritic cell vaccines, using either acid-eluted peptides from glioblastoma multiforme cell cultures developed from the surgical specimen or from crude tumor lysates (45–48). In several patients, a systemic CTL response was detected, and in some cases, tumor-infiltrating lymphocytes were also observed in the resection specimen obtained at relapse. Despite the occurrence of sporadic objective responses and some modest increase in the patient’s survival, no long-term survivors have emerged from these approaches (45–48).

Presumably, established invasive gliomas escape immune recognition through multiple mechanisms. One of them is low expression of MHC class I molecules, as we have previously reported in all grade 4 human gliomas (9). Findings from studies in other tumor types also emphasize the importance of MHC class I expression by the tumor cells in eliciting an effective immune response. A recent study of 124 stage IV melanoma patients shows that infiltration of tumors by CD4+ and CD8+ T cells correlated with the expression of MHC class I by the tumor cells and was associated with a better prognosis (49). Similar findings were reported in another study of head and neck tumors patients, in which a positive correlation was found between MHC class I expression, the presence of tumor-infiltrating lymphocytes, and a better prognosis (50). These data support the hypothesis that a strategy to enhance the expression of MHC class I molecules on tumor cells may increase the efficacy of cancer vaccines.

In summary, our results show that IR increases the expression of MHC class I on GL261 glioma cells in vitro and increases the expression of β2-microglobulin in vivo. When radiotherapy is combined with peripheral vaccination, it achieves significant long-term survival rates in animals bearing established, measurable i.c. tumors with an invasive phenotype. These results also show that the GL261 glioma model is a relevant preclinical model for further exploring the mechanisms associated with radiation-induced antitumor immunity. The preclinical evidence presented offers promise to a clinical translation. The fact that modest doses of radiation were required to recover MHC class I expression supports the safety of combining radiotherapy in trials of immunotherapy for patients with recurrent high-grade gliomas who are likely to have already received first-line radiotherapy.
References

The Combination of Ionizing Radiation and Peripheral Vaccination Produces Long-term Survival of Mice Bearing Established Invasive GL261 Gliomas

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