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Ionizing radiation augments glioma tropism of mesenchymal stem cells

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Abstract

OBJECTIVE—Mesenchymal stem cells (MSCs) have been shown to localize to gliomas after intravascular delivery. Because these cells home to areas of tissue injury, the authors hypothesized that the administration of ionizing radiation (IR) to tumor would enhance the tropism of MSCs to gliomas. Additionally, they sought to identify which radiation-induced factors might attract MSCs.

METHODS—To assess the effect of IR on MSC migration in vitro, transwell assays using conditioned medium (CM) from an irradiated commercially available glioma cell line (U87) and from irradiated patient-derived glioma stem-like cells (GSCs; GSC7-2 and GSC11) were employed. For in vivo testing, green fluorescent protein (GFP)-labeled MSCs were injected into the carotid artery of nude mice harboring orthotopic U87, GSC7-2, or GSC17 xenografts that were treated with either 0 or 10 Gy of IR, and brain sections were quantitatively analyzed by immunofluorescence for GFP-positive cells. These GSCs were used because GSC7-2 is a weak attractor of MSCs at baseline, whereas GSC17 is a strong attractor. To determine the factors implicated in IR-induced tropism, CM from irradiated GSC7-2 and from GSC11 was assayed with a cytokine array and quantitative ELISA.

RESULTS—Transwell migration assays revealed statistically significant enhanced MSC migration to CM from irradiated U87, GSC7-2, and GSC11 compared with nonirradiated controls and in a dose-dependent manner. After their intravascular delivery into nude mice harboring orthotopic gliomas, MSCs engrafted more successfully in irradiated U87 ($p = 0.036$), compared

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with nonirradiated controls. IR also significantly increased the tropism of MSCs to GSC7-2 xenografts ($p = 0.043$), which are known to attract MSCs only poorly at baseline (weak-attractor GSCs). Ionizing radiation also increased the engraftment of MSCs in strong-attractor GSC17 xenografts, but these increases did not reach statistical significance. The chemokine CCL2 was released by GSC7-2 and GSC11 after irradiation in a dose-dependent manner and mediated in vitro transwell migration of MSCs. Immunohistochemistry revealed increased CCL2 in irradiated GSC7-2 gliomas near the site of MSC engraftment.

CONCLUSIONS—Administering IR to gliomas enhances MSC localization, particularly in GSCs that attract MSCs poorly at baseline. The chemokine CCL2 appears to play a crucial role in the IR-induced tropism of MSCs to gliomas. <https://thejns.org/doi/abs/10.3171/2016.9.JNS16278>

Keywords

glioma; mesenchymal stem cells; radiation; CCL2; MCP-1; oncology

Glioblastomas (GBMs) are the most common malignant primary brain tumors in adults. Patients with GBM have a dismal prognosis, surviving on average only 14 months despite maximal standard therapy.³⁵ This poor outcome is due not only to the infiltrative growth pattern of GBMs and the presence of glioma stem-like cells (GSCs), which are resistant to most therapies, but also to the inability to deliver most therapies to the tumor, because systemically administered treatments are insulated by the blood-brain barrier (BBB) or the blood-tumor barrier.^{1,3} In an effort to overcome this delivery problem, we and others have used bone marrow–derived mesenchymal stem cells (MSCs) as vehicles to deliver anti-glioma agents to GBMs based on accumulating evidence that MSCs are capable of homing to brain tumors after local and systemic administration.³⁰ MSCs are well suited for both experimental and clinical purposes because they can be easily isolated from patients, rapidly cultured and expanded in vitro, and efficiently engineered and because autologous or allogeneic transplantation can be performed with simpler ethical concerns than with other stem cells.

We have shown that MSCs selectively localize to gliomas after intravascular injection, and this property has been exploited in orthotopic glioma models to deliver anti-glioma therapies such as interferon- β ,³⁰ s-TRAIL,^{31,39} and oncolytic viruses.^{30,40} We have shown that MSCs home not only to xenografts grown from commercially available “professional” mouse or human glioma lines^{16,30,40} (for example, U87), but also to xenografts derived from human GSCs.³² This finding is important because commercial cell lines fail to mimic several key features of human gliomas, particularly the infiltrative growth pattern, whereas GSCs recapitulate the genotypic and phenotypic characteristics of human gliomas seen clinically.^{7,33} GSCs are capable of self-renewal, grow as spheroids in culture, and initiate tumors in vivo even after implantation of a small number of cells (100–1000 cells).^{7,33} Additionally, GSCs are resistant to most conventional therapies and represent the population of cells responsible for tumor recurrence.^{7,8,33}

Mesenchymal stem cells are known to home to sites of tissue injury or inflammation. Because tumors mimic “wounds that do not heal,”¹² we reasoned that mechanisms underlying the homing of MSCs to sites of tissue injury may be similar to those underlying

the homing of MSCs to tumors. In this context, it appears that MSCs home to gliomas in response to tumor-derived factors similar to those found in nonhealing wounds. For example, we have shown that MSCs migrate toward growth factors, such as platelet-derived growth factor (PDGF),¹⁶ or inflammatory factors, such as transforming growth factor- β 1 (TGF- β 1).³² Therefore, we hypothesized that perturbations that enhance tissue injury and/or inflammation, thereby facilitating the release of growth factors and chemokines, will enhance MSC homing to gliomas. This hypothesis is particularly relevant to GSCs as we previously reported that some GSCs are very strong attractors of MSCs (called “strong attractors,” such as GSC17), whereas other GSCs attract MSCs less efficiently in vivo (called “weak attractors,” such as GSC7-2).³² Therefore, there is a need to develop clinically applicable methods that will enhance MSC homing, particularly to weak-attractor GSCs.

One therapeutic modality that can effectively stoke local injury or inflammation in the tumor microenvironment is ionizing radiation (IR). In response to radiation-induced injury, tumors release a variety of growth factors, chemokines, and cytokines. Indeed, reports have shown enhanced biodistribution of MSCs following radiation treatment in murine models of breast cancer, due at least in part to an increase in inflammatory cytokines.²⁴ Additionally, radiation can enhance vascular permeability,¹⁴ potentially facilitating the translocation of MSCs from the brain vasculature into the tumor.

In this study, we test the hypothesis that IR is capable of enhancing the localization of intravascularly delivered MSCs to gliomas. We use a commercially available human glioma line (U87) as well as several patient-derived GSC lines (GSC7-2, GSC11, GSC17). Subsequently, we use these models to identify factors secreted by the tumor and involved in mediating the tropism of MSCs to gliomas.

Methods

Mesenchymal Stem Cells

Bone marrow–derived human MSCs (hMSCs) from a male were obtained from Lonza. Cells were positive for CD44, CD73, CD90, and CD105 and negative for CD34, CD45, and CD133. Cells were numerically expanded in a 37°C, 5% CO₂ incubator in α -minimum essential medium (α -MEM) containing 10% fetal bovine serum (Sigma), 1% 2-mmol/L L-glutamine (Invitrogen), and 1% penicillin-streptomycin (Lonza) and were used from passages 3 to 6.

Tumor Cells

Glioma stem-like cells GSC7-2, GSC11, and GSC17 were established from fresh surgical specimens from patients with GBM by using our standard protocol, as described elsewhere.^{17,20} They were grown as neurospheres and expanded in DMEM/F12 (Mediatech) supplemented with vitamin B27 (GIBCO, Invitrogen), 20 ng/ml of human epidermal growth factor (EGF; Sigma-Aldrich), 20 ng/ml of human basic fibroblast growth factor (Sigma-Aldrich), and 1% penicillin-streptomycin (Lonza) in a 37°C, 5% CO₂ incubator. The cells were used from passages 5 to 8. The GBM cell line U87 was obtained from the American Type Culture Collection and was grown in α -MEM containing 10% fetal bovine serum

(Lonza), 1% nonessential amino acids (HyClone Laboratories), and 1% penicillin-streptomycin (Lonza).

Labeling of MSCs

Human MSCs were transduced with *GFP* using a replication-incompetent Ad5/F35-CMV-GFP (Ad-GFP; Vector Development Laboratory, Baylor College of Medicine). Monolayers were treated with a multiplicity of infection of 50 in 3 ml of serum-free MSC medium and were shaken every 10 minutes at 37°C. After 1 hour, MSC medium containing 10% fetal bovine serum was added.

In Vitro Transwell Migration Assays

In vitro migration was assayed using Matrigel-coated transwell plates as previously described.¹⁶ The GSC7-2, GSC11, GSC17, and U87 tumor cells were plated in serum-free medium at a concentration of 10^5 cells/ml. Twenty-four hours later, the plates were subjected to 0, 2, 5, or 10 Gy of IR from a ^{137}Cs irradiator. Eight, 24, or 48 hours later, conditioned medium (CM) was collected and placed in the lower well of 24-mm tissue culture plates (Corning Inc.). Then 10^5 MSCs in 1 ml of serum-free medium were plated in the upper well (8- μm pore, Corning Inc.) coated with Matrigel (BD Biosciences). All plating was done in triplicate.

For assessment of in vitro CCL2 chemoattraction, 0-, 0.02-, 0.2-, 2-, and 20-ng/ml concentrations of human CCL2 (RayBiotech) were placed in the lower wells of 24-mm tissue culture plates, and 10^5 MSCs in 1 ml of serum-free medium were plated in the upper 8- μm pore transwell plates coated with Matrigel. After 48 hours of incubation, the migration of MSCs through the Matrigel was determined by fixing the membrane, staining the cells using the Hema3 staining kit (Fisher Diagnostics), directly counting the number of migrated cells in 10 hpf ($\times 400$), and calculating the average.

Chemokine Assay and CCL2 ELISA

The expression levels of various chemokines in the CM from irradiated GSC7-2 and GSC11 cells were assessed using a chemokine antibody array (R&D Systems). The CCL2 in the CM was quantified using an ELISA kit (Quantikine, R&D Systems) according to the manufacturer's protocol. Briefly, GSC7-2 or GSC11 cells were plated at 10^5 cells/ml in serum-free medium. Twenty-four hours later, the plates were irradiated with 0, 5, or 10 Gy. Eight, 24, or 48 hours later, the CM was collected and assayed in duplicate.

Animal Subjects

Male athymic nude mice (nu/nu) were purchased from the Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center. Animals were anesthetized with intraperitoneal injections of ketamine (100 mg/kg)/xylazine (10 mg/kg) during all procedures. All animal manipulations were performed in the veterinary facilities in accordance with institutional, state, and federal laws and ethics guidelines under an approved protocol.

Intracranial Xenografting

Intracranial glioma xenografts were implanted as previously described.^{25,30} After dissociation, 5×10^5 U87, GSC7-2, or GSC17 cells suspended in 5 μ l of serum-free medium were implanted into the right frontal lobe of the nude mice.

Irradiations of Xenografts

At Day 49 postimplantation of GSC7-2 cells and Day 7 postimplantation of GSC17 and U87 cells, mice were placed in a lead head holder and were subjected to 0- or 10-Gy irradiations delivered to the whole brain using a cobalt-60 source.

Internal Carotid Artery Injections

One day after irradiations in the mice implanted with U87 and GSC17 cells and 6 days after in those implanted with GSC7-2 cells, 10^6 GFP-labeled MSCs were injected into the right internal carotid artery of each mouse, as previously described.^{16,30,40}

Brain Tissue and Tumor Preparation

Three days after intracarotid injections, mice were sacrificed and their brains were harvested and processed for frozen or paraffin sections, as previously described.¹⁶ Staining with H & E or DAPI (FluoroPure grade, Invitrogen) was performed to visualize the tumor. The GFP-labeled cells were visualized in frozen sections using fluorescence microscopy and in paraffin sections after deparaffinization and antigen retrieval with 1:1000 rabbit anti-GFP primary antibody (Novus) and 1:200 Donkey anti-rabbit green secondary antibody (Invitrogen). Detection of endothelial cells was performed with 1:250 goat anti-CD31 primary antibody (R&D) and 1:200 donkey anti-goat red secondary antibody (Invitrogen). For U87 xenografts, specimens were fixed in 10% formalin for 48 hours, placed in 30% sucrose until they sank, and then embedded in optimal cutting temperature medium and frozen.

Histological Quantification of hMSCs Homing Toward Gliomas

Quantitative assessments of GFP-labeled MSCs in vivo engraftment after intracarotid injection was performed in histological sections by counting the number of GFP-positive cells in the tumor, calculating the tumor area in 5- μ m coronal sections every 100 μ m through the tumor, and then dividing the total number of counted cells by the total tumor area. For GSC tumors (which were generally larger than U87 tumors), we chose 6 sections 100 μ m apart in the approximate center of the tumor. Cells were counted as positive if there was fluorescein isothiocyanate (FITC) fluorescence in the cytoplasm of a cell whose DAPI-stained nucleus was also observed under the high power field of view. The tumor area was calculated by manually outlining the tumor with digital microscope software as described previously.¹⁶

Antagonism of CCR2 for the Transwell Assay

RS504393 (Santa Cruz Biotechnology, a small molecule inhibitor of CCR2, was dissolved in dimethyl sulfoxide (DMSO) and added at 1:1000 dilution to CM from GSC7-2 cells that had undergone 0- or 5-Gy irradiation 24 hours earlier, resulting in a 3- μ M concentration. Control

CM from irradiated and nonirradiated tumor cells with DMSO alone and with no DMSO was used. Transwell assays using this CM were then performed.

Immunohistochemistry for CCL2

Paraffin sections were deparaffinized in 3 changes of xylene and washed in decreasing ethanol concentrations (100% to 95%), followed by sterile deionized water (dH₂O) and phosphate-buffered saline (PBS). The slides were processed for antigen retrieval by microwaving in citrate buffer (0.01 M, pH 6.0) for 10 minutes. After washing the slides in 3 series of dH₂O, endogenous peroxidase was inactivated with 1% H₂O₂ in methanol, then washed in PBS. The slides were blocked overnight at 4°C with 2.5% bovine serum albumin (Invitrogen)/0.1% Triton X-100. The slides were then incubated overnight with rabbit anti-human CCL2 antibody (Abcam) at a 1:50 dilution with 2.5% bovine serum albumin/0.1% Triton X-100. After being rinsed with PBS, the slides were incubated with an avidin-conjugated horse anti-rabbit antibody (Vector Laboratories) at a 1:200 dilution for 1 hour and then treated with a solution of avidin-biotin-peroxidase complexes (Vectastain ABC kit; Vector Laboratories) for 30 minutes. The DAB substrate kit (Vector Laboratories) was used to develop the stain. Slides were counterstained with hematoxylin.

Statistical Analysis

Statistical differences were assessed utilizing unpaired 2-tailed t tests. Differences were determined to be statistically significant at $p < 0.05$. The data were represented as the means $\pm$ standard deviation or standard error for at least 3 replicate determinations for each experiment.

Results

Increased Tropism of MSCs to Irradiated Glioma Cells In Vitro

The effects of IR on MSC tropism to gliomas were first tested in U87 cells using in vitro transwell migration assays. The MSCs were plated on Matrigel-coated upper wells, and the bottom wells were filled with serum-free CM collected from cultured U87 cells 24 hours after treatment with mock (0 Gy) or 5 Gy of IR. After incubating the plates for 48 hours, cell counting under high power field magnification revealed that significantly more MSCs had migrated toward the CM from irradiated U87 cells compared with CM from the nonirradiated U87 cells (65.3 vs 40 cells, $p = 0.016$; Fig. 1A).

Next, the effects of IR on in vitro MSC tropism to GSCs was similarly tested using Matrigel-coated trans-well assays with serum-free CM collected from GSC7-2 cells 48 hours after receiving 0, 2, or 5 Gy of radiation. After incubating the transwell plates for 48 hours, significantly more MSCs migrated toward the CM from irradiated GSC7-2 cells (2-Gy CM: 18.7 cells/10 hpf, $p = 0.054$; 5-Gy CM: 29.7 cells/10 hpf, $p = 0.0014$) than the CM from nonirradiated GSC7-2 cells (0 Gy: 13.3 cells/10 hpf; Fig. 1B). Transwell assays were similarly used with the GSC11 line exposed to 0, 2, 5, or 10 Gy of radiation. Compared with MSC migration in nonirradiated GSC11 (11.0 cells/10 hpf), migration in the irradiated groups was increased: 2-Gy CM: 21.7 cells/10 hpf, $p = 0.091$; 5-Gy CM: 25.3 cells/10 hpf, $p = 0.25$; and 10-Gy CM: 26.5 cells/10 hpf, $p = 0.024$ (Fig. 1C).

Increased Tropism of MSCs to Irradiated Gliomas In Vivo After Intravascular Delivery

To determine whether the IR-induced augmentation of the tropism of MSCs to gliomas in vitro would also occur in vivo, radiation-induced tropism was first tested using U87 xenografts. Nude mice were implanted with U87 cells and 7 days later underwent either 0- (4 mice) or 10-Gy (4 mice) irradiation. On Day 1 postirradiation, carotid injections of 10^6 GFP-labeled MSCs were performed, and after 3 days, the mice were sacrificed and brain specimens were collected. After brain sectioning, the density of MSCs (that is, GFP-positive cells) per tumor area was compared between irradiated and nonirradiated specimens (Fig. 2A). The average GFP-positive cell density was higher in irradiated U87 specimens than in nonirradiated U87 specimens (19.0 vs 7.1, $p = 0.036$; Fig. 2B). There were scant GFP-positive cells outside the tumor in either group, with an average of 3.7 extratumoral cells per section in the irradiated group compared with 4.0 extratumoral cells per section in the nonirradiated group ($p = 0.80$).

We next explored the potential of IR to enhance the homing of MSCs to intracranial xenografts derived from GSCs. We first tested gliomas arising from GSC17 cells, a GSC line that is known to elicit strong engraftment of MSCs following intravascular delivery.³² The GSC17 cells were implanted in the brains of nude mice. After 7 days, the mice were treated with either 10 Gy (5 mice) or 0 Gy (control, 4 mice) of IR. The next day, mice were injected with GFP-labeled MSCs (10^6 cells). Three days later, brain specimens were collected, paraffin sections were cut, and immunofluorescence was used to calculate the density of GFP-positive cells per area of tumor in 6 representative sections for each specimen. The density of MSC engraftment in the tumor was higher in the irradiated specimens than the nonirradiated tumors, although the difference did not reach statistical significance (11.8 vs 6.5 MSCs/mm², $p = 0.11$; Fig. 2C and D). There was no significant difference in the average tumor cross-sectional area (3.1 vs 3.3 mm², $p = 0.86$).

We next assessed GSC7-2, a GSC line that poorly attracts intravascularly delivered MSCs at baseline (a weak-attractor GSC). Specifically, GSC7-2 cells were implanted in the brains of nude mice, and after 49 days (when tumors were 3–5 mm in diameter), the mice were treated with either 10 Gy of IR (5 mice) to the brain or with 0 Gy (control, 5 mice). Six days after irradiation, the mice were injected with GFP-labeled MSCs (10^6 cells). Three days later, brain specimens were collected, paraffin sections were cut, and immunofluorescence was performed. Consistent with our previous experience, nonirradiated specimens attracted few MSCs (0.4 MSCs/mm² tumor). However, irradiated tumors attracted high numbers of MSCs (3.4 MSCs/mm²). This increase was statistically significant ($p = 0.021$; Fig. 2E and F). The average tumor area per cross section was smaller in the irradiated group than the nonirradiated group (14.4 vs 19.5 mm², $p = 0.04$). Because radiation effects on the intratumoral blood vessels could affect intravascular MSC homing and engraftment, sections were also stained for the endothelial cell marker CD31 to observe patterns of MSC transmigration and to qualitatively compare blood vessel size, density, and structure across the specimens. There did not appear to be any qualitative difference in the vasculature between irradiated and nonirradiated specimens.

Increased Expression of CCL2 in a Dose-Dependent Fashion Following GSC Irradiation

To identify factors that may be responsible for IR-induced tropism, CM was collected 8, 24, and 48 hours after the treatment of GSC7-2 cells with 0, 5, or 10 Gy of IR and was assayed using a cytokine/chemokine antibody array capable of detecting 38 candidate molecules. Of the factors tested, only CCL2 was increased in the CM from irradiated cells, and this increase was dose dependent (Fig. 3A). To confirm and quantify this dose response, CM from GSC7-2 cells treated with 0, 5, or 10 Gy of IR was analyzed using ELISA for CCL2. This analysis confirmed that CCL2 levels increased with increasing radiation dose (Fig. 3B left). These results were confirmed in another cell line, GSC11 (Fig. 3B right).

Migration of MSCs In Vitro Enhanced by CCL2

To determine whether CCL2 causally mediates MSC migration, a transwell Matrigel migration assay was performed. Specifically, MSCs were plated on Matrigel in the upper chambers of transwell plates and increasing concentrations of CCL2 were placed in the bottom chambers. The MSCs were then assayed for migration through the Matrigel after 48 hours. Migration of MSCs increased in response to CCL2 in a dose-dependent manner (Fig. 3C).

To provide further evidence of a causal role for CCL2 in mediating the migration of MSCs to irradiated GSCs, MSCs were plated on Matrigel in the upper well of trans-well plates, and CM collected from GSC7-2 cells treated with either 0 Gy (control) or 5 Gy of IR was placed in the lower wells. The CM was also treated with 0 or 3 μ M of RS504393, a small molecule inhibitor of CCR2 (the receptor for CCL2). As expected, without the inhibitor, CM from irradiated GSC7-2 significantly increased the migration of MSCs compared with CM from nonirradiated cells ($p = 0.034$). Importantly, the addition of the CCR2 antagonist significantly inhibited MSC migration to the CM from both nonirradiated and irradiated tumor cells (Fig. 3D; $p = 0.0013$ and $p < 0.0001$ respectively). In fact, MSC migration toward medium from nonirradiated or irradiated cells was similar when RS504393 was present ($p = 0.80$).

CCL2 in Irradiated Glioma Xenografts That Contain Engrafted MSCs

Paraffin sections from irradiated and nonirradiated mouse brains harboring GSC7-2 tumors (weak-attractor GSC) that had been treated with intravascularly injected GFP-labeled MSCs were prepared and stained for CCL2. Speckled staining was found in the irradiated samples in the extracellular space close to clusters of MSC engraftment (detected on an adjacent section by immunofluorescence), but no staining was seen in nonirradiated samples, which had few MSCs (Fig. 3E).

Discussion

Mesenchymal stem cells display a robust capacity to home to sites of tissue injury such as myocardial infarction,²¹ muscular dystrophy,¹⁰ stroke,⁶ and parkinsonism.²⁷ Because tumors behave as wounds that do not heal, mechanisms similar to those mediating homing to injured tissue are thought to be responsible for the tropism of MSCs to tumors. Therefore, we hypothesized that perturbing the tumor with IR might increase tissue injury and induce

the release of chemoattractive factors, thereby increasing the capacity of tumors to attract MSCs. Indeed, MSCs have displayed enhanced tropism to irradiated tumors in experimental models of breast cancer²⁴ and colon cancer.⁴³ Because breast and colon tumors nest in a fibroblastic stroma rather than an astrocytic stroma as do gliomas, and because breast and colon tumors are not excluded from the circulation by the BBB or blood-tumor barrier (whereas gliomas are), it was unclear whether IR would have similar effects on MSC homing to gliomas. One previous study qualitatively suggested that after MSC tail-vein injection, MSC homing to U87 intracranial xenografts might be enhanced by IR.²³ However, in our experience,³⁰ we have found that intravenous injection of MSCs results in inefficient tumor engraftment, as most of the cells are filtered by the lungs en route to the arterial side of systemic circulation.¹³

In this report, we show that MSCs home more efficiently to irradiated gliomas after intravascular delivery than to nonirradiated gliomas. This effect was demonstrated in a commercially available “professional” glioma cell line (U87) and, more importantly, in GSCs, which closely mimic the histopathological features and biological behavior observed in clinical gliomas and are therefore considered to be the current gold-standard preclinical model for human gliomas. Additionally, GSCs are thought to represent the treatment-resistant subpopulation of cells responsible for tumor recurrence,^{7,8,33} therefore, effectively targeting these cells is imperative.

In contrast to the uniformly high levels of MSC homing after intravascular delivery to xenografts from commercial glioma lines,^{30,40} the GSC-derived xenografts support MSC homing to a variable extent depending on the GSC line, possibly from differing intrinsic expression of chemoattractive cytokines.³² Importantly, in this study we showed that IR enhanced the homing of MSCs to a cell line that was capable of attracting MSCs at baseline (strong-attractor GSC: GSC17). But even more importantly, IR converted a weak-attractor GSC (GSC7-2) into a GSC that more robustly attracted MSCs. Although the increase in MSC homing after IR did not reach statistical significance at a level of $p < 0.05$ ($p = 0.11$) in the strong-attractor GSC (GSC17), probably because of the high baseline attraction that resulted in more variability in the controls, the increase in MSC homing in the nonattractor GSC (GSC7-2) was highly significant ($p = 0.02$). Therefore, it appears that the inability of some GSC tumors to attract MSCs can be overcome to a great extent by IR administration.

The majority of GBMs observed clinically are treated with radiation, which has important implications in potential translational and clinical studies using MSCs as therapeutic delivery vehicles. The timing of the intravascular administration of such therapies may be most ideal soon after radiation is administered, as this appears to allow superior tumor homing without a significant increase in nonspecific engraftment in normal brain. Furthermore, IR can potentially act synergistically with MSC-delivered therapies. For example, MSCs have been shown to effectively deliver the oncolytic adenovirus 24-RGD to intracranial xenografts, including those derived from GSCs.^{32,40} 24-RGD is a tumor-selective replication-competent adenovirus with augmented infectivity.^{15,40} 24-RGD adenoviral proteins directly target key DNA repair genes to allow replication in the host cell,^{19,34} and this may induce susceptibility in its tumor host cell to DNA-damaging treatments such as IR.

The mechanism for the glioma tropism of MSCs has not been fully elucidated, but numerous chemotactic factors have been implicated, including PDGF-BB, TGF- β 1, EGF, stromal cell-derived factor-1 α , vascular endothelial growth factor, and interleukin-8.^{23,24,30,32} We used a global approach with antibody arrays, looking at numerous potential cytokines to determine which factors mediated irradiation-induced MSC tropism to gliomas. We found that CCL2 protein levels exhibited a dose-dependent response with increasing radiation, and we confirmed that CCL2 plays a causal role in the migration of MSCs. Chemokines are potent cytokines capable of selectively recruiting a distant population of inflammatory cells into the bloodstream toward the site of release. Therefore, these factors are promising candidates for mediating MSC migration. Known as monocyte chemoattractant protein-1, CCL2 attracts monocytes, memory T cells, natural killer cells, and dendritic cells to sites of tissue injury.^{2,5} Its key functional receptor is CCR2, a G-protein-coupled receptor that is expressed by MSCs^{24,38} and appears to promote MSC migration in vitro.^{38,42} The role of CCL2 in human cancers and MSC migration still requires further elucidation, but tumor-derived CCL2 has been implicated in chemotactic migration of myeloid suppressor cells¹⁸ and in rat bone marrow-derived MSCs.³⁸ Additionally, CCL2 is expressed in gliomas,^{9,36} with increasing expression in higher grade tumors,^{9,26} and has been shown to facilitate in vitro migration of rat bone marrow-derived MSCs to gliomas.³⁸ Moreover, CCL2 is released by colon cancer cells after irradiation,⁴³ and MSCs exposed to irradiated breast cancer cells exhibit increased CCR2 expression.²⁴ Additionally, CCL2 is rapidly released in the territory of ischemia after middle cerebral artery occlusion and is thought to promote crossing of the BBB and subsequent engraftment of injected MSCs in rat models.³⁷

Though our in vitro studies implicate the release of CCL2 by irradiated glioma cells as a causative factor for increased MSC tropism, in the in vivo setting in which tumor cells are integrated with supporting cells, the mechanism is more uncertain. Ionizing radiation can increase vascular permeability via disruption of the BBB,^{4,11,28,41} decreased tight junction proteins,²² or endothelial cell damage.²⁹ Additionally, proinflammatory chemoattractants may be released by the supporting cells rather than the tumor itself. Note that CCL2 staining was seen in the extracellular space, not intracellularly.

Conclusions

In summary, the administration of IR to GSC-derived gliomas increases the tropism of intravascularly injected MSCs, and the chemokine CCL2 appears to contribute to this augmented tropism.

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ABBREVIATIONS

α-MEM	α -minimum essential medium
BBB	blood-brain barrier
CM	conditioned medium
dH₂O	deionized water
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
GBM	glioblastoma
GFP	green fluorescent protein
GSC	glioma stem-like cell
hMSC	human MSC
IR	ionizing radiation
MSC	mesenchymal stem cell
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
TGF-β1	transforming growth factor- β 1.

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FIG. 1.

Matrigel-coated transwell assay of MSC migration from the upper well to CM in the bottom well, which was performed in triplicate for U87 (**A**), GSC7-2 (**B**), and GSC11 (**C**).

Increased migration was seen with medium from irradiated cells for all lines. *Error bars* indicate standard error. * $p < 0.05$, compared with the nonirradiated sample.

FIG. 2.

A: Photomicrographs of U87 xenografts treated with 0 Gy (*left*) or 10 Gy (*right*) of IR 7 days after tumor implantation. Bars = 100 μ m. **B:** There was a higher density of MSCs in irradiated U87 tumors than in nonirradiated tumors ($p = 0.036$). *Error bars* indicate standard error. * $p < 0.05$, compared with nonirradiated sample. **C:** Photomicrographs of GSC17 xenografts treated with 0 Gy (*left*) or 10 Gy (*right*) of IR 7 days after tumor implantation. Bars = 50 μ m. **D:** The MSC migration tended to be greater toward irradiated GSC17 tumors rather than nonirradiated specimens, though the difference between the two was not statistically significant ($p = 0.11$). **E:** Photomicrographs of GSC7-2 xenografts treated with 0 Gy (*left*) or 10 Gy (*right*) of IR 49 days after implantation in nude mice. Tumor was assessed by immunofluorescence alongside staining for the endothelial cell marker CD31 and DAPI nuclear counterstain. Note the sparse transmigration of MSCs across CD31-positive endothelial cells (*left*) and the robust MSC engraftment in a more clustered pattern (*right*). Bars = 50 μ m. **Insets** show H & E staining of brain specimens with tumor. **F:** There was a significantly higher density of MSCs in irradiated GSC7-2 tumors than in nonirradiated tumors ($p = 0.021$). *Error bars* indicate standard error. * $p < 0.05$, compared with nonirradiated sample.

FIG. 3.

A: Chemokine antibody array of medium alone (*upper left*) and CM from GSC7-2 cells treated with 0 Gy (*upper right*), 5 Gy (*lower left*), or 10 Gy (*lower right*) of IR 24 hours prior to collection. The corners of the array are the appropriate positive and negative controls. Increasing signal strength for the chemokine CCL2 with increasing radiation dose is highlighted in *red boxes*. **B:** Quantitative ELISA assays for CCL2 were performed on CM from GSC7-2 (*left*) and GSC11 (*right*) irradiated with 0, 5, or 10 Gy of IR. In medium from GSC7-2, CCL2 release increases with increasing radiation dose ($p < 0.001$) and plateaus at 24 hours. In medium from GSC11, CCL2 release increased with the 10-Gy radiation dose at 24 hours ($p = 0.06$) and at 48 hours ($p = 0.03$). *Error bars* indicate standard deviation. **C:** Matrigel-coated transwell assay results show that MSC migration increases with increasing concentrations of CCL2 in the bottom well. *Error bars* indicate standard error. * $p < 0.05$ in unpaired t test comparison. **D:** Antagonism of CCR2, the receptor for CCL2, inhibits MSC migration. Medium from untreated irradiated cells (GSC7-2 + 5 Gy IR + 0 μ M) induced increased migration compared with untreated nonirradiated cells (GSC7-2 + 0 Gy IR + 0 μ M; $p = 0.034$). Conditioned medium with the inhibitor showed a marked decrease in migration especially in the medium from irradiated cells ($p < 0.0001$), reducing migration

down to unconditioned medium levels. $*p < 0.05$. **E:** Photomicrographs of GSC7-2 xenografts subjected to 0 (*upper row*) or 10 (*lower row*) Gy of IR 49 days after implantation in nude mice. Six days post-IR, GFP-labeled MSCs were injected into the carotid artery. Three days later, brain specimens were collected. Immunohistochemistry for CCL2 shows CCL2 positivity (*lower left*) in areas of irradiated tumor where MSCs have migrated (*lower right*), but no CCL2 staining (*upper left*) or MSC migration (*upper right*) from nonirradiated tumor.

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