Human Bone Marrow–Derived Mesenchymal Stem Cells in the Treatment of Gliomas

Akira Nakamizo, Frank Marini, Toshiyuki Amano, Asadullah Khan, Matus Studeny, Joy Gumin, Julianne Chen, Stephen Hentschel, Giacomo Vecil, Jennifer Dembinski, Michael Andreeff, and Frederick F. Lang

Abstract

The poor survival of patients with human malignant gliomas relates partly to the inability to deliver therapeutic agents to the tumor. Because it has been suggested that circulating bone marrow–derived stem cells can be recruited into solid organs in response to tissue stresses, we hypothesized that human bone marrow–derived mesenchymal stem cells (hMSC) may have a tropism for brain tumors and thus could be used as delivery vehicles for glioma therapy. To test this, we isolated hMSCs from bone marrow of normal volunteers, fluorescently labeled the cells, and injected them into the carotid artery of mice bearing human glioma intracranial xenografts (U87, U251, and LN229). hMSCs were seen exclusively within the brain tumors regardless of whether the cells were injected into the ipsilateral or contralateral carotid artery. In contrast, intracarotid injections of fibroblasts or U87 glioma cells resulted in widespread distribution of delivered cells without tumor specificity. To assess the potential of hMSCs to track human gliomas, we injected hMSCs directly into the cerebral hemisphere opposite an established human glioma and showed that the hMSCs were capable of migrating into the xenograft in vivo. Likewise, in vitro Matrigel invasion assays showed that conditioned medium from gliomas, but not from fibroblasts or astrocytes, supported the migration of hMSCs and that platelet-derived growth factor, epidermal growth factor, or stromal cell–derived factor-1c, but not basic fibroblast growth factor or vascular endothelial growth factor, enhanced hMSC migration. To test the potential of hMSCs to deliver a therapeutic agent, hMSCs were engineered to release IFN-β (hMSC-IFN-β). In vitro coculture and Transwell experiments showed the efficacy of hMSC-IFN-β against human gliomas. In vivo experiments showed that treatment of human U87 intracranial glioma xenografts with hMSC-IFN-β significantly increase animal survival compared with controls (P < 0.05). We conclude that hMSCs can integrate into human gliomas after intravascular or local delivery, that this engraftment may be mediated by growth factors, and that this tropism of hMSCs for human gliomas can be exploited to therapeutic advantage.

Introduction

There is currently no optimal treatment for glioblastoma multiforme, the most common malignant brain tumor in adults, and patients typical survive <1 year (1, 2). This poor outcome relates at least in part to the inability to deliver therapeutic agents to the tumor (3). Delivery problems have especially slowed the development of novel gene therapy strategies (4). Indeed, intratumoral injection of viral vectors has proven incapable of delivering therapeutic genes to many tumor cells (4), and systemic i.v. or intra-arterial administration has been limited by the neutralizing effects of antibodies and by immune-mediated organ toxicity (5, 6). Methods for achieving widespread distribution of therapeutic agents throughout infiltrative gliomas would substantially improve brain tumor therapy.

Recent evidence suggests that stem cells are useful delivery vehicles for brain tumor therapy. Several laboratories have shown the potential of neural stem cells to function as delivery vehicles for brain tumor therapy (7–10). Aboody et al. were among the first to show that after intracranial injection neural stem cells have a tropism for brain tumors that could be exploited therapeutically (7). Likewise, Ehtesham et al. have shown that locally injected neural stem cells engineered to deliver interleukin-12 or tumor necrosis factor–related apoptosis-inducing ligand could slow the growth of brain tumors (9, 10). However, the clinical application of neural stem cells will be limited undoubtedly by logistic and ethical problems associated with their isolation and by potential immunologic incompatibility due to the requirement for allogenic transplantation. Because of the significant limitations associated with isolating human neural stem cells, to date, only murine neural stem cells have been evaluated in experimental settings. These inherent problems with neural stem cells led us to ask whether other types of stem cells that are more readily accessible (and thus more clinically applicable) may be used as vehicles for delivering therapeutic agents to brain tumors.

Bone marrow is an alternative source of stem cells (11–14). Human bone marrow–derived stem cells are well suited for clinical application because they are easily obtained from patients and because autologous transplantation, which obviates immunologic incompatibilities, is possible (15). Of the various progenitor cells that exist within bone marrow, human mesenchymal stem cells (hMSC) are particularly attractive for clinical use because they are easily isolated, can be expanded in culture, and can be genetically manipulated using currently available molecular techniques (14, 16–23). hMSCs are precursors that cause bone marrow stroma by differentiating into adipocytes, chondrocytes, and osteoblasts (11, 20, 24). However, MSCs have also been shown to be capable of differentiating into nonsesdermal tissues, including neurons and astrocytes (25, 26).
The rationale for using bone marrow–derived stem cells for delivering therapies to brain tumors is based on the developing current concept that bone marrow is a source of circulating stem cells that are recruited from the blood into peripheral solid organs in times of tissue stress or injury (27–31). Because the microenvironments of solid tumors is similar to the environment of injured/stressed tissue (32, 33), it is logical to hypothesize that solid tumors may provide a permissive environment for the engraftment of exogenously given hMSCs (27). In this context, we have shown previously that systemically delivered hMSCs are capable of integrating into human tumors grown within the lungs of nude mice (27). However, the unique features of the microenvironment of the brain and gliomas, including their highly specialized vasculature and gliu-derived stroma, led us to evaluate whether brain tumors would also provide a permissive environment for the selective engraftment of hMSCs. Using an intracranial model of gliomas, we now show that hMSCs have a tropism for human gliomas after intravascular and local delivery and that this tropism can be exploited therapeutically by engineering hMSCs to release a soluble antiglioma factor.

Materials and Methods

Mesenchymal stem cell isolation and culture. Human MSCs were isolated as described previously (27) from the bone marrow of normal individuals undergoing bone marrow harvest for autologous bone marrow transplantation after informed consent according to institutional guidelines under an approved protocol. Briefly, mononuclear cells were separated by centrifugation over a Ficoll-Hypaque gradient (Sigma Chemical Co., St. Louis, MO) and suspended in α-MEM containing 20% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD), 2-(glutamine, and penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD) followed by plating at an initial seeding density of 1 × 10^5 cells/cm^2. After 3 days, the nonadherent cells were removed by washing with PBS, and monolayers of adherent cells were cultured until they reached confluence. Cells were then trypsinized (0.25% trypsin with 0.1% EDTA), subcultured at densities of 5,000 to 6,000 cells/cm^2, and used for experiments during passages 3 to 4.

Mouse MSCs (mMSC) were isolated from long bones of C57BL/6-Tg(NACTbeGFP)10ob/GFPg mouse (The Jackson Laboratory, Bar Harbor, ME) using the methods described by Peister et al. (34). Briefly, cells from each long bone were plated in 40 mL complete isolation medium (see ref. 34). After 24 hours, nonadherent cells were removed and adherent cells were washed with PBS, and fresh complete isolation medium was added every 3 to 4 days for 4 weeks. Cells were collected by trypsinization and replated in 30 mL complete isolation medium in 175 cm^2 flasks. After 1 to 2 weeks, cells were trypsinized and plated in complete expansion medium (see ref. 34). After another 1 to 2 weeks, passage 3 cells were either frozen or expanded further by plating at 50 cells/cm^2 and incubating in complete expansion medium.

Cell lines. Glioblastoma multiforme cell lines U87 and LN229 were obtained from the American Type Culture Collection (Manassas, VA). U251 cells were obtained from W.K. Alfred Yung (M.D. Anderson Cancer Center, Houston, TX). U87 and U251 cells were maintained in α-MEM supplemented with 10% FBS, and LN229 cells were maintained in α-MEM supplemented with 20% FBS and 2-(glutamine. Normal human astrocytes (NHA) were obtained from Cambrex (Walkersville, MD). The fibroblast line C29 was obtained from Juan Fueyo (M.D. Anderson Cancer Center). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2/95% air.

Animal subjects. Male athymic nude mice (nu/nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). All animal manipulations were done in accordance with institutional guidelines under approved protocols.

Intracranial xenografting of human glioma cells. Monolayers of human glioma cell lines were detached by trypsinization, washed, and resuspended in PBS at a concentration of 1 × 10^6 cells in 5 μL. Cells were injected into the right frontal lobe of nude mice using a guide-screw system implanted within the skull as described previously (35). To increase uniformity of xenograft take and growth, cells were injected into 10 animals simultaneously using a multiport Microinfusion Syringe Pump (Harvard Apparatus, Holliston, MA). Animals were anesthetized with xylazine/ketamine during the procedure.

Internal carotid artery injection of human mesenchymal stem cells. hMSCs were injected into the internal carotid artery of xenograft-bearing nude mice according to the previously described method (36). Briefly, animals were anesthetized with ketamine/xylazine and the internal carotid artery was surgically identified under microscopic visualization. Monolayer cultures of hMSCs were trypsinized and suspended in 100 μL α-MEM supplemented with 10% FBS and injected into the internal carotid artery using a prefabricated injection cannula. Injections were done manually over 3 to 5 minutes. Animals were monitored continuously until awakening and then daily for neurologic deterioration related to the injection.

Human mesenchymal stem cell labeling with SP-DiI. The fluorescent dye SP-DiI (Molecular Probes, Eugene, OR) was dissolved in dimethylfor-mamide (Sigma Chemical) to the concentration of 2.5 mg/mL as described previously (27). SP-DiI dye was then added directly to culture medium to a final concentration of 10 μg/mL. hMSCs were incubated with 25 mL medium with SP-DiI in T175 flask for 48 hours, after which time cells were washed with PBS, incubated with dye-free medium for 4 hours, and used for experiments.

Brain tissue/tumor preparation. At indicated time points, animals were sacrificed by CO2 inhalation, and 6 μm serial coronal cryosections from frozen brains were processed for light and fluorescent microscopy. Adjacent sections were stained with H&E for visualization of the tumor mass. In some sections, nuclei were stained with fluorescence-conjugated antibody [4',6-diamidino-2-phenylindole (DAPI), Molecular Probes]. Imaging was done with a Nikon microscope equipped with a CCD camera. Images were merged using Adobe Photoshop software version 7.0 (Adobe Systems, Inc., San Jose, CA).

In vivo migration assay. The ability of hMSCs to migrate toward gliomas was assessed in vivo by implanting U87 glioma cells [10^5 cells, stably transfected with plasmid containing green fluorescent protein (GFP) gene, gift of Charles Conrad, M.D., Anderson Cancer Center] into the frontal lobe of nude mice as described above. Seven days later, SP-DiI-labeled hMSCs (10^5 cells) were implanted in the opposite hemisphere. Migration toward the tumor was assessed at 14 days by direct visualization using fluorescent microscopy.

In vitro migration assay. The tropism of hMSCs for tumor cells and growth factors was determined using an in vitro migration assay according to previously described methods (37). hMSCs in serum-free medium were placed in the upper well of 24 mm tissue culture Transwell plates (12 μm, Nunc, Naperville, IL) coated with polylysine and Matrigel (1 mg/mL in α-MEM). U87 cells, fibroblasts, or NHAs were incubated in serum-free medium for 48 hours, and the resulting conditioned medium was aspirated and placed in the lower well of the Transwell plates. In selected experiments, growth factor [epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), or stromal cell–derived factor-1α (SDF-1α), 100 mg/mL] was added to the lower compartment. In other experiments, a cocktail of antibodies that blocked the activity of specific growth factors [i.e., anti-PDGF-BB (Sigma Chemical), anti-EGF (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-SDF-1α (Chemicon, Temecula, CA)] each at 0.8 μg/mL was added to the conditioned medium from gliomas cells. hMSCs were incubated for 48 hours at 37°C, and the migration ratio was determined using colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] as described previously (38) or by fixing the membrane, staining the cells using the Hema3 staining kit (Fisher Diagnostics, Pittsburgh, PA), directly counting the number of migrated cells in 10 high-power fields, and calculating the mean. All experiments were done in triplicate.
Adenoviruses carrying the IFN-β gene or the β-galactosidase (β-gal) were engineered using the bacterial plasmid recombination AdEasy system (Qbiogene, Irvine, CA) as described previously (27). To transfect hMSCs, cells were incubated with adenoviruses at specified multiplicity of infection for 2 hours, cells were washed and used as described in each experiment. IFN- expression was detected by ELISA (R&D Systems, Minneapolis, MN).

In vitro efficacy experiments of hMSC-IFN-β. For coculture experiments, U87 glioblastoma cells (10^5 cells) were plated onto 6 cm dish along with increasing ratios of IFN-β-secreting hMSCs. As a control, MSCs-β-gal were used. Cells were trypsinized, stained with 0.04% trypan blue solution, and counted using a hematocytometer. Growth curves were generated by combining data from three independent experiments.

For efficacy experiments involving Transwell plates, U87 cells were grown as monolayers in the lower well of 24 mm tissue culture Transwell plates on porous inserts (12 μm). hMSCs, hMSC-β-gal, or hMSC-IFN-β were plated in the upper well of the Transwell plates. U87 cells were trypsinized and viable cells were counted using a hemocytometer after trypan blue staining. All experiments were done in triplicate.

In vivo efficacy experiments of hMSC-IFN-β. To evaluate the effects of hMSCs in vivo, direct intratumoral injection of hMSC-IFN-β was undertaken. U87 cells (5 × 10^5 cells in 5 μL PBS) were implanted into the right frontal lobes of nude mice as described above. After 10 days, when tumors where well established, hMSC-IFN-β were injected directly into the tumor using the permanently implanted guide-screw system or into the ipsilateral carotid artery as described above. Animals were followed until tumor using the permanently implanted guide-screw system as described previously (35). Seven days after tumor establishment, hMSCs (10^6 cells in 200 μL PBS) were injected directly into the carotid artery ipsilateral to the implanted tumor (n = 8). To determine whether hMSCs could localize to gliomas other than U87, xenografts were injected into the internal carotid artery contralateral to the hemisphere bearing the U87 xenograft (n = 5). Similar to the ipsilateral injection, contralateral injection resulted in the localization of SP-DiI-labeled hMSCs exclusively within the tumor, including (in one example) within small tumor nodules away from the main mass but not in the normal brain (Fig. 2). In addition to supporting the concept that hMSCs are selective for gliomas compared with normal brain tissue, these results also indicate that the localization within the xenograft was not merely the result of preferential blood flow to the tumor mass.

To show that injected hMSCs remained as whole cells within the xenograft, engrafted SP-DiI-labeled hMSCs were counterstained with DAPI, a nuclear-specific probe. Dual DAPI-positive and SP-DiI-labeled cells were seen exclusively within the xenografts, indicating that engrafted hMSCs were morphologically intact (Fig. 4).

To determine whether hMSCs could localize to gliomas other than U87, xenografts were established in the frontal lobe of nude mice using the U251 (n = 5) or LN229 (n = 4) glioma cell lines. After injection into the carotid artery ipsilateral to the implanted tumor, SP-DiI-labeled hMSCs were identified within the LN229 and U251 xenografts (Fig. 5), suggesting that the ability to support the integration of hMSCs was not a unique property of U87 cells.

To show that the tropism of hMSCs for gliomas was a unique property of these stem cells and not a property of other human cells, SP-DiI-labeled human fibroblasts were injected into the peritumoral normal brain or in the hemisphere opposite to the implanted tumor in all animals assessed, indicating that hMSCs specifically localize in the tumor but not in the normal brain. The specificity of hMSCs for U87 xenografts was seen best in whole mounts of the entire brain in which SP-DiI-labeled hMSCs were confined specifically within the borders of the irregularly shaped tumor but were not seen in the surrounding brain (Fig. 2).

Results
Isolation and expansion of human mesenchymal stem cells. Bone marrow aspirates were obtained from normal human donors, isolated, and expanded according to previously described methods (27). Cells had a typical spindle shape, consistent with the morphology reported by others (11, 39–42). Although hMSCs do not have a specific antigen profile, for each culture, we verified that isolated cells were negative for typical hematopoietic antigens CD45, CD34, and CD38 and were positive for CD44 and CD105 (data not shown). The doubling time of our cultures varied between 30 and 40 hours and cells could be expanded to 10 to 11 passages before developing a large flat morphology. Thus, based on available criteria, the cells used in our experiments had the properties of hMSCs as described previously (11, 39–42).

Localization of human mesenchymal stem cells in human glioma xenografts after regional intravascular administration. To determine the extent to which hMSCs are capable of selectively integrating into human gliomas after systemic delivery in vivo, intracranial xenografts of the human glioma cell line U87 were established in the frontal lobes of nude mice using a guide-screw system as described previously (35). Seven days after tumor inoculation when xenografts were established, hMSCs (10^6 suspended in 200 μL α-MEM with 10% FBS) were injected into the carotid artery ipsilateral to the implanted tumor (n = 8). To visualize them on postmortem histologic sections, hMSCs were stained before injection with a fluorescent vital dye SP-DiI as described in Materials and Methods. Animals were sacrificed 1 and 7 days after injection, the brains were removed, and frozen sections were analyzed by light and fluorescent microscopy. SP-DiI-labeled hMSCs were seen exclusively within the U87 tumor mass both 1 and 7 days after injection (Fig. 1). Essentially no hMSCs were seen in the peritumoral normal brain or in the hemisphere opposite to the implanted tumor.
ipsilateral carotid artery of mice bearing U87 gliomas. Fibroblasts were chosen because hMSCs were originally described as fibroblast colony-forming cells and because fibroblasts are morphologically similar to hMSCs (14). However, injections of fibroblasts into the carotid artery consistently resulted in the death of animals (n = 8) presumably due to intravascular cellular embolization and secondary brain ischemia, a finding suggestive of widespread distribution of fibroblasts in the brain without tumor specificity. As an alternative approach, U87 tumor cells were used. In contrast to hMSCs, ipsilateral carotid injection of SP-DiI-labeled U87 tumor cells into the carotid artery of animals bearing U87 xenografts resulted in widespread distribution of injected cells throughout the brain with no cells colocalizing to the tumor (Fig. 6A). These studies show that not all human cells are capable of localizing to human gliomas after intracarotid injection; thus, the capacity of hMSCs to integrate into gliomas is a specific property of these stem cells. These studies also indicate that the observed localization of hMSCs within human glioma xenografts was not due to the fact that human cells were injected into mice (i.e., that the results were due to a species-specific interaction between human xenografts and human stem cells in a mouse brain background), because not all injected human cells have the capacity to localize to human xenografts in this model system.

To further show that the observed localization of hMSCs within gliomas was not a function of the model system (i.e., a species-specific interaction), mMSCs were harvested from the bone marrow of C57 mice (see Materials and Methods). mMSCs were labeled with SP-DiI and injected into the ipsilateral carotid artery of nude mice bearing established U87 xenografts (n = 3). Similar to the hMSCs, mMSCs were found exclusively within the xenografts 7 days after injection (Fig. 6B).

Human mesenchymal stem cells migrate toward gliomas after intracranial injection. Although the above studies indicate that hMSCs can localize to human gliomas after intravascular delivery, it is also of interest to determine the extent to which hMSCs have the capacity to migrate toward gliomas once within the brain. To better define the extent to which hMSCs are capable of migrating toward human gliomas in vivo, local delivery experiments were carried out. Specifically, intracranial xenografts of the human glioma cell line U87 were established in the right frontal lobe in mice using a guide-screw (35). Seven days after tumor inoculation, SP-DiI-labeled hMSCs (10^5 cells in 10 μL medium/FBS) were injected directly into the opposite cerebral lobe (n = 5). As a control, a group of tumor-bearing animals received intracranial injections of human fibroblasts (10^5 cells in 10 μL medium/FBS). Animals were sacrificed 14 days after injection, their brains were removed, and frozen sections were analyzed by light and fluorescent microscopy. By 14 days after administration,
SP-DiI-labeled hMSCs were seen extending from the site of injection, across the brain between the tumor and the injection site, and within the tumor (Fig. 6C). In contrast, fibroblasts remained within the injection site. Thus, hMSCs have an intrinsic attraction for gliomas and are capable of migrating between hemispheres toward gliomas.

Factors mediating tropism of human mesenchymal stem cell for gliomas. We hypothesized that a factor released by the glioma cells may be a potential mediator of the tropism of hMSCs for human gliomas. To test this hypothesis, in vitro Matrigel invasion assays using Transwell plates were done as a surrogate assay for the tropism of hMSCs for gliomas. We first investigated if human glioma U87 cell lines were capable of stimulating the migration of hMSCs. Thus, hMSCs were placed in the upper wells on Matrigel, and conditioned medium from U87 gliomas and C29 fibroblasts grown in serum-free medium were placed in the lower wells. Conditioned medium from NHAs was used as another control to better mimic the normal brain milieu. A semiporous membrane (8 μm pores) separated the wells. Cell-free medium without and with 20% FCS was also used as controls. Migration was quantified by directly visualizing and counting migrated cells under the microscope after cell staining. Whereas exposure to cell-free medium or to conditioned medium from fibroblasts or NHAs resulted in low levels of migrating hMSCs, exposure to conditioned medium from U87 cells produced significant hMSC migration (Fig. 7A). The observed differences in migration were not due to increases in hMSC proliferation because the total number of hMSCs (invading plus noninvading) was the same for each condition.

Because exposure to 20% FCS stimulated significant hMSC migration (Fig. 7A), we analyzed the effects on hMSC migration of several growth factors (specifically PDGF-BB, EGF, basic FGF, and VEGF) that are commonly present in serum and that have been implicated in glioma growth. Maximal hMSC migration occurred with exposure to PDGF-BB (100 ng/mL). Intermediate levels of migration were observed after exposure to EGF (100 ng/mL) and SDF-1α (100 ng/mL), whereas basic FGF (100 ng/mL) and VEGF (100 ng/mL) had no significant effect compared with serum-free medium (Fig. 7B and C).

To document that the increase in migration of hMSCs that was seen after exposure to conditioned medium from U87 cells was due to the presence of PDGF-BB, EGF, or SDF-1, conditioned medium from U87 cells was treated with a cocktail containing anti-PDGF-BB, anti-EGF, and anti-SDF-1 antibodies (see Materials and Methods) that are capable of blocking the activity of each growth factor. Whereas conditioned medium from U87 cells resulted in a significant increase in hMSC migration, treatment with the blocking antibody cocktail significantly attenuated the migration of hMSCs through Matrigel (Fig. 7D). These results suggest that specific growth factors may at least in part mediate the tropism of hMSCs for gliomas.
Therapeutic potential of genetically engineered human mesenchymal stem cells on human gliomas: in vitro studies.

As a proof of principle that hMSCs are capable of delivering a therapeutic agent to brain tumors, we transfected hMSCs with an adeno-viral vector containing the cDNA of the IFN-β gene (Ad-IFN-β) as described previously (27). A quantified ELISA assay revealed that monolayers of these engineered hMSCs (designated hMSC-IFN-β) released IFN-β into the medium dependent on the number of viral particles used to transfect the cells (Fig. 8A). Based on these results, hMSCs were typically
infected with 3,000 multiplicities of infection for all subsequent experiments.

To determine whether hMSC-IFN-β are of therapeutic benefit, U87 glioma cells were cocultured with increasing ratios (0.1-50%) of hMSC-IFN-β. As a control, hMSCs transfected with adenovirus containing the β-gal cDNA (hMSC-β-gal) were cocultured with U87 cells at a ratio of 2:1 (U87:hMSC). hMSC-IFN-β significantly inhibited the growth of human gliomas even when the ratio of U87 to hMSC-IFN-β was 1,000:1 (0.1%; Fig. 8B).

To prove that this growth inhibition was specifically due to the release of soluble IFN-β, hMSCs (untransfected), hMSC-β-gal, or hMSC-IFN-β were grown in the upper well of Transwell plates in increasing numbers and U87 glioma cells were grown in the lower well. A semiporous membrane (8 μm pores) separated the cells. Whereas treatment with hMSCs or hMSC-β-gal resulted in progressive increase in cell numbers, exposure to hMSC-IFN-β resulted in a dose-dependent growth inhibition of U87 glioma cells (Fig. 8C). To verify that this effect was due to release of soluble IFN-β, the concentration of IFN-β in the medium of the lower well was determined at each time point (Fig. 8D). There was a dose-dependent increase in the amount of soluble IFN-β that directly correlated with the inhibition of tumor cell growth.

Therapeutic potential of genetically engineered human mesenchymal stem cells on human gliomas: in vivo studies.

To determine if hMSC-IFN-β-induced growth inhibition also occurs in vivo, U87 cells ($5 \times 10^5$) were implanted into the frontal lobe of nude mice, and after 10 days, animals were treated with a single intratumoral injection of PBS, hMSC-β-gal (2.5 × $10^5$ cells), or hMSC-IFN-β (2.5 × $10^4$ or 2.5 × $10^5$ cells). In a group of animals, hMSC-IFN-β (2.5 × $10^5$ cells) were given s.c. Compared with animals treated with PBS or with hMSC-β-gal, treatment with 2.5 × $10^5$ hMSC-IFN-β resulted in a significant ($P < 0.05$) increase in animal survival (Fig. 9A). Interestingly, injection of 2.5 × $10^4$ hMSC-IFN-β did not prolong survival, suggesting that in this model >2.5 × $10^4$ hMSC-IFN-β must be present within the tumor for growth inhibition to occur. Furthermore, s.c. administration of hMSC-IFN-β had no effect on survival compared with controls, suggesting that local (intratumoral) release of IFN-β is required for an antitumoral effect.

To determine whether regional delivery of hMSC-IFN-β is an effective antiglioma approach, U87 cells were implanted into the frontal lobe of nude mice ($n = 6$/group). Ten days later, animals were treated with PBS, hMSC-β-gal (10⁶ cells), or hMSC-IFN-β (10⁶ cells) by injection into the internal carotid artery (Fig. 9B). A group of animals were also treated with human IFN-β (50,000 IU) given i.v.
and another group received a s.c. injection of hMSC-IFN-β (10^6 cells). Only intra-arterial treatment with hMSC-IFN-β significantly extended the survival of the animals (P < 0.05). Neither systemic treatment with IFN-β (not carried by hMSCs) nor s.c. injection (i.e., distant from the tumor) of hMSC-IFN-β altered animal survival compared with controls. Thus, hMSC-IFN-β are effective against intracranial gliomas when delivered regionally.

**Discussion**

In this study, we provide evidence that human bone marrow-derived MSCs can localize to human gliomas after regional intra-arterial delivery and can migrate toward human gliomas after local intracranial delivery. We also show that the tropism of hMSCs for gliomas may be mediated at least in part by specific growth factors/chemokines. Most importantly, *in vitro* and *in vivo* efficacy...
studies show that hMSCs can be engineered to release a soluble factor (e.g., IFN-\textbeta) and that these engineered hMSCs can be exploited to therapeutic advantage against gliomas.

The finding that hMSCs localize to human gliomas is of interest because it suggests that the capacity for integration into tumors is an intrinsic property of these stem cells. This observation is consistent with the hypothesis that the intra-tumoral integration of exogenously delivered hMSCs is a recapitulation of the natural recruitment of endogenous, circulating hMSCs to aid in the process of stroma formation and tissue remodeling and suggests that hMSCs may contribute to the stroma of tumors (27, 30). Initial work from our group showed that bone marrow–derived hMSCs are capable of integrating into the stroma of metastatic melanoma grown either s.c. or in the lungs of nude mice (27). We now show that human gliomas grown in the brain of nude mice also support the engraftment of hMSCs delivered by an intravascular route. This finding in brain tumors is surprising because the stroma of primary brain tumors is composed of glial/astrocytic cells (ectodermal origin) and is thus distinct from the fibroblast-based (mesenchymal) stroma of most systemic (extracerebral) cancers. However, it has been shown that MSCs are capable of differentiating into glial cells (25, 26), including astrocytes, and it is thus possible that this property may explain the intrinsic capacity of hMSCs to integrate into the stroma of gliomas. Whether hMSCs that have localized to gliomas differentiate into astrocytes in our system is currently under investigation. Alternatively, human gliomas, similar to other cancers, require the elaboration of mesodermal elements, specifically endothelial cells and pericytes. It has been suggested that MSCs are a main source of pericytes within the bone marrow stroma (11, 16, 17, 40, 43); thus, hMSCs may integrate into gliomas to contribute to the mesenchymal elements of the tumor.

Figure 9. 

A, survival of mice after intracranial intratumoral injection of hMSC-IFN-\textbeta into established U87 gliomas. U87 cells (10^6) were implanted into the frontal lobe of nude mice. After 10 days, tumors were injected with a single dose of hMSC-IFN-\textbeta, hMSC-\textbeta-gal, or PBS (n = 5/group). A significant increase in survival is evident with treatment with 2 × 10^5 hMSC-IFN-\textbeta (star), Diamond, PBS; triangle, 2 × 10^5 hMSC-\textbeta-gal; cross, 2 × 10^5 hMSC-IFN-\textbeta s.c.; square, 2 × 10^5 hMSC-IFN-\textbeta; star, 2 × 10^5 hMSC-IFN-\textbeta. 

B, survival of mice after intra-arterial treatment with hMSC-IFN-\textbeta. U87 cells (10^5) were implanted into the frontal lobe of nude mice (n = 6/group). After 10 days, animals were treated with hMSC-IFN-\textbeta or controls by injection of 10^5 cells into the carotid artery. Treatment with hMSC-IFN-\textbeta significantly increased survival. Circle, PBS; triangle, IFN-\textbeta i.v.; diamond, hMSC-IFN-\textbeta i.v.; square, hMSC-IFN-\textbeta flank; cross, hMSC-\textbeta-gal intra-arterial; dark circle, hMSC-IFN-\textbeta intra-arterial.

![Graph A](attachment:image1.png)

![Graph B](attachment:image2.png)

P<0.05
previous work (27) suggest that hMSCs seem to have the capacity to engraft themselves into a variety of histologically disparate tumors, including gliomas, and thus may be a cellular vehicle that is universally applicable for delivery of therapeutic agents to most tumor types.

Several lines of evidence suggest that the localization of hMSCs to gliomas was not merely a consequence of the in vivo model system used in our studies. First, the integration of hMSCs is seen with several xenografts, including the U87, U251, and LN229 cell lines. These cell lines are genetically and phenotypically distinct. In addition, the LN229 cell line is fairly invasive and grows phenotypically similar to invasive gliomas in situ. Second, the engraftment of gliomas by hMSCs was seen after injecting the stem cells into the carotid artery either ipsilateral or contralateral to the hemisphere harboring the tumor. Thus, the results are not merely a fortuitous outcome of cerebral blood flow patterns but rather are due to a selectivity of hMSCs for the tumor compared with the normal brain. Third, intra-arterial injection of human fibroblasts resulted in death of most animals presumably due to widespread arterial occlusion. Furthermore, intracarotid injection of U87 tumor cells did not result in localization of delivered U87 cells within established U87 xenografts. Thus, the capacity to localize to a glioma after intravascular delivery seems to be a specific property of hMSCs and does not seem to be a function of all human cells. Fourth, similar to hMSCs, mMSCs that were delivered intravascularly also were capable of localizing to human gliomas. Taking these findings together, it is unlikely that the localization of hMSCs within human gliomas grown in nude mice was merely the result of a species-specific (human cell-human cell) tropism. Instead, hMSCs seem to have an intrinsic, cell-specific capacity to localize to human gliomas.

The isolation of MSCs from human subjects is an important aspect of our study. In fact, to our knowledge, this is the first demonstration that MSCs obtained from the bone marrow of human subjects can engraft themselves into human gliomas. Although Nakamura et al. reported recently the use of MSC in glioma therapy, these investigators isolated the stem cells from rat species (44). This is important because differences between nonhuman (i.e., murine or rat MSCs) and human MSCs have been noted (42). In addition, all studies using neural stem cells have by necessity relied on murine-derived neural stem cells (7–10). Because the MSCs used in our studies were derived from human subjects, our results support the application of these cells in an autologous transplantation setting in patients.

Although we show that hMSCs can migrate toward gliomas after intracranial delivery, an important aspect of our study from the perspective of clinical application is the use of intravascular delivery. Although direct intratumoral (intracranial) delivery has been reported for rat MSCs (44) and mouse neural stem cells (4, 7–10), intravascular delivery has the advantage that it obviates invasive surgical interventions and that repeated injections over an extended period are clinically feasible. To our knowledge, no other report has shown the propensity of hMSCs to localize to brain tumors after intravascular delivery. It should be noted, however, that we originally sought to deliver hMSCs systemically by tail vein injection but found that the majority of cells were filtered by the lung and only rare hMSCs were integrated into the tumor (at least when examined 7 days after injection; data not shown). Aboody et al. reported that after tail vein injection murine neural stem cells localized to tumors, albeit with low efficiency (45). In our studies, filtering of hMSCs within the lungs of nude mice may have been due at least in part to species incompatibilities. Whether trapped pulmonary hMSCs eventually recirculate and localize to intracerebral tumors is currently under investigation. Whether similar intrapulmonary entrapment will also occur in patients treated with hMSCs will ultimately require studies in human subjects.

Our results indicate that the tropism of hMSCs for gliomas may be mediated at least in part by growth factors/chemokines. This observation is consistent with the concept that hMSCs are attracted to the tumor milieu because tumors mimic tissue injury (31–33). Similar to damaged tissue, human gliomas express EGF, PDGF, VEGF, and FGF as well as the chemokine SDF-1α (see refs. 46, 47 for review). Despite this wide array of growth factors within tumors, however, we show that there is selectivity of hMSCs for specific factors. Whereas FGF and VEGF had little effect on hMSC migration, PDGF, EGF, and SDF-1α enhanced hMSC tropism. Moreover, a cocktail of antibodies that block PDGF-BB, EGF, and SDF-1α was able to attenuate the migration of hMSCs toward condition medium derived from U87 cells. Indeed, hMSCs are known to express EGF and PDGF receptors on their surface (43). It should be noted, however, that the in vitro invasion assay employed in this study may not directly mimic the in vivo conditions necessary for migration of hMSCs from the vasculature to the tumor. In vivo models that better recapitulate this process are currently under development. Further elucidation of the mechanism underlying the tropism of hMSCs for gliomas may provide insights into methods for increasing the efficiency of the engraftment process.

Our studies of IFN-β indicate that hMSCs can be used to deliver a diffusible molecule that can be released form hMSCs to achieve tumoricidal effects. IFN-β is a particularly good choice to show the proof of principle of this approach because phase I clinical trials of recombinant human IFN-β have shown that although responses do occur, systemic delivery of high doses of IFN-β is associated with toxicity and a narrow therapeutic index that limits the overall efficacy (48–52). Because IFN-β functions physiologically as a paracrine factor, its antitumoral effects can be enhanced and its toxicity is reduced if it is given locally (53, 54). We reasoned that hMSCs engineered to produce IFN-β would provide a high degree of local intratumoral delivery, with reduced systemic toxicity. In this context, we used an adenoviral vector to transfer the IFN-β gene into hMSCs and found that these engineered hMSCs (hMSC-IFN-β) released high levels of IFN-β and were capable of directly killing human glioma cell lines grown in vitro. Indeed, in Transwell experiments in which hMSC-IFN-β were physically separated from the glioma cells, there was a dose-dependent tumoricidal effect, the amount directly correlated with the concentration of soluble IFN-β released by the engineered hMSCs. Most importantly, regional delivery of hMSC-IFN-β by injection into the internal carotid artery in vivo significantly extended the survival of animals harboring established intracranial gliomas. These results were due to the local delivery of IFN-β by the engrafted hMSCs because IFN-β given i.v. did not extend animal survival compared with saline-treated controls, and hMSC-IFN-β implanted s.c. (i.e., at a site distant from the tumor) also had no effect. Thus, these studies provide the proof of principle that hMSCs can be engineered to release a soluble factor into brain tumors. Although our studies suggest that IFN-β is itself a good therapeutic agent worthy of assessment in patients with glioma, the same approach can be exploited in the delivery of other agents with antitumor activity.

Comparing the experiments in which hMSC-IFN-β were directly injected in the tumor with those in which hMSC-IFN-β were
delivered intravascularly provides insights into the number of hMSCs that may integrate into a tumor after intravascular delivery. Specifically, we found that a significant increase in animal survival required a direct intratumoral injection of at least $2.5 \times 10^5$ hMSC-IFN-γ; intratumoral injection of $2.5 \times 10^4$ did not extend animal survival (Fig. 9.4). In this context, it is reasonable to assume that at least $2.5 \times 10^5$ cells integrated into the tumor after intravascular delivery; otherwise, we would not have observed the reported increase in survival that occurred after administering hMSC-IFN-γ by this route. Because we saw a significant increase in survival after intra-arterial injection of $10^5$ hMSCs, we estimate that at least 25% of the cells ($2.5 \times 10^5$) must have integrated into the tumor.

As mentioned above, these in vivo studies revealed that animals bearing U87 xenografts that received hMSC-γ-gal (i.e., nonsenseecting hMSCs) had shorter survival than did animals who received saline injections. Although this observation implies that hMSCs may have a role in tumor development under physiologic conditions (see above), the fact that animal survival was increased when hMSCs were engineered to secrete IFN-γ indicates that this physiologic role can be exploited and tumor growth is reversed when a protein with antiangioma effects is released by the hMSCs. From a therapeutic perspective, these observations imply that relatively pure populations of stem cells engineered to carry a therapeutic gene (i.e., populations free of untransfected cells) will be needed to achieve maximal antitumor effects. Methods for maximizing transfection of therapeutic genes to hMSCs and for separating transfected from nontransfected cells are challenges for the ultimate application of this and other stem cell approaches to tumors.

Although our studies have focused on bone marrow–derived hMSCs, recent work has suggested that other cells in the bone marrow may also be useful as delivery vehicles for brain tumors (55). Specifically, Fine et al. reported recently that a neural stem-like cell could be isolated from the bone marrow and that these bone marrow–derived stem cells can be used to deliver therapeutic biological agents to brain tumors (55). Interestingly, using cDNA microarray technology, these investigators showed that the profile of expressed genes of bone marrow–derived hMSCs (i.e., the same cells used in our studies) is distinct from the expression profile of bone marrow–derived neural stem-like cells used in their studies, indicating that the two cell populations are unique. Thus, the bone marrow may be a rich source of several stem cell populations that may be useful in the treatment of brain tumors. Future studies comparing the functional properties of these distinct bone marrow–derived stem cell population will be of great interest for the clinical application of this type of stem cell therapy.

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References


16. Lal S, Lacroix M, Tofilon P, Fuller GN, Sawaya R, Lang FF. An implantable guide-screw system for brain...
Photomicrograph Showing Migration of hMSCs toward Glioma

In the article on human bone marrow–derived mesenchymal stem cells and gliomas in the April 15, 2005 issue of Cancer Research (1), Fig. 6C did not indicate that the middle figure was a montage created from five individual photomicrographs taken from regions between the stem cell injection site and the implanted tumor xenograft. For illustration purposes, the individual figures were postprocessed to highlight the red stem cells and the individual photomicrographs were placed on a black background, giving the incorrect impression that this was a single image. These data acquisition methods were not described in the figure legend or in Materials and Methods. Any manipulation of scientific illustrations is inconsistent with both previous journal standards and recently defined standards for electronic image collection, compilation, and alteration (2), and is not acceptable.

The authors have now corrected the figure to show the five individual photomicrographs, thereby eliminating the montage; the authors also have revised the figure legend. The new figure is free of any postprocessing, except where red and green images were merged with each other. This corrected version of the figure appears below. The authors note that the correction does not alter their conclusion that hMSCs are capable of migrating toward the tumor after intracranial injection.


Figure 6. Photomicrographs showing migration of hMSCs toward glioma. U87 cells (gfp labeled) were implanted in the right frontal lobe (day 0) and SP-DiI-labeled hMSCs were implanted in the left lobe (day 7). Top, photomicrograph of brain section taken 14 days after cell injection. The boxes show the location of the images represented at the bottom. The numbers in the box correspond to the photomicrographs at the bottom. The corner where the number is located is on the top is the same corner of the corresponding photomicrograph at the bottom. Note the overlap of photomicrographs 1, 2, 4, and 5. Bottom, individual photomicrographs taken under the fluorescent microscope from regions shown on the top. Images 1 to 3, 4R, and 5R were taken with the red fluorescent filter. Images 4G and 5G were taken under the green filter. No green tumor cells were seen in the left brain (not imaged). 4R, 4G, 5R, and 5G were merged using Photoshop to produce images 4M and 5M, respectively. Specifically, the corresponding red and green images were merged by copying the red image onto the green image and then using the Layer Style function and adjusting the Change Blend option, thus producing a merged picture. Red-fluorescent hMSCs are seen around the injection site (1), in between the injection site (2 and 3), and within the green-fluorescent tumor (4R, 4G, 4M, 5R, 5G, and 5M).
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Akira Nakamizo, Frank Marini, Toshiyuki Amano, et al.


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