In vivo magnetic resonance imaging of cell tropism, trafficking mechanism, and therapeutic impact of human mesenchymal stem cells in a murine glioma model

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ABSTRACT

Stem cells have offered much promise as delivery vehicles for brain tumor therapy, with the development of modalities to track the tumor tropism of stem cells receiving intense focus. Cellular magnetic resonance imaging (MRI) allows serial high-resolution in vivo detection of transplanted stem cells’ tropism toward gliomas in the mouse brain once these cells are internally labeled with iron oxide particles, but has been impeded by low labeling efficiencies. In this study, we describe the use of ferucarbotran and protamine (Fer-Pro) complexes for labeling human mesenchymal stem cells (hMSCs) for MRI tracking of glioma tropism in vivo. We found that Fer-Pro was not toxic and was highly efficient for labeling in vitro. Cell labeling with Fer-Pro promoted the migration of hMSCs toward glioma U87MG cells in vitro, which was mediated by stromal-derived factor-1/CXCR4 (SDF-1/CXCR4) signaling. Fer-Pro-labeled hMSCs could migrate specifically toward gliomas in vivo, which was observed with a clinical 1.5-T MRI system. The efficient labeling of Fer-Pro also allowed a tropic mechanism mediated by SDF-1/CXCR4 signaling to be detected by MRI in vivo. Additionally, the potential intrinsic inhibitory effect of hMSCs on glioma progression was estimated simultaneously. This is the first report to have used a clinical MRI modality to simultaneously study the migration, the therapeutic impact on tumors, and above all the trafficking mechanism of bone marrow-derived mesenchymal stem cells from human in a murine glioma xenograft model. The use of Fer-Pro for stem cell labeling may have potential clinical applications in stem cell guided therapy.

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1. Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumor, with a high mortality rate and the worst prognosis [1–3]. Despite advances in surgical and adjuvant therapies, patients carrying a primary GBM typically have a mean survival period of less than a year following diagnosis [3]. The poor outcome of patients with GBM is related to the resistance to current therapy, radiation, and chemotherapeutic approaches. Also, it is difficult to completely resect high-grade gliomas because of the infiltration of malignant cells into surrounding brain parenchyma [4,5]. Moreover, some new therapeutic modalities have failed to achieve significant therapeutic effect due to an inability to effectively deliver therapeutic agents to the invasive tumor cells [5]. Therefore, new strategies for selective tumor-targeting of therapeutic agents are needed to substantially improve brain tumor therapy.

Stem cells have offered much promise as delivery vehicles for brain tumor therapy because of their tropism toward tumor cells. For instance, the use of neural stem cells (NSCs) as vehicles for gene therapy in brain tumors has been of great interest [6–9]. In addition, due to the intrinsic tumor-inhibition effect of NSCs on gliomas [7,10], this NSC-based therapeutic approach would overcome current limitations of conventional clinical therapies. Because of ethical and technical problems associated with neural stem cells, an alternative type of is being sought. Mesenchymal stem cells (MSCs) are increasingly regarded as attractive candidate for delivering vesicles of therapeutic agents [11,12]. Among the main reasons for this are that MSCs can be easily isolated and expanded in culture [13–15], that MSCs can possibly overcome immunological incompatibilities because of autologous transplantation [16], and, above
all, that MSCs can migrate toward gliomas in vivo [11,12,17]. Also, there is evidence that MSCs can exert an inhibitory effect on several tumors [17–19]. Taken together, these facts indicate that MSCs could replace NSCs as a therapeutic vehicle against gliomas.

Development of any cell-based therapy requires monitoring the fate and distribution of transplanted stem cells to maximize the therapeutic benefit. Traditional techniques for examining stem-cell transplantation in animal models, often performed by postmortem histological analysis, cannot be appropriate in clinical studies; hence, it is important to develop noninvasive in vivo stem cell tracking modalities. Cellular magnetic resonance imaging (MRI) is an ideal imaging modality for serial tracking and identifying the fate of transplanted cells once the cells are internally labeled with suitable MRI-visible particles prior to transplantation. Currently most studies employ superparamagnetic iron oxide (SPIO) nanoparticles as cellular MRI probes. The efficacy of cellular MRI largely depends on the internalizing efficiency of SPIO nanoparticles into stem cells; therefore, considerable efforts have been devoted to improving their cellular uptake efficiency. Recently it was reported that ferucarbotran (Resovist), anionic SPIO nanoparticle with carboxydextran coating, can more efficiently magnetically label human MSCs (hMSCs) than ferumoxide (Feridex) without cytotoxicity [20]. Our study has also demonstrated that ferucarbotran-labeled hMSCs can be easily detected in vitro and in a mouse brain model with a clinical 1.5-T MRI system [21]. Thus, ferucarbotran seems to be an ideal labeling probe to investigate the tropism of MSCs toward malignant gliomas using MRI.

Proteamine is a low molecular weight polycationic peptide that has been approved by the U.S. FDA as an antidote for heparin anticoagulation [22]. Arbab et al. showed an enhanced uptake of ferumoxide by proteamine for cell labeling [23]. Moreover, a recent study used proteamine to accelerate hMSC labeling with ferucarbotran [24]. Thus, in this study we investigated the use of ferucarbotran as MRI contrast probe combined with proteamine for efficiently cellular labeling to study the detailed tropism behavior of hMSCs in a murine glioma model. Using a clinical 1.5-T MRI system we observed that hMSCs labeled by ferucarbotran and proteamine complexes could specifically migrate toward gliomas in vivo. The efficient labeling of ferucarbotran and proteamine complexes allowed a tropic mechanism mediated by stromal-derived factor-1/CXCR4 (SDF-1/CXCR4) signaling to be detected also by MRI in vivo. Additionally, the potential intrinsic inhibitory effect of hMSCs on glioma progression was estimated simultaneously.

2. Materials and methods

2.1. Cells and culture conditions

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow of normal donors as described in our previous study [25] with informed consent approved according to the procedures of the institutional review board and were cultured in regular growth medium consisting of low-glucose DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The cells were immunophenotypically characterized as described in our previous study [25] by flow cytometry for many markers common to human MSCs [26] and were able to give rise to adipocytes, osteocytes, and chondrocytes when incubated with adequate differentiation media [25], which shows their multipotentiality (data not shown). hMSCs were used at passages 6–8 in all experiments.

U87MG cells from American Type Culture collection (Manassas, VA) were maintained in high-glucose DMEM (Gibco) supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

All cultures were kept in an atmosphere of 5% CO2 and 95% air at 37 °C.

2.2. Formation of ferucarbotran and proteamine complexes (Fer-Pro) and magnetic cellular labeling

Ferucarbotran (Bayer Schering Pharma AG, Berlin, Germany) is provided as a 1.4-ml ready-to-use solution of 28 mg iron/ml (0.5 w). A ferucarbotran stock solution of 1 mg iron/ml was prepared with phosphate buffered saline (PBS). Proteamine sulfate (Sigma-Aldrich, St. Louis, MO) was also prepared with PBS in a stock solution of 1 mg/ml. Complex formation was performed by mixing ferucarbotran with a final concentration of 100 μg iron/ml and proteamine with a final concentration of 100 μg/ml and then under sonication for 10 min in the labeling media (serum-free DMEM), after which the ferucarbotran and proteamine complexes (Fer-Pro) were used.

For magnetic labeling, hMSCs were seeded at 1.2 × 10^6 cells/cm^2 in growth media and then cultured for 24 h. After PBS wash, the cells were labeled with or without ferucarbotran (100 μg iron/ml), proteamine (100 μg/ml), or Fer-Pro complexes (100 μg iron/ml ferucarbotran and 100 μg/ml proteamine) in the labeling media for 1 h, after which cells were washed once with 2% FBS-containing PBS and twice with fresh PBS, and then processed for further experiments.

2.3. Cell viability assay

The cytotoxic effect of cellular labeling was assessed using 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) reduction. After labeling, cells in 96-well plates (6 × 10^3 cells per well) were either immediately incubated with fresh serum-free medium containing 0.5 mg/ml of MTT for 1 h at 37 °C for acute cytotoxicity assay or allowed to grow in regular growth medium for 24 h, followed by incubation with fresh serum-free medium containing 0.5 mg/ml MTT for 30 min at 37 °C for growth effect assay. After MTT incubation, 400 μl of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to the cells to extract the dark blue formazan dye generated by the live cells, and the absorbance at 570 nm was measured using a microplate reader.

2.4. Prussian blue staining

Magnetic labeling efficiency of hMSCs was also qualitatively examined by Prussian blue staining as previously described [27,28]. After labeling, cells in 12-well plate (4.8 × 10^4 cells per well) were stained with 1% potassium hexacyanoferrate (II) trihydrate (Sigma-Aldrich) and 20% hydrochloric acid (HCl) for 30 min. Then the cells were washed twice with distilled water and visualized under a light microscope (CX41, Olympus, Tokyo, Japan).

Fig. 1. The effect of magnetic labeling on cytotoxicity. After labeling for 1 h, the effects of 100 μg iron/ml ferucarbotran (Fer), 100 μg/ml proteamine (Pro), and the ferucarbotran and proteamine complexes (Fer-Pro) on the acute MTT reduction activity (A) and the cell proliferation (B) of hMSCs, respectively. All data are expressed as mean ± standard error of five determinations (each in quadruplicate) of three donors. (*, p < 0.05; ***, p < 0.001).
2.5. ICP-MS

Magnetic labeling efficiency of hMSCs was quantitatively determined by ICP-MS for iron content. After labeling, cells in 6-well plates (1.2 × 10^5 cells per well) were washed twice by PBS for removing residual culture media, collected by trypsinization, and centrifuged in 1-ml eppendorf tubes. The cell pellets were lysed with 0.5 ml of double deionized water. Hereafter, nitric acid (0.1 ml, ultrapure reagent grade, Mallinckrodt Baker Inc., Phillipsburg, NJ) was admixed to digest the cell lysate for 10 min, which was then diluted to 5 ml with double deionized water. All of the digested cell lysates were further analyzed by ICP-MS (Agilent 7500cx, Agilent Technologies Inc., Tokyo, Japan) for quantifying the concentration of iron.

2.6. In vitro cellular MRI protocol

MRI was performed using a clinical 1.5-T MR system (Signa Excite, GE Healthcare). After magnetic labeling, cells in 6-well plates (1.2 × 10^5 cells per well) were collected by trypsinization, centrifuged, and placed in 300-µl eppendorf tubes (1.2 × 10^5 cells per tube) positioned in a water tank. Next, the tank was placed in an 8 channel head coil. Gradient echo pulse sequences provided by the vendor were used (TR/TE = 550/15 ms, Flip angle = 15°, matrix size = 256 × 192). The slice thickness was 1.4 mm with a 0.3 mm gap; and the field of view (FOV) was 14 × 10.5 cm for coronal scanning of the test tubes and 8 min and 2 s for sagittal scanning at the NEX of 3. The images were then analyzed at the workstation provided by GE healthcare.

2.7. In vitro migration assay

The migration capacity of hMSCs was analyzed using Costar Transwell chambers with polycarbonate membrane filters of 6.5 mm diameter and 8 µm pore size (Corning, NY). After Fer-Pro labeling, cells in 6-well plates (1.2 × 10^5 cells per well) were harvested, and then added to the top chambers at 2 × 10^5 cells/100 µl of 15% FBS-containing DMEM per chamber. The bottom chambers were added with U87MG cells at 5 × 10^5 cells/600 µl of 10% FBS-containing DMEM per chamber. Then the top chambers and bottom chambers were separately incubated at 37 °C, 5% CO₂, overnight for cell seeding. After seeding, the media in the top chambers and the bottom chambers were replaced with 0.5% FBS-containing DMEM, and the top chambers were transferred to the bottom chambers. After that the chamber system was incubated overnight at 37 °C, 5% CO₂ for 24 h, hMSCs on the top surface of the filters were wiped off with cotton swabs after a careful wash with cold PBS. Cells that had migrated into the lower compartment of the top chambers and attached to the lower surface of the filter were counted after staining with crystal violet dye. The bottom chambers without U87MG cells (0.5% FBS-containing DMEM only) were used as negative control. For neutralization assays, anti-CXCR4 (100 µg/ml) (clone 12G5, R&D Systems, Lille, France) and anti-SDF-1 antibodies (250 µg/ml) (clone 79104, R&D Systems) were added with hMSCs in the top chambers or with U87MG cells in the bottom chambers, respectively. Five replicates of each sample were counted. The assay was done six times for each condition. The migration rate was expressed as the percentage of migrated cell number of anti-CXCR4 antibody-treated condition or anti-SDF-1 antibody-treated group compared with that of vehicle control (hMSCs without anti-CXCR4 antibodies in the top chambers and U87MG cells without anti-SDF-1 antibodies in the bottom chambers).

2.8. Animal study

Seven- to 8-week-old male nude mice were obtained from the Animal Center of National Taiwan University and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee. Stereotaxic implantation of cells was performed with a NARISHIGE apparatus (51600 Single Manipulator Model, Stoelting Co., Wood Dale, IL), and a WPI syringe (Hamilton Co., Reno, NV) with a bevel-tipped 26-gauge needle. Animals were anesthetized with ketamine/xylazine during the following cell implantation.

U87MG cells maintained in high-glucose DMEM were labeled with Vybrant DyeCycle Green stain (Molecular Probes, Eugene, OR) (5 µg/ml medium) for 48 h, harvested, and suspended in PBS at a concentration of 1 × 10^5 cells/ml. DyeCycle Green-labeled U87MG cells were injected into the right frontal lobe of mice. The injection coordinates were (relative to bregma: AP, the midline [ML], and the dura [DV]); AP: +1 mm, ML: 1.5 mm (right), DV: 2 mm. Injections were performed at the rate of 1 µl/min; and the needle was left in place for 5 min before withdrawal.

Fig. 2. The efficiency of magnetic labeling. (A) Qualitative detection of intracellular iron of unlabeled cells (Control cells), Fer-labeled cells, and Fer-Pro-labeled cells by Prussian blue staining. (B) Intracellular iron content was quantitatively determined by ICP-MS. Data are expressed as mean ± standard error of three independent experiments. ***Statistically significant difference (p < 0.001) as compared with unlabeled cells (Control). Fer-Pro demonstrated higher cellular uptake in hMSCs compared to Fer (#, p < 0.05). (C) Representative MRI images of centrifuged hMSCs pellets in tubes placed in a water bath.
Fig. 3. Imaging of tumor tropism of hMSCs toward glioma cells in vitro and in vivo. (A) In vitro migration of hMSCs in Transwell assay. Unlabeled hMSCs in the top chambers migrated toward the bottom chambers with 0.5% FBS-containing media (a), 30% FBS-containing media (b), or U87MG cells in 0.5% FBS-containing media (c). Fer-Pro-labeled hMSCs in the top chambers migrated toward the bottom chambers with U87MG cells in 0.5% FBS-containing media (d). Comparing (c) with (d), Fer-Pro labeling did not prohibit but promoted the migration capacity of hMSCs toward U87MG cells (e). Data are expressed as mean ± standard error of five determinations (each in quadruplicate) of three donors (***, p < 0.001). (B) In vivo MRI of mouse that received Vybrant DyeCycle Green-labeled U87MG cells (green arrow) in the right frontal lobes for 14 days. (C) In vivo MRI of mouse that received Vybrant DyeCycle Green-labeled U87MG cells alone (green arrow) in the right frontal lobes for 28 days. (D) In vivo MRI of mouse injected with Vybrant DyeCycle Green-labeled U87MG cells (green arrow) in the right frontal lobes for 28 days and SP-Dil-Fer-Pro colabeled hMSCs (yellow arrow) into the left lateral ventricles for 14 days. Red arrow denotes the migrated SP-Dil-Fer-Pro colabeled hMSCs toward DyeCycle Green-labeled U87MG cells in the right frontal lobes. (E) In vivo MRI of mouse injected...
hMSCs in growth media (10% FBS) were labeled with fluorescent dye SP-Dil (Molecular Probes) (10 μg/ml medium) for 48 h, harvested, and subcultured onto 6-well plates at 1.2 × 10^5 cells/well for magnetic cellular labeling. At day 14 post-implantation, SP-Dil-Fer-Pro colabeled hMSCs in serum-free DMEM were resuspended in serum-free DMEM at a concentration of 3.6 × 10^5 cells/5 μl and injected into the left lateral ventricles of nude mice. For in vivo tropism blocking, SP-Dil-Fer-Pro colabeled hMSCs in serum-free DMEM were mixed with anti-CXC4R1 antibodies (200 μg/ml) before the implantation. The injection coordinates were (relative to bregma [AP], the midline [ML], and the dura [DV]): AP: 0 mm, ML: 1 mm (left), and DV: -2.0 mm. Injections were performed at the rate of 1 μl/min; and the needle was left in place for 5 min before withdrawal.

2.9. In vivo MRI protocol

After implantation of hMSCs, the clinical 1.5-T MR system applied in vitro was also used to observe the tropism of hMSCs in vivo. Under gas anesthesia with 2% isoflurane, the mouse was placed in a homemade resonance coil with an inner diameter of 3.7 cm. Fast spin echo pulse sequences provided by the vendor were used (TR/TE = 8000/101.4 ms, Matrix size = 288 × 192). The slice thickness was 0.8 mm with a 0.2 mm gap, and the FOV was 5 × 2.5 cm. Total scan time was 3 min and 20 s at the NEX of 8. The images were then analyzed at the workstation provided by GE healthcare.

2.10. Histological analysis of mouse brains

All animals were sacrificed 2 weeks after the implantation of hMSCs with an overdose of ketamine/xylazine. The brains were harvested, embedded in OCT, and stored at −80 °C. Brains were cryosectioned at −20 °C by a cryostat (CM1900, Leica, Heerbrugg, Switzerland), and 20-μm thick sections from representative areas were directly examined with a confocal microscope (LSM510, Carl Zeiss, Jena, Germany) to observe DyeCycle Green-labeled U87MG cells and SP-Dil-labeled hMSCs. The sections were then stained with hematoxylin and eosin (H&E, Sigma-Aldrich) for visualization of gliomas, or processed for Prussian blue staining to determine the localization of Fer-Pro-labeled hMSCs.

2.11. Quantitative MRI for glioma size analysis

We measured glioma size by performing MRI scanning with T2 weight pulse sequences described earlier. The tumor growth rate was observed by imaging identical rat twice at 6–11 day intervals from the beginning of 21–29 days after tumor implantation. The images were analyzed by two investigators (L.-Y. Chien and J.-K. Hsiao). The tumor size was measured with plotting the region of interest of each significant slice. All of the tumor slices were summed up; and the signal intensities of the slices were recorded for statistical analysis. Up to 8 mice in three group+− with tumor, including SP-Dil-Fer-Pro colabeled hMSCs (N = 3), with tumor-alone group (N = 3), and with CXC4R1 antibody pretreated SP-Dil-Fer-Pro colabeled hMSCs (N = 2)—were scanned, with the image data used for further statistical analysis.

2.12. Statistical analysis

Data are presented as the mean ± standard error of mean (SEM) for the indicated numbers of separate experiments. The results were compared using Student’s t-test. Statistical significance was assigned if the p-value was less than 0.05. For correlation between observed signal intensity of glioma and its growth rate, linear regression model was used and F-test was used for accept or reject null hypothesis (α = 0.05).

3. Results

3.1. Viability and proliferation potential of magnetic–labeled hMSCs

In our previous study, we showed that ferucarbotran was not cytotoxic and that it promoted the proliferation of hMSCs [21]; but the effect of protamine on the cellular viability and proliferation potential of hMSCs was not tested. Although some studies have suggested that protamine has no significant deleterious effect on hMSC survival [23,29,30], the cytotoxicologic concern of using polycationic transfection agents for cellular labeling has not been fully addressed; hence, in this study we used MTT reduction assay to verify the cytotoxic effect of protamine labeling on hMSCs. As in our previous study [21], ferucarbotran (100 μg iron/ml) induced greater MTT reduction in the acute cytotoxicity (Fig. 1A) and proliferation (Fig. 1B) assays; however, protamine at a high dose (100 μg/ml) was shown to significantly decrease the MTT reduction activity in both assays. Interestingly, the ferucarbotran and protamine complexes (Fer-Pro) did not affect cell viability in the acute cytotoxicity assay (Fig. 1A) but caused a slight and significant increase in cell proliferation (Fig. 1B). Thus, these results suggested that ferucarbotran and Fer-Pro were not cytotoxic for labeling hMSCs and prompted us to investigate the labeling efficiency in vitro and in vivo.

3.2. Magnetic cellular labeling efficiency

In order to test the utility of ferucarbotran or Fer-Pro for imaging cell tropism, we used Prussian blue staining and ICP-MS to determine the cellular iron uptake and employed a clinical 1.5-T MRI system to examine the MRI-detectable efficiency. By a simple incubation for 1 h, qualitative immunostaining showed that ferucarbotran-labeled hMSCs obviously displayed Prussian blue positive; and a stronger staining was observed in Fer-Pro-labeled cells than cells with ferucarbotran-labeling (Fig. 2A). The iron amount engulfed by hMSCs was also quantitatively determined by ICP-MS. The iron contents increased significantly in hMSCs labeled with ferucarbotran (71.6 ± 8.2 pg/cell), and greatly in Fer-Pro-labeled cells (157.5 ± 27.5 pg/cell) (Fig. 2B). Under T2-weighted image mode, the MRI images of ferucarbotran-labeled or Fer-Pro-labeled cell pellets in test tubes placed in a water bath were easily and obviously detected as dark spots with hypointensities; moreover, the MRI signal intensity loss of hMSCs labeled with Fer-Pro was greater than that of those treated with ferucarbotran (Fig. 2C). The fact that a higher iron uptake in qualitative Prussian blue staining and ICP-MS quantification correlated with the finding of marked contrast effect in MRI suggested the potential of Fer-Pro labeling for detailed MRI in vivo.

3.3. Tumor tropism of hMSCs: in vitro and in vivo visualization

To confirm whether the hMSCs in our study could migrate toward glioma cells, an in vitro migration assay using Transwell system was first employed. The migration capacity of hMSCs in the presence of 0.5% FBS-containing medium alone in the bottom chamber was low (negative control; Fig. 3A, a); it increased when 30% FBS-containing medium was in the bottom chamber (positive control; Fig. 3A, b). U87MG cells in the bottom chamber (with 0.5% FBS-containing medium) significantly stimulated the migration of non-labeled hMSCs (Fig. 3A, c) and Fer-Pro-labeled hMSCs (Fig. 3A, d). By counting crystal violet-stained cells, we found that Fer-Pro labeling did not prohibit but promoted the migration capacity of hMSCs toward U87MG cells (Fig. 3A, e).

Because the migration capabilities of hMSCs and Fer-Pro-labeled hMSCs in vitro were established in the study, we next investigated whether intraventricularly-transplanted Fer-Pro-labeled hMSCs are capable of migrating toward human gliomas and then being imaged by MRI in vivo. For postmortem histological examination, U87MG cells and hMSCs were stained before injection with Vybrant DyeCycle Green and SP-Dil, respectively, as described in Section 2. Fourteen days after intracranial inoculation of Vybrant DyeCycle with SP-Dil-Fer-Pro colabeled hMSCs (yellow arrow) alone into the left lateral ventricles for 14 days. (F–H) Immunohistological analyses of mice from (C–E), respectively. (F): SP-Dil (a), DyeCycle Green (b), merge image (c), and Prussian blue staining (d) of right brain samples. (G): SP-Dil (a), DyeCycle Green (b), merge image (c), and Prussian blue staining (d) of right brain samples. (H): SP-Dil (a) and Prussian blue staining (c) of left brain samples; SP-Dil (b) and Prussian blue staining (d) of right brain samples. L: left site; R: right site. Scale bar 50 μm.
Green-labeled U87MG cells in the right frontal lobes of nude mice when xenografts were established and an intrinsic bright positive signal (a white spot) of glioma cells could be observed under T2-weighted images in vivo (Fig. 3B green arrow), SP-Dil-Fer-Pro colabeled hMSCs were injected into the left lateral ventricles. The stem cell images of MRI in animals were acquired 14 days after injection of SP-Dil-Fer-Pro colabeled hMSCs, animals were sacrificed, and the brains were removed for being multi-observed. Twenty-eight days after intracranial inoculation of Vybrant DyeCycle Green-labeled U87MG cells, the area of intrinsic bright positive signal of glioma cells increased (green arrows in Fig. 3C vs. 3B) in the right frontal lobe of the control animal that only received Vybrant DyeCycle Green-labeled U87MG cells but without SP-Dil-Fer-Pro colabeled hMSCs; however, in the animal that received both Vybrant DyeCycle Green-labeled U87MG cells and SP-Dil-Fer-Pro colabeled hMSCs, the area of intrinsic bright positive signal of glioma in the right frontal lobe was obviously less than that of control (green arrows in Fig. 3D vs. C). Interestingly, although few of intraventricularly-transplanted SP-Dil-Fer-Pro colabeled hMSCs remained at the original injection site (left ventricle) (Fig. 3D yellow arrow), most of the cells appeared as hypointense regions in the right ventricle (Fig. 3D red arrow). The animal injected with SP-Dil-Fer-Pro colabeled hMSCs but without Vybrant DyeCycle Green-labeled U87MG cells showed a main area of hypointensities on the left ventricle (Fig. 3E yellow arrow). These results suggest the migration capacity of SP-Dil-Fer-Pro colabeled hMSCs to the glioma cells and the therapeutic impact of the migrated SP-Dil-Fer-Pro colabeled hMSCs on gliomas.

Correlating the MRI findings with histological and immunostaining evidence provide more evidence of the migratory and therapeutic capacities of hMSCs. From the animal of Fig. 3C, no SP-Dil-Fer-Pro colabeled hMSCs could be seen because of no implantation of these cells (Fig. 3F; panel a and panel d for SP-Dil and Prussian blue staining, respectively), and Vybrant DyeCycle Green-labeled U87MG cells were clearly observed in the right frontal lobe (Fig. 3F; panel b). As shown in Fig. 3G (the identical animal sample in Fig. 3D), SP-Dil-Fer-Pro colabeled hMSCs migrated and were detected around Vybrant DyeCycle Green-labeled U87MG cells in the right frontal lobe (Fig. 3G; panel a for SP-Dil, panel b for Vybrant DyeCycle Green, panel c for the merge image of panels a and b, and panel d for Prussian blue staining). In addition, there was no migration of SP-Dil-Fer-Pro colabeled hMSCs in the animals without receiving the 1st stereotaxic injection of Vybrant DyeCycle Green-labeled U87MG cells (Fig. 3E and H). Colocalization of SP-Dil and Prussian blue staining was observed in the left ventricle (Fig. 3H, left brain samples; panel a for SP-Dil and panel c for Prussian blue staining respectively) but not in the right side of brain samples (Fig. 3H, right brain samples; panel b for SP-Dil, and panel d for Prussian blue staining), indicating the migration of SP-Dil-Fer-Pro colabeled hMSCs is due to a specific attraction of gliomas.

3.4. Mechanism study of glioma tropism of hMSCs in vitro and in vivo

To further explore the possibility of using the labeling of ferucarbtronan combined with protamine (Fer-Pro) for identifying the tropism mechanism in vivo, using in vitro migration assay we tested one of the candidate pathways (SDF-1/CXCR4 signaling axis) that has is widely believed to be involved in the tropism of hMSCs toward tumors. First, we demonstrated that hMSCs in the study did express CXCR4 and that the labeling of Fer-Pro did not diminish its expression (Fig. 4A). As shown in Fig. 3A, Fer-Pro labeling did not prohibit the migration of hMSCs toward U87MG glioma cells; Fer-Pro-labeled hMSCs were used as control in the mechanism experiments. By the incubation of Fer-Pro-labeled hMSCs with an anti-CXCR4-blocking antibody in the top chamber or with the addition of anti-SDF-1 neutralization antibody in the bottom chamber, the migration of Fer-Pro-labeled hMSCs toward U87MG cells in the bottom chamber significantly decreased (Fig. 4B). The inhibitory effect on Fer-Pro-labeled hMSCs migration was not observed with isotype-matched nonspecific antibodies (data not shown). These data suggest that the SDF-1/CXCR4 signaling axis was involved in the tropism of expanded hMSCs toward U87MG gliomas in the study. In the in vivo experiments, similar to those in Fig. 3D and G, the migration of intraventricularly-transplanted SP-Dil-Fer-Pro colabeled hMSCs to the gliomas and the therapeutic impact of the migrated SP-Dil-Fer-Pro colabeled hMSCs on gliomas were observed in Fig. 4C and E. However, as shown in Fig. 4D, the treatment of anti-CXCR4-blocking antibodies not only inhibited the in vivo migration of SP-Dil-Fer-Pro colabeled hMSCs toward gliomas but also weakened the therapeutic efficacy. In vivo MRI showed that anti-CXCR4-blocking antibodies-treated SP-Dil-Fer-Pro colabeled hMSCs failed to migrate but were still imaged in the original injection site (Fig. 4D yellow arrow) and that a greater area of intrinsic bright positive signal represented the expansion of gliomas (Fig. 4D green arrow). Histological examinations also showed that SP-Dil-Fer-Pro colabeled hMSCs stayed left in the animal brain (Fig. 4F, left brain samples; panel a for SP-Dil and panel c for Prussian blue staining respectively) and that Vybrant DyeCycle Green-labeled U87MG cells spread in the right frontal lobe (Fig. 4F, right brain samples; panel b for Vybrant DyeCycle Green).

3.5. Evaluation of therapeutic impact of migrated hMSCs on gliomas

As above shown in Figs. 3 and 4, the therapeutic impact of hMSCs on gliomas could be observed in vivo by MRI due to the intrinsic bright positive signal of gliomas that originate from edematous change, we analyzed the area of tumor to quantify the antitumor effect of hMSCs. The tumor growth rate of SP-Dil-Fer-Pro colabeled hMSCs group shows shrinkage of the glioma after the migration of SP-Dil-Fer-Pro colabeled hMSCs into the tumor part. The mean tumor growth size is ~0.82 mm3 whereas the tumor-alone group is 6.67 mm3 (p < 0.03) (Fig. 5A). The measured signal intensity of glioma at first MRI observation was 3.37 absolute units (AU) in the SP-Dil-Fer-Pro colabeled hMSCs group and 8.17 AU in the tumor-alone group; the results reached statistical significance. We also found the tumor growth and glioma signal intensity of the CXCR4-pretreated SP-Dil-Fer-Pro colabeled hMSCs group was between that of the SP-Dil-Fer-Pro colabeled hMSCs and tumor-alone groups (Fig. 5B). We further analyzed the relationship between measured signal intensity and tumor size. Interestingly, the signal intensity correlated well with glioma tumor growth under the linear regression model, with a regression coefficient of 0.82, which is statistically significant under the F-test (Fig. 5C). Histological examination by H&E staining also qualitatively demonstrated that migrated hMSCs inhibited the expansion of gliomas in vivo. As typically shown in Fig. 5D, enormous glioma masses (denoted by black dotted line) not only occupied the right hemisphere and extended the growth into the left hemisphere in the mouse that only received Vybrant DyeCycle Green-labeled U87MG cells. In the animal that received both Vybrant DyeCycle Green-labeled U87MG cells and SP-Dil-Fer-Pro colabeled hMSCs (Fig. 5E), the gliomas retained in the right hemisphere and were obviously smaller than those in Fig. 5B. However, when SP-Dil-Fer-Pro colabeled hMSCs were pretreated with anti-CXCR4-blocking antibodies before their transplantation, the gliomas occupation of the right hemisphere shifted the midline toward the left hemisphere (Fig. 5F). These results confirmed the antitumor effect of hMSCs and the involvement of SDF-1/CXCR4 in the migration and antitumor mechanisms and showed that antitumor effect could be determined by a clinical MRI system.
4. Discussion

The use of tumor-tropic stem cells for targeted delivery of tumoricidal therapeutic agents to tumor sites is generally believed to be able to improve therapeutic efficacy in glioma patients. MRI represents an attractive approach for monitoring the trafficking of transplanted stem cells in vivo in order to achieve effective use of stem cell-based cancer therapy; however, the cells of interest have to be internally labeled with suitable MRI contrast agents, such as SPIO nanoparticles, prior to transplantation. Therefore, it is highly required to develop efficient and biosafe labeling strategies, manifested as the requirements of short-term incubation and low

Fig. 4. Imaging of the mechanism of tumor tropism of hMSCs. (A) The immunofluorescent detection of CXCR4 expression in hMSCs. (B) In vitro migration of Fer-Pro-labeled hMSCs was blocked by anti-CXCR4-blocking antibodies and anti-SDF-1 neutralization antibodies. Data are expressed as mean ± standard error of seven independent experiments. ***Statistically significant difference (p < 0.001) as compared with untreated cells (Control). (C) In vivo MRI of mouse injected with Vybrant DyeCycle Green-labeled U87MG cells (green arrow) in the right frontal lobes for 28 days and SP-Dil-Fer-Pro colabeled hMSCs (yellow arrow) into the left lateral ventricles for 14 days. Red arrow denotes the migrated SP-Dil-Fer-Pro colabeled hMSCs toward DyeCycle Green-labeled U87MG cells in the right frontal lobes. (D) In vivo MRI of mouse injected with Vybrant DyeCycle Green-labeled U87MG cells (green arrow) in the right frontal lobes for 28 days and anti-CXCR4-blocking antibodies-pretreated SP-Dil-Fer-Pro colabeled hMSCs (yellow arrow) into the left lateral ventricles for 14 days. No tropism of SP-Dil-Fer-Pro colabeled hMSCs was observed. (E) and (F): immunohistological analyses of mice from (C) and (D), respectively. (E): SP-Dil (a), DyeCycle Green (b), merge image (c), and Prussian blue staining (d) of right brain samples. (F): SP-Dil (a) and Prussian blue staining (c) of left brain samples; DyeCycle Green (b) of right brain samples. L: left site; R: right site. Scale bar 50 μm.
concentration of labeling agents, the employment of low number of labeled cells and a clinical-used MRI, and lower toxicity. Whereas protamine has been employed to improve the labeling efficiencies of SPIO nanoparticles in many cell types [23,24,31,32], in this study we have demonstrated that the combination of ferucarbotran and protamine would be a clinically applicable strategy for achieving good MRI performance when studying cellular behaviors in stem cell-mediated glioma therapy. Although several MRI studies show promise for the monitoring of cellular trafficking in animal models of regenerative medicine as well as stem cell-mediated tumor therapy [33–35], to our knowledge this is the first report in which a clinical MRI modality was used to simultaneously study the migration, the therapeutic impact on tumors, and above all the trafficking mechanism of bone marrow-derived mesenchymal stem cells from human in a murine glioma xenograft model.

The primary concern associated with using MRI for cellular tracking is the uptake/labeling efficiency of the MRI-visible particles selected for use. Up to now, of the two main classes of MRI-visible agents for labeling cells—i.e., SPIO nanoparticles and Gd-based chelates—most studies have used the former class as the cellular imaging probe. SPIO nanoparticles act as negative contrast agents, producing strong hypointensities (dark spots) on T2- or T2*-weighted images. Gd-based contrast agents, however, produce bright positive signal intensity in T1-weighted MRI images. Due to the intrinsic bright

Fig. 5. Imaging and evaluation of therapeutic impact of hMSCs on gliomas. The tumor growth observed during two MR imaging observation in SP-Dil-Fer-Pro colabeled hMSCs (hMSCs), anti-CXCR4-blocking antibodies-pretreated SP-Dil-Fer-Pro colabeled hMSCs (CXCR4), and tumor-alone (Tumor) groups. The U87MG glioma growth shows statistical significance between hMSCs treated group and U87MG tumor-alone group (p < 0.03). The anti-CXCR4-blocking antibodies-pretreated SP-Dil-Fer-Pro colabeled hMSCs group exhibit intermediate tumor growth rate between the other groups (A). The signal intensity of U87MG glioma at first MRI observation among SP-Dil-Fer-Pro colabeled hMSCs group (hMSCs), anti-CXCR4-blocking antibodies-pretreated SP-Dil-Fer-Pro colabeled hMSCs (CXCR4), and tumor-alone (Tumor) groups. Statistical significance could be found in the hMSCs treated group and U87MG cell alone group (p < 0.005). The findings suggest more stem cell tropism toward glioma in the SP-Dil-Fer-Pro colabeled hMSCs group (B). Relationship between observed U87MG glioma signal intensity at first MRI session and the U87MG cells growth rate was visualized by MRI. Strong relationship (Relation coefficient = 0.83, p < 0.05) was observed between these two parameters (C). Macroscopic view (H&E staining) of brain specimens of mice that were injected with Vybrant DyeCycle Green-labeled U87MG cells alone in the right frontal lobes for 28 days (D), Vybrant DyeCycle Green-labeled U87MG cells in the right frontal lobes for 28 days and SP-Dil-Fer-Pro colabeled hMSCs into the left lateral ventricles for 14 days (E), or Vybrant DyeCycle Green-labeled U87MG cells in the right frontal lobes for 28 days and anti-CXCR4-blocking antibodies-pretreated SP-Dil-Fer-Pro colabeled hMSCs into the left lateral ventricles for 14 days (F). Gliomas are denoted by black dotted lines.
positive signal of gliomas in T2-weighted images in vivo, SPIO nanoparticles are more suitable than Gd-based nanoparticles in distinguishing labeled stem cells in targeted sites with high MRI signals. Although the internalization mechanisms were not examined in this study, an important role of endocytosis has been strongly suggested in the uptake/labelling process of ferucarbotran and Fer-Pro complexes [20,21,24]. High cellular labeling can be achieved by combining several strategies, including using simple incubation with SPIO nanoparticles alone for a longer incubation time, combination with a transfection agent but a shorter incubation period, surface modifications of SPIO nanoparticles with other materials, HIV tat peptide, or monoclonal antibody [36–39], and electroporation [40].

In this study we used higher doses of ferucarbotran and protamine to perform Fer-Pro complexes but a shorter incubation time to label hMSCs than previously reported by Golovko et al. [24], resulting in a huge cellular loading of iron (15.7 ± 27.5 pg/cell in Fig. 2B). The method utilizing high concentrations of ferucarbotran and protamine in our case offers an instant but still biosafe labeling for hMSCs in the glioma model (as discussed below); moreover, this method may prove profitable.

With regard to the toxicity of labeling strategies of the combination of protamine and SPIO particles, protamine is usually believed to be biosafe for enhancing SPIO nanoparticle labeling. Studies with protamine, however, have focused mainly on the effect of complexes of SPIO nanoparticles and protamine on cellular attributes; thus, the potential of polycationic transfection agents to harm stem cell behavior remains uncertain [41,42]. This prompted us to examine the cytotoxic effects of all the labeling agents, especially protamine itself, in hMSCs. The labeling of hMSCs with protamine alone at a high concentration (100 µg/ml) in this study resulted in the cell number decreases, suggesting a cytotoxic potential of protamine for stem cell labeling. However, the promoting activity of ferucarbotran on cell growth alleviated the threat of protamine to cellular viability, suggesting that protamine may be an uptake-enhancing adjuvant with ferucarbotran for stem cell labeling. In addition to protamine, SPIO nanoparticles (such as, in this study, ferucarbotran) play an important role in toxicity concerns, although they are generally believed to be inert and biocompatible. Previously we have shown that ferucarbotran can promote cell growth of hMSCs [21], as verified in the present study, leaving the question of whether this growth promotion is good or bad for stem cells themselves. Recently we demonstrated that ferucarbotran could activate the migration of hMSCs in the osteogenic medium and subsequently induce the abolishment of cellular osteogenesis [43], suggesting the need for caution about using ferucarbotran to label stem cells for osteogenic MRI tracking. Furthermore, the applicability of protamine in combination with ferucarbotran for stem cell labeling would be greatly dependent on the mission of stem cells in different circumstances. In the glioma models, the migration capacity toward gliomas and the expression activity of therapeutic transgene but not multiple-potent differentiation are of vital importance for stem cells to be used as a tool for targeted therapeutic transgene delivery and then tumor therapy. In the present study the glioma tropism of hMSCs is demonstrated by in vitro Transwell assay and by in vivo MRI; and the labeling of Fer-Pro complexes has been shown to activate the in vitro migration of hMSCs toward glioma cells (Fig. 3A). Although it is not clear whether Fer-Pro complexes (or ferucarbotran itself) can promote the tropism of hMSCs in vivo for that unlabeled hMSCs cannot be observed by MRI, the caution of migration activation for using stem cells in osteogenesis or other regenerative medicines would advantage Fer-Pro complexes to label/image hMSCs in stem cell–mediated tumor therapy. More comprehensive analyses of the effects of Fer-Pro complexes on the in vivo tropism and the expression of engineered genes are warranted.

NSCs as well as MSCs from various species (e.g., mouse, rat, and human) have been demonstrated to be able to migrate toward gliomas in vitro and in vivo [6–12,17,33–35]. Although several growth factors/chemokines may be possible candidates responsible for the tropism of NSCs or/and MSCs toward gliomas, the interaction of CXCR4 and SDF-1 is known to be involved in the tropism of hMSCs. Although the tropism behaviors in above studies can be determined by histochemical staining, fluorescent images, or noninvasive and dynamic MRI, no report has shown the utility of MRI to examine the tropism mechanism in vivo. In the present study we not only demonstrate the tropism of hMSCs toward gliomas in vitro and in vivo by histochemical staining, fluorescent images, and a clinical MRI system but also indicate that the SDF-1/CXCR4 system responsible for in vitro mechanism is indeed involved in the tropism of hMSCs in vivo. Additionally, it should be noted that MSCs in the study were from human subjects, suggesting an autologous transplantation of these cells in patients. Furthermore, the observed SDF-1/CXCR4 mechanism may provide insights into strategies for enhancing the glioma tropism of hMSCs for better therapeutic efficiency.

The strong relationship between signal intensity of U87MG glioma was detected under MRI and tumor growth. The signal intensity of glioma is determined by the tumor itself and the migrated hMSCs. The finding reinforces that hMSCs implantation is feasible as a treatment method and evaluation technique for understanding stem cell behavior in vivo. As our data show, the tropism of hMSCs could be visualized clearly under 1.5 T clinical MR system after SPIO labeling.

5. Conclusions

Labeling of hMSCs by ferucarbotran and protamine complexes (Fer-Pro) is not toxic to cells but can promote the migration of hMSCs toward glioma cells in vitro. Fer-Pro-labeled hMSCs can specifically migrate toward gliomas in vivo, which was observed with a clinical 1.5-T MRI system. The efficient labeling of Fer-Pro allows a tropic mechanism mediated by SDF-1/CXCR4 to be also detected by MRI in vivo. Additionally, the potential intrinsic inhibitory effect of hMSCs on glioma progression can be estimated simultaneously. This MRI technique described in the study has clinical application potential for stem cell guided therapy.

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Appendix

Figures with essential color discrimination. Figs. 2–5 in this article are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2011.01.042.

References
