Magnetic Resonance Imaging of Mesenchymal Stem Cells Homing to Pulmonary Metastases Using Biocompatible Magnetic Nanoparticles

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Abstract

The ability of mesenchymal stem cells (MSC) to specifically home to tumors has suggested their potential use as a delivery vehicle for cancer therapeutics. MSC integration into tumors has been shown in animal models using histopathologic techniques after animal sacrifice. Tracking the delivery and engraftment of MSCs into human tumors will need in vivo imaging techniques. We hypothesized that labeling MSCs with iron oxide nanoparticles would enable in vivo tracking with magnetic resonance imaging (MRI). Human MSCs were labeled in vitro with superparamagnetic iron oxide nanoparticles, with no effect on differentiation potential, proliferation, survival, or migration of the cells. In initial experiments, we showed that as few as 1,000 MSCs carrying iron oxide nanoparticles can be detected by MRI one month after their injection with breast cancer cells that formed subcutaneous tumors. Subsequently, we show that i.v.-injected iron-labeled MSCs could be tracked in vivo to multiple lung metastases using MRI observations that were confirmed histologically. This is the first study to use MRI to track MSCs to lung metastases in vivo. This technique has the potential to show MSC integration into human tumors, allowing early-phase clinical studies examining MSC homing in patients with metastatic tumors. [Cancer Res 2009;69(23):8862–7]

Introduction

The poor survival of both lung cancer patients and those with other forms of pulmonary metastatic disease relates partly to the inability to deliver locally targeted therapeutic agents. A recent body of work has used exogenous mesenchymal stem cells (MSC) from the bone marrow compartment to attenuate several carcinoma models (1–6). In some of these studies, the MSCs carrying antitumor therapies have been delivered locally (2), whereas in others, they have been delivered systemically and migrate to the site of the tumor where they contribute to tumor reduction (1, 3–6).

We have shown previously in murine cancer studies that human MSCs expressing tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) can provide targeted delivery of this proapoptotic agent to breast cancer metastases (1). Similarly, MSCs transduced to express IFNβ or the immunostimulatory chemokine CX3CL1 have also been shown to reduce tumor burden in murine glioma (3), breast (5), melanoma (4), and colorectal models (6) with an improvement in survival.

The ability of bone marrow–derived stem cells to migrate to areas of injury in a range of pathologic conditions suggests that they may be ideal vectors for therapeutic delivery. MSCs possess a number of properties that make them suitable candidates. They are easily obtained from a simple bone marrow aspirate and are readily expanded in culture without losing their multilineage potential. They are readily transducible, allowing for simple ex vivo modification (7). Finally, they seem to be relatively nonimmunogenic (8) due to their lack of MHC2 and costimulatory molecules CD80, CD86, and CD40 (9). This may allow the delivery of genetically dissimilar MSCs without the need for immunomodulation or subsequent immunosuppressive therapy for the recipient. Because of these properties, MSCs have considerable therapeutic potential in tumor therapy.

To deliver cell therapy for cancer in the clinical setting, there is a need for imaging confirmation of targeted delivery. Novel imaging contrast agents have emerged that open up the possibility of visualizing stem cell transplants in vivo using magnetic resonance imaging (MRI). Superparamagnetic iron oxide (SPIO; Fe3O4) nanoparticles have been used for tracking engrafted cells in a variety of tissues (10), as well as targeted cell delivery (11). The nanoparticles generate a local magnetic field perturbation, which leads to a marked shortening of the MRI parameter T2*. This is exhibited as hypointensity on magnetic resonance images, leading to the possibility of imaging the localization of these particles (10, 12). We have exploited this phenomenon using MRI to track the fate of MSCs labeled with magnetic nanoparticles in a metastatic lung cancer model.

Here we have introduced biocompatible iron oxide nanoparticles into MSCs to enable localized cellular-level sensing while retaining full viability. Using a combination of cancer cells and iron nanoparticle–containing MSCs, we have shown in a subcutaneous tumor model that the MSCs can be imaged down to very low numbers. Finally, we show in a metastatic cancer model that systemically delivered cells can be tracked to pulmonary metastases, which is subsequently confirmed histopathologically.

Materials and Methods

Cell culture. Tissue culture reagents were purchased from Invitrogen unless otherwise stated. MDAMB231 breast cancer cells were obtained from Cancer Research UK, London Research Institute and were cultured in DMEM and 10% fetal bovine serum (FBS). Human adult MSCs were

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Labeling, phenotyping, and visualization of MSCs with iron nanoparticles. Labeling of MSCs with iron nanoparticles was done by overnight incubation with 0.5 mg/mL of nanoparticles in cell culture medium. The cells were vigorously washed with PBS eight times to remove any free particles before use.

Adipogenic and osteogenic differentiation of MSCs was done as previously described (13, 14). Cell viability was done using an MTS NAD(P)H-dependent assay (15) according to the manufacturer’s guidelines (Promega). Cell apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) assay (ApoTarget, Invitrogen) 72 h after labeling. Ten samples were analyzed using a flow cytometer (FACSCalibur), and 6 x 10^3 to 8 x 10^3 cells were scored per analysis (CellQuestPro, Becton Dickinson). Annexin V−/PI− cells were judged to be viable. Annexin V+/PI− cells were considered to be undergoing apoptosis, and Annexin V+/PI+ cells were considered late apoptotic or necrotic and recorded as dead (1).

Cell migration was done as previously described (1). Briefly, 1.5 x 10^5 MDAMB231 cells were plated in 800-μL medium on the bottom well of a transwell plate (Becton Dickinson), with 4 x 10^4 MSCs in 300 μL plated in the upper well. The MSCs were allowed to migrate across the 8-μm pore membrane for 24 h at 37°C. The cells that attached to the upper side of the membrane were removed with a cotton bud, and the cells on the lower side that had migrated through the membrane were fixed, stained (Rapid Romanowsky, Raymond Lamb), and counted (five fields per well, triplicate wells) at x10 magnification (Olympus BX40).

Prussian blue staining (1.2% potassium ferrocyanide with 1.8% hydrochloric acid) was done on fixed cells (4% paraformaldehyde) 96 h after labeling. Confocal microscopy was done on a Leica TCS SP2 microscope (Leica Microsystems Ltd.). Reflectance was used to visualize iron as previously described (12), and images were processed using Image J. For electron microscopy, cells were fixed with 2% paraformaldehyde, 1.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.3). They were then osmicated in 1% O₃O₄/0.1 mol/L phosphate buffer, dehydrated in a graded ethanol-water series, cleared in propylene oxide, and infiltrated with Araldite resin. Ultrathin sections were cut using a diamond knife, collected on 300 mesh grids, and then stained with uranyl acetate and lead citrate.

Figure 1. MSCs take up SPIO nanoparticles without affecting their phenotype. A, i, MSCs in culture (bar, 20 μm); ii, Prussian blue staining of MSCs after 24 h of culture with SPIO nanoparticles (bar, 20 μm); iii, electron microscopy image showing cytoplasmic location of SPIO nanoparticles (bar, 1 μm); iv, confocal image showing colocalization of SPIO nanoparticles (yellow), DiI (red), and the nuclear counterstain DAPI (blue; bar, 3 μm); v, differentiation to osteoblasts, Alizarin Red S staining (bar, 40 μm); vi, differentiation to adipocytes, Oil Red O staining (bar, 5 μm). B, i, MSCs stained on the underside of the transwell membrane (bar, 50 μm at x4 magnification, 10 μm at x20 magnification). ii, the SPIO-loaded MSCs (Fe) migrate toward MDAMB231 breast cancer cells through a transwell membrane at the same rate as nonlabeled MSCs (Non-Fe); iii, the SPIO-loaded MSCs proliferate at the same rate as control MSCs. C, there is no increase in death and apoptosis of the SPIO-loaded MSCs compared with the nonlabeled MSCs. D, these are consistent with there being no increase in death and apoptosis of the SPIO-loaded MSCs compared with the nonlabeled MSCs.

Magnetic resonance imaging. Images were acquired on a 9.4-T horizontal bore Varian (VNMRS) system using a 39-mm RF coil (RAPID Biomedical GmbH). Lung in vivo images were obtained before and 1 and 24 h after MSC injection, at day 35 after the metastatic model had been initiated (n = 4 mice). They were acquired using a fast spin-echo sequence with cardiac and respiratory gating [repetition time (TR) = 1 s, effective echo time (TE) = 5 ms, 100-μm in-plane resolution, 1-mm slice thickness, number of signals averaged (NSA) = 4]. Subcutaneous tumor images were obtained 28 d after s.c. injection of MDAMB231 cells and MSCs, and were acquired ex vivo using the same sequence and similar parameters (TR = 1.5 s, effective TE = 5 ms, 100-μm in-plane resolution, 1-mm slice thickness, NSA = 4; n = 14 mice; 2 per group). Signal-to-noise ratios (SNR) were obtained from three consecutive coronal slices for four lung areas (right and left, upper and lower) using the same sequence and similar parameters (TR = 1.5 s, effective TE = 5 ms, 100-μm in-plane resolution, 1-mm slice thickness, NSA = 4).
upper and lower), using the average signal intensity of each area, the signal intensity of shoulder muscle, and the SD of the noise within each slice.

Immunohistochemistry. Mice were sacrificed by CO2 asphyxiation followed by exsanguination following the MRI at day 28 in the subcutaneous tumor experiment and after final MRI (1 or 24 h after MSC delivery) in the metastatic experiment. Subcutaneous tumors were removed and fixed in 4% paraformaldehyde for histology. The lungs were excised and inflated with a fixed 20-cm pressure of 4% paraformaldehyde and then bathed in 4% paraformaldehyde for histology (1).

Fixed specimens were embedded in paraaffin and cut into 3-μm sections for H&E staining. Prussian blue staining was used to detect iron, and fluorescence microscopy was used to detect DiI-positive cells with 4',6-diamidino-2-phenylindole (DAPI) counterstain. Macrophages were stained with a monoclonal rat anti-mouse Mac-2 primary antibody (1/50 dilution; Cedarlane) for immunohistochemistry and a monoclonal rat anti-mouse F4/80 primary antibody (1/10,000 dilution; Ebiosciences) for immunofluorescence. Microscopy was done using a light (Olympus BX40) or a fluorescent (Axioskop 2, Carl Zeiss Ltd.) microscope.

Statistics. Statistical analysis was done using GraphPad Prism v4 (GraphPad Software). Multiple groups were analyzed by ANOVA. Single-group data were assessed using Student’s t test or Mann-Whitney test. Results were considered to be statistically significant at P < 0.05.

Results

Iron labeling of MSCs. The MSCs readily internalized the iron nanoparticles. This was confirmed by Prussian blue staining, electron microscopy, and confocal microscopy (Fig. 1Ai–iv). Cells contained up to 30 pg of iron oxide per MSC, quantified using superconducting quantum interference device magnetometry. The labeled cells retained their MSC characteristics, with the ability to differentiate into stromal tissues, including bone and fat (Fig. 1Av–vi).

Furthermore, the iron nanoparticle–labeled and unlabeled MSCs showed equivalent in vitro tumor homing (104.4 ± 5.6 versus 113.1 ± 16.1 cells per field) in transwell migration studies [nonsignificant (ns), t test; Fig. 1B]. There was also no effect of iron nanoparticles on MSC proliferation (ns, two-way ANOVA), as shown by the MTS proliferation assay (Fig. 1C), or on cell viability, as shown by Annexin V flow cytometry apoptosis assay [33.0 ± 4.2% dead cells or apoptotic cells with no iron nanoparticles, compared with 29.4 ± 2.1% cells with iron nanoparticles (ns, Mann-Whitney); Fig. 1D].

Detection and sensitivity of MRI to iron-labeled cells. To determine the sensitivity of MRI in visualizing MSCs carrying iron nanoparticles, we used subcutaneous tumors, rather than lung tissue, in combination with our lung imaging MRI sequence to assess the dose response of iron-labeled cells, as the air spaces in the lung could confound this assessment. We grew subcutaneous MDAMB231 tumors (2 × 106 cells) in nonobese diabetic/severe combined immunodeficient mice with increasing numbers of DiI-labeled human MSCs carrying nanoparticles (100, 1 × 103, 1 × 104, and 1 × 105) for 28 days (n = 2 per group). Using a 9.4-T MRI system, we were able to visualize as few as 1,000 MSCs labeled with nanoparticles in tumors 28 days after injection of the MDAMB231 cells (Fig. 2Ai–ii). Signal voids were not visible at 28 days when non–iron-labeled MSCs, dead iron-labeled MSCs (Fig. 2Av), or free iron (Fig. 2Aiv) were coadministered with the tumor cells. Histopathologic examination confirmed that iron was present only in the tumors injected with live nanoparticle-labeled MSCs. This was shown by colocalization of the Prussian blue staining of iron and Dil fluorescence with the MSCs (Fig. 2B).

Figure 2. SPIO-loaded MSCs can be visualized by MRI in tumors at low concentrations. A, 2 × 106 MDAMB231 cells were coinjected with 1 × 105 (i), 1 × 104 (ii), 1 × 103 (iii), and 100 (iv) SPIO-loaded MSCs and visualized using a 9.4-T MRI scanner 28 d later. The subcutaneous tumors can be seen in all mice (asterisks). SPIO-loaded MSCs are visualized (arrow) when as few as 1 × 105 cells were originally injected. There were no hypointensities on MRI with 1 × 104 dead SPIO-loaded MSCs (v) or free iron particle injection (vi; 100 ng; n = 2 in all groups). B, Prussian blue histochemistry (i and ii) and Dil (red) immunofluorescence [with DAPI nuclear counterstain (blue); iii and iv] corresponding to the coinjection of 1 × 105 (i and ii) and 1 × 104 (iii and iv) SPIO-loaded MSCs, confirming that the iron stain colocalizes with the Dil-labeled MSCs. Bar, 50 μm at ×4 magnification, 20 μm at ×10 magnification.
Homing and in vivo detection of iron-labeled MSCs to lung tumors. In the following experiments, 2 × 10^6 MDAMB231 cells were injected into the tail vein. This model reproducibly forms pulmonary metastases throughout all lung lobes. We were able to detect lung metastases using MRI, visualized as focal regions of increased signal. These areas correspond to metastases on H&E histologic sections (bar, 100 μm). One hour after SPiO-loaded MSC injection, there is a decrease in signal intensity caused by the iron oxide in MSCs (+, ribcage; *, trachea; ^, diaphragm with upper abdomen below; ∼, fissure separating lobes).

As we have shown previously, MSCs show tropism to pulmonary tumors (1). Therefore, 35 days after i.v. delivery of MDAMB231 cells, MSCs double-labeled with DiI and iron nanoparticles were injected i.v. We used MRI to confirm the fate of the i.v.-injected, iron-labeled MSCs within metastases in vivo by acquiring magnetic resonance images before and 1 and 24 hours after injection. MRI images after MSC injection showed a decrease in signal intensity in areas of metastatic deposits detected in pre-MSC delivery images, which correlated with the iron-labeled MSCs integrating or lodging into these tumors (n = 4; Fig. 3A). To examine MSC engraftment throughout the lung, the signal intensity across the lung was examined before and after MSC injection in three consecutive slices. This was compared with the SD of the signal noise of each slice, giving a within-slice SNR for each examined area, which was averaged across the three slices. There was a significant reduction in the SNR following the MSC injection, which was consistent in all lung areas (P = 0.005, two-way ANOVA; n = 3; Fig. 3B). There were no differences in the SNR decrease between the lung areas (ns, two-way ANOVA). Immunohistochemistry confirmed our previous findings that DiI/Fe staining cells were found within or adjacent to tumors (Fig. 3Ci and ii). As previous studies have suggested that iron-labeled cells may represent macrophages, we performed immunohistochemistry and immunofluorescence for macrophages and iron or DiI (18, 19). There was no colocalization of the macrophage marker with the iron nanoparticles or DiI-positive cells with either technique (Fig. 3Ci and iv).

Discussion

MSCs have enormous potential as vehicles for directed cancer delivery. The mechanism responsible for the homing of MSCs to tumors is thought to involve chemokine ligands and receptors as with the recruitment of leukocytes to areas of inflammation. However, unlike with leukocytes, the specific chemokines responsible...
for MSC migration are less well characterized (1, 20). Nevertheless, homing to tumors has been confirmed by several studies using the labeling of MSCs and subsequent immunohistochemistry (21). Identification of the MSCs in these previous studies has necessitated histologic tissue and animal sacrifice. In this preliminary study, we have shown the ability to detect and visualize homing of iron-labeled MSCs in real-time in vivo.

For clinical applications, the ability to track MSC homing to primary tumors and metastases using a simple noninvasive scan would be of great benefit. Although murine models have shown a lot of promise for transduced MSCs in cancer therapy, many uncertainties still remain. The ability to systematically visualize the therapy and the response of the tumor will allow for more informed decisions about the optimum timing of MSC therapy, as well as the number of treatments.

We have previously described the use of MSCs in delivering the proapoptotic protein TRAIL (1). In these experiments, the expression of TRAIL was sensitively controlled by doxycycline via an inducible lentivirus. The ability to detect the proximity of the transduced MSCs to the tumors with MRI could be used in such a model, expressing the antitumor antigen only when the MSCs are in their optimal position. Although the spatial proximity of the MSC homing to the tumors and the benefit of magnetic resonance monitoring for TRAIL therapy were not investigated as part of this study, we envisage that MSC tracking will help define the optimal time window by detection of MSCs in the lung and any regression of the metastases.

The noninvasive tracking of MSCs has previously been studied with the use of bioluminescence (22) and whole-body microscopic emission tomography (23) with MSCs labeled with firefly luciferase or transduced to express HSV1-TK, respectively. The use of SPIO particles has the advantage of labeling MSCs without transduction but with the use of agents and facilities that are now frequently used in medical practice, thus providing direct clinical applicability. In our study, we found that iron particles had no effect on MSC differentiation, migration, survival, and proliferation capacity.

Previous groups have studied the use of nanoparticles for detecting MSCs in vivo with direct injection into a cardiac scar (18) and direct injection into the brain (24). MSCs have also been tracked after i.v. injection in a Kaposi’s sarcoma model (25). This is the first study, to our knowledge, that assesses the applicability of a systematic delivery of SPIO nanoparticle–labeled MSCs to metastatic disease.

As a noninvasive imaging modality that uses nonionizing radiation, MRI and, in general, the use of iron nanoparticles may have an important future in human applications. The approach described here could augment tracking of cells in other cancer models and will be crucial in the monitoring of cell localization before clinical gene therapy studies.

One of the limitations of this approach is that the detection of iron nanoparticles by MRI does not ensure that these cells are the labeled MSCs. Indeed, other studies have suggested that some of the MRI signal may be the result of either the release of free iron or the uptake of iron by macrophages after labeled-MSC death (18, 19). In our experiments, there was no MRI signal with the use of free iron or dead iron-labeled MSCs, suggesting that the iron is cleared in both these situations and that the MRI signal is generated exclusively by viable, labeled cells, as also shown in other studies (26, 27). The MSCs containing the iron nanoparticles were also labeled with the fluorescent dye DiI, and histochemistry for iron of both subcutaneous and metastatic tumors showed the persistent colocalization of the iron with the fluorescent marker DiI. DiI labeling has been validated as a technique for tracking human cells transplanted into mice in a study whereby DiI-stained cells were characterized as of human origin by in situ hybridization (28), and we have previously shown that the transplanted DiI cells retain characteristics of MSCs with the use of ex vivo vimentin staining in the same in vivo models as used in this study (1). This present study has also shown that there was no colocalization of the iron with macrophage markers, again suggestive that the iron signal represents MSCs.

In conclusion, targeted technologies, using proapoptotic methods such as TRAIL in conjunction with noninvasive magnetic resonance monitoring, may become an important adjunct to the use of ionizing radiation and chemotherapeutic agents, opening up a variety of possibilities for the future of cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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