Serial Cardiac Magnetic Resonance Imaging of Injected Mesenchymal Stem Cells

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Background—Delivery and tracking of endomyocardial stem cells are limited by the inability to image transplanted cells noninvasively in the beating heart. We hypothesized that mesenchymal stem cells (MSCs) could be labeled with a iron fluorophore particle (IFP) to provide MRI contrast in vivo to assess immediate and long-term localization.

Methods and Results—MSCs were isolated from swine. Short-term incubation of MSCs with IFP resulted in dose-dependent and efficient labeling. Labeled cells remained viable for multiple passages and retained in vitro proliferation and differentiation capacity. Labeled MSCs (10^4 to 10^6 cells/150 μL) were injected percutaneously into normal and freshly infarcted myocardium in swine. One, 3, and 1 animals underwent serial cardiac MRI (1.5T) for 4, 8, and 21 days, respectively. MRI contrast properties were measured both in vivo and in vitro for cells embedded in agar. Injection sites containing as few as 10^5 MSCs could be detected and contained intact IFP-bearing MSCs on histology.

Conclusions—IFP labeling of MSCs imparts useful MRI contrast, enabling ready detection in the beating heart on a conventional cardiac MR scanner after transplantation into normal and infarcted myocardium. The dual-labeled MSCs can be identified at locations corresponding to injection sites, both ex vivo using fluorescence microscopy and in vivo using susceptibility contrast on MRI. This technology may permit effective in vivo study of stem cell retention, engraftment, and migration. (Circulation. 2003;108:1009-1014.)

Key Words: cells ■ magnetic resonance imaging ■ myocardial infarction ■ contrast media

Cellular agents such as mesenchymal stem cells (MSCs), endothelial progenitor cells, and skeletal myoblasts are under investigation as potential treatments for myocardial dysfunction. Once delivered to the heart, injected cells are difficult to visualize and track in vivo. Fluorescent or genetic marking identifies transplanted cells only after explantation. Nuclear scintigraphic tracking is limited by poor spatial resolution and radionuclide decay. In this study, we describe labeling cells with contrast agent, permitting identification under both MRI in vivo and fluorescence microscopy ex vivo.

Ultrasmall (nanometer-scale) superparamagnetic iron oxide particles have been used to track stem cells transplanted into stationary organs such as brain, in small animals or to demonstrate macrophage activity within atherosclerotic plaque. Comparably sized magnetodendrimers, which transfect cells with MRI contrast agents, have been used for static MRI of neural cell transplants. To date, no approach has permitted in vivo MRI of transplanted cells in a beating heart. Iron oxide can generate MRI contrast by disturbing the local magnetic field near excited spins, a property termed T2* relaxation. Hinds et al recently demonstrated efficient labeling of hematopoietic stem cells using relatively large micron-scale iron oxide particles by simple incubation. The particles generate significantly greater T2* effect (and more MRI contrast) than an equivalent weight of smaller nanometer-scale particles. The particles also contained a fluorophore, permitting both MRI and fluorescence imaging.

We hypothesized that such iron fluorescent particle (IFP) labeling of cells, specifically of mesenchymal stem cells, imparts sufficient MRI contrast that cells can be detected in vivo in a beating heart after direct injection. We show that IFP-labeled MSCs have a concentration-dependent T2* effect in vitro, can be visualized noninvasively in vivo after transplantation using a conventional scanner, can be observed serially over a period of weeks, and can then be identified ex vivo using fluorescence microscopy.
Methods

Iron Fluorescent Particle
The contrast agent consisted of iron oxide (62.4%) microparticles and a fluorescein-5-isothiocyanate analogue (Dragon Green) embedded in 0.9-μm inert polystyrene microspheres (Bangs Laboratories).

Cell Preparation, Labeling, and Characterization
MSCs were derived from bone marrow aspirates of healthy adult Yorkshire swine. Mononuclear cells were isolated using density gradient centrifugation (Ficoll-Paque, Amersham Biosciences), and culture was expanded based on plastic adherence. Nonadherent cells were removed every 5 days, and the adherent cells were washed and resuspended at a plating density of 1000 cells/mm² in MSC basal media supplemented with bullet kits (MSCGM, Poteatis, BioWhittaker).

For cell-labeling procedures, early passage nonconfluent MSCs were incubated overnight with IFP (10 μL of 1% stock solution per mL medium) at 37°C in 5% CO₂. Excess particles were removed after 18 hours by repeated washing. To measure intracellular IFP density, samples labeled separately were lysed with water and IFPs were counted on a hemocytometer under light and fluorescent microscopy for morphological assessment of viability and apoptosis. The effect of the particles on cell proliferation, growth kinetics (MTT Roche Diagnostics) were determined at a range of IFP concentrations (0.1 to 50 μL IFP stock per mL medium). The effect of labeling on MSC differentiation ability was tested using lineage-specific assays and immunostains including osteogenic, adipogenic, myogenic, and chondrogenic induction.

Animal Studies
Animal protocols were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee. Allogeneic MSCs were isolated from Yorkshire swine and delivered into Hanford mini-swine. Myocardial infarctions were created in 3- to 6-month-old Hanford mini-swine (30 to 40 kg) by transaortic occlusion of the left anterior descending coronary artery using platinum emboli (VortX, Boston Scientific). MSCs were injected under x-ray guidance percutaneously using steerable guiding catheters to position a spring-actuated biocompatible 27G needle (Stiletto). MRI was performed at 1.5T with a head coil (C-Vi, General Electric) using 3D fast gradient echo (v/0.47×0.47×1 mm, 256×256x128 matrix, repetition time 300 ms, echo time 3.3 ms, flip angle 45 degrees, bandwidth ±13kHz, imaging time 1 hour). Histology specimens were stained with oil-red O, and for 3D MRI, fresh explants were retroperfused with 4% formaldehyde, and gadolinium-filled reference markers were sutured onto the epicardium for subsequent registration of MR images with pathology sections. Ex vivo MRI was performed at 1.5T with a head coil (C-Vi, General Electric) using 3D fast gradient echo (v/0.47×0.47×1 mm, 256×256x128 matrix, repetition time 300 ms, echo time 3.3 ms, flip angle 45 degrees, bandwidth ±13kHz, imaging time 1 hour). Hearts were then sectioned to examine for the presence of IFP-bearing cells.

Relaxometry and In Vivo MRI
MRI contrast characteristics of IFP-labeled MSCs were studied by measuring spin-lattice (T₁) and spin-spin (T₂ and T₂*) relaxation times in cell suspensions and in vivo after endomyocardial injection. IFP-labeled MSCs were embedded in 2 mL of 1% low-melting-point agarose at a range of densities from 10³ to 10⁵ cells/mL in 24-well plates and imaged at 1.5T (Sonata, Siemens) using the spine coil. The T₁, T₂, and T₂* relaxation rates of in vitro preparations were measured at 37°C. T₁ was measured using steady-state free precession (SSFP) inversion recovery pulse sequences and multiple inversion times (196 to 1000 ms), T₂ by fast spin echo with multiple effective echo times (3.4 to 90 ms), and T₂* by fast gradient echo (FGRE) with multiple echo times (2.6 to 60 ms). The field of view was 360 mm, which yielded voxel sizes of 1.4×1.9×6.0 mm. Voxel by voxel relaxation curves were generated and fitted using a nonlinear least-squares algorithm (Matlab v6.1, Mathworks). In vivo relaxation rate measurements and MRI were performed using both SSFP and FGRE pulse sequences in standard segmented, ECG-gated, and breath-hold examinations. In vivo T₂* relaxation rates were determined using gated FGRE with multiple echo times (3 to 20 ms). Signal to noise ratio and contrast to noise ratio (CNR) were measured for normal, infarcted, and injected myocardium according to the relation (CNR=|SIFP−SIFP|/SD₅₀₀), where SIFP represents the signal intensity (in arbitrary units) of normal myocardium, SIFP represents the signal intensity of IFP-labeled cell injection sites, and SD₅₀₀ represents the standard deviation of background noise.

Results

Cell Labeling and Effect on Viability and In Vitro Differentiation
MSCs were efficiently labeled (>99%) by confocal microscopy in culture at a range of exposure times and particle concentrations, optimally 10 μL IFP stock per mL of medium for 18 to 24 hours. At higher concentrations or exposure times, there was dose-dependent attenuation of cell proliferation (data not shown). Labeled MSCs maintained viability by Trypan Blue exclusion (Vector Laboratory) as well as IFP before injection. Viability was determined by Trypan Blue exclusion. Cell death was determined by light and confocal microscopy for morphological evidence of apoptosis and chromatin abnormality. To assess the effects of the particles on cell proliferation, growth kinetics (MTT Roche Diagnostics) were determined at a range of IFP concentrations (0.1 to 50 μL IFP stock per mL medium). The effect of labeling on MSC differentiation ability was tested using lineage-specific assays and immunostains including osteogenic, adipogenic, myogenic, and chondrogenic induction. Adipogenic differentiation was induced with 1-methyl-3-isobutylxanthine, insulin, indomethacin, and dexamethasone. Osteogenic differentiation was measured by staining for calcium deposition by the von Kossa method and for alkaline phosphatase (Fast Violet/naphthol, Sigma 85L-3R). We are unable to reproduce in vitro 5-azacytidine–induced cardiomyocyte differentiation in our laboratories.

Tissue Handling and Ex Vivo MRI
Explanted hearts were snap-frozen to examine for IFP-labeled MSCs or IFP alone. Frozen sections were fixed with cold methanol, washed with PBS, and mounted with medium containing DAPI. Photographs were obtained using a Leica TCS-SP upright confocal microscope.
Relaxometry: Contrast Effects of IFP Labeling

Figure 1 shows $T_1$, $T_2$, and $T_2^*$ relaxation time constants for IFP-labeled and unlabeled MSC agar suspensions at a range of cell concentrations. Unlabeled refers to average of a range of cell concentrations from $10^4$ to $10^6$ MSCs without IFP. B, Comparison of relaxation time constants for labeled and unlabeled cell suspensions.

<table>
<thead>
<tr>
<th>Relaxation time constants (ms)</th>
<th>Suspension of unlabeled cells (10^5/150 µL)</th>
<th>Suspension of IFP-labeled cells (10^5/150 µL)</th>
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<tbody>
<tr>
<td>$T_1$</td>
<td>4.50 ± 0.04</td>
<td>4.75 ± 0.04</td>
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<tr>
<td>$T_2$</td>
<td>4.75 ± 0.03</td>
<td>5.00 ± 0.04</td>
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<tr>
<td>$T_2^*$</td>
<td>5.00 ± 0.04</td>
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Serial In Vivo MR Imaging of IFP-Labeled MSCs

Labeled cells could be identified after injection into both normal and infarcted myocardium. A magnetic susceptibility artifact was seen as a signal void (dark region) corresponding to the injection sites (Figure 2). Injection volumes of $1 \times 10^6$ IFP-labeled MSCs in 150 µL (0.15 cm³) generated signal void volumes of $0.36 \pm 0.38$ cm³. The CNR between normal myocardium and the signal void created by the injected IFP was $10.2 \pm 3.1$ (n=6) and between infarcted myocardial and IFP signal voids was $16.5 \pm 2.3$ (n=2). The $T_2^*$ values for in vivo endomyocardial injections of $10^4/150$ µL IFP-labeled MSCs are far shorter than for normal myocardium (9 versus 27 ms) and facilitate ready visualization.

Figure 3 shows representative myocardium before and after injections of $4 \times 10^5$ IFP-labeled MSCs into normal and infarcted myocardium on day 1, imaged using SSFP MRI. The same slices are shown after intravenous Gd-DTPA injection and imaged using inversion recovery FGRE to show delayed hyperenhancement.

Serial MRI studies were conducted in all animals. Four pigs were followed for 4 to 8 days and 1 for 21 days. Each had at least 2 follow-up MRI studies before euthanasia. A total of 18 injection sites were analyzed. Nine were into the infarct and 9 into normal myocardium. Injections of $1 \times 4 \times 10^6$ MSCs were seen reproducibly and serially (Figure 4). The minimum detectable injection dose was $1 \times 10^5$ MSCs. Injections of 1 to $3.3 \times 10^4$ MSCs were not visualized. To investigate whether IFPs released from cells would be likely to show the same pattern after injection, up to 100 µL of IFP solution mixed in 150 µL of saline vehicle without cells was delivered in an identical manner. Only injections of 10 µL were detectable on the first day (data not shown), and there was no signal detected on subsequent MRI studies at days 4 or 7.

Ex Vivo Correlation

To register in vivo images with histopathology sections, we used high-resolution 3D MRI (Figure 4D). Explanted hearts (n=3)
had fiducial MRI markers applied over dye marks on the epicardial surface, and these were subsequently used to guide sectioning.

Figure 5 is a representative photomicrograph of a single IFP-labeled MSC under fluorescence (panel A) and differential phase (panel B) illumination. There is heavy perinuclear accumulation of IFP. Figure 6 shows correlation of labeled MSC injection sites under confocal fluorescence and differential interference microscopy. IFP-labeled MSCs are found within myocardium immediately after injection and appear rounded (Figures 6A through 6C). Other inflammatory cells are evident within needle tracts as described previously. When the animal shown in Figure 4 is studied after 3 weeks, IFP-labeled MSCs remain detectable, albeit with a more elongated morphology (Figures 6D through 6F). In this animal, MSCs were labeled with both IFP and DAPI before injection. Injected cells appeared viable and had preserved DAPI nuclear stain, normal chromatin pattern, and no membrane blebbing or vacuolization characteristic of apoptotic or necrotic death. Few free IFP particles appeared in the extracellular space.

Bare IFP injection sites were all identified 7 days after injection using tissue-fast dye. Free IFPs were identified on histopathology at the highest dose (10^12 μL), sparse IFP at the medium dose (1 μL), and none at lower doses. Of note, 10^12 μL of IFP stock corresponds to the number of particles in 3.1×10^5 IFP-labeled MSCs.

Discussion

We demonstrate that MSCs, labeled with a large (micron-scale) iron-fluorophore intracellular contrast particle, can readily be detected in vivo within beating hearts using MRI and that their incorporation into myocardial tissue can be confirmed ex vivo using confocal fluorescence microscopy.

An ideal agent for noninvasive tracking of therapeutic cells would have several essential characteristics. It should be nontoxic without altering cell viability, growth, differentiation, or other biological activity. It should not affect surrounding tissue if released by the carrier cell. It should be durable but ideally should have some elimination pathway. It should also permit repeated, nondestructive, noninvasive detection and should be detectable with satisfactory CNR and spatial and temporal resolution at realistic doses. It should accurately reflect the behavior of the cells it labels and should indicate the location, migration, and quantity of labeled cells. Finally, an ideal agent should indicate the true disposition of labeled cells after emigration, death, or phagocytosis; nonspecific interstitial deposition should not misrepresent target cell bioactivity.

The larger IFP we used to label MSCs exhibits many of the above characteristics. Porcine MSCs can be labeled with preserved in vitro viability, proliferation, and differentiation capability as well as in vivo viability after allogeneic transplantation. IFP effects on surrounding cells and the characteristics of IFP elimination are not yet understood. We have demonstrated that IFP-labeled MSCs have useful contrast characteristics, because we can distinguish labeled MSCs from unlabeled MSCs in vitro and in fresh myocardial tissue. IFP-labeled MSCs can be detected in beating myocardium with millimeter-scale spatial resolution and 40-ms temporal resolution immediately after direct injection in pigs. The contrast is satisfactory within normal or infarcted myocardium, both of which may be targeted in future MSC therapies.

We have identified a minimum detectable quantity (10^5...
cells/injection) of cells using conventional cardiac MRI on a commercial scanner. This is at least one order of magnitude lower than projected injection doses of cellular agents\textsuperscript{14} and can accommodate tracer quantities of labeled MSCs admixed with unlabeled MSCs.

Moreover, we are able to detect labeled cells serially and noninvasively when animals are kept alive for up to 3 weeks. Animals in an ongoing study reproducibly have detectable signal voids after 12 weeks (data not shown). The label precisely colocalizes with recovered cells ex vivo when histologic sections are registered with high-resolution ex vivo MRI. Bare label injections seem to exit the myocardium soon after direct injection. When we recovered myocardium 3 weeks after IFP-labeled MSC injections, we found most IFP to remain incorporated within MSCs and not free in the interstitium. However, IFPs impart contrast based on their presence, not based on whether host cells are viable. In 2 animals, injected MSCs were labeled both with DAPI and IFP. Intracellular IFPs were found within DAPI-labeled cells, suggesting retention by administered MSCs rather than ingestion by host cells. However, we cannot exclude the possibility that resident or recruited phagocytes have reincorporated free IFP after MSC death or lysis.

The IFP generated significant T$_2$* contrast in vitro and in vivo, with T$_2$* values significantly shorter than neighboring normal and infarcted myocardium, enabling ready detection using conventional cardiac MR to assess both immediate and long-term localization. This is consistent with the known bulk magnetic susceptibility effects of iron oxide within IFP.\textsuperscript{15,16} We observed less T$_1$ or T$_2$ contrast effect of IFP. This is not surprising for magnetite particles embedded in polystyrene, which cannot associate as freely with nearby water protons. Cell injections were detected as signal voids (dark regions) on MRI using both FGRE and SSFP pulse sequences. Signal voids can result from a variety of causes and are thus less specific than signal enhancement methods in MRI and can be additionally obscured by signal averaging within voxels. Nevertheless, T$_2$* effects influence a larger volume than that occupied by the iron label itself (“blooming”), providing useful image amplification of small injections. However, whereas T$_2$* in vitro was linearly related to the concentration of labeled cells, in vivo cellular redistribution and aggregation will probably confound quantitation of retained or expanded stem cells. Alternative contrast agents using gadolinium chelates, although attractive for their T$_1$-shortening (signal enhancement) properties, require direct association with water protons (precluding sequestration within polymer beads) and are potentially toxic should free gadolinium be liberated during prolonged intracellular exposure. Polystyrene microspheres seem biocompatible but may be unattractive for implantation into patients, because they are not degraded and may not be excreted. Clinically approved ultrasmall iron oxide particles (Feridex, Berlex) generate significantly less MRI contrast than the IFP we describe.
here. Other biodegradable polymers such as dextran may provide a more suitable shell for magnetite labels should they be applied to human cellular therapeutics.

Although MSCs or marrow stromal cells, isolated based on density gradient centrifugation and plastic adherence, may contain both mature and progenitor cell population, there is evidence that these preparations contain many cells with multipotent capability in vitro as well as desirable effects when delivered to regions of myocardial injury. It is unlikely that our injection samples contain large numbers of other nonspecific phagocytes, such as macrophages. Most cells we inject into the myocardium have undergone multiple passages yet retain in vitro differentiation capacity. In this preliminary experience, we observed IFP-containing cells with preserved nuclear structure that have elongated and aligned with host myocardial fibers. Whether IFP-labeled MSCs indeed migrate, differentiate, and improve myocardial function after transplantation remains to be demonstrated in longer-term studies including careful controls.

This dual-fluorescence MRI contrast agent, incorporated into MSCs, imparts contrast characteristics favoring ready MRI detection and permits serial in vivo tracking of MSCs after endomyocardial delivery to the beating heart. In addition, this technology also allows accurate localization of injection sites and of retained cells within both normal and infarcted myocardium. It may prove useful for real-time MRI-guided therapeutic endomyocardial injection of labeled MSCs. This new technique has potential to provide insight into stem cell retention, engraftment, and homing for cardiovascular cell therapy.

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