Efficient Tracking of Non–Iron-Labeled Mesenchymal Stem Cells With Serial MRI in Chronic Stroke Rats

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Background and Purpose—Although stem cell-based treatments for neurodegenerative diseases have advanced rapidly, there is currently no clinically available method to monitor the fate of transplanted cells in the brain.

Methods—To use magnetic resonance imaging for tracking transplanted stem cells in the ischemic rat brain, we used the cellular labeling substance Effectene to transfect a standard contrast agent (Gd-DTPA) into immortalized human bone marrow stromal cells.

Results—The transfection efficiency of this method was up to 90%, which is substantially better than pure spontaneous endocytosis or other transfection agents. In addition, cellular uptake of Gd-DTPA in vitro was maintained for >28 days. Therefore, we could follow transplanted stem cell migration and homing into the penumbric area. Using double immunofluorescence, the transplanted cells were seen to differentiate into glial cells, neurons and vascular endothelial cells. Cortical neurochemical activity as evaluated by proton magnetic resonance spectroscopy (1H-MRS) also increased considerably after immuno-labelled human bone marrow stromal cell transplantation.

Conclusion—This method of tracking immortalized human bone marrow stromal cells is highly efficient and allows for nontoxic labeling of cells. (Stroke. 2007;38:367-374.)

Key Words: Effectene ■ Gd-DTPA ■ immortalized human bone marrow stromal cells ■ in vivo monitoring ■ magnetic resonance imaging

Stem cells have been used to treat neurological diseases in which neuronal death is the major pathogenetic mechanism, including cerebrovascular and other neurodegenerative diseases.1 Because the application of stem cell-based therapies is potentially wide-ranging, specific methods are needed to continuously and noninvasively monitor stem cell survival. Three-dimensional imaging and in vivo cell tracking capabilities allow magnetic resonance imaging (MRI) to provide high-resolution visualization of the fate of cells after transplantation, and the migration of cells after injection.2 To visualize stem cells in the brain using MRI, recent advances in cell-labeling techniques with iron oxide and paramagnetic particles (gadolinium-diethylene triamine penta-acetic acid [Gd-DTPA]) have also been developed.3 However, pure cell endocytosis and lipofectamine-mediated methods of transfection have low labeling efficiencies,4 and superparamagnetic substances, such as iron oxide, have been demonstrated to have harmful effects on cell signaling and function.5

Here, we present a method whereby immortalized human bone marrow stromal cells (IhMSCs), labeled with Gd-DTPA using Effectene and transplanted into the rat ischemic brain, are tracked with 3.0-Tesla MRI. We also demonstrated that transplanted IhMSCs preserved their differentiation potential in the ischemic brain after being labeled with the MRI contrast agent Gd-DTPA.

Materials and Methods

IhMSC Preparation and Labeling

IhMSCs, donated by Dr Toguchida,6 were cultured and expanded as described.6 Gd-DTPA (Megnevist, Germany), which has a molecular weight of 547 Da, is the standard MR contrast medium for clinical use. Effectene (QIAGEN) was used to transfect Gd-DTPA into IhMSCs (Gd-hMSCs) as previously described.7 Immortalized hMSCs (6×10^5 cells) were labeled with 50 µL 0.5 mol/L Gd-DTPA using 10 µL Effectene in 6-well plates with serum-containing DMEM medium following the manufacturers’ instructions. After discarding the supernatant, the labeled cells were recovered in original medium and incubated with 1 µg/mL bis-benzimide (Hoechst 33342; Sigma) for 24 hours at 37°C.

Cell Viability and Longevity of Contrast Medium Maintenance

After Gd-DTPA labeling, the viability of cells (1×10^5) was determined by trypan blue exclusion assays. To evaluate the longevity of Gd-DTPA maintenance in stem cells, labeled cells were cultured and
propagated under standard conditions, and MRI was performed 3, 7, 14, and 28 days after the initial labeling procedure. Before MRI, cells were washed 3 times with phosphate-buffered saline to eliminate residual contrast agent particles in the supernatant.

Spectrophotometric Analysis of Cell Labeling Efficacy

The Gd-DTPA concentration within labeled cells was investigated by spectrophotometric measurement of the cellular uptake of Gd-DTPA particles using an atomic absorption spectrometer (ZeeMan spectrometer model Z-8200; Japan) as previously described.4,5

Animal Brain Ischemia/Reperfusion Model

Adult male Sprague-Dawley rats (weighing 250 to 300 grams; Experimental Animal Center, Tzu-Chi University, Hualien, Taiwan) were subjected to 3-vessel ligation. Ligation of the right middle cerebral artery and bilateral common carotids were performed by modified methods as described previously.8

Gd-hMSC Transplantation

Before transplantation, Gd-hMSCs with bis-benzimide were trypsinized and resuspended in phosphate-buffered saline. One week after ischemia, adult male Sprague-Dawley rats were anesthetized with chloral hydrate (0.4 g/kg, intraperitoneally). They were then injected stereotactically with $1 \times 10^6$ Gd-hMSC cells into 3 cortical areas adjacent to the right middle cerebral artery,9 3.5 to 5.0 mm lateral to the midline. Rat hosts did not receive any immunosuppressive medication.11

Gd-hMSCs Tracing With MRI

To assess Gd-hMSC migration, animals were imaged 3, 7, 14, and 28 days after intracerebral Gd-hMSC injection using high resolution 3-Tesla MRI (whole-body Sigma EchoSpeed MR scanner, General Electric, Milwaukee, Wis) as described previously.13 T1-weighted fast spin echo sequences were optimized to detect Gd-hMSCs. Acquisition parameters were TE/TR 59.4/600 ms, echo train length 53, and NEX 8. Each image was determined by a consensus of 2 observers blinded to the Gd-hMSC injection.

Proton MR Spectroscopy Assessment

Proton MR spectroscopy (H-MRS) was performed using the same MRI scanner with a single-voxel technique, and then T2-weighted transverse, coronal, or sagittal images were used to localize the volume of interest as previously described with modification.13 Volume of interest indicates the region measured by the three parameters (N-acetylaspartate [NAA], creatine [Cr], and choline and choline-containing compounds [Cho]) in the MRI computer software, which includes both the core and the penumbra regions of the infarcted brain. The volume of interest ($3 \times 3 \times 3$ mm) was localized centrally to the infarcted region using 2 or 3 images (transverse and sagittal/coronal). Spectroscopic acquisition parameters were as follows: water suppression was provided for by CHESS pulses and localization by a standard PRESS-type sequence (TR=2000 ms; TE=68, 136, and 272 ms). Spectra were processed using the NMR1 program (NMR1, Syracuse, NY). Metabolic peaks were fitted by the Lorentzian line shape at the known frequencies of NAA at 2.02 ppm, Cr at 3.03 ppm, and Cho at 3.22 ppm. From this, NAA/Cr and NAA/Cho ratios were calculated. Metabolic ratios are presented as mean±SE.

Histological Evaluation of Brain Tissue

Animals were anesthetized with chloral hydrate (0.4 g/kg, intraperitoneally) and their brains fixed by transcardial perfusion with saline, followed by perfusion and immersion with 4% paraformaldehyde as previously described.13

Laser-Scanning Confocal Microscopy for Immunofluorescence Colocalization Analysis

To demonstrate the differentiation potential of transplanted cells, the expression of cell type-specific markers in bis-benzimide–labeled IhMSCs were identified by immunofluorescence analysis for each brain section as previously described.13 Because bis-benzimide–labeled cells showed spontaneous blue fluorescence in their nuclei, cell-type-specific antibodies, such as GFAP (1:400; Sigma), MAP-2 (1:200; BM), von Willebrand factor (1:20; Sigma), Nestin (1:400; Sigma), and Neu-N (1:200; Chemicon) conjugated with Cy-3 (Jackson Immunoresearch), were stained to determine whether they colocalized with bis-benzimide in the same cell. The total number of differentiated cells that colocalized with bis-benzimide–labeled cells was measured as previously described.13

Quantitative Reverse-Transcription Polymerase Chain Reaction of Growth Factors Synthesis

Experimental rats were anesthetized with chloral hydrate (0.4 g/kg, intraperitoneally) at 3, 7, 14, and 28 days after cell or vehicle transplantation. Ischemic cortical and striatal areas were immediately removed on ice. Subsequently, brain tissue samples were homogenized by a plastic homogenizer in 1 g/mL lysis buffer (Promega), and total RNA and cDNA synthesis were performed as previously described.13

Results

Unaltered Cell Viability after Gd-DTPA Labeling

To determine whether there was any detrimental effect on cells labeled with contrast agent, cell viability tests were performed after each labeling procedure. Evaluation of cell viability by trypan blue exclusion tests showed an initial transient reduction in cell numbers. After 24-hour incubation under the same culture conditions, cellular viability was 93±5% for nonlabeled cells and 91±4% for Gd-hMSCs. No signs of apoptosis were detected by DNA fragmentation assays (data not shown).

Improved Labeling Efficiency and Increased Longevity of Gd-DTPA Cell Maintenance

To determine whether Effectene affected the transfection of Gd-DTPA into cells, the level of gadolinium in cells was measured by spectrophotometry. Effectene-mediated labeling efficiency was determined to be 90±3%, which was higher than that obtained (52±4%) by pure endocytosis. Saturation of the system was defined by adding 50 μL 0.5 mol/L Gd-DTPA. Results of the longevity of Gd-DTPA in cells showed that passed cells remained healthy and retained Gd-DTPA intracellularly for a period of 28 days (data not shown).

Gd-hMSC Migration: In Vivo Tracing With MRI

To examine whether Gd-hMSCs could migrate throughout the ischemic brain, Gd-hMSCs were injected intracerebrally into 3 holes and tracked with MRI at 3, 7, 14, and 28 days without the use of immunosuppressive agents (n=6 at each time point).9 Within 2 to 3 days, 3 strong spots of increased signal intensity became visible, showing a white tract through the cerebral cortex from the anterior to posterior portion of the rat brain under MRI (Figure 1B through 1E). Controls (IhMSCs without labeling, n=6) lacked spots of increased
signal intensity (Figure 1A). The area of one spot of increased signal intensity over each plane was measured at \( \approx 0.5 \times 0.5 \) mm\(^2\) from an MRI coronal view (Figure 1F through 1I). Sequentially from days 3 to 28 after immortalized hMSC transplantation, the confluent (versus dispersed) area of increased signal intensity (Figure 2A) rapidly spread from the striatum to the ipsilateral corpus callosum and hippocampus toward the peri-infarcted cortical area (Figure 2B through 2C), and even migrated across the midline to the contralateral corpus callosum and hippocampus (Figure 2D). Fluorescent histological examination confirmed that this developing MRI signal was caused by transplanted Gd-hMSCs (Figure 2A through 2J). MRI showed that many Gd-hMSCs gathered near the subventricular zone, giving an area of increased signal intensity (Figure 2G). Generally, in animals treated with Gd-hMSCs, this cell accumulation extended over time to line the lateral ventricular wall and the peri-infarcted area (Figure 2H through 2J). Furthermore, Gd-hMSCs were observed on the choroid plexus of the lateral ventricle in the ischemic hemisphere (Figure 2G).

**IhMSC Treatment Increases Neurochemical Activity**

To evaluate any improvement in neuronal metabolism after transplantation, experimental rats were studied using \(^1\)H-MRS to assess the neurochemical activity of ischemic rats. Seven days after cell transplantation, \(^1\)H-MRS showed a significant decrease in the metabolic ratio of NAA/Cho and NAA/Cr (1.61±0.03 and 1.79±0.04, respectively) (n=6) in IhMSC-treated rats (Figure 3C) and 1.52±0.03 and 1.63±0.03, respectively, in untreated rats (n=5) compared with the prestroke stage (2.6±0.11 and 2.1±0.08, respectively) (Figure 3D). Consistent with the recovery of neurological behavior test scores (data not shown), significant improvement in neurochemical activity under \(^1\)H-MRS was observed specifically with regard to NAA/Cho and NAA/Cr (1.72±0.04 and 1.90±0.06, respectively) at 14 days (n=6) and NAA/Cho and NAA/Cr (1.84±0.08 and 2.08±0.13, respectively) at 28 days (n=6) in the treated group (Figure 3F and 3H) in comparison to NAA/Cho and NAA/Cr (1.59±0.06 and 1.71±0.14, respectively) at 14 days (n=6) and NAA/Cho and NAA/Cr (1.66±0.07 and 1.80±0.13, respectively) at 28 days (n=6) in the control group (Figure 3E and 3G). Measurement data for NAA/Cho and NAA/Cr are displayed graphically for the IhMSC-treated and control groups (Figure 3I).

**Neuroplasticity After Intracerebral Transplantation of Gd-hMSCs After Cerebral Ischemia**

To study whether the Effectene-labeling procedure impeded the potential of stem cells to differentiate, fluorescent immu-
nohistochemistry was used to analyze the colocalization between cell-type specific markers and bis-benzimide–labeled cell nuclei. The results showed that some bis-benzimide–labeled cells colocalized with antibodies for GFAP and MAP-2 (Figure 4A and 4B), and Neu-N and Nestin (Figure 4C and 4D). Some bis-benzimide–labeled cells showing vascular phenotypes of von Willebrand factor (Figure 4E) were also found around the perivascular and endothelial regions in the ischemic hemispheres of Gd-hMSC–treated rats. The transplanted cells did not induce any inflammatory cell infiltration (immune reaction) or tumor formation in the host brain.

Figure 2. Continuous monitoring of Gd-hMSC migration in rat brain. A, A strong white signal caused by transplanted Gd-hMSCs labeled in MRI was found in the right striatum 3 days after cell transplantation. B, Transplanted stem cells spread rapidly to the ipsilateral corpus callosum and hippocampus toward the peri-infarcted cortical area (C) and even migrated across the midline to contralateral corpus callosum and hippocampus (D) 7 days after Gd-hMSC transplantation. E, Many of the implanted Gd-hMSCs migrated to the peri-infarcted area and gathered near the subventricular zone and choroid plexus (white arrow) (F) 14 days after transplantation (G). H, Generally in Gd-hMSC–treated animals, stem cell accumulation extended to the peri-infarcted area (I) and corpus callosum (J). Bar =150 μm.

Intracerebral Transplantation of IhMSCs Modulates Neurotrophic Factor Expression in the Ischemic Hemisphere

To identify molecular mechanisms for improvement of neurological dysfunction after cerebral ischemia in IhMSC–treated animals, we examined the expression of neurotrophic factors known to neuroprotect the ischemic cortical area (n=4). The results revealed significantly increased expression levels of SDF-1 and BDNF in the ischemic rats treated with stem cells in comparison to vehicle controls (Figure 5A and 5B). The ratio of SDF-1 and BDNF to GAPDH peaked at ~2-fold increase in comparison to the
control 14 days after transplantation of stem cells (Figure 5C and 5D).

**Discussion**

In molecular biological experiments, standardized transfection protocols with either liposomes or viral vectors are widely used to transport extracellular DNA or other substances into targeted cells. Here we used Effectene to transfect the MRI contrast agent Gd-DTPA into stem cells, because it is relatively more efficient and less toxic than liposome, calcium phosphate, or viral vectors for transfection into primary cells. There was a possibility that phagocytosed or dead transplanted cells with Gd-DTPA were detected by the MRI. However, a previous pharmacokinetic study reported that Gd-DTPA in dead cells or interstitial spaces was washed out within 24 hours. Furthermore, we did not find any phagocytosed implanted cells or fusion cells in our Gd-hMSC–treated rat brain. In addition, our experimental

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**Figure 3.** IhMSC treatment increases neurochemical activity. 1H-MRS examination was used to assess neurochemical activity. A, In 1H-MRS of the cerebral cortex of nonischemic rats, 3 important signals were consistently detected: Cho, Cr, and NAA. B, Representative figure of a rat possessing a cortical infarction (blue circle area) under MRI, square area indicates volume of interest located precisely central to the infarcted brain. C and D, A decrease in NAA peak ratio was observed. Seven days after treatment, the NAA peak ratio (white arrow) showed a significant increase compared with vehicle control. E and G, Subsequently, improvement of neurochemical activity under 1H-MRS was also observed 14 days and 28 days after stem cell transplantation in the vehicle control group, and (F and H) in the stem cell treated group, respectively. I, Statistical results of NAA/Cho and NAA/Cr were depicted against temporal sequence after IhMSC transplantation. The mean±SEM is shown. *P<0.05 vs control.
results showed improvement in neurological dysfunction, increased neurotrophic factor synthesis, and enhanced neuronal activity after stem cell transplantation. On the basis of these pieces of evidence, we conclude that the possibility of having detected dead or phagocytosed transplanted cells in the ischemic brain was very low.

Many experiments have used other paramagnetic substances to track cell movement under MRI, including iron-containing agents (eg, Feridex) and a new generation of contrast agents magnetodendrimers and particles conjugated with Tat peptides.20 However, magnetodendrimers and particles conjugated with Tat peptides require complicated methods of manufacture. Furthermore, iron-containing agent-labeled stem cells transplanted into brain are visualized as signal void images (black signal) using these paramagnetic substances. This can create problems because enlarged “false” black signaling effects of iron-labeled cells can exceed true stem cell mass.20 In addition, iron can be toxic at high concentrations,21 with its accumulation in tissue also catalyzing the Fenton reaction and potentiating oxygen toxicity through the generation of a wide range of free radical species. Furthermore, leakage of iron or the death of labeled cells may cause the release of iron oxide crystals into tissue, which can result in a potentially toxic uptake in surrounding healthy cells.22 In contrast, the pharmacological properties of Gd-DTPA have been extensively investigated17 and clinically applied. Under MRI, Gd-DTPA–labeled cells in the brain show increased signal intensity (white signal) rather than void signals. Although the MR detection thresholds in stem cell labeling were lower in the iron-containing particle ($2.5 \times 10^5$ cells) than that of Gd-DTPA ($5 \times 10^5$ cells), it is highly desirable for clinical applications to have the choice of

Figure 4. Neuroplastic effects after intracerebral transplantation of Gd-hMSCs after cerebral ischemia. A, The result shows that many bis-benzimidylabeled cells expressed the cellular marker GFAP. B, Several bis-benzimide-labeled cells also expressed cellular marker MAP-2. C, Some bis-benzimide-labeled cells expressed the cellular marker Neu-N. D, Some bis-benzimide-labeled cells also expressed the cellular marker Nestin. E, Many bis-benzimide-labeled cells expressed the cellular marker von Willebrand factor in Gd-hMSC–treated rat brains. Bar=50 μm.
identifying labeled stem cells with high signal intensity on MRI such as Gd-DTPA. In this study, the increased signal intensity seen in the MRI of ischemic rat brains correlated well with the true size of engrafted stem cells and corresponding anatomical brain structures. However, one major advantage of using iron-based contrast agents rather than Gd-DTPA would be their much lower detection threshold.

In summary, the present study demonstrates a strategy to detect implanted stem cells using an imaging system in an intact animal. We speculate that MRI tracking of grafted cells might become a powerful tool for understanding the molecular mechanisms that are ultimately responsible for the successful migration and expansion of neurotransplanted cells.

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References

Figure 5. Intracerebral transplantation of IhMSCs modulates neurotrophic factor expression in the ischemic hemisphere of stem cell-treated rats. A and C, Result of conventional reverse-transcription polymerase chain reaction analysis. Using gene-specific primers, reverse-transcription polymerase chain reaction was performed for BDNF and SDF-1 in brain samples from the cortex and striatum of experimental rats at different time-points after euthanasia (3, 7, 14, and 28 days). C, Vehicle-control group. B and D, The result of quantitative reverse-transcription polymerase chain reaction was present with the ratio of BDNF and SDF-1 mRNA relative to GAPDH mRNA in IhMSC-treated and control group. The mean±SEM is shown. *P<0.05 vs control.

Disclosures
None.


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