MR tracking of magnetically labeled mesenchymal stem cells in rats with liver fibrosis

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Abstract

**Purpose:** In vivo magnetic resonance (MR) tracking of magnetically labeled bone marrow mesenchymal stem cells (BMSCs) administered via the mesenteric vein to rats with liver fibrosis.

**Materials and Methods:** Rat BMSCs were labeled with superparamagnetic iron oxide (SPIO) and the characteristics of the BMSCs after labeling were investigated. Eighteen rats with CCL4-induced liver fibrosis were randomized to three groups to receive SPIO-labeled BMSCs (BMSC-labeled group), cell-free SPIO (SPIO group), or unlabeled BMSCs (control group). MR imaging of the liver was performed at different time points, and signal-to-noise ratio (SNR) of the liver was measured. In vivo distribution of delivered BMSCs was assessed by histological analysis.

**Results:** Labeling of BMSCs with SPIO did not significantly alter cell viability and proliferation activity. In BMSC-labeled group, the liver SNR immediately decreased from 8.56±0.26 to 3.53±0.41 at 1 h post injection and remained at a significantly lower level till 12 days (P<0.05 versus the level before). By contrast, the liver SNR of the SPIO group almost recovered to the preinjection level (P=0.125) at 3 days after a transient decrease. In control group, the liver SNR demonstrated no significant difference at the tested time points. Additionally, Prussian blue-positive cells were mainly distributed in the liver parenchyma, especially in injured areas.

**Conclusion:** The magnetically labeled BMSCs infused through the mesenteric vein can be detected in the fibrotic liver of rats using in vivo MR imaging up to 12 days after injection.

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**Keywords:** Mesenchymal stem cells; Superparamagnetic iron oxide; Magnetic resonance imaging; Liver fibrosis

1. Introduction

Liver fibrosis and its end-stage, cirrhosis, represent the common pathogenesis of chronic liver diseases [1,2]. Advanced fibrosis is characterized by an excessive deposition of extracellular matrix rich in fibrillar collagens. Currently transplantation is the only curative treatment for cirrhosis; however, the shortage of donor organs limits its utility. Mesenchymal stem cells (MSCs) may offer a potentially alternative therapy to organ transplantation, because of their capacity to differentiate into multiple types of cells. Importantly, human MSCs xenografted directly to rat liver have been reported to differentiate into hepatocytes, as revealed by positive immunostaining for hepatocyte-specific markers [3]. Moreover, MSCs can effectively rescue experimental liver failure and contribute to liver regeneration [4]. In models of experimental liver fibrosis, transplanted MSCs have been documented to prevent the fibrotic process [5–7], suggesting the potential for therapeutic applications.

For clinical applications of MSCs, labeling and tracking are critical to assess cell distribution and homing. Magnetic resonance (MR) imaging is ideally suited for high-resolution whole-body in vivo imaging. Two methods of cell labeling have been developed for further MR imaging, i.e., labeling with paramagnetic agents like gadolinium [8] and with superparamagnetic agents like superparamagnetic iron oxide (SPIO) [9–11]. Much interest has recently been focused towards SPIO applications, as cells labeled with these compounds are more readily detectable with MR imaging [12]. In addition to high sensitivity, the SPIO particles
exhibit nontoxic and biodegradable properties, and do not affect proliferation and multilineage differentiation capacity in vitro [13–15]. SPIO labeling of MSCs, for instance, does not alter the function or differentiation capacity of the target cells [13]. The purpose of this study was to evaluate in vivo MR tracking of SPIO-labeled MSCs after intravascular injection in a rat model of liver fibrosis.

2. Materials and methods

2.1. MSC isolation and culture

The study was approved by the Institutional Committee on Animal Research of Sun Yat-sen University. Four male Sprague-Dawley rats, aged 3–4 weeks and weighing 70–80 g, were sacrificed, and bone marrow was harvested in sterile conditions from rat femurs and tibiae. MSCs were then isolated from bone marrow as described previously [16,17]. Briefly, bone marrow was resuspended in phosphate-buffered saline (PBS) to a final volume of 10 ml and layered over an equal volume of 1.077 g/ml Percoll solution (Pharmacia, Piscataway, NJ, USA). After centrifugation at 2000 rpm for 20 min, the mononuclear cells were recovered, transferred to a final volume of 10 ml and layered over an equal volume of 1.077 g/ml Percoll solution (Pharmacia, Piscataway, NJ, USA). After centrifugation at 2000 rpm for 20 min, the mononuclear cells were recovered, transferred to a final volume of 10 ml and layered over an equal volume of 1.077 g/ml Percoll solution (Pharmacia, Piscataway, NJ, USA). After centrifugation at 2000 rpm for 20 min, the mononuclear cells were recovered, transferred to a final volume of 10 ml and layered over an equal volume of 1.077 g/ml Percoll solution (Pharmacia, Piscataway, NJ, USA). After centrifugation at 2000 rpm for 20 min, the mononuclear cells were recovered, transferred to a 100-mm culture dish (Corning, Schiphol-Rijk, the Netherlands) and incubated (37°C, 5% humidified CO2) with low-glucose Dulbecco’s Modified Eagle Medium containing 0.2 mmol/ml L-glutamine (Gibco, BRL, Karlsruhe), 100 IU/ml penicilllin (Gibco, BRL, Karlsruhe), 100 μg/ml streptomycin (Gibco, BRL, Karlsruhe), 10 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ, USA) and 10% fetal calf serum (PAA, Pasching, Austria). Nonadherent cells were removed after 24 h and the medium was changed every 3 days. At subconfluence (90%), the cells were detached with 0.25% trypsin and passaged at a split ratio of 1:2.

2.2. In vitro cell labeling

Bone marrow-derived MSCs (BMSCs) were magnetically labeled with a combination of SPIO (Resovist; Schering, Germany) and poly-L-lysine (PLL, Sigma, St. Louis, MO, USA), as previously described [14]. The PLL is used as a transfection agent that provides efficient labeling. Cells were incubated with the labeling medium containing 50 μg/ml iron and 0.75 μg/ml PLL for 48 h at 37°C with 5% CO2. After labeling, cells were counted, and cell viability was examined by means of the dye exclusion test (0.04% trypan blue solution). To assess the effect of iron labeling on cell proliferation, labeled and unlabeled control cells were seeded in 96-well plates at 1×10^3 cells per well, and viable cells were counted at 6 days after seeding.

To examine labeling efficiency, Prussian blue staining of iron particles and total iron load (TIL) analysis were performed, as described previously [18]. For Prussian blue staining, the cells were fixed for 10 min in methanol, incubated for 30 min with 2% potassium ferrocyanide in 6% hydrochloric acid, and then counterstained with nuclear fast red for 30 min. For TIL analysis, the labeled cells were harvested and counted; the cell pellet was then dried for 2 h at 80°C. Samples were subsequently incubated overnight at room temperature and for another 2 h at 60°C in perchloric acid and nitric acid at a 3:1 ratio to expose iron oxide from the dextran-coated nanoparticles. The solution was diluted 10-fold with PBS and analyzed by using a polarized atomic absorption spectrometer (HG-9602A; Shengyang Huaguan, Shengyang, China). The location of the SPIO particles within the cells was studied with an 80-kV electron microscope (Philips, CM10, The Netherlands).

2.3. Model of rat liver fibrosis and cell transfer

Liver fibrosis was induced as previously described [19]. Briefly, 20 female Sprague-Dawley rats weighing 150–160 g were intraperitoneally administered with carbon tetrachloride (CCl4) at a dose of 0.033 ml per 100 g body weight twice a week. After 10 weeks, rats developed liver fibrosis confirmed by histological analysis. Eighteen of the rats were randomly divided into three groups (n=6 for each group): BMSC-labeled group (administration of SPIO-labeled BMSCs), SPIO group (injection of SPIO) and the control group (transplantation of unlabeled BMSCs). After being anesthetized, animals underwent an abdominal incision to expose the mesenteric vein, by which approximately 4×10^6 labeled or unlabeled BMSCs or 4 μl Resovist (112 μg iron) in 2 ml PBS were slowly administered.

2.4. Assessment of the signal-to-noise ratio of the liver

MR imaging of the liver was carried out before and after injection of BMSCs into rats at 1.5 T using a 3.5-inch dual surface coil. The imaging parameters were as follows: field of view, 80×80 mm²; section thickness, 2 mm, spacing 0.5 mm; base resolution matrix, 256×128. Imaging sequences included: transverse T1-weighted spin-echo [repetition time (ms)/echo time (ms), 400/10; Nex, 6, 0.0], T2-weighted fast spin-echo (2500/85; Nex, 8, 0.0), and T2*-weighted gradient-echo (450/15.0; Nex, 6.0; flip angle, 20°). To minimize respiratory artifacts, the abdominal wall of rats was compressed with a non-magnetic pressure plate. The total area of the liver was covered with contoured regions of interest on each section. The signal-to-noise ratio (SNR) for each section was calculated as (SNR=SIL/S.D.noise), where SIL represents the signal intensity of the liver, and S.D.noise represents the S.D. of the background noise. The overall liver SNR was then obtained by determining the weighted average of the SNR values in the individual sections.

2.5. Histological analysis

At 7 and 15 days after injection, rats were sacrificed and the liver tissues were histologically evaluated. The liver samples were fixed with 4% buffered formalin, embedded in paraffin and sectioned with a microtome (5 μm thick). Finally, the sections were subjected to hematoxylin-eosin staining, the cells were fixed for 10 min in methanol, incubated for 30 min with 2% potassium ferrocyanide in 6% hydrochloric acid, and then counterstained with nuclear fast red for 30 min. For TIL analysis, the labeled cells were harvested and counted; the cell pellet was then dried for 2 h at 80°C. Samples were subsequently incubated overnight at room temperature and for another 2 h at 60°C in perchloric acid and nitric acid at a 3:1 ratio to expose iron oxide from the dextran-coated nanoparticles. The solution was diluted 10-fold with PBS and analyzed by using a polarized atomic absorption spectrometer (HG-9602A; Shengyang Huaguan, Shengyang, China). The location of the SPIO particles within the cells was studied with an 80-kV electron microscope (Philips, CM10, The Netherlands).
Fig. 1. SPIO incorporation into BMSCs. Rat BMSCs were labeled with SPIO-PLL complex. (A) Iron staining after 48 h of labeling shows that almost 100% of the cells incorporated SPIO and are stained blue (original magnification ×1000). The iron-oxide particles were mainly dispersed in the cytoplasm. (B) An electron micrograph demonstrates the presence of SPIO particles (indicated by arrowhead) in BMSCs (original magnification ×3900).

Fig. 2. T2*-weighted MR imaging of the liver. Rats with CCL4-induced liver fibrosis received intravascular administration of SPIO-labeled BMSCs, SPIO or unlabeled BMSCs (as a control). MR imaging of the liver was performed before and after injection (1 h, Days 3 and 15). The liver SI of both the SPIO-labeled BMSC and SPIO groups dramatically decreased at 1 h post injection, and recovered to the pretreatment levels at Days 15 and 3, respectively. In the control group, the liver SI demonstrated no significant difference at the tested time points.
2.6. Statistical analysis

All statistical analyses were performed using the SPSS program 11.5 for Windows (SPSS, Chicago, IL, USA). Data were presented as means±S.D. Student’s unpaired, paired t test and repeated-measures analysis of variance were used for group comparison. A P value of less than .05 was considered to indicate a significant difference.

3. Results

3.1. Labeling of BMSCs with SPIO

BMSCs isolated from rat bone marrow were labeled with SPIO and the labeling efficiency was determined by Prussian blue staining. The result demonstrated that almost 100% of the cells incorporated SPIO particles manifested as numerous blue granules in the cytoplasm (Fig. 1A). By contrast, no Prussian blue-positive product could be detected in the unlabeled cells. Electron microscopy further revealed that SPIO particles were present in endosomal vesicles (Fig. 1B).

The mean TIL of the unlabeled cells was 0.25±0.19 pg per cell, while incubation with SPIO-PLL for 48 h increased TIL to 28.23±7.52 pg per cell. Consistent with previous studies [10,18], no significant difference was observed in cell viability (97.9%±0.04% vs. 98.4%±0.03%, P>.05) as well as average proliferation rate (43%±0.3 vs. 46%±0.8 per day, P>.05) between SPIO-labeled and unlabeled BMSCs during 6 days of culture.

3.2. Stem cell tracking by MR imaging

Signal intensity (SI) was obtained with T1-weighted spin-echo, T2-weighted fast spin-echo, and T2*-weighted gradient-echo sequences in the liver tissue. T2*-weighted imaging seemed to be the most sensitive among them (data not shown). The liver SI of both the BMSC-labeled and SPIO groups considerably decreased at 1 h post injection, and returned to the preinjection level at Days 15 and 3 respectively (Fig. 2). No apparent change in liver SI was noted in the control group at the tested time points. Measurement of SNRs further revealed that injection of SPIO-labeled BMSCs or SPIO caused a significant decline in liver SNRs at both 1 h (P=.001 and P=.000, respectively) and 1 day (P=.011 and P=.019, respectively), compared with the levels before injection (Fig. 3). The liver SNR of the SPIO group recovered to the pretreatment level at Day 3 (P=.125), while that in the BMSC-labeled group remained at a considerably lower level until 12 days after injection (P<.05 versus the level before). No statistically significant difference in the liver SNR was detected in the control group at the tested time points after cell injection compared with before.

3.4. Histological observation of SPIO-labeling BMSCs

Histological examination showed an established liver fibrosis, as evidenced by fibrotic septa arising from the portal area and hydropic degeneration (Fig. 4B). Prussian blue staining further revealed that at 3 days after transplantation the staining-positive cells were distributed in the portal triad region (Fig. 4C). At 15 days, the positive cells decreased in number and mainly retained in the liver intralobular parenchyma (Fig. 4D).

4. Discussion

BMSCs possess pluripotent-differentiation capability and can differentiate into multiple mesoderm type (e.g., osteoblasts and chondrocytes) and nonmesoderm type (e.g., neuronal-like cells) lineages [20]. Generation of functional hepatocytes from BMSCs has been documented in several studies [3,21,22], suggesting the potential therapeutic implications of BMSCs in liver diseases. One important issue concerning stem cell transplantation is efficient labeling and tracking of them. Noninvasive MR imaging has proven effective to in vivo monitor cells labeled with magnetically visible contrast agents [8,23]. In this study, rat BMSCs were labeled with SPIO, one of the most sensitive existing contrast agents. The labeling efficiency was approximately 100%. The TIL of the labeled cells was 100-fold higher than that of the unlabeled control cells. In addition to high efficiency, this labeling with SPIO did not considerably alter cell viability and proliferation activity, consistent with previous studies [14,24]. These findings suggest that SPIO has excellent biocompatibility.
In the setting of diffuse disease such as liver fibrosis, local intravascular injection is usually accomplished for delivery of stem cells [25,26]. One of the advantages of this administration approach is to achieve a dispersed distribution of stem cells through the whole organ. Infusion of magnetically labeled BMSCs via the hepatic artery has been documented to yield a significant signal loss of the liver in MR images for at least 7 days [25]. A comparable or slightly longer duration (i.e., 12 days) of signal decay of the liver was observed following delivery of labeled stem cells through the portal vein [26]. Liver fibrosis is frequently accompanied by portal hypertension and coagulation abnormality and direct portal vein injection may cause embolization. Given that the blood of the mesenteric vein directly flows into the portal vein (data not shown) and the mesenteric vein is more readily exposed, herein we chose the mesenteric vein rather than the portal vein for delivery of stem cells. Consistently, injection of SPIO-labeled BMSCs through the mesenteric vein also allows for efficient tracking by MR imaging for around 12 days. Notably, the duration of signal loss in the liver seems to be longer than that reported in a previous study [27], whereby intrasplenic transplantation of SPIO-labeled MSCs caused a significant reduction of signal intensity in the liver for 3 days followed by gradual recovery. The differences in delivery route and other experimental parameters such as labeling efficiency, cell purity and cell dose may account for the longer persistence of signal loss in this study.

At 1 h post infusion, SPIO-labeled BMSCs migrated to the liver as evidenced by a considerable diminution in the liver SNR. Moreover, the signal loss persisted over 12 days, suggesting that donor BMSCs could be retained and incorporated into the liver. Similar to intracellular counterparts, free SPIOs also accumulated in the fibrotic liver where they were likely uptaken by activated macrophages as shown previously [28,29]. This accumulation significantly caused signal loss in the MR images of the liver. After 12 days in the BMSC-labeled group and 3 days in the SPIO group, the liver SNR returned to the pre-injection level. However, the excretion route for the SPIOs is still unclear. It may be due to mobilization of labeled cells out of the liver and/or clearance of dead cells. Alternatively, SPIO particles may exit the host cells via exocytosis and enter the blood circulation. In the BMSC-labeled group, some labeled cells remained in the liver parenchyma, primarily in the periporal and injured areas at 15 days after delivery. However, it is unclear whether the SPIO particles were retained in the BMSCs or in other types of cells, as they could be taken up by the resident macrophages when released from the stem cells [9]. Nevertheless, these findings suggest that SPIO-labeled BMSCs can home to the damaged liver and be tracked by MR imaging for at least a short period of time.

Several open questions remain to be addressed in the future work. First, what is about the long-term fate of the injected BMSCs in vivo? In this study, only a limited time (i.e., 15 days) of follow-up was performed. Second, it is unclear whether and to what extent the donor cells migrate from the liver to adjacent tissues such as the lung, which may partially account for the recovery of the liver SNR with

Fig. 4. Micrographs of histologic specimens of liver tissue (original magnification ×100). Hematoxylin-eosin staining shows (A) normal and (B) fibrotic rat livers. (C) Prussian blue staining demonstrates SPIO-labeling BMSCs in the portal triad region at 3 days post transplantation. (D) At 15 days, the stain-positive cells are decreased in number and mainly distributed in the liver intralobular parenchyma.
time. And finally, the differentiation capacity of the labeled cells in vivo is still unknown.

In conclusion, labeled BMSCs can be retained in the fibrotic liver of rats following delivery through the mesenteric vein. SPIO labeling allows for efficient tracing of the donor cells with in vivo MR imaging.

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