Biomaterials 34 (2013) 4914–4925

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

MRI of iron oxide nanoparticle-labeled ADSCs in a model of hindlimb ischemia

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ARTICLE INFO

Article history:
Received 19 January 2013
Accepted 6 March 2013
Available online 25 March 2013

Keywords:
Cell tracking
Adipose-derived stem cells (ADSCs)
Limb ischemia
Iron oxide nanoparticles
Magnetic resonance imaging (MRI)

ABSTRACT

Adipose-derived stem cells (ADSCs) exhibit tremendous potential for repair of ischemic diseases. However, studies on the fate, migration, differentiation, and body distribution of the labeled ADSCs are rarely reported. In this study, magnetic iron oxide nanoparticles were designed, synthesized, and coated with meso-2,3-dimercaptosuccinic acid (DMSA) to produce DMSA nanoparticles (DMSA-NPs). The properties, size distribution, and characterization of DMSA-NPs were evaluated. Green fluorescent protein expressing ADSCs (GFP-ADSCs) were obtained and labeled with DMSA-NPs. The viability, cytotoxicity and multi-differentiation capacity of labeled GFP-ADSCs were evaluated in vitro. Labeled and non-labeled GFP-ADSCs were injected into a mouse model of hindlimb ischemia, and 3T magnetic resonance imaging (MRI) was acquired. The synthesized DMSA-NPs efficiently labeled the GFP-ADSCs in vitro and in vivo without affecting cell viability, proliferation, cell cycle, and multi-differentiation capacity. The MRI showed hypointense spots in the labeled GFP-ADSCs that lasted up to 8 weeks. Prussian blue staining and immunofluorescence assay at 4 and 8 weeks indicated that the labeled GFP-ADSCs were in and around the ischemic sites and some differentiated into capillaries. This observation is identical to that seen for transplants of unlabeled cells. Labeled cells were also identified mainly in the liver and spleen, with significantly smaller amounts in the lungs, intestines, heart, and kidney. Developed DMSA-NPs were shown to exhibit a considerable potential for use as nanoprobes for MRI of stem cells, which will enhance our understanding of cell-based therapeutic strategies for ischemic diseases.

1. Introduction

Peripheral vascular disease, which is caused by atherosclerosis, is increasingly becoming a major health care problem in an aging society [1,2]. Early and rapid revascularization of injured and ischemic tissues is of great importance for the restoration of their physiological function [3]. Cell-based therapies have shown promise for the treatment of myocardial infarction, hindlimb ischemia, and stroke [4–6]. Adipose-derived stem cells (ADSCs) reportedly possess pluripotency, and can differentiate into adipocytes, osteoblasts, chondrocytes, neurons, and endothelial cells [7,8], which share similar characteristics with bone marrow stromal cells [9,10]. With their simple isolation methods considered, easy expand ability, and multipotency, ADSCs have been considered as ideal candidates for the treatment of ischemic diseases.

The location, distribution, and long-term viability of the transplanted cells must be non-invasively evaluated in vivo to develop effective stem cell-based therapies for the repair of ischemic tissues

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http://dx.doi.org/10.1016/j.biomaterials.2013.03.014

[11,12]. Magnetic resonance imaging (MRI) of cells labeled with magnetic contrast agents can achieve this goal [13]. The frequently used magnetic contrast agents are the superparamagnetic iron oxide and its derivatives. These agents have been successfully applied in monitoring various cells [11]. Some transfection reagents must be added to enhance the labeling efficiency [14]. However, transfection agents reportedly inhibit stem cells from differentiating into chondrocytes [15], emphasizing the demand for non-transfectant magnetic nanoparticles that have no cytotoxic effect on stem cell biology and property.

Magnetic iron oxide nanoparticles (Fe_2O_3, Fe_3O_4) are water-dispersible, stable, and non-cytotoxic when they were coated with meso-2,3-dimercaptosuccinic acid (DMSA) [16]. As previously reported, DMSA nanoparticles (DMSA-NPs) settle in phagocytic vacuoles or lysosomes, enhancing MRI sensitivity to depict the labeled cells. DMSA-NPs show a highly efficient label for various cell types [16–18]. However, whether ADSCs can be labeled by DMSA-NPs remain unclear and the fate, migration, differentiation, and body distribution of labeled ADSCs are rarely reported.

Despite the superior sensitivity and resolution of MRI in tracking iron oxide nanoparticle-labeled stem cells, some investigators cast doubts that hypointense spots blur the distinction among iron-labeled cells and the surrounding air, hemorrhage, dead cells, and macrophages [19]. To help address these significant questions, we conducted head-to-head comparisons of tracing ADSCs by using Green fluorescent protein (GFP) gene and iron oxide nanoparticles for imaging. GFP is an excellent transgene marker because it can be easily tracked and identified in living cells in an in vitro culture and has been used in in vivo transplantation [20,21].

In a previous study, we demonstrated that magnetically labeled ADSCs can dwell in mouse vascular injury sites [22]. In the current study, DMSA-NPs were designed, synthesized and evaluated. GFP-ADSCs were labeled with DMSA-NPs. The viability, cytotoxicity, and multi-differentiation capacity of the labeled GFP-ADSCs were evaluated in vitro. The labeled and non-labeled cells were injected into a mouse model of hindlimb ischemia, and 3T MRI was performed to investigate the migration of the transplanted GFP-ADSCs to the ischemic muscles in live animals. We also investigated whether the implantation of labeled GFP-ADSCs would exhibit an identical characterization of survival, migration, differentiation, and biodistribution as their unlabeled counterparts after transplantation into the ischemic limb.

2. Materials and methods

2.1. Materials

Nanohydroxyapatite was purchased from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). DMSA was obtained from J and K Chemical Co., Ltd. (Shanghai, China). Acetic anhydride and succinic anhydride were from Sigma–Aldrich (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO Company and SIGMA Company. All other chemicals were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used as received. Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ cm.

2.2. Synthesis and characteristics of DMSA-NPs

The procedure to synthesize DMSA-Fe_2O_3 was adopted from previously reported techniques [16–18]. Briefly, a solution consisting of $FeCl_3$ and $FeSO_4$ with a molar ratio of 2:1 was mixed under N_2 protection, followed by gradual addition of excess ammonia aqueous solution under vigorous stirring for 30 min to obtain black Fe_3O_4 precipitates. Then, the obtained Fe_3O_4 was washed immediately with distilled water for five times and separated by magnet. After that, the Fe_3O_4 was redispersed in distilled water and oxidized by air at the temperature of 90 °C to become more stable NPs (c-Fe_2O_3). Finally, the NPs were coated with DMSA and stable aqueous DMSA-NPs (DMSA–Fe_2O_3) were obtained.

The hydrodynamic size of the formed DMSA-Fe_2O_3 dispersed in water was analyzed using a Zetasizer Nano ZS system (Malvern, UK) equipped with a standard 633 nm laser. The morphologies of the DMSA–Fe_2O_3 were viewed under a scanning electron microscope (SEM) (JSM-5600LV, JEOL Ltd., Japan) at an operating voltage of 15 kV.

2.3. Characterization of GFP-ADSCs

GFP-ADSCs were isolated from the inguinal adipose tissues of adult transgenic C57/BL6-GFP mice (the Model Animal Research Center of Nanjing University, Nanjing, China) and expanded as we previously described [22]. All animal procedures were performed in accordance with the Guidelines of the Animal Experiment and Care Committee of Shanghai JiaoTong University School of Medicine.

To characterize the phenotypes of GFP-ADSCs, passage 3 (P3) GFP-ADSCs were washed with phosphate buffer solution (PBS) and incubated with phycoerythrin (PE)-conjugated anti-mouse antibodies against CD11b, CD31, CD34, CD45, CD83, CD90, CD105, CD133, Sca-1, and major histocompatibility complex (MHC)-II for 30 min at 4 °C in the dark. Isotype control antibodies were used as the control group (eBioscience, San Diego, USA). After washing, the cells were then analyzed by flow cytometry (Beckman Coulter, Fullerton, CA).

To test the characterization of GFP-ADSCs, P3 GFP-ADSCs were fixed with 4% paraformaldehyde, the pluripotency marker Sox2 (Epitomics, Abcam, USA), and extracellular matrix (ECM) markers laminin (Abcam, UK), Fibronectin (Abcam, UK), and endothelium cell marker CD31 (Abcam, UK) were performed by immunofluorescence staining, as we previously described [22].

2.4. Viability and toxicity of labeled GFP-ADSCs

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) as we previously described [22] to assess whether the DMSA-NPs treatment would adversely affect the biology and property of the labeled GFP-ADSCs. The final DMSA-NPs concentrations were 0, 12.5, 25, and 50 μg/mL.

The toxicity of the DMSA-NPs on the labeled GFP-ADSCs was further examined by conducting a flow cytometric detection of the cell cycles and apoptosis. Labeled or unlabeled cells were stained with propidium iodide (PI) for the cell cycle tests and Annexin V (eBioscience, San Diego, USA) for the apoptosis tests. The cell cycle profiles, including G0–G1, G2–M, S phases, and apoptosis were analyzed.

2.5. Differentiation assays of labeled GFP-ADSCs

Both the labeled GFP-ADSCs and unlabeled control cells were subjected to stimulation for adipogenic and osteogenic differentiation according to cell supplier's instructions to determine whether GFP-ADSCs retained the ability to differentiate into adipocytes and osteocytes after labeling with DMSA-NPs, as we previously described [22]. After 3 weeks, the expression of the adipogenic representative gene, PPARr, and the osteogenic representative gene, Runx2, was analyzed. PCR with cDNA was performed using the following primers: 5′ CAC TCG CAT TCC TTT GAC AT 3′, 5′ TTG ATC GCA CTT TGG TAT TCT 3′ (PPARr), and 5′ GGA CTG TGG TTA CCG TCA T 3′, 5′ GGA GGA TTT GTG AAG ACT GTT 3′ (Runx2).

2.6. Prussian blue staining and TEM assays of labeled cells

Prussian blue staining was performed to localize the iron particles in the labeled cells. The P3 GFP-ADSCs were incubated with 25 μg/mL DMSA-NPs for 24 h according to previously established methods [23]. The unlabeled cells were used as the control groups. The cells were fixed with 4% paraformaldehyde for 20 min and stained with 2% potassium ferrocyanide in 6% hydrochloric acid for 30 min. After washing with PBS thrice, the cells were counterstained with nuclear fast red.

The labeled cells were analyzed by transmission electron microscopy (TEM) according to instructions as we previously described [22,23] to evaluate further the DMSA-NPs internalization in GFP-ADSCs.

2.7. In vitro MR imaging of labeled cells

Different numbers of labeled GFP-ADSCs (0, 0.5×10^6, 1×10^6, 2×10^6, and 5×10^6 cells/mL suspended in 1% agarose gel) were scanned by a 3T clinical MRI system (GE Medical Systems, Milwaukee, WI, USA) as previously described [23] to determine the number of labeled cells required to cause a sufficient decrease in the MRI signal intensity.

2.8. Cell transplantation and in vivo MRI tracking of labeled cells

The P3 GFP-ADSCs were incubated with 0 and 25 μg/mL DMSA-NPs for 24 h. After washing with PBS for 3 times, cells were trypsinized, centrifuged, and resuspended with 100 μL PBS in a 0.5 mL Eppendorf tube (containing approximately 1.0×10^6 GFP-ADSCs for each tube) at 4 °C in the dark. Ischemia to the left hindlimb was induced in male C57BL/6J mice (6–8 weeks old, $n = 24$, Shanghai SLAC Laboratory Animal Co. LTD) as described previously [24,25] to investigate whether the DMSA-NPs labeled GFP-ADSCs may have any adverse effect on the neovascularization of a mouse model of hindlimb ischemia. All animal protocols were