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In vivo real-time visualization of mesenchymal stem cells tropism for cutaneous regeneration using NIR-II fluorescence imaging

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ABSTRACT

Mesenchymal stem cells (MSCs) have shown great potential for cutaneous wound regeneration in clinical practice. However, the *in vivo* homing behavior of intravenously transplanted MSCs to the wounds is still poorly understood. In this work, fluorescence imaging with Ag_2S quantum dots (QDs) in the second near-infrared (NIR-II) window was performed to visualize the dynamic homing behavior of transplanted human mesenchymal stem cells (hMSCs) to a cutaneous wound in mice. Benefiting from the desirable spatial and temporal resolution of Ag_2S QDs-based NIR-II imaging, for the first time, the migration of hMSCs to the wound was dynamically visualized *in vivo*. By transplanting a blank collagen scaffold in the wound to help the healing, it was found that hMSCs were slowly recruited at the wound after intravenous injection and were predominantly accumulated around the edge of wound. This resulted in poor healing effects in terms of slow wound closure and thin thickness of the regenerated skin. In contrast, for the wound treated by the collagen scaffold loaded with stromal cell derived factor-1α (SDF-1α), more hMSCs were recruited at the wound within a much shorter time and were homogenously distributed across the whole wound area, which enhances the re-epithelialization, the neovascularization, and accelerates the wound healing.

1. Introduction

Poor healing of cutaneous wounds has been a significant clinical problem [1]. It has been well documented that wound healing is an intricate process in which the heterogeneous stem cell pool and their niche play important roles [2,3]. Thus, stem cell-based tissue engineering has attracted great attention as an alternative strategy to autologous and xenogenous transplantation for cutaneous regeneration [4–7]. Mesenchymal stem cells (MSCs) have been identified as an effective cell source in cutaneous wound healing because of their active roles in improving neovascularization and re-epithelialization in wounds and consequently the improved cutaneous regeneration [5,6,8,9]. The population of MSCs in the wound site, that is the local concentration of MSCs, plays a critical role in healing the cutaneous wounds. Therefore, great efforts have been paid on enriching MSCs at wound sites to accelerate tissue regeneration. Stromal cell derived factor-1α (SDF-1α) is a vital chemokine in regulating stem cell migration and homing by binding to its complementary receptor CXC chemokine receptor type4 (CXCR4). Hence, collagen scaffold in combination with SDF-1α has been developed as a desirable strategy for tissue repair, including cartilage defects repair and tendon regeneration, in which collagen scaffold provides a suitable niche for MSCs attachment and proliferation [10–12] and the chemotactic factor SDF-1α is used to recruit stem cells from surrounding [13,14]. However, limited information on the migration behavior of the MSCs in response to the SDF-1α and the pathophysiological course involved in the cutaneous wound healing was available. Therefore, a high-resolution imaging technique to monitor the temporal and spatial homing of transplanted MSCs to the wounded tissues and the dynamic healing process is urgently needed for a better understanding of the therapeutic mechanism.

Recently, it is reported that fluorescence imaging in the second near-infrared window (NIR-II, 1.0–1.4 μm) is an ideal strategy for

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in vivo imaging due to its deeper tissue penetration, higher temporal and spatial resolution in comparison with the fluorescence imaging in visible and the first near-infrared window (NIR-I, 650–950 nm) [15,16] and tomographic imaging such as magnetic resonance imaging (MRI) and positron emission tomography (PET) [17–20]. By using NIR-II emitting agents, for example, single-walled carbon nanotubes (SWNTs) and Ag_2S quantum dots (QDs), a dynamic imaging of mouse hind limb vasculatures with an ultrahigh spatial resolution of 30 μm and a temporal resolution of less than 50 ms have been unprecedentedly obtained [19,21].

In this contribution, Ag_2S QDs-based NIR-II imaging was employed to dynamically visualize the migration and distribution of human mesenchymal stem cells (hMSCs) in response to SDF-1α on the cutaneous wound site and therefore the healing effect. In order to achieve this goal, the effects of Ag_2S QDs on the proliferation and differentiation behaviors of the labeled hMSCs on the three-dimensional (3D) collagen scaffolds were firstly systematically studied. Then, *in vivo* imaging of the dynamic homing behavior of intravenously transplanted hMSCs was performed in a mouse wound healing model implanted with a blank collagen scaffold and a SDF-1α-loaded collagen scaffold, respectively, followed with comprehensive investigation on how the concentration and distribution of hMSCs on the wound site affect the healing effect. This study provides the first-hand knowledge of the temporal and spatial homing behavior of hMSCs *in vivo* in response to cutaneous wound and the external SDF-1α chemokine for cutaneous regeneration, which betters our understanding on the mechanism of stem cell-based regeneration medicine and will promote its potential clinic applications.

2. Materials and methods

2.1. Cell culture and labeling

hMSCs were obtained as a generous gift from Dr. Jianwu Dai at Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. hMSCs were cultured in DMEM/F-12 (Hyclone, UT, US) medium containing 10% fetal bovine serum (FBS) (Hyclone, UT, US), 1% streptomycin and penicillin (S-P) (Hyclone, UT, US) in 5% direct heat autoflow CO_2 incubator at 37°C. Cells were cultured with medium change every 3 days.

Tat-Ag_2S QDs were prepared according to previous description [22]. To label hMSCs with Ag_2S QDs, hMSCs were incubated in DMEM/F-12 medium containing 12.5 μg/mL Tat-Ag_2S QDs for 12 h. After incubation, hMSCs were washed twice with PBS to remove unbound Tat-Ag_2S QDs. The NIR-II photoluminescence (PL) imaging of Ag_2S QDs-labeled hMSCs were performed by using the NIR *in vivo* imaging system with an 808 nm external diode laser (Starway Laser Inc., China) as the excitation source and an InGaAs/SWIR CCD camera (Photonic Science, UK) [21].

2.2. Assessment of the cytotoxicity of Ag_2S QDs on 3D-cultured hMSCs

2.2.1. Proliferation assay

The collagen scaffolds were kind gifts from Dr. Jianwu Dai, and prepared as described previously [23,24]. Pieces of collagen scaffolds (4 × 4 mm) were placed in a 24-well culture plate, and then 5×10^3 hMSCs suspended in 15 μL medium was seeded into the scaffold. The viabilities of Ag_2S QDs-labeled and unlabeled hMSCs were measured by MTT assay after 24 h, 10, 20 and 30 days growth. To further confirm the growth and viability of hMSCs in collagen scaffold, hMSCs were stained with 4 μM calcein-AM (Sigma, MO, US) which was specific for living cells. Then the living hMSCs were imaged by a Nikon confocal laser scanning microscope (CLSM).

2.2.2. Differentiation assay

Differentiation capacities of Ag_2S QDs-labeled and unlabeled hMSCs were assessed by osteogenic and adipogenic differentiation experiments. For osteogenic differentiation assay, each scaffold (4 × 4 mm) was seeded with 5×10^3 hMSCs and cultured with osteogenic induction medium containing 10% FBS, 1% S-P, 0.1 μM dexamethasone, 0.05 mg/mL ascorbic acid and 10 mM β-glycerophosphate in DMEM/F-12 medium for 3 weeks, the induction medium was changed every 3 days. Scaffolds were stained with calcein-AM to determine the viability of hMSCs in collagen scaffold after osteogenic induction. Osteogenic differentiation was characterized by alizarin red S staining. To further quantify the osteogenic cells in collagen, alizarin red S was extracted by 10% cetylpyridinium chloride, the calcium concentrations were then quantified by measuring the absorbance at 562 nm.

For adipogenic differentiation, collagen scaffolds-cultured hMSCs were treated with adipogenic differentiation supplements containing 10% FBS, 1% S-P, 1 μM dexamethasone, 5 μg/mL insulin, 60 mM indometacin and 0.5 mM isobutylmethylxanthine in DMEM/F-12 medium for 2 weeks. Scaffolds were stained with calcein-AM and 0.36% Oil-red O for viability and adipogenic differentiation assay respectively. Quantification measurement was performed by measuring the Oil-red O at 404 nm that extracted from adipogenic cells by dimethyl sulfoxide (DMSO).

2.2.3. Expression of the stemness genes

1×10^6 Ag_2S QDs-labeled and unlabeled hMSCs were seeded into collagen scaffolds (10 × 10 mm) respectively, scaffolds were cultured with DMEM/F-12 complete medium for 24 h. Total RNA was extracted by the Total RNA Kit (Omega, US), DNaseI (Thermo, US) was used to remove the genomic DNA and total RNA was reverse transcribed by using Revert Aid First Strand cDNA Synthesis Kit (Thermo, US). The polymerase chain reaction (PCR) program of Nanog, Rex-1 was 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 47 °C for 30 s and 72 °C for 30 s, finally, 72 °C for 5 min. For Oct4, Sox2 and GAPDH, PCR amplifications include 95 °C for 5 min of denaturation, followed by 25 cycles of 95 °C for 50 s, 54 °C for 50 s, 72 °C for 50 s, then 72 °C for 10 min of extension. The primer sequences were listed in Supplementary Table S1. The PCR products were analyzed by agarose gel electrophoresis.

2.3. Ag_2S QDs on subcutaneously transplanted 3D-cultured hMSCs

Mice were purchased from Changzhou Cavens Lab Animal Co. Animal handling was carried out at the animal laboratory of Soochow University, China. All animal experiments were conducted under the guidelines approved by Soochow University Laboratory Animal Center.

hMSCs were seeded into collagen scaffolds (4 × 4 mm) and cultured with DMEM/F-12 complete medium for 24 h. After that, blank scaffolds, scaffolds seeded with Ag_2S QDs-labeled hMSCs (1×10^5 hMSCs per scaffold) or scaffolds seeded with unlabeled hMSCs (1×10^5 hMSCs per scaffold) were subcutaneously transplanted into the dorsum of three BALB/cASlac-nu mice, respectively. The NIR-II PL images of each mouse were collected at day 0, 10, and 30 after subcutaneous transplantation. All images were obtained by using the NIR *in vivo* imaging system according to previous description [21,22]. Briefly, the excitation power density was 123.8 mW/cm^2 during imaging when using the 808 nm diode laser, and the exposure time was 100 ms. Scaffolds were collected at day 0, 10, and 30 after transplantation, then sectioned and further stained with hematoxylin and eosin (HE) staining. Images were taken by using Nikon Ti Microscope.

For immunofluorescence assay, scaffolds were fixed with 4% paraformaldehyde and cut into 10 μm sections, followed by BSA blocking for 1 h at room temperature. Primary antibody was mouse anti-human nuclei (1:100, HuNu; Millipore, US), a fluorescent Cy3 conjugated goat anti-mouse IgG (1:400, Abbkine, US) second antibody was used after Hoechst staining.

2.4. In vitro migration assay of hMSCs

1×10^5 Ag_2S QDs-labeled and unlabeled hMSCs in 200 mL DMEM/F-12 medium with 2% FBS were placed in upper chambers of a 24-well transwell system (8 μm pore diameter) respectively. To induce migration, 120 ng SDF-1α in 1 mL DMEM/F-12 medium with 2% FBS was added to lower chamber, and negative control was treated without SDF-1α. After 24 h incubation at 37 °C, the upper surface of the filter was scraped, migrated cells were stained with crystal violet. The number of migrated cells were counted in 5 freedom selected filed (100×). Quantification of average cell numbers per high power filed was performed by 3 independent experiments.

2.5. Dermal regeneration assay

Six weeks old female nude mice (BALB/cASlac-nu) were anesthesia by 10% chloral hydrate. Two 5-mm full-thickness skin wounds were excised on each side of the dorsum. Collagen fragments were soaked with 400 ng human recombinant SDF-1α (BioVision, US) and then filled in the left wound site (n = 3), the collagen fragments that without SDF-1α were filled in the right wound site (n = 3). Matrigel (BD, NJ, US) was injected into the wounds site to ensure the materials exchange, and Tegaderm (3M, MN, US) was used to wrap the wounds. After one day, 1×10^6 Ag_2S QDs-labeled hMSCs were intravenously injected via tail vein, PL emission signals were collected by NIR *in vivo* imaging system with a power intensity of 123.8 mW/cm^2 and 100 ms exposure time. The wound sizes were measured by vernier caliper and the healing degrees were analyzed every two days. Tissues at wounds site were sampled at day 4 and day 10, HE staining and immunohistochemical analysis were performed to determine the re-epithelialization and vascular endothelial growth factor (VEGF) expression respectively.

2.6. Statistical analysis

Numerical values are expressed as the mean ± SD. Each experiment was repeated three times. Statistical significance was evaluated using ANOVA analysis; p-values <0.05 were considered to be statistically significant.