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The promotion of human mesenchymal stem cell proliferation by superparamagnetic iron oxide nanoparticles

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ABSTRACT

Superparamagnetic iron oxide (SPIO) nanoparticles are very useful in cell imaging; meanwhile, however, biosafety concerns associated with their use, especially on therapeutic stem cells, have arisen. Most studies of biosafety issues focus on whether the nanoparticles have deleterious effects. Here, we report that Ferucarbotran, an ionic SPIO, is not toxic to human mesenchymal stem cells (hMSCs) under the conditions of these experiments but instead increases cell growth. Ferucarbotran-promoted cell growth is due to its ability to diminish intracellular H_2O_2 through intrinsic peroxidase-like activity. Also, Ferucarbotran can accelerate cell cycle progression, which may be mediated by the free iron (Fe) released from lysosomal degradation and involves the alteration of Fe on the expression of the protein regulators of the cell cycle.

1. Introduction

Stem cell therapy is a rapidly evolving area of research in regenerative medicine. Being able to monitor the fate of transplanted cells in vivo is crucial for developing successful cell therapies. Furthermore, an effective, noninvasive, nontoxic method is required for ideal cell tracking. Recently, magnetic resonance imaging (MRI) has afforded the superb spatial resolution and repeated noninvasive imaging of magnetically labeled cells and has been the most attractive modality for cellular imaging [1,2]. For MRI detection, however, the cells need to be internally labeled with contrast agents. Of the two main classes of contrast agents, superparamagnetic iron oxide (SPIO) nanoparticles and gadolinium-based chelates, most approaches are with the usage of the former class as cellular MRI probe. SPIO nanoparticles are commercially available and already FDA-approved for use in humans [3,4], but not yet in intracellular labeling. To achieve sufficiently intracellular uptake of SPIO nanoparticles for efficient MRI, most studies use transfection agents [3–6]; most of these agents, however, are cationic and toxic [7,8].

Regardless of the toxicity of transfection agents, the potential effects of SPIO nanoparticles on stem cells remain vitally important. Although it is believed that SPIO nanoparticles are inert, biocompatible nanomaterials capable of being eventually metabolized, the term "biocompatible" can be misleading due to a lack of criteria for evaluating the toxic effects of nanomaterials. Furthermore, the cytotoxicity of SPIO nanoparticles remains an unresolved issue [9–16]. A recent study showed that Resovist (Ferucarbotran) without using a transfection agent was nontoxic and more efficiently taken up by human mesenchymal stem cells (hMSCs) than Feridex (Ferumoxides) [17]. Therefore, Ferucarbotran would be an ideal candidate for studying the precise action of SPIO nanoparticles on cellular responses.

In view of the present biocompatibility definition of nanoparticles, most studies have focused on whether cytotoxicity was observed [7–17]. In this study, however, we examined the cellular effects of Ferucarbotran labeling in different views on hMSCs to

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explore the biosafety of SPIO nanoparticles for cellular MRI. The recent finding that magnetic nanoparticles possess intrinsic peroxidase-like activity [18] and the important role of H_2O_2 in cell growth led us to investigate whether SPIO nanoparticles can affect cell growth by decreasing intracellular H_2O_2.

In addition, after the internalization into cells, SPIO nanoparticles could be transferred to lysosomes, in which degradation of SPIO nanoparticles may occur and free iron (Fe) could be released into the cytoplasm [19,20]. It is well known that Fe plays an important role in cell cycle progression, cell proliferation and apoptosis. Therefore, we also examined the effects of Ferucarbotran labeling on the cell cycle proceeding and the expression of several proteins critical for cell cycle progression.

2. Materials and methods

2.1. Culture of hMSCs from bone marrow

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow of normal donors. The bone marrow aspirate was added to low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 25 U/ml heparin in 1:1 ratio, fractionated by Ficoll-Paque density gradient centrifugation. The hMSC-enriched low-density fraction was collected, rinsed with DMEM, and plated in T25 flasks at 5×10^7 nucleated cells per flask in 5 ml regular growth medium consisting of low-glucose DMEM supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich). During the first two-week incubation for cell adherence and initial expansion, 5 ml of fresh growth medium was added twice weekly for the first week. And that, medium changes were carried out twice weekly. When adherent cells reached ~60% to 70% confluence, they were detached with 0.25% trypsin–EDTA (ethylenediaminetetraacetic acid; Gibco) and replated at 1:3 in regular growth medium to allow for continued passaging. All cultures were kept in atmosphere of 5% CO_2, 95% air at 37 °C.

2.2. Antibodies

Mouse anti-human cyclin A (SC-239), cyclin B1 (SC-245), cyclin D1 (SC-8396), cyclin E (SC-247) and CDK2 (SC-6248) monoclonal antibodies, rabbit anti-human p21 (SC-756) and p27 (SC-528) polyclonal antibodies, and goat anti-human pRb (SC-16670) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at a dilution 1:200. Rabbit anti-human CDK4 (SC-601) polyclonal antibody was from Santa Cruz and was used at a dilution 1:1000. Mouse anti-human p53 (610183; used at a dilution 1:200) was from BD Biosciences. Mouse anti-actin monoclonal antibody (MAB1501; used at a dilution 1:1000) was from Chemicon.

2.3. MRI in vitro and in vivo

MRI was performed using a clinical 1.5-T MR system (Signa Excite, GE Healthcare, USA). hMSCs were incubated with various concentrations of Ferucarbotran (Schering AG, Berlin, Germany) in serum-free medium for 1 h; the cells were then collected by trypsinization, centrifuged, and placed in a water tank. Next, the tank was placed in an 8 channel head coil. Gradient echo pulse sequences provided by the vendor were used (TR/TE = 550/15 ms, FA = 15, matrix size = 256 × 192). The slice thickness was 1.4 mm with a 0.01 mm gap; and the field of view (FOV) was 14 × 7 cm. Total scan time was 2 min 42 s at the NEX of 3. The images were then analyzed at the workstation provided by GE healthcare.

Male NOD/SCID mice (8 weeks of age) were obtained from the National Laboratory Animal Center, Taiwan, and maintained in accordance with the Institutional Animal Care and Use Committee's procedures and guidelines. hMSCs were treated with Ferucarbotran for 1 h at the concentration of 100 μg/ml, harvested by trypsinization, and suspended in PBS at 1×10^7 cells/ml. The mice were anesthetized by injecting 100 mg/kg ketamine mixed with 5 mg/kg xylazine into intraperitoneal space. Stereotaxic injection of hMSCs was performed with a Narishige apparatus and a WPI syringe with a bevel-tipped 26.5-gauge needle. Ferucarbotran-labeled hMSCs (1.2×10^5) were infused in 5 μl PBS. The injection coordinates were (relative to bregma [AP], the midline [ML], and the dura [DV]): AP: −2.5 mm, ML: +1.0 mm, and DV: −2.0 mm. Injections were performed at the rate of 1 μl/min, and the needle was left in place for 5 min before withdrawal. After 7 and 21 days of hMSC implantation, animal MR imaging studies were carried on under isoflurane gas anaesthesia (Forane, Abbott). The animal was then scanned under the same clinical 1.5-T MR system. A custom-made radiofrequency (RF) coil for RF excitation and signal reception was used. The RF coil was made as a cylindrical volume resonator with an inner diameter of 38 mm and length of 82 mm. Gradient echo (GRE) pulse sequences were used (TR/TE = 550/25 ms, echo chain = 1/1, bandwidth = 15.6 kHz, matrix size = 288 × 192). The slice thickness was 1.4 mm. The scan time was 4 min 1 s in coronal plane. The images were then analyzed at the workstation provided by the vendor.

2.4. Cellular effects of SPIO labeling on hMSCs

The cellular effects of SPIO labeling were determined as follows: MTT reduction, trypan blue dye exclusion, and SRB assays.

After incubation with various concentrations of Ferucarbotran in serum-free medium for 1 h, cells were incubated with fresh serum-free medium containing 0.5 mg/ml of MTT for 1 h at 37 °C for acute cytotoxicity assay. For growth assay, the cells after treatment with Ferucarbotran for 1 h were allowed to grow in regular growth medium for 24 h, followed by incubation with MTT reagent or by haemocytometer counts of trypan blue-excluding cells.

In SRB assay, cells were inoculated into 24-well microtiter plates in DMEM containing 15% FBS and maintained in a humidified incubator at 37 °C in 5% CO_2/95% air. After 24 h, one plate of cells was fixed with trichloroacetic acid to represent a measurement at the time of Ferucarbotran labeling (T_0). Additionally, various concentrations of Ferucarbotran were added to the cells of the other plates in serum-free medium for 1 h and then incubated with 15% FBS-containing DMEM for an additional 24 or 48 h. The assay was terminated by the addition of cold trichloroacetic acid. After rinsing three times with tap water, SRB solution at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. The unbound dye was removed by washing three times with 1% acetic acid, and the plates were air-dried. Bound SRB was subsequently solubilized with 10 mM trizma base, and the absorbance was read at a wavelength of 515 nm. Using such absorbance measurements as time zero (T_0), control growth (C), and cell growth in the presence of Ferucarbotran labeling (T_x), the percentage growth was calculated at each of the Ferucarbotran concentration levels as: $[(T_x - T_0)/(C - T_0)] \times 100$ for concentrations for which $T_x \geq T_0$.

2.5. In vitro peroxidase-like activity assay

The peroxidase-like activity assays were carried out in 1.5-ml tubes with various concentrations of Ferucarbotran or 0.5 ng HRP for positive control in 500 μl of reaction buffer (0.2 M NaAc, pH 3.5) in the presence of 530 mM H_2O_2 for Ferucarbotran or 8.8 mM for HRP, using 816 μM TMB as the substrate. Immediately after the tubes were incubated at 40 °C, color reactions were observed; the reactions were then stopped by adding 200 μl of 0.5 M H_2SO_4.

Also, the peroxidase-like activity was determined with the reaction mixture of 75 mg ammonium nickel sulfate in 2.5 ml of 0.1 M NaAc (pH 6.0), 50 μl of DAB (50 mg/ml) in 2.5 ml of low salt tris-base buffer, and 1.76 mM H_2O_2. Each assay was carried out in 500 μl of the reaction mixture with 40 μg Ferucarbotran or 5 ng HRP at room temperature.

2.6. Determination of intracellular H_2O_2

For measurement of intracellular H_2O_2 level, hMSCs after treatment with the indicated concentrations of Ferucarbotran for 1 h were immediately incubated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) at 37 °C in the dark for 30 min. Thereafter the cells were washed twice and resuspended in PBS; the fluorescence intensity was detected by FACSCalibur flow cytometry and CellQuest Pro software (Becton Dickinson, Mississauga, CA). The mean fluorescence intensity of Ferucarbotran-labeled cells was normalized to that of unlabeled cells as control.

2.7. Flow cytometric analysis of cell cycle distribution

hMSCs after treatment with Ferucarbotran (300 μg/ml) for 1 h were allowed to grow in regular growth medium. After 2, 4, 6, and 24 h of growth, cells were harvested by trypsinization, fixed in 70% ethanol on ice for 1 h, and washed once with PBS. After centrifugation, the cells were incubated at room temperature in the dark for 30 min in 0.5 ml hypotonic solution containing 50 μg/ml PI, 0.1% Triton X-100, 0.1% sodium citrate solution, and 100 μg/ml RNase. The DNA content was analyzed using a FACSCalibur flow cytometer. The mean of counting cells in S and G_2/M phases of Ferucarbotran-labeled cells was normalized to that of unlabeled cells as control.

2.8. Preparation of whole cell extracts and Western blot analysis

After treatment with Ferucarbotran (300 μg/ml), cells were washed twice with ice-cold PBS and then collected by trypsinization followed by centrifugation. The cell pellet was resuspended in lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin. After incubation for 30 min on ice, cell homogenates were centrifuged at 13,000 rpm for 30 min at 4 °C. The protein concentration of the supernatant was assessed by the Bio-Rad protein assay kit.

Proteins (50–100 μg) were separated by electrophoresis in a 6–15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After incubation at room temperature in PBS plus 3% bovine serum albumin (BSA) for 1 h, the membrane was washed three times with PBS plus 0.5% Tween 20 (PBST).

Antibodies were added to PBST containing 1% BSA and incubated with the membranes at 4 °C overnight. Membranes were then washed 3 times in PBST, for 10 min each time. After washing, horseradish peroxidase (HRP)-conjugated anti-