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Magnetic targeting of cardiosphere-derived stem cells with ferumoxytol nanoparticles for treating rats with myocardial infarction

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ABSTRACT

Stem cell transplantation is a promising therapeutic strategy for acute or chronic ischemic cardiomyopathy. A major limitation to efficacy in cell transplantation is the low efficiency of retention and engraftment, due at least in part to significant early "wash-out" of cells from coronary blood flow and heart contraction. We sought to enhance cell retention and engraftment by magnetic targeting. Human cardiosphere-derived stem cells (hCDCs) were labeled with FDA-approved ferumoxytol nanoparticles Feraheme® (F) in the presence of heparin (H) and protamine (P). FHP labeling is nontoxic to hCDCs. FHP-labeled rat CDCs (FHP-rCDCs) were intracoronarily infused into syngeneic rats, with and without magnetic targeting. Magnetic resonance imaging, fluorescence imaging, and quantitative PCR revealed magnetic targeting increased cardiac retention of transplanted FHP-rCDCs. Neither infusion of FHP-rCDCs nor magnetic targeting exacerbated cardiac inflammation or caused iron overload. The augmentation of acute cell retention translated into more attenuated left ventricular remodeling and greater therapeutic benefit (ejection fraction) 3 weeks after treatment. Histology revealed enhanced cell engraftment and angiogenesis in hearts from the magnetic targeting group. FHP labeling is safe to cardiac stem cells and facilitates magnetically-targeted stem cell delivery into the heart which leads to augmented cell engraftment and therapeutic benefit.

1. Introduction

Most families in the United States are impacted by cardiovascular disease. Cardiovascular disease remains the leading cause of death and disability in Americans. On average, cardiovascular disease kills one American every 37 s. Death rates have improved, but new treatments are urgently needed. Stem cell transplantation is a promising therapeutic strategy for acute or chronic ischemic cardiomyopathy [1]. A major limitation in cell transplantation is the low efficiency of retention and engraftment [2]. Acute (<24 h) cell retention in the heart is usually <10%, regardless of the cell type and delivery route [3]. Long-term cell engraftment is even lower. Coronary blood flow and heart contraction account for significant early "wash-out" of cells immediately after transplantation [2]. We hypothesize that physical approaches that reduce wash-out will cause cells to linger sufficiently to enable biological integration, thereby enhancing cell retention and augmenting the functional benefits of cell therapy.

Ours and others' work have shown that stem cells can be loaded with superparamagnetic iron oxide nanoparticles (SPIONs) and magnetically-guided cell delivery into the heart can boost short-term cell retention, long-term engraftment and function benefit of the cell therapy [4–8]. Magnetic targeting has also been studied for efficient homing of SPIONs-labeled endothelial cells to stents [9]. Following the success of magnetic cell delivery in various animal models, translation into clinical applications would be markedly facilitated by the use of FDA-approved SPIONs. The anemia drug Feraheme® (ferumoxytol), as the only FDA-approved SPIONs currently available in the US market, becomes a logical choice for

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stem cell-labeling. Previous work indicates multiple stem cell types can be loaded with Feraheme particles in the presence of heparin and protamine [10]. However, this particle has not been extensively studied for cardiac stem cell-labeling or for magnetic targeting applications. Moreover, the cytotoxicity of this particle for magnetically-targeted stem cell therapy is largely unknown. Our group has been studying cardiosphere-derived stem cells (CDCs) for the last 5 years. CDCs are a natural mixture of cardiac stem cells and supporting cell types. The potency of CDCs for cardiac regeneration after myocardial infarction has been concurred in numerous animal studies as well as in a recently completed clinical trial [11].

The purpose of this study is to report on the effects of ferumoxytol labeling on human and rat CDCs and the feasibility of using ferumoxytol-labeled CDCs for magnetically-targeted cell delivery into the rat heart. Given that both ferumoxytol and CDCs are already in human use, the methodology tested here offers the potential for rapid clinical translation.

2. Materials and methods

2.1. Human and rat CDC culture

Human CDCs were generated and expanded as previously described from human myocardial tissues [12]. Rat CDCs were obtained from myocardial tissues from male syngeneic Wistar-Kyoto rats [4]. Unless otherwise noted, IMDM basic medium (Gibco) supplemented with 10% FBS (Hyclone) and 20 mg/ml gentamycin was used to culture all CDCs. Passage 2 CDCs were used for all *in vitro* and *in vivo* experiments. To facilitate fluorescent imaging and histological detection, a group of rat CDCs were also transduced with red fluorescent protein (RFP) lentiviral particles.

2.2. Ferumoxytol loading into CDCs

We followed a ferumoxytol loading method developed by the Frank group at NIH [10]. Briefly, ferumoxytol ("F", Feraheme®, AMAG Pharmaceuticals; Lexington, MA), heparin sulfate ("H", 1000 IU/ml, Hospira; Lake Forest, IL) and protamine sulfate ("P", 10 mg/ml, APP Pharmaceuticals; Schaumburg, IL) were used to form FHP nanocomplexes. The FHP nanocomplexes were prepared by sequentially adding F at 50 μg/ml, H at 2 IU/ml, P at 60 μg/ml and in serum free IMDM media for cell culture studies. Human or rat CDCs were grown to >75% confluence. FHP nanocomplexes dissolved in serum-free media were added to the cells in culture and incubated for 12 h. Following incubation in serum free media, an equal amount of complete media containing 20% Fetal Bovine Serum (FBS) was then added and the cells were incubated overnight for each cell type. Cells were washed with Phosphate-Buffered Saline (PBS) (Invitrogen) to remove the residual FHP particles. For brevity, the FHP nanocomplex-labeled human CDCs and rat CDCs were hereafter referred as FHP-hCDCs and FHP-rCDCs, respectively.

2.3. Prussian blue staining

For Prussian blue staining [4], cells or tissue sections were fixed in 2% glutaraldehyde for 10 min at 4 °C, and then immersed in 1% potassium ferrocyanide and 3% HCl solution (Sigma) for 30 min. After several washes with D.I. water, cell nuclei were counter-stained with nuclear fast red (Sigma) for 5 min. Then the cells were washed again with D.I. water twice. After dehydration with methanol (3 washes, 70% once and 100% twice) and xylene, the slides were finally mounted in DPX mounting media (Sigma) before observation.

2.4. Transmission electron microscopy

In preparation for TEM, cells were spun down, media removed, and resuspended in 4% formaldehyde and 1% gluteraldehyde fixative (4F:1G) and stored at 4 °C. At a later date, the samples were stained following the procedure in Ref. [13]. Some samples were not post-stained to allow for easier detection of FHP nanoparticles. Sections of cell samples were imaged using a FEI/Philips EM 208S/Morgagni transmission electron microscope at the NCSU Laboratory for Advanced Electron and Light Optical Methods (LAELOM).

2.5. In vitro cytotoxicity assay

In vitro cytotoxicity assays were performed using FHP-hCDCs and control (non-labeled) hCDCs. Cell viability was assessed by Trypan blue exclusion. To evaluate apoptosis, cells were fixed, and apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The proliferation rates of FHP-CDCs and control CDCs were assessed with by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Rockville, MD), and cell cycling was evaluated by immunocytochemistry staining of Ki67 (cell proliferation marker). Cell migration was evaluated with a trans-well cell migration assay plate (BD Biosciences) [14]. Reactive oxygen species (ROS) generation was measured with an Image-IT® LIVE Green Reactive Oxygen Species Detection Kit (Life Technologies) [4]. As positive controls for the TUNEL and ROS assay, human CDCs were incubated with 100 μM H_2O_2 in the medium for 24 h to induce cell apoptosis and ROS generation.

2.6. Microarray gene expression analysis

Total RNA was isolated from FHP-hCDCs (3 days after FHP loading) and control hCDCs using Qiagen RNeasy Kits (Qiagen) according to the manufacturer's standard protocol. The quantity of mRNA isolated from each sample was determined using the adsorption of each solution at 260 nm and 280 nm. The purity of each sample was monitored using the A260/A280 ratio. Samples then underwent Human Whole Genome U133A 2.0 Plus GeneChip Expression Analysis (Affymetrix). The raw fluorescence data was normalized using Affymetrix Expression Console. The fold changes and *p*-values were calculated using Affymetrix Transcriptome Analysis Console. This data was then organized using R software and plotted with the ggPlot2 package. Thresholds were set at a linear fold change ≥ 2 or ≤ 2, and *p*-value ≤ 0.05. Any genes contained in the microarray data that did not include a gene name were excluded from analysis. Genes exceeding both thresholds were considered to have significantly changes in expression.

2.7. Differentiation assay

FHP-hCDCs and control hCDCs were cultured in cardio-differentiation media (Millipore) or vascular differentiation media (ATCC) for 14 days for cardiac and endothelial differentiation, respectively. Cells were then fixed with 4% PFA, blocked/permeabilized with Protein Block Solution (DAKO, Carpinteria, CA) containing 1% saponin (Sigma–Aldrich), and then stained with the following antibodies: rabbit anti-sarcomeric actin (Sigma), rabbit anti-von Willebrand factor (Abcam), rabbit anti-ckit (Sigma–Aldrich), and chicken anti-CD105 (endoglin, Sigma) antibodies. FITC, or Texas-Red secondary antibodies was obtained from Abcam. Cell nuclei were counter-stained with DAPI. Images were taken by a Zeiss LSM710 laser scanning confocal microscopy system.

2.8. Finite element analysis of the NdFeB magnet

The magnetic field simulation was performed using the open-source software Finite Element Method Magnetics (http://www.femm.info/wiki/HomePage). Finite element analysis was performed on the 1.3 T NbFeB magnet (Edmund Scientifics, Tonawanda, NY; diameter = 9.5 mm; height = 5 mm) to be used in subsequent rat studies. A density plot of the field lines was produced to show the shape of the field. The field magnitude along the *z*-axis directly above the magnet was calculated as well to show the decay of the field with distance.

2.9. Rat model of ischemia/reperfusion

Animal care was in accordance to the Institutional Animal Care and Use Committee (IACUC) guidelines. A rat ischemia/reperfusion model was used [5]. Female WKY rats (Charles River Laboratories) ($n = 58$ total) underwent left thoracotomy in the 4th intercostal space under general anesthesia. The heart was exposed and myocardial infarction was produced by 45 min ligation of the left anterior descending coronary artery, using a 7-0 silk suture. The suture was then released to allow coronary reperfusion. Intracoronary injection was achieved by injection into the left ventricle cavity during a 25 s temporary aorta occlusion with a looped suture. Animals were randomized into three treatment groups: 1) Control, intracoronary injection of 200 μL PBS; 2) FHP-CDC, intracoronary injection of 500,000 FHP-labeled syngeneic CDCs in 200 μL PBS; 3) FHP-CDC + Magnet, intracoronary injection of 500,000 FHP-labeled syngeneic CDCs in 200 μL PBS with a superimposed 1.3 T circular NdFeB magnet during and after the cell injection for 10 min [4]. The chest was closed and the animal was allowed to recover after all procedures.

2.10. Magnetic resonance imaging (MRI)

24 h after injection, magnetic resonance imaging (MRI) of the heart was accomplished by a gradient echo sequence to produce a T_2^* weighting with a Bruker Biospec 9.4T small animal MRI system. A 60 mm diameter linear transmit/receive coil was utilized for excitation and a 4 channel array coil designed for rat heart imaging was used for improved signal-to-noise data acquisition. Retrospective gating was accomplished with Bruker IntraGate® software to produce 10 cine images for each slice. In addition, one coronal cine image was acquired which dissected the heart to verify the presence of iron particles.

2.11. Fluorescence imaging (FLI)

24 h after cell injection, the hearts and lungs were excised, washed with PBS, and placed in a Xenogen IVIS imaging system (Caliper Life Sciences, Mountain View, CA) to detect RFP fluorescence. Excitation was set at 550 nm and emission was set at 580 nm. Exposure time was set at 5 s and kept the same during the entire imaging session.