Biomaterials 32 (2011) 5558–5567

Contents lists available at ScienceDirect

Biomaterials

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

Enhancement of cell retention and functional benefits in myocardial infarction using human amniotic-fluid stem-cell bodies enriched with endogenous ECM

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ARTICLE INFO

Article history:
Received 17 February 2011
Accepted 12 April 2011
Available online 8 May 2011

Keywords:
Methylcellulose hydrogel
Cell body
Cell therapy
Tissue regeneration
Myocardial infarction

ABSTRACT

Stem cell transplantation may repair the infarcted heart. Despite the encouraging preliminary results, an optimal cell type used and low retention of the transplanted cells remain to be overcome. In this study, a multiwelled methylcellulose hydrogel system was used to cultivate human amniotic-fluid stem cells (hAFSCs) to form spherically symmetric cell bodies for cellular cardiomyoplasty. The grown hAFSC bodies enriched with extracellular matrices (ECM) were xenogenically transplanted in the peri-infarct area of an immune-suppressed rat, via direct intramyocardial injection. Results of bioluminescence imaging and real-time PCR revealed that hAFSC bodies could considerably enhance cell retention and engraftment in short-term and long-term observations, when compared with dissociated hAFSCs. Echocardiography and magnetic resonance imaging showed that the enhanced cell engraftment in the hAFSC-body group could significantly attenuate the progression of heart failure, improve the global function, and increase the regional wall motion. At the infarct, expressions of HGF, bFGF and VEGF were significantly up-regulated, an indication of the significantly increased vessel densities in the hearts treated with hAFSC bodies. The injected hAFSC bodies could undergo differentiation into angiogenic and cardiomyogenic lineages and contribute to functional benefits by direct regeneration. The aforementioned results demonstrate that hAFSC bodies can attenuate cell loss after intramuscular injection by providing an adequate physical size and offering an enriched ECM environment to retain the transplanted cells in the myocardium, thus improving heart function.

1. Introduction

Stem cell transplantation is a promising therapeutic strategy for ischemic heart diseases [1,2]. Several studies have shown that cell therapy administered after ischemia can augment the recovery of cardiac function in animal models [3–6]. Despite the encouraging preliminary results, an optimal cell type used and low retention of the transplanted cells remain to be overcome [7,8]. Autologous stem cells such as induced pluripotent stem (iPS) cells and bone marrow mesenchymal stem cells (MSCs) have been used for cellular cardiomyopalsty [6,9]. Although these stem cells have functional benefits for cardiac repair, iPS cells have the potential risk of teratoma formation [9], while MSCs are low efficient for cardiomyogenic differentiation [10].

Human amniotic-fluid stem cells (hAFSCs) have been isolated in recent years [11–13]. They are obtained from amniocentesis samples and thus raise no ethical controversy. hAFSCs express embryonic as well as adult stem cell markers; they expand rapidly without feeders and are not tumorigenic [12,14]. Our previous study has shown that when cultured in EGM-2 medium or cocultured with rat neonatal cardiomyocytes, hAFSCs could be differentiated into endothelial and cardiomyogenic lineages and therefore may be an appropriate cell source for cardiac regenerative therapy [15].

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doi:10.1016/j.biomaterials.2011.04.031

Low retention of transplanted cells has been reported as a major obstacle in accomplishing functional benefits in cellular cardiomyoplasty [8,16]. Typical cell transplantation involves the administration of dissociated cells by direct injection into the myocardium, in which a large portion of transplanted cells are either washed away by local bleeding or squeezed out from the injected sites by cardiac contracting [17,18]. Different approaches, including magnetizing cells with an external magnetic field [17] and using biodegradable scaffolds as vehicles [19,20], have been reported to improve cell retention. Although magnetic control can enhance cell retention, its long-term engraftment was promoted to approximately 2% only. On the other hand, scaffold biodegradation results in inflammation and fibrous tissues in ischemic heart diseases and dilated cardiomyopathy [21].

In the present study, a multiwelled methylcellulose (MC) hydrogel system was used to cultivate hAFSCs to form spherically symmetric cell bodies with a relatively uniform size for cellular cardiomyoplasty. We hypothesize that the use of cultivated hAFSC bodies enriched with endogenous extracellular matrices (ECM) and adhesion molecules may enhance cell retention and be beneficial for cell therapy. Transplantation of hAFSC bodies, via direct intramyocardial injection, to induce tissue regeneration and improve cardiac function in a rat model with infarcted myocardium was investigated. The dissociated hAFSCs obtained by traditional trypsinization were used as a control.

2. Materials and methods

2.1. Isolation, characterization and transfection of hAFSCs

hAFSCs were isolated from second-trimester amniotic fluid according to our previous report [11]. The protocol was approved by the Institutional Review Board (IRB ref. no. CT9558) of Cathay General Hospital, Taipei, Taiwan. hAFSCs were positive for SSEA-4, Nanog, CD44, CD73, CD90, CD105, CD166 and slightly positive for Oct-4, CD29, while negative for CD31, CD34, CD45 and CD117 [15].

To develop an imaging assay for tracking transplanted cells, hAFSCs were transfected overnight with pooled viral supernatant generated by transfection of 293 T cells with the lentiviral vector pLKO_AS2.neo carrying a cytomegalovirus (CMV) promoter driving the expression of a firefly luciferase together with the packaging vector pCMVΔR8.91 and envelope vector pMD.G. Cells were washed and incubated in fresh medium for 48 h with G418 (1 mg/ml), to select stable Fluc expressing hAFSCs.

2.2. Construction and characterization of hAFSC bodies

Preparation of the multiwelled MC hydrogel system was reported previously by our group [22]. hAFSCs were dissociated from culture dishes with 0.05% trypsin and then seeded in the prepared multiwelled hydrogel system at different cell densities (3×10^3, 5×10^3, 7×10^3 and 1×10^4 cells/well) and cultivated for 24 h to form cell bodies. Photomicrographs of hAFSC bodies grown in the multiwelled hydrogel system were taken and their diameters were measured ($n = 10$ batches), using a computer-based image analysis system (Image-Pro® Plus, Media Cybernetics, Silver Spring, MD, USA).

The collected hAFSC bodies were fixed and stained with a monoclonal antibody against SSEA-4, Nanog, Oct-4, vimentin, fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), collagen III (Sigma–Aldrich, St Louis, MO, USA) or laminin (Abcam, Cambridge, MA, USA). Different Alexa Fluor secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used to obtain fluorescent colors. Cell bodies were costained to visualize F-actins and nuclei by phalloidin (Alexa Fluor 488 phalloidin, Invitrogen) and propidium iodide (PI, Sigma–Aldrich), respectively, and examined using an inverted confocal laser scanning microscope (TCS SL, Leica, Germany).

2.3. Cell transplantation

Animal care and use was performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council and published by the National Academy Press in 1996, and approved by the IRB of Veterans General Hospital (Taichung, Taiwan). Acute myocardial infarction (MI) was created in male Lewis rats weighing 250–300 g [15]. Animals meeting the echocardiographic inclusion criterion [fractional shortening (FS) < 35%] were injected intramyocardially with saline, dissociated hAFSCs (using a 27 G needle) or hAFSC bodies (using a 26 G needle, 3×10^6 cells per rat) at the border zone of the infarct area. To prevent graft rejection, the rats receiving cell transplantation were treated with cyclosporine A (10 mg/kg/day) via the intramuscular route, starting 3 days before treatment.

2.4. Bioluminescence imaging (BLI)

hAFSCs were seeded into a 6-well plate at different concentrations, and D-luciferin (Caliper, Hopkinton, MA, USA) was added at 1 μg/ml. Bioluminescent signals expressed as photons per second per square centimeter per steradian were measured using a Xenogen IVIS 200 system (Caliper). *In vivo* BLI images were taken in rats on days 1, 7, 14 or 28 after cell transplantation ($n = 5$ per group). Ten min after the intraperitoneal injection of D-luciferin (150 mg/kg), rats were scanned for 1–5 min using the IVIS system. Bioluminescence intensities from regions of interesting were evaluated using the Living Image 3.1 software (Caliper).

2.5. Echocardiography

Echocardiography was performed at baseline (5 days after MI) and at 4 weeks after cell transplantation with a SONOS 5500 system equipped with a 12-MHz broadband sector transducer (Hewlett–Packard, Palo Alto, CA, USA). Rats ($n = 10$ per group) were anesthetized using 2% isoflurane with oxygen. The left ventricular end-systolic dimension (LVESD) and end-diastolic dimension (LVEDD) were obtained from M-mode tracings at the midpapillary level. The left ventricular fractional shortening (LVFS) was calculated as follows: $\text{LVFS}\,(\%) = [(\text{LVEDD} - \text{LVESD})/\text{LVEDD}] \times 100\%$.

2.6. Magnetic resonance imaging (MRI)

Four weeks after cell transplantation, cardiac MRI was performed using a 7 T small-animal scanner (ClinScan, Bruker BioSpin, Billercia, MA, USA) with a phased array ^{1}H rat heart coil ($n = 5$ per group). The electrocardiographic (ECG) gating was optimized with two cardiogram electrodes attached to the animal's forelimbs with respiratory motion and body temperature monitors (Small Animal Instruments, Stony Brook, NY, USA). LV functions were evaluated using an ECG-triggered cine sequence (TE 1.67 ms, TR 5.5 ms, flip angle 15°, field of view 45 mm × 45 mm, matrix 125 × 192, slice gap 0 mm, slice thickness 1.0 mm, number of excitation: 8, and 12 cardiac phases). A total of 8 contiguous short-axis slices were acquired from apex to base. Data were analyzed using the Syngo MR software (Siemens). The LV ejection fraction (LVEF) was calculated by tracing the endocardial and epicardial borders at end-systole and end-diastole. The LV wall thickening fraction was calculated by the equation [(diastolic wall thickness − systolic wall thickness)/diastolic wall thickness] × 100%.

2.7. Morphometric and histological analyses

LV myocardium specimens of each studied group were retrieved at 4 weeks post-transplantation ($n = 5$ per group). The hearts were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin and cut into 4 slices (~2 mm thick) from the apex. Sections were prepared every 100 μm (5 μm in thickness) and stained with Masson's Trichrome. Images were acquired and analyzed by Image-Pro® Plus. From Masson's trichrome-stained images, morphometric parameters including LV cavity area, total LV area, risk region, scar area, and LV wall thickness in the infarct and non-infarcted regions were measured in each section [23]. To quantify the degrees of LV dilation and infarct-wall thinning, the LV expansion index was calculated: (LV cavity area/total LV area) × (non-infarcted region wall thickness/risk region wall thickness) [23]. The percentage of viable myocardium as a fraction of the risk region was quantified [17].

For vessel density analysis, additional sections were stained for endothelial cells with an immunohistological technique with a rabbit anti von Willebrand factor (vWF, Dako, Denmark) or for smooth muscle cells (SMCs) with a rabbit anti smooth muscle actin (SMA, Abcam). The capillary and arteriole densities at the peri-infarct and infarct regions were then quantified [15]. For immunofluorescent staining, the primary antibodies used were mouse anti human nuclei antibody (HNA, Millipore, Billerica, MA, USA), rabbit anti-vWF (Dako), rabbit anti-SMA, mouse anti α-sarcomeric actin IgM and rabbit anti cardiac Troponin I (cTnI, Abcam). Appropriate Alexa Fluor conjugated secondary antibodies were applied to obtain the fluorescent colors. The stained sections were counterstained to visualize nuclei by Sytox blue (Invitrogen) or PI [15].

2.8. Real-time polymerase chain reaction (real-time PCR)

For quantification of engraftment, real-time PCR was performed at 24 h and 4 weeks post cell transplantation ($n = 5$ per group) using the 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA) in triplicate for each sample. Genomic DNA was extracted from the LV free wall using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA). Human Alu primers and TaqMan probe (Applied Biosystems) used were as follows: forward: 5′-GAG ATC GAG ACC ATC CCG GCT AAA-3′; reverse: 5′-CTC AGC CTC CCA AGT AGC TG-3′; TaqMan probe: 5′-GGG CGT AGT GGC GGG-3′ [24]. For each reaction, 250 ng of template DNA was used, and the PCR condition used was previously described [24]. A calibration curve was derived with multiple dilutions of genomic DNA isolated from hAFSCs and rat hearts for the calculation of hAFSC/total DNA ratio. Assuming a value of 5 pg per cell [3], the number