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Generation of human secondary cardiospheres as a potent cell processing strategy for cell-based cardiac repair

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ABSTRACT

Cell therapy is a promising approach for repairing damaged heart. However, there are large rooms to be improved in therapeutic efficacy. We cultured a small quantity (5–10 mg) of heart biopsy tissues from 16 patients who received heart transplantation. We produced primary and secondary cardiospheres (CSs) using repeated three-dimensional culture strategy and characterized the cells. Approximately 5000 secondary CSs were acquired after 45 days. Genetic analysis confirmed that the progenitor cells in the secondary CSs originated from the innate heart, but not from extra-cardiac organs. The expressions of Oct4 and Nanog were significantly induced in secondary CSs compared with adherent cells derived from primary CSs. Those expressions in secondary CSs were higher in a cytokine-deprived medium than in a cytokine-supplemented one, suggesting that formation of the three-dimensional structure was important to enhance stemness whereas supplementation with various cytokines was not essential. Signal blocking experiments showed that the ERK and VEGF pathways are indispensable for sphere formation. To optimize cell processing, we compared four different methods of generating spheres. Method based on the hanging-drop or AggreWell™ was superior to that based on the poly-D-lysine-coated dish or Petri dish with respect to homogeneity of the product, cellular potency and overall simplicity of the process. When transplanted into the ischemic myocardium of immunocompromised mice, human secondary CSs differentiated into cardiomyocytes and endothelial cells. These results demonstrate that generation of secondary CSs from a small quantity of adult human cardiac tissue is a feasible and effective cell processing strategy to improve the therapeutic efficacy of cell therapy.

1. Introduction

Cell therapy is a promising approach for repairing or regenerating infarcted myocardium. However, recent clinical trials using stem and progenitor cells have shown marginal benefits in patients with acute myocardial infarction (MI) and chronic ischemic or non-ischemic cardiomyopathy [1–5]. The majority of studies have applied patient-derived autologous bone marrow (BM)-derived cells to the ischemic myocardium through intracoronary infusion or endomyocardial injection using catheters. Although the delivery of heterogeneous BM cells that do not require an *in vitro* culture process has been shown to be safe, the resulting clinical and hemodynamic improvements have been modest [6,7]. Beyond simple fractionation by centrifugation and injection thereafter, the challenge remains to identify the optimal cell type for myocardial repair and regeneration and an effective *in vitro* cell processing method prior to transplantation.

Phase 1 trials recently demonstrated that *in vitro* expansion of patient-derived autologous cardiac progenitor cells and intracoronary infusion in patients with LV systolic dysfunction after MI is effective in cardiac repair and scar reduction [8,9]. These results suggest that *in vitro* culture and subsequent transplantation for myocardial regeneration is a potent approach compared to using extra-cardiac cells. However, there are several barriers that must be overcome to apply these techniques in a clinical setting. To acquire a sufficient number of cells, surgical resection of cardiac atrial

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tissues is required to isolate and expand c-kit (+) progenitor cells [8]. Repeated plucking of the ventricular endomyocardium using biopsy forceps is necessary to culture cardiosphere-derived cell (CDCs) [9]. In addition, *in vitro* expansion using a two-dimensional monolayer in culture could limit cell-to-cell and cell-to-matrix interactions, which may subsequently reduce the cellular potency for effective cardiac repair.

Stem and progenitor cells exist in a specialized microenvironment *in vivo* [10,11]. We hypothesized that formation of cardiospheres (CSs) *in vitro* could mimic this environment and maintain and enhance stemness. Recently we demonstrated that formation of 'secondary' CSs by suspension culture of single cells derived from 'primary' CSs of murine heart tissue increased stemness, cardiovascular differentiation potential, and cell number, and that it enhanced cellular engraftment, differentiation and paracrine effects when transplanted *in vivo* [12]. However, human data are necessary to determine whether three-dimensional culture as secondary CSs prior to use is better than progenitor cells cultured in a two-dimensional environment in clinical applications.

In this study, we tested the murine findings of secondary CSs to be reproducible or extrapolatable to a small quantity of human cardiac tissues by assessing the feasibility and applicability of this process. We also examined the ability of *in vivo* cardiovascular differentiation of human secondary CSs. Furthermore, we compared different techniques to generate secondary CSs in order to optimize the protocol for the future clinical applications.

2. Materials and methods

2.1. Collection of human cardiac tissues

Less than 10 mg (one or two pieces) of human right ventricular septal tissue was collected by endomyocardial biopsy from heart transplant patients who had undergone the scheduled procedures for rejection surveillance (Fig. 1A). The tissue sampling was approved by the Institutional Review Board (IRB) at Seoul National University Hospital, and written informed consents were obtained from all participants.

2.2. Culture methods for primary CSs, PCDCs, and secondary CSs

A timeline of the culture process is shown in Fig. 1B. Human heart tissues were mechanically minced with microscissors and digested 3 times with 0.2% trypsin and 0.1% collagenase type IV for 5 min. The tissues were cultured on fibronectin (FN)-coated dishes in basal media (IMDM, 20% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and antibiotics) [12,13,27]. After observation of phase-bright cells, the cells were detached with 0.05% trypsin and collected. Contamination with non-phase-bright cells was minimized. The cells (about 1.5×10^5) were plated on poly-D-lysine (PDL)-coated dishes (BD Biosciences) with growth media (35% IMDM/65% DMEM/F12, 3.5% FBS, 2% B27, 25 ng/mL epidermal growth factor, 40 ng/mL basic fibroblast growth factor, 4 ng/mL cardiotrophin-1, 1 unit/mL thrombin, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and antibiotics) [12,13,27]. Within 3 days, the cells aggregated and formed 'primary' cardiospheres (CSs). Floating primary CSs were collected and plated on 0.125 mg/mL FN-coated dishes with growth media (483 ± 120 primary CSs per dish, which were expanded through subculturing). The resulting monolayer of cells was referred to as 'primary cardiosphere-derived cells' (PCDCs). Following trypsinization, approximately 1000 PCDCs were grown in a hanging-drop culture for 48 h to generate 'secondary' cardiospheres [12,18].

The Petri dish and AggreWell™ (Stemcell™ Technologies) methods were also used to generate secondary CSs. Sphere formation was observed through time-lapse imaging at a rate of one frame per minute using a JuLI™ microscope and a 1.3 mega pixel CMOS camera (NanoEnTek, Inc., Korea).

2.3. Gene and protein expression

2.3.1. RT-PCR

Total RNA was extracted from the cells using TRIZOL (Life Technologies). Quantitative real-time PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems) as previously described [12,28].

2.3.2. Immunofluorescence staining and confocal microscopic examination

The cells or tissue sections were stained for detection using immunofluorescence [29,30]. Primary antibodies against Oct4 (Santa Cruz Biotechnologies), VEGF (Abcam), alpha sarcomeric actinin (Sigma), VE-cadherin (Santacruz) and GFP (Abcam) were used with appropriate fluorescence-conjugated secondary antibodies. SYTOX blue (Invitrogen) was used for nuclear counterstaining. Confocal microscopy (LSM710, Zeiss) was used to acquire images.

2.3.3. Flow cytometry

Single-dissociated cells were incubated with the following fluorescence-labeled monoclonal antibodies: CD34 (AbD Serotec), CD44 (eBioscience), CD45 (DAKO), CD105 (AbD Serotec) and c-kit (eBioscience). An IgG isotype was used to define the percentage of positive cells. Analyses were performed using FACS Aria™ (Becton Dickinson).

2.4. Molecular pathways to generate CSs

For signal blocking experiments, U0126 (A.G. Scientific) was used to block ERK [31], LY294002 (A.G. Scientific) was used to block AKT [32] and bevacizumab (Avastin®, anti-VEGF antibody, Genentech/Roche) was used to block human VEGF.

2.5. Analysis of short tandem repeats

Genomic DNA was extracted from the peripheral blood mononuclear cells and heart tissue of patients and the cultured secondary cardiospheres. Origins were identified using real-time PCR amplification of fifteen STR loci [23] and gender-determining amelogenin (X,Y) [24] using specific primers and probes (AmpFlSTR® Identifiler® PCR Amplification Kit, Applied Biosystems). The genotypic data were analyzed using GeneMapper® software (Applied Biosystems).

2.6. Induction of myocardial infarction and cell transplantation using nude mice

Balb/c background athymic nude mice (Orient Bio Inc., Korea) were anesthetized with Zoletil® (91 mg/kg, Virbac, i.p.) and Xylazine® (11.65 mg/kg, Bayer, i.p.), intubated and artificially ventilated (Harvard Apparatus). The left anterior descending artery was ligated through a thoracotomy under a dissecting microscope (Olympus, Japan) using 8-0 polypropylene sutures. Akinesia of the anterior wall and apex of the left ventricle were observed. Secondary cardiospheres generated from 3×10^5 CDCs were directly injected into three peri-infarct sites [12]. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC).

2.7. Statistical analysis

All data are presented as the mean ± S.E.M. One-way ANOVA with Bonferroni's correction were applied for intergroup comparisons using SPSS version 17.0. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Generation of adult human secondary cardiospheres

We enrolled 16 patients who received heart transplantation and were scheduled for routine rejection surveillance. The characteristics of the patients are described in Table 1. We obtained one or two pieces (less than 10 mg) of right ventricular septal tissues by endomyocardial biopsy (Fig. 1A).

We generated sequentially primary cardiospheres (CSs), primary CS-derived cells (PCDCs), and secondary CSs from adult human cardiac tissues (Fig. 1B). First, at thirteen to fifteen days after culturing the cardiac explants on fibronectin (FN)-coated dishes, we could observe phase-bright cells (Fig. 1C). In a previous study using young murine heart tissue, the phase-bright cells were observed three days after starting culture [12]. To generate primary CSs, phase-bright cells were collected at day 21 using trypsin and reseeded on poly-D-lysine (PDL)-coated dishes. On day 24, approximately 100 primary CSs were obtained (Fig. 1C). These floating primary CSs were transferred and plated on FN-coated dishes and grew as adherent cells with a mesenchymal stromal cell-like (or fibroblast-like) morphology.

Flow cytometry analysis was performed to characterize these PCDCs (Fig. 1D). The majority of adherent cells expressed the mesenchymal lineage markers CD44 and CD105, but less than 1% expressed the hematopoietic cell-related marker CD45. A small proportion of cells expressed c-kit and CD34. These results are consistent with the findings of our previous murine study [12] and