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Human cardiac stem cells exhibit mesenchymal features and are maintained through Akt/GSK-3β signaling

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Abstract

Recent evidence suggested that human cardiac stem cells (hCSCs) may have the clinical application for cardiac repair; however, their characteristics and the regulatory mechanisms of their growth have not been fully investigated. Here, we show the novel property of hCSCs with respect to their origin and tissue distribution in human heart, and demonstrate the signaling pathway that regulates their growth and survival. Telomerase-active hCSCs were predominantly present in the right atrium and outflow tract of the heart (infant > adult) and had a mesenchymal cell-like phenotype. These hCSCs expressed the embryonic stem cell markers and differentiated into cardiomyocytes to support cardiac function when transplanted them into ischemic myocardium. Inhibition of Akt pathway impaired the hCSC proliferation and induced apoptosis, whereas inhibition of glycogen synthase kinase-3 (GSK-3) enhanced their growth and survival. We conclude that hCSCs exhibit mesenchymal features and that Akt/GSK-3β may be crucial modulators for hCSC maintenance in human heart.

Keywords: Cardiac stem cells; Mesenchymal cells; Proliferation; Survival; Akt/GSK-3β

The postmitotic heart was shown to exhibit a previously unappreciated self-renewing phenotype, in which primitive cells proliferated and differentiated into specific progeny under acute or chronic workloads [1,2]. Recent studies have challenged this paradigm and shown the existence of intrinsic cardiac stem or progenitor cells in the mammalian heart [3–5]. CSCs expressing c-kit were clonogenic and multipotent [4,6], and were also able to be isolated from human heart in the floating culture system [7]. Furthermore, hCSCs were reported to be activated in response to myocardial ischemia and increased workload [8,9]. These cells have a significant impact on future clinical application to treat patients with heart failure. However, it is necessary to further examine the property and regulatory mechanism of hCSC growth to obtain a sufficient number of stem cells from a small amount of tissue samples to achieve an efficient regenerative-therapy.

Recent reports have suggested that bone marrow-derived mesenchymal stem cells (MSCs) enhanced with Akt, a serine/threonine protein kinase, can repair infarcted myocardium, prevent remodeling, and normalize cardiac performance through the prevention of apoptosis as well as a paracrine effect on resident cells [10,11]. Recently, insulin-like growth factor-1 (IGF1) has been shown to maintain murine CSC (mCSC) viability and growth through activation of Akt [12,13]; however, the downstream signals of

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Akt pathway in hCSC growth remain to be investigated. In the present study, we characterized the property of hCSCs and clarified the role of Akt/GSK-3β signaling pathway in hCSC growth and survival. These results suggest that pharmacological inhibition of GSK-3β may have practical application in hCSC transplantation therapy in human heart failure.

Materials and methods

Tissue samples. The heart samples were obtained from 18 patients undergone cardiac surgery (9 males and 9 females aged from 9 days to 77 years old) in confirmation with the guidelines of the Kyoto University Hospital and Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Isolation of hCSCs. The heart samples were excised, minced, and digested with 0.4% type II collagenase and 0.01% DNAse. Obtained cells were then plated at 20 cells/μl in ultra-low culture dishes to generate cardiospheres with growth medium containing DMEM/F12, 5% FBS, 20 ng/ml EGF (Sigma), and 40 ng/ml bFGF (Promega). For the analyses described below, generated cardiospheres were dissected into single cells to obtain hCSCs by exposure to a 0.05% Trypsin/EDTA solution.

hCSC differentiation. For cardiac differentiation, hCSCs were cultured in differentiation medium containing 10% FBS, insulin-transferrin-selenium, and 10 nM dexamethasone. Differentiation medium containing DMEM/F12 supplemented with 10 ng/ml VEGF or 50 ng/ml PDGF-BB (R&D Systems) and 10% FBS was used to induce endothelial or smooth muscle cell differentiation, respectively. For the assay of cell proliferation and survival, specific inhibitors for Akt and GSK-3 (BIO) were purchased from Calbiochem.

FACS analysis. hCSCs were labeled with the following antibodies; phycoerythrin-conjugated antibodies against c-kit, CD45, CD34, CD31, CD90, CD29, CD73, CD71 (BD Biosciences), CD105 (Ancell Corp), and Stro-1 (R&D Systems). Cell events were collected by FACS Calibur flow cytometer and data were analyzed by Cell Quest (BD Biosciences).

RT-PCR and telomerase activity. Total RNA was extracted from cells using TRIzol and RT-PCR was performed with a SuperScript III First-Strand Synthesis System. The primer sequences are available upon request. Telomerase activity was measured with a TRAP assay kit, TRAPEZE (Chemicon).

Immunochemistry. Fixed cells and sections were stained with primary antibodies against cardiac troponin-I (Scripps), CD31, Ki67 (DAKO), α-SMA, connexin 43 (Sigma), collagen type1 (LSL), vimentin, and human nuclei (Chemicon). Secondary antibodies were conjugated to Alexa 488 and Alexa 555, and nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Apoptotic hCSCs were evaluated by TUNEL assay with ApopTag kit (Chemicon). Images were captured with a BZ-8000 (Keyence) and IX71 (Olympus Corporation).

Myocardial infarction (MI) and cell grafting. MI was created in 12- to 24-week-old NOD/*scid* mice (Jackson Laboratories) in accordance with the animal care and use guidelines at Kyoto University Hospital. MI was induced by ligation of the left anterior descending coronary artery. One hour after MI, 3×10^5 hCSCs were injected into two sites of the infarcted border zone. In the control group, mice were sham-operated on receiving a thoracotomy but no ligation of coronary artery.

Echocardiography. Two-dimensional and M-mode recordings (Sonos 5500, PHILIPS) were obtained from the short-axis view at the midpapillary muscle level.

Western blotting. Cell lysates were extracted with lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1× protease inhibitor, 1 mM Na_3VO_4, and 1 mM NaF. Transferred membranes were incubated with primary antibodies against GSK-3β (BD Biosciences), phospho-GSK-3β (Ser9), phospho-Akt (S473), and Akt (Cell Signaling). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were used as secondary antibodies.

Statistics. Data are means ± SE, and were analyzed by ANOVA and Scheffe's test, using a significance level of $p < 0.05$ (StatView).

Results

Identification and distribution of hCSCs in human heart

To characterize the hCSCs in human heart, primary heart-derived cells from patients were cultured at low density with low serum condition in a floating culture system using a modification of the method previously reported [7]. At day-14, spherical colonies were generated at a frequency of 63.1 ± 16.5 spheres per 200,000 viable cells (Fig. 1A). The initial yield of digested cells was proportional to the number of spheres, and the number of isolated cells was significantly increased in heart tissues from the right atrium (RA) and outflow tract (OFT) than in tissue from the left ventricle (LV) (Fig. 1B). Moreover, the isolated cells were 5-fold greater and had higher telomerase activity in the infant heart than the adult heart (Fig. 1C and D).

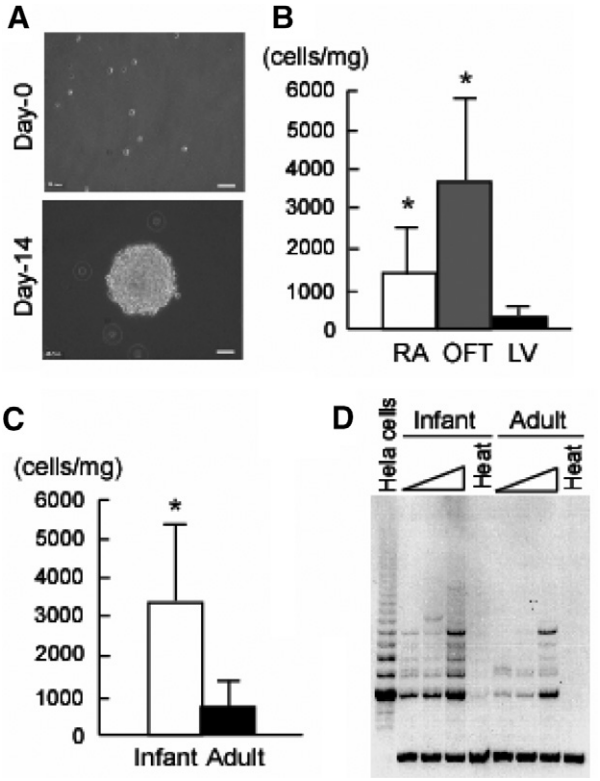

Fig. 1. Isolation and distribution of hCSCs. (A) Generation of cardiosphere from human heart. Bars, 20 μm. (B, C) The initial progenitor cell number harvested by primary isolation as indicated. Total yield was corrected by tissue weight (mg). Distribution of hCSCs corresponding to the parts of the heart (B) or the patients' age (C). $^*p < 0.05$ versus LV in (B); $^*p < 0.01$ versus adult in (C). (D) Telomerase activity in hCSCs. Threefold serial dilutions of hCSCs isolated from infant and adult hearts were treated with or without heat and used as templates. HeLa cells were used as a positive control ($n = 3$).