Cardiac Stem Cells Differentiate into Sinus Node-Like Cells

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The advent of stem cell therapy brings about the hope to restore the loss of cardiac pacemaker cells. However, it is largely unknown whether cardiac stem cells are able to differentiate into pacemaker cells. The purpose of this study was to determine whether the heart of large juvenile mammals contains cardiac stem cells (CSCs), which are suitable as seed cells for restoration of cardiac pacemaker cell. The c-kit CSCs were isolated from one-month-old mongrel dogs. CSCs that we sorted were self-renewing, and they could proliferate by clonal expansion. CSCs could differentiate into cardiac muscle, smooth muscle and endothelial cells at rates of 10.5 ± 4.2%, 13.5 ± 5.1% and 12.9 ± 3.5%, respectively, at week 4, as judged by the expression of respective differentiation markers: cardiac troponin I, smooth muscle actin, and CD31. At week 8, the differentiation rates were further increased to 23.2 ± 3.6%, 25.9 ± 6.6% and 28.3 ± 6.1% (P < 0.05 for each marker). Some of cells derived from CSCs could express cardiac transcription factor GATA-4 after week 2 and express pacing-related genes, including hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) and HCN4 after week 4. Importantly, a fraction of CSCs demonstrated the presence of inward currents that indicate the expression of inward current channels. In conclusion, c-kit CSCs may differentiate into cardiac muscle cell and sinus node-like cells, suggesting that CSCs would be useful as seed cells in treating sinus bradycardiac disorders or exploring the mechanism of pacemaker activity.

Keywords: cardiac stem cell; expansion; differentiation; c-kit; dog

In the past a few years, research evidence has suggested that the cardiac tissue has resident stem cells with regenerative potential (Messina et al. 2004; Wang et al. 2006; Smith et al. 2007). This discovery has dramatically changed the traditional view of all heart cells as terminally differentiated cells (Beltrami et al. 2001; Bearzi et al. 2007). It is greatly hoped that an improved understanding of the physiological function of CSCs will promote the development of novel therapeutic strategies (stem cell therapy) for treating heart disease and perhaps even for preventing cardiac senescence (Bearzi et al. 2007; Gonzalez et al. 2008; Rota et al. 2008). Meanwhile, many researchers now pay great attention to biological re-establishment of cardiac pacemaker cells so as to replace electronic pacemaker for treating patients suffering from loss of cardiac pacemaker cells that results in sinus bradycardiac disorders (Miake et al. 2002; Plotnikov et al. 2004). So, the CSCs therapy of sinus bradycardiac disorders, which aims to restore pacemaker cells, would be promising. Although some investigations suggested that CSCs had the potential to reconstitute dead myocardium (Beltrami et al. 2003; Leri et al. 2005; Smith et al. 2007), detailed exploration of CSCs’ characteristics related to pacemaker cell is rare and a number of fundamental issues need to be elucidated before this reconstruction approach could be considered for biological pacemaker studies.

Finding a way to get more, purified CSCs with fine function is significant to both study now and clinical treatment in the future. In the past several years, population of CSCs (c-kit+, Sca-1+) were isolated from hearts by cell sorting from enzymatically digested hearts based on cell surface markers (Beltrami et al. 2003; Oh et al. 2003; Tang et al. 2007). In order to make sure the integrity of important surface antigens of resident CSCs and avoid dysfunctional cells and fibroblasts’ contamination, we have developed a three-step procedure to isolate and expand pure CSCs. First, by expansion of endogenous CSCs through primary heart tissue explants, second, by isolation of CSCs from fibroblasts by cell sorting with stem cell marker (c-kit), and third, by expansion and differentiation of sorted CSCs after sorting.

We intend to use CSCs as seed cells for biological pacemaker study. First and foremost, we need to identify the biological characteristics and function of CSCs, including electrophysiological properties of the cells. It is impor-
tant to know whether CSCs can differentiate into sinus node–like cells that express pacing-related genes.

Stem cell related antigen c-kit, also called CD117, which is a cytokine receptor expressed on the surface of stem cells, is a marker of stem cell. The cells, which come from heart tissue and express c-kit, can be considered as CSCs (Beltrami et al. 2003; Gonzalez et al. 2008; Rota et al. 2008). Cardiac transcription factor GATA-4 is a zinc-finger transcription factor that acts as a critical regulator of the cardiac differentiation-specific gene program (Pu et al. 2004). Hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) and HCN4 are pacing-related genes (Ludwig et al. 2008). In this study, we investigated the differentiation of the CSCs by analyzing mRNA expression of GATA-4, HCN2 and HCN4. We also measured inward currents of CSCs in order to make clear whether c-kit+ CSCs have the potential of pacemaker cells.

Materials and Methods

Isolation and expansion of CSCs

All experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University. Four one-month-old mongrel dogs were obtained for the experiments.

After anesthetization with sodium pentobarbital (30mg/Kg intraperitoneal injection), the heart tissues of the dog were excised and washed twice with cold Ca2+-Mg2+ free phosphate-buffered solution (PBS) to remove the blood cells. According to previous reports with minor modification (Messina et al. 2004; Tang et al. 2007), the tissues from atrium, cardiac apex and middle of the heart were minced into 1 to 2 mm3 pieces respectively. Then, the minced tissues were digested alternately three times for 5 min with 0.25% trypsin (HyClone, Logan, UT, USA) and two times for 5 min with 0.1% collagenase II (Sigma, St. Louis, Missouri, USA) at room temperature. After initial treatment, the remaining tissue fragments were cultured as explants in explant medium (IMDM with 10% fetal calf serum, 0.1 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 U/mL penicillin G and 100 ng/ml streptomycin) (HyClone, Logan, UT, USA) at 37°C and 5% CO2. 3-5 days later, we observed small, bright cells migrating from adherent explants. These phase-bright cells were collected repeatedly. To avoid damaging the integrity of cell surface antigen, we collected these phase-bright cells by washing with 0.05% trypsin and 0.53 mmol/L EDTA (HyClone, Logan, UT, USA) for 2 min at room temperature under visual control. After collecting the washed cells, we filtered the cells through a 40-µm cell strainer and counted the number of filtered single-cell under microscope.

Further expansion of CSCs after sorting

We selected 12-well and 6-well plates in succession for the sorted cell culture. After cell sorting, the resultant cell suspensions were plated in 12-well plates with serum-free growth medium (35% IMDM/65% DMEM–Ham F-12 mix containing 2% B27, 0.1 mmol/L 2-mercaptoethanol, 10 ng/mL epidermal growth factor [EGF], 20 ng/mL basic fibroblast growth factor [bFGF], 0.1 U/mL thrombin, antibiotics, and L-Glu, as in explant medium). 1 day later, the still suspending cells and loosely adherent cells were moved to 6-well plates. The growth medium was replaced every 3 days.

To identify the clonal growth pattern of CSCs, we employed an approach to culture CSCs by seeding the cells at a density of 1 cell per well in growth medium in 96-well plates. After single-cell deposition analysis was performed by the limiting dilution technique, each well was inspected for the presence of single cell by phase contrast microscope.

The growth curves of c-kit+cells

To compare the proliferation speed of the CSCs from atrium, cardiac apex and middle of the heart, the sorted cells were seeded in 6 cm (diameter) culture dishes at the seeding density of 1 × 104 with cell growth medium. The medium was replaced every 3 days. The growth curves of sorted cells from different parts of the heart were constructed according to mean values measured by cell counting on day 1, day 3, day 6, day 9, day 12, day 15 and day 18. The cell growth curves were drawn with the culture time as the abscissa and the cell number as the ordinate.

Immunofluorescence

Immunofluorescence staining was performed to explore differentiation lineages of the CSCs. Cardiac troponin I (cTnI), smooth muscle actin (SMA), and CD31 are regarded as markers of cardiomyocyte, smooth muscle cell and endothelial cell, respectively. At week 4 and week 8 after cell sorting, immunofluorescent analyses on the cells cultured on slides were performed as previously described (Tang et al. 2007). At first, the cells were fixed with 4% paraformaldehyde for 30 min. After blockage with goat serum for 30 min and permeabilization with 0.2% Triton X-100 (Sigma, USA) for 10 min, the fixed cells were incubated with primary antibodies overnight at 4°C, then incubated with rhodamine-conjugated secondary antibodies for 1 h at room temperature. Nuclei were counterstained with 4′,6-diamidino-2′-phenylindole (DAPI) (Sigma, St. Louis, Missouri,
USA) for 30 min. Specimens were examined under a fluorescence microscope (Leica, Wetzlar, Germany). Finally, we counted the number of all cells (blue coloration) and the cells which were stained both red and blue at each specimen. The percentage of cells being stained both red and blue can be regarded as different lineage's differentiation rate.

Primary antibodies contained anti-cardiac troponin I (sc-98830, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-smooth muscle actin (sc-53142, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD31 (bs-0468R, 1:100, Biosynthesis Biotechnology, Beijing, China). Isotype-matched antibodies were used as control. Rhodamine-conjugated second antibodies (sc-2091, sc-2092, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect the primary antibodies which aimed directly at cTnI, SMA and CD31.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed to study the expression of GATA-4, HCN2, HCN4 mRNA. At week 2, week 4 and week 8 after cell sorting, the total mRNA was extracted with Trizol Reagent (Invitrogen, San Francisco, CA, USA) from the cultured cells. Transcriptional expression of GATA-4, HCN2, HCN4 genes was determined by semi-quantitative RT-PCR according to the manufacturer’s instructions. Transcript levels were standardized to the corresponding dog β-actin level. The primers for RT-PCR were as follows: GATA-4, For: 5´-CAATGCGGAAAGGGGAT-3´; Rev: 5´-GAGAGGACTGGG TGGATGGA-3´ (GenBank accession #NM_104811.2). HCN2, For: 5´-ATCGTGAGAGGGGACATTGAC-3´; Rev: 5´-TCCCCACTGG TGGATGAGCG-3´ (GenBank accession #XM_850140.1). HCN4, For: 5´-GAGTCAACAAATTATCCCAGAG-3´; Rev: 5´-GAATTGCAATGCCAGA-3´; Rev: 5´-GAG AGG ACT GGG TGGATGGA-3´ (GenBank accession #NM_104811.2). HCN2, For: 5´-CAATGCGGAAAGGGGAT-3´; Rev: 5´-GAGAGGACTGGG TGGATGGA-3´ (GenBank accession #NM_104811.2). HCN4, For: 5´-ATCGTGAGAGGGGACATTGAC-3´; Rev: 5´-TCCCCACTGG TGGATGAGCG-3´ (GenBank accession #XM_850140.1). HCN4, For: 5´-ATCGTGAGAGGGGACATTGAC-3´; Rev: 5´-TCCCCACTGG TGGATGAGCG-3´ (GenBank accession #XM_850140.1). HCN4, For: 5´-ATCGTGAGAGGGGACATTGAC-3´; Rev: 5´-TCCCCACTGG TGGATGAGCG-3´ (GenBank accession #XM_850140.1).

Statistics were performed with the SPSS (version 11.0) (Chicagao, Illinois, USA) and Graphpad Prism (version 5). P-value smaller than 0.05 was considered to be statistically significant. Values were given as mean ± s.d..

Results

Isolation and expansion of c-kit+ cells

By 3-5 days after explanting of the minced dog heart tissue, we observed that a layer of fibroblast-like cells covered the culture dish, and round, phase-bright cells with different size migrated from the adherent explants (Fig. 1A). Successful outgrowth of the phase-bright cells was obtained in 5 of 10 culture dishes from atrium, 4 of 10 culture dishes from middle of the heart and 10 of 10 culture dishes from cardiac apex respectively. These cells were loosely attached to the fibroblast-like cell layer, and could be collected periodically by simply washing and centrifuging. After cell sorting, some c-kit+ cells clustered and formed three-dimensional spheres (Fig. 1B). From our visual observation, each cluster shown in figure 1 was a result of proliferation of one single CSC through many times division. 1 month later, more and more c-kit+ cells differentiated into fusiform shape or irregular shape. Nevertheless, some descendants of c-kit+ cells still kept round and bright shape. In Fig.1E, growth curves of sorted cells from atrium, cardiac apex and middle of the heart were showed to evaluate the proliferation ability of the c-kit+ cells from different tissues. From the curves, we could see that the proliferation speed of the c-kit+ cells from cardiac apex was faster than those from atrium and middle of the heart. The proliferation speed of the cells from middle tissue of the heart was the lowest.

To observe the clonal growth pattern of CSCs, we cultured CSCs by seeding the cells at a density of 1 cell per well in growth medium. Altogether, 200 single cells were deposited, and about 80% of the cells survived and expanded within 7 days and about 50% of the cells formed spherical clusters.

Characterization of c-kit+ cell

As shown in Fig. 2A, before cell sorting, 20.4 ± 7.5% of detected cells were c-kit positive by means of flow cytometry. After cell sorting based on c-kit+ via FACS, we detected the positive rate of CD34 and CD45 respectively in purified c-kit+ cells. Marker expression analysis indicated that the c-kit+ cell hardly expressed CD34 (0.7%, Fig. 2D) and CD45 (0.5%, Fig. 2C).

Immunophenotype characterization of c-kit+ cells

To characterize the differentiation of the c-kit+ cells, at week 4 and week 8 after cell sorting, we examined the expression of cTnI, SMA and CD31 by immunostaining. In Fig. 3, the representative images and frequencies of cTnI, SMA and CD31 were shown. Fluorescence microscopy analysis revealed that the CSCs differentiated into cardiac muscle cell (cTnI), smooth muscle cell (SMA) and endothelial cell (CD31) at rates of 10.5 ± 4.2%, 13.5 ± 5.1% and
12.9 ± 3.5% respectively at week 4. Subsequently, the differentiation rates were 23.2 ± 3.6%, 25.9 ± 6.6% and 28.3 ± 6.1%, respectively at week 8. The difference of each lineage’s differentiation rate between week 4 and week 8 was of significance ($P < 0.05$ for each marker).

The mRNA expression of GATA-4, HCN2 and HCN4 in CSCs

We investigated the mRNA expression of GATA-4, HCN2 and HCN4 in the cultured cells generated from CSCs at week 2, week 4 and week 8 respectively. There was clear mRNA expression for GATA-4, HCN2 and HCN4 at week 4 and week 8. The expression of each gene at week 8 was stronger than that at week 4 respectively, $P < 0.05$. In addition, we detected no mRNA for HCN2 and HCN4 in the cultured cells at week 2. The reason why the expression of HCN2 and HCN4 could not be explored perspicuously at week 2 might be the slow growth and differentiation speed of CSCs at early stage after cell sorting under our culture condition.

Inward currents of CSCs

At first, we tried to detect sodium inward currents in undifferentiated CSCs. The results were that sodium inward current was small in CSCs being tested (11 out of 60 cells). Then, we tested for the presence of functional Ca$^{2+}$ channels. At 2 mM external CaCl$_2$, it was hard to identify clearly inward current and the recorded current was very low.
However, after switching to 10 mM BaCl₂, we could record inward higher currents (10 out of 50 cells). The currents were activated around −35 mV and the current peak was at 20 to 30 mV (Fig. 5), similar to Ba²⁺ currents conducted by L-type Ca²⁺ channels of other cell types (Heubach et al. 2000, 2004). The strongest current was −60.5 ± 4.8 pA at 30mV. These results verified the presence of functionally inward channels.

Discussion

The discovery that stem cells reside in the heart and constantly give rise to a cardiomyocyte progeny has changed dramatically our interpretation of heart tissue and offered novel therapeutic options for the management of heart disease (Linke et al. 2005; Smith et al. 2007). The c-kit⁺ cells isolated from the heart behaved like stem cells since they were self-renewing, clonogenic, and multipotent (Beltrami et al. 2003). Study showed that after many doublings in culture, cardiac c-kit⁺ cells retained differentiation competence (Beltrami et al. 2003). We conducted the study to know whether the CSCs were fit for biological pacemaker study as seed cells.

The c-kit positive cells we isolated seemed to be a mixture of CSCs and cardiac progenitor cells. Although c-kit is a kind of non-special surface marker of stem cell, the results that purified c-kit⁺ cells were negative for hematopoietic cell lineage marker (CD45) and endothelial cell lineage marker (CD34) confirmed that the c-kit⁺ cells we sorted were not hematopoietic or endothelial progenitor cells. Meanwhile, the c-kit⁺ cells came from heart and could differentiate into cardiac muscle, smooth muscle and endothelial cells. So, we could consider the c-kit⁺ cells we sorted as CSCs. From the result of growth curves, we could see that the proliferation speed of the CSCs from cardiac apex is faster than those from atrium and middle of the heart.

The c-kit⁺ cells isolated from the dog heart via our three-step procedure were found to be self-renewing. The cells could proliferate by clonal expansion and differentiate spontaneously into cardiomyocytes in vitro but fail to contract automatically. The cardiac transcription factor GATA-4, which is essential for normal heart morphogenesis and regulates the survival, growth and proliferation of cardiomyocytes (Armiñán et al. 2009; Zaglia et al. 2009; Singh et al. 2010), could be considered as early marker of differentiated cardiomyocyte. Hyperpolarization-activated nucleotide-gated (HCN) channel family genes, which figure prominently in physiological automaticity, are related to automatic pacing activity. The transfer of HCN genes into quiescent heart tissue had been explored as a way of creat-
Fig. 3. Immunophenotype characterization of purified c-kit+ cells grown on coated wells. Immunofluorescence staining of the cells with anti-cTnI, SMA and CD31 (red A-D). The cTnI staining colocalized with single cells (A). Cells were counterstained with DAPI (blue) staining the nucleus. Bars, 20 µm. Experiments were done in triplicate with similar results.

Fig. 4. RT-PCR analysis of GATA-4, HCN2 and HCN4 for CSCs at different time. The picture (upper) was electrophoretogram of GATA-4, HCN2 and HCN4 PCR amplification product. The graph (lower) showed that quantitative densiometric analysis of the GATA-4, HCN2 and HCN4 mRNA amount normalized β-actin mRNA at different time.

*P < 0.05, #P < 0.01, vs. group at week 4.
Differentiation of Cardiac Stem Cells

HCN2 and HCN4 channels are expressed in the sinus node, determine the pacemaker current $I_f$ and regulate the heart rate (Ludwig et al. 2008; DiFrancesco D. 2010). So, the cells expressing HCN2 and HCN4 could be considered sinus node-like cells. Although low levels of HCN2 and HCN4 expression could be observed in cultured cells generated from the CSCs, the findings provide new insights into the endeavours for restoration of pacemaker cell using the CSCs.

There were limited currents observed in undifferentiated CSCs. In spite of preliminary observation, the existence of inward current channels reinforced the feasibility of using the CSCs as seed cells for biological pacemaking study. We could suppose that the expression of current channels vary at different phases of the cell cycle. The modification of present current and/or the occurrence of new current could be suitable markers of CSC differentiation in vitro.

In conclusion, the c-kit$^+$ CSCs could express GATA-4 and differentiate into cardiac muscle, smooth muscle and endothelial cell. It is therefore feasible to select CSCs for further research about repairing cardiac muscle. Because of expression of pacing-related genes (HCN2 and HCN4) and existence of inward current channels, CSCs seem to be promising to change into pacemaker cells in the future study. The findings raise the possibility to use the cells for the management of sinus bradycardiac disorders and the exploration of pacemaker activity mechanism.

References


