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A novel two-step procedure to expand Sca-1+ cells clonally

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Abstract

Resident cardiac stem cells (CSCs) are characterized by their capacity to self-renew in culture, and are multi-potent for forming normal cell types in hearts. CSCs were originally isolated directly from enzymatically digested hearts using stem cell markers. However, long exposure to enzymatic digestion can affect the integrity of stem cell markers on the cell surface, and also compromise stem cell function. Alternatively resident CSCs can migrate from tissue explant and form cardiospheres in culture. However, fibroblast contamination can easily occur during CSC culture. To avoid these problems, we developed a two-step procedure by growing the cells before selecting the Sca1+ cells and culturing in cardiac fibroblast conditioned medium, they avoid fibroblast overgrowth.

Keywords

Stem cells; Differentiation; cell transplantation; myocardial infarction

INTRODUCTION

Ischemic heart disease accounts for 50% of all cardiovascular deaths and is the leading cause of congestive heart failure[1]. A new approach to cardiac repair is cellular therapy with stem cells, such as bone marrow-derived mesenchymal stem cells[2–4], skeletal myoblast[5], adipose derived mesenchymal stem cells[6], and hematopoietic stem cells[7]. Recently, resident cardiac progenitor/stem cells (CSCs) have been discovered in the adult heart[8–13], which hold great promise for clinical applications because they are normal components of adult heart and capable of differentiating into cardiomyocyte or vascular lineages.

Over the past 3 years, populations of cardiac stem cells (c-kit+, Sca-1+) were isolated from adult hearts by cell sorting from enzymatically digested hearts based on cell surface markers [8;9;11]. However, enzymatic digestion of myocardium compromises the integrity of

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important surface antigens of resident cardiac stem cells, and leads to dysfunctional sorted cells, which makes the method hard to be reproduced. More recently, Messina et al.[10] demonstrated that CSCs can be clonally expand from mouse and human myocardial biopsies and form cardiospheres which expressed Sca-1, c-kit, Flk and CD31. The primary tissue explant technique can facilitate the proliferation of endogenous cardiac stem cells in vitro, which are rare in adult heart. However, cardiospheres contain a mixture of cells, including cardiac fibroblasts, which grow faster than endogenous cardiac stem cells in the cardiosphere. Contamination of fibroblasts in cardiospheres would limit the therapeutic use of CSCs because the fibroblasts could facilitate the scar dilation and less efficient for heart repair[14].

To avoid these problems, we have developed a two-step procedure to isolate and expand pure cardiac progenitor/stem cells. First, by expansion of endogenous cardiac stem cells through primary heart tissue explant, second, by isolation of stem cells from fibroblasts by cell sorting with stem cell markers, and cloning. A fibroblast-free culture system is used for the proliferation of cardiac progenitor/stem cells. We demonstrated that Sca-1+ cells isolated by this method keep their self-renewal and clonogenic character in vitro, and can directly differentiate into normal cells in myocardium, including cardiomyocytes, endothelial cells, and smooth muscle cells, after transplantation in ischemic hearts of mice.

MATERIAL AND METHODS

Isolation of Sca-1+ stem cells using two-step procedure

There are two stages to isolate and culture Sca-1+ cardiac stem cells: the primary tissue explant and the cell enrichment. Mouse heart tissue were cultured according to a previous report[10] with minor modification. Mouse heart tissues from male mice (2 month old; www.harlan.com) were obtained with approval by the Institutional Animal Care and Use Committee of University of South Florida. Tissues were minced into small pieces, and subjected to enzymatic dissociation with a mixture of 0.2% trypsin and 0.1% collagenase IV (Worthington Biochemical Corp. www.worthington-biochem.com) in PBS three times for 5 minutes at 37 °C. After treatment, the remaining tissue fragments were cultured as explants in explant medium (Iscove's Modified Dulbecco's IMDM with 10% fetal calf serum (FBS), 100U/mL penicillin G, 100ug/ml streptomycin, 2 mmol/L L-glutamine, and 0.1mmol/L 2-mercaptoethanol) at 37°C and 5% CO₂. After 2–3 weeks, small, phase-bright cells migrate above the fibroblast layer formed from adherent explants. To avoid damaging the integrity of cell surface antigen, we collected these phase-bright cells simply by washing with D-Hanks. The cell suspension was filtered through a 40-µm cell strainer (BD Falcon, www.bdbiosciences.com), and cell numbers were counted. To enrich the cardiac progenitor cells, we sorted Sca-1+ cells from collected cells by positive selection with anti-Sca-1-microbeads (Miltenyi Biotec, www.miltenybiotec.com) using a magnetic cell sorter device from Miltenyi Biotec.

Newly isolated cardiac Sca-1+ cells were seeded at multiwell plates precoated with Matrigel (BD Biosciences, www.bdbiosciences.com) in media designated for cell growth medium (CGM) (DMEM/F12, 10% FBS, 200 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1% nonessential amino acids, 1000 units/ml LIF, 0.1 unit/ml thrombin and 5 ng/ml basic fibroblast growth factor (bFGF)). This medium was conditioned by exposing it for 48 hours to fibroblast culture to dissolve paracrine factors from fibroblasts. The conditioned medium (CCGM) was mixed with fresh CGM medium at a 3:1 ratio, sterilized by filtration, and supplemented with additional bFGF at 5 ng/ml.

The growth curves of Sca-1+ cells

Sca-1+ cells or unsorted cells were seeded in a 100 cm² dish at the seeding density of 1×10^4 with CGM medium or conditional CGM medium, and the medium was replaced every 3 days. The cell growth curve was constructed according to mean values measured by cell counting on day 4, day 7, day 10, day 14 and day 17 (n=4/time point). The cell growth curves were drawn with the culture time as the abscissa and the cell number as the ordinate.

Characterization of Cells: Flow Cytometry

Cultured Sca-1+ cells were blocked with 5% BSA, and stained with a panel of antibodies including Sca-1; CD45; c-kit; CD31; and CD34 (BD Pharmingen, www.bdbiosciences.com), or isotype controls respectively. Cells were subjected to flow cytometry using BD LSRII flow cytometer and BD FACSDiva™ software.

Immunocytochemistry

Immunofluorescent analyses on cultured slides and tissue sections were performed as previously described[3]. Cells and tissue were fixed with 4% paraformaldehyde. Primary antibodies specific for ABCG2(Biotinylated anti-human, 1:100; R&D System, www.rndsystems.com), c-kit (Biotinylated anti-mouse, 1:100; BD Pharmingen), VEGF R2 (Flk-1) (Biotinylated anti-mouse, 1:100; R&D System), GATA4 (goat polyclonal, 1:100; Santa Cruz, www.scbt.com), Phospho-Histone H3 (Ser10) (rabbit polyclonal, 1:100; Upstate, www.upstate.com), Myosin (rabbit polyclonal, 1:100; Sigma-Aldrich, www.sigmaaldrich.com), Connexin 43(mouse monoclonal, 1:100; BD Transduction Lab.) for cultured slides were used in immunocytochemistry staining. Isotype-matched antibodies were used as control. In mice treated with LacZ-labeled Sca-1+ cells, the hearts were harvested 1 month after cell injection. The hearts were embedded in OCT compound and sectioned at 5 μ m.

Incorporated LacZ-labeled cells were detected by the FITC conjugated anti-LacZ antibody (1:500, Abcam, www.abcam.com). An anti-cTnI (1:100, Santa Cruz), anti-CD31 biotin antibody (1:100, BD PharMingen) and anti-SMA (1:100, Zymed Lab, www.zymed.com) were used for tissue sections. Primary antibody binding was detected via corresponding Streptavidin Alexa Fluor 488-conjugated, goat anti-rabbit Alexa Fluor 555-conjugated, goat anti-rabbit Alexa Fluor 488-conjugated or donkey anti-goat Alexa Fluor 488 secondary antibodies (1:500; Invitrogen, www.invitrogen.com). Nuclei were counterstained with either Draq5 (Alexis Biochemicals, www.axxora.com) or PE (Vector Lab, www.vectorlabs.com) to show nuclei. Staining was observed by a Leica TCS confocal microscope (www.leica-microsystems.com).

Cell labeling, myocardial infarction and cell transplantation

To track the cells after transplantation in the heart, Sca-1+ cells were genetically engineered to express the LacZ reporter gene. Cells were transduced with the retroviral reporter vector packaged from pCL-MFG-LacZ plasmid (Imgenex, www.imgenex.com). The Retronectin dishes (Takara Bio Inc. www.takara-bio.co.jp) were used to increase the transduction ratio of retrovirus. The transduction efficiency was evaluated by LacZ detection kit for cells (InvivoGen, www.invivogen.com). Male C57/BL6 mice (4 month old) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and mechanically ventilated. Myocardial infarction was induced via ligation of the left anterior descending coronary artery 2 mm from the tip of the normally positioned left auricle as we described previously[2] (n=8). 10 min after ligation, a 200 μ l solution containing 1×10^6 Sca-1+ cells in PBS was intravenously injected into the mice through right jugular vein. Mice were killed 4 weeks later.

RESULTS

In Vitro Studies

Isolation and Expansion of Sca-1+ cells—At 2–3 weeks after explanting of minced mouse heart tissue, we observed that a layer of fibroblasts covered the culture dish, and round, phase-bright cells with different size migrated from the adherent explants (Fig 1, A). These cells were loosely attached to the fibroblast layer, and could be collected periodically by simply washing and centrifuging (Fig 1, B). After cell sorting via MACS, purified Sca-1+ cells were cultured in conditioned CGM medium (CCGM), which is sufficient to maintain Sca-1+ cells in a proliferative state without the need for fibroblasts. Contrast microscope examinations showed that Sca-1+ cells were bright, round without fibroblast contamination. Some of them proliferated and became 2- to 3-cell aggregates in suspension after 3 to 5 days. These aggregates slowly increased in size, and gradually attached to the plate. The adherent cells sprout new round bright cells at their cell body. After 2 weeks, the Sca-1+ cells form three-dimensional spheres (Fig 1, C). Therefore, CCGM culture medium can be used to expand Sca-1+ cells from mouse myocardium. In Fig. 1D, we show growth curves to evaluate the effect of CCGM medium on proliferation dynamics of Sca-1+ cell. Compared to CGM medium, we observed that the absence of cardiac fibroblast inhibit the proliferation of Sca-1+ cells, but the presence of CCGM medium improves the growth of Sca-1+ cell, and makes cell growth similar to the growth curve of unsorted cardiosphere derived cells. The CCGM grown Sca-1+ cells are however fibroblast free unlike the unsorted cells.

Characterization of Sca-1+ Cell

The purity of sorted Sca-1+ cells was characterized by flow cytometry. As shown in Fig 2A–B, 97.6% of sorted Sca-1+ cells are Sca-1+CD45[−]. The lack of CD45, the marker for hematopoietic stem cells. *Marker expression analysis indicated that Sca-1+ cells also expressed another stem cell marker c-kit (19.8% Fig 2C). The profile further suggests that a minority of cells expressed CD31 (4.1% Fig 2D); CD34 (6.5% Fig 2E) markers.*

Immunophenotype characterization of Sca-1+ cells

By immunofluorescence confocal microscopy, phenotypic analysis of newly developing mouse Sca-1+ revealed expression of other stem cell marker including c-kit (Fig. 3 A1–3), ATP-binding cassette transporter (ABCG2), a marker for side population stem cells[15] (Fig. 3 B1–3) and endothelial marker (Flk-1) (Fig. 3 C1–3).

To characterize the Sca-1+ cells, we examined GATA4, a cardiac specific transcription factor, by immunostaining. We observed that there were many GATA4-positive cells among the Sca-1+ cells (Fig. 3 D1–3) with approximately 55% of the cells being GATA4 positive. The results demonstrate that cells express both stem cell marker (Sca-1) and cardiac specific transcription marker. This finding is strong evidence that Sca-1+ cells from cardiospheres have entered a differentiation pathway toward a cardiomyocyte phenotype.

We also observed that some of Sca-1+ cells expressed the serine-10 phosphorylation of histone H3 (Fig. 3 E1–3), a marker of mitotic Cdc2 activity, a marker of proliferative potential.

Spontaneous Differentiation of Sca-1+ cell-formed spheres in vitro

To analyze the spontaneous differentiation of Sca-1+ cell-formed spheres, we exposed cells to low-serum medium (2% FBS) for 2 days, and assayed the expression of cardiomyocyte structure protein, including myosin, connexin43, by immunofluorescent staining. As shown in Fig. 3F 1–4, confocal immunofluorescence analysis of cardiosphere with anti-connexin43 (green) and anti-myosin (red) revealed spontaneous differentiation presented in the peripheral

zone of sphere (Fig. 3F arrow). These results suggest that, in the low serum environment, Sca-1⁺ cell-formed spheres tend to differentiate into cardiomyocyte phenotypes.

In Vivo Studies

Differentiation of Sca-1⁺ cells in injured myocardium—To test the ability of Sca-1⁺ cells to differentiate and reconstitute the myocardium, we used retro-MFG- β -gal virus to label Sca-1⁺ cells with genes expressing LacZ, with over 90% efficiency by β -gal staining (Fig. 4A). We injected Sca-1⁺ cells i.v. in syngenic mice 10 min after inducing myocardial infarction. Donor cells were detected in the myocardium by laser confocal microscopy 1 month after cell transplantation. Double staining of sections for LacZ and cardiac-specific proteins indicated that LacZ colocalized with cTnI (Fig. 4B), indicating that Sca-1⁺ cells develop into cardiac myocyte-like cells after transplantation. Graft cells expressing β -gal also integrated into blood vessels and expressed endothelial cell marker-CD31 (Fig. 4C) and smooth muscle cell marker-smooth muscle α -actin (SM-actin) (Fig. 4D), suggesting that grafted Sca-1⁺ cells can also differentiate into endothelium and smooth muscle.

DISCUSSION

These findings suggested that Sca-1⁺ cells extracted and purified by this technique are multipotent and can be used to reconstitute dead myocardium by differentiating to normal components of adult hearts. In this study, we describe a two-step procedure in which a large number of Sca-1⁺ cells can be purified from small amount of heart tissue. We showed that Sca-1⁺ cells keep their capacity for self-renewal and clonogenic in vitro with fibroblast-free conditional CGM medium, and can differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells after being transplanted into ischemia-induced heart of mice.

Myocardial infarction is one of the leading causes of congestive heart failure in the United States, with median survival after onset only 1.7 years in men and 3.2 years in women[16]. The irreversible loss of myocytes induced by myocardial infarction leads to a sequence of congestive heart failure. The longstanding dogma of the heart as a terminally differentiated tissue incapable of regeneration has recently been challenged. Investigators from different laboratories have only recently discovered stem cells in the adult heart[8;10–13;17;18]. These cells are rare, but might have appropriate regenerative potential for repairing injured hearts. However, myocardial failure is usually irreversible. This may be due to the inadequate numbers of resident cardiac stem cells to replace injured heart issue and the negative environment of ischemic heart for stem cell proliferation and survival. Although the small number of resident cardiac stem cells may not be sufficient to restore heart function after MI, their presence has raised the possibility of regenerating damaged heart tissue by using them if they can be expanded and purified in vitro. Over the past 3 years, several populations of cardiac stem cells have been described. Among these cell types, Sca-1⁺ cells are promising for heart repair as sca-1⁺ cells predisposed to become cardiomyocytes. Oh et al.[9;19] has reported cardiac Sca-1⁺ cells lacking hematopoietic stem cell markers CD45, and also lacking transcripts for cardiac structural genes (myosin light chains), but expressing the cardiac transcription factor GATA-4. Matsuura et al.[17] showed that adult cardiac Sca-1⁺ cells can differentiate into beating cardiomyocytes with multipotency. Recently, Wang et al. [11]demonstrated that Sca-1⁺ cells can increase of cardiomyocyte regeneration, and hold therapeutic possibilities with regard to the treatment of ischemic heart disease. However, for treatment, the technical difficulties lie in collecting the endogenous stem cells from adult hearts because they are rare in the heart and enzymatic digestion may destroy the integrity of stem cell markers, which may lead to sorted cells losing their potential as stem cells. Establishing effective methods to isolate and culture large number of resident cardiac stem cells is critical for basic research and future clinical application. Messina et al.[10] recently reported a method to culture cardiospheres from human

myocardial biopsies that expressed Sca-1, c-kit, Flk and CD31. Myocytes constitute the majority of cardiac tissue volume, however, fibroblasts dominate in number, and their proliferation are feature of many of cardiac pathologies[20]. Excluding fibroblasts from cardiac stem cell culture is essential because fibroblasts maintain high proliferative potential, and will overgrow cardiac stem cells in cardiosphere. To avoid the contamination of fibroblast, our two-step procedure combines primary tissue explant with cell sorting to produce large numbers of purified Sca-1+ cells. With this method, we isolate and purify cardiac stem/progenitor cells which migrate from heart explants, not by enzymatic digested heart. This avoids the direct enzymatic digestion of cardiac stem cells, and maintains the integrity of stem cells marker at the cell surface.

Several cell surface markers are used in the isolation and determination of cardiac stem cells, for example Sca-1+ and c-kit+ cells, and both appeared to have similar efficacy profiles. Sca-1+ cells are considered cardiac progenitor cells[21], which are at undifferentiated status, yet predisposed to become cardiomyocytes. Our study isolated that Sca-1+ cells lacking hematopoietic stem cell markers CD45, and expressing GATA-4, which is a cardiac transcription factor[13;22] GATA4 activates the promoters of several cardiac genes, such as myosin light chain, troponin T, troponin I, α -MHC, and ANP[9]. Sca-1+ cells facilitate the expression of cardiac structure proteins, such as myosin and connexin43. These cells also possess multipotency. After being given intravenously to mice post-MI, these stem cells homed to injured myocardium and differentiated into cardiomyocytes, endothelial cells and smooth muscle cells in the border zone 4 weeks after transplantation.

In the cardiosphere culture, cardiac fibroblasts, which are the majority cell population in the normal adult heart[20], tend to spread in sheet-like extensions. They provide a feeder layer for cardiac stem cells proliferation and migration from explants[10]. To separate the cells from the fibroblasts, yet maintain the benefits of the fibroblasts attempts have been made to replace the fibroblasts. In mouse embryonic stem cell (ES) culture, it was reported that the feeder layer can be replaced by addition of leukemia inhibitory factor (LIF) to the growth medium to maintain ES in undifferentiated state[23]. Considering that the soluble paracrine factors produced by cardiac fibroblasts may be important for the proliferation of cardiac stem cells, we evaluated the ability of cardiac fibroblast-free conditioned medium to support cardiac stem cell growth. We conditioned the cell culture medium by exposing it for 48 hours to fibroblast culture to extract paracrine factors from fibroblasts. Pure Sca-1+ cells were grown on Matrigel, which provides the required adhesion for survival and growth of cardiac stem cell because it contains mostly laminin, collagen IV, and heparin sulfate proteoglycan. Addition of the fibroblast-free conditioned cell medium demonstrated that cultures on Matrigel coated plate fostered cardiac stem cell growth while avoiding the contamination of fibroblasts.

In conclusion, we have developed two-step procedure suitable for the isolation of intact cardiac Sca-1+ cells. This method avoids the possible enzymatic destruction of functional stem cell markers at cell surface. Using a fibroblast-free culture system, with the conditioned medium improved the growth of cardiac stem cells. The Sca-1+ cells were demonstrated to be functional in vitro and in vivo. After been injected into mice with myocardial ischemia, Sca-1+ cells were detectable in the heart for up to one month after injection, and differentiated to cardiomyocytes, endothelial cells and smooth muscle cells. This two-step procedure can also be used to isolate other possible types of cardiac stem cell, such as c-kit or ABCG2, and makes purified stem cells an attractive source for cardiac regeneration from any species.

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Figure 1.

Isolation and expansion of Sca-1+ cells. **A:** Phase-contrast microscopy of cells migrated from primary culture of mouse ventricular explants. **B:** migrated cells aggregated and proliferated above the coating of fibroblasts (white arrow: new round bright cells migrated off the explant; green arrow: fibroblast layer). **C:** Culture of purified Sca-1+ cells after MACS enrichment. Isolated Sca-1+ cells form spheres without contamination of cardiac fibroblastic cells. **D:** Cell growth curves of unsorted cardiosphere derived cells, Sca-1+ cells with CGM medium and Sca-1+ cells with conditioned CGM (CCGM) medium.

Figure 2. Flow cytometric analysis of Sca-1⁺ cell population. Representative marker expression analyses are indicated for the following: A: Sca-1 and CD45; B: Sca-1; C: c-kit; D: CD31; E: CD34.

Figure 3.

Immunophenotype characterization of purified Sca-1+ cells grown on coated wells. Cells were counterstained with Draq5 (blue). **A–C:** Immunofluorescent imaging demonstrated that some cells in sca-1+ cells express other stem cell markers (c-kit and ABCG2) and endothelial cell marker (FLK-1). **D:** Sca-1+ cells expressed GATA4, a marker for cardiac specific transcription factor. **E:** sca-1+ cells expressed phosphor-H3 in nuclei, a marker of mitotic Cdc2 activity. **F:** Spontaneous differentiation of Sca-1+ cell-formed sphere in vitro. F1–F3: Immunofluorescence staining of sphere with anti-connexin43 (green) and anti-myosin (red) after 2 days of exposure to low serum medium. Cells were counterstained with Draq5 (blue).

Figure 4.

Engraftment and multilineage differentiation of graft Sca-1+ cells in ischemic myocardium.

A: β-gal-expressing Sca-1+ cells. β-gal staining in vitro demonstrated that most of cells express β-gal. **B:** Merged images of double staining of sections for β-gal (green) and for cardiac-specific proteins: cTnI (red) demonstrated the colocalization of reporter with cardiac-specific protein (yellow). **C–D:** Merged images of double staining of sections for β-gal (green) and for CD31 (red) and SM-α-actin (red) demonstrated that Sca-1+ cells integrated into new blood vessels (yellow). Arrow: grafted LacZ-labeled Sca-1+ cells.