International Journal of Cardiology 227 (2017) 378–386

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Proliferation, differentiation and migration of SCA1$^-$/CD31$^-$ cardiac side population cells *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 3 June 2016
Received in revised form 3 September 2016
Accepted 5 November 2016
Available online 9 November 2016

Keywords:
Stem cells
Side population cells
Myocardial ischemia
Cell migration
CXCR4 receptor

ABSTRACT

Background: Side-population (SP) cells, identified by their capacity to efflux Hoechst dye, are highly enriched for stem/progenitor cell activity. They are found in many mammalian tissues, including mouse heart. Studies suggest that cardiac SP (CSP) cells can be divided into SCA1$^+$/CD31$^-$, SCA1$^+$/CD31$^+$ and SCA1$^-$/CD31$^-$ CSP subpopulations. SCA1$^+$/CD31$^-$ were shown to be cardiac and endothelial stem/progenitors while SCA1$^+$/CD31$^+$ CSP cells are endothelial progenitors. SCA1$^-$/CD31$^-$ CSP cells remain to be fully characterized. In this study, we characterized SCA1$^-$/CD31$^-$ CSP cells in the adult mouse heart, and investigated their abilities to proliferate, differentiate and migrate *in vitro* and *in vivo*.

Methods and results: Using fluorescence-activated cell sorting, reverse transcriptase/polymerase chain reaction, assays of cell proliferation, differentiation and migration, and a murine model of myocardial infarction we show that SCA1$^-$/CD31$^-$ CSP cells are located in the heart mesenchyme and express genes characteristic of stem cells and endothelial progenitors. These cells were capable of proliferation, differentiation, migration and vascularization *in vitro* and *in vivo*. Following experimental myocardial infarction, the SCA1$^-$/CD31$^-$ CSP cells migrated from non-infarcted areas to the infarcted region within the myocardium where they differentiated into endothelial cells forming vascular (tube-like) structures. We further demonstrated that the SDF-1α/CXCR4 pathway may play an important role in migration of these cells after myocardial infarction.

Conclusions: Based on their gene expression profile, localization and ability to proliferate, differentiate, migrate and vascularize *in vitro* and *in vivo*, we conclude that SCA1$^-$/CD31$^-$ CSP cells may serve as endothelial progenitor cells in the adult mouse heart.

1. Introduction

The prevailing view of the heart, as a terminally differentiated organ, has been challenged by the discovery of resident cardiac stem cells (CSCs), potentially bringing the treatment of cardiac diseases into a new era. Various subsets of resident CSCs have been identified that are capable of self-renewal, proliferation, and differentiation into cardiomyocytes, endothelial and smooth muscle cells. These findings were based on: (i) expression of stem cell-associated markers, such as c-kit and SCA1 [1,2], (ii) a specific culture system to obtain cardiospheres that expressed cardiac and smooth muscle genes [3], (iii) expression of the homeodomain transcription factor, Isl-1 [4], and (iv) discovery of cardiac side-population (SP) cells. After labeling with Hoechst 33342 dye, SP cells can be distinguished by their weak labeling compared to the main population (MP) of labeled cells [5–7]. This unique property of SP cells can be explained by their expression of the p-glycoprotein multidrug/ATP-binding cassette transporter protein ABCG2, which pumps Hoechst dye out of the cell [8,9]. Side-population cells isolated from adult mammalian tissues and organs, including heart, are enriched for stem/progenitor cell activity [7,9–11].

Studies from different groups, including ours suggested that cardiac SP (CSP) cells are capable of differentiation into cardiomyocytes as well as endothelial and smooth muscle cells [12–15]. Based on expression of SCA1 and CD31, murine CSP cells can be divided into SCA1$^+$/CD31$^-$, SCA1$^+$/CD31$^+$ and SCA1$^-$/CD31$^-$ cells [7,14,16]. *In vitro* and *in vivo*

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http://dx.doi.org/10.1016/j.ijcard.2016.11.047
0167-5273/

studies have shown that SCA1$^+$/CD31$^-$ CSP cells are able to differentiate into cardiomyocytes and endothelial cells, while SCA1$^+$/CD31$^+$ CSP cells differentiate into endothelial cells [7,14,15]. However, the nature and differentiation potential of SCA1$^-$/CD31$^-$ CSP cells has remained unclear.

Cell migration is an important character of stem cells. We have shown that, after myocardial infarction (MI) in mice, SCA1$^+$/CD31$^-$ and SCA1$^+$/CD31$^+$ CSP cells migrate from the healthy myocardium into the infarcted area. Our studies further suggested that the SDF-1α/CXCR4 pathway plays an important role in this process [14,15]. However, the capacity of SCA1$^-$/CD31$^-$ CSP cells to migrate and the role of SDF-1α/CXCR4 pathway in their behavior remain unknown.

In this study, we set out to characterize SCA1$^-$/CD31$^-$ CSP cells in the adult mouse heart, and to investigate their capacity for proliferation, differentiation, vascularization and migration *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6 J mice (female, 8–10 weeks) were purchased from Beijing HFK-Bio-Technology (Beijing, China). Mice were group-housed in individually-ventilated cages under pathogen-free conditions. The general health status of the animals was monitored daily. All animal experiments described herein were conducted strictly according to ethical standards approved by the institutional animal ethical committee of Jinzhou Medical University (approval ID: LY2014D001). We have consulted the ARRIVE guidelines in preparing this report [17].

2.2. CSP cell isolation, fluorescence-activated cell sorting and analysis, and magnetic depletion of CD45$^+$ cells

Isolation of CSP cells was performed as described [14,15]. Briefly, cardiac tissue was digested with 0.1% collagenase B, 2.4 U/ml dispase II and 2.5 mmol/l $CaCl_2$ for 45 min. Cell suspensions were incubated for 90 min with Hoechst 33342 (5 μg/ml; Sigma-Aldrich, USA) at a density of 10^6 cells/ml with or without verapamil (50 mM; Sigma-Aldrich, USA) [9]. The suspension was subsequently incubated with one or more fluorescently-conjugated monoclonal rat anti-mouse antibodies including anti-SCA1-FITC, anti-CD31-PE and anti-CXCR4-APC (BD, USA). For negative control, cells were incubated with isotope/control antibodies to establish gating parameters for positively-stained cells. All incubations were performed for 30 min at 4 °C in the dark.

To remove CD45$^+$ cells from cell suspensions of 3-day post-MI hearts, biotin-conjugated anti-CD45 antibody and streptavidin-conjugated microbeads (Miltenyi Biotech, Germany) were used as described [14,15]. Purification of the cells was analyzed by flow cytometry using PE-conjugated anti-CD45 antibody (BD, USA) staining. The cells were stained with Hoechst 33342 following by SCA1, CD31 and CD184 as mentioned above.

Fluorescence-activated cell sorting (FACS) on a FACS Vantage-SE-Cell Sorter (BD, USA) was used to isolate SCA1$^+$/CD31$^-$, SCA1$^+$/CD31$^+$, SCA1$^-$/CD31$^-$ CSP and SCA1$^-$/CD31$^-$ main population (MP) cells for all experiments. The following 6 parameters were used to discriminate cells in the sorts: Hoechst 33342 (red), Hoechst 33342 (blue), forward scatter, side scatter, FITC-SCA1 and PE-CD31 reactivity (green) as described [14,15]. This protocol is illustrated in Supplementary Fig. S1. The Hoechst dye was excited at 350 nm. A 488-nm argon laser was used for exciting PE and FITC, and a 633-nm HeNe laser for APC. Data were analyzed using BD FACSDiva™ software v4.12 (BD, USA).

2.3. Methylcellulouse assay (colony forming unit assay)

SCA1$^-$/CD31$^-$ CSP and MP cells (7000 cells/ml) were plated in Methocult GF M3534 media following the manufacturer's instructions (StemCell Technologies, Canada) as described [5,7,15]. Cell colonies consisting of more than 30 cells were scored after 14 day in culture. In order to determine whether the cells retained their SCA1$^-$/CD31$^-$ CSP phenotype, Methocult media was cut into pieces and incubated with DMEM at 37 °C with shaking for 35 min. The resuspended cells were collected and expression of the ABCG2, CD133 and SCA1 genes was examined by semi-quantitative real-time RT-PCR (qRT-PCR).

2.4. Primary cell culture

SCA1$^-$/CD31$^-$ CSP cells were plated into fibronectin-coated 8-well chamber slides (10,000 cells/well) and were cultured with a defined endothelial differentiation medium (EGM-2) from Cambrex (Baltimore, MD) and antimicrobial agents. They were maintained in humidified 5% CO_2/air at 37 °C. Culture medium was changed regularly. After 14 days, cells were collected and expression of the ABCG2, CD133, vWF and Tie2 genes was examined by qRT-PCR.

2.5. Immunofluorescence staining

The heartswere harvested 14 days after MI. Hearts embedded in OCT (Tissue-Tek, USA) were frozen and were cryosectioned at 5 μm. Slides were stored at −20 °C before staining. Immunofluorescence labeling of heart tissue was performed as described [11,15]. Primary antibodies included polyclonal rabbit anti-mouse vWF, monoclonal rat anti-mouse ABCG2 (Chemicon, USA), polyclonal rabbit anti-mouse SCA1 (R&D System, USA), and monoclonal anti-mouse α-actinin FITC (Sigma-Aldrich, USA). Secondary antibody combinations included goat anti-rabbit IgG-FITC (Invitrogen, USA) goat anti-rat IgG biotin (Vector-Lab, USA) and streptavidin-Cy3 (BD, USA). Nuclei were counterstained with 4-6-diamidino-2-phenylindol-dihydrochloride (DAPI). Non-specific IgG-isotypes were employed as negative controls (Vector-Lab, USA). Slides were imaged using an Olympus-DP70 microscope and images were captured with a digital camera BX51.

2.6. Cellular uptake of dil-labeled acetylated low-density lipoprotein (DiI-Ac-LDL)

SCA1−/CD31- CSP cells were isolated and incubated for 8 h at 37 °C with endothelial differentiation medium (EGM-2) containing 10 mg/ml of DiI-Ac-LDL (Molecular Probes, USA) in fibronectin-coated 8-well chamber slides (7000 cells/well). After washing with PBS, cells were fixed and counterstained with DAPI as described [15]. Slides were imaged as described above.

2.7. Semi-quantitative real-time RT-PCR

Total RNA was extracted from distinct CSP cell isolates using a RNAeasy-Mini Kit according to the manufacturer's instruction (Qiagen, Germany). Complementary DNA was generated using M-MLV MicroRNA Reverse Transcription Kit based on the manufacturer's instruction (Promega, USA). qRT-PCR was performed with Rotor-gene 3000 (Corbett-Research, Australia) and a SYBER Green qPCR-SuperMix-UDG kit (Invitrogen, USA) based on the manufacturers' instructions. Primers for the qRT-PCR were purchased from Qiagen, including ABCG2 (ID: QT00173138), VEGFR2 (ID: QT00097020), VEGFR1 (ID: QT00096292), Tie2 (ID: QT00114576), vWF (ID: QT00116795), CD133 (ID: QT01065162), Nkx2.5 (ID: QT00124810), SCA1 (ID: QT00293167), E-cadherin (ID: QT00121163) and β-actin (ID: QT01136772). The thermal cycler conditions were as follows: Cycle 1 (95 °C for 3 min) ×1, Cycle 2 (95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s) ×45, Cycle 3 (72 °C for 4 min). All samples were amplified in triplicate. Gene expression levels of ABCG2, VEGFR2, VEGFR1, Tie2, vWF, CD133, Nkx2.5, SCA1, and E-cadherin were normalized to a housekeeper gene, β-actin. Amplification data was analyzed with Corbett Rotor-gene 3000 software (Corbett Research, Australia) as described [15]. Gene expression data are presented in relative units.

2.8. Mouse model of myocardial infarction and in vivo cell migration study

Experimental MI was induced by permanent ligation of the left anterior descending coronary artery (LADCA) in C57BL/6 J mice (female, 8–10 weeks) as we previously described [14,15]. Briefly, mice were anesthetized with an intraperitoneal injection of 0.1 ml/10 g body weight of a cocktail consisting of 1 part Hypnorm, 1 part Midazolam and 2 parts distilled water by volume. Unconsciousness was monitored based upon complete suppression of the foot withdrawal full reflex. They were ventilated with a standard rodent ventilator (Harvard Ventilator) which was set to the rate of 110 ± 5 respirations per minute with a tidal volume of 2 ml. The thoracic cavity was opened by incision between the second and the third intercostal space, a rodent rib spreader was introduced to permit visualization of the heart and the LAD coronary artery was ligated with suture. Approximately 150,000 SCA1$^-$/CD31$^-$ CSP cells, labeled with the red fluorescence tracker dye, PKH26 (Sigma-Aldrich, USA), were injected into the (non-ischemic) right ventricle wall. The needle was directed toward the right auricle to ensure that the cells were delivered away from the ischemic area. Finally, surgical stitching was used to appose subcutaneous tissue and skin. After surgery, animals were placed on heating pads maintained at 37 °C until they regained mobility. Ten hours post-operation, they received buprenorphine for analgesia (0.03 mg/kg, intraperitoneal injection). Nerve reflexes, basic sense of movement and muscle tension were regularly monitored during all the post-operation period, as previously described [14,15]. Fourteen days later the mice were sacrificed by cervical dislocation and the hearts were harvested. In some experiments, hearts with LADCA ligation were harvested 3 days post-MI to study expression of CXCR4 or E-cadherin in CSP cells. Sham-operated mice underwent identical surgical procedures except without LADCA ligation.

2.9. Chemotaxis assay

The *in vitro* migration of SCA1$^-$/CD31$^-$ CSP cells was appraised using polycarbonate membrane Transwell® inserts (Millipore, USA) with 8 μm pores size as previously described [14,15]. Briefly, 30,000 cells in 50 μl DMEM without serum were added to the upper surface of the membrane in each chemotaxis chamber. SDF-1α (R&D Systems, USA) at a concentration of 0, 50, 100 or 500 ng/ml was placed in the lower chamber. After 4 h of incubation at 37 °C in 5% CO_2 and 95% humidity, the upper membrane surface was scraped free of cells and debris, and gently washed with PBS. Membranes were then fixed and stained as described [14]. Cell migration was measured by counting the number of cells that had migrated through pores and adhered to the lower surface of the membrane in five adjacent high-power fields (40×). To block the SDF-1α signal, the cells were incubated with anti-CXCR4 antibody (10 μg/ml, eBioscience, USA) [14] for 30 min