FEBS Letters 581 (2007) 4681–4684

VEGF receptor Flk-1 plays an important role in c-kit expression in adipose tissue derived stem cells

Xiaowen Bai[1], Sanga Sadat[1], Sebastian Gehmert, Eckhard Alt, Yao-Hua Song*

Department of Molecular Pathology, University of Texas, MD Anderson Cancer Center, SCRB2, Box 951, 7435 Fannin Street, Houston, TX 77054, United States

Received 4 August 2007; revised 24 August 2007; accepted 27 August 2007

Available online 5 September 2007

Edited by Veli-Pekka Lehto

Abstract **It is known that c-kit$^+$ cells are increased in heart after infarction. The exact origins of the cardiac c-kit$^+$ cells remain to be determined. We asked whether adipose tissue could be a potential source of c-kit$^+$ cells. Our data show that the number of c-kit$^+$ cells increased in adipose tissue derived stem cells when cultured with conditioned medium from neonatal cardiomyocytes grown under serum deprivation and hypoxia condition. We also found that VEGF receptor Flk-1 is involved in c-kit up regulation via ERK-mediated pathway.**

Keywords: Stem cell; c-Kit; Infarction

1. Introduction

Beltrami et al. reported the existence of Lin$^-$ c-kit$^+$ cells with the properties of cardiac stem cells [1]. These cells are self-renewing, clonogenic, and multipotent, giving rise to myocytes, smooth muscle, and endothelial cells. Recently, the origin of the cardiac c-kit$^+$ was traced back to bone marrow [2]. However, it is known that bone marrow derived c-kit$^+$ cells are CD45 positive whereas cardiac c-kit$^+$ cells are CD45 negative. We reasoned that cardiac c-kit$^+$ cells could also be derived from other tissue/organs. It has been shown that adipose tissue derived stem cells (ASCs) contain CD45$^-$ plastic adherent mesenchymal stem cells that have both mesodermal and ectodermal capacity [3–5]. ASCs engrafted into injured myocardium and that this engraftment is associated with expression of cardiomyocytic markers by donor-derived cells [6]. We demonstrated VEGF receptor Flk-1 activation is responsible for spontaneous differentiation of human ASCs into a cardiomyocyte phenotype via paracrine mechanism [7]. In the present study, we asked whether adipose tissue could also be a potential source of c-kit$^+$ cells and whether c-kit$^+$ expression can be regulated by soluble factors released by neonatal cardiomyocytes exposed to serum deprivation/hypoxic condition and whether VEGF receptor Flk-1 plays a role in c-kit$^+$ expression in ASCs.

2. Materials and methods

2.1. Isolation and culture of hASCs

Subcutaneous adipose tissue was obtained from patients undergoing elective operations following the University Institutional Guidelines. Cells were isolated from the fat tissue as described previously [8] with modifications. Fat tissue was minced and incubated for 90 min at 37 °C on a shaker with Liberase Blendzyme 3 (Roche) at a concentration of 4 units per gram of fat tissue in PBS. The digested tissue was sequentially filtered through 100 μm and 40 μm filters (Fisher Scientific) and centrifuged at ($450 \times g$) for 10 min. The supernatant containing adipocytes and debris was discarded and the pelleted cells were washed twice with Hanks Balanced Salt Solution (Cellgro) and finally resuspended in growth media. Growth media contained α-modification of Eagle's medium (αMEM, Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine (Cellgro), 100 U/ml penicillin with 100 μg/ml streptomycin (Cellgro). Plastic adherent cells were designated human adipose tissue-derived stem cells (hASCs) and grown in α-MEM containing 20% fetal bovine serum. Flk-1 and Flt-1 neutralizing antibodies were purchased from R&D Systems. Kinase inhibitors (PD098059 and JNK II) were purchased from Calbiochem.

2.2. Flow cytometric analysis of phenotype in hASCs

hASCs of the third passage from 3 donors were used for phenotype analysis. Cell aliquots (1×10^6 cells/1 ml) were stained with primary antibodies at room temperature for 30 min. The primary antibodies were fluorescein isothiocyanate (FITC)-conjugated anti-human CD44 (Chemicon), CD34, CD90, HLA-DR (USBiological) or phycoerythrin (PE)-conjugated anti-human CD11b, CD105 (eBioscience), CD14, CD45 (USBiological). Monoclonal antibody against c-kit was purchased from Sigma. Isotype-matched normal mouse IgGs (Chemicon) were used as controls. Flow cytometry was performed on a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences, San Jose, CA) and data analysis was performed with Cell Quest software (Becton-Dickinson).

2.3. Adipogenic differentiation

The adipogenic differentiation potential for stem cells was analyzed as described previously [9,10]. The cells of passage three were harvested using trypsin/EDTA and plated in 24-well plates at 30,000 cells/cm^2 for 16 h to allow attachment. At 100% confluency, cells were then switched to adipogenic medium containing low glucose Dulbeco's modification of Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Cellgro) 100 μM L-ascorbate acid (Sigma), 1 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 100 μM indomethacin and 10 μg/ml human recombinant insulin (Sigma) for 21 days. Cells of the control group were cultured in low glucose DMEM plus 10% FBS (control medium). Medium were changed every three days. Adipogenesis of ASCs was assessed by staining cells with Oil Red O (Sigma).

*Corresponding author. Fax: +1 713 834 6083.
E-mail address: ysong@mdanderson.org (Y.-H. Song).

[1]These authors contributed equally to this work.

Abbreviation: ASCs, adipose tissue derived stem cells

0014-5793/$32.00
doi:10.1016/j.febslet.2007.08.063

2.4. Osteogenic differentiation

Osteogenic differentiation [11] was assessed by incubating the cells of 100% confluence with high-glucose DMEM plus 10% FBS supplemented with 0.1 μM dexamethasone, 200 μM L-ascorbic acid, and 10 mM β-glycerol phosphate (Sigma). Media was changed every three days for three weeks. Cells of the control group were cultured in high-glucose DMEM plus 10% FBS for three weeks. To assess mineralization, calcium deposits in cultures were stained with Alizarin red stain (Sigma).

2.5. Neurogenic differentiation

Trypsinized cells at passage 3 were added on the coverslips coated with fibronectin in wells at a density of 3×10^4 cells per cm^2 in αMEM containing 20% FBS and antibiotics. The medium was replaced the following day by induction medium containing DMEM/F12, 1% FBS, 2% B27 (Invitrogen), 10 ng/ml bFGF (Chemicon), 10 ng/ml EGF, 10 μM forskolin, 1 mM cAMP (for human 2 Mm), 5 μg/ml insulin, 0.5 mM 1-methyl-3-isobutylxanthine, 1 mM 2-mercaptoethanol, 50 μM Vitamin C (Sigma), 10 ng/ml NGF (R&D) for 21 days. Cells were fixed with methanol at −20 °C for 10 min and washed, and immunocytochemistry performed using standard methods. In brief, cells were blocked with 10% donkey serum and then incubated with a polyclonal antibody against a MAb against microtube related protein-2 (MAP-2, abcam) overnight at 4 °C. After washing, cells were incubated with Alex-488 donkey anti-rabbit antibody (1:1000, Invitrogen). A Zeiss microscope (Germany) with a camera and controller software was used to capture images.

2.6. Hepatogenic differentiation

Trypsinized cells at passage 3 were added on the coverslips coated with fibronectin at a density of 3×10^4 cells per cm^2 in αMEM containing 20% FBS and antibiotics. The medium was replaced the following day by induction medium containing DMEM (1 g/l glucose), 1% FBS, 10 ng/ml bFGF, 20 ng/ml aFGF (Chemicon), 1% ITS, 10 ng/ml EGF (Sigma), 10 ng/ml OSM, 20 ng/ml HGF (R&D) for 21 days. Hepatogenesis was verified by immunofluorescence staining of hepatocytes specific antigen albumin (abcam).

2.7. Cardiomyocyte extraction

Cardiomyocytes were obtained from neonatal Sprague-Dawley rats following the guidelines of Veterinary Medicine and Surgery at MD Anderson Cancer Center. For primary culture of Neonatal Rat Cardiomyocytes (NRCMs) a commercial kit was used (Worthington). Briefly, the hearts were rinsed in HBSS, minced to pieces less than 1 mm^3 in size. Trypsin (50 μg/ml) was added and the minced tissue was incubated overnight at 2–4 °C. Twenty-four hours later trypsin inhibitor was added and the tissue was subsequently digested with collagenase (300 U/ml) by incubating at 37 °C for 45 min. The supernatant was filtered through 70 μm cell strainer, centrifuged at $80 \times g$ for 5 min and resuspended in Leibovitz L-15 Media and plated at a density of 100,000 cardiomyocytes per cm^2. Beating myocytes were observed within 18 h and used immediately for subsequent experiments. Conditioned medium was concentrated with Centriprep Centrifugal Filter Unit (Millipore).

2.8. Statistics

All values are presented as means ± standard deviation of the mean (S.D.). Statistical analysis was performed using Student's *t*-tests. A level of $p < 0.05$ was considered as statistically significant.

3. Result

3.1. ASC characterization

These cells are plastic adherent, spindle shaped cells (Fig. 1). The ASCs are positive for CD44 (99.36 ± 0.75%), CD90 (97.59 ± 2.45%), CD105 (98.51 ± 1.83%) and negative for CD11b (0.33 ± 0.18%), CD14 (0.51 ± 0.11%), CD34 (1.09 ± 0.16%), CD45 (0.39 ± 0.29%) and HLA-DR (0.68 ± 0.92%) (Fig. 2).

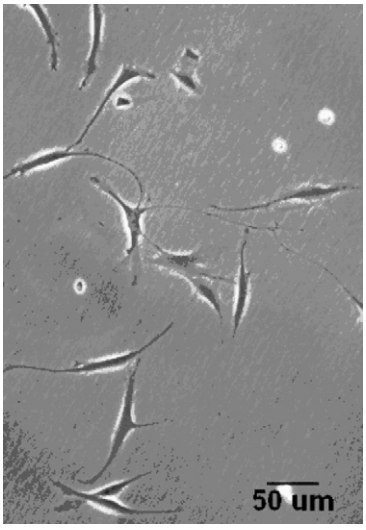

Fig. 1. Characterization of human ASCs. Human ASCs are spindle shaped and plastic adherent when cultured in α-MEM containing 20% FBS.

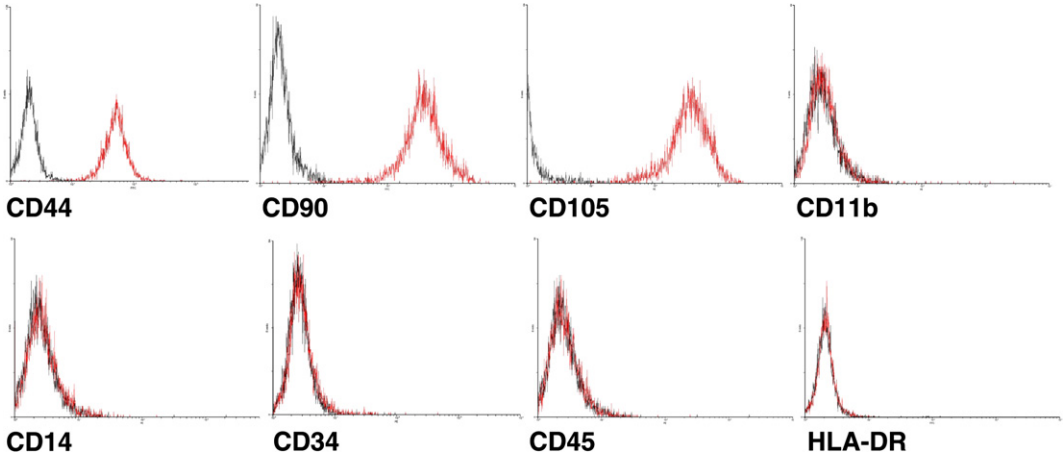

Fig. 2. Flow cytometric analysis of surface markers. Flow cytometric analysis of surface markers demonstrated that ASCs express CD markers that are typical for mesenchymal stem cells. Black histograms indicate isotype-matched controls; red histograms show surface antigen expression level.