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Hiding inside? Intracellular expression of non-glycosylated c-kit protein in cardiac progenitor cells

Huilin Shi [a], Christopher A. Drummond [a], Xiaoming Fan [a], Steven T. Haller [a], Jiang Liu [b], Deepak Malhotra [a], Jiang Tian [a,*]

[a] Department of Medicine, Division of Cardiovascular Medicine, College of Medicine and Life Sciences, University of Toledo, Toledo, OH 43614, USA
[b] Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755, USA

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ABSTRACT

Cardiac progenitor cells including c-kit$^+$ cells and cardiosphere-derived cells (CDCs) play important roles in cardiac repair and regeneration. CDCs were reported to contain only small subpopulations of c-kit$^+$ cells and recent publications suggested that depletion of the c-kit$^+$ subpopulation of cells has no effect on regenerative properties of CDCs. However, our current study showed that the vast majority of CDCs from murine heart actually express c-kit, albeit, in an intracellular and non-glycosylated form. Immunostaining and flow cytometry showed that the fluorescent signal indicative of c-kit immunostaining significantly increased when cell membranes were permeabilized. Western blots further demonstrated that glycosylation of c-kit was increased during endothelial differentiation in a time dependent manner. Glycosylation inhibition by 1-deoxymannojirimycin hydrochloride (1-DMM) blocked c-kit glycosylation and reduced expression of endothelial cell markers such as Flk-1 and CD31 during differentiation. Pretreatment of these cells with a c-kit kinase inhibitor (imatinib mesylate) also attenuated Flk-1 and CD31 expression. These results suggest that c-kit glycosylation and its kinase activity are likely needed for these cells to differentiate into an endothelial lineage. In vivo, we found that intracellular c-kit expressing cells are located in the wall of cardiac blood vessels in mice subjected to myocardial infarction. In summary, our work demonstrated for the first time that c-kit is not only expressed in CDCs but may also directly participate in CDC differentiation into an endothelial lineage.

1. Introduction

Studies have shown that progenitor cells exist in adult hearts, including aged and diseased hearts (Mercola et al., 2011; Olson and Schneider, 2003). Different types of cardiac progenitor cells were discovered including c-kit$^+$, Sca-1$^+$, Islet-1$^+$, SSEA-1$^+$ cells, as well as side population and cardiosphere-derived cells (CDCs) (Nigro et al., 2015). Animal and clinical studies have shown that c-kit$^+$ cells and CDCs play important roles in cardiac repair and regeneration (Leri et al., 2015; Marban and Cingolani, 2012; Ellison et al., 2013; Hariharan et al., 2015; Fransioli et al., 2008). C-kit$^+$ progenitor cells were first identified in rat cardiac tissues (Beltrami et al., 2003). Despite debate on the role of c-kit$^+$ cells in myogenesis (Leri et al., 2015; Goldstein et al., 2015; van Berlo et al., 2014), c-kit protein expression is the most recognized progenitor cell marker. CDCs were initially isolated from human and murine cardiac tissue (Messina et al., 2004) and were introduced as a candidate progenitor cell for regenerative therapy after myocardial infarction (MI) (Smith et al., 2007). Clinical trials such as CADUCEUS (Makkar et al., 2012) demonstrated that injection of CDCs improved cardiac function and increased viable tissue in patients with MI. Finally, it was reported that CDCs contain a small subpopulation of c-kit$^+$ cells, ranging from ~1% to ~25% (Messina et al., 2004; Smith et al., 2007; Cheng et al., 2014).

C-kit protein, first identified as a virus proto-oncogene, v-kit (Besmer et al., 1986), is a tyrosine kinase and a receptor for stem cell factor (SCF), containing nine N-glycosylation sites in its sequence (Nigro et al., 2015; Yarden et al., 1987). It is known that c-kit undergoes N-linked glycosylation in the endoplasmic reticulum (ER) before being transported to the Golgi apparatus where it is modified by further complex glycosylations and subsequently transported to the cell surface (Aebi, 2013). Two forms of c-kit protein, a non-glycosylated form (~100–120 kDa) and a glycosylated form (~140 kDa), were detected previously in cancer cells (Blume-Jensen et al., 1991; Rubin et al., 2001; Schmidt-Arras et al., 2005). In studies of cardiac progenitor cells, cellular distribution and glycosylation of c-kit have not been evaluated.

Increased c-kit$^+$ cell number was observed in different disease states, and conditional knockout of c-kit was found to abolish cardiac

* Corresponding author at: University of Toledo, College of Medicine and Life Sciences, Department of Medicine, Cardiology Division, Health Science Campus, Mail Stop 1025, 3000 Arlington Ave., Toledo, OH 43614-2598, USA.
E-mail address: Jiang.Tian@utoledo.edu (J. Tian).

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regeneration in experimental heart failure (Ellison et al., 2013; Ellison et al., 2007). Our previous study also showed that c-kit$^+$ cells were increased in cardiac tissue from mice with chronic kidney disease, particularly in transgenic mice with reduced Na/K-ATPase-Src mediated signaling capability (Drummond et al., 2014). However, the specific role of c-kit protein and its regulatory mechanism in these progenitor cells remain elusive. The current work studied c-kit expression and its potential role in cardiac progenitor cell differentiation into an endothelial lineage.

2. Materials and methods

2.1. Animals

Animal experiments were conducted in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee at the University of Toledo. Mice from an inbred C57BL6/J strain (Moseley et al., 2004) were maintained at the University of Toledo. Adult male mice which were two months of age were used for isolation of cardiosphere-derived progenitor cells and for myocardial infarction experiments.

2.2. Isolation of cardiac progenitor cells

Cardiosphere-derived cells (CDCs) were obtained following procedures previously described (Messina et al., 2004; Smith et al., 2007) with minor modifications. Briefly, gross connective tissue was removed by blunt dissection to obtain pure mouse heart muscle tissue, which was then cut into small explants (~1 mm in dimension). The explants were washed and partially digested enzymatically with 0.025% Trypsin/0.01% ethylenediaminetetraacetic acid (EDTA. Gibco Inc., Grand Island, NY, Cat. No.: R001-100) for 10 min. The explants were then cultured on dishes coated with fibronectin (Santa Cruz Inc., Santa Cruz, CA, Cat. No.: sc-29011) in complete explant medium (CEM), which contains: 500 mL Iscove's Modified Dulbecco's Medium (IMDM, Gibco Inc., Cat. No.: 12440), 125 mL Fetal bovine serum (FBS) (Gibco Inc., Cat. No.: 10437), 1% penicillin–streptomycin (Mediatech Inc., Manassas, VA, Cat. No.: 30-002-Cl), 1 mmol/L L-glutamine (Gibco Inc., Cat. No.: 25030), and 1 mmol/L 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, Cat. No.: M6250). After 14 days, a layer of stromal-like cells grew out of adherent explants, over which small, round, phase-bright cells migrated. These phase-bright cells were harvested by sequential Versene (Gibco Inc., Cat. No.: 15040) and 0.025% Trypsin-0.01% EDTA digestion and seeded on poly-D-lysine coated (Sigma-Aldrich, Cat. No.: P6407) dishes in cardiosphere growth medium (CGM) that contains: 175 mL IMDM, 325 mL DMEM/F12 (Gibco Inc., Cat. No.: 11330), 3.5% FBS, 1% penicillin–streptomycin, 1 mmol/L L-glutamine, 2% B27 supplement (Gibco Inc., Cat. No.: 17504), 1 mmol/L 2-mercaptoethanol, 80 ng/mL basic Fibroblast growth factor (bFGF, PeproTech Inc., Rocky Hill, NJ, Cat. No.: AF-450-33), 25 ng/mL epidermal growth factor (EGF, PeproTech Inc., Cat. No.: 315-09), 4 ng/mL cardiotrophin-1 (PeproTech Inc., Cat. No.: 250-25), 1 unit/mL α-Thrombin (Haemtech Inc., Essex Junction, VT, Cat. No.: HCT-0020). The seeded cells formed cardiospheres on poly-D-lysine coated dishes following 7 to 21 days. Subsequently, the cardiosphere forming cells were collected and plated on fibronectin-coated dishes in CGM where they expand as monolayers. CDCs were expanded and stocks were collected and stored in liquid nitrogen for later experiments.

2.3. Inducing endothelial lineage differentiation of CDCs

CDCs at the 5th–15th passages were used for endothelial differentiation. CDCs were seeded on matrigel-coated dishes or glass coverslips. For Western blotting, CDCs grew in CGM for three days at which time they reached 80% confluence. Then media was changed to complete mouse endothelial cell culture medium (MECM, Cell Biologics Inc., Chicago, IL, Cat. No.: M1168) to start endothelial differentiation. For immunostaining, CDCs were allowed to attach to coverslips in CGM overnight, at which point the media was changed to MECM to start endothelial differentiation. MECM was changed every two days during differentiation until cells were collected for Western blot or immunostaining. Following the start of endothelial differentiation cells were collected on days 1, 3, 7, 10, 14, 21, and 28.

For inhibition of N-glycosylation in CDCs, 1-deoxymannojirimycin hydrochloride (1-DMM, Sigma-Aldrich, Cat. No.: D9160), an inhibitor of N-linked glycosylation, was added to MECM at 5, 50 and 500 μM concentrations during induction of endothelial differentiation. CDCs in MECM without 1-DMM were used as control. The induction media was changed every two days. Cells were collected on day 7 and 14 for Western blot.

Specific inhibition of c-kit kinase activity was achieved through the use of the c-kit tyrosine kinase inhibitor imatinib mesylate (Selleckchem, Cat. No.: S1026) which was was added to MECM at 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM concentrations during the 14 day endothelial differentiation induction period. Cells treated with MECM alone without imatinib mesylate were used as controls. The induction media was changed every two days. Cells were collected on day 14 for Western blot.

2.4. Immunocytochemistry

Cells were fixed in 3% paraformaldehyde. Following fixation, cells were washed with 1× Dulbecco's phosphate buffered saline (DPBS; Gibco, Cat. No: 14190-144) three times and were then blocked with 2% Bovine Serum Albumin (BSA; Sigma-Aldrich Inc., Cat. No: A4503) in DPBS containing 0.3% Triton X-100 (DPBS-Tr) for 1 h at room temperature. Subsequently, cells were incubated with primary antibodies diluted in blocking buffer in a humidified chamber overnight at 4 °C. The next day, cells were washed three times with DPBS-Tr. Cells were then incubated with secondary antibodies diluted in blocking buffer at room temperature for 1.5 h. Following incubation with secondary antibody, the cells were washed with DPBS-Tr one time. Cells were then incubated with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies Inc., Cat. No.: D1306) solution for 5 min and were washed three times in DPBS-Tr. The coverslips were then mounted with Prolong Gold anti-fade reagent (Life Technologies Inc., Cat. No.: 36,930). Immunofluorescence was visualized on a confocal microscope (TCS SP5 LCSM, Leica, Buffalo Grove, IL).

The primary antibodies used in immunostaining include: anti-c-kit-FITC antibody, 1:100 dilution (Abcam Inc., Cambridge, MA, Cat. No.: ab24870); rabbit anti-CD31, 1:20 dilution (Abcam Inc., Cat. No.: ab28364); mouse anti-Flk-1, 1:50 dilution (Santa Cruz Inc., Cat. No.: sc-6251); rat anti-CD90, 1:100 dilution (Abcam Inc., Cat. No.: ab3105); rat anti-mouse CD105, 1:50 dilution (R&D Systems, Minneapolis, MN, Cat. No.: MAB1320); rabbit anti-Oct3/4, 1:200 dilution (Santa Cruz Inc., Cat. No.: sc-9081); rabbit anti-Nanog, 1:200 dilution (Santa Cruz Inc., Cat. No.: sc-33760); rabbit anti-Nkx2.5, 1:400 dilution (Abcam Inc., Cat. No.: ab22611); rabbit anti-GATA4, 1:200 dilution (Santa Cruz Inc., Cat. No.: sc-9053); mouse anti-α-actinin, 1:1000 dilution (Sigma-Aldrich Inc., Cat. No.: A7811); rabbit anti-cardiac troponin I, 1:100 dilution (Abcam Inc., Cat. No.: ab47003); rabbit anti-α-smooth muscle actin, 1:500 dilution (Abcam Inc., Cat. No.: ab5694); chicken anti-vimentin, 1:200 dilution (Abcam Inc., Cat. No.: ab24525); rat anti-mouse CD31, 1:100 dilution (AbD Serotec Inc., Cat. No.: MCA2388GA). The secondary antibodies included: goat anti-rabbit IgG (H + L), Alexa Fluor 594 conjugate (Life Technologies Inc., Cat. No.: A-11012); goat anti-mouse IgG (H + L), Alexa Fluor 594 conjugate (Life Technologies Inc., Cat. No.: A-11005); goat anti-mouse IgG (H + L), Alexa Fluor 488 conjugate (Life Technologies Inc., Cat. No.: A-11001); goat anti-rat IgG (H + L), Alexa Fluor 488 conjugate (Life Technologies Inc., Cat. No.: A-11006); goat anti-chicken IgG (H + L), Alexa Fluor 647 conjugate