International Journal of Cardiology 265 (2018) 173–180

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Preinduction with bone morphogenetic protein-2 enhances cardiomyogenic differentiation of c-kit$^+$ mesenchymal stem cells and repair of infarcted myocardium

Yong-li Wang [a,1], Guitao Zhang [a,b,1], Hai-jie Wang [a,*], Yu-zhen Tan [a,*], Xin-yan Wang [a]

[a] Department of Anatomy, Histology and Embryology, Shanghai Medical School of Fudan University, Shanghai 200032, China
[b] Department of Anatomy, Histology and Embryology, Capital Medical University, Beijing, China

ARTICLE INFO

Article history:
Received 8 September 2017
Received in revised form 27 January 2018
Accepted 31 January 2018

Keywords:
c-Kit
Mesenchymal stem cells
Bone morphogenetic protein-2
Stem cell transplantation
Myocardial infarction

ABSTRACT

Background: Preclinical and clinical trails show that c-kit$^+$ cardiac stem cells can differentiate towards cardiovascular cells and improve cardiac function after myocardial infarction (MI). However, survival and differentiation of the engrafted stem cells within ischemic and inflammatory microenvironment are poor.
Methods: c-Kit$^+$ cells were isolated from mesenchymal stem cells (MSCs) of rat bone marrow. Reliability of preinduction with bone morphogenetic protein-2 (BMP-2) in promotion of survival and differentiation of c-kit$^+$ MSCs was assessed in vitro and after transplantation.
Results: c-Kit$^+$ MSCs have a potential to differentiate towards cardiomyocytes. BMP-2 promotes proliferation, migration and paracrine of the cells, and protects the cells to survive in the hypoxic condition. After induction with 10 ng/mL BMP-2 for 24 h, the cells can differentiate into cardiomyocytes at four weeks. The electrophysiological characteristics of the differentiated cells are same as adult ventricular cardiomyocytes. In rat MI models, cardiac function was improved, the size of scar tissue was reduced, and regeneration of the myocardium and microvessels was enhanced significantly at four weeks after transplantation of BMP-2-preinduced cells. The survived cells and cardiomyocytes differentiated from the engrafted cells were increased greatly.
Conclusion: The results suggest that transient treatment with BMP-2 can induce c-kit$^+$ MSCs to differentiate into functional cardiomyocytes. Preinduction with BMP-2 enhances survival and differentiation of the cells. BMP-2-primed cells promote repair of the infarcted myocardium and improvement of cardiac function. Transplantation of BMP-2-preinduced c-kit$^+$ MSCs is a feasible strategy for MI therapy.

1. Introduction

Myocardial infarction (MI) is a leading cause of death of the cardiovascular disease worldwide [1] and projected to worsen with the current trends in obesity and aging of the population [2]. In recent years, stem cell-based therapies have become promising approaches in myocardial regeneration [3]. Cardiac stem cells (CSCs) and mesenchymal stem cells (MSCs) are particularly promising cell populations in improving cardiac function and attenuating adverse ventricular remodeling of the ischemic myocardium [4]. Main population of CSCs expresses tyrosine kinase c-kit (CD117, marker of stem cell). c-Kit$^+$ CSCs are self-renewing, clonogenic and multipotent, giving rise to cardiomyocytes, endothelial cells, and smooth muscle cells [5,6]. Preclinical and clinical trails prove that CSCs can improve cardiac function and structure [4,7]. Regardless of the debated potential to generate cardiomyocytes in vivo [8,9], c-kit$^+$ CSCs are a desirable source of seed cells for stem cell transplantation. MSCs have shown similar cardiac regenerative capacity with CSCs in cell therapies [10]. However, whether bone marrow-derived MSCs contain c-kit$^+$ cells and therapeutic efficacy of c-kit$^+$ MSCs is unclear. MSCs exhibit cardiomyocyte, vascular smooth muscle and endothelial cell differentiation [11,12]. MSCs have been considered a very promising cell population for cardiac transplantation [13]. Therefore, it is necessary to select optimal c-kit$^+$ cells from bone marrow-derived MSCs for myocardium repair.

Stem cell-based therapies have been limited by poor survival and differentiation of the engrafted cells within ischemic and inflammatory microenvironment [14]. Approaches priming stem cells into prosurvival state include heat shock, hypoxia, anoxia, cytokine stimulation, genetic modification and treatment with pharmacologics [4,10]. However, initiating differentiation of stem cells before transplantation remains to be explored. Bone morphogenetic protein-2 (BMP-2) is a number of the transforming growth factor-β (TGF-β) super family. On binding preferentially to type II serine–threonine kinase receptor, BMP-2 activates Smads 1, 5, and 8 signaling pathways, which leads to transcription of

* Corresponding authors at: Department of Anatomy, Histology and Embryology, Shanghai Medical School of Fudan University, 138 Yixueyuan Road, Shanghai 200032, China.
E-mail addresses: hjwang@shmu.edu.cn (H. Wang), yztan@shmu.edu.cn (Y. Tan).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijcard.2018.01.134
0167-5273/

the target genes [15]. BMP-2 can induce differentiation of embryonic stem cells [16], cardiomyocyte precursor cells [17] and c-kit$^+$/Sca-1$^+$ marrow-derived cells [18] into cardiomyocytes. Therefore, optimizing stem cell transplantation with BMP-2-preinduction would be beneficial to tolerance of the engrafted cells against the local hostile environment.

This investigation was designed to sort c-kit$^+$ MSCs from rat bone marrow-derived MSCs, and examine biological characteristics of the cells and evaluate effectiveness of BMP-2-preinduced c-kit$^+$ MSCs on repairing the infarcted myocardium after transplantation.

2. Materials and methods

2.1. Isolation of c-kit$^+$ MSCs

The protocol was approved by the Institutional Animal Care Committee of Fudan University. Isolation of MSCs from bone marrow of male Sprague-Dawley (SD) rats was performed as described previously [19]. In brief, bone marrow in the femurs and tibias was flushed out with PBS containing heparin (100 U/mL). After centrifugation, the bone marrow cells were incubated with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Invitrogen). After three days, the non-adherent cells were discarded by vigorous washing, and the adherent cells were used as MSCs. For sorting c-kit$^+$ cells from the first and sixth passages of bone marrow-derived MSCs, the cells were incubated with mouse anti-rat c-kit antibody (1:200; Santa Cruz, Dallas, TX, USA) for 50 min at 4 °C, followed by incubation with goat anti-mouse IgG conjugated with Alexa 647 (1:400; Jackson, West Grove, PA, USA) for 30 min. c-Kit$^+$ cells were analyzed and sorted using a Beckman MoFlo™ XDP FACS (fluorescence-activated cell sorter; Beckman Coulter, Fullerton, CA, USA). Expression of c-kit in the sorted cells was determined by immunostaining.

2.2. Epitope analysis of the cells

The first and sixth passages of c-kit$^+$ MSCs and primary MSCs were incubated with rabbit anti-rat CD29, rabbit anti-rat CD90, or rabbit anti-rat CD105 (1:100, Bioss, Beijing), mouse anti-rat CD34 (1:100, Santa Cruze), or rabbit anti-rat CD45 antibodies (1:100, Bioss) for 1 h at 4 °C. Subsequently, the cells were incubated with Alexa fluor 647-labelled goat anti-mouse IgG or Alexa Fluor 488-labelled goat anti-rabbit IgG (1:400; Jackson) for 30 min at 4 °C. Epitopes of the cells were analyzed using BD FACSVerse™ flow-cytometer (BD Biosciences, San Jose, CA, USA).

2.3. RNA sequencing and analysis of differentially expressed genes (DEGs)

To clarify characteristics of transcriptome of c-kit$^+$ MSCs and their potential of differentiation towards cardiomyocytes, total RNA was extracted from the cells of the first and sixth passages respectively using TRIzol reagent (Life technologies, New York, USA). RNA quantity and quality were examined by a NanoDrop-1000 spectrophotometer (LabTech, Boston, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) [20]. Paired-end deep-transcriptome sequencing was performed at the BGISEQ-500 platform from Beijing Genome Institute (Shenzhen, China). DEGs of c-kit$^+$ MSCs were identified as described in the Supplemental materials and methods 1.

2.4. Assays of viability, proliferation and migration of c-kit$^+$ MSCs

c-Kit$^+$ MSCs were treated with 10 ng/mL BMP-2 (PeproTech, London, England) for 24 h. Then, the cells were treated with 1% O_2 for 12 h. Subsequently, the cells were incubated with 10 μL of cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) for 2 h. Absorbance at 450 nm was measured using Infinite 200 PRO Microplate Reader (Tecan, Mannedort, Switzerland). Effect of BMP-2 on cell viability in the hypoxic condition was evaluated. To assess effect of BMP-2 on cell proliferation, the cells were incubated with rabbit anti-Ki-67 antibody (1:200, Abcam, Cambridge, MA, USA) and Alexa Fluor 488-labelled goat anti-rabbit IgG. Percentage of Ki-67-positive cells was calculated using an Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). To determine effect of BMP-2 on cell migration, a straight scratch was made with a p200 pipette tip when the cells were grown into confluence. Then, the cells were incubated for another 24 h. The area of the migrated cells was measured using Image-Pro Plus 6.0 software.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Paracrine of HGF, IGF-1, SCF, SDF-1 and VEGF from BMP-2-induced c-kit$^+$ MSCs was examined with ELISA as described in the Supplemental materials and methods 2.

2.6. Quantitative reverse transcription PCR (qRT-PCR) analysis

Differentiation of c-kit$^+$ MSCs towards cardiomyocytes was induced with 10 ng/mL BMP-2 for 24 h. To evaluate differentiation of the cells towards cardiomyocytes, qRT-PCR analysis and cTnT or Cx43 immunostaining were performed respectively as described in the Supplemental materials and methods 3 and 6. The genes detected with qRT-PCR and their primer sequences were described in Supplemental Table 1.

2.7. Whole cell patch clamp

To assess electrophysiological features of the differentiated cells, action potential (AP) and L-type calcium ion current ($I_{Ca,\ L}$) of the cells at three or four weeks after induction were recorded with the whole cell patch clamp as described [21]. AP and $I_{Ca,\ L}$ of cardiomyocytes isolated from LV wall of adult rat were used as positive control.

2.8. Cell transplantation

For tracing the transplanted cells, MSCs and c-kit$^+$ MSCs were transfected with lentiviruses carrying GFP (green fluorescent protein) expression cassette before transplantation. Fifty female SD rats were used, forty-five rats for modeling, five rats for sham surgery. The animal protocol complied with the institution's guidelines. MI models were established with ligation of the left anterior descending (LAD) coronary artery [22]. At one week after MI, five rats were kicked out because of the lack of an ideal echocardiogram, the remaining forty rats were divided randomly into control, MSC, c-kit$^+$ MSC and BMP-2 groups (ten rats in each group). Two or three passages of cells were used for transplantation. In BMP-2 group, c-kit$^+$ MSCs were induced with 10 ng/mL BMP-2 for 24 h before transplantation. The cells were suspended in 80 μL PBS, and injected into the border of the infarcted myocardium at four spots. The same volume of PBS was injected as control group.

2.9. Echocardiography

Echocardiographic studies were performed before MI, one week after MI and four weeks after cell transplantation as described in the Supplemental materials and methods 4.

2.10. Histological section of the heart

At four weeks after cell transplantation, all hearts were harvested after echocardiographic analysis. Histological examination was performed as described in the Supplemental materials and methods 5.

2.11. Immunostaining

Differentiation of c-kit$^+$ MSCs towards cardiomyocytes after induction with BMP-2 was evaluated with cTnT and Cx43 immunostaining, cardiomyogenesis and angiogenesis were examined with GFP and cTnT or CD31, and cTnT and Cx43 double immunostaining respectively as described in the Supplemental materials and methods 6.

2.12. Statistical analysis

Data were expressed as mean ± SD and analyzed using GraphPad Prism (version 6.0, La Jolla). To analyze the data statistically, Student's *t*-test and one-way analysis of variance were performed with Scheffe's post hoc multiple comparison analysis. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Phenotype and gene expression of c-kit$^+$ MSCs

Flow cytometry analysis showed that percentage of c-kit$^+$ MSCs at the first and sixth passages were 11.3% and 2.5% respectively (Fig. 1A). Immunostaining demonstrated that purity of c-kit$^+$ cells sorted from both passages of MSCs >95% (Fig. 1B). The flow cytometric results demonstrated that the first and sixth passages of c-kit$^+$ MSCs and primary MSCs expressed mesenchymal lineage markers (CD29, CD90 and CD105) and were negative for hematopoietic markers (CD34 and CD45) (Supplemental Fig. 1). Compared with MSCs, expression of CD29, CD90 and CD105 in c-kit$^+$ MSCs at the first and sixth passages has no significant different.

The numbers of the expressed genes in c-kit$^+$ MSCs at the first and sixth passages were 12,542 and 12,476 respectively, and the coverage was 72.05% for the first passage and 71.67% for the sixth passage compared with total gene number in the database (Supplemental Table 2). Both passages of c-kit$^+$ MSCs expressed genes related with mesoderm and early myocardial formation (*Msx1*, *Mixl1*, *Isl-1*, *Mesp1*, *Hand2*, *Sca-1*, *Gata4*, *Tbx5*, *Tbx2*, *Nkx2.5* and *MEF2c*) (Fig. 1C, D). Expression of Sca-1 was lower at the sixth passage than that at the first passage. Expression of cardiac-specific genes (*TNNT2*, *TNNI3*, *Mybpc3* and *Pln*) and endothelial-specific genes (*Cdh5*, *Emcn*, *Eng*, *Pecam1* and *Vwf*) was minimally observed in both passages. Expression of Eng was lower at the sixth passage than that at the first passage. Compared with the first passage,