Single Cell Gene Profiling Revealed Heterogeneity of Paracrine Effects of Bone Marrow Cells in Mouse Infarcted Hearts

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Abstract

It is now recognized that transplantation of bone marrow cells (BMCs) into infarcted hearts has the capacity to improve the cardiac function through paracrine effects. However, detailed expression levels of paracrine factors in BMCs in infarcted hearts are poorly described. By use of laser capture microdissection combined with real-time PCR, we depicted the expression profiles of paracrine factors in infarcted hearts versus normal hearts. Consistent with the in vivo observation, a similar expression pattern was evidenced in cultured BMCs. Furthermore, BMCs displayed heterogeneity of paracrine effects in infarcted hearts as analyzed at the single cell level using single cell PCR. Interestingly, the CD45+ subpopulation showed higher expression levels of angiogenic factors compared to other subpopulations. Finally, most angiogenic factors were induced under the microenvironment of infarction. Our study demonstrated the heterogeneity of paracrine effects in BMCs at single cell level in infarcted hearts, highlighting preferential expression of angiogenic factors in the CD45+ subpopulation. These findings broaden our understanding of paracrine effects of BMCs in vivo, and offer new insights into BMCs therapy in myocardial infarction (MI).

Introduction

Despite advances in our understanding and treatment of coronary artery disease, myocardial infarction (MI) remains a major cause of morbidity and mortality worldwide. Recently, stem cell therapy is recognized as a promising therapeutic strategy in the protection and repair of damaged myocardium after infarction [1,2,3]. Growing experimental studies and clinical trials have been carried out using BMCs, displaying encouraging outcomes in the improvement of cardiac function, which offered the fascinating possibility of the application of BMC-based therapy in MI [4,5,6,7,8,9].

In order to ensure the safety and effectiveness of BMC therapy, extensive studies were performed to dissect the mechanisms underlying their therapeutic actions. Transdifferentiation into cardiomyocytes and vascular lineage cells has been originally proposed as the principal mechanism accounting for improved cardiac function. Anversa and colleagues illustrated that Lin-c-Kit+ (Hematopoietic stem cells enriched) cells from BMCs can give rise to cardiac cells, and regenerate 68% of the infarcted area with novel cardiomyocytes [5]. Conversely, however, other groups failed to detect cardiac transdifferentiation in hearts transplanted with BMCs derived hematopoietic stem cells (HSCs) [10,11]. Given the ongoing scientific debates, a different hypothesis has been raised, emphasizing paracrine effects of stem cells in the treatment of MI [12]. For example, Takahashi et al. injected conditioned medium from bone marrow derived mononuclear cells (BM-MNCs) into infarcted hearts and observed an overall improvement of cardiac function [13]. Simultaneously, a considerable number of studies focused on the regulation of paracrine effects in cultured BMCs [14,15,16]. However, the paracrine effects of BMCs in vivo, as well as responses of paracrine actions challenged by infarction, are not fully understood. In addition, given that BMCs are composed of different cell populations, analyses of the paracrine effects that distinguish the different subpopulations were rarely reported.

In this study, we depicted paracrine effects of BMCs in vivo, with particular focus on the comparison between infarcted and normal hearts. Moreover, we uncovered the heterogeneity of paracrine effects of BMCs in infarcted hearts using single cell PCR, and revealed that the CD45+ subpopulation in BMCs preferentially expressed angiogenic factors.

Materials and Methods

Ethics Statement

All animal studies have been approved by the Animal Care and Use Committee of the General Hospital of Chinese PLA.

Animals

Myocardial infarction (MI) surgery in female SCID mice, 12 weeks old, was used in this study. Male green fluorescent protein
paracrine factors. Afterwards, the total RNA was extracted for evaluation of various
whole BMCs. The cultured BMCs were exposed to either hypoxia

**Isolation and Culture of BMCs**

In brief, mice femurs were removed immediately after sacrifice, and
flushed by phosphate saline buffer (PBS) with 2% fetal bovine
serum (FBS). The obtained cells were lysed by ammonium-
chloride-potassium (ACK) lysis buffer (Gibco) to eliminate red
blood cell contamination. The resulting cells were either cultured
in BMC culture medium (STEMCELL Technologies) or subjected
to isolation of CD45+ cells. Magnetic activated cell sorting
(MACS, Miltenyi) was employed to collect CD45+ cells from
whole BMCs. The cultured BMCs were exposed to either hypoxia
(1% O2, 5% CO2, 94% N2) or normoxia condition for 48 hours.
After that, the total RNA was extracted for evaluation of various
paracrine factors.

**Fluorescence Activated Cell Sorting (FACS) and Single Cell Isolation**

SCID mice were sacrificed 5 days after surgery. Hearts were
removed and chopped up in microfuge tubes as fine as possible,
then small pieces were moved to 10 cm culture dishes followed by
collagenase type I (1 mg/ml, Sigma) digestion for 1 hour at 37°C.
Afterwards, cells were spun down to form a pellet, which was
further digested with 0.25% trypsin at 37°C for another 5-10 min.
The resulting cells were subjected to FACS to sort GFP+ cells.

**High through-put Single-Cell qPCR Analysis**

Inventoried TaqMan assays (20×, Applied Biosystems) were
pooled and diluted to a final concentration of 0.2× for each of the
48 probes. Individual cells were collected directly into 10 μl RT-
PreAmp Master Mix (5.0 μl CellsDirect 2× Reaction Mix
(Invitrogen); 2.5 μl 0.2× assay pool; 0.5 μl RT/Taq enzyme
(CellsDirect qRT-PCR kit, Invitrogen); 2.0 μl TE buffer). After
that, the mixture of single cell was applied to sequence specific
reverse transcription at 50°C for 20 min. Subsequently, cDNA
was used as a template for partial sequence-specific amplification
by following protocol: denaturing at 95°C for 15 s, and annealing
and amplification at 60°C for 4 min for 18 cycles. These
preamplified products were analyzed with Universal PCR Master
Mix and inventoried TaqMan gene expression assays (ABI) in
48.48 Dynamic Arrays on a BioMark System (Fluidigm). Ct values
were calculated from the system’s software (BioMark Real-time
PCR Analysis; Fluidigm).

**Single-Cell Data Processing**

All Ct values of genes were converted into relative expression
levels by subtracting the values from the assumed baseline value
of 32. The resulting values were then normalized to the endogenous
controls by subtracting the average of its β-actin and GAPDH
expression levels.

**Statistical Analysis**

Data are expressed as mean±standard error of the mean
(SEM). Statistical analysis involved use of the Student’s t-test for
comparison of two groups. A P<0.05 was considered statistically
significant.

**Results**

**Gene Expression Profiling of Paracrine Effects of BMCs in Normal Versus Infarcted Hearts**

Using a LAD ligation model, we first examined gene expression
levels of different paracrine factors of BMCs in vivo. 5 days after
surgery, injected cells were evidenced by GFP signal. To assess
their paracrine effects in hearts, GFP+ cells (200 cells) were
captured using LCM in both sham and infarcted hearts
(Figure 1A). The expression profile of paracrine effects of the
injected cells was evaluated by real-time PCR array. When the
cells were analyzed as a single population, they displayed similar
expression patterns in terms of gene expression of paracrine factors
(Figure 1B). Among those factors, VEGF and IL1 were
significantly increased in infarcted hearts (Figure 1C). Conversely,
IGF1, TNFα, TGFβ, CSF1, BMP4, MMP2, MMP9, TIMP1 and
TIMP2 were markedly decreased (Figure 1E). No significant
changes were detected regarding the expression of VEGF, HGF,
FGF1, FGF2, Angiogenin1 [Ang1], Angiogenin2 [Ang2], PDGF-
BB, NGF, BMP2 or IL6 (Figure 1D). The above results suggested
that BMCs failed to enhance most of the paracrine factors under
hypoxia challenge in vivo.

**Heterogeneity of Paracrine Effects of BMCs**

SCID mice were sacrificed 5 days after surgery. Hearts were
removed and chopped up in microfuge tubes as fine as possible,
then small pieces were moved to 10 cm culture dishes followed by
collagenase type I (1 mg/ml, Sigma) digestion for 1 hour at 37°C.
Afterwards, cells were spun down to form a pellet, which was
further digested with 0.25% trypsin at 37°C for another 5-10 min.
The resulting cells were subjected to FACS to sort GFP+ cells.
Heart Function was Improved by BMCs Injection

To assess cardiac function after BMCs transplantation, echocardiograms were applied in our study at pre-surgery, 1, 4, and 10 days post-surgery (Figure 2A & B). At day 1 postsurgery, both BMCs injected group and PBS group displayed significant reduction in cardiac function, characterized by decreased fractional shortening (FS), which dropped from 41% to 20%, suggesting successful MI surgery. Intriguingly, compared to PBS injected group, BMCs injected group displayed moderate but statistically significant increase in FS at 4 days (16.97% vs. 20%) and 10 days (18.35% vs. 21.88%) after surgery, respectively. To further confirm the effect of BMCs injection, HE staining was employed in our experiments to determine the ventricular wall thickness. At day 10, ventricular wall thickness was increased in BMCs injected group compared to PBS group (153.6±67.6 vs. 117.0±53.8 μm, n = 8 mice for each group).

Figure 1. Gene expression profiling of paracrine effects of BMCs in normal versus infarcted hearts. (A) Representative pictures of LCM performed in frozen slides of normal and infarcted hearts. Removed GFP+ cells (white arrow) were indicated by white lines. (magnification 100×). (B) An array displayed overall expression profile of paracrine factors synthesized by BMCs in normal and infarcted hearts 5 days post surgery. (n = 8 per group). (C–E) Quantitative analysis of gene expression in normal and infarcted hearts. Values are mean of 8 mice per each group.

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each group, P<0.05; Figure 2C & D). These results suggested that BMCs injection was capable of improving cardiac function after infarction.

Cultured Bone Marrow Cells Displayed Similar Expression Pattern of Paracrine Factors in Response to Hypoxia Stimulation

*In vitro* experiments were utilized to further confirm the paracrine response of BMCs induced by hypoxia. Consistent with the observation in *vivo*, the expression of VEGFα and IL1α was induced by 48-hour hypoxia stimulation (1.55- and 1.94-fold increase, respectively. Figure 3A, D). In contrast, the expression of IGF1, TNFα, MMP2, MMP9 and TGFβ was inhibited under hypoxia condition (decreased by 45.3%, 75.7%, 71.5%, 74.9% and 31.5%, respectively. Figure 3C, E, F, G, K). However, there were no significant changes in the expression of VEGFβ, TIMP1 or CSF1 (Figure 3B, H, J).

BMCs Displayed Heterogeneity Regarding Paracrine Effects in Infarcted Hearts

Since BMCs comprise different subpopulations of cells, and thus display multilineage differentiation potential, we then assessed the heterogeneity of BMCs with regard to paracrine effects. Transplanted GFP+ BMCs were collected 5 days after infarction surgery.
by FACS, followed by analysis of paracrine effects at single cell level (Figure S1B). To exclude local cell contamination and cell fusion, we applied probes against SRY (data not shown), GFP and H2-K1 in following single cell PCR (Figure S1A). A total of 300 transplanted BMCs were harvested from 6 infarcted hearts (50 cells per mouse), and analyzed by single cell real-time PCR array with a total of 31 specific probes. Compared to analysis as a single population, the transplanted cells exhibited heterogeneity of paracrine effects at single cell level (Figure 4A). In addition, the frequency distribution was performed to evaluate the heterogeneity of paracrine effects in single cells, which was evidenced by the horizontal spread of a histogram plot (Figure 4B–J). The expression of most of major paracrine factors, including VEGFα, VEGFβ, IGF1, IL6, MMP2, MMP9, PDGF-BB, TGFβ and TNFα, which are involved in the pathophysiological process after MI, showed variation among different cells.

Figure 3. Paracrine effects of cultured BMCs in response to hypoxia. (A–K) Quantification of relative mRNA level of paracrine factors in cultured BMCs 48 hours after hypoxia stimulation. *P<0.05 versus normoxia condition. doi:10.1371/journal.pone.0068270.g003
Figure 4. BMCs displayed heterogeneity regarding paracrine effects in infarcted hearts. (A) A heat map showing that gene expression levels of different paracrine factors from 300 individual BMCs collected from infarcted hearts. (B–J) Population distribution plots (horizontal axis represents expression level; vertical axis represents percentage of total cell population) uncovered that single BMCs collected from infarcted hearts possessed considerable heterogeneity with respect to synthesis of paracrine factors.

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Various Angiogenic Factors were Preferentially Highly Expressed in CD45+ BMCs in Infarcted Hearts

Due to the diversity of components, as well as the heterogeneity of paracrine effects in BMCs, different markers of subpopulations, such as CD31, CD54, CD45 and CD90, were also employed in our single cell PCR study to compare the differentially expressed genes among different subpopulations (Figure 4A). Compared to other subpopulations, the CD45+ subpopulation was intriguing in that it highly expressed angiogenic factors. 5 days after MI and BMCs transplantation, various factors implicated in angiogenesis, including VEGFα, VEGFβ, HGF, IGF1, FGF2, PDGF-BB, IL1, TNFα, TGFB and BMP4, were highly expressed in CD45+ cells relative to CD45- cells (Figure 5A). Interestingly, some of the factors were involved in the degradation and remodeling of extracellular matrix (ECM), such as MMP2, MMP9 and TIMP1, were highly expressed in CD45+ cells (Figure 5C). However, no significant differences in the expression levels of FGF1, Ang2, NGF, CSF1, BMP2 and TIMP2 were observed between these two populations (Figure 3B). Collectively, these data suggested that the CD45+ subpopulation plays a critical role in angiogenesis after MI.

CD45+ BMCs Enhanced Angiogenesis and Cardiac Function after Infarction

In order to examine angiogenic effect of CD45+ BMCs in vivo, myocardial infarction surgery was performed followed by injection of PBS, CD45+ BMCs (5 x 10^6) or CD45- BMCs (5 x 10^6), respectively. Five days after surgery, CD45+ BMCs injection group showed higher density of CD31 positive staining compared to PBS and CD45- BMCs groups, revealing that CD45+ BMCs possessed higher angiogenic effect per se (Figure 5D & E). In agreement with this angiogenic effect, cardiac function was improved in CD45+ BMCs group compared to PBS and CD45- BMCs groups at day 4 (17.5% vs. 12.5% and 14.4%) and day 10 (19.4% vs. 14.3% and 15.9%), respectively (Figure 5F).

The Expression of Angiogenic Factors were Induced in CD45+ Cells in Infarcted Hearts

Based on the above results, we asked how angiogenic factors are regulated in response to hypoxia condition in vivo. After isolation of CD45+ BMCs in vitro, 5 x 10^6 cells were immediately injected into hearts with MI or without myocardial infarction (Sham). The transplanted cells were collected by LCM 5 days after surgery. Real-time PCR revealed that the expression of most of angiogenic factors, including VEGFα, VEGFβ, IGF1, FGF2, PDGF-BB, IL1, TNFα and TGFB, were enhanced in infarcted hearts compared to sham group (Figure 6A). By contrast, the expression of a few factors was inhibited in infarcted hearts (Figure 6C). There were no significant changes in the expression of FGF1, Ang1, Ang2, NGF, CSF1, BMP2, BMP4, TIMP1 and TIMP2 (Figure 6B). Taken together, these results revealed that, compared to CD45- cells, CD45+ BMCs were more responsive to hypoxia stimulation in vivo with regard to secretion of angiogenic factors, which may promote angiogenesis and myocardial recovery after MI.

Discussion

Clinical trials have been carried out worldwide on cell-based therapy, with particular interest in the delivery of autologous cells derived from bone marrow to treat ischemic heart diseases [17,18]. In order to obtain safe and effective therapeutic effects, considerable studies were performed to investigate the underlying mechanisms of BMC therapy in ischemic hearts. Rather than direct cell transdifferentiation, there is a growing body of evidence supporting the hypothesis that paracrine mechanisms mediated by factors secreted from BMCs play a crucial role in the protection and recovery of damaged myocardium. However, due to limited approaches for assessing paracrine effects in vivo, there was lack of proof of how BMCs secret paracrine factors in hearts, especially under the challenge of ischemia. Our study, for the first time to our knowledge, delineated the regulation of paracrine factors released by BMCs in vivo, uncovered the heterogeneity of paracrine effects of BMCs at a single cell level in infarcted hearts, and revealed preferential secretion of angiogenic factors in the CD45+ subpopulation in ischemic myocardium. Although some positive outcomes of paracrine effect-based BMCs therapy, such as improved capillary density and cardiac function [8,19,20,21], have been reported in human clinical trials and experimental studies, the precise expression levels of paracrine factors in BMCs in infarcted hearts remain unclear. By use of GFP transgenic mice and LCM, we had the unique opportunity to monitor the expression of various paracrine factors in vivo, and showed that BMCs expressed a broad spectrum of paracrine factors in infarcted hearts. Given the hypoxic microenvironment in infarcted hearts, it is important to study the response of BMCs challenged by hypoxia. Many studies elucidated the regulation of gene expression of paracrine factors in BMCs under hypoxia stimulation in vitro, as well as enhanced level of paracrine factors in infarcted hearts treated with BMCs transplantation [15,22,23]. However, few studies investigated the response of BMCs in infarcted hearts compared to normal hearts. Surprisingly, in our study, we found that only a few paracrine factors were upregulated in infarcted hearts, whereas most of the paracrine factors were downregulated or not affected by hypoxia stimulation. Our results were not controversial with other groups showing increased VEGF, bFGF, HGF, IGF1 and adrenomedullin in injured hearts treated with BMCs [22], because their results were based on the comparison between cell transplantation and non-transplantation groups, rather than cell transplantation in infarcted hearts versus normal hearts. To confirm the observations in vitro, we studied the regulation in cultured cells, where only a few paracrine factors were induced upon hypoxia stimulation, which was consistent with results from other groups, as well as with our in vivo data. Since bone marrow cells exemplify a typical adult stem cell source containing different cell populations with diverse characteristics under various stimuli, studying the response to hypoxia of subpopulations in the heart after BMCs administration facilitates our understanding of the complex process of heart recovery, and helps optimize therapeutic strategies using different subpopulations. By application of single cell PCR, we uncovered the heterogeneity of BMCs in expression of paracrine factors in infarcted hearts. Based on the results, one would speculate that different subpopulations in BMCs possess different expression profiles of paracrine factors. Identification of firemen, bystanders or criminals in BMCs may facilitate the selection of specific subpopulations for different therapeutic purposes in MI. Using identified markers of different subpopulations in BMCs, we were able to analyze the characteristics of paracrine effects among different subpopulations. Our results revealed a preferential expression of angiogenic factors in CD45+ cells. The CD45 antigen is expressed on all cell surfaces of hematopoietic origin except for erythrocytes and platelets. Using CD45 as the marker, Balsam L. et al. reported that BMC-derived stem cells adopted mature haematopoietic [10]. However, since BMCs were capable of transdifferentiating into cells of diverse phenotypes in infarcted hearts, more specific markers should be applied in future studies to define the exact cell type of CD45+ cells. Besides higher expression levels in CD45+ cells, most angiogenic factors were also induced.
under hypoxia stimulation in vivo, indicating a desirable response of CD45+ cells in infarcted hearts. Due to the small number of CD45+ cells in transplantation, we were not able to compare the outcomes of stem cell transplantation between BMCs and CD45+ cells. Further studies need to be conducted to address the questions regarding heart protection, remodeling, repair, and regeneration using CD45+ cells.
In summary, our study demonstrated the heterogeneity of paracrine effects in BMCs, and revealed that the CD45 \(^+\) subpopulation of BMCs have higher expression levels of angiogenic factors and a more desirable response under hypoxia challenge compared to CD45 \(^-\) cells. Together, these findings broaden our understanding of stem cell therapy at the single cell level, and offer new insights into optimization of stem cell type selection for therapeutic applications.

**Supporting Information**

**Figure S1** Strategy used in our study to isolate single injected cell without local cell contamination and cell fusion (A). (B) Representative images of FACS in single cell collection. GFP cells were sorted and analyzed afterwards. (TIF)

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