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Original Article

Absence of myocyte regeneration by mobilization of bone marrow stem cells after myocardial infarction

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Abstract: The study aimed to test the potential for bone marrow stem cells (BMSC) mobilized by granulocyte macrophage colony stimulating factor (GM-CSF) to promote neovascularization and cardiomyocytes regeneration in a rat model of myocardial infarction. The myocardial infracted rats were randomly assigned to receive GM-CSF injection as GM-CSF group or received saline injection as control group. Evaluation of $CD34^+$ stem cells was performed by flow cytometry. Cardiac functions were monitored using a multiple channel recorder via cardiac catheterization. Immunobiological staining including factor VIII and Ki67, and phosphotungstic acid-hematoxylin (PTAH) staining, were performed to assess angiogenesis and myogenesis and calculated myocardial infarction size. The $CD34^+$ stem cells in blood and bone marrow of GM-CSF group increased significantlyon day 7 and day 14 comparing with control group, and declined on day 28. Immunobiological staining showed neovasculature formation and more Ki67 expression in the infracted regions of theGM-CSF group.Ki67 and PTAH double staining showed Ki67 positive signals were overlap with lymphocytes, fibroblasts and endothelial cells but not myocytes. No significant decrease of infracted size occurred in the GM-CSF group. These results suggested BMSC could be mobilized effectively by GM-CSF after myocardial infarction, which could only promote neovascularization without myogenesis.

Keywords: Myocardial infarction, stem cells, bone marrow

Introduction

In the past years, it was believed that after myocardial infarction, myocardial cells could not regenerate and be replaced by fiber scar tissue [1, 2]. With the rise of stem cell research, it has been found that there is some proliferative myocardial cell in the infarction area, and autologous bone marrow stem cells (BMSC) has the potential to differentiate into myocardial cells [3]. Some stem cell cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF) has been widely used to mobilize BMSC into peripheral blood for the treatment of blood disease. By this method, it also has been discovered that the mobilized BMSC can migrate to the ischemic and infracted myocardial tissue to promote neovascularization and cardiomyocytes regeneration [4].

With the deepening of the research, different effects of BMSC transplantation on myocardial infarction have been found [5-8]. Especially whether mobilization of BMSC promotes mycardial tissue regeneration is controversial. To solve the problem, we used GM-CSF to mobilize BMSC after myocardial infarction in a rat model to survey its influence on myocardial tissue regeneration.

Materials and methods

Experimental animals

Sprague-Dawley [9] rats weighing 280 g to 300 g were used in this experiment. All animals received humane care and the animal protocols complied with the institution's guidelines. SD rats were random divided into GC group as a group of control [10] and GM-CSF group (A group of using Granulocyte Macrophage Colony Stimulating Factor to mobilize bone marrow stem cells), and each group had 7~8 viable rats at each check point (3 d, 7 d, 14 d, and 28 d).

Reagents and instruments

Anti-rat CD34 antibody was purchased from Santa Cruz, America; rabbit anti-factor VIII polyclonal antibody from Zymed, America; Ki67 polyclonal antibody from Neomakers, America; GM-CSF from Schening-Plough Pharmaceuticals, America. FACSCAlibur (BD, America), small animal Ventilator (Harvard 683, USA), and SW2001 electro cardiogram monitor (Simens, Germany) were also used in the study.

Establishment of myocardial infarction model

Adult SD rats were narcotized by intraperitoneal injection of 30 mg/kg ketamine and 5 mg/kg diazepam, and rodent ventilator was used to assist respiration by intubating 16G sheath needle into weasand. Cut open the chest after sterilized by iodine and revealed their hearts. Ligated the left anterior descending coronary artery and the marker for successful establishment of myocardial model was that electrocardiogram(ECG) monitor showed ST segment elevation or occurrence of ventricular rhythm, and the anteroinferior myocardium appeared to be pale. GM-CSF (50 $\mu g \cdot kg^{-1} \cdot day^{-1}$) was subcutaneously injected for 7 days in the GM-CSF group, and the GC group would be received an equal volume of saline.

Peripheral blood and bone marrow stem cell assay

The methods were described previously [4]. For each experimental groups and control groups after myocardial infarction at 3 day, 7 day, 14 day, 28 day, five SD rats were random selected and used the bone marrow and peripheral blood to make cell suspension. Pipetted 100 μL bone marrow or peripheral blood cell suspension into centrifuge tube, added phycoerythrin-conjuncted CD34 antibody into the tube and incubated at room temperature in dark room. Added red cell lysis buffer into the tube and then centrifuged at 1500 rpm/min. Then resuspended cell with PBS and used flow cytometry to evaluate the percentages of $CD34^+$ stem cells. The exciting light was 514 nm and the emission light was 550 nm.

Cardiac functions test

The multipurpose polygraph was utilized to record the hemodynamics and cardiac functions after the establishment of myocardial infarction model at 14 day and 28 day. SD rats were narcotized by intraperitoneal injection of 30 mg/kg ketamine and 5 mg/kg diazepam. Made the rat lie on its back and fastened all fours and head. Cut open lateral thoracotomy the skin along the cervical median incision, separated the subcutaneous tissue and neck muscles to right part, detached the right carotid artery and ligated the proximal end of the artery. Used the 20G venous sheath needle that filled with diluted heparin to puncture the right carotid artery. Pulled out the needle and leaved the sheath, then injected heparin-saline (40 mg/100 ml) 1 ml into the artery to anticoagulate. The end of the sheath and heparin-saline filled plastic conduit was conjuncted, which related to the pressure transducer of multi-lead purpose polygraph. Balanced with atmosphere and continuously recorded the systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP). Retrograded the sheath into the ventriculus sinister (left ventricle) and continuously recorded the Left ventricle systolic pressure (LVSP), left ventricle diastolic pressure (LVEDP), max left ventricular pressure rise rate (+dp/dt_{max}), minimum left ventricular pressure drop rate (-dp/dt_{min}).

Histological examination

At each time point mentioned above, sacrificed SD rats by intravenous injection of 2 mL 15% KCl solution, then fixed the heart tissue with paraformaldehyde and embedded with paraffin. Sliced different part of the heart tissue continuously at 5 μm thick, then immunohistochemically stained by hematoxylin-eosin (HE), phosphotungstic acid hematoxylin (PTAH), anti-factor VIII polyclonal antibody and Ki67 polyclonal antibody. Anti-factor VIII was diluted at 1:100 to stain the vascular endothelial cell and the anti-factor VIII positive cells would show brown cytoplasm [8]. Randomly selected five myocardial sections in each group and picked five fields under microscope for each section, used image analysis software UTHSCSA3.0 and square microscopic measurement tape to calculate the number of capillary blood in the infarction scar area and normal myocardial tissue. Ki67 was diluted at 1:50 to stain the dividing cells and the Ki67 positive cells had brown nucleus, used the same methods to calculate the number of Ki67 positive cell as the capillary blood. PTAH could specific stain the rhabdium of myocardial cell to be bluish violet [11], so

Table 1. The percentages of $CD34^+$ stem cells in bone marrow and peripheral blood (n = 5, $\bar{x}$ ± SD, unit: %)

	7 d	14 d	28 d
Blood stem cells in GC group	0.17%±1.52E-02	0.15%±1.05E-02	0.13%±1.60E-02
Blood stem cells in GM-CSF group	0.35%±2.61E-02*	0.26%±2.24E-02*	0.15%±2.07E-02
Bone marrow stem cells in GC group	1.24%±6.35E-02	1.17 %±5.42E-02	1.23%±9.74E-02
Bone marrow stem cells in GM-CSF group	2.25%±0.14%*	2.06%±0.11%*	1.29%±5.12E-02

Comparison between GC group and GM-CSF group, *P<0.01.

Table 2. Number of blood capillary and Ki67 positive cell in myocardial infarction tissue (n = 5, $\bar{x}$ ± SD)

Group	7 d		14		28 d	
	Ki67 positive cell	Blood capillary	Ki67 positive cell	Blood capillary	Ki67 positive cell	Blood capillary
GC	236±7.52	272±8.21	207±9.59	264±10.27	200±6.24	271±9.18
GM-CSF	89±9.15*	301±7.62#	275±5.26*	313±9.96*	246±4.53*	321±8.65*

Comparison between GC group and GM-CSF group, #P<0.05; *P<0.01.

combination staining of PTAH and Ki67 could be used to evaluate whether or not myocardial cell divided in the myocardial infarction tissue after mobilization of bone marrow stem cells. The measurement of myocardial infarction area was referred to the methods followed [12], HE stained heart slice was analyzed by image analysis software to measure the outer perimeter, inner perimeter of the left ventricle section and arc length of scar tissue. Myocardial infarction area was calculated as: Arc length of scar tissue/[(outer perimeter + inner perimeter)/2].

Results

$CD34^+$ stem cells increased in the GM-CSF group

The increased tendency of stem cells was compared. After myocardial infarction, the percentages of $CD34^+$ stem cells at 7 days and 14 day in the GM-CSF group were greater than those of control group (P<0.01), and Ki67 positive cells at 7 day and 14 day in the GM-CSF group were more than those at the 7 day and 14 day in the GC group (P<0.01). At the time point of 28 day, the percentage of $CD34^+$ stem cells in peripheral blood and bone marrow decreased and no difference was found between the GM-CSF group and GC group (P>0.05), but the Ki67 positive cells in infarction area in the GM-CSF group were greater than those in GC group (P<0.01) (**Tables 1** and **2**). The correlation analysis revealed that, at 7 day and 14 day, the numbers of Ki67 positive cells were positively correlated with the peripheral blood $CD34^+$ stem cells and the correlation coefficients were r = 0.956 (P = 0.017) and r = 0.921 (P = 0.026) respectively in the GM-CSF group. And the numbers of Ki67 positive cells were also positive correlated with the bone marrow $CD34^+$ stem cells and the correlation coefficients were r = 0.961 (P = 0.019) and r = 0.975 (P = 0.005), respectively. The result indicated that BMSC might migrate into the blood circulation system, reach the ischemic myocardium and settle down there.

Cardiac functions had no significant improvement in the GM-CSF group

Hemodynamic parameters were recorded and compared. The systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), left ventricle systolic pressure (LVSP), left ventricle diastolic pressure (LVEDP), max left ventricular pressure rise rate (+dp/dt_{max}), and minimum left ventricular pressure drop rate (-dp/dt_{min}) of GC group (group of control) and GM-CSF group were recorded after myocardial infarction at 14 day and 28 day. The parameters of cardiac functions in the GM-CSF group had been improved comparing to the GC group, but no significance difference (P>0.05, **Table 3**). Cardiac functions had no significant improvement in GM-CSF group.

Angiogenesis was detected in the GM-CSF group

Angiogenesis was also compared. The blood capillary number in the GM-CSF group in the

Table 3. The influence of bone marrow stem cell mobilization on the cardiac functions of myocardial infarction rats ($\bar{x} \pm SD$)

Group	Time	SBP mHg	DBP mmHg	MBP mmHg	LVSP mmHg	LVEDP mmHg	+dp/dt mmHg/s	-dp/dt mmHg/s	HR number/min
GC N = 7	2	104±11	82±14	89±15	104±13	11±3.7	4942±291	3742±355	421±20
	4	107±12	81±9	90±12	109±10	7.8±2.1	5025±177	3525±322	414±25
GM-CSF N = 8	2	110±13	87±10	96±10	111±12	9.1±3.2	5150±327	4125±338	407±23
	4	112±10	83±9	95±8	114±8	6.8±3.9	5350±126	3975±258	408±21

The cardiac function of GC group and GM-CSF group had no difference, $P>0.05$.

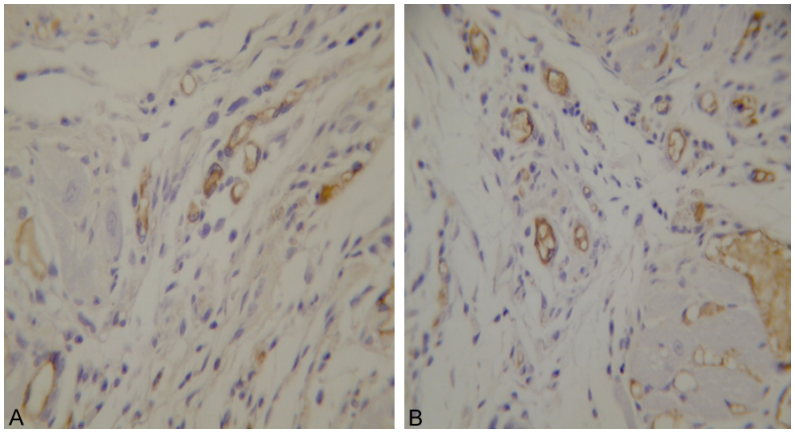

Figure 1. Experimental group (B) had more blood capillary than control group (A).Both were factor VIII staining (400×).

infarction area had no significant difference with the GC group ($P>0.05$) at 3 day post myocardial infarction, but was significantly increased at 7 day and 14 day ($P<0.05$ and $P<0.01$, respectively. **Figure 1**). Angiogenesis increased in the GM-CSF group.

Absence of myocardium regeneration in the GM-CSF group

The nucleus of divided cells can be assessed by staining of Ki67 staining. Double staining for Ki67 and PTAH in successive myocardial biopsy revealed that the number of Ki67 positive cells in the GM-CSF group was greater than that in control group. However, they did not stain the same cell, the positive Ki67 nucleus were negative for cytoplasm PTAH staining, while the nuclei were negative for Ki67 in cytoplasm PTAH positive cells. Most of the Ki67 positive cells were in the infarction area and a few were scattered in the interstitial space but not in the cardiomyocytes. These Ki67 positive nuclei overlapped with lymphocytes and monocytes in the early stage of myocardial infarction, and most with mesenchymal cells and a few with vascular endothelial cells in the late stage. But no Ki67 positive nuclei overlapped with myocardial cells (**Figure 2**). The results revealed that myocardial cells did not divide and regenerated.

Myocardial infarction area was not decreased

The area of myocardial infarction was also measured. In the GM-CSF group (45.21-%±1.92%), it had no significant different from the GC group (44.80%±1.46%, $P>0.05$) at 28 day after myocardial infarction.

Discussion

Mobilization of BMSC is a special form of BMSC transplantation. The self-transplantation of BMSC is realized by using some cytokines to promote BMSC proliferation. The BMSC then migrate into the blood circulation system, reach the ischemic myocardium and settle down there [3, 4]. In our study, we used GM-CSF to mobilize BMSC. Along with increases in the percentage of bone marrow and peripheral blood $CD34^+$ stem cells, the Ki67 positive cells also increased with obvious correlation, revealing that the mobilized BMSC migrated to the myocardial infarction area. The mechanism of BMSC homing was unclear. It was possible that, after myocardial infarction, the white blood cell and monocytes in the myocardium were activated and released chemokines, such as monocyte chemoattractant protein (MCP) and stromal cell derived factor, thereby promoting the migration and homing of BMSC [13-15].

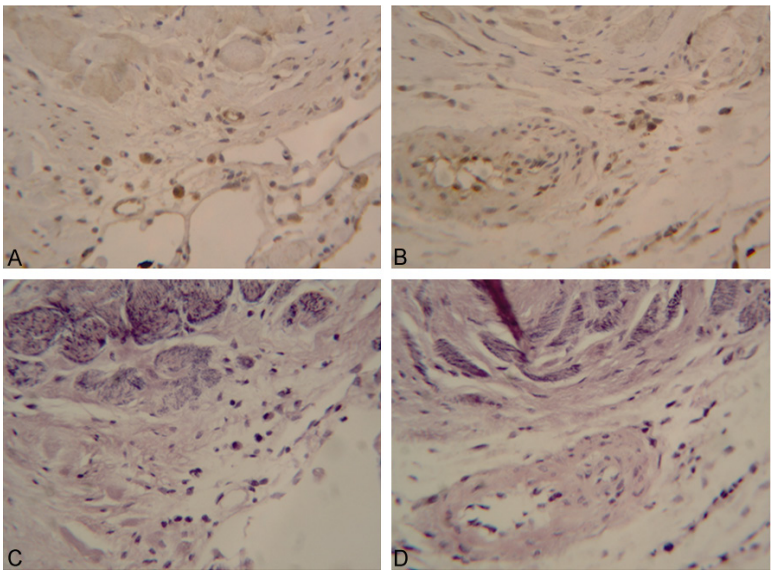

Figure 2. Most Ki67 positive cells (brown nucleus) located at the scar of myocardial infarction tissue, and the GM-CSF group (B) had more Ki67 positive cells than those in GC group (A). But the nucleus of myocardial cells in both GC group and GM-CSF groups (deep violet cytoplasm, C and D) were Ki67 negative, only a few lymphocytes, mesenchymal cells and vascular endothelial cells had Ki67 positive nucleus. (A and B) were Ki67 staining, while (C and D) were PTAH staining (400×).

It has been reported that BMSC could promote the regeneration of blood vessel [16]. We also found a great number of new generated blood vessels in the infarction area of GM-CSF group. After the mobilization of BMSC, $CD34^+$ stem cells, including hematopoietic and progenitor cells and endothelial progenitor cells (EPC) in bone marrow and peripheral blood increased. Like the mobilization of BMSC, bone marrow EPC could be mobilized to the peripheral blood, then migrated to myocardial tissues, and took part in vasculogenesis [4]. Besides, some scientists propose that BMSC has limited effect on the vasculogenesis. The functions of BMSC might be duo to the secretion of vascular endothelial growth factor (VEGF) and then promote the neovascularization from the original blood vessel [16, 17]. Our study also revealed that GC-CSF mobilization could increase the number of $CD34^+$ stem cell in bone marrow and peripheral blood, but this was a late effect. Though it did promote vasculogenesis in myocardial infarction tissue, the optimal time to cure the acute myocardial infarctionwas missed. So, the blood supply could not be restored in time, the myocardial infarction area was not decreased significantly and the cardiac functions were not improved.

BMSC can not only promote vasculogenesis, but also has an amazing rolein myocardial cell regeneration. Orlic et al [3] has first reported that both BMSC transplantation and BMSC mobilization could promote myocardial cell regeneration. However, Norol et al [5, 8] has found that, after the mobilization of BMSC, the number of blood capillary in crease but myocardial cell does not regenerate. Murry [7] and Balsam [6] have verified that BMSC does not differentiate into myocardial cell by transplanting genetic marked mouse bone marrow into the myocardial infarction tissue. Our findings had also revealed that the mobilization of BMSC after myocardial infarction could improve the number of Ki67 positive cells in myocardial tissue. But double staining of Ki67 and PTAH discovered that Ki67 positive nucleus did not overlapped with myocardial cell. This illustrated that BMSC could not promote the regeneration of myocardial cell after myocardial infarction. Our study also found that the mobilization of BMSC could not decrease the myocardial infarction area and improve the cardiac functions, which might be due to the reason that the new generated blood vessel could not improve the blood supply for heart tissue in time and the myocardial cell did not regenerate. The previous papers [18, 19] suggest that Orlic's staining for the stem cell with fluorescent antibody hashigh background fluorescence because of high myocardial protein density and non-specific binding is observed, which confuses the results. In addition, if the transplanted BMSC is closed to the myocardial cell, it is easy to mistake the two cells as one cell because the two cells overlap under fluorescence microscope.

In brief, mobilizing BMSC by GM-CSF could efficiently promote the migration and homing of stem cells to myocardial infarction tissue. And the benefit of BMSC mobilization was more due to the promotion of angiogenesis but not the regeneration of myocardial cell. So, it is necessary to gain more convinced experimental evi-

dence before the application of BMSC to the clinic trial.

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Disclosure of conflict of interest

None.

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