Erythropoietin supports the growth and function of Sca-1+ stem cells and promotes myocardial infarction repair in mice

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Research

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Abstract

Background Myocardial infarction (MI) is the leading cause of death in the world-wide population. With the improvement of clinical therapy, the mortality in acute MI cases has been significantly reduced. This study was to demonstrate that erythropoietin (EPO) is an effective supporter for Sca-1⁺ stem cells (SCs) and can promote the repair of myocardial infarction (MI) partially via way of enhancing Sca-1⁺ SCs activities.

Methods: Darbepoetin alpha (a long-acting EPO analog, EPO_{anlg}) (30 mg/kg) was injected into the border zone of MI in adult mice. Infarct size, cardiac remodeling and performance, cardiomyocytes apoptosis and regenerations and microvessels density were measured. Lin⁻ Sca-1⁺ SCs were isolated from neonatal and adult mice hearts and were respectively used to identify the colony forming ability and the supporting effect of EPO on these Sca-1⁺ SCs.

Results: Compared to MI alone, EPO_{anlg} reduced the infarct percentage and cardiomyocyte apoptosis ratio and LV chamber dilatation, improved cardiac performance, increased the regenerated cardiomyocyte ratio in the border zone and the numbers of coronary microvessels, while without obvious adverse effects in vivo. In vitro, EPO increased the proliferation, migration and clone formation of Lin⁻ Sca-1⁺ SCs likely via the EPO receptor and Stat5-p38MAPK signaling.

Conclusions: EPO promotes Sca-1⁺ SCs activities and MI repair. The study enlightens the prospects of Sca-1⁺ SC supporters in the treatment of MI.

Background

Myocardial infarction (MI) is the leading cause of death in the world-wide population. With the improvement of clinical therapy, the mortality in acute MI cases has been significantly reduced [1]. However, current clinical treatments for MI can only improve myocardial ischemia/reperfusion induced injury, while to a larger extent, cannot resolve the loss of cardiomyocytes and subsequent cardiac remodeling and failure in late phase MI [2].

In recent years, the research of stem cells has provided a new direction to address this problem. Stem cells can be divided into embryonic stem cells and adult stem cells based on their developmental stages [3]. Compared to embryonic stem cells, adult stem cells are easier to differentiate into specific tissue cells without immune rejection and ethical implications [4]. Among them, cardiac stem cells (CSCs) have been observed to have a higher ability than other adult stem cells in treating MI [4]. In vivo, CSCs could be differentiated to cardiomyocytes, fibroblasts and endothelial cells with high efficiency and specificity [5]. Alternative study shows that CSCs may improve MI repair via exosomal transfer [6]. However, disputes arise in recent years in the curative values of CSCs on MI. The leading arguments are whether CSCs exist in the heart and whether these assumed resident CSCs can differentiate to cardiomyocytes. It has been demonstrated that c-kit⁺ CSCs have a poor therapeutic effect on MI because this kind of CSCs are difficult

to differentiate to cardiomyocytes in adult hearts [7]. We also noticed in our preliminary flow cytometry experiments that the population of c-kit⁺ CSCs in mice hearts was very low compared with the stem cell antigen 1 (Sca-1) positive (Sca-1⁺) SCs. A study even denied the existence of regenerative SCs in adult mammalian hearts [8]. Therefore, it is necessary to consider and test alternative SCs for the purpose of MI therapy. Here, we suggest that Sca-1⁺ SCs may be a choice.

Sca-1⁺ SCs exist in many organs including the hearts and have an advantage in amount [9]. Researchers have demonstrated that functional decline of SCs was the main cause of poor cardiac repair [10]. Therefore, for stem cell therapy of MI, the key question is how to keep SCs alive and how to promote or support the proliferation, migration and differentiation of the target SCs. Theoretically, one live stem cell should be enough to support the damage repair of MI. But in fact, cardiac local microenvironment after MI is very harsh and is not suitable for stem cell survival, although the stem cell has strong vitality. Based on this consideration, the selected type of SCs with a larger population is an advantage in stem cell therapy of MI. In this study, we found that erythropoietin (EPO), a glycoprotein cytokine that promotes hematopoiesis, was an effective supporter of Sca-1⁺ SCs, and potentially by this way, promoted MI repair. EPO has been demonstrated to protect ischemic myocardium [11] and improve MI repair by promoting cardiac progenitor cells (CPCs) [12], but there is no report to show that EPO promote MI repair by way of supporting Sca-1⁺ SCs, or at least partially associated with the activities of Sca-1⁺ SCs. Another reason for EPO not been extensively used in treating MI is that systemic application of EPO could cause a variety of adverse effects, including secondary hypertension, venous thrombosis, brain disease and muscle infection [13]. In the present study, we tested intracardiac (*i.c.*) injection of EPO in a mouse MI model aiming to limit these adverse effects. We focused on the long-term curative effects of EPO (*i.c.*) on MI and the underlying mechanisms especially whether EPO exerted the repairing effect on MI via stimulating the growth and function of cardiac resident Lin⁻ Sca-1⁺ SCs, or partially due to the activities of Sca-1⁺ SCs. Results support our expectation that EPO (*i.c.*) was effective in promoting MI repair and enhancing the proliferation and migration of Lin⁻ Sca-1⁺ SCs, and there was likely an association between Sca-1⁺ SCs activities and MI repair. The study suggests that EPO is an effective supporter of Sca-1⁺ SCs and is efficient to treat cardiomyocyte loss and cardiac remodeling after MI without obvious adverse effects.

Methods

Creation of MI and intracardiac injection of EPO analog

We established a mouse MI model by coronary ligation according to a novel procedure [14], and intracardiac (*i.c.*) injection of darbepoetin alpha (a long-acting EPO analog, EPO_{anlg}) (30 mg/kg) was performed during MI. Detailed methods can be found in the Supplementary Material.

Echocardiography

Echocardiography was performed in each mouse to measure cardiac structure and function at the first, second and fourth week post MI using an echocardiographer for small animals (Vevo 2100, VisualSonics, Toronto, Canada). To measure the responsiveness of infarct heart to isoproterenol (ISO) challenge, left ventricular (LV) catheterization was performed to monitor hemodynamics parameters. ISO was injected into the external jugular vein after inhalation anesthesia with isoflurane at the fourteenth day post MI to check the response of MI hearts to β -adrenoceptor activation, as we reported previously [14].

Masson's trichrome staining and determination of infarct size

Masson's trichrome staining was used to identify cardiac infarct size. Detailed methods can be found in the Electronic Supplementary Material.

TUNEL staining

TUNEL staining was used to determine cardiomyocyte apoptosis at the seventh day post MI according to a reported method [15].

Immunofluorescent staining

Immunofluorescent staining was used to identify CD31 of endothelial cells in the myocardium and EPO receptors in $\text{Lin}^- \text{Sca-1}^-$ SCs and $\text{Lin}^- \text{Sca-1}^+$ SCs. Detailed methods can be found in the Electronic Supplementary Material.

Incorporation of BrdU into post-MI hearts

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) (5 mg/ml in 150 mM NaHCO_3) was injected (50 mg/kg *i.p.*) twice a day for five days beginning at the seventh day after the onset of MI, followed by two days of no injection prior to harvesting of hearts. Mice were euthanized 48 hours after the final injection and hearts were flushed with PBS, fixed with 4% paraformaldehyde and embedded in paraffin. Five mm tissue sections were cut with a microtome. To determine BrdU incorporation, tissue slides were deparaffinized, rehydrated and placed in antigen retrieval solution and kept boiling for 10 min. Tissue sections were rinsed with PBS, blocked with 5% serum, incubated with troponin I (Trop I) antibody (dilution 1:200) at 4 °C overnight, then were incubated with BrdU primary antibody (dilution 1:10) for 2 h and rinsed again with PBS. Sections were incubated with secondary antibody (1:300 in block solution) for 1 h. Tissue slides were then rinsed with PBS for 3 × 5 min and stained with DRAQ5 for 5 min, then mounted. Trop I- and BrdU-positive cells, which represented newly regenerated cardiomyocytes, were counted under a fluorescence microscope. The ratio of BrdU-positive cardiomyocyte numbers over total nuclei was calculated.

Isolation and culture of $\text{Lin}^- \text{Sca-1}^+$ SCs

For cloning study, $\text{Lin}^- \text{Sca-1}^+$ SCs were isolated from the hearts of neonatal (1 - 3 days old) C57BL/6J mice. For other cellular experiments, $\text{Lin}^- \text{Sca-1}^+$ SCs were isolated from the hearts of adult (8-week old)

male C57BL/6J mice. Detailed methods for isolating these SCs can be found in the Electronic Supplementary Material.

Above two kinds (neonatal and adult) of filtered cells were centrifuged again at 1300 rpm for 7 min at 4 °C. Cell pellets were resuspended and cell suspension were incubated with biotin-antibody cocktail (10 μ l/ 10^7 cells) at 4 °C for 10 min. Cell pellets were then incubated with anti-biotin microbeads (20 μ l/ 10^7 cells) at 4 °C for 15 min. Cells were purified by the magnetic column and Lin⁻ cells were obtained. Lin⁻ cell suspension was incubated with anti-Sca-1-FITC (10 μ l/ 10^7 cells) for 10 min at 4 °C, washed with PBS, and incubated with anti-FITC microbeads (20 μ l/ 10^7 cells) for 15 min at 4 °C. Sca-1⁺ SCs were collected by positive magnetic sorting. After sorting, the percentage of Lin⁻ Sca-1⁺ SCs was (85.03 $\pm$ 5.28)% which was significantly higher than the Lin⁻ Sca-1⁻ SCs (7.64 $\pm$ 3.31)% ($p < 0.001$) (Additional file 1: Fig. S1). Therefore, by magnetic sorting, high purity cardiac resident Lin⁻ Sca-1⁺ SCs were obtained.

Flow cytometry to detect cell cycle

Lin⁻ Sca-1⁺ SCs isolated from adult mice were used. Purified Lin⁻ Sca-1⁺ SCs suspension was centrifuged and 2 ml pre-cooled 70% ethanol was added to the pellet for overnight at 4 °C. The immobilized cells were centrifuged at 2000 rpm for 4 min. Cells pellets were rinsed with PBS for 3 times, and then incubated with 100 μ l RNase A and 400 μ l propyl iodide (PI) for 15 min at RT. Flow cytometry was used to detect fluorescence signal at the excitation wavelength of 488 nm. The percentages of Lin⁻ Sca-1⁺ SCs at G₀/G₁, S and G₂/M phases were calculated.

Measurement of cell proliferation

The third passage of Lin⁻ Sca-1⁺ SCs isolated from adult mice were seeded to 24-well plates (5 $\times 10^3$ per well). The Lin⁻ Sca-1⁺ SCs were divided into EPO group and PBS (vehicle) group (6 wells per group) randomly. EPO (0.5 μ mol/l) was added to each well of EPO group every 48 hours. Meanwhile, same volume of PBS was added to the wells of vehicle group. Cells were cultured in Cytation 5 cell imaging microtiter plate and cell numbers were counted every 4 h.

Assessment of SCs clonality

The third passage of Lin⁻ Sca-1⁺ SCs isolated from neonatal mice were seeded to 6-well plates (1 $\times 10^5$ per well). EPO (0.5 μ mol/l) was added to each well of EPO group every 48 hours, and the same volume of PBS was added to each well of vehicle group. At the seventh day of treatment, cloning numbers were counted under a microscope.

Transwell assay to measure SCs migration

The third passage of Lin⁻ Sca-1⁺ SCs isolated from adult mice were recovered and seeded into the upper chamber of a transwell (200 μ l, 5 $\times 10^4$ cells/ml). Stem cell culture medium (500 μ l) was added into the bottom chamber. After cells were adhered, serum-free medium was used to starve cells for overnight.

Then the culture media with different concentrations of EPO (0, 0.05, 0.1, 0.5, 1, 5 $\mu\text{mol/l}$) were added to the bottom chamber. After 24 h, cells in the upper chamber removed to the bottom surface. The transwell chamber was immersed in 4% paraformaldehyde for 30 min at RT and rinsed with PBS for 3×5 min. The transwell chamber was placed in 0.1% crystal violet solution for 20 min at RT. After rinsing with water for 3×5 min, transwell chamber was placed under a microscope for data collection. Finally, the transwell chamber was decolored with 1 ml of 33% glacial acetic acid for 10 min and then the optical density (O.D.) values representing migrated $\text{Lin}^- \text{Sca-1}^+$ SCs were determined in different groups with a microplate reader at the wavelength of 570 nm.

Western blotting

Western Blotting was used to evaluate the expression levels of EPO receptor in $\text{Lin}^- \text{Sca-1}^-$ and $\text{Lin}^- \text{Sca-1}^+$ SCs, and the expression levels of p38, p-p38, Stat-5, p-Stat-5, Erk-1/2 and p-Erk-1/2 in $\text{Lin}^- \text{Sca-1}^+$ SCs. Detailed methods for Western blotting can be found in the Electronic Supplementary Material.

Reagents

Reagents used in this study were purchased from the following suppliers: EPO, Sigma; darbepoetin alpha, Amgen, Thousand Oaks, CA; mouse lineage depletion kit, Miltenyi Biotec; anti-Sca-1 MicroBead Kit, MACS; LS column, Miltenyi Biotec; Masson's trichrome stain kit, propyl iodide (PI), ribonuclease A, BrdU, DAPI and anti-p-Stat-5A/B (pSer725/730), Sigma; transwell, Corning; anti-CD31, BD; anti-Stat-5 (c-17) and secondary antibodies, Santa Cruz; anti-phospho-p38 MAPK, anti-p38 MAPK (Thr180/Tyr182), anti-phosphoPlus® p44/42 MAPK (Erk-1/2) (Thr202/Tyr204), anti-p44/42 MAPK (Erk-1/2) and anti-troponin I, Cell Signaling. All other chemicals were purchased from Sigma (St. Louis, MO).

Statistical analysis

All values are presented as mean $\pm$ standard error (S.E.M.) of independent experiments from given n-sizes. Statistical significance was determined by student's *t*-test or one-way ANOVA followed by the Bonferroni post-hoc test where appropriate. The survival rate was analyzed by Gehan Breslow-Wilcoxon test. *p* value of < 0.05 were considered statistically significant.

Results

EPO improves MI-induced cardiac remodeling and dysfunction

Echocardiographic results revealed that EPO_{anlg} (*i.c.*) rescued the decline of cardiac pumping function after MI (Fig. 1a). At the fourth week post MI, LV ejection fraction (LVEF) and LV fraction shortening (LVFS) in the EPO_{anlg} group were respectively ($28.9 \pm 0.49\%$) and ($20.1 \pm 0.57\%$), which were respectively better than those in the PBS group ($(16.4 \pm 0.27\%)$ and ($12.1 \pm 0.38\%$)) ($p < 0.05$) (Fig. 1b, 1c). In addition,

LV end-diastolic internal diameter (LVDI, d) in the EPO_{anlg} group (5.0 ± 0.06 mm) was shorter than that in the PBS group (5.7 ± 0.09 mm) ($p < 0.05$) (Fig. 1d), suggesting improvement of LV chamber dilatation after MI in the EPO_{anlg} treated mice.

At the second week after MI, hemodynamic data showed that aortic systolic pressure (ASP), LV $+dP/dT_{\max}$ and $-dP/dT_{\max}$ in the EPO_{anlg} group were all greater than those in the PBS group after isoproterenol (ISO) challenge ($p < 0.05$) (Fig. 1e, 1f, 1 g, 1 h). In addition, $-dP/dT_{\max}$ (-6528 ± 90.1 mmHg/sec) in the EPO_{anlg} group was also higher than that in the PBS group (-4174 ± 201.2 mmHg/sec) ($p < 0.05$) (Fig. 1h). Moreover, ASP (92.6 ± 1.41 mmHg and 90.2 ± 2.55) in the EPO group respectively at the 14th day and the 28th day post MI were higher than those in PBS group (82.1 ± 3.74 mmHg and 82.2 ± 3.40) ($p < 0.05$) (Fig. 1e). There was no significant difference in the heart rate (HR) between EPO_{anlg} group and PBS group (Fig. 1i).

Note that the mean ASP in the EPO_{anlg} group were less than 100 mmHg (Fig. 1e), suggesting that intracardiac injection of EPO_{anlg} did not induce hypertension. We also did not find thrombosis in larger blood vessels and coronary arteries in the EPO-treated mice (not shown).

Figure 1 Echocardiographic results showing the protective effects of EPOanlg on cardiac function in mice with MI. Echo experiments were performed at the twenty-eighth day post MI. (a) Representative M-mode cardiac echo recordings from sham, PBS-treated and EPOanlg-treated mice. (b-d) Statistical results of left ventricular ejection fraction (LVEF), left ventricular fraction shortening (LVFS) and left ventricular diastolic internal diameter (LVDI, d) in the PBS and EPOanlg groups measured at different times post MI. (e-i) Hemodynamic parameters in response to isoproterenol (ISO) challenge measured 14 days after MI. B, baseline. ASP, aortic systolic pressure. * $P < 0.05$ vs PBS group; $n = 6-8$ mice per group.

EPO reduces MI scar area and improves loss of myocardial mass

At the 7th, 14th and 28th day after the onset of MI, mice were executed and cardiac tissues were harvested and Masson's trichrome staining was performed to measure scar area and heart size. Results showed that the percentage of LV scar area in the EPO_{anlg} group was smaller than that in the PBS group (Fig. 2a, 2b). At the 28th day post MI, LV infarct percentage was (42.7 ± 2.6)% in the EPO_{anlg} group, which was lower than in the PBS group (55.8 ± 1.7)% ($p < 0.05$) (Fig. 2b). Mice in the EPO_{anlg} group showed a smaller infarct size (Fig. 2b) and a smaller LV chamber (Fig. 2c) compared with those of the PBS group, indicating that EPO_{anlg} rescued MI-induced LV chamber dilatation. In addition, the ratio of heart weight to body weight (HW/BW) at the second and the forth week post MI in the EPO_{anlg} group (6.22 ± 0.19 mg/g and 5.82 ± 0.13 mg/g, respectively) were higher than those in the PBS group (5.18 ± 0.11 mg/g and 4.78 ± 0.18 mg/g) ($p < 0.05$) (Fig. 2d), and the ratio of heart weight to tibial length (HW/TL) at the second week post MI in the EPO_{anlg} group (8.15 ± 0.25 mg/mm) was also higher than that in the PBS group (6.87 ± 0.14 mg/mm) ($p < 0.05$) (Fig. 2e), suggesting that EPO improved the loss of myocardial mass after MI. Mice in the EPO_{anlg} group showed a higher survival rate after MI than in the PBS group (Fig. 2f).

Figure 2 Effects of EPO_{anlg} on infarct size, cardiac structural remodeling and survival rate in mice with MI. (a) Representative Masson's trichrome stains of hearts obtained at the first, second and fourth week after MI from EPO_{anlg} and PBS groups showing infarct area (blue) and heart size. Scale bar, 1000 μ m. (b) Percentage infarct size. (c) Calculated LV area representing the size of LV chamber and wall. (d, e) Statistical HW/BW and HW/TL ratios, respectively. (f) Mice survival rate post MI. * $P < 0.05$ vs PBS group; $n = 6-8$ mice per group.

Discussion

This study demonstrated that EPO promoted mice MI repair partially via supporting the growth and function of cardiac resident Sca-1⁺ SCs. EPO is a major regulator of red blood cell generation and plays a protective role in cardiovascular diseases, such as decreasing cardiomyocyte apoptosis in vitro [16], reducing cardiomyocyte damage during cardiac ischemia/reperfusion in vivo [17], reducing MI scar area and improving cardiac function [18]. However, subcutaneous and intravenous injection, the usual clinical administration route of EPO, cannot avoid the adverse effects of systemic administration [19–21]. The present study showed that intracardiac (*i.c.*) injection of EPO_{anlg} to the infarct border zone of myocardium after coronary occlusion exerted significant curative effects on MI while without obvious side effects. In clinical practice, intracardiac injection of EPO might be accomplished by catheter technique.

Although EPO has been found to protect ischemic myocardium and reduce cardiomyocytes apoptosis, the underlying mechanisms have not been well clarified. Specifically, it is unknown whether these cardiac protective effects of EPO are associated with Sca-1⁺ SCs. In the early stage of this study, we observed that EPO_{anlg} increased the generation of cardiomyocytes after MI. Based on this observation, we expected that EPO may exert its cardioprotective effects via stimulating the growth and function of a certain type of SCs. We first observed the protective effect of EPO_{anlg} on cardiac function after MI. Echo data indicated that LVEF and LVFS in the EPO_{anlg} group were all better than those in the PBS group four weeks after MI; LVID_d in the EPO_{anlg} group was shorter than that in PBS group, suggesting that EPO_{anlg} improved LV performance and chamber dilatation. Hemodynamic results showed that LVSP and $\pm$ dP/dT_{max} were higher in the EPO_{anlg} group than those in the PBS group. At the fourteenth day post MI, the responses of hearts to isoproterenol (ISO) in the EPO_{anlg} group was better than that in the PBS group, suggesting a restore of the cardiac response to catecholamines. All these results indicate that intracardiac injection of EPO_{anlg} improves cardiac remodeling and dysfunction after MI, a similar curative effect as observed by EPO systemic administration in an alternative study [22].

We further investigated the effects of EPO_{anlg} on cardiac structural remodeling after MI using differential methods. It is known that MI can induce irreversible cardiac remodeling, and the remodeling will sustain throughout the whole pathological process [23] till heart failure. Preventing cardiac pathological remodeling is a key treatment of MI and subsequent heart failure. In the present study, Masson's trichrome staining at the first, second and fourth week of MI revealed that the infarct area in the EPO_{anlg}

group was smaller than that in the PBS group at the same time points. The ratios of HW/BW and HW/TL in the EPO_{anlg} group were higher than those in the PBS group at the fourth week after MI, but the mean heart size was smaller in the EPO_{anlg} group than that in the PBS group, suggesting that EPO_{anlg} significantly improved cardiomyocytes loss, wall thinning, LV chamber dilatation and myocardial mass decline. Moreover, EPO_{anlg} treatment increased the survival rate of MI mice compared with the mice treated with PBS. This result is coincident with an alternative study of systemic EPO administration [24]. All these results indicate that EPO_{anlg} intracardiac injection improved cardiac remodeling towards heart failure after MI.

Progressive loss of cardiomyocytes is the main cause of cardiac function decline and remodeling after MI [25]. In present clinical practices, there is no effective drug to solve this problem. In the ways of cell death, necrosis is irreversible, but apoptosis is a programmed cell death which can be regulated. It is reported that inhibition of cardiomyocyte apoptosis could reduce infarct size by 50% – 70% [26]. In the present study, EPO treatment decreased cardiomyocyte apoptosis after MI. More importantly, we observed that EPO_{anlg}-treated MI hearts were heavier (with higher HW/BW and HW/TL ratios) than that treated with PBS, and EPO increased the numbers of live cardiomyocytes and microvessels in the border zone and even in the remote area of MI. These results suggest that EPO rescues the loss of myocardial mass after MI. Unfortunately, we were unable to identify whether EPO promoted the differentiation of Sca-1⁺ SCs to cardiomyocytes. More accurate detection methods are needed to identify this differentiation. The increase of cardiomyocyte numbers in response to EPO may mainly be a result of reduced apoptosis rather than differentiation of Sca-1⁺ SCs to cardiomyocytes. Immune or exosome mechanisms may also be involved in the cardioprotective effect of EPO. Increased cardiomyocyte numbers may resist cardiomyocyte apoptosis and hinder wall thinning and heart failure after MI, and thus improve cardiac function.

We performed transwell assay, flow cytometry and dynamic cell proliferation assay to confirm the promoting effects of EPO on the proliferation and migration of Sca-1⁺ SCs. In the transwell assay, we observed that EPO promoted the migration of Lin⁻ Sca-1⁺ SCs roughly in a concentration-dependent manner, EPO at 0.5 μ mol/l yielded the strongest migration-stimulating effect. Flow cytometry and dynamic cell proliferation assay indicated that EPO promoted the proliferation of Lin⁻ Sca-1⁺ SCs, as shown by the increase of clone formation in neonatal Lin⁻ Sca-1⁺ SCs. These results, together with the BrdU incorporation results, suggest that EPO promoted the proliferation and migration of Lin⁻ Sca-1⁺ SCs, and these effects might contribute to the repairing effect of EPO on MI.

We found that EPO receptor (EPOR) was expressed in Sca-1⁺ SCs. It is possible that the delivered EPO may bind to EPOR and thus activate Sca-1⁺ SCs to proliferate and migrate to the injured area. We isolated and purified Lin⁻ Sca-1⁺ SCs from mice hearts by magnetic bead sorting. The purity of Lin⁻ Sca-1⁺ SCs in primary isolation was about 85%. The lineage cell deletion kit we used is a labeling system for deleting the mature hematopoietic cells (lineage positive, Lin⁺), including T cells, B cells, macrophages, granulocytes, erythrocytes and their committed precursor cells from bone marrow. The cocktail

biotinylated antibodies can recognize the antigens include CD5, CD45R, CD11b and Gr-1, etc. Therefore, the SCs used in the study were mostly Lin⁻ Sca-1⁺ SCs which were plentiful in the heart, suggesting a perspective of Lin⁻ Sca-1⁺ SCs in treating MI.

We further examined the potential signaling pathways underlying the above beneficial effects of EPO. It is known that EPO participates in cell proliferation by activating JAK2/Stat5, PI3K/Akt and Ras/Erk signal pathways [27], and JAK2 is thought to play an important role in promoting cell proliferation [28]. Stat5 is activated by JAK2, and it then translocates to the nucleus, recognizes a specific base sequence in the promoter region of its target gene, and initiates transcription [29]. However, it is unknown whether EPO promotes the proliferation of Lin⁻ Sca-1⁺ SCs via the same signaling pathways described above. We found that p-Stat5 and p-p38 MAPK were significantly increased 30 min after EPO treatment, while EPO did not significantly increase the expression level of p-Erk. These results suggest that EPO activates JAK2-Stat5 and p38 MAPK signal pathways via the EPOR and promoted the growth and function of Lin⁻ Sca-1⁺ SCs.

Study limitations. The first limitation of the study was that we could not solidly demonstrate the contribution of neo-vascularization and increased amounts of cycling cardiomyocytes after EPO administration in the cardioprotective action of EPO. The reduction in infarct size may be due to reduced apoptosis rather than new cardiomyocytes formed by Sca-1⁺ cells. A second limitation was that we could not clarify the derivation of cycling cardiomyocytes from Sca-1⁺ cells. It is possible that the cycling cardiomyocytes may not derive from Sca-1⁺ cells but may cycle due to paracrine factors secreted from Sca-1⁺ cells and/or the other cell types affected by EPO in the heart. A third limitation was that we did not carefully compare the differences in the potential side effects of EPO (hypertension, thrombosis, neuronal adverse reactions, allergy, etc.) between intracardiac (i.c.) injection and intravenous (i.v.) infusion. Further investigations are warranted to address these questions.

Conclusions

In summary, the present study demonstrated that intracardiac injection of EPO_{anlg} improved cardiac dysfunction and remodeling towards heart failure, reduced the death rate in mice with MI, decreased apoptosis and increased the numbers of cardiomyocyte, while without obvious adverse effects. These curative effects of EPO are potentially associated with the promoting effects of EPO on the proliferation and migration of Lin⁻ Sca-1⁺ SCs, although we cannot exclude the possibility that EPO may has effects on a variety of cells including stem/progenitor cells. EPO exerted these effects likely via the activation of EPOR and the downstream Stat-5 and p-38 MAPK signal pathways in Lin⁻ Sca-1⁺ SCs. The study provides a new mechanism by which EPO rescues the infarct hearts, and suggests that supporting the resident Lin⁻ Sca-1⁺ SCs might be an effective therapeutic strategy in the treatment of MI.

Abbreviations

MI

Myocardial infarction; EPO:Erythropoietin; SCs:Stem cells; Sca1:Stem cell antigen-1; LV:Left ventricular; CSCs:Cardiac stem cells; CPCs:Cardiac progenitor cells; BrdU:Bromodeoxyuridine; PBS:Phosphate buffer saline; Trop I:Troponin I; PI:Propyl iodide; RT:Room temperature; O.D.:Optical density; MAPK:Mitogen-activated protein kinase; Stat:Signal transducers and activators of transcription; S.E.M.:Mean $\pm$ standard error; ASP:Aortic systolic pressure; HR:The heart rate; EPOR:EPO receptors; ERK:Extracellular regulated protein kinases; LVEF:Left ventricular ejection fraction; LVFS:Left Ventricular Fraction Shortening; ISO:Isoproterenol; Lin+:Linage positive; JAK2:Janus Kinase 2; PI3K:Phosphoinositide 3-kinase;

Declarations

Availability of data and materials

All supporting data are included in the article and its additional files.

Ethics approval and consent to participate

All surgical procedures and animal care protocols in this study were approved by the Laboratory Animal Research Committee of Soochow University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.Z., E.G. and J.C. led and designed the project; E.G. carried out the in vivo MI and EPO injection experiments and provided suggestions to the manuscript structure; L.Z., D.L., X.M., S.S., D.L., J.S. and W.Z. performed the remaining experiments; L.Z., D.L. and X.M. analyzed the data; L.Z. and J.C. co-wrote the paper.

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Figures

Figure 1

Echocardiographic results showing the protective effects of EPOanlg on cardiac function in mice with MI. Echo experiments were performed at the twenty-eighth day post MI. (a) Representative M-mode cardiac echo recordings from sham, PBS-treated and EPOanlg-treated mice. (b-d) Statistical results of left ventricular ejection fraction (LVEF), left ventricular fraction shortening (LVFS) and left ventricular diastolic internal diameter (LVID, d) in the PBS and EPOanlg groups measured at different times post MI. (e-i) Hemodynamic parameters in response to isoproterenol (ISO) challenge measured 14 days after MI. B, baseline. ASP, aortic systolic pressure. * $P < 0.05$ vs PBS group; $n = 6-8$ mice per group. EPO reduces MI scar area and improves loss of myocardial mass At the 7th, 14th and 28th day after the onset of MI, mice were executed and cardiac tissues were harvested and Masson's trichrome staining was performed to

measure scar area and heart size. Results showed that the percentage of LV scar area in the EPOanlg group was smaller than that in the PBS group (Fig. 2a, 2b). At the 28th day post MI, LV infarct percentage was $(42.7 \pm 2.6)\%$ in the EPOanlg group, which was lower than in the PBS group $(55.8 \pm 1.7)\%$ ($p < 0.05$) (Fig. 2b). Mice in the EPOanlg group showed a smaller infarct size (Fig. 2b) and a smaller LV chamber (Fig. 2c) compared with those of the PBS group, indicating that EPOanlg rescued MI-induced LV chamber dilatation. In addition, the ratio of heart weight to body weight (HW/BW) at the second and the fourth week post MI in the EPOanlg group (6.22 ± 0.19 mg/g and 5.82 ± 0.13 mg/g, respectively) were higher than those in the PBS group (5.18 ± 0.11 mg/g and 4.78 ± 0.18 mg/g) ($p < 0.05$) (Fig. 2d), and the ratio of heart weight to tibial length (HW/TL) at the second week post MI in the EPOanlg group (8.15 ± 0.25 mg/mm) was also higher than that in the PBS group (6.87 ± 0.14 mg/mm) ($p < 0.05$) (Fig. 2e), suggesting that EPO improved the loss of myocardial mass after MI. Mice in the EPOanlg group showed a higher survival rate after MI than in the PBS group (Fig. 2f).

Figure 2

Effects of EPOanlg on infarct size, cardiac structural remodeling and survival rate in mice with MI. (a) Representative Masson's trichrome stains of hearts obtained at the first, second and fourth week after MI from EPOanlg and PBS groups showing infarct area (blue) and heart size. Scale bar, 1000 μ m. (b) Percentage infarct size. (c) Calculated LV area representing the size of LV chamber and wall. (d, e) Statistical HW/BW and HW/TL ratios, respectively. (f) Mice survival rate post MI. * $P < 0.05$ vs PBS group; $n = 6-8$ mice per group. EPO suppresses cardiomyocyte apoptosis TUNEL staining showed that the percentage of apoptotic cardiomyocytes 7 days after the onset of MI were much lower in the EPOanlg group ($1.45 \pm 0.23\%$) than that in the PBS group ($3.74 \pm 0.87\%$) ($p < 0.01$, $n = 10$ in each group) (Fig. 3).

Figure 3

TUNEL staining of mice LV tissues at the seventh day post MI, reflecting cardiomyocyte apoptosis in the EPO and PBS groups. TUNEL positive stains were green. DAPI stains (nuclei) were blue. Troponin I stains representing cardiomyocytes were red. * $P < 0.05$ vs PBS group; $n = 10$ hearts per group. Scale bar, 100 μm . EPO likely promotes the regeneration of cardiomyocytes and coronary microvessels after MI Intracardiac (i.c.) injection of EPOanlg was performed at three points in the border zone after coronary occlusion. At the second week post MI, BrdU and troponin I were used to label the regenerated cardiomyocytes, and CD31 was used to mark endothelial cells representing coronary microvessels. Results indicated that the percentage of potential neo-cardiomyocytes (BrdU- and troponin I-positive, nucleus and cytoplasm matched) in the border zone were $(0.020 \pm 0.011)\%$ in the EPOanlg group (Fig. 4a, 4e), which was significantly higher than that in the PBS group $(0.002 \pm 0.001)\%$ ($p < 0.05$) (Fig. 4b, 4e). In the non-infarct area, the percentage of newly generated cardiomyocytes was $(0.009 \pm 0.004)\%$ in the EPOanlg group (Fig. 4c, 4e), which was also higher than that in the PBS group $(0.002 \pm 0.001)\%$ ($p < 0.05$) (Fig. 4d, 4e). CD31 staining revealed that the density of microvessels was $72.5 \pm 0.25/\text{field}$ in the EPOanlg group (Fig. 4f, 4h), which was higher than that in the PBS group $(46.1 \pm 0.65/\text{field})$ ($p < 0.05$) (Fig. 4g, 4h). These results suggest that EPO promotes the regeneration of cardiomyocytes and endothelial cells potentially due to

the differentiation of SCs to cardiomyocytes and endothelial cells or due to the paracrine activities of Sca-1+ SCs or other cell types.

Figure 4

Immunofluorescent staining of mice myocardium showing the promoting effects of EPOanlg on the regeneration of cardiomyocytes and microvessels. The staining was performed two weeks after MI. Regenerated cardiomyocytes were only those doubly marked by BrdU (green) and Troponin I (red) (framed cells). Those marked by BrdU alone were not newly regenerated cardiomyocytes. Nuclei were stained blue by DAPI. (a, b) Representative images of regenerated cardiomyocytes (framed) in the border zone of MI in the EPOanlg group and the PBS group, respectively. (c, d) Representative images of regenerated cardiomyocytes in the remote area of MI in the EPOanlg group and the PBS group, respectively. A magnified regenerated cardiomyocyte was shown in the center of a circle in each group. (e) Statistical results of regenerated cardiomyocyte percentage in EPOanlg and PBS groups after MI. Note that more regenerated cardiomyocytes were observed in the EPOanlg group than in the PBS group. (f, g)

CD31 stains representing endothelial cells in the LV walls respectively from the EPOanlg group and PBS group after MI. (h) Quantitative analysis of microvessel density (MVD) after MI in the two groups. * $P < 0.05$ vs PBS group; $n = 8$ hearts per group. EPO promotes the proliferation and migration of Lin⁻ Sca-1⁺ SCs of adult mice and enhances the clone formation of Lin⁻ Sca-1⁺ SCs from neonatal mice in vitro. The purity of isolated Lin⁻ Sca-1⁺ SCs from adult mice hearts reached about 85% (Additional file 1: Fig. S1). Isolated Lin⁻ Sca-1⁺ SCs were cultured using stem cell culture medium. After cell adherence and starvation for 24 hours, the EPO- or PBS-containing culture medium was added to the wells for 48 hours. PI staining and flow cytometry was used to detect the status of cell cycle in different groups. In addition, dynamic cell proliferation was measured by Cytation 5. Results showed that, in the EPO group, the cell percentages at S phase and G2/M phase were $(15.81 \pm 0.31)\%$ and $(14.04 \pm 0.75)\%$, respectively, which were higher than those in the PBS group $((7.89 \pm 0.73)\%$ and $(8.27 \pm 0.57)\%$) ($p < 0.05$) (Fig. 5a, 5b, 5c). Results of dynamic cell proliferation showed that Lin⁻ Sca-1⁺ SCs proliferated faster in the EPO group than in the PBS group ($p < 0.05$) (Fig. 5d). In the experiments of clone formation ability, the Lin⁻ Sca-1⁺ SCs from neonatal mice hearts formed more clones in the EPO group than that in the PBS group in vitro ($p < 0.01$) (Fig. 5e, 5f, 5g). To check the migration of Lin⁻ Sca-1⁺ SCs from adult mice hearts, cells (1×10^5 cells/well) were seeded into the upper chamber of the transwell and cultured with the stem cell culture medium. EPO were added to the bottom wells. Results of transwell assay and crystal violet staining showed that EPO concentration-dependently increased the migration of Lin⁻ Sca-1⁺ SCs (43.24 ± 2.77 , 51.35 ± 3.42 , 63.22 ± 3.55 , 49.43 ± 4.19 and 50.83 ± 2.67 cells/field respectively corresponding to EPO concentrations of 0.05, 0.1, 0.5, 1 and 5 $\mu\text{mol/L}$) (Fig. 6a, 6b), which were all more than that in the PBS group (23.24 ± 2.99 cells/field) ($p < 0.05$). EPO at 0.5 $\mu\text{mol/L}$ likely had the best effect in promoting SCs migration. Results from the microplate reader (O.D. value of crystal violet) were similar with the above cell count data. In the EPO group, the O.D. values representing migration levels were 0.1520 ± 0.0020 , 0.1575 ± 0.0015 and 0.1530 ± 0.0030 , respectively corresponding to EPO concentrations ($\mu\text{mol/L}$) of 0.1, 0.5 and 1.0; these O.D. values were also all higher than that in the PBS group (0.1415 ± 0.0015) ($p < 0.05$) (Fig. 6c).

Figure 5

Flow cytometry results showing the effects of EPO on cell cycle of adult Lin⁻ Sca-1⁺ SCs and clone formation of neonatal Lin⁻ Sca-1⁺ SCs. (a, b) Typical cell distribution reflecting cell cycle in the PBS group (A) and EPO (B) group. (c) Percentage of cells at different phases of cell cycle. (d) cell proliferation curves recorded by Live Cell Imaging System. (e, f) Representative images of neonatal Sca-1⁺ SCs clone formation respectively in the PBS group and EPO group. Scale bar, 100 μ m. (g) Statistical results of SCs clone formation. ** $P < 0.01$ vs PBS group; $n = 6$ per group.

Figure 6

Transwell assay showing the promoting effect of EPO on the migration of Lin⁻ Sca-1⁺ SCs in vitro. (a) Representative images of migrated Lin⁻ Sca-1⁺ SCs treated with different concentrations of EPO. The migrated Lin⁻ Sca-1⁺ SCs was stained purple blue with crystal violet. (b) Cell migration analyzed by cell counting assay. (c) Cell migration analyzed by quantitative crystal violet elution assay. * $P < 0.05$ vs PBS group; $n = 8$ per group. Scale bar (yellow), 50 μm . EPO receptors and p38-MAPK and Stat-5 signaling pathways participate in the EPO effects on Lin⁻ Sca-1⁺ SCs. Immunofluorescent staining showed that EPO receptors (EPOR) were expressed in the surface membrane of Lin⁻ Sca-1⁺ SCs (Additional file 1: Fig. S2). Western blotting indicated that 30 min after EPO treatment, the expression levels of p-STAT5 and p-p38 MAPK in adult mice Lin⁻ Sca-1⁺ SCs were significantly increased compared with PBS treatment ($p < 0.05$) (Fig. 7a, 7b, 7c). There was no significant change of p-ERK expression after EPO treatment compared with the PBS group (Fig. 7d).

Figure 7

Western blotting to identify the potential signaling pathway(s) by which EPO activates Lin⁺ Sca-1⁺ SCs. (a) Representative electrophoresis bands showing the protein expression levels of Stat-5, p-STAT-5, p38 MAPK, p-p38 MAPK, ERK, p-ERK and β-actin in Lin⁺ Sca-1⁺ SCs treated with different concentrations of EPO and PBS (vehicle). (b-d) Statistical ratios of p-Stat-5/Stat-5, p-p38 MAPK/p38 MAPK and p-ERK/ERK in the EPO treated (for different times) Lin⁺ Sca-1⁺ SCs and PBS treated Lin⁺ Sca-1⁺ SCs. * P < 0.05, *** P < 0.001 vs vehicle (PBS); n = 6 per group.

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