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ORIGINAL ARTICLE

Combining erythropoietin infusion with intramyocardial delivery of bone marrow cells is more effective for cardiac repair

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Summary

We postulated that combining erythropoietin (EPO) infusion with bone marrow mesenchymal stem cells (MSC) delivery may give better prognosis in a rat infarcted heart. Acute myocardial infarction (MI) model was developed by coronary artery ligation. Animals were grouped ($n = 18$) to receive intramyocardial injection of 30 μl saline solution without cells (EPO and control groups) or with 3×10^6 MSC from transgenic green fluorescent protein (GFP)+ male mice (MSC and MSC-EPO groups). The animals received either 5000 U/kg body weight EPO (EPO and MSC-EPO groups) or saline solution (MSC and control groups) for 7 days after MI. Cardiac functions were measured by echocardiography and cardiac tissue was harvested for immunohistological studies 3 weeks after surgery. We observed regeneration of MSC in and around the infarcted myocardium in MSC and MSC-EPO groups. Capillary density was markedly enhanced with significantly smaller infarct size and reduced fibrotic area in MSC-EPO group as compared with other three groups. A smaller left ventricular (LV) diastolic dimension and a higher LV fractional shortening were observed in MSC-EPO group than in other three groups. Transplantation of MSC combined with cytokine EPO is superior to either of the monotherapy approach for angiomyogenesis and cardiac function recovery.

Introduction

The prevalence of ischemic heart failure remains markedly high despite several recent therapeutic advances in the treatment of acute myocardial infarction (MI) [1]. Although myocytes mitosis and the presence of cardiac precursor cells in adult hearts have recently been reported [2], the death of large numbers of cardiomyocytes results in the development of heart failure. Thus, restoring lost myocardium would be desirable for the treatment of heart infarction.

Bone marrow mesenchymal stem cells (MSC) have generated a great deal of excitement and promise in cell and gene therapy applications, because of their multipotentiality and capacity for extensive self-renewal [3,4]. MSC have been used for cardiomyoplasty and to induce neovascularization when they are injected into infarcted myocardium [5]. Preclinical studies investigating bone marrow-derived MSC as treatment for ischemic myocardium have been performed [6]. However, optimization of strategies to enhance both the generation and integration of bone marrow-derived cells into the heart may augment application of this technology for the treatment and possible prevention of heart disease.

Erythropoietin (EPO) was first characterized as a hematopoietic factor. It has been shown to stimulate differentiation and proliferation of erythroid progenitor cells [7]. The primary clinical use of EPO is to increase the production of red blood cells in certain anemia. Independent of its hematopoietic effect, EPO was recently shown to be protective in heart disease [8]. Recently, it is

reported that EPO and other signaling factors involved stem cells regenerating the infarcted heart [9].

Taken these findings together, we investigated whether the combination therapy with MSC transplantation and EPO infusion would promote functional neovascularization in a rat model of heart ischemia.

Materials and methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Expansion of bone marrow MSC

The MSC expansion was performed according to previously described methods [10]. In brief, we humanely killed male transgenic mice expressing green fluorescent protein (GFP-mice, provided by State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, China) and harvested bone marrow by flushing their femoral and tibial cavities with phosphate-buffered saline (PBS). Bone marrow cells were cultured in Low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplement with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics. A small number of cells developed visible symmetric colonies by day 5–7. Hematopoietic cells, fibroblasts and other nonadherent cells were washed away during medium changes. The remaining purified MSC population was further expanded in culture.

Flow cytometry

Cultured MSC were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). Cells were incubated with fluorescence isothiocyanate (FITC)-conjugated rat anti-mouse CD45, CD34 (Becton Dickinson), phycoerythrin (PE)-conjugated rat anti-mouse CD117, CD44 (Becton Dickinson). Cells were also stained with purified rat anti-mouse CD29 and CD31 (Becton Dickinson) and then counterstained with FITC-conjugated goat anti-rat IgG. The cells stained with FITC- or PE-labeled goat anti-rat IgG were used as negative controls.

Myocardial infarction model and animal groups

Female Wistar rats weighing 210–250 g (State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, China) were used in this study. Rats were anesthetized with a combination of ketamine (30 mg/kg intraperitoneally, i.p.) and xylazine (10 mg/kg i.p.). The rats were shaved, intubated and ventilated mechanically with humidified room air supplemented with oxygen. In a sterile procedure, the chest was opened through the fourth and fifth intercostal space. After the pericardium was opened, A 5-O silk suture with attached atraumatic needle was placed around the left descending coronary artery. MI model was deemed successful when the anterior wall of the left ventricular turned pale and the ST-T wave elevated >0.05 mV than baseline.

After MI was created, animals were randomly divided into four groups ($n = 18$ in each group): (i) MSC group (MSC transplantation plus vehicle infusion), (ii) EPO group (vehicle transplantation plus EPO infusion), (iii) MSC-EPO group (MSC transplantation plus EPO infusion), (iv) control group (vehicle transplantation and vehicle infusion).

MSC transplantation and EPO infusion

Transplantation of MSC and/or 7-day infusion of EPO was performed immediately after coronary ligation. MSC (3×10^6 cells in 30 μl saline solution) was injected into three sites with a 26-gauge needle bent, one within the infarct area and two in the myocardium bordering the ischemic area. EPO (5000 U/kg body weight) was subcutaneously administered for 7 days, whereas control animals were injected saline solution for the same time duration.

Functional assessment by echocardiography

Echocardiographic studies were performed by an experienced technician, blinded to the treatment group 3 weeks after operation. Two-dimensional, targeted M-mode tracings were obtained at the level of papillary muscles with an echocardiographic system equipped with 12 MHz phased array transducer (Hewlett Packard Inc, Andover, MA, USA). The left ventricular ejection fraction (LVEF) and LV dimensions were measured according to the American Society for Echocardiology leading-edge method from at least three consecutive cardiac cycles. Fractional shortening (FS) was calculated as $[(\mathrm{LVDd} - \mathrm{LVDs})/\mathrm{LVDd}] \times 100$, where LVDd, LV diastolic dimension and LVDs, LV systolic dimension.

Histological examination

Three weeks after surgery, rats were sacrificed and hearts were harvested for following histological examination. Heart infarct size was measured by tetrazolium method [11] ($n = 8$ from each group). IA, RA and LA were calculated from enlarged digital micrographs according to different colors (pale white = infarct area; red = living at risk; blue = normal; risk area = pale white + red). The ratios (%, heart weight integrated) of IA to RA and RA to LA were calculated.

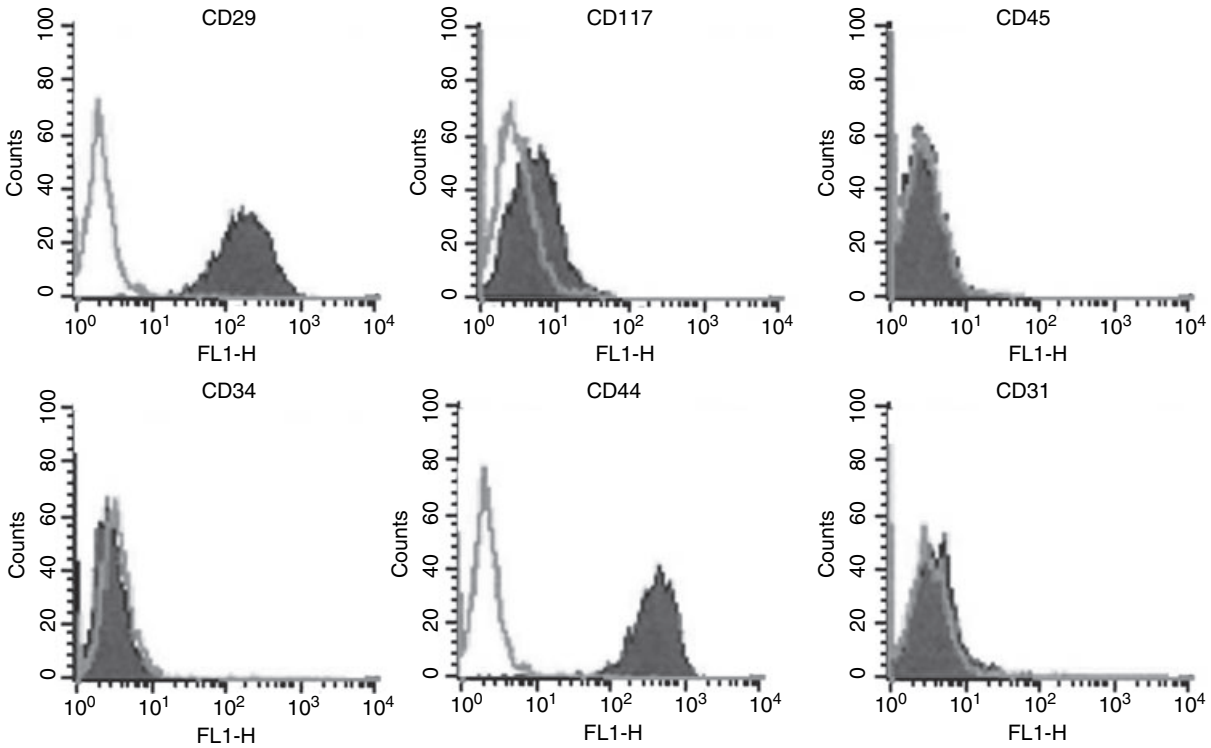

Figure 1 Flow-cytometric analysis of the adherent, spindle-shaped MSC population expanded to three to five passages. CD34+ 0.9%; CD31+ 0.7%; CD45+ 0.5%; CD117+ 1.2%; CD29+ 92.9%; CD44+ 93.2%.

To detect capillary endothelial cells in the peri-infarct area, samples of the harvested muscle ($n = 5$ each) were embedded in optimum cutting temperature (OCT) compound, snap frozen in liquid nitrogen, and cut into transverse sections. Tissue sections were immunostained for the endothelial cell marker factor von Willebrand factor (Dako, Carpinteria, CA, USA). The number of capillary vessels was counted in the peri-infarct area under a light microscope in five random fields. Capillary density was expressed as the area of blood vessels in μm^2 per mm^2 of each ventricular section.

To detect fibrosis in cardiac muscles, the LV myocardium ($n = 5$, each) was fixed in 4% paraformaldehyde, cut transversely, embedded in paraffin, and stained with Masson's trichrome. Ten anterolateral sections from each heart were evaluated in their entirety and quantified. The results were expressed as μm^2 of fibrosis per mm^2 of each ventricular section. These morphometric studies were performed by two examiners who were blinded to treatment assignment.

Monitoring of implanted MSC in ischemic heart

Left ventricular samples were embedded in OCT compound, snap-frozen in liquid nitrogen, and cut into sections. Potential transformation to cardiac-like cells from engrafted MSC was verified by antibody immunostaining for cardiac troponin I (Abcam, Cambridge, UK). Neovascular transformation of engrafted MSC was verified by antibody immunostaining for α-smooth muscle actin (α-SMA; Dako) and von Willebrand factor (vWF; Dako) according to the manufacturer's recommendations. Tetraethyl rhodamine isothiocyanate (TRITC)-conjugated IgG antibody (BD Pharmingen, San Diego, CA, USA) was used as a secondary antibody. All morphometric studies were performed by two examiners blinded to treatment.

Table 1. Echocardiographic findings (mean ± SD, $n = 8$ in each group).

	MSC-EPO	MSC	EPO	Control
LVEF (%)	84.4 ± 3.9*,†,‡	79.4 ± 1.8*	75.2 ± 3.6*	69.0 ± 2.2
LVDd (mm)	3.0 ± 0.3*,†,‡	5.6 ± 0.5*	5.5 ± 0.3*	6.3 ± 0.3
LVSd (mm)	5.6 ± 0.4*,†	3.3 ± 0.3*	3.5 ± 0.2*	4.2 ± 0.2
%FS (%)	26.6 ± 1.2*,†,‡	22.0 ± 1.7*	20.8 ± 0.8*	14.4 ± 0.9

*$P < 0.05$ vs. control group.
†$P < 0.05$ vs. MSC group.
‡$P < 0.05$ vs. EPO group.
LVEF, LV ejection fraction; LVDd, LV diastolic dimension; LVDs, LV systolic dimension; %FS, LV fractional shortening.

Statistical analysis

All data are expressed as mean ± SD. Statistical significance was evaluated with Student's *t*-test for comparisons between two means, with ANOVA, followed by Scheffe's procedure for more than two means, and with repeated-measures ANOVA to test for interaction. Data were considered significant when the *P*-value was <0.05.

Results

Characterization of cultured MSC

Cultured MSC were identified by surface markers: CD34+ 0.9%; CD31+ 0.7%; CD45+ 0.5%; CD117+ 1.2%; CD29+ 92.9%; CD44+ 93.2%. Flow cytometric analysis showed that they were strongly positive for CD29 and CD44, but negative for CD31, CD34, and CD45 (Fig. 1).

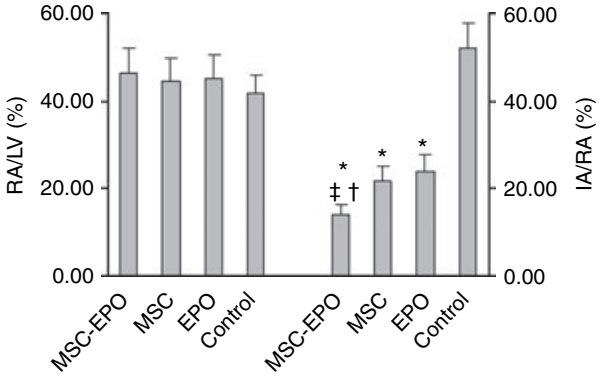

Figure 2 Bone marrow cells and erythropoietin myocardial infarct size. **P* < 0.05 vs. control group, †*P* < 0.05 vs. MSC group, ‡*P* < 0.05 vs. EPO group.

Echocardiographic study

The LVEF was highest in MSC-EPO group, followed by MSC and EPO groups, when compared with control group. LV diastolic dimension was smallest, in the MSC-EPO group, followed by the MSC, EPO, and control groups. LV FS in the MSC-EPO group was also higher than that in the control, MSC, and EPO groups

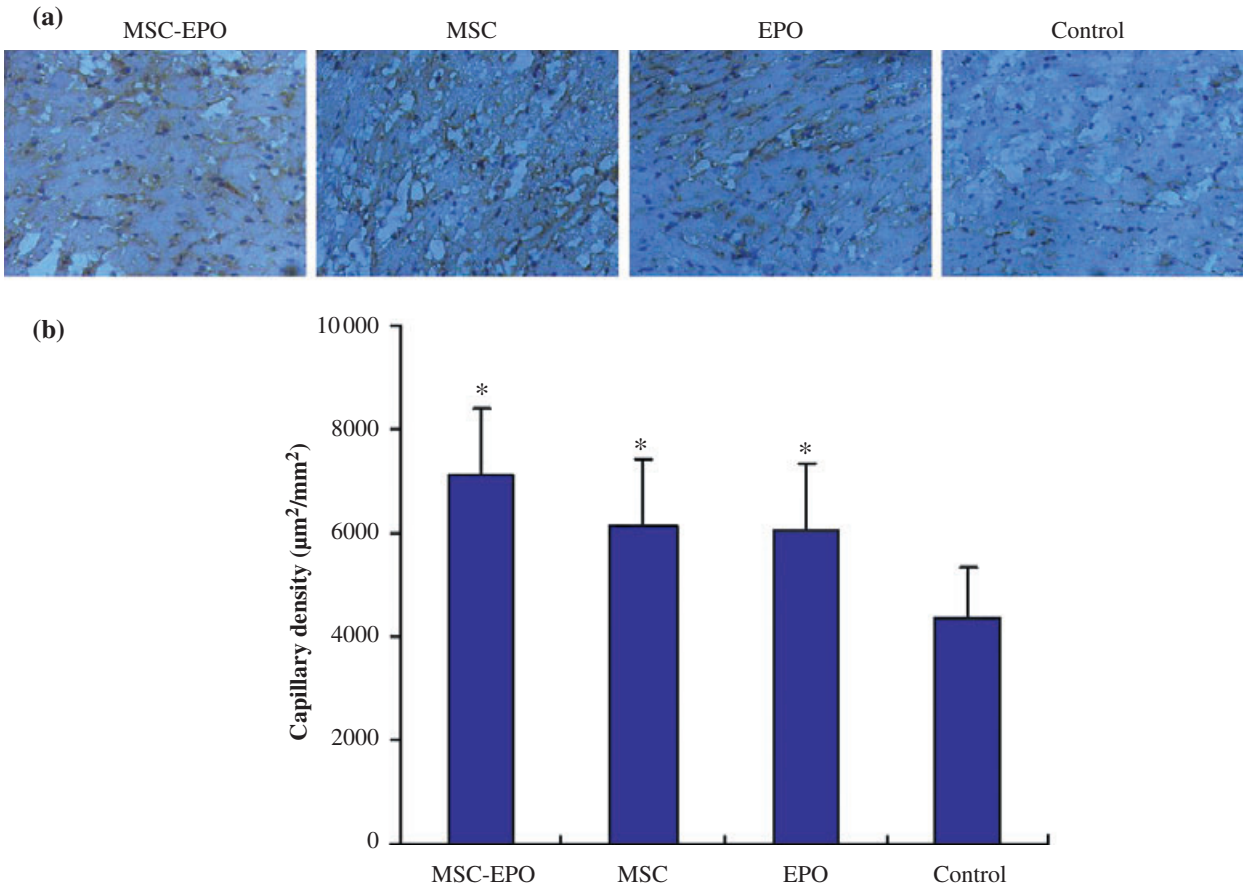

Figure 3 (a) Representative samples of von Willebrand factor (vWF) staining in peri-infarct area. Magnification ×400. (b) Quantitative analysis of capillary density in peri-infarct area. Values are mean ± SD. **P* < 0.05 vs. control group.

(Table 1). Diastolic thickness of the anterior wall was significantly attenuated in the MSC, EPO, and MSC-EPO groups compared with the control group.

Infarct size

The ratio (heart weight integrated) of risk area (RA) to total left ventricle (LA) was equivalent in MSC-EPO, MSC, EPO and control groups (46.5 ± 5.5, 44.3 ± 5.5, 45.2 ± 5.3 and 41.7 ± 4.3, respectively, $P > 0.05$; Fig. 2). The maximal ratio (heart weight integrated) of infarct area (IA) to RA was observed in the control group (51.8 ± 5.8%; Fig. 2). MSC alone decreased IA/RA by 30.1 ± 6.8% compared with 37.9 ± 6.2% by MSC-EPO.

Capillary density

Capillary density was markedly higher in the MSC-EPO group, followed by MSC and EPO groups, when compared with control group (Fig. 3). Cartilage, bone, or fat was not observed in the transplanted area. No tumor-like cells were seen.

Percentage of myocardial fibrosis

Masson's trichrome staining demonstrated modest myocardial fibrosis in the control group (Fig. 4). MSC alone significantly reduced fibrosis, whereas MSC plus EPO reduced fibrosis furthermore. EPO infusion alone attenuated the development of myocardial fibrosis, but did not reach statistical significance.

Stem cell differentiation in ischemic myocardium

Three weeks after transplantation, GFP-labeled transplanted MSC were more frequently observed in the MSC-EPO group than in the MSC group (6.8 ± 0.5 to 3.1 ± 0.7, $P < 0.05$). Some transplanted MSC formed vascular structures in the myocardium and were positive for vWF (Fig. 5). Other MSC were positive for α-SMA and participated in vessel formation (Fig. 6). Semiquantitative analysis demonstrated that the number of vWF positive cells to GFP/TRITC double-positive cells was significantly higher in the MSC-EPO group than in the MSC group (68.0 ± 9.3% to 51.1 ± 10.4%, $P < 0.05$). The ratio of GFP/TRITC double-positive cells to

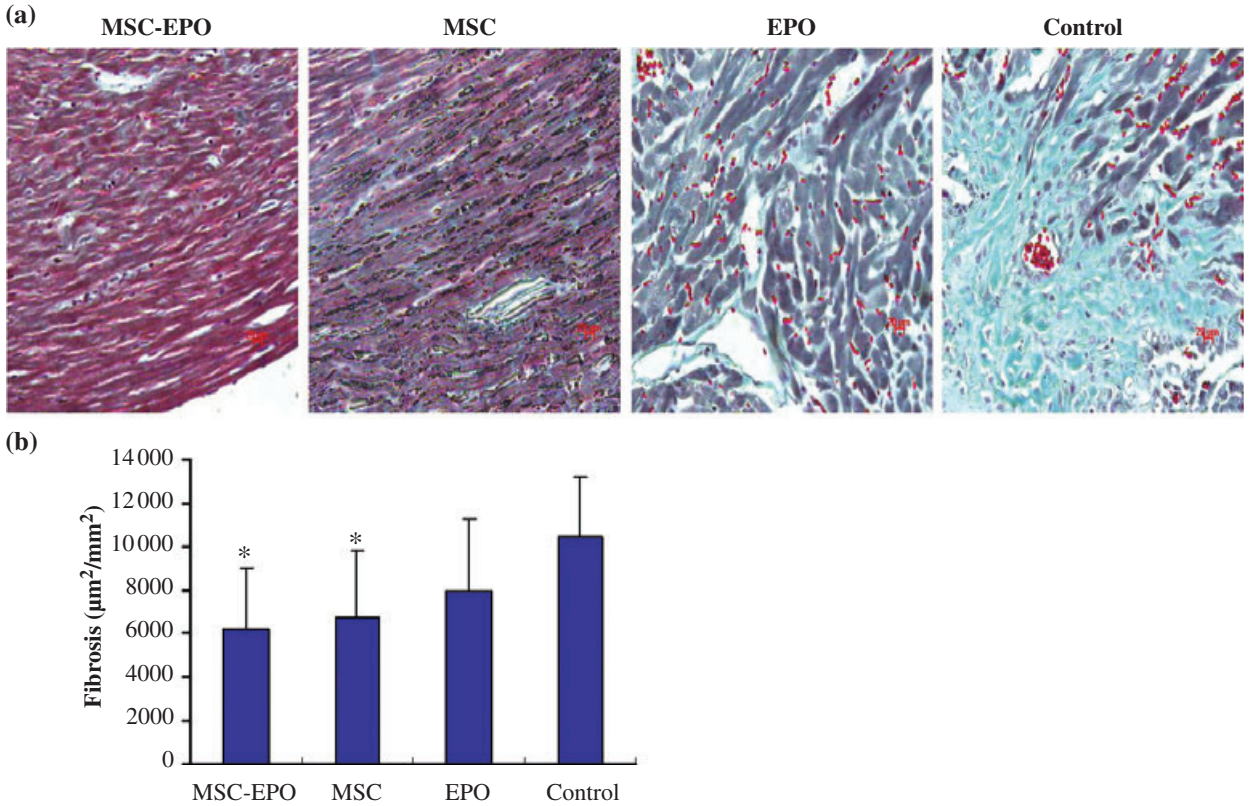

Figure 4 (a) Representative samples of myocardial fibrosis stained with Masson's trichrome. Scale bar = 20 μm. (b) Quantitative analysis of myocardial fibrosis. Values are mean ± SD. *$P < 0.05$ vs. control group.

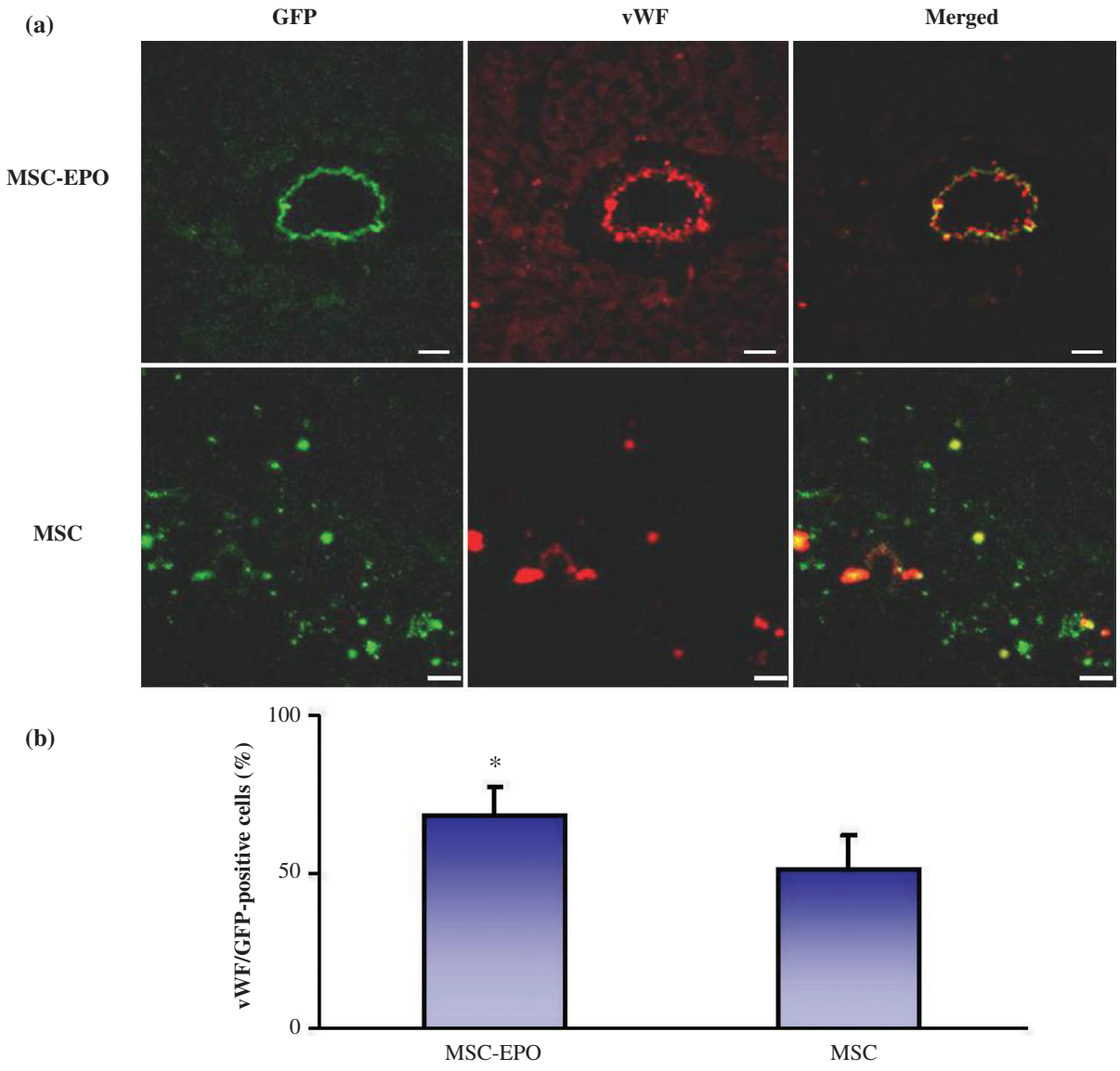

Figure 5 (a) Representative examples of MSC differentiation into endothelial lineage. Green fluorescence (GFP) marks transplanted cells; red fluorescence indicates von Willebrand factor (vWF), a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial cells in the MSC-EPO group and formed vascular structure. Scale bar = 20 μm. (b) quantitative analysis of transplanted cells differentiation. The ratio of vWF-positive cells to transplanted cells was significantly higher in the MSC-EPO group than in the MSC group. Values are mean ± SD. *$P < 0.01$ vs. MSC group.

α-SMA-positive cells was small, but significantly higher in the MSC-EPO group than in the MSC group (30.9 ± 12.2% to 15.2 ± 7.6%, $P < 0.01$) A few of the transplanted MSC were positive for troponin I (Fig. 7), a marker of cardiac cells, but no significant difference was observed in two groups (1.2 ± 1.1% to 1.3 ± 1.2%, $P > 0.05$).

Discussion

The results of the present studies demonstrate the following: (i) MSC transplantation were capable of engraftment in the ischemic myocardium and the engrafted MSC differentiated into cardiomyocytes and vascular endothelial cells, resulting in myogenesis and angiogenesis; (ii) EPO decreased myocardial infarct size and improved cardiac function after acute MI in rats; (iii) EPO enhanced the angiogenesis potency of MSC in a rat model of acute MI, resulting in decreased infarct size and improved cardiac function.

The main advantage of using MSC in treating MI is that they can be isolated from adult bone marrow by aspiration and expanded *ex vivo* before implantation. Under special-

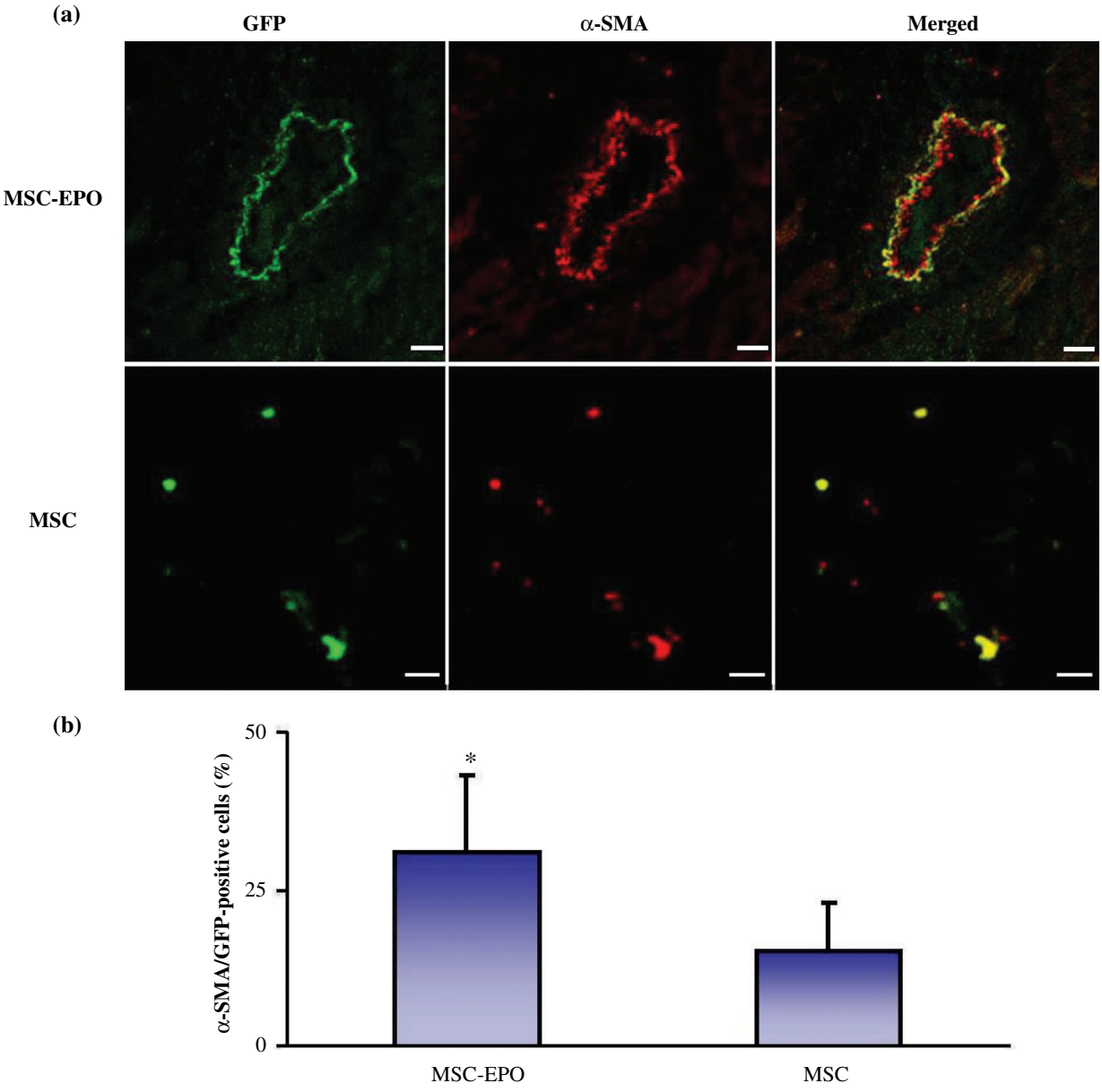

Figure 6 (a) Representative examples of MSC differentiation into smooth muscle lineage. Green fluorescence (GFP) marks transplanted cells; red fluorescence indicates α-smooth muscle actin (α-SMA), a marker for vascular smooth muscle cells. Some of the transplanted cells differentiated into smooth muscle cells in the MSC-EPO group and participated in vessel formation. Scale bar = 20 μm. (b) quantitative analysis of transplanted cells differentiation. The ratio of α-SMA –positive cells to transplanted cells was significantly higher in the MSC-EPO group than in the MSC group. Values are mean ± SD. $*P < 0.05$ vs. MSC group.

ized culture conditions, MSC have the capacity to differentiate into cells such as bone, cartilage, adipocytes, myocytes, and even cardiomyocytes [12,13]. These results suggest that MSC may be good candidates for cell transplantation. However, some patients are refractory to this cell therapy. Thus an approach to augment the angiogenic potency of MSC transplantation is required. In the present study, we demonstrated that combination of MSC transplantation and EPO infusion provided advanced benefits on treatment for acute MI, compared with MSC transplantation alone.

In the present study, engrafted MSC present in vessel walls were positive for α-smooth muscle actin, suggesting their transdifferentiation into smooth muscle cells. Yet, MSC were present predominantly in the luminal face of the endothelium of several vessels and expressed factor vWF, suggesting their transdifferentiation into endothelial cells.

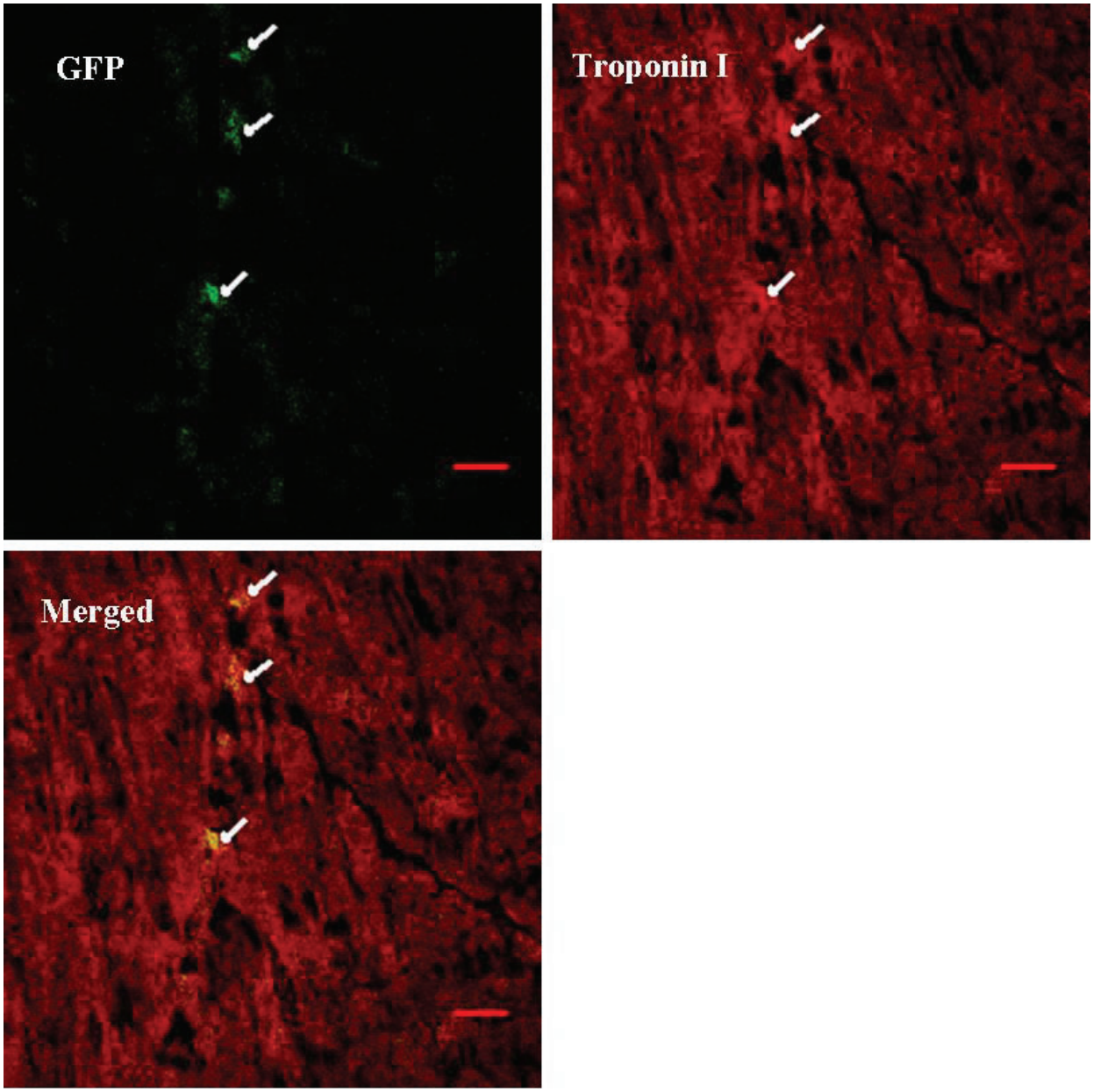

Figure 7 Differentiation of transplanted MSC in ischemic myocardium. Engrafted MSC were positive (arrows) for troponin I. Scale bar = 20 μm.

This transdifferentiation might have contributed to the significantly higher capillary density in the stem cell-treated groups, although several MSC were positive for troponin I, a special maker of cardiac cells. Therefore, MSC may have participated in or triggered an angiogenic process. Earlier studies have shown MSC transplantation induced therapeutic angiogenesis and preserves LV function [14]. More specifically, MSC have been demonstrated to produce a wide array of arteriogenic cytokines and improve perfusion and remodeling in a mouse model of hindlimb ischemia [15], and these effects appear to be mediated through paracrine mechanisms associated with local release of the arteriogenic cytokines [16–18]. This suggests that acute infarction, as in the present study, may drive MSC to differentiate into vascular cells. Thus, our findings added to the evidence in the literature that MSC might induce angiogenesis in the setting of acute MI.

In our study, we evaluated the functional improvement seen after MSC implantation by echocardiography. At 3 weeks after MI, a higher LVEF and LV FS was observed in MSC group than in control group. Angiogenesis may contribute to the maintenance of cardiac

function by preserving residual, viable cardiomyocytes, and neovascularization might also restore contractility in hibernating areas of myocardium [19]. Furthermore, the grafting of MSC may augment or preserve the myocardial elasticity after ischemia. Overall, the increase in capillary density and the transdifferentiation into endothelial cells and smooth muscle seen in the treated group might have contributed to the preservation of LVEF and LVFS.

The present study showed that EPO infusion significantly increased capillary density in ischemic myocardium. Furthermore, EPO infusion plus MSC transplantation demonstrated a further increase in capillary density compared with EOP or MSC alone. Contribution of transplanted MSC to neovascularization was significantly greater in the MSC-EPO group than in the MSC group. Moreover, immunohistological examination demonstrated that infusion of EPO increased the number of vWF-positive (endothelial) cells and α-smooth muscle actin-positive (smooth muscle) cells in transplanted MSC. A recent study has reported that EPO has similar angiogenesis ability as vascular endothelial growth factor (VEGF) [20] and promotes proliferation and migration of endothelial progenitor cells [21,22]. These findings suggest that the beneficial effects of combination therapy using EPO and MSC may be attributable, in part, to the angiogenic properties of EPO itself and EPO induced-differentiation of MSC. Thus it is possible that EPO infusion and MSC transplantation induce additive effects on myocardial damage after MI.

This study includes some study limitations. The doses used in the present study (5000 U/kg body weight) are higher than what humans would receive to treat anemia. High dose EPO administration might result in potential hazards, such as augmenting platelet aggregation, increasing the likelihood of microinfarctions and macroinfarctions. Although Laura Calvillo *et al.* have demonstrated the same high dose of EPO repeatedly administered i.p. for 7 days was not associated with any evident adverse consequences in rat myocardial ischemic model [23]. The further study of lower dose of EPO on ischemia disease model should be performed in future.

The MSC transplantation as a promising treatment for ischemic heart disease in the clinical setting is under way. Because their use does not require immunosuppression and has no ethical problems [24,25]. EPO has been used in cardiovascular medicine for many years [26]. Currently, the safety of EPO infusion in patients with congestive heart failure has been demonstrated [8,26]. Thus, combination therapy with EPO infusion and MSC transplantation may be a novel and promising therapeutic strategy for the treatment of severe cardiac vascular disease.

In conclusion, these findings emphasize that infusion of EPO combined with MSC transplantation can achieve superior therapeutic neovascularization and LV-functional recovery. Therefore, combination therapy using EPO infusion and MSC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

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