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Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction

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Summary

Both cell therapy and angiogenic growth factor gene therapy have been applied to animal studies and clinical trials. Little is known about the direct comparison between cell therapy and angiogenic growth factor gene therapy. The goal of this study was to compare the effects of human bone marrow-derived mesenchymal stem cells (hMSCs) transplantation and injection of angiogenic growth factor genes in a model of acute myocardial infarction in mice. The hMSCs were obtained from adult human bone marrow and expanded *in vitro*. The purity and characteristics of hMSCs were identified by flow cytometry and immunophenotyping. Immediately after ligation of the left anterior descending coronary artery in male severe combined immunodeficient (SCID) mice, culture-expanded hMSCs or angiogenic growth factor genes were injected intramuscularly at the left anterior free wall. The engrafted hMSCs were positive for cardiac marker, desmin. Infarct size was significantly smaller in the hMSCs-treated group than in the angiopoietin-1 (Ang-1) or vascular endothelial growth factor (VEGF)-treated group at day 28 after infarction. hMSCs transplantation was better in decreasing left ventricular end-diastolic dimension and increasing fractional shortening than Ang1 or VEGF gene therapy. Capillary density was markedly increased after hMSCs transplantation than Ang1 and VEGF gene therapy. In conclusion, intramyocardial transplantation of hMSCs improves cardiac function after acute myocardial infarction through enhancement of angiogenesis and myogenesis in the ischemic myocardium. hMSCs are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. Transplantation of MSCs may become the future therapy for acute myocardial infarction for myocardial regeneration.

Introduction

Cardiovascular disease is the leading causes of mortality in developed countries. The prognosis and quality of life for patients with severe ischemic cardiovascular disease are poor. Modern interventional and surgical therapies are not suitable for many of them because the anatomic extent and distribution of arterial occlusion are too severe. The need for alternative treatment strategies is compelling. Stem cell therapies hold promise for

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the treatment of ischemic cardiovascular disease [1–4]. Stem cells are pluripotent and have the property of self-renewal as well as multilineage differentiation [5]. Therefore stem cells make it possible to regenerate damaged cells [6].

The bone marrow is home to mesenchymal stem cells (MSCs) that are able to differentiate into many different cell types [7]. Bone marrow-derived cells are autologous, abundant, and relatively easy to harvest; they do not require immune suppression, and they are associated with less ethical concern than are fetal or neonatal heart cells. These characteristics make bone marrow-derived cells uniquely suited to the task of restoring structure and function in the wake of a myocardial infarction [8]. As damaged tissue may lose anatomical cues for functioning organ neovascularization, *in vitro* manipulation of stem cells may be essential to facilitate *in vivo* incorporation.

Cell transplantation, growth factors, and gene therapy represent emerging biologic treatments conceived to improve myocardial function in ischemic heart disease. Both MSCs cell therapy and angiogenic growth factor gene therapy have been applied to animal studies and clinical trials [9]. Direct comparison between cell therapy and angiogenic growth factor gene therapy is rarely reported. The goal of this study was to compare the effects of MSCs transplantation and injection of angiogenic growth factor genes in a model of acute myocardial infarction in mice.

Methods

Isolation and culture of human bone marrow-derived MSC

Bone marrow-derived hMSCs were isolated and cultured according to a previously reported method by Pittenger et al. [10]. Briefly, after informed consent, the donor marrows obtained from healthy volunteers were suspended with buffered PBS supplemented with 12.5 U/ml heparin, 50 U/ml penicillin, 50 mg/ml streptomycin. Mononucleated cells were isolated by ficol density gradient, washed and resuspended in Dulbecco's modified Eagle's medium-low glucose supplemented with 10% fetal bovine serum (Hyclone), and cultured at 37 °C and 5% CO_2. Seven days later, individual colonies were collected, isolated, cultured and expanded. When the cultures reached 80% of confluence, cells were recovered by 0.25% trypsin-1 mM EDTA and followed by passages.

Immunophenotyping of cultured MSCs

Analysis of cell surface molecules was made on hMSCs using flow cytometry (Becton Dickinson, USA) by following the manufacture's procedure. hMSCs were analyzed by direct or indirect immunofluorescence. To detect surface antigens, cells were detached with 0.25% trypsin-1 mM EDTA in PBS, washed with PBS containing 2% bovine serum albumin and 0.1% sodium azide (Sigma, USA), and incubated with the respective antibody at a concentration previously established by titration. For indirect immunofluorescence assays, cells were washed and incubated with secondary antibody (FITC-conjugated goat anti-mouse second antibody). In each case, 1×10^4 cells were acquired and analyzed by using the Cell Quest software.

Growth characteristic analysis

The cell-doubling growth kinetics of hMSCs was measured. Cell-doubling counts at each passage were taken upon the cell harvest trypsin treatment.

Recombinant plasmids and preparation of AAV-vector

The angiopoietin-1 plasmid (pAng1) and human vascular endothelial growth factor 165 plasmid (pVEGF) were constructed as described previously [11]. Human $VEGF_{165}$ cDNA and Ang1 cDNA were ligated into AAV vector (pAAV-IRES-hrGFP) (Stratagene, La Jolla, CA), named as AAV/VEGF and AAV/Ang1, respectively. The pAAV-IRES-hrGFP vector contains the cytomegalovirus promoter and other elements for high-level gene expression in mammalian cells when a gene of interest is cloned into the multiple cloning sites. The vector contains AAV-2 inverted terminal repeats, which direct viral replication and packaging.

Mouse model of acute myocardial infarction

A male severe combined immunodeficient (SCID) mouse (aged 6–8 weeks) model of left anterior descending coronary artery (LAD) occlusion was

used. After induction of anesthesia with isoflurane (3%), a tracheotomy was performed, and the animal was ventilated on a Harvard Rodent Respirator. An anterior thoracotomy was performed to open the pericardium. The heart was then rapidly exteriorized, and an 8-0 silk suture was tightened around the proximal LAD (before the first branch of diagonal artery). Sham-operated control animals were prepared in a similar manner, except that the LAD was not occluded. Positive end-expiratory pressure was applied to fully inflate the lungs. The muscle layer and skin were closed separately after stem cell transplantation. The study conforms with *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The surgical mortality rate after coronary ligation and intramyocardial injection was around 50% in each group. No mortality difference among the four groups was found.

Intramyocardial injection of stem cells or plasmids

After ligation of the LAD, 1×10^6 culture-expanded hMSCs resuspended in 50 μl of normal saline were injected intramuscularly at the left anterior free wall by using an insulin syringe with a 30-gauge needle. Naked plasmid DNA containing Ang1 (pAng1) or $VEGF_{165}$ cDNA (pVEGF) at 50 μg in 50 μl of normal saline was also injected in a similar way as the hMSCs. After the left ventricle was accessed, the needle was advanced along the left ventricular free wall and hMSCs or plasmids were injected over a period of 5–10 s at three separate sites. The injected sites were chosen at least 5 mm away from the left ventricular apex. Aspiration was done to confirm that the injection was given into the left ventricular wall and not into the left ventricular cavity. After injection, the chest was closed and the animals were allowed to recover.

Infarct size determination

Two weeks and 28 days after myocardial infarction, mice were deeply anesthetized with isoflurane (3%). Evans blue dye (1%) was perfused into the aorta and coronary arteries with distribution throughout the left ventricular wall proximal to the site of coronary artery ligation as described previously [12]. The non-ischemic area was stained blue. Hearts were excised and sliced into cross-sections below the ligature. These sections were weighed and then incubated with a 1% triphenyltetrazolium chloride solution at 37 °C for 20 min. Each heart was cut in cross-section at four levels from apex to base and prepared for routine histology. Infarct size, the area at risk, and total left ventricular area from each section were measured as described previously [13].

Physiological assessment of LV function

Transthoracic echocardiography (Acuson Sequoia 512 machine using a 15-MHz probe) was performed just before (baseline) and 28 days after myocardial infarction. Left ventricular diastolic and systolic dimensions and fractional shortening were measured at the midpapillary muscle level. The echocardiographic examination was performed by a blinded observer.

Histological assessment of transplanted myocardium

At the day of sacrifice (14 and 28 days after intramyocardial transplantation), the left ventricle was harvested and fixed in methanol and sliced into 5 μm paraffin sections. To block endogenous peroxide activity and non-specific binding, sections were incubated with 3% hydrogen peroxide followed by 10% normal horse serum. Specimens were incubated with a monoclonal anti-mouse CD31 antibody or anti-desmin antibody at 4 °C overnight. Bound primary antibody was detected with the avidin–biotin–immunoperoxidase method (Signet Laboratories, Dedham, MA, USA). Non-immune normal rabbit IgG was used to confirm specificity. The number of capillaries was counted in regions with transversely sectioned myocytes in the border zone and in the area of infarction. Capillary density was counted as described previously [13]. Incorporation of injected hMSCs was verified by staining for HLA class I-APC (BD Pharmingen).

The specimens were also stained with desmin, Tie-2, c-kit, and PCNA monoclonal antibody to confirm that transplantation of MSCs differentiate to cardiomyocytes.

Human and endogenous Ang1 and VEGF gene expression in ischemic muscle

Gene expression was evaluated by detecting mRNA level using real time polymerase chain reaction (RT-PCR) with SCID mice after ligation of LAD that were put to death at 7 and 28 days after the transfection with pAng1 and pVEGF ($n = 3$ at each time point). To ensure specificity and avoid amplification of endogenous mice Ang1 and VEGF, each primer was selected from a region that was not conserved among different species. To detect the endogenous gene response to the ischemic change, RT-PCR was performed with mice specific primers for Ang1 and VEGF. The primers used and the procedure of RT-PCR was described previously [11].

Statistical analysis

All results were expressed as mean ± SEM. Statistical significance was evaluated using analysis of variance followed by Tukey–Kramer multiple comparisons test (GraphPad Software Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered to denote statistical significance.

Results

Adherent, fibroblast-like bone marrow-MSC

Human adult marrow nucleated cells separated by density gradient were cultured at low density ($12{,}000/cm^2$), and after 5–7 days incubation, formed individual colonies displayed fibroblast-like morphology. Six out of 24 selected colonies were formed into an adherent layer, and these adherent cells could be readily expanded *in vitro* by successive cycles of trypsinization, seeding, and culture every 3 days for 19 passages without visible morphologic alteration (Figure 1).

Immunophenotypic characterization of MSCs

We confirmed that the major population of adherent cells used in this study were MSCs. Flow cytometric analyses demonstrated that the bone marrow-MSCs were CD29, CD44, CD49, CD90, CD105, CD106, CD166, and SH positive, but were negative for Cd31, CD45, KDR, Flt1 and Flt3

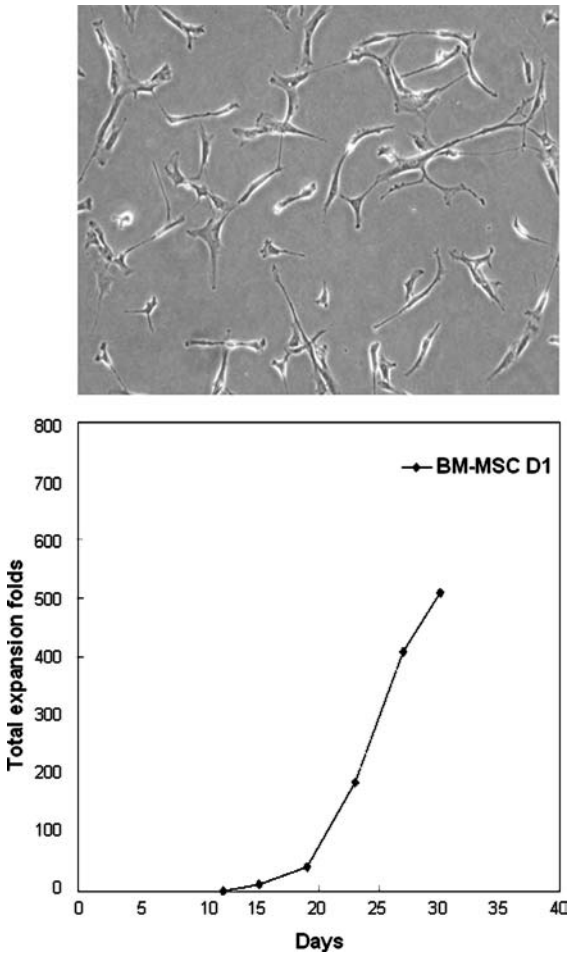

Figure 1. Cell morphology and growth kinetics of hMSCs. Cultured bone marrow-derived adherent cells showed similar morphologic appearance as that of bone marrow stromal cells. The growth kinetics of hMSCs expressed by doubling folds was shown on the lower panel.

(Figure 2). These results were consistent with the properties of the documented bone marrow-derived MSCs. All isolated colonies-derived MSCs maintained a similar phenotype even at passage 15.

Evidence of incorporation of mesenchymal stem cells into ischemic myocardium

Five mice in each group were used to investigate the incorporation of MSCs into the heart tissue. hMSCs incorporated into the heart at days 5 and 14 after transplantation, as evidenced by the presence of HLA-positive myocytes within the mouse myocardium. The cells positive for HLA were also positive for desmin, a cardiac myocyte marker (Figure 3).

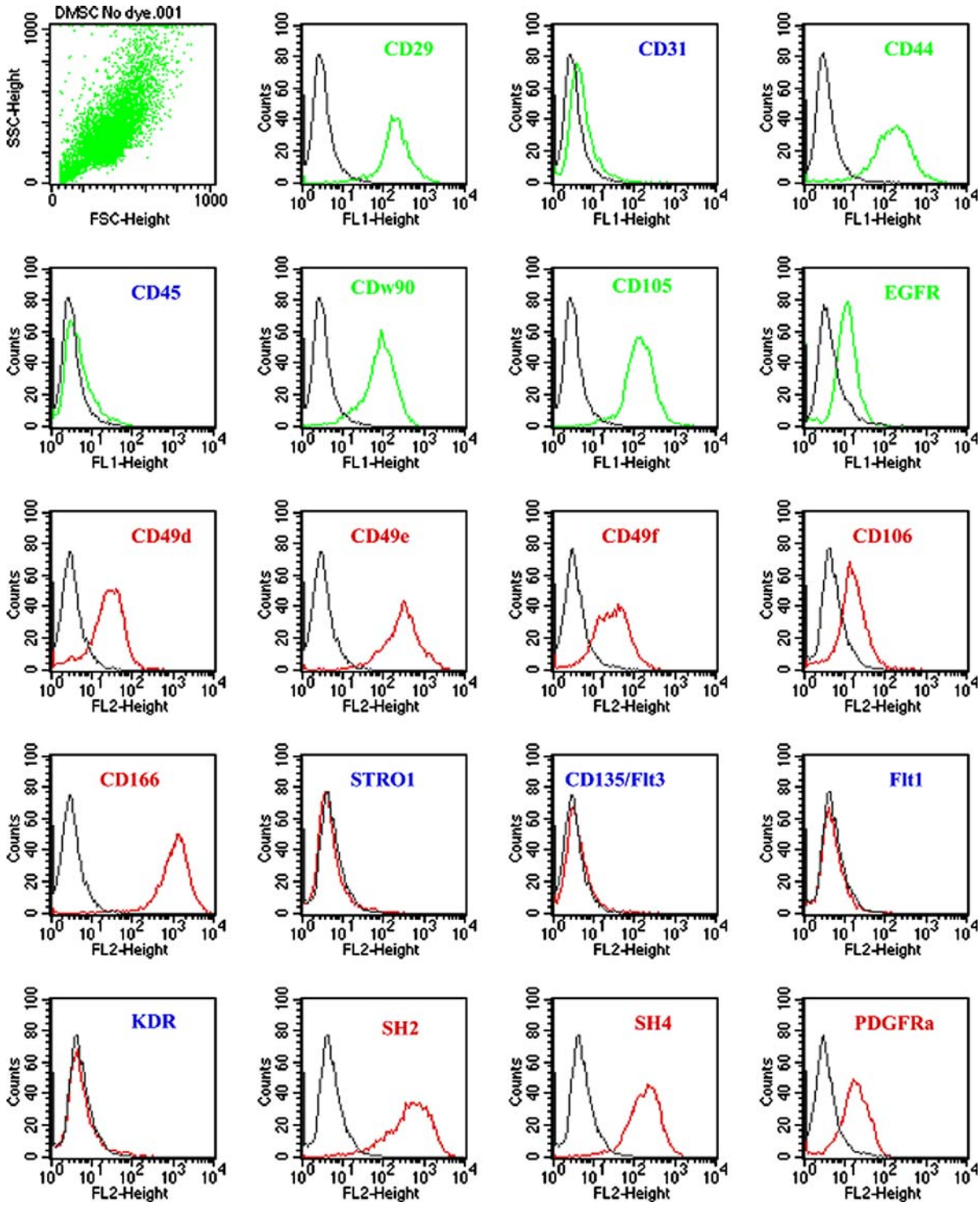

Figure 2. Flow cytometric analysis of hMSCs immunophenotyping showing that they were CD29, CD44, CD49, CD90, CD105, CD106, CD166, and SH positive; but were negative for CD31, CD45, STRO1, KDR, Flt1, and Flt3.

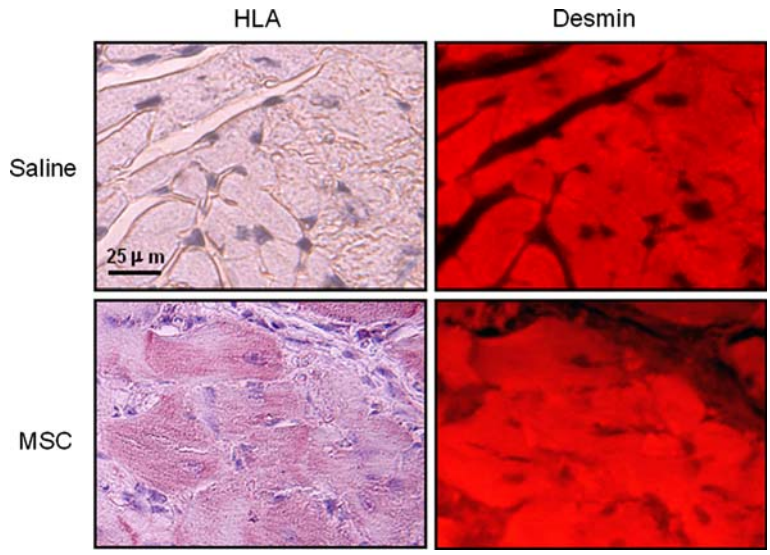

Figure 3. Representative photographs of immunohistochemistry analysis by use of HLA-APC and desmin antibodies. Positive stain for HLA was shown in MSC-treated myocardium, but not in saline-treated myocardium. Desmin was stained positively in both groups. Similar results were observed in another three independent experiments.

Reduced infarct size by mesenchymal stem cells

Percentages of left ventricle at risk (risk area/total left ventricular area) measured after 14 days of infarction was similar among 4 groups ($61 \pm 2\%$ for control group, $60 \pm 1\%$ for hMSCs group, $63 \pm 3\%$ for pVEGF group, and $61 \pm 4\%$ for pAng1 group). Infarct size (infarct area/total left ventricular area) was significantly smaller in the hMSCs-treated animals than in the control group at day 14 ($34.8 \pm 0.7\%$ vs. $40.5 \pm 0.9\%$, $p < 0.001$) and day 28 (32.8 ± 0.8 % vs. $42.7 \pm 0.4\%$, $p < 0.001$) as shown in Figure 4. Infarct size for mice treated with hMSCs was reduced by 14% at day 14 and by 23% at day 28. The infarct size corrected for areas at risk in mice treated with hMSCs and saline was $58.1 \pm 1.1\%$ and $66.1 \pm 1.5\%$ ($p < 0.01$), respectively. Infarct size was also significantly reduced by pVEGF ($36.8 \pm 0.8\%$) at day 28 as compared to control group ($p < 0.01$). Treatment with pAng1 did not reduce the infarct size for either time recovery points measures (14 and 28 days post-myocardial infarction). These findings for pVEGF- and pAng1-treated animals were the same when infarct size was corrected for area at risk hMSC is better in reducing infarct size than Ang1 at both days 14 and 28 and is better than VEGF at day 28.

Improved cardiac function by mesenchymal stem cells and angiogenic growth factor genes

The cardiac functional parameters evaluated by echocardiography, 4 weeks after LAD ligation, are shown in Table 1. In the control group, markedly decreased fractional shortening (FS) with dilated left ventricular cavity was clearly seen. In the group that underwent hMSCs transplantation, significantly higher FS and smaller left ventricular cavity were observed compared with the control groups (saline). The FS of hMSCs-treated mice increased by 30%. Both FS and left ventricular ejection fraction improved after treatment with pAng1 and pVEGF compared to the control group. FS and LVEF improved to $40 \pm 2\%$ and $76 \pm 2\%$, respectively after treatment with AAV/Ang1 and to $39 \pm 2\%$ and $74.6 \pm 2.2\%$, respectively after treatment with AAV/VEGF. Treatment with hMSCs got better improvement of cardiac function than treatment with either pAng1, pVEGF, AAV/Ang1, or AAV/VEGF.

Increased capillary density by mesenchymal stem cells

At 14 days after infarction, capillary density of the ischemic myocardium in the hMSC-treated group ($814 \pm 28/mm^2$) was significantly higher ($p < 0.001$,

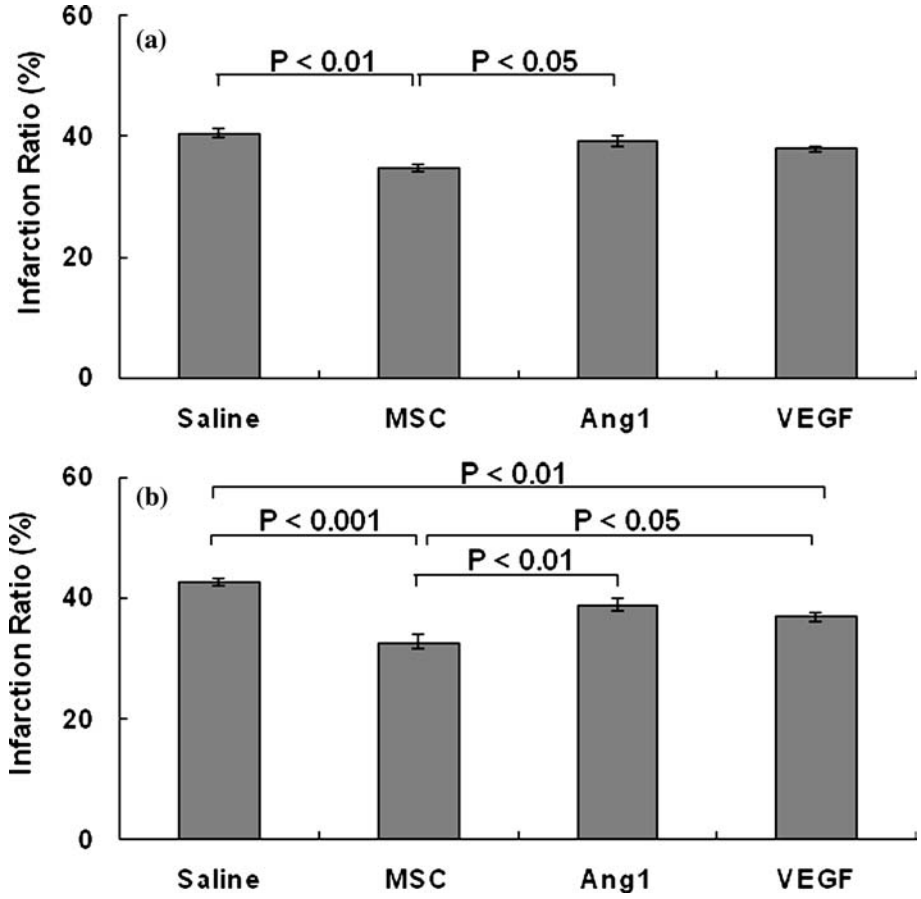

Figure 4. Reduced infarct size by mesenchymal stem cells at day 14 (a) and 28 (b). $n = 7$ in each group. The infarct ratio represents infarction area divided by total left ventricular area.

Table 1. Evaluation of cardiac function by echocardiography 4 weeks after infarction.

	LVEDd (mm)	LVEDs (mm)	FS (%)	LVEF (%)
Sham ($n = 7$)	3.4 ± 0.5	1.5 ± 0.1	55.1 ± 1.9	90.0 ± 1.3
Saline ($n = 7$)	4.6 ± 0.2^{a}	3.1 ± 0.3^{a}	32.9 ± 1.4^{a}	67.9 ± 1.9^{a}
MSC ($n = 7$)	3.6 ± 0.1^{b}	$2.2 \pm 0.1^{b, c}$	$44.4 \pm 2.3^{a, b}$	$80.7 \pm 2.3^{b, c}$
Ang1 ($n = 7$)	3.9 ± 0.2^{b}	$2.5 \pm 0.1^{b, c}$	34.9 ± 1.2^{d}	71.2 ± 2.1^{e}
VEGF ($n = 7$)	4.2 ± 0.2	$2.6 \pm 0.1^{b, c}$	$37.1 \pm 1.4^{b, d}$	$73.8 \pm 2.3^{d, e}$

Data indicate mean ± SD; LVEDd = left ventricular end-diastolic diameter; LVEDs = left ventricular end-systolic diameter; FS = fractional shortening; LVEF = left ventricular ejection fraction.
[a] $p < 0.001$ vs. sham group.
[b] $p < 0.001$ vs. saline group.
[c] $p < 0.001$ vs. sham group.
[d] $p < 0.001$ vs. MSC group.
[e] $p < 0.01$ vs. saline group.

$n = 7$) than that in the control group (561 ± 26/mm^2). The capillary density of the ischemic myocardium at 28 days after infarction in the MSC-treated group (1119 ± 17/mm^2) was also significantly higher ($p < 0.001$, $n = 7$) than that in the control group (439 ± 27/mm^2) (Figure 5). The

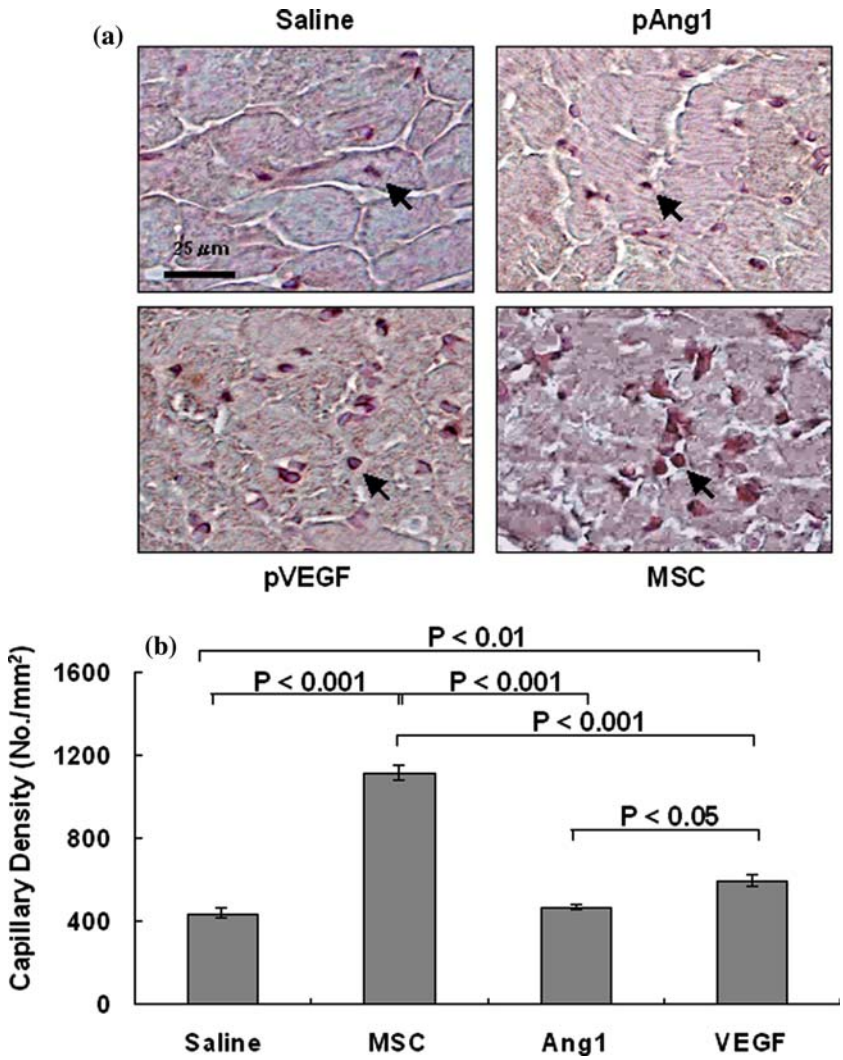

Figure 5. Increased capillary density by mesenchymal stem cells. (a) Photomicrography shows representative immunohistochemical CD31 staining of ischemic myocardium from border zone harvested at day 28. Dots (arrow) indicate capillaries. (b) Quantitative analysis of capillary density. $n = 7$ per group.

capillary density in the control group decreased from day 14 ($561 \pm 26/mm^2$) to day 28 ($439 \pm 27/mm^2$) ($p < 0.05$), while the capillary density in the hMSC-treated group increased from day 14 ($814 \pm 28/mm^2$) to day 28 ($1119 \pm 17/mm^2$) ($p < 0.001$). A higher capillary density was also observed in the pVEGF-treated group than in the control group. Capillary density at day 28 was $589 \pm 25/mm^2$ and $622 \pm 21/mm^2$, respectively after treatment with AAV/Ang1 and AAV/VEGF. Treatment with hMSCs had significantly higher ($p < 0.001$) capillary density at day 28 than treatment with either pAng1, pVEGF, AAV/Ang1 or AAV/VEGF.

Myogenesis induced by hMSCs

Five heart tissues from mice in each group were used for the immunohistochemical study at 14 days after infarction. As shown in Figure 6, immunohistochemical analysis showed that the stem cell markers including Tie2, and PCNA were all positive in the ischemic myocardium treated with hMSCs. These positive cells for the markers were cardiomyocytes. Since bone marrow-derived stem cells express Tie2, these data suggest that hMSCs are capable of differentiating into cardiomyocytes. Stem cell markers were not stained in the ischemic myocardium treated with pAng1 or pVEGF.

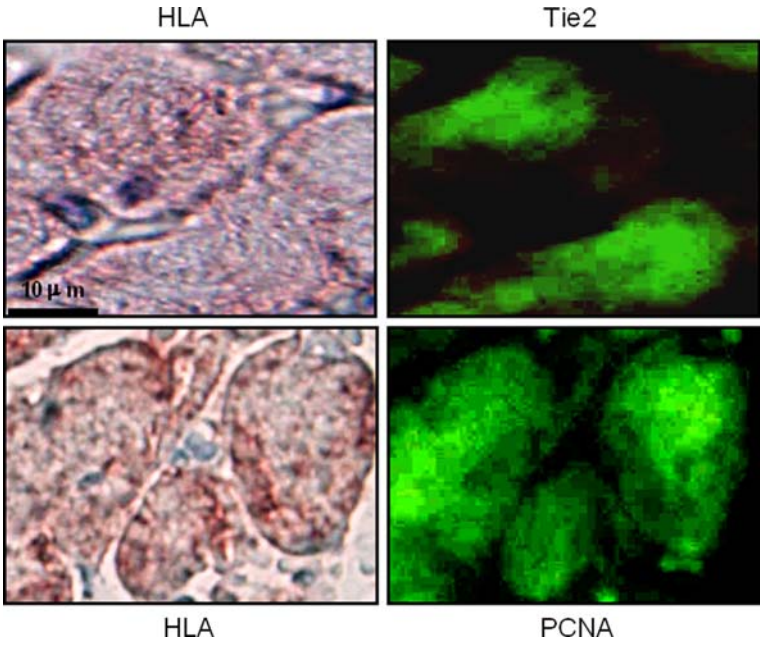

Figure 6. Representative microscopic photography for immunohistochemical staining. The positive staining myocytes for Tie2 and PCNA was also stained positively for HLA. Similar results were observed in another two independent experiments.

Ang1 and VEGF gene expression in ischemic myocardium

Human Ang1 and VEGF mRNA were detected in the ischemic area of the myocardium at days 7 and 28 in the group treated with pAng1 or pVEGF, respectively. The level of human Ang1 and VEGF mRNA at day 7 was 2- and 3-fold higher than that at day 28, respectively. The mRNA of endogenous Ang1 and VEGF at 7 days after myocardial infarction in each treated group increased 3- and 5-fold, respectively as compared to sham group. These data implicated that transfection of pAng1 and pVEGF into the ischemic myocardium activated the expression of endogenous Ang1 and VEGF gene in mice that suffered an induced myocardial infarction. The transfection efficiency of gene transfer using β-galactosidase detection was $3.9 \pm 0.6\%$. The efficiency of transfer of AAV-Ang1 and AAV/VEGF was 20%.

Discussion

The present study demonstrates that transplantation of adult hMSCs induces myocardial myogenesis and angiogenesis in a mouse model of myocardial infarction. This model of therapy reduced infarction size, improved left ventricular function, and increased capillary density. We have shown that highly purified $CD29^+CD90^+CD166^+CD45^-$MSCs can be isolated and expanded from human adult bone marrow by immunoselection. Some of the engrafted hMSCs stained by cardiac proteins such as desmin were stained positive for Tie2 and PCNA. These results suggest that hMSCs differentiate into cardiomyocytes. However, without high-power laser-scanning confocal microscope, it is very difficult to determine whether these double stain positive cells are newly differentiated endogenous cells or the product of cell fusion. Both transdifferentiation and cell fusion are two possible ways of human stem cells to differentiate into cardiomyocytes and endothelial cells [14]. Recently, Murry et al. have reported that hematopoietic stem cells do not transdifferentiate into cardiomyocytes in myocardial infarcts [15]. The controversy may be related to differences in cell origin, cell preparation, and detection methodology. Transplantation of hMSCs leads to a significantly improved function in the post-infarction left ventricle.

Angiopoietin-1 has been shown to enhance angiogenesis and to attenuate progression of cardiac dysfunction after myocardial infarction [16, 17]. In our study, the angiogenesis effect of

Ang1 was not obvious. However, cardiac function improved after intramyocardial delivery of pAng1. Cell therapy using skeletal myoblast has been shown to be superior to VEGF protein therapy for myocardial regeneration [18]. In the present study, we demonstrated that cell therapy using hMSCs was superior to VEGF and Ang1 gene therapy in enhancing angiogenesis and improving cardiac function after myocardial infarction in mice. The clinical results reported from two recent clinical angiogenesis trials [19, 20] suggest that strategies involving administration of a single angiogenic agent may not result in optimal angiogenesis. Stem cells express and secrete Ang1, VEGF, and other cytokines [21, 22], which are important contributors for angiogenesis. The efficiency of using plasmid in gene transfer for acute myocardial ischemia is low [13] as compared to cell transplantation. These mechanisms may provide an explanation for why cell therapy is superior to gene therapy.

In the present study, we demonstrated that capillary density after infarction in the MSCs treatment group was further increased from 2 to 4 weeks. The infarction size also showed a trend to decrease from 2 to 4 weeks after infarction in the MSC group. On the contrary, infarct size and capillary density deteriorate from 2 to 4 weeks after infarction in the control group. The enhancement of cardiac function in MSCs therapy may be attributed to the ability of self-renewal of the MSCs and cytokines production of MSCs [23]. Prevention of apoptosis in ischemic myocardium may also represent another important mechanism for cardiomyocyte recovery by MSCs transplantation [24, 25].

MSCs have been injected directly into the infarct site, or they have been administered intravenously and seen to home to the site of injury [23, 24]. Most of the cell therapies using MSCs for myocardial infarction are of isogenic or allogenic origins [26, 27]. Rare studies used the human MSCs to treat myocardial infarction in mice model. Although bone marrow mononuclear cells have been used in clinical trials [28–31], these cells are not purified MSC population. Moreover, these trials using bone marrow mononuclear cells are not without risks [32–34].

Heeschen et al. have demonstrated that patients with chronic ischemic heart disease have profoundly reduced neovascularization capacity in their bone marrow mononuclear cells [35]. Furthermore, Rauscher et al. have demonstrated the progressive progenitor cell deficits in older animals [36]. Several studies have demonstrated that risk factors for coronary artery disease correlate with a reduced number and functional activity of circulating endothelial progenitor cells [37]. These data may implicate that MSCs from young and healthy donors would be the ideal source for cell therapy. MSCs are defined self-renewal, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages [26]. Although MSCs are a rare population of cells in the bone marrow, they can be readily grown in culture. It would be desirable to induce angiogenesis and myogenesis by MSCs for the treatment of acute ischemic heart disease.

In summary, the present study demonstrated that intramyocardial transplantation of human MSCs improves cardiac function after acute myocardial infarction through enhancement of angiogenesis and myogenesis in the ischemic myocardium. hMSCs are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. Transplantation of MSCs provides a promising opportunity in developing future therapies in myocardial regeneration for persons who have suffered an acute myocardial infarction.

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