Combined Therapy With Simvastatin and Bone Marrow–Derived Mesenchymal Stem Cells Increases Benefits in Infarcted Swine Hearts

Yue-Jin Yang, Hai-Yan Qian, Ji Huang, Jian-Jun Li, Run-Lin Gao, Ke-Fei Dou, Guo-Sheng Yang, James T. Willerson, Yong-Jian Geng

Objective—Widespread death of implanted cells hampers stem cell therapy for acute myocardial infarction (AMI). Based on the pleiotropic beneficial effects of statins, we examined whether simvastatin (SIMV) increased the efficacy of mesenchymal stem cell (MSC) transplantation after AMI.

Methods and Results—Chinese miniswine (n=28) were randomized to 1 of 4 groups (n=7 per group): control, SIMV (0.25 mg/kg·d), MSC transplantation, and SIMV+MSCs. AMI was created by ligating the left anterior descending coronary artery; MSCs were injected immediately into the cyanotic myocardium. At 6 weeks, MRI showed the number of dysskinetic segments and the infarct size were significantly decreased in the SIMV group. Cardiac function improved and the perfusion defect decreased significantly in the SIMV+MSC group but not in the MSC-only group (P<0.05, versus control group). MSC survival and differentiation were significantly better in the combination group than in the MSC-only group (P<0.01). Cell apoptosis decreased significantly in both the SIMV and the SIMV+MSC groups but not in the MSC-only group when compared with controls (P<0.05). Furthermore, oxidative stress and inflammatory response was significantly reduced in the infarcted regions in both the SIMV and the SIMV+MSCs groups.

Conclusions—SIMV treatment improves the therapeutic efficacy of MSC transplantation in acutely infarcted hearts by promoting cell survival and cardiovascular differentiation. (Arterioscler Thromb Vasc Biol. 2009;29:2076-2082.)

Key Words: mesenchymal stem cells ■ acute myocardial infarction ■ transplantation ■ HMB-CoA reductase ■ simvastatin

Cellular cardiomyoplasty (CCM), or cell-based repair, is emerging as a potential novel therapy for acute myocardial infarction (AMI). CCM can help regenerate damaged cardiovascular tissue by implanting stem/progenitor cells or by mobilizing peripheral blood- or bone marrow–derived stem cells into the site of cardiac injury.1-3 Among donor cell types used for CCM, bone marrow–derived mesenchymal stem cells (MSCs) have been the focus of much research because of their plasticity and availability.4 However, the efficacy of cell-based therapy is hindered by the presence of proinflammatory and proapoptotic factors in the injured heart that increase cell death. Successful cellular therapy requires survival, proliferation, and differentiation of implanted stem cells. The in vivo fate of implanted cells is determined by many environmental factors. However, the most important factor in determining donor cell fate is the local postinfarction milieu of the myocardium.5

As the most prescribed drug, statins have many cardioprotective effects independent of their lipid-lowering ability. Statins can protect endothelial function and the integrity of the microvasculature, increase nitric oxide bioavailability, exert antioxidant and antiinflammatory effects, and stabilize atherosclerotic plaques.6-10 Based on these beneficial effects of statins, this study tested whether simvastatin improves the fate of MSCs in vivo, thus further enhancing cardiac function.

Methods

Animals

Ten-month-old domestic miniswine (30±5 kg) were obtained from the Laboratorial Animal Center of the Chinese University of Agriculture, China, or Texas A&M University (College Station, Tex). All animals received humane care in compliance with the Guide for the
Domestic miniswine (n/H1100528) were divided into 4 groups of 7 each: group 1 (control), group 2 (simvastatin administration only, SIMV), group 3 (transplantation of MSCs only), and group 4 (combination of simvastatin and MSC transplantation).

Other experimental procedures are listed in supplemental materials (available online at http://atvb.ahajournals.org).

**Results**

All the animals operated on survived to the end of the study except for 3 (1 in the control group, 1 in group SIMV, and 1 in group MSCs). Measurements were successfully obtained and analyzed from all the living animals. Phenotypic characterization showed that pig bone marrow–derived MSCs were negative for the CD34 and CD45 antigens but positive for CD29 (99.94±0.05%) and CD90 (97.43±1.29%; supplemental Figure I). In MSCs labeled with DAPI, cell viability was consistent at about 98%, which is similar to that in control MSCs labeled without DAPI.

Statin Improves Morphology of Infarcted Myocardium With MSC Implantation

Hematoxylin and eosin (Figure 1A) and Masson trichrome staining (Figure 1B) showed the development of severe fibrosis and chronic inflammation with the infiltration of numerous leukocytes in the infarcted regions in control pigs and in those treated with MSCs only. In contrast, reduced fibrosis and inflammatory cell infiltration were seen in the hearts of the groups treated either with SIMV alone or with both SIMV and MSCs. (Figure 1C and 1D).

Statin Enhances Survival and Myogenesis of Implanted MSCs

Immunofluorescent microscopy revealed higher numbers of DAPI-labeled cells in the hearts of pigs treated with SIMV+MSCs than in pigs treated only with MSCs (310.6±83.8 versus 70.5±22.3, P<0.01; Figure 2A and 2E), indicating increased survival of implanted cells in postinfarction hearts treated with both SIMV and MSCs. Further immunofluorescent analyses showed that DAPI-labeled cells expressed cardiac- and vessel-specific proteins, including VWF and SM-actin (Figure 2B), α-sarcomeric actin (Figure 2C), and cardiac troponin T (Figure 2D), in the hearts of pigs treated with MSCs. The efficiency with which DAPI-labeled cells differentiated into cardiomyocytes was greater in the combination group than in the group treated only with MSCs (46.0±5.2% versus 9.6±3.5%, P<0.01; Figure 2F).
Statin Improves Cardiac Perfusion and Performance

Initial baseline SPECT at 1 week after cell transplantation demonstrated no significant differences among the 4 groups ($P=0.984$). At end point, follow-up SPECT showed no significant changes in the reduction of perfusion defect in the MSC only group (52.2±16.3%, at baseline versus 49.8±13.6% at end point, $d=2.3±4.1%$) compared to that in the control group (50.7±14.5% at baseline versus 47.8±11.1% at end point, $d=2.8±6.1%$, $P>0.05$). However, significantly reduced defect was observed in both the SIMV-treated and combination groups compared with the control group (52.7±15.5% at baseline to 39.3±12.4% at end point $d=13.3±6.2%$ [$P<0.05$] and 49.7±16.8% to 28.3±7.4% $d=21.4±12.0%$ [$P<0.01$], respectively). There was no obvious synergy in restoring perfusion between SIMV and MSCs ($P=0.192$; Figure 3).

At baseline, no significant differences in MRI parameters were found among the groups. At end point, the combination group showed significantly improved left ventricular ejection fraction (LVEF). The average increase in LVEF was about 6.5±4.4% ($P<0.01$) compared to that in the control group. The change in end-diastolic volume (EDV) was not significantly different among the 4 groups ($P=0.846$); the change in end-systolic volume (ESV) was significant only in comparison between the combination group and the control group ($P<0.05$), indicating that the systolic function of the left ventricle was significantly improved by MSC transplantation facilitated by SIMV administration. The size of the infarct region was reduced in both the SIMV group and the combination group compared with the control group ($P<0.01$), and the number of dyskinetic segments was lower in both the SIMV and combination groups than in the control group ($P<0.05$, respectively). Wall thickening significantly increased only in the combination group compared with the control group ($P<0.01$; Table). A synergism appeared to occur between SIMV and MSCs, in the infarcted area (interaction term $P=0.001$), dyskinetic segments (interaction term $P=0.021$), and wall thickening (interaction term $P=0.000$).

Statin Inhibits Apoptosis in the Perifarct Myocardium

Analysis of the heart sections by TUNEL (Figure 4A) indicated significantly fewer apoptotic myocytes in the infarcted left ventricles of SIMV-treated pigs (both SIMV only and the combination groups) than in the left ventricles of controls (apoptotic index, 5.6±1.5 in the SIMV group, 2.3±0.3 in the combination group, and 10.1±1.8 in the control group, respectively; $P<0.01$). Furthermore, the apoptotic index was significantly lower in the combination group than in the SIMV group ($P<0.01$; Figure 4B). However, the apoptotic index in the MSC only group (9.2±1.4) did not differ significantly from that in the control group ($P=0.262$). In addition, Western blot analysis indicated that the proapoptotic protein, Bax, was significantly increased in the control group compared with sham animals at end point ($P<0.01$). However, SIMV treatment significantly decreased Bax expression in the SIMV group and combination group when compared with control animals ($P<0.01$). In contrast, MSC transplantation alone did not decrease Bax levels significantly ($P=0.069$). Levels of the antiapoptotic protein, Bcl-2, were lower in control animals than in the sham group ($P<0.05$), but Bcl-2 was upregulated by statin treatment...
Statin Treatment Reduces Oxidative Stress

At the end point, SOD activity in the postinfarction myocardium was significantly higher in the SIMV and combination groups than in the control group (100.8±12.1, 108.5±10.2, and 83.4±8.8 U/mg protein, respectively, P<0.05), indicating that statin administration enhanced the ability to scavenge free radicals in the postinfarction myocardium. However, there was not a significant difference between the control group and the MSC only group (87.4±10.2 U/mg protein, P=0.515). In contrast, the MDA content of the myocardium was lower in the SIMV and combination groups than in the control group (5.8±0.7, 5.6±0.9, and 9.0±0.8 nmol/mg protein, respectively; P<0.01), suggesting statin treatment reduced lipid peroxidation and subsequent cell damage. MDA content did not differ significantly between the control and the MSC only groups.

Statin Treatment Reduces Expression of Proinflammatory Cytokines

Increased expression of IL-1, IL-6, and TNF-α was found in the postinfarction myocardium of the control group compared with sham animals (P<0.01), whereas β-actin levels remained unchanged (Figure 5A). However, SIMV treatment reduced the induction of IL-1β, IL-6, and TNF-α in the SIMV group (P<0.01) and the combination group (P<0.01) when compared with the control group. In addition, MSC transplantation alone decreased the levels of IL-1β (Figure 5B).

**Table. Left Ventricular Function and Geometry at Baseline and End Point by MRI**

<table>
<thead>
<tr>
<th>Test</th>
<th>Control (n=6)</th>
<th>SIMV (n=6)</th>
<th>MSCs (n=6)</th>
<th>SIMV+MSCs (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>End Point</td>
<td>d</td>
<td>Baseline</td>
</tr>
<tr>
<td>Global LVEF, %</td>
<td>43.9±6.6</td>
<td>42.0±7.1</td>
<td>-2.0±5.0</td>
<td>43.3±7.9</td>
</tr>
<tr>
<td>EDV, ml</td>
<td>57.8±5.8</td>
<td>67.2±6.6</td>
<td>9.3±2.7</td>
<td>58.2±6.0</td>
</tr>
<tr>
<td>E/ED</td>
<td>32.7±6.6</td>
<td>39.2±7.3</td>
<td>6.5±4.1</td>
<td>33.2±7.7</td>
</tr>
<tr>
<td>Dyssynchronous segments</td>
<td>8.2±2.9</td>
<td>8.2±2.2</td>
<td>-0.1±0.3</td>
<td>8.8±1.9</td>
</tr>
<tr>
<td>Wall thickening, %</td>
<td>-26.5±16.3</td>
<td>-27.2±16.0</td>
<td>-0.7±6.4</td>
<td>-25.3±8.8</td>
</tr>
<tr>
<td>Infarct size, cm²</td>
<td>6.7±1.9</td>
<td>6.6±2.1</td>
<td>-0.1±0.3</td>
<td>7.0±2.1</td>
</tr>
<tr>
<td>LV mass index, g/m²</td>
<td>64.2±7.0</td>
<td>77.3±8.1</td>
<td>13.2±8.1</td>
<td>63.2±8.1</td>
</tr>
</tbody>
</table>

D indicates difference between the values at baseline and end point; EDV, end-diastolic volume; ESV, end-systolic volume; LVEF, left ventricular ejection fraction.

*P<0.05 vs control; #P<0.05 vs control; §P<0.05 vs SIMV; §§P<0.05 vs SIMV.

Figure 4. Detection of apoptosis in infarcted hearts receiving SIMV and MSCs. A, Apoptotic cells with intranuclear DNA fragmentation were detected by the TUNEL assay in infarcted hearts of the control, MSC, SIMV, and SIMV+MSCs groups. B, Quantification shows that at end point apoptotic myocytes were significantly reduced in the SIMV group vs control group (P<0.0001), and in the SIMV+MSC group vs the SIMV group (P<0.0001). However, there was no significant difference between the MSC only group and the control group (P>0.05). C, Western blot analysis and densitometry of Bax, Bcl-2, and β-actin. D, Bax was significantly increased in the SIMV group, but was increased in the SIMV only group and the control group (P<0.05). E, Bcl-2 was significantly decreased in the control group compared with sham animals (P<0.0001). However, simvastatin treatment significantly decreased Bax expression in the SIMV group and SIMV+MSC group when compared with control animals (P<0.0001). There was no significant difference between the MSC only group and the control group (P>0.05). E, Bcl-2 was significantly decreased in the control group compared with sham animals (P<0.0001) but was increased in the SIMV group, MSCs, and SIMV+MSC group when compared with control animals (all P<0.0001).
The milieu for implanted cells differs, depending on the environment just after AMI and reperfusion. Li and colleagues reported that the local environment just after AMI and reperfusion differs at various time points after infarction. The heart is complicated and dynamic, and the local microenvironment differs after AMI. The repair process of the postinfarction myocardium may differentiate into resident cells in the hearts, including cardiomyocytes. Bone marrow–derived MSCs colonizing the myocardium and survive. In our study, most of the implanted MSCs did not survive in the postinfarction myocardium, indicating that the local environment just after AMI and reperfusion was unsuitable for MSC engraftment. Moreover, our study indicates that the mechanisms resulting in MSC death may include inflammation, oxidative stress, apoptosis, and ischemia/reperfusion. It is unclear why MSC transplantation alone decreased IL-1β and increased Bcl-2 expression in the postinfarction myocardium but did not produce functional benefits.

In our study, low-dose short-term administration of simvastatin around the time of infarction and reperfusion decreased the number of dyskinetic segments as detected by MRI and reduced the area of perfusion defect as seen on SPECT. These findings are consistent with those from previous reports of statins in experimental myocardial infarction. Statins may provide benefits after AMI and reperfusion via multiple mechanisms, including anti-inflammatory and antioxidative stress reactions. We found that simvastatin administration alone did not increase capillary density in or around the infarcted region; therefore, under experimental conditions in the porcine model, treatment with simvastatin alone provides limited benefits in terms of angiogenesis (supplemental Figure II). In a study in mice with permanent ligation of the LAD arteries, low-dose simvastatin (0.25 mg/kg/d) administered for 28 days after infarction improved survival and cardiac function and inhibited cardiac hypertrophy and pulmonary edema. Moreover, in a previous study, we found that pretreatment with simvastatin decreased the area at risk after AMI and reperfusion by activating ATP-sensitive potassium channels. Our current study provides evidence of the multiple benefits of simvastatin and suggests that statin administration may improve the local milieu after AMI and reperfusion.

Perhaps the most important finding in our study is that simvastatin treatment may significantly increase the in vivo survival and differentiation of implanted cells in the myocardium after infarction over that seen with MSC transplantation alone. Furthermore, simvastatin treatment appears to provide significant benefits in cardiac function, indicating that stem cell engraftment, survival, and differentiation are enhanced in our experimental model.

A previous study showed that DAPI that was released from labeled cells that had been killed by heat would falsely stain other unlabeled cells. However, the in vivo conditions of that study differ from those found in vivo and do not represent the environment in which implanted MSCs survived. In addition, there have not been other studies until now that have shown that DAPI was taken up by host cells, after DAPI-labeled MSCs were implanted in vivo. Furthermore, in our study, if DAPI that was released from dead or mitotic implanted MSCs falsely labeled host cardiomyocytes, then the total number of DAPI-positive cells would not differ significantly between groups because we implanted the same number of cells (3 × 10^7 MSCs per animal) in all animals in the MSC and combination groups. However, our results showed significant differences between the 2 implantation groups. Based on these findings, we believe that the difference in the DAPI-positive cell numbers between the 2 groups resulted from the different survival potential of the implanted cells.

**Figure 5.** Analysis of proinflammatory cytokines in infarcted myocardium receiving SIMV and MSCs. A, Western blot assays of IL-1β, IL-6, and TNF-α in infarcted hearts of sham, control, SIMV, MSCs, and SIMV+MSCs groups. Beta-actin was used as an internal loading control. B through D, Densitometry of the IL-1β, IL-6, and TNF-α protein bands. *P < 0.0001 compared with sham, #P < 0.0001 compared with control, **P > 0.05 compared with control.

and IL-6 (Figure 5C) compared with control (P < 0.01) but exerted no significant effect on TNF-α expression (Figure 5D). These data indicate that combined treatment with SIMV and MSCs reduces the inflammatory response in the myocardium after AMI.

**Discussion**

Bone marrow–derived MSCs colonizing the myocardium may differentiate into resident cells in the hearts, including cardiomyocytes. The repair process of the postinfarction heart is complicated and dynamic, and the local microenvironment differs at various time points after infarction. Thus, the milieu for implanted cells differs, depending on the time of injection. Li and colleagues reported that the effects of cell transplantation on scar expansion and cardiac function were better when cells were injected 2 weeks after cryoinjury than when injected immediately or 4 weeks after injury. They proposed that excessive cell death caused by early postinfarction inflammation hinders improvement in remodeling or function. To achieve benefits, the donor cells should engraft in the targeted region and survive. In our study, most of the implanted MSCs did not survive in the postinfarction myocardium, indicating that the local environment just after AMI and reperfusion...
MSCs. Detecting the mitotic potential of stem cells after their implantation in infarcted myocardium is important, as is determining the impact of mitosis on DAPI labeling. However, visualizing or determining cell mitosis in cardiac or other tissue is technically difficult because of the limited capability of tracking cells.

Our findings indicate that the local milieu plays a critical role in cell engraftment. Although the usefulness of stem cells for cardiac repair has been examined in clinical studies, progress in cellular cardiomyoplasty is hampered by the poor survival of implanted cells. A high proportion of engrafted cells die after implantation in injured hearts. Our results suggest that the molecular mechanisms of stem cell death in acutely ischemic hearts include apoptosis, oxidative stress, and inflammation in the postinfarction myocardium, a finding supported by previous studies. Protecting grafted cells from acute death in ischemic myocardium is important for clinical applications. To date, several methods have been used to improve the fate of implanted cells, such as heat-shock treatment, Akt-engineering, and overexpression of hemeoxygenase-1. We believe that intervention aimed at improving the quality of the local microenvironment may improve survival and facilitate the biological behavior of implanted cells.

In the present study, low-dose simvastatin alone did not produce detectable benefits in cardiac function, except for a possible decrease in infarcted area; however, it significantly increased the potential of survival and differentiation of implanted MSCs. Many studies including our own work have shown that inflammatory cytokines can induce apoptosis of cardiovascular cells. During AMI, myocytes are exposed to a variety of proinflammatory and proapoptotic factors, and ultimately undergo high degrees of apoptosis. The statin-mediated reduction in inflammatory cytokines may therefore improve survival of both implanted MSCs and resident cardiovascular cells in the infarcted hearts. The beneficial effect can be further enhanced by the statin-mediated inhibition of expression of the proapoptotic protein Bax or increase in the expression of the antiapoptotic protein Bcl-2. However, Bax and Bcl-2 are not the only apoptosis regulation factors that could explain our findings in the TUNEL assay.

Reactive oxygen species (ROS) contribute to cardiomyocyte apoptosis and inflammation, especially under the conditions of AMI. After AMI, ROS can activate nuclear factor kappa B, which then triggers gene expression of proinflammatory cytokines, such as TNF-α and interleukins, and initiates inflammatory reactions. Several studies have shown that antioxidant treatment reduces infarct size in the rat AMI model. The acute release of large amounts of ROS has been implicated in the cell death associated with AMI, whereas the chronic release of ROS may contribute to the development of left ventricular hypertrophy and heart failure. Antioxidant treatment appears to attenuate cardiac fibrosis. Our results indicate that simvastatin administration may inhibit the oxidative stress level in the infarcted myocardium during the chronic stage, which is consistent with previous findings. Nonetheless, we have not fully defined the precise signaling pathway by which SOD and MDA regulate tissue repair and regeneration.

In conclusion, our study has provided in vivo evidence showing that short-term administration of low-dose simvastatin may increase the efficacy of cellular cardiomyoplasty and improve cardiac function after MSC transplantation.

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Disclosures
None.

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Supplemental Material

Methods

Acute Myocardial Infarction Model and Treatment Procedures

Porcine bone marrow–derived MSCs were isolated and cultured as previously described with some modifications [1]. In brief, about 50 ml of bone marrow aspirated from the iliac crest was used for preparation of mononuclear cells by centrifugation through 1.077g/ml Percoll (Sigma). Cells were then suspended at a density of $5 \times 10^5$/cm$^2$ in a low-glucose DMEM medium containing 10% fetal bovine serum (Gibco). The medium was changed every 3 days. At 80% confluence, cells were detached, labeled with 4',6-diamidino-2-phenylindole (DAPI; Sigma) and kept in warm DMEM for transplantation ($3 \times 10^7$ cells per animal). The labeling efficiency was determined by fluorescent microscopy, which confirmed that all the cell nuclei were labeled. The viability of DAPI-labeled MSCs in vitro was detected by trypan blue dye exclusion assay. The purity of the injected MSCs was determined by fluorescent flow cytometry.

Swine were sedated with ketamine (25mg/kg intramuscularly) and valium (1mg/kg), endotracheally intubated, and connected to a Narkomed ventilator. A midline sternotomy was performed, and the left anterior descending (LAD) coronary artery was dissected free just distal to the first diagonal branch and isolated with a vessel loop. The LAD coronary artery was occluded, and at the end of 90 minutes, the snare loop was released and reperfusion was visually confirmed. Thirty minutes after reperfusion, autologous bone marrow–derived MSCs ($3 \times 10^7$ cells per animal) were
injected into the left ventricular wall of the infarcted hearts (300 μl into 12 foci) and the periinfarct zones (200 μl into 8 foci) with a 28-gauge needle. Animals in the control group received intramyocardial injections of 500 μl DMEM. After cell or DMEM injection, the chest was closed, and the animal was extubated. Animals were treated postoperatively with an antibiotic (cephazoline, 1.0 gram intramuscularly, twice daily for 3 days) and an analgesic (buprenorphine, 0.3 mg intramuscularly, twice daily for 3 days). For Western blot analysis, 5 miniswine were included in the study as sham animals.

According to the dose determined by our previous experiments and the data reported by other investigators [2, 3], low-dose SIMV (kindly donated by Merck Pharmaceutical Company, 0.25mg. kg⁻¹.d⁻¹) was administered orally via animal feed from the 3rd day before transplantation of MSCs to the 4th day after transplantation.

**Cardiac Perfusion and Function**

The therapeutic effects of stem cell transplantation in infarcted hearts do not usually become apparent until at least 2 to 4 weeks after transplantation; therefore, the status of the heart 1 week after cell transplantation is usually similar to that of the baseline status. In addition, our preliminary experiments showed no differences in cardiac function and morphology during the first week after transplantation. Thus, we assessed the perfusion defect and cardiac function at 1 week (baseline) and 6 weeks (endpoint) after cell transplantation by using single photon emission computed tomography (SPECT) and cine magnetic resonance imaging (MRI). For SPECT

\(^{99m}\text{Tc-sestamibi, about 296 MBq (8mCi) was administered to the pigs, and the hearts}
were imaged 45-60 minutes later with a gamma camera. The dual-head gamma camera (Varicum, GE) with a low-energy and high-resolution collimator at a 20% energy window was set to 140 KeV gamma peak. Quantitative analysis was performed using Cedars quantitative perfusion SPECT (QPS). Perfusion defects were calculated using a scintigraphic bull’s eye technique.

In addition, all the animals were studied at both baseline and endpoint by cine MRI and contrast-enhancement MRI. MRI was performed by using a 1.5 T clinical MRI scanner (Siemens Avanto) with a phase-array radiofrequency receiver coil. The cardiac function and geometry were detected by using an MRI scanner according to the previous study [4].

**Histologic Analyses**

For histologic analysis, the left ventricle of every heart was cut into 8 fragments from the apex to the base, and 5 5-μm–thick sections were randomly chosen from regions where cells or placebo were injected in every fragment.

The fibrotic tissue area in cross sections of the hearts was measured with the use of a computer software package (Leica QWin) after Masson’s trichrome staining as described previously [5]. To determine the inflammatory cell infiltrates, heart sections were cut (5 μm) and stained with hematoxylin and eosin. Under 400X magnification, the densities of polymorphonuclear neutrophils and macrophages in the infarcted border zones and in the infarcted area of the heart were determined manually from 10 randomly selected areas in a blinded fashion based on morphology of nuclei and cell size, as described previously [6, 7].
To evaluate in vivo cardiomyogenic differentiation and angiogenesis derived from implanted MSCs, the tissue was prepared for immunofluorescent analyses by freezing in O.C.T. compound and slicing serial sections at 5μm. Sections were immunolabeled with antibodies against the following: von Willebrand factor (VWF, 1:50, DAKO), α-smooth muscle actin (SM-actin, 1:50, DAKO), α-sarcomeric actin (1:50, DAKO), and cardiac troponin T (cTn-T, 1:50, Sigma). The sections were washed with PBS and incubated with a goat anti-mouse antibody conjugated with rhodamine-labeled IgG or a goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC)-labeled IgG. Finally, fluorescent staining was detected and photographed by using a laser-scanning confocal microscope.

To determine the in vivo survival and differentiation potential of implanted MSCs, 5 5-μm–thick sections were randomly chosen from regions of the 8 corresponding fragments of the left ventricle where cells or placebo were injected. Cells positive for DAPI and cTn-T were counted from 5 random fields of every frozen section under the fluorescence microscope. Cells positive for cTn-T were considered to have differentiated into cardiomyocyte-like cells.

**TUNEL Assay**

The TUNEL assay (Roche, Germany) was used for in situ detection of apoptosis at the single-cell level. Myocardial tissue sections were obtained from periinfarct regions of all pigs at endpoint and from healthy swine as negative controls. Briefly, tissues were deparaffinized, digested with trypsin, and incubated with TdT and fluorescein-labeled dUTP in a humid atmosphere for 60 min at 37 °C. After the
sections were incubated for 30 min with an antibody specific for fluorescein-conjugated alkaline phosphatase, the TUNEL stain was visualized with a substrate system in which nuclei with fragmented DNA stained blue. Tissue sections were examined microscopically (400X magnification) and at least 100 cells were counted in a minimum of 8 high-power fields. The percentage of apoptotic cells was called the apoptotic index.

**Measurement of Oxidative Stress**

Myocardial tissues within the infarcted myocardium were harvested at endpoint, and the oxidative stress level was detected as previously described [8]. The enzymatic activities of superoxide dismutase (SOD) were measured by using a xanthine-oxidation method according to the instructions (Jiancheng Institute, Nanjin, China). The degree of lipid peroxidation was determined by measuring the level of malondialdehyde (MDA) in the myocardial tissues with the use of the thiobarbituric acid–reactive substances assay (TBARS, Jiancheng Institute, Nanjin, China) [9].

**Western Blot Analysis**

For Western blot analyses of Bax, Bcl-2, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, equal amounts of protein (60 μg protein/lane) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis in a Tris/HCl buffer system, and sequentially electrophoretically transferred to a polyvinylidene difluoride microporous membrane (Millipore, Billerica, Massachusetts, USA). Then, blotting was conducted according to standard procedures with a polyclonal rabbit anti-Bax (1:500) or anti-Bcl-2 (1:500, both from Santa Cruz) antibody or a monoclonal goat
anti-IL-1β (1:1,000), IL-6 (1:1,000), or TNF-α (1:1,000, all from R&D system) antibody, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (1:4,000, Jackson Immuno Research Laboratories, PA). The specific bands of target proteins were visualized by chemiluminescence. Membranes were then stripped and rebotted with monoclonal anti-actin (1:10 000, Advanced ImmunoChemical). Target signals were normalized to actin signal and analyzed semiquantitatively with an NIH Image system.

**Histologic Analyses: Capillary Density**

To determine capillary density in the injured myocardium and the peri-infarction zone, tissue was prepared as described previously [6]. Sections were stained using polyclonal anti-VWF antibody (1:200, DAKO). For quantification of positively stained vessels, 5 sections from within the injury zone and 8 from within the peri-infarction zone of each pig were analyzed by an investigator blinded to the treatment. Capillaries were counted in 5 randomly chosen high-power fields (HPFs) in every section. The results were expressed as number of capillaries per HPF.

**Statistical Analyses**

Continuous variables are presented as means ± standard deviation. Differences in the survival and differentiation efficiency of implanted MSCs between the SIMV + MSCs group and MSCs only group were analyzed by Student’s t test and chi-square (χ²) test, respectively. After the homogeneity of variance and normal distribution of data were evaluated, analysis of variance was performed to determine the differences in MRI and SPECT parameters between baseline and endpoint among groups. Least
significant difference (LSD) tests were used for multiple comparisons between groups. The synergistic effect of SIMV+MSC was tested in the ANOVA framework including the two interventions (SIMV and MSCs), the corresponding interaction term as well as the value of the outcome variable at baseline. All statistical tests were two-tailed, and $P<0.05$ was considered statistically significant. All statistical analyses were conducted with SPSS 13.0.

**Results**

**Phenotype Detection of Bone Marrow-MSCs**

The purity of the injected MSCs was determination by fluorescent flow cytometry. We observed that bone marrow-MSCs isolated by the above method were negative for CD34 and CD45, but positive for CD29 (99.94±0.05%) and CD90 (97.43±1.29%) (Supplemental Figure 1).

**Statin Enhances Survival and Myogenesis of Implanted MSCs: Capillary Density**

Capillary density in the infarcted zone was not significantly different among the control, the SIMV, and the MSC only groups (1.7 ± 0.6/HPF, 2.1 ± 0.6/HPF, and 1.8 ± 0.8/HPF, respectively, $P>0.05$). However, capillary density was significantly increased in the infarcted zone of the MSC-SIMV combination group (3.8 ± 1.0/HPF, $P<0.01$) when compared with that in the MSC only group. In the peri-infarct regions, capillary density was 8.9±1.9/HPF in the combination group, which was significantly higher than that seen in the other groups ($P<0.01$) (Supplemental Figure 2).
References


6. Gao XM, Dilley RJ, Samuel CS, Percy E, Fullerton MJ, Dart AM. Lower risk of postinfarct rupture in mouse heart overexpressing β2-adrenergic receptors:


Figure Legends

Supplemental Figure 1

The purity of the injected MSCs was determined by fluorescent flow cytometry, and results showed that bone marrow-MSCs isolated by the above method were negative for CD34 and CD45, but positive for CD29 (99.94±0.05%) and CD90 (97.43±1.29%).

Supplemental Figure 2

Quantification of neovascularization in and around the infarcted cardiac area in miniswine treated with simvastatin (SIMV) and mesenchymal stem cells (MSCs). Miniswine treated with a combination of SIMV + MSCs had significantly increased vascular density in the peri-infarct and infarcted areas when compared with the other groups (**P <0.0001, ##P <0.0001, respectively). However, MSCs alone and SIMV alone did not increase vascular density compared with control group (*P > 0.05, #P > 0.05, respectively).
Supplemental Figure

Supplemental Figure 1

Supplemental Figure 2
Changes to the Manuscript

According to the editor’s requests, we have made several changes as follows:

1. We have transferred the “Section of Method” except “Animal” into the Supplemental Material, as a result, the number of word of manuscript body is 5000, and abstract is 204, which meets the request of instructions.

2. We have increased the font in Figure 1.

3. In the Supplemental Material, we have listed separate references.