Stem cell therapies hold promise for 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 fetal or neonatal heart cells. These

Human Mesenchymal Stem Cells Improve Myocardial Performance in a Splenectomized Rat Model of Chronic Myocardial Infarction

Jen-Fu Liu,1 Bao-Wei Wang,1 Huei-Fong Hung,1 Hang Chang,2 Kou-Gi Shyu1,3*

Background/Purpose: Cellular therapy has been applied to animal studies and clinical trials for acute or subacute myocardial infarction. Little is known about the effect of cell therapy on chronic myocardial infarction. The goal of this study was to investigate myocardial performance after human bone marrow-derived mesenchymal stem cell (hMSCs) transplantation in rats with chronic myocardial infarction.

Methods: 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. Splenectomy in male rats was performed to prevent immune reaction. One week after splenectomy, ligation of the left anterior descending coronary artery was performed to induce myocardial infarction. Four weeks after ligation of the coronary artery, culture-expanded hMSCs were injected intramyocardially at the left anterior free wall. Left ventricular function measured by echocardiography, infarct size and immunohistochemical stain were performed to evaluate the effect of the therapy.

Results: The engrafted hMSCs were positive for the cardiac marker troponin T. Infarct size (35.4 ± 3.4% vs. 53.3 ± 3.0%, p < 0.001) and fibrotic area (2.6 ± 0.1% vs. 5.9 ± 0.2%, p < 0.001) were significantly smaller in the hMSC-treated group than in the control group at 28 days after therapy. hMSC transplantation resulted in smaller left ventricular end-diastolic dimension (6.5 ± 0.1 mm vs. 7.9 ± 0.7 mm, p < 0.001) and better left ventricular ejection fraction (88.7 ± 1.2% vs. 65.8 ± 2.5%, p < 0.001) than in the control group. Capillary density was markedly increased after hMSC transplantation compared with the control group.

Conclusion: This study demonstrates that intramyocardial transplantation of hMSCs improves cardiac function after chronic myocardial infarction through enhancement of angiogenesis and myogenesis in the ischemic myocardium. Transplantation of hMSCs for myocardial regeneration may become the future therapy for chronic myocardial infarction. [J Formos Med Assoc 2008;107(2):165–174]

Key Words: cellular therapy, mesenchymal stem cells, myocardial infarction, myocardial regeneration

Stem cell therapies hold promise for 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 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. As damaged tissue may lose anatomic cues for functioning organ neovascularization, in vitro manipulation of stem cells may be essential to facilitate in vivo incorporation.

MSCs have been shown to improve heart function in acute myocardial infarction and chronic myocardial ischemia. However, little is known about the effect of MSCs on heart function in chronic myocardial infarction. Therefore, the aim of the present study was to investigate whether MSC therapy can improve cardiac performance after chronic myocardial infarction.

To investigate the potential therapeutic utility for clinical application, we used human MSCs (hMSCs) in the present study.

**Methods**

**Isolation and culture of human bone marrow-derived MSCs**

Bone marrow derived hMSCs were isolated and cultured according to a previously reported method by Pittenger et al. 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, and 50 mg/mL streptomycin. Bone marrow was obtained from healthy donors because of ethical problem and risk factor for coronary artery disease reducing capacity of progenitor cells. Subjects were informed that their genetic material would be transferred to animals. Mononucleated cells were isolated by ficoll 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% CO2. Seven days later, individual colonies were collected, isolated, cultured and expanded. When the cultures reached 80% confluence, the 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, Franklin Lakes, NJ, USA) by following the manufacturer’s instructions. 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, St Louis, MO, 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 secondary antibody). In each case, 1 x 10^4 cells were acquired and analyzed by using the CellQuest software (Becton Dickinson).

**Rat model of chronic myocardial infarction**

To prevent immune reaction to the transplanted hMSCs, splenectomy was performed in adult male Sprague-Dawley rats. A week after splenectomy, permanent occlusion of the left anterior descending coronary artery (LAD) was performed to induce myocardial infarction. After induction of anesthesia with isoflurane (3%), 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. Splenectomy was also performed in the sham-operation control group. Four weeks after induction of myocardial infarction, the pericardium was opened again using the procedure described above. Animals were randomized to either direct injection of hMSCs or normal saline to the left ventricular free wall. After the procedure, the chest wound was sutured and the rats were allowed to recover. The study conformed to the guidelines issued in the Guide for the Care and Use of Laboratory Animals published by the
Intramyocardial injection of stem cells
The rats after chronic myocardial infarction were randomly chosen to receive hMSCs or saline therapy. Four weeks after ligation of the LAD, $1 \times 10^6$ culture-expanded hMSCs resuspended in 50 µL of normal saline were injected intramyocardially at the left anterior free wall by using an insulin syringe with a 30-gauge needle. After the left ventricle was accessed, the needle was advanced along the left ventricular free wall and hMSCs or normal saline were injected over a period of 5–10 seconds at three separate sites. The injected sites were chosen to be at least 5 mm away from the left ventricular apex. Aspiration was done to confirm that the injection had been into the left ventricular wall and not into the left ventricular cavity.

Physiologic assessment of left ventricular function
Transthoracic echocardiography (Acuson Sequoia 512 machine using a 15-MHz probe; Siemens Medical Solutions USA Inc., Malvern, PA, USA) was performed just before (baseline) and 28 days after saline injection or hMSC transplantation. 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.

Infarct size determination
Four weeks after intramyocardial transplantation, mice were deeply anesthetized with isoflurane (3%) and killed by rapid excision of the heart. The excised hearts were immediately soaked in cold saline for 10 seconds to remove excess blood from the ventricle and fixed in neutral-buffered 4% formalin for 48 hours. Paraffin-embedded samples were sectioned at 10 µm, and Masson’s trichrome staining was performed to delineate scar tissue from viable myocardium. Masson’s trichrome-stained sections were captured as digital images and analyzed by NIH Image software. The infarct size was determined by triphenyltetrazolium chloride method as previously described. Each heart was cut in cross-section at four levels from apex to base and prepared for routine histology. The examiner of the tissue sections was blinded to the treatments. Infarct size was determined as described previously.

Immunohistochemical assessment of transplanted myocardium
On the day of sacrifice (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 nonspecific 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-troponin T antibody at 4°C overnight. Bound primary antibody was detected with the avidin-biotin-immunoperoxidase method (Signet Laboratories, Dedham, MA, USA). Nonimmune 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. Incorporation of injected hMSCs was verified by staining for HLA class I-APC (BD Pharmingen).

Specimens were also stained with troponin-T, Tie-2, and PCNA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to confirm that transplantation of MSCs differentiate to cardiomyocytes. The anti-Tie-2 and anti-PCNA antibodies do not cross-react with human and rat myocardium.

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

**Immunophenotypic characterization of hMSCs**

We used flow cytometric analysis and immunophenotyping to confirm that the majority of adherent cells used in this study were MSCs. The bone marrow-derived MSCs were positive for CD29, CD44, CD49, CD90, CD105, CD106, CD166 and SH, but were negative for CD31, CD45, KDR, Flt1 and Flt3. These data were consistent with the properties of documented bone marrow-derived MSCs. These results have been shown in our previous study.9

**Evidence of incorporation of hMSCs into ischemic myocardium**

A total of 79 rats received splenectomy and 75 survived the procedure (5% mortality rate). Seven splenectomized rats in each group were used to investigate the incorporation of hMSCs into heart tissue. hMSCs were incorporated into the heart by 14 days after transplantation, as evidenced by the presence of HLA-positive myocytes within the rat myocardium. The cells positive for HLA were also positive for troponin-T, a cardiac myocyte marker (Figure 1). There were 27 ± 4% of HLA-positive myocytes in the left ventricle under high power field microscope examination as calculated by area.

**hMSCs reduced infarct size**

The infarct size was similar in each group at 4 weeks after LAD ligation and before randomization for therapy (41 ± 5% for saline group and 42 ± 4% for MSC group). A total of 26 rats received saline injection and 20 rats received hMSC injection. The survival rate after chronic infarction and intramyocardial injection was 46% for the control group and 55% for the hMSC group. Although there was a trend toward better survival for the hMSC group compared to the control group, the difference did not reach statistical significance. Infarct size (infarct area/total left ventricular area) was significantly smaller in the hMSC-treated animals (35.4 ± 3.4%) compared to the control animals (53.3 ± 3.0%) or animals injected with saline (54.9 ± 3.3%) (Figure 2). Infarct size in splenectomized rats treated with hMSCs was reduced by 34% compared to control rats without treatment. Infarct size was similar between the control group without treatment and the saline-treated group. As shown in Figure 2, the fibrotic area as determined by Masson’s trichrome stain decreased significantly from 5.9 ± 0.2% in the saline-treated group to 2.6 ± 0.1%

![Figure 1](image1.png)

**Figure 1.** Representative photographs of immunohistochemical analysis using HLA-APC and troponin T antibodies. Positive staining for HLA was shown in mesenchymal stem cell (MSC)-treated myocardium, but not in saline-treated myocardium. Both groups stained positive for troponin T. Similar results were observed in another six independent experiments. The left panel is hematoxylin and eosin (H&E) stained myocardium.
in the hMSC-treated group ($p < 0.001$). The fibrotic area was similar between the control group without treatment and the saline-treated group.

**hMSCs improved cardiac function**

The left ventricular ejection fraction (LVEF) was similar in each group at 4 weeks after LAD ligation and before randomization for therapy (68 ± 3% for saline group and 67 ± 4% for MSC group). The cardiac functional parameters evaluated by echocardiography, 4 weeks after therapy, are shown in the Table. In the control group, markedly decreased fractional shortening and LVEF with dilated left ventricular cavity (Figure 3) were clearly seen. In the group that underwent hMSC transplantation, significantly higher fractional shortening and LVEF and smaller left ventricular cavity were observed compared with the control group without treatment and the saline-treated group. The LVEF of hMSC-treated rats increased by 35% compared to control rats without treatment and increased by 37% compared to rats treated with saline.

**hMSCs increased capillary density**

At 28 days after therapy, capillary density of the ischemic myocardium in the hMSC-treated group was increased compared to the control group without treatment and the saline-treated group.

![Figure 2. Mesenchymal stem cells (MSCs) reduced infarct size and fibrosis area. (A) Representative Masson’s trichrome stain for cross sections of myocardium. AMI = chronic myocardial infarction without any treatment; Saline = treatment with normal saline; MSC = treatment with human MSCs. (B) Quantitative analysis of fibrosis area. (C) Quantitative analysis of infarct ratio. The infarct ratio represents infarction area divided by total left ventricular area. $N = 10–12$. *$p < 0.001$ vs. MSC group.](image-url)

**Table. Evaluation of cardiac function by echocardiography 4 weeks after cell therapy**

<table>
<thead>
<tr>
<th></th>
<th>LVEDd (mm)</th>
<th>LVEDs (mm)</th>
<th>FS (%)</th>
<th>LVEF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham ($n = 7$)</td>
<td>6.8 ± 0.5</td>
<td>3.2 ± 0.1</td>
<td>52.3 ± 3.6</td>
<td>87.6 ± 2.8</td>
</tr>
<tr>
<td>Control ($n = 10$)</td>
<td>7.9 ± 0.7†</td>
<td>5.3 ± 0.5†</td>
<td>32.0 ± 1.8†</td>
<td>65.8 ± 2.5†</td>
</tr>
<tr>
<td>Saline ($n = 12$)</td>
<td>7.8 ± 0.2†</td>
<td>5.4 ± 0.6†</td>
<td>31.5 ± 6.5†</td>
<td>64.7 ± 9.4†</td>
</tr>
<tr>
<td>MSC ($n = 11$)</td>
<td>6.5 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>53.5 ± 1.7</td>
<td>88.7 ± 1.2</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± standard deviation; †$p < 0.001$ vs. sham group; ‡$p < 0.001$ vs. MSC group. LVEDd = left ventricular end-diastolic diameter; LVEDs = left ventricular end-systolic diameter; FS = fractional shortening; LVEF = left ventricular ejection fraction.
**Figure 3.** Representative echocardiographic images at 4 weeks after intramyocardial therapy. Smaller left ventricular end-diastolic dimension and better contractility were observed in the mesenchymal stem cell (MSC)-treated group. AMI = chronic myocardial infarction without any treatment; Saline = treatment with normal saline.

**Figure 4.** Mesenchymal stem cells (MSCs) increased capillary density. (A) Photomicrographs show representative immunohistochemical CD31 and hematoxylin and eosin (H&E) staining of ischemic myocardium from the border zone. The red dots indicate capillaries. (B) Quantitative analysis of capillary density. N = 10–12. *p < 0.001 vs. MSC group.
Mesenchymal stem cells in chronic MI

J Formos Med Assoc | 2008 • Vol 107 • No 2

171

group (1110 ± 61/mm²) was significantly higher than that in the control group without treatment (396 ± 40/mm²) and the saline-treated group (476 ± 61/mm²) (Figure 4). Capillary density in the border zone was 1300 ± 70/mm² for the hMSC-treated group, 940 ± 50/mm² for the control group without treatment, and 930 ± 46/mm² for the saline-treated group. Capillary density in the infarct zone was 938 ± 42/mm² for the hMSC-treated group, 140 ± 12/mm² for the control group without treatment, and 144 ± 16/mm² for the saline-treated group. Capillary density in the border and infarct zones was significantly higher in the MSC-treated group than the control and saline-treated groups.

hMSCs induced myogenesis

Seven heart tissues from splenectomized rats in each group were used for immunohistochemical study at 28 days after therapy. As shown in Figure 5, immunohistochemical analysis showed that stem cell markers, including Tie-2 and PCNA, were all positive in the ischemic myocardium treated with hMSCs. The myocytes that stained positive for Tie-2 and PCNA also stained positive for HLA. There were 55 ± 3% and 53 ± 3% of HLA-positive MSCs in the myocardium that were also Tie-2 and PCNA positive, respectively. These marker-positive cells were cardiomyocytes. This result indicates that the transdifferentiation percentage of MSCs is around 50%. Since bone marrow-derived stem cells express Tie-2, these data suggest that hMSCs are capable of differentiating into cardiomyocytes. Stem cell markers were not stained in the ischemic myocardium treated with saline. Some of the positive HLA-stained cells were also positive for CD31 (Figure 5B), indicating that some engrafting MSCs incorporate into vasculature to participate in vasculogenesis in addition to enhancing angiogenesis. Since capillary density was significantly higher in the MSC-treated group, our results indicate that the origin of the capillaries is mostly from local endothelial cells and only a few are from HLA-positive MSCs.

Discussion

The present study demonstrates that transplantation of adult hMSCs induces myocardial myogenesis and angiogenesis in a rat model of chronic myocardial infarction. This model of therapy reduced infarction size and fibrosis area, 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 troponin T also stained positive for Tie-2 and PCNA. These results suggest that hMSCs differentiate into cardiomyocytes. Our study also demonstrated that some of the engrafted hMSCs stained by cardiac proteins such as troponin T also stained positive for CD31. This finding indicates that engrafting MSCs incorporate into vasculature to participate in both vasculogenesis and angiogenesis. When demonstrating two phenotypes in the same cells, it is better to use a confocal microscope for immunostaining. In the present study, without a high-power laser-scanning confocal microscope, it was very difficult to determine if these double-stained cells were newly differentiated endogenous cells or the product of cell fusion or transdifferentiation.

Myocardial dysfunction resulting from myocardial infarction is a widespread and important cause of morbidity and mortality in adults. Clinical manifestations are enormous and heterogeneous because of scar- and ischemia-related postinfarction events. Bone marrow-derived cells have been used to regenerate damaged myocardium in patients with myocardial infarction. However, clinical bone marrow-derived cell therapy resulted in mixed results from mixed cells. Bone marrow-derived cells have been shown to improve left ventricular function and metabolism after chronic infarction in a small clinical study. The effect of hMSCs in chronic myocardial infarction is poorly understood. MSCs have the capacity to engraft and survive long-term in distinctive target tissue and can be isolated from several human
Figure 5. Representative microscope photographs for immunohistochemical staining. (A) The cells that stained positive for Tie-2 and PCNA also stained positive for HLA. Similar results were observed in another six independent experiments. (B) Some of the HLA-positive cells were also positive for CD31. Positive stain for HLA was shown in mesenchymal stem cell (MSC)-treated myocardium but not in sham or saline-treated myocardium. Similar results were observed in another six independent experiments.
sources. MSCs exhibit a high ex vivo expansion capacity. These characteristics make MSCs a good choice of cell for cell therapy in dysfunctional myocardium.

MSCs have been injected directly into the infarct site, or they have been administered intravenously and observed to home in to the site of injury. Most of the cell therapies for myocardial infarction use MSCs of isogenic or allogeneic origins. Rarely have studies used hMSCs to treat myocardial infarction in a rat model. Although Makkar et al reported that allogeneic bone-marrow-derived MSCs preserved cardiac function without immunosuppression, Grinnemo et al demonstrated that transplant rejection of hMSCs can occur in a xenogenic model. Therefore, in this study, we used splenectomized rats to prevent immune reaction to the transplanted hMSCs. hMSCs could be found in the transplanted myocardium at 4 weeks after therapy. This finding indicates that the hMSCs were not rejected.

Heeschen et al demonstrated that patients with chronic ischemic heart disease have profoundly reduced neovascularization capacity in their bone marrow mononuclear cells. Furthermore, Rauscher et al have demonstrated the progressive progenitor cell deficits in older animals. Several studies have demonstrated that risk factors for coronary artery disease correlate with a reduced number and functional activity of circulating endothelial progenitor cells. These data may indicate that MSCs from young and healthy donors would be the ideal source of cells for cell therapy. MSCs are defined as self-renewing multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages. 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.

MSCs have been shown to improve regional and global left ventricular function and to increase microvessel formation. Recently, MSCs have also been demonstrated to improve myocardial compliance after acute myocardial infarction. However, the effect of MSCs on myocardial regeneration and neoangiogenesis after chronic myocardial infarction is not known. Miyahara et al have demonstrated that monolayered MSCs from adipose tissue, using cell sheet technology, repaired scarred myocardium after chronic myocardial infarction. In the present study, we demonstrated that direct intramyocardial injection of hMSCs from bone marrow without any tissue engineering technique improved cardiac performance after chronic myocardial infarction in rats. Taken together, MSCs from different sources may have the same therapeutic effect on chronic myocardial infarction.

A previous study has demonstrated that infarct expansion starts early after myocardial infarction and cardiac remodeling is continued chronically in a rat model of myocardial infarction. Infarct healing is usually completed by 3 weeks after myocardial infarction in rats, suggesting that MSCs may be given late after myocardial infarction, when the infarct expansion and inflammation is completed and the infarct scar is fully matured. Therefore, we injected hMSCs 4 weeks after myocardial infarction.

In summary, the present study demonstrated that intramyocardial transplantation of hMSCs improves cardiac function after chronic myocardial infarction through enhancement of angiogenesis and myogenesis in the ischemic myocardium. MSC transplantation holds promise in the development of future therapies for myocardial regeneration for persons who have suffered acute myocardial infarction.

Acknowledgments

This study was sponsored in part by a grant from the National Science Council, Taipei, Taiwan.

References


20. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004;94:678–85.


