The effects of mesenchymal stem cells transduced with Akt in a porcine myocardial infarction model

Sang Yup Lim\textsuperscript{a}\textsuperscript{,1}, Yong Sook Kim\textsuperscript{a}\textsuperscript{,1}, Youngkeun Ahn\textsuperscript{a}\textsuperscript{,*}, Myung Ho Jeong\textsuperscript{a}, Moon Hwa Hong\textsuperscript{a}, Soo Yeon Joo\textsuperscript{a}, Kwang Il Nam\textsuperscript{b}, Jeong Gwan Cho\textsuperscript{a}, Peter M. Kang\textsuperscript{c}, Jong Chun Park\textsuperscript{a}

\textsuperscript{a} Department of Cardiovascular Medicine, The Heart Center of Chonnam National University Hospital, 8 Hak Dong, Dong Ku, Gwangju 501-757, South Korea
\textsuperscript{b} Research Institute of Medical Sciences, Chonnam National University Medical School, Gwangju, South Korea
\textsuperscript{c} Cardiovascular Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, USA

Received 4 December 2005; received in revised form 2 February 2006; accepted 13 February 2006

Available online 6 March 2006

Time for primary review 29 days

Abstract

Objective: This study was designed to examine whether mesenchymal stem cells (MSCs) transduced with Akt enhance cardiac repair after transplantation into the ischemic porcine heart.

Methods: MSCs isolated from porcine bone marrow and transduced with myr-Akt were transplanted into porcine hearts after experimental myocardial infarction (MI) using intracoronary injection [Group I, vehicle; Group II, MSCs; Group III, Akt-MSCs]. Myocardial single photon emission tomography (M-SPECT) was performed to assess myocardial function and the infarcted area. Pigs were also sacrificed for immunohistochemical characterization and histologic analysis. In addition, in vitro assays were performed to examine the resistance of Akt-MSCs to H2O2 stimulation.

Results: Transplantation of MSCs into the ischemic porcine myocardium (Group II) increased the left ventricular ejection fraction (\(\Delta\)LV EF; \(6.3\pm15.1\% \) versus \(0.5\pm6.4\%, P<0.001\)) and decreased the \(\Delta\)area of MI (6.8\pm5.6\% versus 5.0\pm5.3\%, \(P<0.001\)) compared with the vehicle control (Group I). Transplantation of MSCs transduced with myr-Akt (Group III) resulted in further improvement in \(\Delta\)LV EF (\(-6.3\pm15.1\% \) versus 5.8\pm11.3\%, \(P<0.001\)) and in \(\Delta\)area of MI (6.8\pm5.6\% versus 17.0\pm7.6\%, \(P<0.001\)). Akt-MSCs were more resistant to apoptosis, and the levels of extracellular signal-regulated protein kinase (ERK) activation and vascular endothelial growth factor (VEGF) were higher in H2O2-stimulated Akt-MSCs.

Conclusion: Cellular transplantation of Akt-MSCs further enhances the repair of injured myocardium compared to MSC transplantation alone by increasing the number of viable MSCs after cellular transplantation.

© 2006 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Mesenchymal stem cell; Akt; Myocardial infarction; Porcine

1. Introduction

Loss of myocardium after myocardial infarction (MI) is a major cause of infarct-related heart failure and death [1]. Recent experimental studies have shown that bone marrow-derived mesenchymal stem cells (MSCs) are self-renewing and capable of regenerating infarcted myocardium by inducing myogenesis and angiogenesis [2–4]. However, studies on large animals showed only marginal improvement in cardiac function after transplantation of MSCs into infarcted porcine hearts. One of the reasons for marginal improvement after cellular transplantation could be a significant cell death rate of implanted cells after grafting into injured hearts [5]. Therefore, a
strategy to overcome the poor survival rate of implanted cells is critical for improving the efficiency of stem cell therapy. Activation of Akt, a serine–threonine kinase, in cardiomyocytes has been shown to protect against apoptosis after ischemia/reperfusion injury [6]. In this regard, Mangi et al. [7] have reported that MSCs transduced with Akt enhance the efficacy of stem cell therapy in a rat model.

Despite several promising results, some issues remain before stem cells can actually be used to treat patients with damaged hearts [8–13]. The questions that still need to be answered are related to what cell type to use, the best method to deliver the cells, and the optimal time for transplantation. In addition, most animal experiments in the field of stem cell therapy are limited to studies performed in small rodents, such as rats or mice. Therefore, further validation of the efficacy of Akt-transduced MSCs in a large animal model represents a step towards the clinical applicability of this method. In this study, we examined whether transplantation of MSCs made resistant to apoptosis by transduction with Akt, in comparison to MSC alone, enhances cardiac repair after transplantation into the ischemic porcine heart.

2. Methods

2.1. Porcine myocardial infarction model

All animal experimental protocols were approved by the Ethics Committee of Chonnam National University Medical School and Chonnam National University Hospital, and conformed 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). In this study, we used 25–35 kg thoroughbred female pigs obtained 3–5 days before the experiments. The animals were housed at the Research Institute of Medical Science of Chonnam National University under conditions of constant temperature and humidity.

The pigs were pre-treated with 100 mg aspirin per day, and fasted overnight prior to the experiments. General anesthesia was induced by intramuscular injection of ketamine (12 mg/kg) and xylazine (8 mg/kg). After local anesthesia with 2% lidocaine, a 7 Fr. arterial sheath was inserted through the carotid artery, and a 7 Fr. coronary guiding catheter was inserted into the coronary ostium under fluoroscopic guidance using a Phillips C-arm system (BV-25 Gold). During the experiment, oxygen by facial oxygen mask and intravenous saline solution through an ear vein were supplied constantly, and appropriate levels of anesthesia maintained by intravenous injection of 5 mg midazolam as needed. Experimental MI was created by balloon occlusion (3.0 or 3.5 mm diameter) for 30 min at 8–10 atm of the left anterior descending artery (LAD) just distal to the 1st diagonal branch. Coronary angiograms were recorded and quantitative analysis performed using the Phillips DCI program. After experimental MI, the carotid artery was ligated and the pigs were returned to the animal quarters.

2.2. MSCs isolation and culture

Bone marrow (10–20 mL) was aspirated from iliac crest of the female pigs under local anesthesia 14 days before the experiment. The aspirates were heparinized, centrifuged at 800 × g for 10 min at room temperature, and the serum layers discarded. The cells were diluted 1:1 with phosphate-buffered saline (PBS) and layered over an equal volume of Lymphoprep (1.077 g/mL, Technoclones) and centrifuged at 800 × g for 30 min. Mononuclear cells were recovered from theuffy coat at gradient interface, and washed twice with PBS (300 × g for 5 min at room temperature). They were seeded at 2 × 10⁵/cm² in Dulbecco’s modified Eagle’s medium containing 1 g glucose/L (DMEM-LG) (Gibco) with 10% fetal bovine serum (FBS) (Gibco). The cells were then cultured at 37 °C in 5% CO₂ in air, and non-adherent cells removed after 5 days. The culture medium was replaced every 3–4 days. After about 7 days, when isolated colonies of MSCs were apparent, the cells were trypsinized and replated at 8000/cm². The mean number of MSCs was about 1.0–5.0 × 10⁵. The characteristics of MSCs were demonstrated by immunophenotyping. To verify the nature of cultured MSCs, cells were labeled against various surface and intracellular markers, and analyzed by flow cytometry. The cells were harvested, washed with PBS, and labeled with the following antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Becton-Dickinson): CD14, CD29, CD31, CD44, CD45 and CD90. Mouse-IgG1-FITC and IgG1-PE were used as isotype controls. Labeled cells were assayed by flow cytometry and analyzed with System II Software.

2.3. Adenoviral transduction

Ad.myr-Akt-HA encodes separate cytomegalovirus-driven expression cassettes for Akt and green fluorescent protein (GFP). It was constructed by subcloning the myr-Akt cDNA into pAdTrackCMV and co-transfecting with pAdEasy1 in E. coli BJ 5183 cells (a generous gift from Dr. Anthony Rosenzweig, Massachusetts General Hospital, Boston, MA). Adenoviruses were amplified in 293 cells, the particle count was estimated under the fluorescent microscope. In addition, Western blot against HA and...
phosphorylated Akt antibodies was performed to confirm the viral transduction.

2.4. Intracoronary delivery of MSCs and evaluation of cardiac functions

After experimental MI, pigs were divided into three groups; Group I= vehicle-delivered ($n=12$), Group II=MSC-delivered ($n=12$), and Group III=Akt-MSC-delivered ($n=12$). Three days after MI, allogenic mesenchymal stem cells were transplanted into the left anterior descending artery using an over-the-wire balloon catheter placed within the LAD just distal to the 1st diagonal branch. With 4 to 6 atm pressure inflation in the balloon catheter, 8 to 10 mL of cell suspension ($1.0 \times 10^7$ cells) was infused over 5 min. Ballooning prevented cell backflow and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. This procedure prolonged contact time for cellular migration. After intracoronary delivery of MSCs, coronary angiography was performed to assess the patency of the coronary artery.

For the efficacy of intracoronary delivery, MSCs were isolated from male pigs and 8–10 mL ($1.0–5.0 \times 10^7$) of MSCs was delivered by intracoronary method into the female porcine myocardium ($n=3$). The female porcine heart was excised and in situ hybridization was performed 7 days after intracoronary delivery. And for the safety of intracoronary delivery, 12-lead electrocardiogram was performed during and after procedure and histologic analysis was done in the myocardium 10 days after MSC delivery ($n=3$).

To determine LV systolic function and infarct area, myocardial single photon emission computerized tomograms (M-SPECT) were acquired both before the experiment and 4 weeks after MSC implantation. For Gated M-SPECT$^{99m}$Tc-tetrofosmin, about 1110 MBq (30 mCi) was administered to the pigs and the hearts were imaged 45 min later with a gamma camera. The dual head gamma camera (DST, SMV, Buc, France) with a low energy, a high resolution collimator was used. The images were performed using a computed visual analysis system in Vision Workstation (SMV, Buc, France). Perfusion defects were calculated using a scintigraphic bull’s eye technique. Homogeneous uptake of the radiopharmaceutical throughout the myocardium is considered normal and regarded as 100% uptake. A localized myocardial area with radiotracer uptake less than 80% of normal was regarded as an area of defective perfusion.

2.5. In situ hybridization

The efficacy of intracoronary delivery of MSCs into the myocardium was assessed by in situ hybridization using porcine Y chromosome specific gene (SRY). Genomic porcine DNA was extracted with the DNeasy tissue kit (Qiagen). The primers for expanding SRY sequences by PCR were F-CTTGAGAAATGGGTAGTGGTGTCGGG and 970 R-GTAGCTCTGTGCTCCTCGAAGAA. PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide (10 ng/mL). The corresponding DNA band was eluted with the Qiaquick gel extraction kit (Qiagen). PCR product was DIG-labeled with the PCR DIG probe synthesis kit (Roche). The labeled probe was purified by ethanol precipitation according to the manufacturer’s protocol.

For in situ hybridization [14], sections were deparaffinized in xylene and rehydrated in PBS. After incubation with PBS containing 0.3% Triton X-100, slides were incubated with TE buffer containing 2 μg/mL protease K for 15 min at 37 °C and rinsed again three times for 5 min. In order to reduce non-specific background, slides were acetylated with TEA buffer containing 0.25% (v/v) acetic anhydride twice for 5 min. To reduce hybridization background, slides were hybridized with hybridization buffer [50% formamide in 5× SSC, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 2% blocking reagent (Roche)] for 3 h at 85 °C, slides were incubated with fresh hybridization buffer containing the denatured DIG-labeled DNA probe (10–200 ng/mL) for an additional 5 min at 96 °C. Then slides were transferred to ice for 5 min and incubated overnight at 40 °C. Pre-hybridization and hybridization steps were performed in a moist chamber containing 50% formamide. After hybridization, slides were briefly rinsed in 2× SSC at room temperature and three times in 0.5× SSC for 30 min at 42 °C. Visualization of DIG-labeled DNA probe was performed according to the protocol of the DIG nucleic acid detection kit (Roche). The slides were blocked for 30 min with blocking buffer (1% blocking reagent (Roche) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5)) and then incubated with alkaline phosphate-conjugated antibody solution (anti-sheep, 1:2000 in blocking buffer containing 0.1% Triton X-100) for 2 h. Following four washes with maleic acid buffer for 15 min, slides were equilibrated for 5 min in Tris buffer pH 9.5. The color development was carried out with freshly prepared substrate solution (nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X phosphate) (Roche) in Tris buffer pH 9.5). After 10 min, enzymatic reaction was terminated with...
stop buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Slides were washed three times for 15 min and counterstained by 0.1% nuclear Fast Red (Aldrich Chemical Corp.) for 2 min. Afterwards, slides were rinsed three times again with PBS.

2.6. Immunohistochemistry and histologic analysis

For immunohistochemistry, the pigs were sacrificed at 14 days of follow-up, and the segments were embedded in paraffin block. Serial paraffin sections were deparaffinized, dehydrated, and for antigen retrieval, pressure-cooked for 4 min in citrate buffer (10 mM, pH 6.0), followed by blocking of endogenous peroxidase (1% H2O2/methanol) and preincubation with 4% skim milk. Primary antibodies against CD117 (1:50, Santa Cruz), CD71 (1:50, Santa Cruz), CD90 (1:50, Santa Cruz), and vimentin (1:50, Santa Cruz) were used. The paraffin-sectioned hearts were stained with Hematoxylin and Eosin (H&E) and Masson’s Trichrome to assess the extent of fibrosis using Visual Image Analysis System 2000. The value was expressed as the ratio of Masson’s Trichrome stained area to infarct area.

2.7. Western blot

MSCs were serum-starved for 24 h and treated with H2O2 (1 mM, Sigma) for 30 min or 3 h. Cells were washed with ice-cold PBS, resuspended in lysis buffer (20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 μg/mL leupeptin, 1 mM Na3VO4), and sonicated briefly. After centrifugation, the supernatant was prepared as

---

**Fig. 1.** The characteristics of MSCs. (A) MSCs from pig bone marrow were labeled with antibodies against the indicated antigens and analyzed by flow cytometry. The MSCs were positive for CD29, CD44, and CD90, but negative for CD14, CD31, and CD45. (B) Photographs showing GFP fluorescence only in MSCs transduced with Ad.GFP,β-gal or Ad.myr-Akt-HA. Western blot analysis revealed that expression of HA-tagged myr-Akt was detected only in MSCs transduced with Ad-Akt.
protein extract, and protein concentrations were measured with a BCA protein assay reagent (Pierce). Whole cell extracts were fractionated by electrophoresis on 4–12% gradient gel (Invitrogen) and transferred onto a PVDF (Millipore). Nonspecific binding was blocked by soaking the PVDF in TTBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk (Difco). Primary antibodies against phosphorylated Akt (1:1000, Cell Signaling), Akt (1:1000, Santa Cruz), phosphorylated ERK (1:1000, Santa Cruz), ERK (1:1000, Santa Cruz), VEGF (1:1000, Santa Cruz), and β-actin (1:1000, Sigma) were used. The protein levels were determined using Western Breeze reagents (Santa Cruz) and Image Reader (LAS-3000 Imaging System, Fuji Photo Film).

2.8. Measurement of intracellular reactive oxygen species (ROS) level

DCFH-DA (Sigma) is a non-polar compound which enters the cell and is cleaved to form DCFH. Trapped

Fig. 2. The electrocardiogram and histopathologic findings. (A) The electrocardiogram was performed before (upper) and after (lower) intracoronary MSC delivery with the induction of experimental MI. (B) H&E staining showing no evidence of microinfarct pathology in the pig myocardium 10 days after MSC implantation.
DCFH is oxidized by oxygen free radicals to produce fluorescent DCF. MSCs were preincubated in serum-free DMEM for 24 h, treated with H$_2$O$_2$ for 30 min, and preloaded with 10 μM DCFH-DA for 30 min at 37 °C. Fluorescence intensity was analyzed by fluorescence reader (Fluoroscan Ascent FL, Labsystems, Finland) using 485 nm excitation and 538 nm emission filter.

2.9. Measurement of cell death induced by H$_2$O$_2$

Nuclear morphology for apoptosis was assessed by DAPI, the fluorescent dye 4',6-diamidine-2'-phenylindole (Roche Molecular Chemicals), which intercalates specifically into adenine–thymidine base pairs of DNA. Fixed cells were immersed in 0.1 μg/mL DAPI in PBS for 20 min at room temperature and then examined with a microscope equipped with a Nikon digital still camera and DXM1200 software (Nikon Corp, Melville, NY). DAPI in aqueous solution has an absorbance maximum at 340 nm and an emission maximum at 448 nm.

The viability of MSCs was determined by MTT (3-(4,5-dimethylthiazol-2-yl)tetrazolium bromide) (Sigma) assay. MSCs were cultured in a 96-well microplate. After incubation with H$_2$O$_2$ for 1 h, the media were replaced with MTT solution (5 mg/mL in PBS). Incubation was further continued for 4 h, and then the supernatant was removed by aspiration. Dimethyl sulfoxide (DMSO, Sigma) was added and absorbance was read at 570 nm on microplate reader (Molecular Devices), and the percentage of cell viability was obtained.

Fig. 3. Representative photographs of H&E staining (A, C, E) and in situ hybridization (B, D, and F) using porcine Y chromosome specific gene, SRY. SRY-reactive cardiomyocytes were observed in the MSC treated group (D, F) but not in the control group (B). Panels E and F are higher (200×) magnifications of panels C and D, respectively.
2.10. Statistical analysis

Statistical analysis was performed with SPSS for Windows (version 12.0). All data were described as mean±standard deviation (S.D.). To analyze the data statistically, we performed Student’s *t*-test and one way ANOVA with post hoc analysis and considered differences significant at *p* < 0.05.

3. Results

3.1. Transduction of MSCs with adenoviral vector expressing Akt

Initial characterization of MSCs demonstrates that the cultured MSCs expressed MSC-related antigens such as CD29 (β1-integrin), CD44, and CD90 (Thy-1) (Fig. 1A). They expressed neither hematopoietic lineage markers, such as CD14 and CD45, nor endothelial markers such as CD31. MSC cultures were infected with vehicle, Ad.GFP,β-gal, and Ad.myr-Akt-HA at 10 MOI. Twenty four hours later the viral transduction efficacy was 80–90%, and the Western blot confirmed the expression of the HA-tagged myr-Akt construct in Akt-MSCs (Fig. 1B).

3.2. Safety and efficacy of intracoronary delivery

To determine the safety of intracoronary delivery of MSCs and the patency of LAD, we performed a coronary angiogram immediately after MSC delivery into the LAD. There was no significant narrowing after delivery. However, there was slow coronary flow after MSC delivery in three cases, which was relieved by administration of intracoronary nitroglycerin, nicorandil, or adenosine. During the

![Fig. 4](https://example.com/fig4.png)

Fig. 4. Left ventricular ejection fraction and myocardial infarction area determined by gated M-SPECT. Representative M-SPECT images for each group before and after MSC delivery.
procedure, no ST-segment elevation was observed in 12-lead electrocardiograms (Fig. 2A). Furthermore, histological analysis revealed no evidence of microinfarct in the myocardium 10 days after MSC delivery (Fig. 2B). In situ hybridization using porcine Y chromosome specific gene, SRY, showed positive signals in the MSC transplanted groups. The signals were mostly limited to the peri-infarct area (34.3%) (Fig. 3).

3.3. Evaluation of LV function

Fig. 4 contains representative M-SPECT images taken before and after MSC delivery to determine LV EF and the area of infarction. Initial baseline M-SPECT findings demonstrate no significant differences among the three groups (Table 1). However, 4 weeks after MSC implantation, follow-up SPECT showed the mean LV EF changed to 36.0 ± 5.4%, 40.8 ± 6.8%, and 49.3 ± 6.6% in Groups I, II, and III, respectively (each n = 12) (Table 2). In addition, the mean MI area changed to 35.7 ± 9.3%, 23.6 ± 8.0%, and 13.3 ± 3.4% in Groups I, II, and III, respectively (each n = 12). Transplantation of MSCs into the ischemic porcine myocardium in Group II increased the ΔLV EF (−6.3 ± 15.1% versus 0.5 ± 6.4%, P < 0.001) and decreased the Δarea of MI (6.8 ± 5.6% versus −5.0 ± 5.3%, P < 0.001) compared with Group I. These changes were significantly more enhanced in Group III with ΔLV EF (−6.3 ± 15.1% versus 5.8 ± 11.3%, P < 0.001) and in Δarea of MI (6.8 ± 5.6% versus −17.0 ± 7.6%, P < 0.001) (Table 3).

3.4. Immunohistochemistry and histologic analysis

Immunohistochemistry revealed that vimentin-positive cells were not observed in Group I, whereas vimentin-positive cells were observed in Group III (Fig. 5A). Also, staining with α-sarcomeric actin in the MSC groups was more intense than in the control group (data not shown). Also, CD117, CD71, and CD90 were only demonstrated in the MSC group. Masson’s Trichrome stained sections displayed significant intense blue staining indicative of collagen deposition characteristic of fibrosis in Group I. However, a significantly decreased area of Masson’s Trichrome staining was observed in Group III compared with Group I (12.8 ± 9.5% vs. 32.9 ± 6.4%, n = 10, P < 0.05) (Fig. 5B).

3.5. In vitro assay in H2O2-treated Akt-MSCs

To determine the mechanism of enhanced cardiac function in Akt-MSC transplanted heart compared to MSC transplanted heart, we determined the effects of the apoptotic stimulus, H2O2, on MSCs transduced with Akt in vitro. The fragmented or condensed chromatin was reduced in Akt-MSCs compared with the control MSCs or β-gal-MSCs after stimulation with 0.5 mM of H2O2 for 5 h (Fig. 6A). In fact, we found that Akt-MSCs were more resistant to H2O2 at different concentrations than control MSCs or β-gal-MSCs (n = 10, P < 0.05, Fig. 6B). The intracellular level of ROS was elevated after H2O2 in control MSCs (144 ± 10% versus vehicle-treated MSCs, n = 6, P < 0.05) and β-gal-MSCs (139 ± 12% versus vehicle-treated MSCs, n = 6, P < 0.05), whereas significantly reduced in Akt-MSCs (119 ± 9.7% versus H2O2-treated β-gal-MSCs, n = 6, P < 0.05) (Fig. 6C). Western blot analysis showed that Akt was phosphorylated in 30 min after H2O2 induced in Akt-MSCs (119 ± 9.7% vs. 32.9 ± 6.4%, n = 10, P < 0.05). In contrast, phosphorylated Akt decreased to near basal level in β-gal-MSCs, but remained significantly elevated after 3 h in Akt-MSCs.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Group I (n=12)</th>
<th>Group II (n=12)</th>
<th>Group III (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction (%)</td>
<td>36.5 ± 5.4</td>
<td>40.8 ± 6.4</td>
<td>49.3 ± 6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>End diastolic volume (mL)</td>
<td>66.5 ± 8.3</td>
<td>71.6 ± 3.7</td>
<td>50.9 ± 12.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>End systolic volume (mL)</td>
<td>42.7 ± 7.2</td>
<td>42.4 ± 6.7</td>
<td>25.4 ± 4.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MI extent (%)</td>
<td>35.7 ± 9.3</td>
<td>23.6 ± 8.0</td>
<td>13.3 ± 3.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total area (cm²)</td>
<td>108.4 ± 10.1</td>
<td>97.8 ± 9.2</td>
<td>99.6 ± 15.8</td>
<td>0.084</td>
</tr>
<tr>
<td>Infarcted area (cm²)</td>
<td>38.9 ± 12.1</td>
<td>23.3 ± 8.5</td>
<td>13.2 ± 5.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Viable myocardium (cm²)</td>
<td>69.5 ± 11.0</td>
<td>74.4 ± 8.8</td>
<td>86.4 ± 14.9</td>
<td>0.004</td>
</tr>
</tbody>
</table>

MSCs, mesenchymal stem cells; MI, myocardial infarction.

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Group I (n=12)</th>
<th>Group II (n=12)</th>
<th>Group III (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔEjection fraction (%)</td>
<td>−6.3 ± 15.1</td>
<td>0.5 ± 6.4</td>
<td>5.8 ± 11.3*</td>
<td>0.049</td>
</tr>
<tr>
<td>ΔEnd diastolic volume (mL)</td>
<td>−0.8 ± 19.2</td>
<td>1.3 ± 6.7</td>
<td>−19.3 ± 20.1***</td>
<td>0.008</td>
</tr>
<tr>
<td>ΔEnd systolic volume (mL)</td>
<td>3.8 ± 15.1</td>
<td>0.3 ± 6.0</td>
<td>−14.4 ± 16.3***</td>
<td>0.005</td>
</tr>
<tr>
<td>ΔArea of MI (%)</td>
<td>6.8 ± 5.6</td>
<td>−5.0 ± 5.3*</td>
<td>−17.0 ± 7.6***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ΔViable myocardium (cm²)</td>
<td>3.3 ± 9.4</td>
<td>6.9 ± 6.6</td>
<td>26.9 ± 10.7***</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

MSCs, mesenchymal stem cells; MI, myocardial infarction.

* P < 0.05 vs. group I.
** P < 0.05 vs. group II by post hoc analysis.
Fig. 5. Histological findings after MSCs delivery. (A) Vimentin was negative in Group I but vimentin-positive cells were observed in Group III. CD117, CD71, and CD90 stainings were negative in Group I, but positive in Group III. (B) Masson’s Trichrome staining in Group I displayed significantly increased fibrosis than in Group III (top panel). The images were photographed at 200× magnification. Quantitative analysis of Masson’s Trichrome stained myocardial area showed significantly decreased fibrosis in Group III compared to Group I (bottom panel). *Indicated significant difference compared with vehicle-transduced MSCs ($p < 0.05$).
(2.2±1.4 fold vs. 5.2±1.5 fold, respectively). In addition, ERK was activated in 30 min (2.7±0.4 fold of control) and remained activated at 3 h (3.8±0.5 fold of control) in Ad-Akt-MSCs (Fig. 6D). VEGF level was not changed in β-gal-MSCs, but there was a significant increase in Akt-MSCs at 3 h (1.7±0.2 fold of control) (Fig. 6D).

4. Discussion

In this study, we validated the efficacy and safety of MSC transplantation after myocardial infarction in a porcine model. We characterized the porcine bone marrow-derived MSCs by immunophenotyping and confirmed that a population of MSCs can be obtained from a MSC fraction of porcine bone marrow by serial passage of adherent cells, as described by other investigators [15,16]. We found significant improvement with regard to infarct region, cardiac geometry (LV end systolic volume), hemodynamics and contractility (LV EF) with MSC transplantation. In addition, we demonstrated that the genetically modified MSC with Akt was more resistant to apoptosis, resulting in the augmentation effect of MSC in a large animal model. Other investigators have found that cultured MSCs could be induced to differentiate into myogenic cells, neurons, skin cells, etc. [17]. Adding 5-azacytidine, a DNA methylation inhibitor, into culture medium can facilitate the differentiation of MSCs into myogenic cells, which may change the superficial type of the MSCs [18]. Wang et al. [19] reported that murine MSCs were related to the formation of new cardiomyocytes in the normal, uninjured heart in vivo. In addition to healthy animal models, MSCs have also been injected into the myocardium of experimental models of cardiac damage. The animals injected with stem cells had a decreased left ventricular cavity size and improved cardiac function compared to the controls [20–23]. Therefore, autologous MSC transplantation represents a potentially effective cellular therapy for MI without the ethical problems seen in embryonic stem cell therapy.

The intracoronary approach represents a novel and effective therapeutic procedure for the repair of infarcted myocardium. We found that intracoronary delivery of MSCs was an efficient method, and may be more efficient than intravenous delivery or intraventricular injection. Intravenous delivery results in only a small fraction of infused cells reaching the infarct region after injection, and requires several circulation passages for infused cells to make contact with the infarct-related artery, potentially reducing...
the homing of cells. Thus, supplying cells by intracoronary administration is likely to be more advantageous for the repair of infarcted myocardium. It may also be superior to intraventricular injection [21], because all cells are able to flow through the infarcted and peri-infarcted tissue during the first passage. Accordingly, infarcted tissue and the peri-infarct zone can be enriched with the maximum available number of cells at all times by the intracoronary procedure.

To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area using an over-the-wire balloon, and the balloon was kept inflated for 2 to 3 min; the cells were not washed away immediately under these conditions. However, a previous report by Vulliet et al. showed that intracoronary injection of MSCs into healthy dogs resulted in myocardial microinfarction after 7 days [24]. However, in our study, we did not find any evidence of microinfarction in either electrocardiographic or pathological studies in healthy pigs 10 days after MSC injection (Fig. 2A and B).

We chose 3 days after experimental MI as the time point for MSC delivery. In animal studies, inflammation is strongest in the first days after infarction. In the first 48 h, debridement and fibrin-based matrix formation predominate before a healing phase starts [25,26]. Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) levels decline about 3 to 4 days after MI [27,28]. We assumed that transplantation of MSCs within 1–2 days after MI might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels. Taking all of this into account, we performed MSC transplantation 3 days after experimental MI. We can conclude that cell transplantation within 3–4 days after infarction shows a significant benefit of cell transplantation.

Akt-overexpressing MSC implantation significantly preserved cardiac function in an ischemic–reperfusion model (Tables 1–3). To verify this protective mechanism in vitro, we evaluated the effects of Akt-MSCs on exogenous oxidative stress-stimulated apoptosis. ROS was produced during ischemia/reperfusion [29], and we treated MSCs with H₂O₂ to induce the oxidative damage that occurs in myocardium after ischemic injury. Both apoptosis and the elevation of intracellular ROS levels induced by H₂O₂ were reduced in Akt-MSCs (Fig. 5A–C). In Western blot analysis, Akt and ERK were found to remain in phosphorlated form in Akt-MSCs longer than in β-gal-MSCs, and VEGF was induced in Akt-MSCs at 3 h after H₂O₂ stimulation. ERK is a well known kinase that activates and promotes cell proliferation by stimulating growth factors. In endothelial cells, VEGF promotes angiogenesis through the phosphorylation of ERK and Akt in rabbit ischemic limb [30] and mouse ischemic hind limb [31]. Therefore, our in vitro results suggest that adenoviral transfer of Akt may contribute to the protection of ischemic myocardium, possibly through enhanced expression of activated ERK and VEGF, as well as by decreasing ROS.

The mechanism of the improvement of cardiac function by MSC implantation is unclear. Tang et al. showed that the para-secretion of cytokines and the differentiation of transplanted MSCs to endothelial cells might contribute to the improvement of cardiac functions [32]. In a canine ischemia model, MSCs differentiated into endothelial and smooth muscle cells but not into myocytes, resulting in increased vascularity and improved cardiac function [33]. Coculturing
rat bone marrow MSCs with cardiomyocytes is obligatory in the differentiation of MSCs into cardiomyocytes or SMCs [34]. Furthermore, a recent study suggests that the paracrine mechanism may play a role distinct from myocardial regeneration. Gnecchi et al. [35] demonstrated that hypoxic cardiomyocytes were protected by paracrine factors released from Akt-modified MSCs. The paracrine factors secreted from Akt-modified MSCs dramatically limited infarct size and apoptosis in myocardial infarcted rats. Characterization of these released factors may have an important implication in elucidating the repair mechanism of stem cells.

Further studies, nevertheless, are needed to address some limitations of our study. First, we showed the resistance of Akt-MSCs to apoptotic cell death induced by oxidative stress in vitro; however, in vivo study is needed to confirm that the mechanism of the enhanced benefits of the Akt-MSC is their increased viability after transplantation. Second, we did not check the occurrence of differentiation of grafted MSCs either into endothelial cells or into cardiomyocytes. Therefore, there is a possibility that the promotion of endothelial differentiation or angiogenesis may play a significant role in improved cardiac function after MSC transplantation. Third, although intracoronary delivery will most likely result in a relatively localized delivery of MSCs into the heart, further study to determine the presence of transplanted MSCs in other tissues is needed. Fourth, the safety of introducing Akt has been cautioned due to the tumorigenic potential of this pathway [36]. Since this study was short term, this issue was not addressed. However, it requires careful attention in long-term studies. Lastly, further experimental studies and controlled clinical trials with variations of cell preparations are needed to validate these findings to be useful in clinical therapy. Nevertheless, we believe that this study further supports the role of MSCs in cellular therapy after MI, and suggest that genetic modification to increase MSC survival may enhance the effectiveness of the therapy.

Acknowledgements

This study was supported by the Korea Research Foundation 2004.

References


[34] Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol in press.
