Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Angiopoietin-1 preconditioning enhances survival and functional recovery of mesenchymal stem cell transplantation*

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Received June 15, 2012; Revision accepted June 26, 2012; Crosschecked July 5, 2012

Abstract: Objective: Mesenchymal stem cell (MSC) transplantation is a promising therapy for ischemic heart diseases. However, poor cell survival after transplantation greatly limits the therapeutic efficacy of MSCs. The purpose of this study was to investigate the protective effect of angiopoietin-1 (Ang1) preconditioning on MSC survival and subsequent heart function improvement after transplantation. Methods: MSCs were cultured with or without 50 ng/ml Ang1 in complete medium for 24 h prior to experiments on cell survival and transplantation. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst staining were applied to evaluate MSC survival after serum deprivation in vitro, while cell survival in vivo was detected by terminal deoxynucleotidyl transferase biotin-dUPT nick end labeling (TUNEL) assay 24 and 72 h after transplantation. Heart function and infarct size were measured four weeks later by small animal echocardiography and Masson's trichrome staining, respectively. Results: Ang1 preconditioning induced Akt phosphorylation and increased expression of Bcl-2 and the ratio of Bcl-2/Bax. In comparison with non-preconditioned MSCs, Ang1-preconditioned cell survival was significantly increased while the apoptotic rate decreased in vitro. However, the PI3K/Akt pathway inhibitor, LY294002, abrogated the protective effect of Ang1 preconditioning. After transplantation, the Ang1-preconditioned-MSC group showed a lower death rate, smaller infarct size, and better heart functional recovery compared to the non-preconditioned-MSC group. Conclusions: Ang1 preconditioning enhances MSC survival, contributing to further improvement of heart function.

Key words: Mesenchymal stem cells, Angiopoietin-1, Preconditioning, Survival, Myocardial infarction
doi:10.1631/jzus.B1201004 **Document code:** A **CLC number:** R541.4

1 Introduction

Mesenchymal stem cell (MSC) transplantation is a promising therapy in regenerative medicine, including the treatment of ischemic heart diseases, due to the multi-lineage trans-differentiation and the capacity of stem cells for continual self-renewal (Williams and Hare, 2011). A large number of basic and clinical studies have shown the efficacy and safety of MSC treatment in myocardial infarction (Tomita *et al*., 1999; Wollert *et al*., 2004; Williams and Hare, 2011). However, only mild to moderate improvement of heart function was observed in the majority of the studies, and even no improvement in a few clinical trials (Meyer *et al*., 2006). Poor cell viability after engraftment into the infarcted myocardium was the main problem (Toma *et al*., 2002; Tang *et al*., 2005). Thus, strategies to improve MSC

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* Project supported by the Natural Science Foundation of Zhejiang Province (No. Y2100362) and the Qianjiang Talents Project of Science and Technology Department of Zhejiang Province (No. 2011R10022), China

survival need to be urgently developed.

Angiopoietin-1 (Ang1) was first identified as a ligand for the Tie2 receptor, which may be involved in endothelial developmental processes (Davis *et al*., 1996). Ang1 is capable of activating Tie2, resulting in receptor autophosphorylation upon binding, which mediates a variety of effects such as inhibition of cell permeability and inflammation, activation of endothelial cell migration, vessel growth, and angiogenesis (Augustin *et al*., 2009; Saharinen *et al*., 2010). In recent years, protective effects of Ang1/Tie2 signaling on cell apoptosis and death have been reported for many cells including endothelial cells, neurons, and cardiomyocytes (Kwak *et al*., 1999; Valable *et al*., 2003; Zeng *et al*., 2012). In accordance with these results, our previous study showed that the Ang1/Tie2 pathway plays an important role in the protection of MSCs against serum deprivation and hypoxia induced apoptosis (Liu *et al*., 2008). However, whether Ang1 can protect MSCs against apoptosis and death or increase the survival of transplanted MSCs in the infarcted myocardium needs to be further investigated.

In the present study, we applied a preconditioning protocol to treat MSCs with Ang1 prior to transplantation into the ischemic myocardium. We then observed the survival rate of engrafted MSCs and the protective effect of Ang1-preconditioned MSCs on heart function in a rat acute myocardial infarction model.

2 Materials and methods

2.1 MSC isolation and culture

Bone marrow MSCs were isolated and harvested from Sprague-Dawley (SD) rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, China). In brief, rats were sacrificed according to the methods of the Animal Care and Utilization Committee of Zhejiang University, Hangzhou, China. Bone marrow samples were then collected by flushing the cavities of tibias and femurs with Dulbecco's modified Eagle's medium (DMEM) (Jinuo, Hangzhou, China). Total bone marrow cells were seeded into 50 cm^2 flasks with DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), and then cultured in a humidified chamber with 5% (v/v) CO_2 at 37 °C. Twenty-four hours later, floating cells were discarded and adherent cells were washed with phosphate buffered saline (PBS) solution three times before addition of fresh complete medium. The culture medium was changed every 3–4 d and cells were subcultured 1:2 or 1:3 at about 80% confluence. All MSCs were cultured for no more than five passages to ensure the accuracy of the study.

To identify the characteristics of the MSCs, fluorescence-activating cell sorting analysis was applied, and $CD90^+/CD34^-/CD45^-$ cells were determined as MSCs.

2.2 Experimental protocol

Our previous study indicated that Ang1 (R&D, Minneapolis, USA) at a concentration of 50 ng/ml exerted the best protective effects on MSCs death (Liu *et al*., 2008). Therefore, we chose the same concentration for this study.

For the in vitro study, MSCs were passaged 1:2 or 1:3 and cultured to 70%–80% confluence before the preconditioning protocol was applied. For preconditioning, MSC cultures were divided into two groups: the Ang1-preconditioned group (AP-MSC) was cultured in fresh complete medium containing Ang1, and the non-preconditioned group (N-MSC) was cultured in fresh complete medium without Ang1. Both groups were cultured for 24 h before subsequent assessments, such as cell survival and Western blot analyses, were carried out. For the in vivo study, cultures were divided into four groups as follows: (1) sham-operated control (Sham); (2) myocardial infarction (MI) with injection of medium; (3) MI with transplantation of N-MSC; and (4) MI with transplantation of AP-MSC. MSCs were labeled with DiI (Molecular Probes, Oregon, USA) by addition into the culture medium to a final concentration of 5 μl/ml, for 20 min before transplantation. One million cells in 150 μl DMEM were injected into the peri-infarct myocardium 30 min after the acute MI model, and the same volume of DMEM was used for the MI group.

2.3 Cell survival and apoptosis evaluation

To assess MSC viability in vitro, a cell apoptosis and death model was established by serum deprivation (SD), mimicking the microenvironment of an in vivo ischemic condition. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Missouri, USA) assay was applied to assess

cell survival. A total of 5×10^3 cells were equally seeded into each well on 96-well plates. After 48 h of serum deprivation, MTT was added to the medium at a final concentration of 0.5 mg/ml for 4 h. Medium was then gently removed and dimethyl sulfoxide (DMSO) was used to solubilize the purple formazan. Results were read using an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm.

Hoechst staining was used for detection of cell apoptosis characterized as chromosomal condensation and nuclear fragmentation. Cells were fixed in freshly prepared 4% (v/v) paraformaldehyde for 15 min after the different treatments, followed by exposure to 1 μg/ml Hoechst 33342 (Invitrogen, California, USA) for 10 min in the dark. All samples were then examined under a fluorescence microscope.

2.4 Western blot analysis

Ang1-preconditioned and non-preconditioned MSCs were washed several times with PBS before being collected and lysed with modified RIPA buffer (50 mmol/L HEPES, pH 7.3, 0.01 g/ml sodium deoxycholate, 1% (v/v) Triton X-100, 0.001 g/ml dodecyl sulfate, sodium salt (SDS), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L Na_3VO_4, 1 mmol/L NaF, and protease inhibitor cocktail; Roche, New Jersey, USA). After repeated vortexing until cells were completely lysed, and centrifugation at 14000×*g* for 20 min, supernatants were acquired. Protein concentration was then determined by Bio-Rad detergent compatible (DC) protein assay (Bio-Rad, California, USA). All of the steps above were performed at 4 °C.

Proteins (30–50 μg) were loaded onto a 6%–15% gradient gel, electrophoresed by SDS-polyacylamide gel electrophoresis (PAGE), and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane using a Bio-Rad wet transfer system. The 5% milk in Tris-buffered saline with 0.1% Tween (TBS-T) was applied for blockage of the membrane for 2 h. The membrane was then incubated with specific primary antibodies, including phosphor-Akt, Akt, Bcl-2, and Bax (Cell signaling, Massachusetts, USA) overnight at 4 °C, followed by conjugation with horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used as references. The proteins were detected with an enhanced chemiluminescent kit (Millipore, Massachusetts, USA) and analyzed by Quantity One software (Bio-Rad, California, USA).

2.5 In vivo cell death assessment

MSC death in vivo was examined by terminal deoxynucleotidyl transferase biotin-dUPT nick end labeling (TUNEL) assay. Heart slices were fixed in 10% formaldehyde for 20 min, and then pretreated with 0.2% Triton X-100 for 15 min. The slices were then incubated in an equilibration buffer followed by incubation with terminal deoxynucleotidyl transferase (TdT) enzyme and a nucleotide mix for 75 min, according to the manufacturer's instructions, in a humidified environment at room temperature. Then, the slices were washed with 2× saline sodium citrate (SSC) washing buffer for 15 min and then with PBS. Finally, the sections were examined under a florescent microscope.

2.6 Heart function analysis

Heart function was evaluated by small animal echocardiography. A transthoracic parasternal short axis echocardiogram at mid-papillary muscle level was recorded at 28 d post-MI, using the Vevo 2100 system (VisualSonics, Toronto, Canada) with a 21-MHz transducer. Rats were anaesthetized by intraperitoneal injection of 4% (v/v) chloral hydrate solution at a dose of 300 mg/kg, and then properly positioned and restrained on a 37 °C heating platform. The left ventricular end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD) were measured according to M-mode tracing images. The percentages of fraction shortening (%FS) and ejection fraction (%EF) were calculated by the in-built software package.

2.7 Infarct size measurement

Masson's trichrome staining was used to examine the infarct size/fibrotic area of the hearts. Rats were humanely sacrificed after echocardiography, four weeks after MI. Hearts were harvested quickly and split into three transverse parts: the apex, mid-LV, and base. Then, the three sections were separately embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc., California, USA). Heart tissues cut at 10-μm thickness were stained with Masson's trichrome and photos of each slide were digitized through the National Institutes of

Health (NIH) image analysis system (NIH, Maryland, USA). The percentage of infarct size/fibrotic area was calculated by dividing the sum of epicardial and endocardial circumferences of the infarcted area by the sum of the total endocardial and epicardial circumferences of the LV (Leenen *et al*., 1995).

2.8 Statistical analysis

SPSS 13.0 was used for statistical analysis and the data were expressed as mean±standard error of mean (SEM). Student's two-tailed *t*-test and one-way analysis of variance (ANOVA) followed by the Tukey post hoc test were used for comparison of pairs of independent experimental groups and multiple comparisons, respectively. Statistical significance was defined as $P<0.05$.

3 Results

3.1 Ang1 preconditioning regulated the expression of survival related proteins

To evaluate the effect of Ang1 preconditioning on MSCs, we analyzed the expression of the survival-related proteins Akt, Bcl-2, and Bax in N-MSC and AP-MSC by Western blotting. Similar to our previous study (Liu *et al*., 2008), we found that Ang1 preconditioning induced Akt phosphorylation of MSCs. The expression of the mitochondrial pathway related Bcl-2 was significantly increased while no obvious change in Bax expression was observed. The ratio of Bcl-2/Bax was much higher after the Ang1 preconditioning treatment of MSCs (Fig. 1).

3.2 Ang1 preconditioning promoted MSC survival both in vitro and in vivo

To assess the effect of Ang1 preconditioning on MSCs, MTT and Hoechst staining were applied to determine MSC survival in vitro, while TUNEL was used in vivo. Cell viability was calculated according to the optical density (OD) value, and we found that Ang1 preconditioning remarkably enhanced MSC survival (70.9±2.3)% vs. (49.5±2.1)%; ($P<0.001$) in comparison with N-MSC (Fig. 2a). Similarly, the

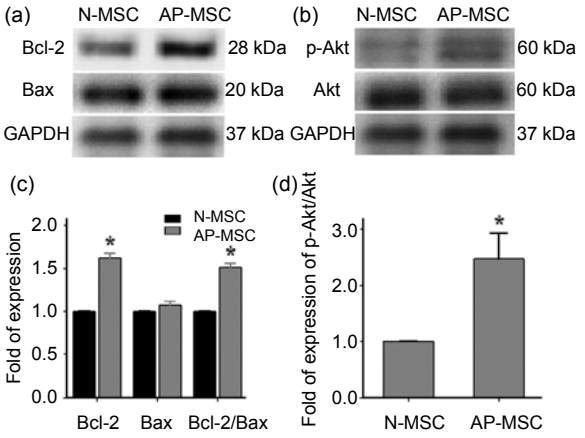

Fig. 1 Effect of Ang1 preconditioning on survival-related proteins

MSCs were cultured with or without Ang1 for 24 h. Then, phospho-Akt (p-Akt), Akt, Bcl-2, and Bax were detected by Western blotting. GAPDH was used as the loading control. Densitometric analysis was applied for comparison of the relative expression levels of different proteins in AP-MSC and N-MSC which was arbitrarily presented as 1 (n=3). $^{*}P<0.05$ compared with the N-MSC group. N-MSC: non-preconditioned mesenchymal stem cells; AP-MSC: Ang1-preconditioned mesenchymal stem cell

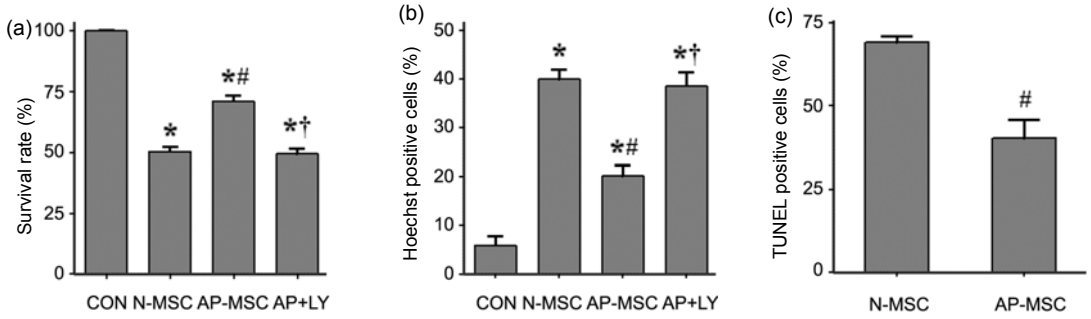

Fig. 2 Effect of Ang1 on MSC survival in vitro and after transplantation

(a) MSCs were cultured with or without Ang1 treatment for 24 h prior to serum deprivation for 48 h, and then MTT was applied to evaluate cell survival in vitro. (b) Hoechst staining was used for detection of apoptosis. The experimental protocol was the same as for MTT. (c) In vivo cell survival was determined by TUNEL assay after MSC engraftment into ischemic heart for 24 h (n=3). $^{*}P<0.05$ compared with the control group. $^{\#}P<0.05$ compared with the N-MSC group. $^{\dagger}P<0.05$ compared with the AP-MSC group. CON: control (without serum deprivation). AP+LY: AP-MSC with LY294002

apoptotic rate induced by serum deprivation was strikingly decreased after Ang1 preconditioning (39.9±1.9)% vs. (20.2±2.1)%; (P<0.001) (Fig. 2b). However, the PI3K/Akt pathway inhibitor, LY294002, abrogated the protective effect, indicating the important role of the PI3K/Akt pathway in the mechanism (Figs. 2a and 2b).

To evaluate whether Ang1 preconditioning might protect MSCs in vivo, N-MSC and AP-MSC were transplanted into the peri-infarcted region 30 min after acute myocardial infarction in rats. Animals were sacrificed 24 h later to identify the MSC death because the majority of cell death after engraftment occurs within 24 h. Cell death was identified by the ratio of TUNEL/Hoechst/DiI co-labeled cells versus Hoechst/DiI positive cells. In the N-MSC group, (69.0±1.9)% of cells were found to be dead. Compared with the N-MSC group, cell death was remarkably suppressed in the AP-MSC group in which the proportion of dead cells was (40.2±5.6)% (P=0.008; Fig. 2c).

3.3 MSC transplantation decreased infarct size

Masson's trichrome staining was used to detect fibrosis and scar formation four weeks after rat myocardial infarction. An obvious scar formation was observed in the MI group that received medium injection without stem cell therapy. In comparison with the MI group, infarct size was significantly decreased from (57.1±3.5)% to (44.4±2.2)% and (29.6±4.2)%, in the N-MSC and AP-MSC implantation groups, respectively. Thus, the AP-MSC group showed the smallest infarct size, indicating a better therapeutic approach (Fig. 3).

3.4 AP-MSC improved cardiac function recovery

Cardiac function recovery after MSC transplantation was examined by small animal echocardiography and the LVEDD, LVESD, fractional shortening (LVFS), and ejection fraction (LVEF) were analyzed four weeks later. Significant differences for all four parameters were observed between the Sham group and the MI group, indicating that the animal MI model was successfully established. Compared to the MI group, N-MSC implantation enhanced LVFS ((19.7±1.6)% vs. (12.7±1.6)%; P=0.003) and LVEF ((38.6±2.9)% vs. (25.8±2.9)%; P=0.001) recoveries while no obvious decrease in LVEDD (P=0.434) and LVESD (P=0.086) was found. There was a significant reduction in LVESD ((6.5±0.3) mm vs. (8.3±0.3) mm; P=0.003) but not in LVEDD (P=0.206) in the AP-MSC group compared to the MI group. In comparison with the N-MSC group, Ang1-preconditioned MSCs further improved heart function as shown by the increased LVFS ((26.2±1.3)% vs. (19.7±1.6)%; P=0.004) and LVEF ((49.3±2.0)% vs. (38.6±2.9)%; P=0.004) (Fig. 4).

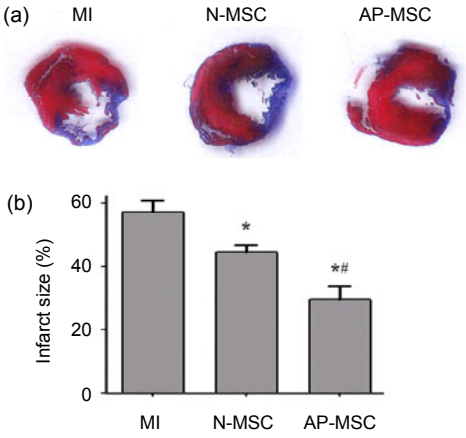

Fig. 3 Effect of MSC transplantation on infarct size
(a) Masson's trichrome staining of MI, N-MSC, and AP-MSC groups. The blue area was defined as the infarcted region and scar formation, while red was defined as normal heart tissue. (b) Quantitative analysis of infarct size in the three groups (n=4). * P<0.05 compared with the MI group; $^{\#}$ P<0.05 compared with the N-MSC group. MI: myocardial infarction without stem cell therapy

4 Discussion

In this study, we demonstrated for the first time that Ang1 preconditioning improves MSC survival both in vitro and in vivo, and eventually further enhances the therapeutic efficacy of MSCs on cardiac functional recovery. Survival-related proteins such as phospho-Akt and Bcl-2 were obviously increased after Ang1 preconditioning, while the PI3K/Akt inhibitor, LY294002, abrogated the protective effect of Ang1 preconditioning, indicating that the PI3K/Akt pathway plays an important role in the mechanism. In this study, we provided new evidence that Ang1 preconditioning enhances MSC survival and subsequent cardiac function recovery after MSC transplantation in the rat myocardial infarction model.

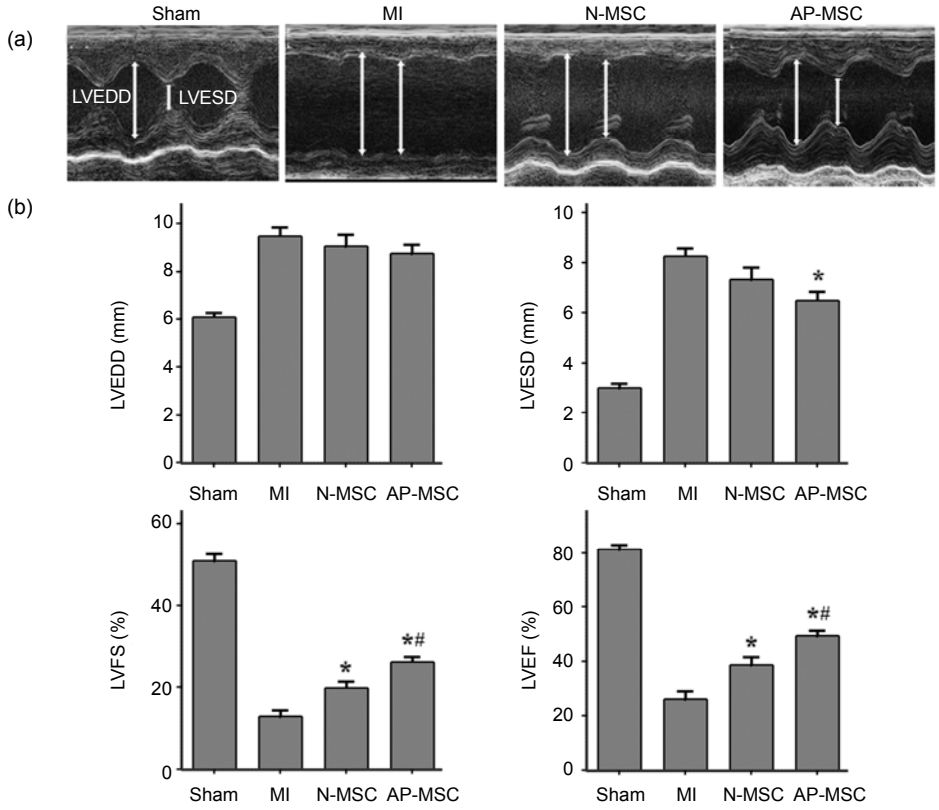

Fig. 4 Effect of MSC transplantation on heart function

(a) M-mode tracing images from Sham, MI, N-MSC, and AP-MSC groups. (b) Analysis of echocardiographic parameters (LVEDD, LVESD, LVFS, LVEF) according to M-mode images (n=8). $^{*}P$<0.01 compared with the MI-only group. $^{\#}P$<0.01 compared with the N-MSC group. LVEDD: left ventricular end diastolic diameter; LVESD: left ventricular end systolic diameter; LVFS: left ventricular fractional shortening; LVEF: left ventricular ejection fraction

After transplantation into the ischemic myocardium, MSCs are able to differentiate into cardiac-like myocytes, endothelial cells, and vascular smooth muscle cells and may have the potential to regenerate the infarcted myocardium (Orlic *et al*., 2001). However, recent studies have shown only modest or even no cardiac function recovery following MSC transplantation (Meyer *et al*., 2006). Besides the timing and route of transplantation, acute-phase cell death was found to be the major factor restricting the protective effect of MSCs (Tang *et al*., 2005). Strategies of gene modification and preconditioning of MSCs are popular and effective manipulations for promoting MSC survival and functional recovery. It is not yet clear whether or not permanent gene modification, which induces long-term gene and protein overexpression, will result in tumorigenesis and other side effects which might limit MSC clinical applications. In contrast, preconditioning induces short but comprehensive enhancements of the transplanted cell quality and probably meets the requirements. Pharmacological preconditioning of MSCs is a novel approach for enhancing cell survival and functional recovery (Haider and Ashraf, 2008). In this study, we focused on the protective effects of pharmacological preconditioning of Ang1 on MSCs and our results confirmed the value of this approach.

Ang1/Tie2 signaling activation stimulates a number of intracellular signaling pathways, notably the PI3K/Akt pathway (Kontos *et al*., 1998). It is well known that the PI3K/Akt pathway plays a pivotal role in the regulation of cell survival/apoptosis, differentiation, and proliferation (Fayard *et al*., 2005). MSCs modified with Akt protect cells against serum deprivation induced apoptosis and prevent remodeling, and restore performance of infarcted hearts (Mangi *et al*., 2003). Our previous study showed that Ang1 activated Akt and decreased MSC apoptosis, while the PI3K/Akt inhibitor wortmannin abrogated the beneficial effect, indicating that PI3K/Akt was very

important in the protective effect of Ang1 on MSCs (Liu *et al*., 2008). This was consistent with observations on endothelial cells and neurons (Kim *et al*., 2000; Valable *et al*., 2003). In accordance with these results, we found that Ang1 preconditioning induced Akt phosphorylation and reduced MSC apoptosis and death while blockage of the PI3K/Akt pathway abolished the beneficial effects.

It has been reported that the intrinsic mitochondrial apoptotic pathway but not the extrinsic death receptor pathway is involved in serum deprivation and hypoxia induced apoptosis of MSCs (Zhu *et al*., 2006). The balance between anti- and pro-apoptotic molecules is crucial to the progress of cell apoptosis and the ratio of Bcl-2/Bax is an important indicator of cell susceptibility to apoptosis (Oltvai *et al*., 1993). Based on this understanding, we examined the effect of Ang1 preconditioning on mitochondria-associated Bcl-2 and Bax expression. Ang1 preconditioning significantly elevated the Bcl-2 expression level and the ratio of Bcl-2/Bax, which was consistent with our previous study (Liu *et al*., 2008) and the results of other groups studying microvascular endothelial cells (Shi *et al*., 2006). The impact of Ang1 on the PI3K/Akt and mitochondrial pathways was confirmed in endothelial cells, but Ang1 had no effect on Bcl-2 family proteins, whereas regulation of Survivin and Smac by Ang1 has been reported (Harfouche *et al*., 2002). This controversy indicates that the influence of Ang1 possibly includes Bcl-2 dependent and independent pathways according to different cell types. In our study, a Bcl-2-dependent manner was confirmed in MSCs.

In conclusion, we demonstrated that Ang1-preconditioned MSCs improved heart function concurrent with enhancing cell survival. PI3K/Akt pathway activation by Ang1 preconditioning may be involved in the mechanism. This study provides a new promising approach for enhancing MSC survival both in vitro and in vivo, and may lead to further improvements in the therapeutic efficacy of MSCs for ischemic heart diseases.

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