Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
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Protective paracrine effect of mesenchymal stem cells on cardiomyocytes*

Mei-xiang XIANG, Ai-na HE, Jian-an WANG†‡, Chun GUI

(*Department of Cardiology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

†E-mail: wang_jian_an@tom.com

Received May 25, 2009; Revision accepted July 3, 2009; Crosschecked July 3, 2009

Abstract: Objective: The aim of this study was to test the protective effect of mesenchymal stem cells (MSCs) on cardiomyocytes in vitro and to investigate the anti-apoptotic signaling pathway. Methods: MSCs from Sprague-Dawley (SD) rats were separated and cultured. MSC medium was collected from MSCs cultured in serum-free Dulbecco's modified eagle medium (DMEM) under hypoxia. Cultured cardiomyocytes from neonatal SD rats were exposed to hypoxia/reoxygenation (H/R) and treated with MSC medium. The apoptotic cardiomyocytes were stained with Annexin-V-fluorescein isothiocyanate (FITC), Hoechst 33342 and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). The mitochondrial transmembrane potential of cardiomyocytes was assessed using a fluorescence microscope. The expression of Bcl-2, Bax, cytochrome C, apoptosis-induced factor (AIF), and caspase-3 was tested by Western blot analysis. Results: Our data demonstrated that MSC medium reduced H/R-induced cardiomyocyte apoptosis, increased the Bcl-2/Bax ratio, and reduced the release of cytochrome C and AIF from mitochondria into the cytosol. Conclusion: MSCs protected the cardiomyocytes from H/R-induced apoptosis through a mitochondrial pathway in a paracrine manner.

Key words: Mesenchymal stem cell (MSC), Apoptosis, Mitochondrial transmembrane potential, Hypoxia/reoxygenation (H/R)
doi:10.1631/jzus.B0920153 **Document code:** A **CLC number:** R54

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (MSCs) are considered effective in myocardial infarction therapies both in basic research studies (Amado *et al*., 2005; Tang *et al*., 2006) and in clinical trials (Janssens *et al*., 2006; Meyer *et al*., 2006). MSCs repair the ischemic myocardium primarily by angioblast-mediated vasculogenesis (Kocher *et al*., 2001), prevention of apoptosis of native cardiomyocytes, or by direct regeneration of the lost cardiomyocytes (Shim *et al*., 2004; Takahashi *et al*., 2006). Gnecchi *et al*.(2005), Kinnaird *et al*.(2004) and Takahashi *et al*.(2006) recently reported that the cardio-protective effect of MSCs is related to a paracrine effect, which could be enhanced by hypoxia (Uemura *et al*., 2006).

However, the compound effect of the cytokines on cultured cardiomyocytes is still unknown. In this study, we use medium prepared from hypoxically cultured MSCs to investigate the effect of the secreted cytokines on cardiomyocytes suffering from hypoxia/reoxygenation (H/R), and further study the potential mechanisms involved.

MATERIALS AND METHODS

Animals

Neonatal and adult Sprague-Dawley (SD) rats were obtained from the Medical Institute Animal Center of Zhejiang University, China. The animal experiments were approved by the Animal Care and Use Committee of Zhejiang Provincial Medical Institute and were in compliance with the Guide for the

‡ Corresponding author

* Project supported by the National Natural Science Foundation of China (No. 30670868) and the Natural Science Foundation of Zhejiang Province, China (No. R206007)

Care and Use of Laboratory Animals as published by the US National Institutes of Health (Institute of Laboratory Animal Research, Commission on Life Science, National Research Council, 1996).

Cell culture

MSCs were obtained from the femora and tibiae of SD rats (each weighing about 80 g), using a modified method as previously described (Dobson *et al*., 1999; Xie *et al*., 2006). After 2~3 passages, MSCs, negative for CD45 and positive for CD44 and CD90, were used. A primary culture of neonatal SD rat cardiomyocytes was prepared using the method described originally by Simpson and Savion (1982) with minor modifications (Xie *et al*., 2006). After 10 d of incubation, cardiomyocytes grew to about 90% confluence.

MSC medium preparation

To prepare MSC medium, MSCs were incubated in a modular incubator chamber (Billups-Rothenberg, USA) for 6 h in serum-free Dulbecco's modified eagle medium (DMEM), where normal air was replaced by 95% N_2 and 5% CO_2. The medium was collected and centrifuged at 4000 r/min to remove cell debris.

H/R-induced cardiomyocyte apoptosis

Cardiomyocytes were randomly divided into two groups: treated with DMEM or treated with MSC medium. Both groups of cells were treated with H/R. To mimic natural hypoxia, the cardiomyocytes were incubated at 37 °C in a modular incubator chamber for 24 h, where normal air was replaced by 95% N_2 and 5% CO_2. They were then moved into a normoxic incubator (95% air and 5% CO_2) for 3 h to mimic the natural reoxygenation process. A control group was incubated in DMEM supplemented with 20% (w/v) fetal calf serum under standard cell culture conditions (95% air and 5% CO_2).

We used three methods to determine apoptosis of cardiomyocytes. The Annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BioVision, USA) was used according to the manufacturer's instructions. Nuclei were stained with the chromatin dye Hoechst 33342 (Sigma, USA). Briefly, cells were fixed for 1 h in 4% (w/v) paraformaldehyde at room temperature, and then exposed to 5 μg/ml Hoechst 33342 for 30 min at 37 °C in the dark. Cells were observed using a fluorescence microscope. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL, Roche Diagnostic, Germany) assays were performed according to the manufacturer's protocol. The percentage of TUNEL-positive cells was determined by counting at least 200 cells in 5 randomly selected fields.

Mitochondrial transmembrane potential assessment

Mitochondrial transmembrane potential was assessed using the lipophilic cationic probe JC-1 (BioVision, USA), a sensitive fluorescent dye, as we had described before (Chen *et al*., 2008). Red emission from the dye is attributed to a potential-dependent aggregation in the mitochondria. Green fluorescence reflects the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization. Cardiomyocytes were incubated with 10 μmol/L JC-1 for 15 min at 37 °C in the dark and monitored using a fluorescence microscope.

Isolation of mitochondria and cytosol

Mitochondrial and cytosolic fractions were prepared using a mitochondria/cytosol fractionation kit (BioVision, USA) according to the manufacturer's protocol. Briefly, cells were collected and resuspended in 100 μl cytosol extraction buffer, incubated on ice for 10 min, and homogenized in an ice-cold tissue grinder. Cell homogenate was subjected to 700×*g* centrifugation for 10 min. The supernatant was further centrifuged at 10000×*g* for 30 min. Supernatants were used as the cytosolic fraction, while the pellets, which were then resuspended in 20 μl of the mitochondrial extraction buffer, were used as the mitochondrial fraction.

Western blot analysis

Protein (20~100 μg) prepared from the disposed cells was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) immobilon-P membrane (Bio-Rad, CA, USA) using a transblot apparatus (Bio-Rad, CA, USA). The membranes were blocked in 10 mmol/L Tri-HCl (pH 8.0), 150 mmol/L NaCl and 0.05% (w/v) Tween 20 (Tris-buffered saline Tween 20, TBST) with 5% (w/v) non-fat milk at room temperature, followed by overnight incubation at 4 °C with primary antibodies

diluted in TBST (1:1000 for Bcl-2, Bax, caspase-3 and β-actin, Cell Signal, USA; 1:1000 for cytochrome C, BD Pharmingen, USA; 1:1000 for AIF, Santa Cruz, USA). After washing with TBST, the membranes were incubated for 1 h with a horse radish peroxidase (HRP)-conjugated secondary antibody diluted 1:5000 in TBST, and the labeled proteins were detected using enhanced chemiluminescence reagents and exposed to film (Kodak, USA).

Data analysis

Data were expressed as the mean±*SEM*. Statistical significance between groups was assessed by one-way analysis of variance (ANOVA) followed by SNK using SPSS 11.5. $P<0.05$ was considered statistically significant.

RESULTS

Morphology of MSCs

MSCs were attached to culture dishes and the majority displayed a spindle-like shape (Fig.1).

MSC medium protected cardiomyocytes from H/R-induced apoptosis

Exposure of cultured cardiomyocytes to H/R led to an increase of cell apoptosis, as assessed by three methods: Annexin V-FITC staining (Figs.2a~2c), Hoechst 33342 staining (Figs.2d~2f) and TUNEL assay (Fig.2g). MSC medium decreased the apoptosis (control: (5.2±0.5)%, DMEM: (23.0±2.1)%, $P<0.05$ vs control; MSC medium: (18.1±3.0)%, $P<0.05$ vs control and DMEM).

MSC medium protected cardiomyocytes from H/R-induced mitochondrial membrane potential loss

To determine whether MSC medium affected H/R-induced cardiomyocyte mitochondrial dysfunction, we assessed mitochondrial membrane potential using the potential-sensitive fluorescent probe JC-1. Normal cardiomyocytes exhibited red fluorescence (Fig.3a) whereas cardiomyocytes after H/R developed a diffuse green staining pattern (Fig.3b), indicative of reduced mitochondrial membrane potential. MSC medium had a marked effect on JC-1 staining, preserving mitochondrial membrane potential (Fig.3c).

MSC medium increased the ratio Bcl-2/Bax in mitochondria

To explore the signaling pathway upstream of the mitochondria, we investigated whether MSC medium would have any impact on the proapoptotic Bcl-2-family members Bcl-2 and Bax. Mitochondria were prepared and analyzed for the expression of Bcl-2 and Bax. Treatment with MSC medium during H/R decreased the Bax level in the mitochondria, resulting in a significant increase of about 3.3-fold in the Bcl-2/Bax ratio (Figs.4a and 4b).

MSC medium reduced the mitochondrial release of apoptosis inducing factor (AIF)

As H/R could impact the mitochondrial transmembrane potential, we testified whether AIF was involved in the caspase-independent proapoptotic effect. The level of AIF in the cytosol increased after H/R, and treatment with MSC medium during H/R prevented AIF release from the mitochondria (Fig.4d).

MSC medium reduced cytochrome C release and caspase-3 activation

Immunoblots of cytochrome C were also studied. There was a significant increase in cytosolic cytochrome C after H/R. MSC medium reduced H/R-induced cytochrome C release (Fig.4c) and the downstream activation of caspase-3 (Fig.4e).

DISCUSSION

The paracrine effect of MSCs has been the focus of many recent studies. It has been reported that MSCs can secrete many growth factors and proteases (Kemp *et al*., 2005; Wang *et al*., 2004), including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factors (IGF)-1, stromal cell-derived factor (SDF), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP), transforming growth factor (TGF)-β, and platelet derived growth factor (PDGF). In this study, we did not target the specific factors, but we found that MSC medium protected cardiomyocytes from H/R-induced apoptosis, inhibited the release of cytochrome C from the mitochondria and reduced caspase-3 activation, suggesting that MSC medium

protects cardiomyocytes by interfering with a mitochondria-mediated apoptotic pathway.

The mechanism by which MSC medium interfered with cytochrome C release was probably by regulating the Bcl-2/Bax ratio. The roles of the Bcl-2 family proteins are oppositional (Fehlberg *et al*., 2003), either antiapoptotic or proapoptotic. Bcl-2 is localized dominantly in the mitochondrial membranes and blocks the mitochondial membrane permeabilization, exerting an antiapoptotic effect. Contrarily, Bax is translocated from the cytosol to mitochondria, and increases the mitochondial membrane permeabilization, which could be blocked by antiapoptotic

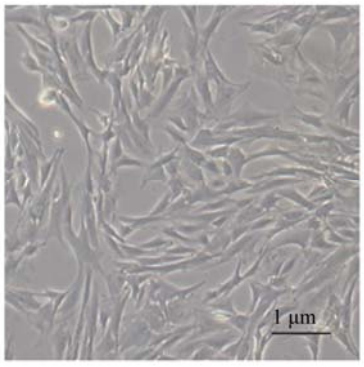

Fig.1 Characteristics of MSCs. Phase-contrast micrographs of the third passage of MSCs

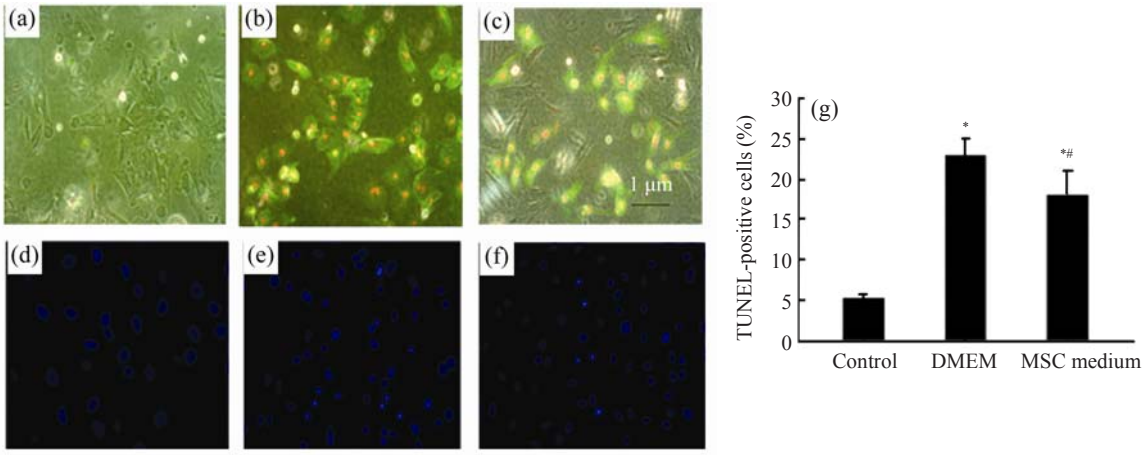

Fig.2 MSC medium reduced H/R-induced apoptosis of cardiomyocytes

(a)~(c) Apoptotic cells were detected by Annexin V-FITC staining for labeling early-stage apoptotic cells (green) and necrotic cells (PI stained, red); (d)~(f) Hoechst 33342 staining of cardiomyocytes: apoptotic cells were characterized by nuclear shrinkage with condensed chromatin structure; (g) Quantification of apoptotic cardiomyocytes measured by TUNEL assay. The fraction of apoptotic cells was determined in five random microscopic fields totalling at least 1000 cells/group. Cardiomyocytes were hypoxic for 24 h and were reoxygenated for 3 h in serum-free DMEM (DMEM group) or the medium abstracted from MSCs (MSC medium group). Control cells were cultured in DMEM containing 20% (w/v) fetal calf serum. Results are representative of three independent experiments. Data are shown as mean±*SEM*. Control group: (a), (d); DMEM group: (b), (e); MSC medium group: (c), (f). $^{*}P<0.05$ vs control group, $^{\#}P<0.05$ vs DMEM group

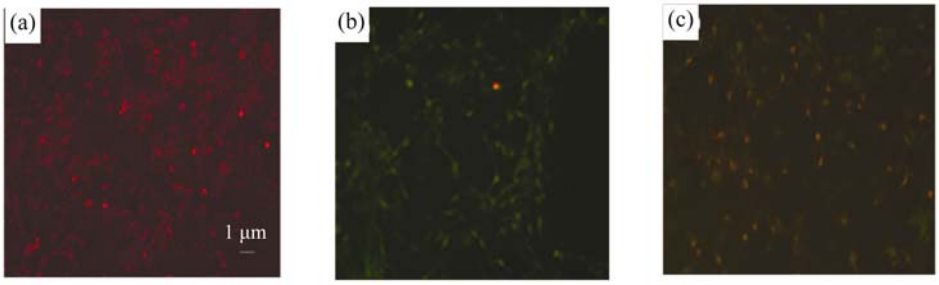

Fig.3 MSC medium attenuated the reduction of mitochondrial membrane potential of H/R-induced cardiomyocytes

Mitochondrial membrane potential was determined using the potential-sensitive fluorescent probe JC-1. (a) Normally cultured cardiomyocytes contained red fluorescent mitochondria in the cytoplasm; (b) Cardiomyocytes after treatment with H/R and serum-free DMEM culture showed green fluorescence, indicating the loss of mitochondrial membrane potential; (c) Cardiomyocytes with H/R and MSC medium culture showed red fluorescent mitochondria in the cytoplasm, indicating the preservation of the mitochondrial membrane potential. Results are representative of three independent experiments

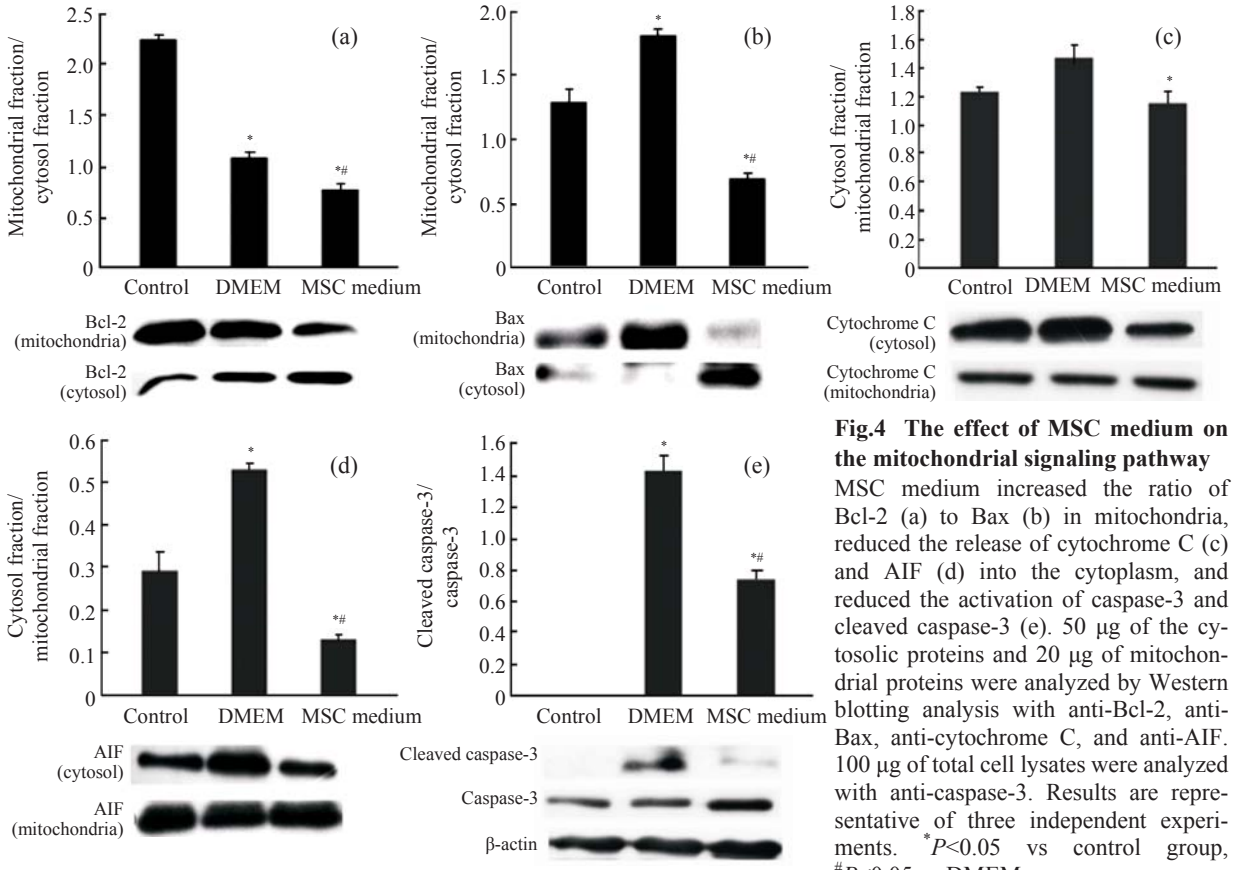

Fig.4 The effect of MSC medium on the mitochondrial signaling pathway

MSC medium increased the ratio of Bcl-2 (a) to Bax (b) in mitochondria, reduced the release of cytochrome C (c) and AIF (d) into the cytoplasm, and reduced the activation of caspase-3 and cleaved caspase-3 (e). 50 μg of the cytosolic proteins and 20 μg of mitochondrial proteins were analyzed by Western blotting analysis with anti-Bcl-2, anti-Bax, anti-cytochrome C, and anti-AIF. 100 μg of total cell lysates were analyzed with anti-caspase-3. Results are representative of three independent experiments. $^{*}P<0.05$ vs control group, $^{\#}P<0.05$ vs DMEM group

protein Bcl-2. The ratio of Bcl-2/Bax could be a key factor in determining the regulation of mitochondrial cytochrome C release, further activation of caspase-3 and in the initiation of apoptosis (Murphy *et al*., 1999).

There are two kinds of apoptotic pathways: caspase-dependent or -independent apoptotic pathways. Mitochondria play an important role between them, like a crossover point (Fehlberg *et al*., 2003). AIF is a key trigger of caspase-independent apoptosis (Daugas *et al*., 2000; Susin *et al*., 1999), which is located in the mitochondrial intermembrane space and is released from mitochondria into the cytosol and nucleus in response to death stimuli. The release of AIF results in the generation of apoptotic phenotypes such as chromatin condensation and phosphatidylserin exposure. As Bax was reported to induce mitochondrial AIF release (Antonsson, 2001) and MSC medium could reduce the Bax level in mitochondria, we concluded that MSC medium protected cardiomyocytes from H/R-induced apoptosis by reducing the translocation of AIF to the cytosol.

In conclusion, in the present study, we demonstrated that H/R induced apoptosis of cardiomyocytes through a mitochondria-mediated pathway, and that the treatment with MSC medium reduced the injury by increasing the ratio Bcl-2/Bax.

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