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RESEARCH ARTICLE

SDF-1/CXCR4 axis modulates bone marrow mesenchymal stem cell apoptosis, migration and cytokine secretion

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ABSTRACT

Bone marrow mesenchymal stem cells (MSCs) are considered as a promising cell source to treat the acute myocardial infarction. However, over 90% of the stem cells usually die in the first three days of transplantation. Survival potential, migration ability and paracrine capacity have been considered as the most important three factors for cell transplantation in the ischemic cardiac treatment. We hypothesized that stromal-derived factor-1 (SDF-1)/CXCR4 axis plays a critical role in the regulation of these processes. In this study, apoptosis was induced by exposure of MSCs to H_2O_2 for 2 h. After re-oxygenation, the SDF-1 pretreated MSCs demonstrated a significant increase in survival and proliferation. SDF-1 pretreatment also enhanced the migration and increased the secretion of pro-survival and angiogenic cytokines including basic fibroblast growth factor and vascular endothelial growth factor. Western blot and RT-PCR demonstrated that SDF-1 pretreatment significantly activated the pro-survival Akt and Erk signaling pathways and up-regulated Bcl-2/Bax ratio. These protective effects were partially inhibited by AMD3100, an antagonist of CXCR4. We conclude that the SDF-1/CXCR4 axis is critical for MSC survival, migration and cytokine secretion.

INTRODUCTION

Bone marrow mesenchymal stem cells (MSCs) are a population of non-hematopoietic and fibroblast-like cells, representing 0.001%–0.01% of the nucleated cells in the marrow (Pittenger et al., 1999). MSCs are considered as a cellular substrate for the cardiac repair because of their presumptive plasticity, genetic stability, and immune tolerance (Trivedi et al., 2010). Furthermore, the accessibility, ease of handling, and enormous expansion potential of MSCs, together with their capacity for self-renewal and amenability to allogenic transplantation (Amado et al., 2005; Dai et al., 2005), provide a nearly unlimited supply of cells for therapeutic applications. The effect of MSCs is thought to be mediated by secreting a number of cardio-protective cytokines and growth factors in addition to differentiation (Kinnaird et al., 2004a, 2004b; Nagaya et al., 2005). Despite initial optimism, MSC transplant therapy has been stymied by the low cellular survival rate after transplantation in the first few days (Toma et al., 2002). The infarcted region usually consists of ischemic and scar tissue which cannot transport enough nutrient and oxygen to support the engraftment and survival of the transplanted stem cells. Most transplanted cells die in the first few hours due to ischemia, inflammation and apoptosis (Menasché, 2008). Thus, it is necessary to reinforce MSC survival against the arduous hypoxic and

hypotrophic microenvironment.

Considering that cell survival is essential for the success of stem cell therapy, many approaches have been utilized to enhance the survival rate of the transplanted cells. Over-expression of pro-survival molecules including Akt (Mangi et al., 2003), Pim-1 (Fischer et al., 2009), Bcl-2 (Li et al., 2007) and stem cell factor (SCF) (Fazel et al., 2008) prevented donor cells from apoptosis. At the same time, pharmacological preconditioning with growth factors and cytokines can also improve the survival of transplanted cells (Haider and Ashraf, 2008).

The stromal-derived factor-1 (SDF-1) plays an essential role in stem cell homing toward the ischemic/hypoxic myocardium by recruiting the progenitor cells that express its cognate receptor, CXC chemokine receptor 4 (CXCR4) (Askari et al., 2003; Abbott et al., 2004). Our previous study showed that overexpression of CXCR4 increased engraftment of MSCs in the infarcted myocardium and improved cardiac performance (Cheng et al., 2008c). We also demonstrated that granulocyte colony-stimulating factor (G-CSF) mobilized MSCs to the infarcted heart through the upregulation of CXCR4 (Cheng et al., 2008a). Beside the role in migration, the SDF-1/CXCR4 axis is also important for cell survival. It has been shown that SDF-1 promoted survival of CD34^{+} cells through increasing the expression of the anti-apoptotic protein bcl-2 (Lataillade et al., 2002). SDF-1 also protected cardiac myocytes against ischemia/reperfusion-induced cell death (Hu et al., 2007). However, the effects and underlying mechanisms of SDF-1 treatment on survival and secretion function of MSCs are largely unknown. In this study, we demonstrated the positive effects of SDF-1 pretreatment on MSC survival, migration, and cytokine production after hypoxia/de-oxygenation.

RESULTS

SDF-1 promotes survival and proliferation of MSCs through the SDF-1/CXCR4 axis

MTT cell proliferation assay was performed to examine whether SDF-1 pretreatment promoted survival and proliferation of MSCs after re-oxygenation. As shown in Fig. 1A, after 16 h of re-oxygenation, the cell number showed a markedly decrease compared to that after only 2 h, indicating an increasingly cell injury with H_2O_2 exposure. Interestingly, SDF-1 treatment (H + SDF) significantly increased the number of MSCs compared with non-treated group (H). The effect of SDF-1 was impaired by pre-treatment with the CXCR4 antagonist AMD3100 (H + AMD + SDF-1), indicating SDF-1 promoted MSC proliferation through the SDF-1/CXCR4 ligation.

We also analyzed cell survival by measuring lactate dehydrogenase (LDH) released into the culture medium. As shown in Fig. 1B, LDH release was decreased by SDF-1 (H + SDF) compared to non-treated cells (H). Pre-treatment with
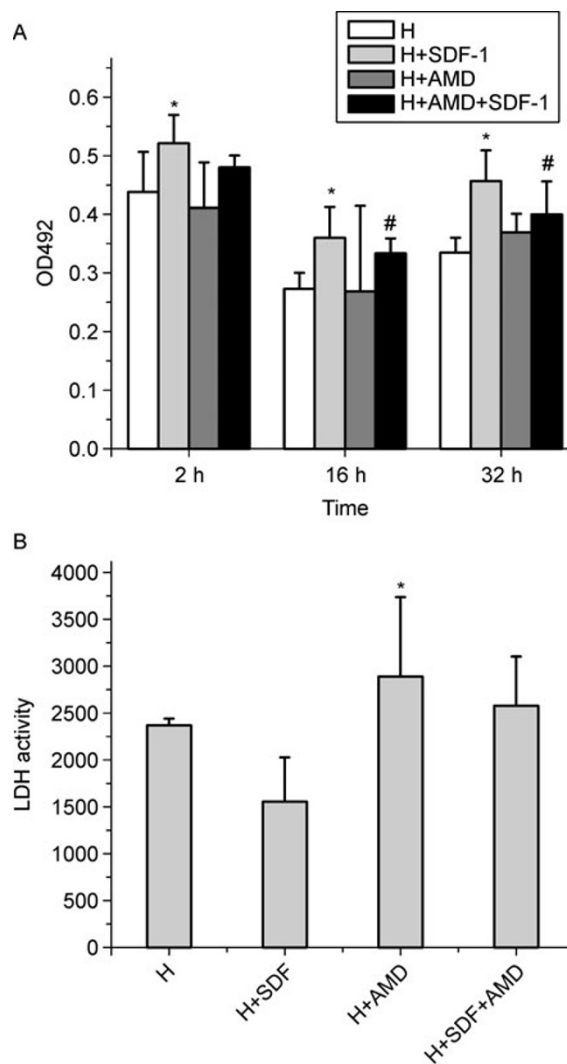

Figure 1.   SDF-1 promotes survival and proliferation of MSCs through the SDF-1/CXCR4 axis. (A) MSC proliferation measured by MTT assay. *: $p < 0.05$ *vs* hypoxia (H); #: $p < 0.05$ *vs* hypoxia, $n = 3$. (B) MSC apoptosis measured by LDH release assay. *: $p < 0.05$ *vs* pretreated with SDF-1, $n = 3$. H, hypoxia, MSCs exposed to H_2O_2 for 2 h; SDF, pretreatment with SDF-1 for 1 h; AMD, pretreatment with AMD3100 for 2 h.

AMD3100 (H + AMD + SDF-1) blocked the protective effect of SDF-1, suggesting SDF-1 promoted survival of MSCs through the SDF-1/CXCR4 axis.

SDF-1 activates the Akt and Erk pathways through the SDF-1/CXCR4 axis

The Akt and Erk signaling pathways are critical for survival and proliferation of MSCs (Mangi et al., 2003; Xu et al., 2008). Thus we postulated that SDF-1 might regulate MSC survival and proliferation through the Akt and Erk pathways. Western blotting showed that SDF-1 induced phosphorylation of both

Akt and Erk, which were also abolished by pretreatment with AMD3100 (Fig. 2).

SDF-1 increases Bcl-2/Bax ratio through the SDF-1/CXCR4 axis

The Bcl-2 family proteins, including the pro-survival Bcl-2 and pro-apoptotic Bax, are key regulators of apoptosis (Misao et al., 1996; Li et al., 2007). To determine whether SDF-1 pretreatment modify the expression of Bcl-2 and Bax, MSCs were pretreated with SDF-1 for 1 h and then exposed to H_2O_2 followed by re-oxygenation for 16 h. Western blotting showed that Bcl-2 level was increased in SDF-1 pretreated cells, but was significantly lower in AMD3100-treated cells (Fig. 3A). Although protein level of Bax was not significantly different among the four groups, SDF-1 treatment significantly increased the Bcl-2/Bax ratio to 1.7 fold compared to the control group. In contrast, pretreatment with AMD3100 completely abolished the effects of SDF-1. At mRNA level (Fig. 3B), SDF-1 treatment also significantly increased Bcl-2/Bax ratio, which was blocked by AMD3100. These results suggested that SDF-1 pretreatment increased Bcl-2/Bax ratio through the SDF-1/CXCR4 axis.

SDF-1 enhances MSC migration

We examined whether SDF-1 pretreatment could influence the chemotaxis of MSCs toward the SDF-1 gradient in a transwell migration assay. As shown in Fig. 4, SDF-1 pretreatment resulted in a 2-fold increase in the number of migrating MSCs ($p < 0.05$), thereby indicating that SDF-1 pretreatment enhanced the response of MSCs to the SDF-1-induced chemotaxis.

Effects of SDF-1 pretreatment on MSC surface CXCR4 expression

Our previous results showed that increased surface CXCR4 expression was associated with enhanced MSC migration (Cheng et al., 2008a, 2008c). To determine whether SDF-1 enhanced MSC migration through modulation of cell surface CXCR4 expression, MSCs were incubated with SDF-1 before hypoxia/re-oxygenation for 16 h. Flow cytometry analysis showed that SDF-1 tended to increase surface CXCR4 expression compared to non-treated cells, but this did not reach statistical significance (Fig. 5; 2.0% vs 1.4%, $p > 0.05$).
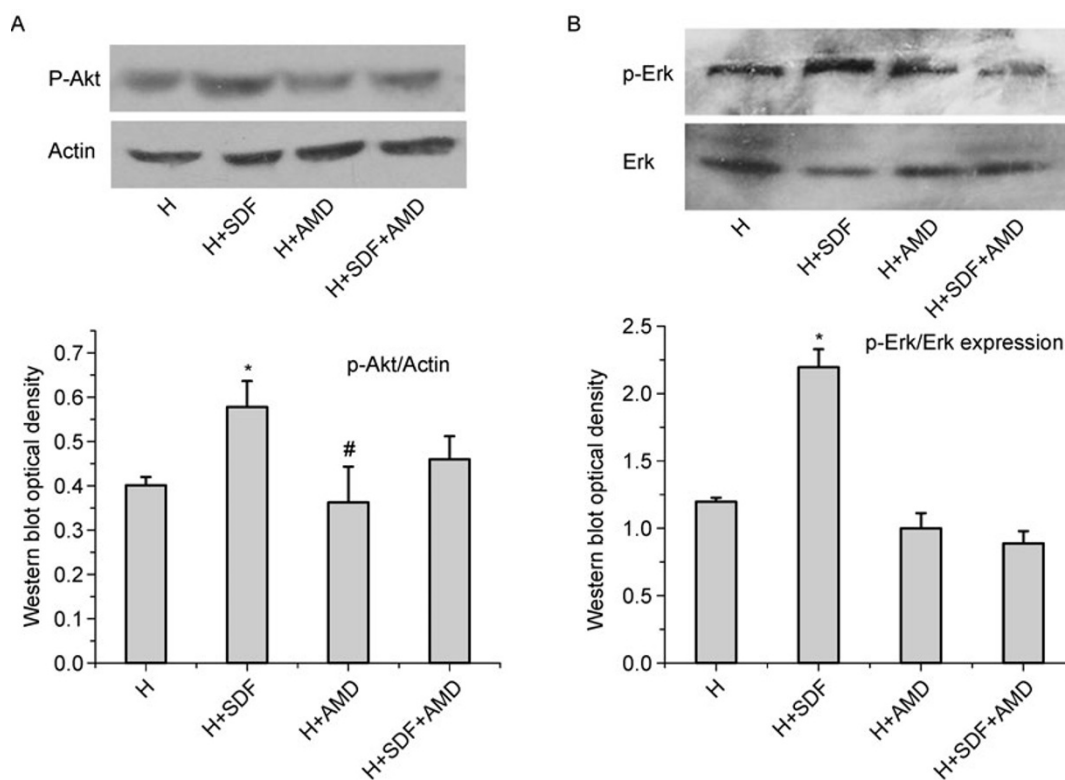

Figure 2. SDF-1 activates the Akt and Erk survival pathways through the SDF-1/CXCR4 axis. (A) SDF-1 pretreatment increased Akt phosphorylation, which could be blocked by AMD3100. (B) SDF-1 pretreatment increased ERK phosphorylation, which could also be blocked by AMD3100.*: $p < 0.05$ vs hypoxia; #: $p < 0.05$ vs SDF-1. Data are representative of three independent experiments.
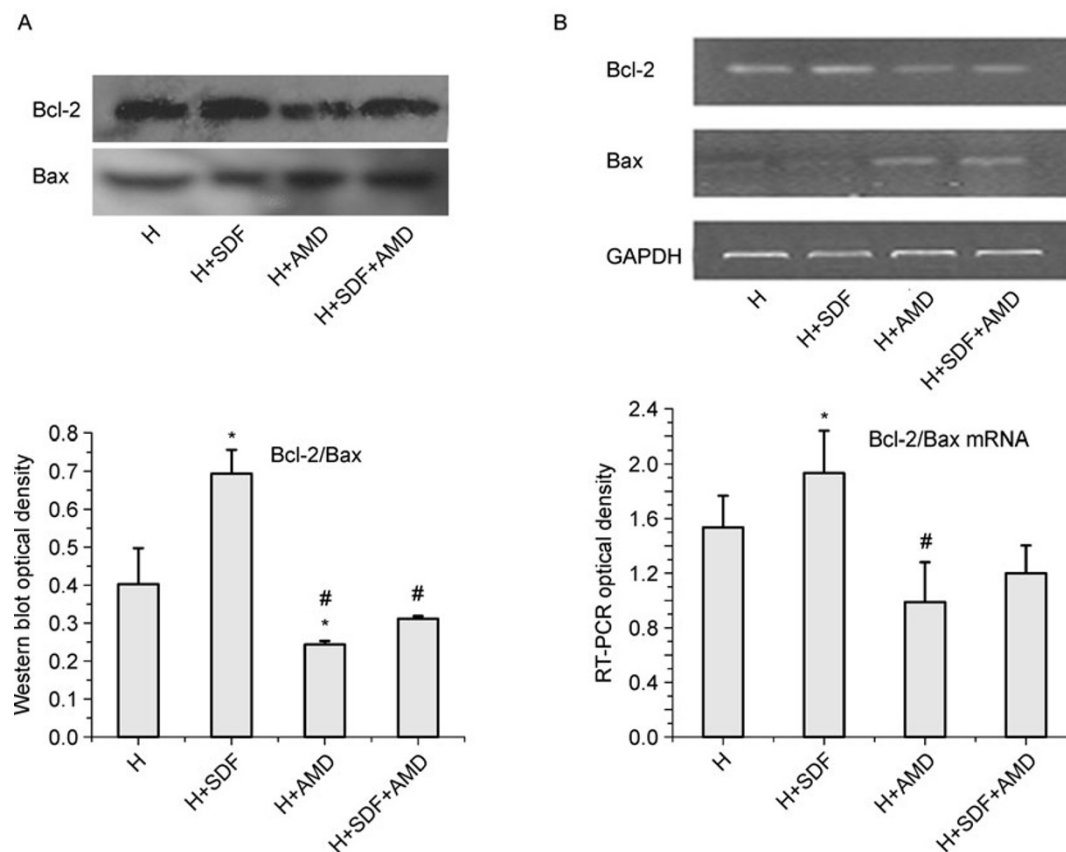
Figure 3. SDF-1 increases Bcl-2/Bax ratio through the SDF-1/CXCR4 axis. (A) Western blot densitometry analysis of Bcl-2 and Bax expression. Representative western blot of Bcl-2, bax and relative Bcl-2/bax ratio are shown. (B) Bcl-2 and Bax mRNA were analyzed by RT-PCR. RT-PCR densitometry showed a significant decrease in bcl-2/bax mRNA ratio when cells were blocked with AMD3100. *: $p < 0.05$ vs hypoxia; #: $p < 0.05$ vs SDF-1. Data are representative of three independent experiments.

SDF-1 promotes the secretion of bFGF and VEGF through the SDF-1/CXCR4 axis

Paracrine secretion of basic fibroblast growth factors (bFGF) and vascular endothelial growth factor (VEGF) is crucial for protecting MSCs in ischemic tissues (Kinnaird et al., 2004a). Therefore we examined the effects of SDF-1 pretreatment on the secretion capacity of MSCs by ELISA. As shown in Fig. 6A, bFGF secretion was significantly enhanced by SDF-1, but the enhancement was almost completely blocked by adding AMD3100 together. Compared to the non-treated cells, SDF-1 only slightly increased VEGF secretion by MSCs (Fig. 6B). However, AMD3100 pretreatment significantly inhibited the VEGF production, indicating MSC secretion of VEGF is regulated, at least in part, by the SDF-1/CXCR4 axis.

SDF-1 pretreatment upregulates SDF-1 mRNA in MSCs

We further examined the effect of SDF-1 treatment on the endogenous SDF-1 expression in MSCs. After re-oxygenation for 16 h, SDF-1-treated MSCs showed an increase of SDF-1 mRNA, which was completely blocked by incubation with AMD3100 (Fig. 7). Our data suggested that SDF-1 pretreatment further increased SDF-1 synthesis through the SDF-1/CXCR4 axis, indicating a possible autocrine positive feedback loop.

DISCUSSION

Stem cell therapy has recently been proposed as a promising strategy for cardiac repair after myocardial infarction (Mangi et al., 2003; Amado et al., 2005; Dai et al., 2005; Li et al., 2007; Xie et al., 2007; Cheng et al., 2008c; Fazel et al., 2008; Fischer et al., 2009). However, most ransplanted stem cells undergo apoptosis in the harsh microenvironment with poor blood and oxygen supply in the infarct zone (Haider and Ashraf, 2008). Therefore, protection of stem cells from apoptosis is crucial for a successful cell therapy. After myocardial infarction and reperfusion, reactive oxygen species (ROS) are generated in the ischemic myocardium and directly cause apoptosis. The in vivo oxidative stress-induced apoptosis can be simulated in vitro by H_2O_2
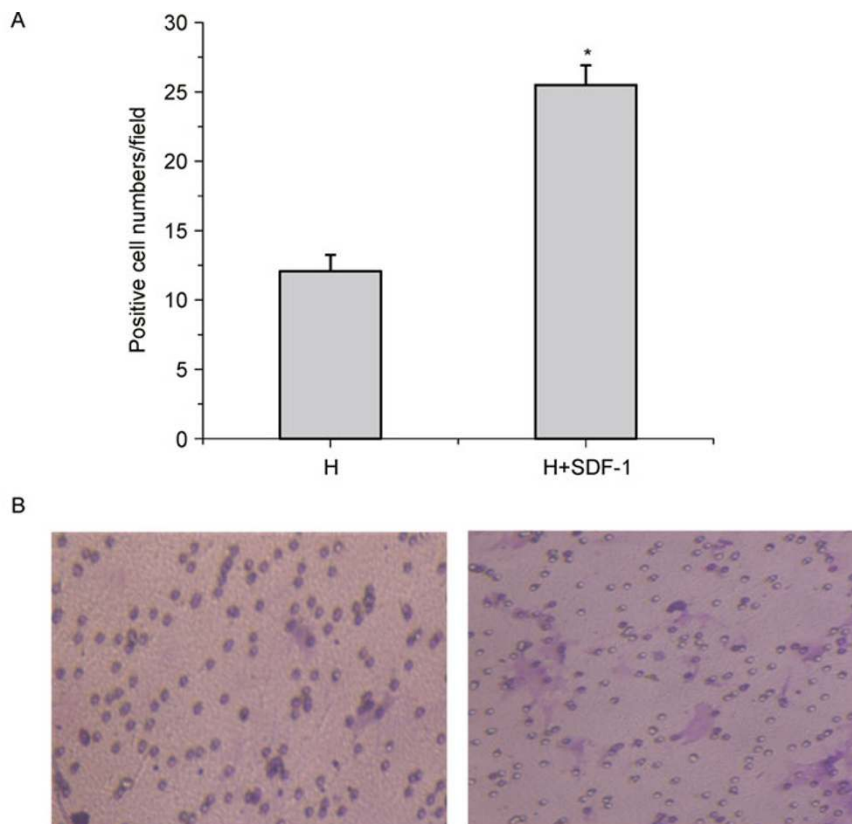
Figure 4. SDF-1 enhances MSC migration. (A) Quantitative analysis of migrated cells in transwell assay. (B) Representative image of hypoxic MSCs (left) and MSCs pretreated with SDF-1 prior to hypoxia (right) in response to 30 ng/mL SDF-1 in the transwell assay. Results are mean values ± SEM of five different fields from four independent experiments. *: $p < 0.05$ *vs* hypoxia.

treatment of both isolated adult cardiomyocytes and MSCs (Kumar and Jugdutt, 2003; Pasha et al., 2008). In the present study, we have shown that SDF-1 pretreatment can protect MSCs from H_2O_2-induced damages. SDF-1 pretreatment significantly attenuates oxidative stress-induced MSC apoptosis; furthermore, SDF-1 markedly activates the Akt and Erk survival pathways and increases the Bcl-2/Bax ratio; besides, SDF-1 pretreatment improves migration capability of MSCs; and finally, SDF-1 enhances the secretion of bFGF and VEGF by MSCs. These findings clearly demonstrate that pretreatment of MSCs by SDF-1 can improve the efficacy of stem cell therapy.

SDF-1 is a CXC chemokine known to be an effective chemotactic factor for progenitor cells and mature blood cells. CXCR4, the cell surface receptor for SDF-1, mediates the homing and migration of stem cells (Cheng et al., 2008c). Previous studies demonstrated that under chronically hypoxic conditions (Rochefort et al., 2006), MSCs were mobilized into the peripheral blood through upregulation of CXCR4 (Schioppa et al., 2003), suggesting that hypoxia potently regulated CXCR4 expression *in vivo*. At the same time, the level of SDF-1 was also elevated after myocardial infarction (Askari et al., 2003; Abbott et al., 2004). In our study, we

showed that SDF-1 pretreatment improved MSC migration through upregulation of cell surface CXCR4 (Fig. 5). The upregulation of endogenous SDF-1 by SDF-1 pretreatment (Fig. 7), a possible autocrine positive feedback effect, might also play a role in promoting MSC migration.

In addition to its role in cell migration, the SDF-1/CXCR4 axis is also associated with enhanced cell survival/proliferation, adhesion, and growth factor secretion (Kucia et al., 2004). SDF-1 has been shown to promote the survival of myeloid, neural, oligodendrocyte and trophoblast precursors (Broxmeyer et al., 2003; Jaleel et al., 2004; Dziembowska et al., 2005). It was reported that overexpression of SDF-1 improved survival of MSCs in the ischemic heart (Zhang et al., 2007). In the present study, we also demonstrated that SDF-1 pretreatment improved MSC survival and proliferation. Pretreatment with the CXCR4 antagonist AMD3100 significantly decreased cell viability (Fig. 1B). These results suggested that the anti-apoptotic effect of SDF-1 was mediated through its cognate receptor, CXCR4.

Although SDF-1 pretreatment improved cell survival and differentiation (Joo et al., 2004; Pasha et al., 2008), the mechanism has not been well elucidated. Previous studies suggested that cell survival and proliferation are mediated
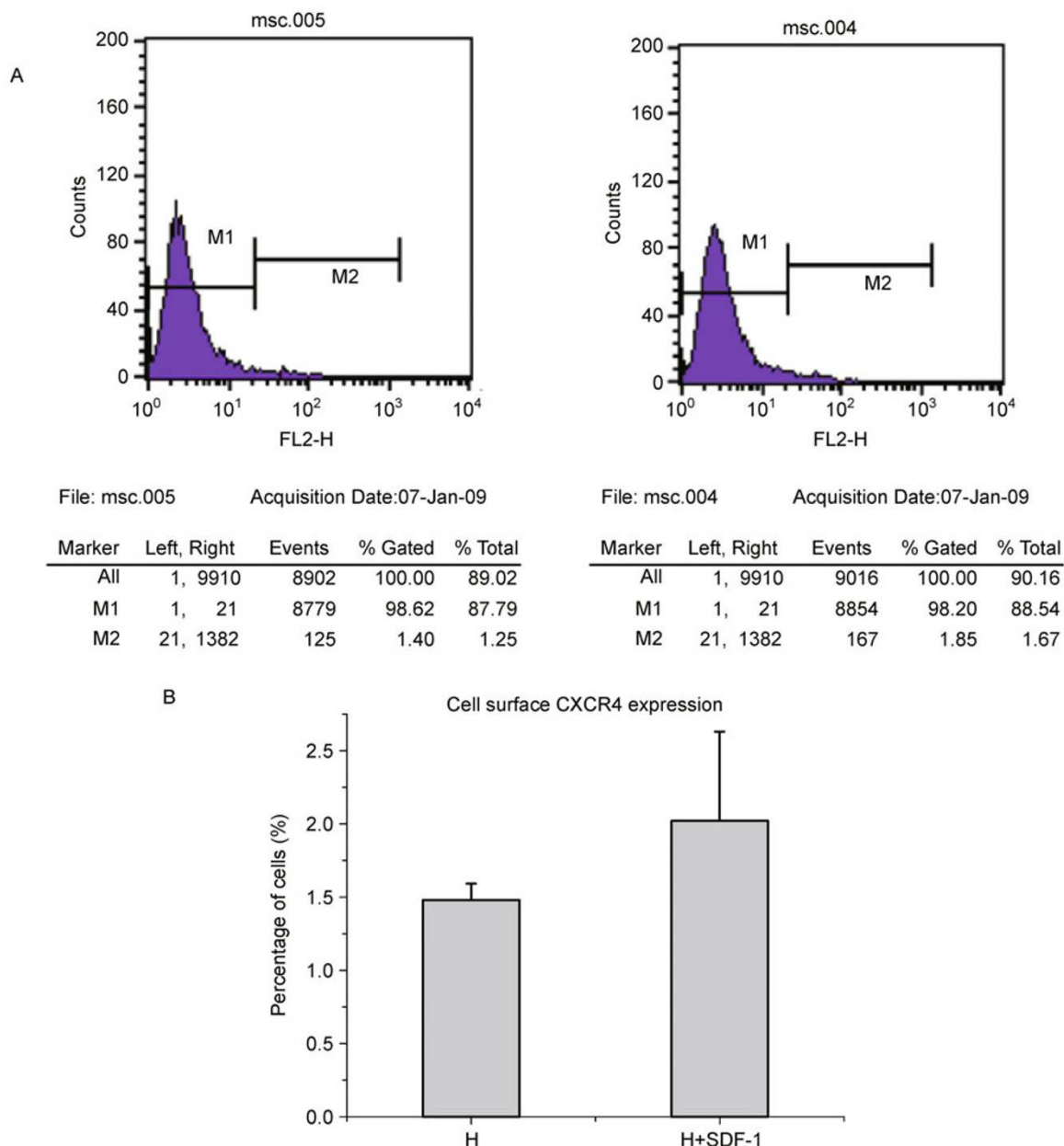
Figure 5. Effect of SDF-1 pretreatment on MSC surface CXCR4 expression. (A) Representative flow cytometry images of surface CXCR4 expression on MSCs pretreated with 0 ng/mL SDF-1 (left) or 50 ng/mL SDF-1 (right) before hypoxia/re-oxygenation for 16 h. (B) Quantitative analysis of surface CXCR4 expression on MSCs. Results are mean ± SEM (n = 5 in each group). The difference was not significant according to the independent t-test.

through activation of the signaling cascades PI3K/Akt and MAPK/ERK (Choi et al., 2008; Zhang and Cai, 2010). Our data showed that SDF-1 mediated MSC survival was associated with increased phosphorylation of Akt and ERK, which can be blocked by AMD3100 (Fig. 2). Our results suggested that SDF-1, acting through its receptor CXCR4, protected MSCs from apoptosis via activation of Akt and ERK.

We also investigated the expression of Bcl-2 family proteins. Bcl-2 is an anti-apoptotic protein which serves as a critical regulator of pathways involved in apoptosis (Reed, 1998), whereas Bax is a pro-apoptotic protein which controls the integrity of the mitochondrial outer membrane (Yang et al., 1997; Jürgensmeier et al., 1998). Here we found pretreatment with SDF-1 increased the expression of Bcl-2 while decreased Bax level. In agreement with our results, SDF-1 has been shown to prevent the apoptosis of hematopoietic precursors through the upregulation of Bcl-2 (Peters et al., 1998).
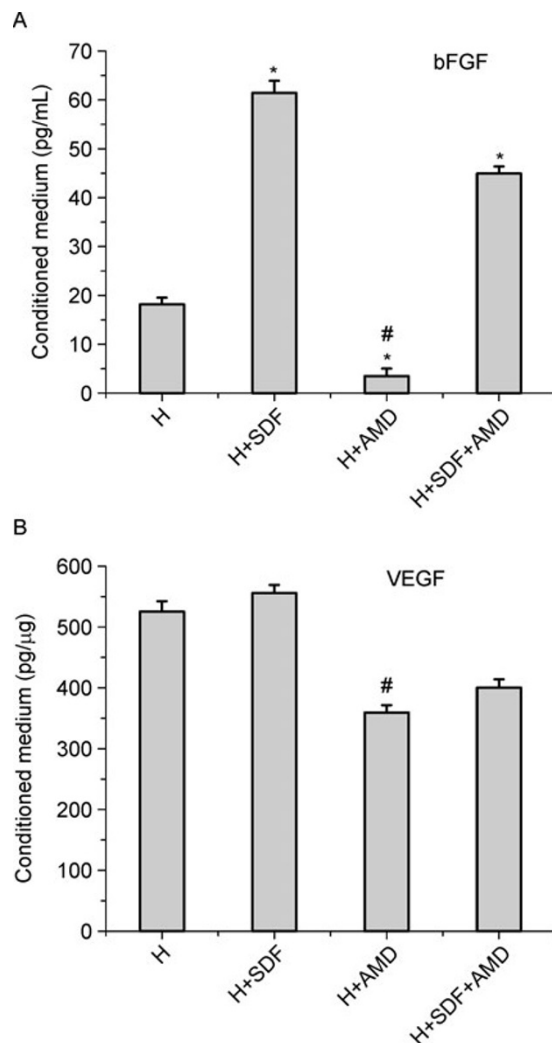
Figure 6. SDF-1 stimulates MSCs to secrete cytokines through the SDF-1/CXCR4 axis. (A) SDF-1 pretreatment increased bFGF secretion, which could be blocked by AMD3100. (B) Secretion of VEGF by MSCs was blocked by AMD3100. *: $p < 0.05$ *vs* hypoxia; #: $p < 0.05$ *vs* SDF-1. Data are representative of three independent experiments.

MSCs can secrete angiogenic cytokines including bFGF and VEGF (Kinnaird et al., 2004a), both of which have been shown to inhibit cellular apoptosis (Gupta et al., 1999; Choi et al., 2008). Our study revealed that SDF-1 pretreatment increased the secretion of bFGF and VEGF by MSCs, whereas blocking of CXCR4 by AMD3100 abolished this effect (Fig. 6). These results suggest that SDF-1 pretreatment can protect the resident cardiomyocytes from apoptosis and improve their survival by paracrine secretion of bFGF and VEGF.

In conclusion, our results showed that SDF-1 pretreatment increases the survival, proliferation, migration and secretion capability of MSCs. These effects of SDF-1 are mediated through its receptor CXCR4.

MATERIALS AND METHODS

Isolation and culture of MSCs

Male Sprague-Dawley (SD) rats were purchased from the Lab Animal Center of The Academy of Military Medical Sciences (Beijing, China). Bone marrow MSCs were isolated as described previously (Cheng et al., 2008c). Briefly, adult male SD rats were euthanatized, and bone marrow from tibias and femurs was flushed with complete culture medium. Mononuclear cells were separated by density-gradient centrifugation with Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). Cells were resuspended in αMEM (Gibco Laboratories, Grand Island, NY, USA) supplemented with 20% fetal bovine serum and antibiotics. Non-adherent cells (hematopoietic cells, fibroblasts, etc.) were washed away during medium changes, while MSCs retained because of their preferential attachment to the polystyrene surfaces. The study protocol was approved by the Ethics Committee on Animal Experimentation of Nankai University (A5521-01), which complies with 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).

SDF-1 pretreatment and hypoxia/re-oxygenation

MSCs were stimulated with SDF-1 (50 ng/mL) for 1 h before exposure to H_2O_2 (200 μmol/L) for 2 h. In control group, cells were cultured with CXCR4-specific antagonist AMD3100 (10 μg/mL) for 2 h. Cells were then cultured in normal growth medium for 2, 16 or 32 h. The supernatant from 16 h culture was collected for ELISA analysis.

MTT cell proliferation assay

Cell proliferation was assessed by MTT method. Briefly, MSCs were seeded in 96 wells at 1×10^5 cells/well in 100 μL complete medium. After hypoxia/re-oxygenation, MSCs were cultured for 2, 16 or 32 h, respectively, followed by the addition of 25 μL MTT (5 mg/mL PBS, pH 7.4, Sigma) for another 4 h. The supernatant was then discarded and 100 μL DMSO was added into each well for 10 min. Finally, absorbance at 492 nm was measured with a Bio-Rad Microplate Reader.

LDH cytotoxicity assay

Lactate dehydrogenase (LDH) is a cytoplasmic oxidoreductase that is released into the culture supernatant when the plasma membrane is damaged. The degree of cellular injury thus can be monitored by measuring the amount of LDH released by the cells. In this study, LDH level in the culture medium was assayed by LDH ELISA according to the manufacturer's protocol (Jiancheng Co., Nanjing, China).

Flow cytometry analysis of surface CXCR4 expression

Surface CXCR4 expression was analyzed as previously described (Cheng et al., 2008a). Briefly, cells were stained with rabbit anti-CXCR4 antibody (Thermo Fisher Scientific, Fremont, CA, USA) followed by Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes,
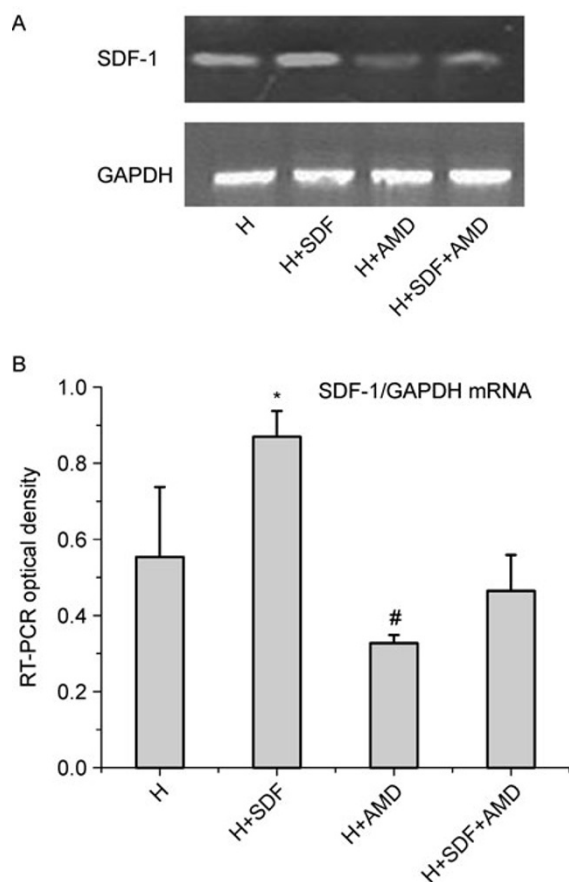
A

SDF-1

GAPDH

H   H+SDF   H+AMD   H+SDF+AMD

B

Figure 7. SDF-1 pretreatment upregulates SDF-1 mRNA in MSCs. (A) Representative images of SDF-1 expression detected by RT-PCR. (B) Quantitative analysis of SDF-1 mRNA by densitometry showed a significant decrease in SDF-1 expression when cells were blocked with AMD3100 for 2 h. *: $p < 0.05$ *vs* hypoxia; #: $p < 0.05$ *vs* SDF-1. Data are representative of three independent experiments.

Eugene, OR, USA). Surface CXCR4 expression was determined by an Epics Altra flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Transwell migration assay

Migration assays were performed as previously described (Cheng et al., 2008c). Briefly, after hypoxia/re-oxygenation, cells were placed in the upper chamber of the transwell assembly (Corning Costar, Cambridge, MA) at a density of $6 × 10^5$ cells/mL. The lower chamber contains 600 µL medium with 30 ng/mL SDF-1 (PeproTech, Rocky Hill, NJ). After incubation at 37°C for 10 h, the membrane was stained with 0.5% crystal violet and the number of migrating cells was determined by counting five random fields per well under the microscope at 100×. Experiments were performed in sets of four for each group.

Reverse transcriptase-polymerase chain reaction analysis

RNA was isolated using Trizol reagent following the manufacturer's instructions (Invitrogen), and cDNA was synthesized from 2 µg of total RNA using reverse transcriptase. Amplification reactions (30 µL) were carried out with 3 µL of template cDNA and 2.4 µmol/L primers for each gene. PCR reaction mixtures were denatured at 94°C for 2 min and cDNA templates amplified as follows: 35 cycles (22 cycles for GAPDH) of denaturation at 94°C for 30 s; annealing temperature and time are different depending on primers: SDF-1 at 61°C for 20 s; Bcl-2 at 60°C for 45 s; Bax at 57°C for 45 s; GAPDH at 55°C for 30 s; and extension at 72°C for 30 s, with a final extension time of 5 min at 72°C. The PCR products were size-fractionated by 1.2% agarose gel electrophoresis. The sequences of primers were: SDF-1 (116 bp): 5′-ATGCCCCTGCCGATTCTTTG-3′ (forward), 5′-TGTTGTTGCTTTTC-AGCCTTGC-3′ (reverse); Bcl-2 (560 bp): 5′-AGATGAA-GACTCCGCGCC-3′ (forward), 5′-GTAGTGAGACCCACGTATG-GACC-3′ (reverse); Bax (94 bp): 5′-TTCATCGAGCCCAGCA-3′ (forward), 5′-CTCGCTCAGCTTCTTGGTC-3′ (reverse); GAPDH (308 bp): 5′-TCCCTCAAGATTGTCAGCAA-3′ (forward), 5′-AGATC-CACAACGGATACATT-3′ (reverse).

Western blot analysis

Western blot was performed as previously described (Cheng et al., 2008b). Briefly, cells were incubated with lysis buffer at 4°C for 30 min, and then centrifuged for 30 min at 12,000 rpm at 4°C. Denatured proteins (40 µg) were separated by SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk in 1 × Tris Buffered Saline Tween-20 (TBST, Tris 1.21 g, NaCl 9.00 g, Tween-20 1 mL, ddH$_2$O 1 L), the membranes were incubated with rabbit anti-bcl-2, rabbit anti-Bax, rabbit anti-p-Erk, goat anti-Erk, rabbit anti-p-Akt and mouse anti-actin (1100, all antibodies were from Santa Cruz Biotechnology Inc., Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, rabbit anti-goat IgG or goat anti-mouse IgG (11000, Zymed, San Francisco, CA) was used as the secondary antibody. Hybridizing bands were visualized using Super-Signal West Pico Substrate (Pierce, Rockford, IL). The signals were quantified by densitometry.

ELISA assay

Conditioned media were collected after re-oxygenation for 16 h, followed by 10 min centrifugation at 12,000 rpm, 4°C. The concentration of VEGF or bFGF was measured with ELISA kits according to the manufacturer's instruction (ADL Company, Shanghai, China).

Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test was used for two-group comparisons and one-way ANOVA was applied for multiple group comparisons. Statistical significance was obtained when $p \leqslant 0.05$.

ACKNOWLEDGEMENTS

ABBREVIATIONS

CXCR4, CXC chemokine receptor 4; G-CSF, granulocyte colony-stimulating factor; LDH, lactate dehydrogenase; MSCs, mesenchymal stem cells; ROS, reactive oxygen species; SCF, stem cell factor; SD, Sprague-Dawley; SDF-1, stromal-derived factor-1

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