Cardiovascular Research (2008) **77**, 134–142
doi:10.1093/cvr/cvm025

Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium

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Received 16 May 2007; revised 10 September 2007; accepted 17 September 2007; online publish-ahead-of-print 22 September 2007

Time for primary review 33 days

KEYWORDS
Preconditioning;
Myoangiogenesis;
Apoptosis;
Infarction;
Stromal-derived factor 1 alpha

Aims We hypothesized that preconditioning (PC) with stromal-derived factor 1 alpha (SDF-1) significantly enhances cell survival, proliferation, and engraftment of bone marrow-derived mesenchymal stem cells (MSCs) via SDF-1/CXCR4 signaling.

Methods and results MSCs were cultured and then incubated in medium for 60 min without SDF-1 (control group) or with SDF-1 0.05 μg/mL (SDF-1 group) or CXCR4-selective antagonist, AMD 3100 (AMD) (5 μg/mL, AMD group) or SDF-1 and AMD (0.05 μg/mL, 5 μg/mL, respectively, SDF-1+AMD group). MSCs were treated for 60 min, washed in normal medium, and then exposed to H_2O_2 (100 μmol/L) for 60 min to determine the effects of various treatments on cell injury, viability, and proliferation. For *in vivo* studies, rats were grouped ($n = 6$) after left anterior descending coronary artery ligation to receive 20 μL Dulbecco's modified Eagle's medium without cells or with 5×10^5 non-preconditioned MSCs (control group), SDF-1 preconditioned MSCs (SDF-1 group), AMD (AMD group), or MSCs treated with SDF-1 plus AMD (SDF-1+AMD group). Heart function, infarct size, fibrosis, and MSC proliferation and differentiation in infarcted myocardium were determined after 4 weeks. *In vitro* data showed a marked increase in cell viability and proliferation following SDF-1 PC. *In vivo* data in preconditioned group showed a robust cell proliferation, reduction in infarct size and fibrosis, and significant improvement in cardiac function. Effects of SDF-1 PC were abrogated by CXCR4 antagonist.

Conclusion We conclude that PC with the chemokine SDF-1 suppresses MSCs apoptosis, enhances their survival, engraftment, and vascular density, and improves myocardial function via SDF/CXCR4 signaling. Chemokine PC is a novel approach for enhancing stem cell survival and regeneration of infarcted myocardium.

1. Introduction

Myocardial infarction (MI) is an important cause of morbidity and mortality among adults in industrialized countries. Recent attempts to repair infarcted myocardium using stem cells derived from bone marrow,[1,2] myoblast,[3] and cardiac tissue[4] have been made. The current data have shown that bone marrow-derived mesenchymal stem cells (MSCs) represent a suitable sub-cell type for regeneration of infarcted myocardium.[1,2]

MSCs are self-renewing and clonal precursors of non-haematopoietic tissues. The promising therapeutic effect(s) of MSCs is dependent on their capacity to survive and engraft in the target tissue. However, the transplantation of as many as 6×10^7 of these putative MSCs into infarcted porcine hearts yielded only marginal improvement in cardiac function.[5] It was reported that a limited number of cells survived past 1 week MSCs injection.[6] This is at least in part by poor viability and increased apoptosis of the transplanted cells in ischaemic environment. Therefore, strategies to enhance their survival are important for their proliferation and differentiation into cardiac phenotypes leading to cardiac regeneration in the infarcted myocardium.

SDF-1 is a member of the chemokine CXC subfamily initially cloned from the murine bone marrow stromal cell lines ST-2 and PA6,[7] then purified from supernatant from the murine MS-5 cell line.[8] Unlike other chemokines that interact with multiple G-protein coupled receptors, SDF-1 mediates its effects through its only known specific receptor, CXCR4.[9,10] Both SDF-1 and CXCR4 are constitutively

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expressed in a variety of tissues and cell types.[11] Local administration of SDF-1 using plasmid vectors promotes collateral development suggesting that SDF-1 mediated recruitment of circulating endothelial progenitor cells is important for collateral vessel development.[12] Mounting evidence thus suggests that the SDF-1/CXCR4 axis may play a role in blood vessel growth and development.

Ischaemic PC is known to be extremely protective against the ischaemic injury.[13,14] The signalling pathways of ischaemic preconditioning (IPC) could be exploited pharmacologically to mimic its protective effects without PC ischaemia. SDF-1/CXCR4 axis activates several signalling pathways in the target cells and plays a crucial role in both cell trafficking and interaction with the intercellular environment. The PI3K/Akt pathway is an important mediator of cell survival in many cell types.[15–17] MSCs transfected with *Akt* (encoding the *Akt* protein) were very effective in PC in preventing apoptosis and cardiac remodelling following MI.[18] However, the role of SDF PC in Akt phosphorylation and its effects on cell pathology are unknown. To exploit the potential of PC in MSCs survival and differentiation, we tested the hypothesis that SDF-1 PC augments MSCs survival and myocardial repair in a rat model of MI.

2. Methods

The present study conforms 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) and animal protocol was approved by the Institutional Animal Care and Use Committee, University of Cincinnati.

2.1 Isolation and expansion of mesenchymal stem cell (*In vitro* and *In vivo* experiments)

MSCs were isolated and harvested on the basis of their preferential adherence to plastic surface of cell culture flasks as previously described by us.[1] In brief, bone-marrow MSCs were extracted from the femur and tibia of 3-month-old rats and resuspended in the Dulbecco's minimum essential medium (DMEM) with 20% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were incubated in 95% air and 5% CO_2 at 37°C for 48 h, and the adherent cells were washed twice consecutively in phosphate-buffered saline (PBS). The cultures were depleted of erythroid progenitor cells through the removal of cells that did not adhere to the culture dishes with medium changes. At 80% confluence, cells were harvested with 0.25% trypsin and passaged at a ratio of 1:3. Subsequent passages were performed similarly. Passage 2–4 MSCs were used in the study. The medium was changed three times per week for 21 days prior to their use.

2.2 Experimental design

2.2.1 *In vitro* experiments

2.2.1.1 Mesenchymal stem cells preconditioning

MSCs in primary culture were randomly assigned to one of four experimental groups as follows: (i) non-preconditioned control; (ii) PC: incubation with SDF-1 (0.05 μg/mL; SDF group); (iii) incubation with AMD, a CXCR4 antagonist (5 μg/mL, AMD group); (iv) incubation with SDF-1 and AMD (0.05 μg/mL, 5 μg/mL; SDF-1+AMD group), respectively. All groups were treated for 60 min and then rinsed for 30 min in normal medium. These groups of MSCs were transplanted in hearts following LAD ligation.

MSCs were treated as described above. At the beginning of the experiment, culture media were replaced with the serum-free DMEM at 37°C during the entire experimental period. The effects of various treatments were determined *in vitro* by exposing MSCs to H_2O_2 (100 μmol) for 60 min. H_2O_2 has been used by several laboratories as an oxidant which induces cellular changes similar to ischaemia or anoxia.[19]

2.2.1.2 Measurement of lactate dehydrogenase and cell viability

The cell viability was evaluated after treatment with H_2O_2 by the trypan blue dye-exclusion method and number of viable cells was calculated by dividing the number of trypan blue negative cells by the total number of cells examined and then multiplied by 100. Cell supernatant was analysed for LDH using a Sigma assay kit at 340 nm.

2.2.1.3 Cell proliferation assay

The cell proliferation assay was performed with the use of the MTS [3-(4,5-dimethylthiazol-2-*yl*)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay according to the manufacturer's recommendations (Promega). Briefly, 96-well plates were coated with human fibronectin and seeded with 5×10^5 cells/well in 100 μL of DMEM medium containing 5% FBS. After 72 h of SDF-1 treatment or PC, 20 μL of CellTiter 96® Aqueous One Solution reagents (Pomega Inc.) was added to each well and cells were incubated for 3–4 h. Subsequently, plates were read at 490 nm using an automated ELISA plate-reader for the quantity of formazan product which was directly proportional to the number of living cells in culture.[20]

2.2.1.4 Enzyme-linked immunosorbent assay (ELISA) for VEGF

To determine whether SDF PC causes an increase in VEGF release from MSCs, an enzyme-linked immunoassay (ELISA, R&D System) was used to quantify VEGF levels in various groups incubated for 60 min according to the manufacturer's protocol as described.[21] The results were compared with a standard curve constructed with murine VEGF (each assay carried out in triplicate for each group). Absorbance was measured at 450 nm by means of a microplate reader.

2.2.1.5 Apoptosis assay

The number of apoptotic cells after H_2O_2 exposure was evaluated by staining with fluorescein isothiocyanate (FITC) Annexin V and quantitated by flow cytometric analysis (FACS Scan, Becton Dickinson; Mountain View, CA, USA).

2.2.1.6 Western blot analysis for Akt

Western blot analysis was performed to determine total Akt and phosphorylated Akt in different treatment groups. Protein samples (30 μg of protein) were mixed with an equal volume of sample buffer (containing 2% SDS, 100 mM Tris, 0.2% bromophenol blue, 20% glycerol, and 200 mM DTT) and boiled for 15 min before loading into each well on 10% polyacrylamide gels (Precast Gels, ISC Bioexpress). These electrophoresed proteins were transferred from the gel to the nitrocellulose membranes (Bio-Rad). Equal loading and transfer of proteins were confirmed by Ponceau's red staining. The membranes were incubated for 60 min with 5% dry milk and Tris-buffered saline to block non-specific binding sites. Membranes were immunoblotted overnight at 4°C with antibodies against Akt (1:1000, cell signalling), Phospho-Akt [Ser473] (1:1000, cell signalling), on a rocking platform overnight. After three 5 min washings, the membranes were incubated for an hour with HRP-conjugated secondary antibody, washed and developed with the ECL plus kit (Bio-Rad, USA).

2.2.2 *In vivo* experiments

2.2.2.1 Myocardial infarction model

MI model was developed in Fisher female rats (200–250 g), as previously described.[1] Briefly, rats were anesthetized with isoflurane. A midline cervical skin incision was performed for intubation. The animals were mechanically ventilated with room air supplemented with oxygen (1.5 L/min) using a rodent ventilator (Model 683,

Harvard Apparatus, South Natick, MA, USA). Body temperature was carefully monitored with a probe (Cole-Parmer Instrument, Vernon Hill, IL, USA) and was maintained at 37°C throughout the surgical procedure. The heart was exposed by left side limited thoracotomy and the left anterior descending (LAD) coronary artery was ligated with a 6-0 polyester suture 1 mm from tip of the normally positioned left auricle. Three days after LAD ligation, rats were re-operated, and MSCs (5×10^5) were injected into two sites in the periphery of infarcted LV. Control animals underwent LAD ligation and only saline was injected. After 4 weeks, the animals were sacrificed and hearts were frozen or fixed with 10% formalin solution, then processed for embedding in paraffin wax.

2.2.2.2 PKH26-labelling of mesenchymal stem cell

Prior to transplantation in the hearts, a cell suspension containing 5×10^5 MSC was labelled with PKH26 (Sigma, Product no.: PKH26-GL) according to manufacturer's instructions. The lipophilic dye PKH26 binds irreversibly to the cell membranes[22] and serves an important marker for tracking MSCs in the infarcted tissue.

2.2.2.3 Measurement of infarct size

Fixed hearts were embedded in paraffin and sections from apex, mid-LV, and base were stained with Trichrome–Masson. Images of LV area of each slide were taken by Olympus BX41 with CCD (MagnaFire™, Olympus) camera.

Fibrosis and total LV area of each image were measured using the Image-Pro Plus (Media Cybernetics Inc., Carlsbad, CA, USA), and the percentage of the fibrotic area was calculated as shown: (fibrosis area/total LV area) $\times$ 100.

2.2.2.4 Reverse transcriptase–polymerase chain reaction analysis for SDF-1 expression in the ischaemic myocardium

Tissue RNA was extracted from frozen heart tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase–polymerase chain reaction (RT–PCR) of the SDF-1 and GAPDH genes was performed using 1 μg of total RNA. The sequences of SDF-1primers: Forward: 5′-GCTCTGCATCAGTG ACGGTA; Reverse: 5′- TAATTTCGGGTCAA- TGCACA. The PCR products were size-fractionated by 1.5% agarose gel electrophoresis.

2.2.2.5 Physiological assessment of cardiac function

Heart function was assessed by transthoracic echocardiography, which was performed 4 weeks after MI using iE33 Ultrasound System (Phillips) with a 15 MHz probe. LV parameters were obtained from two-dimensional images and M-mode interrogation in long-axis view. LV ejection fraction (LVEF) was calculated as: LVEF (%) = [left ventricular end-diastolic dimension $(LVDd)^3$ − left ventricular end-systolic dimension $(LVDs)^3$]/$(LVDd)^3 \times 100$, and interventricular septum thickness (IVST) and left ventricular posterior wall thickness (LVPWT) were calculated. All echocardiographic measurements were averaged from at least three separate cardiac cycles.

2.2.2.6 Immunohistochemical analysis of infarcted hearts

Immunohistochemical analyses were performed on cryo and paraffin LV sections. The stained sections were digitally imaged using a computerized image-analysis system. For the homing assessment of transplanted MSCs, the number of PKH 26 positive cells was counted in ten randomly selected areas. Sections were incubated with primary antibodies specific to α-sarcomeric actin (A2172, Sigma), CD31 (sc-162, Santa Cruz), c-kit (Dako), and treated with respective secondary antibodies. Nuclei were stained with 4′, 6--diamino-2-phenylindole (DAPI) when necessary. Blood vessel density in infarcted myocardium was calculated in at least eight randomly high power fields in each heart section. Fluorescent images were obtained with an Olympus BX 41 microscope equipped with digital camera (Olympus) and Leitz DMRBE fluorescence microscope equipped with a TCS 4D confocal scanning attachments (Leica Inc.).

2.2.2.7 TUNEL analysis

Apoptotic myocytes in the infarcted myocardium were evaluated after 3 days of cell transplantation by TUNEL assay using serial paraffin sections using an MEBSTAIN Apoptosis Kit-ll (Medical and Biological Laboratories Co., Ltd). TUNEL assay was performed in deparaffinized 5 μm thick sections. More than 12 fields per heart in each group were examined in normal, periinfarct, and infarct areas microscopically. In each section, four fields were selected for examination. The number of apoptotic cardiomyocytes was counted per high power field.

2.2.2.8 Statistical analysis

Experiments were performed in quadruplicate and repeated at least three times. Data are expressed as mean ± SEM. Statistical significance was assessed by ANOVA followed by Bonferroni/Dunn testing, or unpaired *t* test. A *P*-value less than or equal to 0.05 was considered statistically significant.

3. Results

3.1 *In vitro* studies

3.1.1 Cytoprotective effects of preconditioning on mesenchymal stem cell

SDF-1 PC cells were markedly protected against H_2O_2, as indicated by reduced LDH release when compared with the control group (*Figure 1A*). Similarly, cell viability evaluated by trypan blue exclusion assay showed the highest number of viable cells in SDF-1 PC group compared with the control and

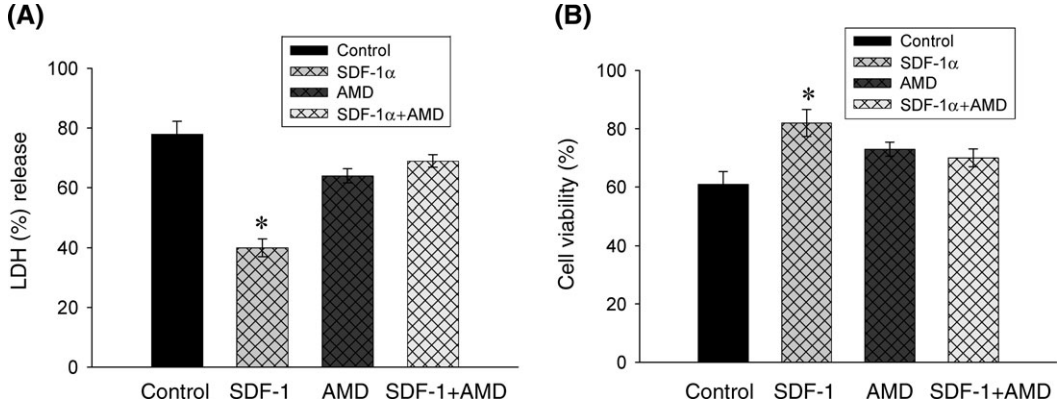

Figure 1 Effects of SDF-1 preconditioning on mesenchymal stem cells (MSCs) viability against oxidant induced injury. (*A*) LDH release was evaluated in the cell supernatant. (*B*) Damaged cells were counted using trypan blue exclusion. LDH release was decreased and cell viability was increased by SDF-1 preconditioning compared with control non preconditioned MSCs. All values were expressed as mean ± SEM. *$P < 0.05$ vs. control.

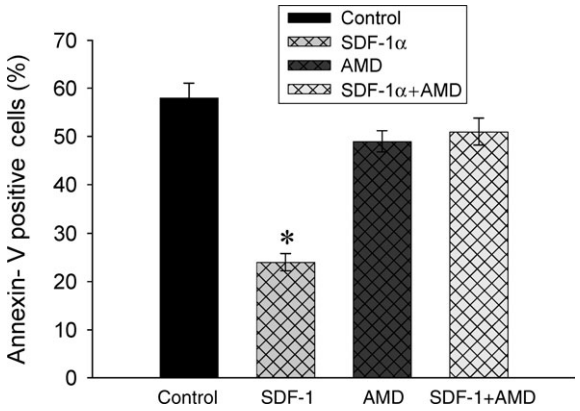

Figure 2 Apoptosis was determined by flow cytometry after staining with annexin V- FITC. Fewer apoptotic cells were observed in SDF-1 pretreated mesenchymal stem cells (MSCs) (24%) when compared with control non-preconditioned MSCs (55%) suggesting that SDF-1 prevents apoptosis of MSCs. All values were expressed as mean ± SEM. *$P < 0.05$ vs. control.

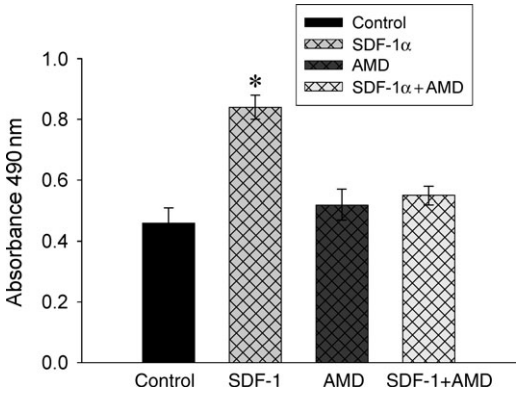

Figure 3 Effects of preconditioning on cell proliferation. An increase in mitogenic response of SDF-1 preconditioning on mesenchymal stem cells was significant compared with non preconditioned and other groups. All values were expressed as mean ± SEM, *$P < 0.05$ vs. control.

other groups (*Figure 1B*). The number of apoptotic cells was significantly reduced in SDF-1 PC group compared with the control and other groups (*Figure 2*).

3.1.2 Stimulation of cell proliferation by preconditioning

To examine whether MSCs exhibited high proliferation rate in preconditioned group, MTS assay was used to determine proliferative activity *in vitro*. With the use of 5% serum-conditioned media, proliferation rate of SDF-1 preconditioned MSCs was significantly higher compared with non-preconditioned MSCs (0.84 ± 0.04 vs. 0.46 ± 0.05 absorbance at 490 nm). The proliferative ability of MSCs by PC was completely abolished by AMD. However no significant changes were observed in other groups (AMD or SDF-1/AMD) (*Figure 3*).

3.1.3 Secretion of vascular endothelial growth factor from preconditioned mesenchymal stem cells

A significant amount of vascular endothelial growth factor (VEGF) was released from SDF-1 preconditioned MSCs (76.37 ± 2.861 pg/mL) when compared with non-preconditioned MSCs (43.16 ± 1.016 pg/mL; $P < 0.05$, *Figure 4*). AMD blocked the release of VEGF from MSCs (76.37 ± 2.861 vs. 43.16 ± 1.016 pg/mL).

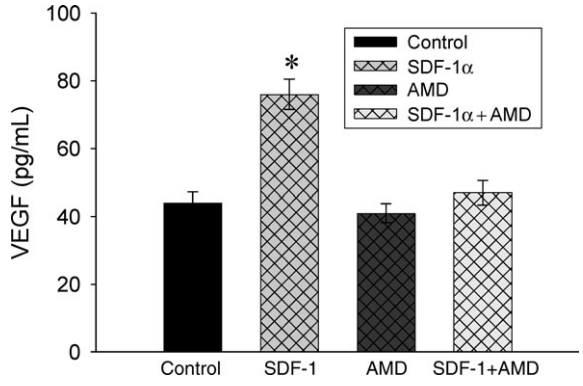

Figure 4 Preconditioning increased vascular endothelial growth factor (VEGF) release from mesenchymal stem cells in culture. A significant release of VEGF from mesenchymal stem cells in response to SDF-1 preconditioning was observed when compared with non-preconditioned control and other treatment groups. All values were expressed as mean ± SEM, *$P < 0.05$ vs. control.

3.2 *In vivo* studies

3.2.1 Preconditioning promotes homing and proliferation of transplanted vascular endothelial growth factors in infarcted myocardium

The number of transplanted MSCs in the recipient heart was identified by PKH 26 labelling. DAPI was used to identify nuclei. SDF-1 expression was markedly upregulated in the ischaemic myocardium 3 days after MSC transplantation (*Figure 5A*). A significant increase in the homing of PKH26 positive cells was observed at the site of infarction and peri-infarct areas in SDF-1 PC group (169.3 ± 23.4 per field, $P < 0.01$ vs. control [21.3 ± 5.7 per field]); (*Figure 5B, F–H, C–E*). Blockade of the SDF-1/CXCR4 interaction with AMD3100 reduced the homing of transplanted MSCs to the infarcted myocardium from 34.2 ± 8.5 to 21.3 ± 5.7 per field, $P < 0.05$, (*Fig.5I–K*). PKH26 remained in the cells and did not fade until 35 days. Migration of c-kit positive cells from the bone marrow to the ischaemic myocardium was also significantly increased by SDF-1 treatment from 2.1 ± 0.88 (cells per field) in control group to 18.2 ± 1.6 (cells per field) in SDF-1 group, $P < 0.05$ (*Figure 5O* and *P*), while no increase in mobilized cells was observed in other groups (*Figure 5P*).

3.2.2 Preconditioning enhances neoangiogenesis in ischaemic myocardium

Hearts transplanted with SDF-1 preconditioned MSCs showed significant neoangiogenesis in the ischaemic area. The number of blood vessels (CD31 positive) was significantly increased in SDF-1 group when compared with control group (27.6 ± 3.1 vs. 12.4 ± 2.5 $P<0.01$) (*Figure 6*, A–D). Approximately 2-fold increase in blood vessel density was observed in SDF-1 preconditioned group (*Figure 6E*) and this increase was abolished by AMD as shown by reduced number of vessels (CD31 positive) (*Figure 6C–E*).

3.2.3 Preconditioning activates phosphoinositide 3-kinase/Akt pathway

Akt is a known downstream effector of the phosphoinositide 3-kinase (PI3K)-dependent signalling cascade. Recent evidence suggests that Akt promotes cell survival by inhibiting apoptosis. *In vitro* experiments showed that SDF-1 PC

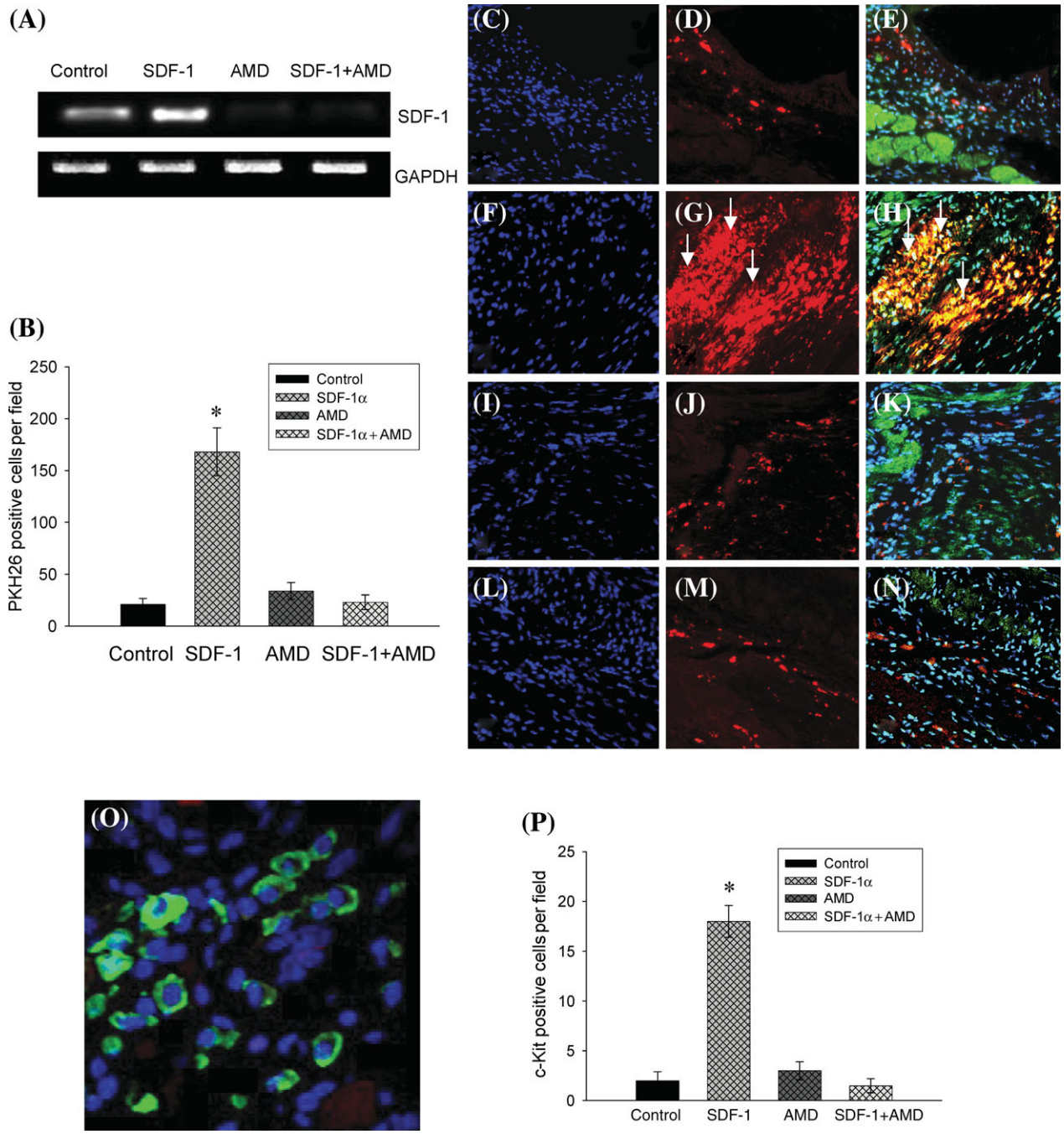

Figure 5 Effect of preconditioning on homing and proliferation of mesenchymal stem cells (MSCs) in infarcted myocardium. Representative immunofluorescent micrographs of hearts transplanted with PKH26 labelled MSCs. DAPI was used to identify nuclei. PKH26 labelling is in red; and α-sarcomeric actin is in green; yellow labelling indicates cardiomyocytes derived from PKH26 positive cells (×200). SDF-1 preconditioning induced up-regulation of SDF-1 in the ischaemic myocardium (*A*) and the number of MSCs was increased by preconditioning (*B*). A limited number of PKH26 labelled MSCs were observed in control group (*C–E*). On the other hand, extensive homing and proliferation of the PKH26 positive MSCs and cardiomyocytes derived from PKH26 labelled MSCs in infarct and peri-infarct regions were observed in SDF-1 preconditioned group (arrow) (*F–H*). PKH26 labelled cardiomyocytes appeared yellow when colocalized with α-sarcomeric actin staining (arrow) and were seen along the native myocytes (H). AMD group (*I–K*); SDF-1+AMD group (*L–N*). (*O*) An increase in c-kit positive cells was observed in the border zone of the hearts pre-treated with SDF-1 (Confocal image, original magnification ×630). (*P*) Average number of c-kit positive cells per field after various treatments. All values were expressed as mean ± SEM. *$P < 0.05$ vs. non-preconditioning control group 1 ($n = 6$ in each group).

stimulated Akt phosphorylation, where no significant change in total Akt expression was observed. To determine whether CXCR4 pathway was involved in SDF-1 PC mediated Akt activation, AMD, a CXCR4 antagonist was included during PC. PC-induced Akt phosphorylation was inhibited by AMD (*Figure 7A–B*). These results suggest that PC-induced-Akt activation was dependent on CXCR4 receptor activation.

3.2.4 Effects of preconditioning on left ventricular remodelling and function

Four weeks after MSCs implantation, a marked reduction in left ventricle fibrosis was observed in SDF-1 preconditioned group compared with the non-preconditioned control group (15.36 ± 2.48% vs. 40.93 ± 1.42%, $P < 0.01$ (*Figure 8*). *Table 1* shows a comparison of the

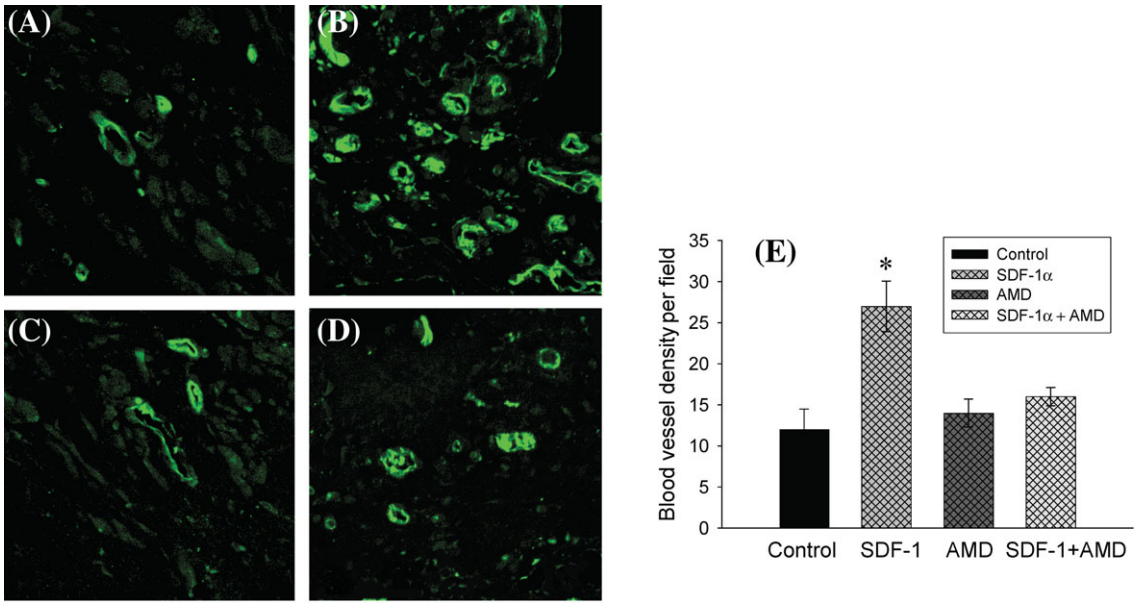

Figure 6 Imaging of blood vessels in various groups by immunohistochemistry (×400). (*A*) Non-preconditioned control group; (*B*) SDF-1 preconditioning group; (*C*) AMD preconditioning; (*D*) SDF-1+AMD preconditioning group. AMD blocked the effect of SDF-1 preconditioning on the increase in vascularization. (*E*) Quantitative estimate of capillary density in infarcted myocardium from various treatment groups. All values were expressed as mean ± SEM. *$P < 0.01$ vs. control.

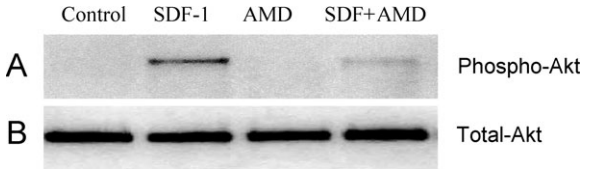

Figure 7 Expression of Akt in preconditioned mesenchymal stem cells. A significant increase in expression of phospho-Akt (*A*) in SDF-1 preconditioned group was observed. AMD inhibited the effect of SDF-1 preconditioning on Phospho-Akt. Total Akt remained unchanged after preconditioning (*B*).

echocardiography findings in various groups. No significant differences in LVDd were noted in control (7.4 ± 0.6 mm), AMD (7.6 ± 0.8 mm), and SDF-1+AMD (7.1 ± 0.5 mm) groups except in SDF-1 group (3.5 ± 0.4 mm) where it was significantly less compared to control group (7.4 ± 0.6 mm). LVDs showed a tendency similar to that of LVDd. LVDs was increased in the control (6.5 ± 0.8 mm), in AMD (6.6 ± 0.5 mm), and in SDF-1+AMD (6.1 ± 0.7 mm) groups and this increase was in significant. Similarly, LVDs in the SDF-1 (2.6 ± 0.3 mm) was significantly less when compared with control group 6.5 ± 0.8 mm).

LVEF and IVST/LVPWT as assessed by echocardiography were significantly improved in SDF-1 group (58.56 ± 3.27% and 0.9 ± 0.07, respectively) when compared with control group (33.23 ± 2.34% and 0.4 ± 0.06, respectively, $P < 0.05$). However, CXCR4 antagonist, AMD blocked the effect of PC on cardiac function. The LVEF was significantly reduced to 34.18 ± 2.70% in AMD group compared with 58.56 ± 3.27% in SDF-1 group. Similarly, AMD given during LAD ligation reduced LV fractional shortening. SDF-1+AMD group was also no different from the control group. The proportion of left ventricular wall thickness (IVST/LVPWT) was 0.4 ± 0.06 in control group, 0.5 ± 0.03 in AMD group, and 0.6 ± 0.04 in SDF-1+AMD group and it was significantly less when compared with SDF-1 group.

3.2.5 Effect of SDF-1 preconditioning on apoptosis

The number of TUNEL-positive cells in the peri-infarct region was significantly reduced in SDF-1 preconditioned group when compared with the control group (14.1 ± 1.8 nuclei per field vs. 21.4 ± 1.5 nuclei per field respectively, $P < 0.05$, *Figure 9A–C*). However, the number of apoptotic cells in other treated groups was similar to the control group (AMD group; 19.3 ± 1.6 nuclei per field; SDF-1+AMD group, 23.1 ± 1.9 nuclei per field).

4. Discussion

The major findings of this study are as follows. (i) PC with SDF-1 significantly improved the survival of MSCs within the ischaemic myocardium via Akt signalling pathway. (ii) PC augmented neovascularization and myogenesis by increased homing and proliferation of MSCs. (iii) SDF-1 PC attenuated LV remodelling and reduction in infarct size. (iv) SDF-1 PC induced MSCs to release paracrine factors, which promoted angiomyogenesis and enhanced MSC survival in the myocardium. Cytokines play an important role in differentiation of stem cells into cardiac phenotypes but the precise effect of different cytokines in cardiac differentiation is unknown. There is substantial evidence for the participation of the SDF-1/CXCR4 axis in the retention of myeloid lineage cells to the bone marrow. SDF-1 is also involved in angiogenesis and induces the formation of capillaries in mice.[23] Accordingly, we postulated that SDF-1, which is secreted in various tissues and organs, acts as an antiapototic factor and participates in regeneration/repair by extensive proliferation and differentiation of MSCs under ischaemic conditions.

IPC is a very powerful protective phenomenon against lethal ischaemic injury. PC of MSCs could result in enhanced survival of MSCs following transplantation in the ischaemic myocardium.[24] Our initial observations suggest that

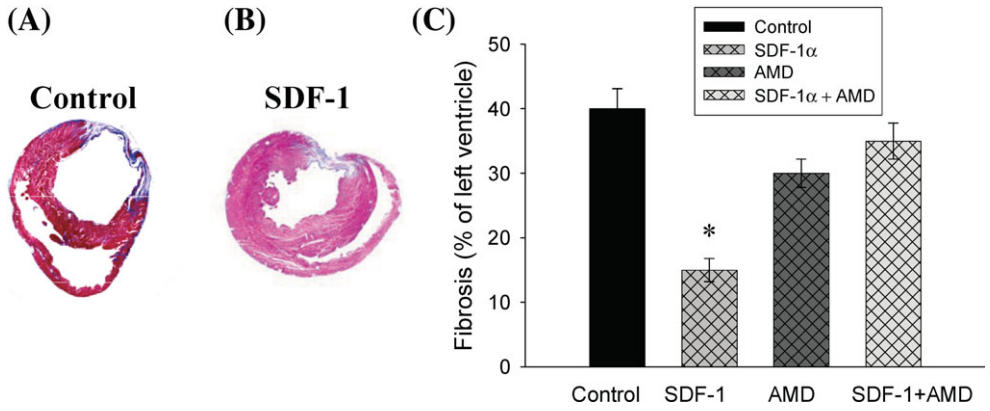

Figure 8 Effects of preconditioning on fibrosis. (*A*) Control and (B) SDF-1 preconditioned groups. (*C*) Quantitative analysis of fibrosis in various treatment groups. *$P < 0.01$ vs. control group ($n = 6$ in each group).

Table 1 Assessment of the cardiac function by echocardiography in various treatment groups

Group	Control	SDF-1	AMD	SDF-1+AMD
LVDd (mm)	7.4 ± 0.6	3.5 ± 0.4*	7.6 ± 0.8	7.1 ± 0.5
LVDs (mm)	6.5 ± 0.8	2.6 ± 0.3*	6.6 ± 0.5	6.1 ± 0.7
EF (%)	33.23 ± 2.34	58.56 ± 3.27*	34.18 ± 2.70	36.74 ± 4.15
IVST/LVPWT	0.4 ± 0.06	0.9 ± 0.07*	0.5 ± 0.03	0.6 ± 0.04

LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular diameter at end-systole; IVST, interventricular septum thickness; LVPWT, left ventricular posterior wall thickness; EF, ejection fraction. AMD, AMD3100. *$P < 0.05$ vs. control group. $n = 6$ in each group.

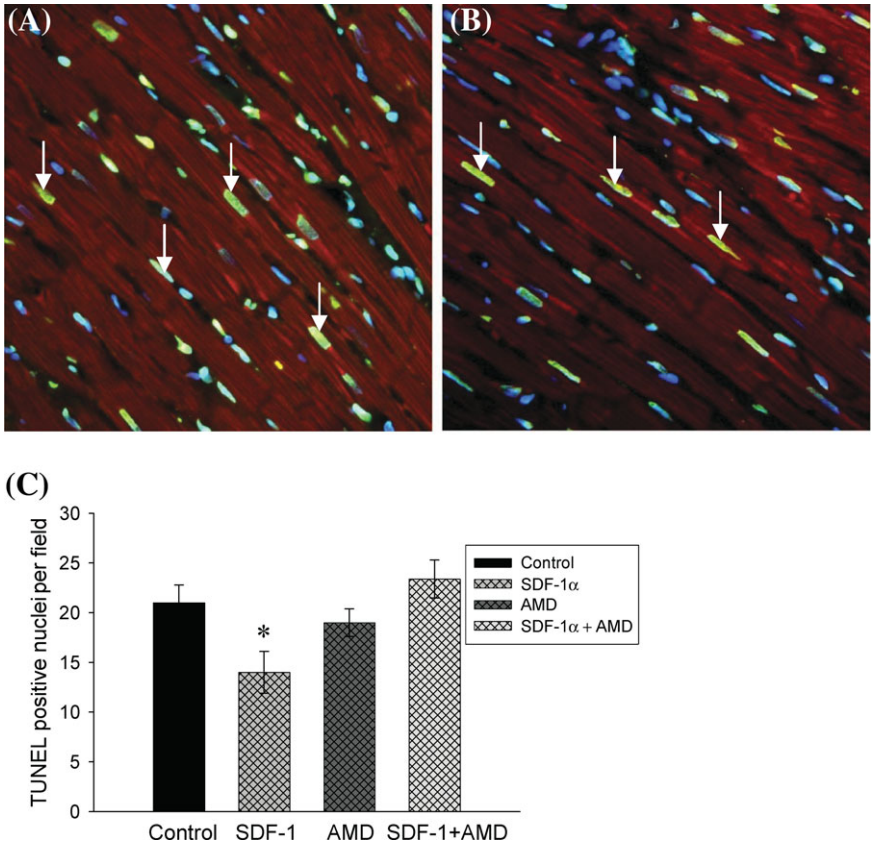

Figure 9 Apoptotic cardiomyocytes in the peri-infarct area at 3 days after LAD ligation. TUNEL positive nuclei (green, arrow, 400×) [(*A*) Non-preconditioned MSCs; (*B*) SDF-1 preconditioned mesenchymal stem cells). (*C*) Semiquantitative estimate of TUNEL-positive cardiac nuclei in heart tissue sections from various treatment groups. A total of 20 sections in each group were analysed. Data shown as mean ± SEM. *$P < 0.05$ vs. control.

stem cells can be preconditioned by growth factors[25,26] to enhance their effectiveness in myoangiogenesis following transplantation in the infarcted myocardium.[26]

Stromal cell derived factor and its receptor CXCR4 are important in the homing of bone marrow derived stem cells to the infarcted myocardium.[27] SDF-1 is involved in angiogenesis[23] and neovascularization[28,29] by recruiting endothelial progenitor cells. SDF-1 enhances cell survival.[29] Our *in vitro* data reported in this study supported the proposal that SDF-1 PC promotes MSC survival and proliferation under anoxic conditions. Accordingly, a significant differentiation of preconditioned MSCs into cardiac myocytes and blood vessels was observed in the infarcted myocardium (*Figures 5* and *6*). In addition, enhanced ability of preconditioned MSCs to survive and repopulate the infarcted myocardium resulted in reduction of infarct size and LV remodelling.

PC-derived effects could be multiple. Our data suggest that PC by SDF-1 leads to differentiation of MSCs into myogenic cells. PC with cytokines may stimulate endogenous genetic machinery of MSCs and promote their commitment to angiomyogenic cells by upregulating cardiac transcription factors. It has been reported that PC of human foetal liver CD133 stem cells with $VEGF_{165}$ enhanced the formation of angiomyogenic cells.[25] Similarly, FGF-2 could regulate the fate and cardiogenic conversion of undifferentiated progenitor stem cells.[30] We recently reported that anoxic PC increased the cardiac transcription factors such as GATA 4 and MEF-2C in MSCs and their transplantation reduced the infarct size and LV wall remodelling.[31] Although cardiac gene expression was not assessed in this study, the formation of new myocytes and blood vessels suggests the upregulation of cardiac transcription factors by cytokine PC.

Our *in vitro* data show a significant increase by MSCs in VEGF secretion which may participate in angiogenesis.[31] Blood vessel density is significantly increased in hearts following transplantation of SDF-1 preconditioned MSCs and this effect is abolished by AMD. Thus, the increased availability of nutrients and oxygen in the treated infarcted hearts may allow robust growth and survival of transplanted MSCs. It is also possible that since PC MSCs secreted large amount of antiapoptotic cytokine, VEGF,[31] the beneficial effects may also be through paracrine mediators. In addition, antiapoptotic effect of VEGF may exert a marked inhibitory effect on pathological myocardial remodelling.[31,32] These cellular responses seem to be unique to the microenvironment of the ischaemic myocardium and the release of paracrine mediators by MSCs as VEGF could lead to *trans*-endothelial migration and proliferation of MSCs. As expected, the mitogenic effect of VEGF was inhibited by CXCR4 blocker, AMD.

Another major effect of SDF-1 PC was the reduction of apoptosis both under *in vitro* and *in vivo* conditions. However, these protective effects were reversed by AMD, an antagonist of CXCR4 suggesting that beneficial effect of SDF-1 is modulated through CXCR4 signalling pathway. Our conclusions are in agreement with several earlier studies that SDF-1 exerts its effects via activation of CXCR4.[33–36]

The underlying mechanism of protection by SDF-1 PC appears to be the activation of Akt (protein kinase B) survival pathway. Akt was upregulated by cytokine PC resulting in prevention of apoptosis. The role of Akt has been well established in a variety of cardiovascular diseases. Akt is an effector molecule for many cellular functions initiated by growth factors[37] and is involved in regulation of gene transcription, protein synthesis, cell signalling, cell hypertrophy, and cell survival.[37,38] Thus, Akt is an important therapeutic factor for preserving MSCs integrity and survival in the early post-transplant period.[24] In this study, we clearly showed SDF-1 PC induced Akt phosphorylation and reduced apoptosis. To further verify whether the PC induced Akt activation was mediated via CXCR4, we preconditioned cells together with AMD, a peptide antagonist known to block the CXCR4/SDF-1 interaction. No Akt phosphorylation was observed in AMD treated group. Our results suggest that SDF-1 induced Akt activation through its interaction with its receptor, CXCR4.

In summary, this is the first study to show that chemokine PC plays a major role in the homing and proliferation of MSCs in the heart after MI and promotes neovascularization and myogenesis. Release of antiapoptotic cytokine by preconditioned MSCs enhance their ability to survive under ischaemic conditions by activation of Akt signalling pathway. The cumulative effect of PC on myocyte regeneration, angiogenesis, and cell survival in the ischaemic myocardium resulted in reduced infarct size and LV remodelling. Thus this novel, cell-based therapeutic approach has the potential in minimizing the adverse effects of ischaemia on cell death and cardiac remodelling.

Funding

This work was supported by National Institutes of Health grants, HL 23597, HL 080686, HL 70062, R 37-HL 074272 and HL 087246 (M.A.); HL-081859-01 (Y.W.).

Conflict of interest: none declared.

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