Co-treating mesenchymal stem cells with IL-1β and TNF-α increases VCAM-1 expression and improves post-ischemic myocardial function

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Abstract. Inflammatory mediators are released by the myocardium following myocardial ischemia as a response to tissue injury, and contribute to cardiac repair and adaptive responses. Treating mesenchymal stem cells (MSCs) with various inflammatory factors activates a series of biological processes that enhance cell-mediated cardioprotection following myocardial infarction (MI). The present study was designed to examine the effect of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) treatment on vascular cell adhesion molecule-1 (VCAM-1) expression in MSCs, and to identify whether cytokine-treated MSCs improve post-ischemic myocardial function in a rat model. MSCs were stimulated with IL-1β and/or TNF-α for 24 h, the production of vascular cell adhesion molecule-1 (VCAM-1) and the adhesion ability of MSCs were assessed by flow cytometry, adhesion assays, quantitative polymerase chain reaction and western blot analysis. The cardiac function was examined by two-dimensional echocardiography. The results demonstrated that in treated MSCs, the secretion of VCAM-1 and the cell adhesion ability were significantly increased, thus markedly improving cardiac function compared with that of the control group (P<0.01). Of all the groups, the rats stimulated with a combination of IL-1β and TNF-α exhibited the greatest cardiac improvements. However, there was no significant difference between the 10 and 20 ng/ml groups which were stimulated with one of the cytokines alone (P>0.05). In conclusion, stimulating MSCs with IL-1β and TNF-α promoted the expression of VCAM-1 and improved post-ischemic cardiac function recovery. Treating MSCs with two cytokines in combination may be a useful method to maximize the potential of cell-based therapy for MI.

Introduction

As a novel therapeutic strategy to prevent or reverse ventricular remodeling, heart failure, arrhythmias and myocardial infarction, mesenchymal stem cell (MSC)-based cell therapy replaces endogenous myocardial repair as an improved approach with marked potential (1-3). However, this approach has a number of limitations that restrict its application, including low efficiency of MSCs in colonization, survival and differentiation towards myocardial tissue, and diminished donor cell-function in an ischemia microenvironment following transplantation (4-6). Therefore, it is crucial that studies focus on devising a mechanism to increase the survival of cells following transplantation to areas of ischemia tissue.

The activated inflammatory response and cytokine elaboration following myocardial infarction together contribute to cardiac remodeling and eventual host outcome (7). Cytokines are released immediately following ischemia in order to modulate tissue repair and adaptation. Previous studies revealed that MSCs treated with inflammatory mediators activate a series of pathophysiological processes, including cell survival, cell migration, cell adhesion, chemokine release, induction of angiogenesis and modulation of immune responses (8-10). Several studies have demonstrated that pretreatment of MSCs with cytokines, which were released by the myocardium following ischemic injury, increased MSC-mediated cardioprotection following acute myocardial infarction (AMI) (11,12). Interleukin (IL)-1β and tumor necrosis factor (TNF)-α are not constitutively expressed in normal myocardium; however, their levels markedly increase in the infarct and non-infarct areas following AMI (13).

Cell adhesion molecules and their ligands, extracellular matrix components, chemokines and specialized bone marrow niches all have roles in the precise regulation of MSC adhesion.

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to endothelial cells (14). It was reported that VCAM-1 together with its ligand very late antigens-4 (VLA-4) was able to bind to stromal or endothelial cells, which subsequently facilitated stem cell homing (14-16). Furthermore, blockage of VCAM-1 or VLA-4 markedly reduced stem cell migration and adhesion ability (15,17,18). Several investigations have also demonstrated that MSCs treated with appropriate cytokines affect the paracrine of cells and then improve cardioprotection (19-21).

In the present study, MSCs with two representative inflammatory cytokines were stimulated alone or in combination to examine the effect on the expression of VCAM-1 in MSCs and the cardiac protective efficiency of cell-transplantation therapy in a rat model of AMI.

**Materials and Methods**

**Animals.** Male Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center of Anhui Province (Anhui, China). The rats received a 12 h light and dark cycle everyday, and were kept at 20-25°C and 40-70% humidity. In addition, the rats were fed standard laboratory rodents feed ad libitum. All animals used in the present study received the appropriate care according to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). The present study was approved by the ethics committee of the Experimental Animal Center of Anhui Province.

**MSCs isolation and culture.** MSCs were isolated from bone marrow of male SD rats (4-6 weeks old) following the standard procedure with certain modifications (22). In brief, bone marrow cells were collected from the bilateral femurs and tibias by removing the epiphyses, flushing the cavity with Dulbecco’s modified Eagle’s medium (DMEM) and centrifuging the suspension for 10 min at 300 x g. The cell pellet was then resuspended and cultured in 25 cm² culture flasks with complete media containing 10% fetal bovine serum (HyClone, Logan, UT, USA), at 37°C, 90% humidity and 5% CO₂. Non-adherent cells in the suspension were discarded following 48 h and fresh complete medium was added and replaced every 3-4 days thereafter. At 90% confluence, the cells were trypsinized (0.25% trypsin) and passed at 1:3 ratios. Cells were identified by flow cytometry as described previously (23). MSCs between passages three and four were used for the following experiments.

**Stimulation of MSCs.** MSCs were stimulated for 24 h with IL-1β (PeproTech, Rocky Hill, NJ, USA; 10 or 20 ng/ml), TNF-α (PeproTech; 10 or 20 ng/ml) or IL-1β (10 ng/ml) combined with TNF-α (10 ng/ml). In the meantime, cells in the control group were incubated in parallel without stimulation. Control and treated cells were used for subsequent experiments. Each experiment was repeated at least three times.

**Flow cytometry.** The control and treated MSCs were harvested and adjusted to a cell density of 10⁶/ml, then resuspended in 100 µl phosphate-buffered saline (PBS; 1x10⁶ cells). The cells were then incubated with phycoerythrin (PE)-VCAM-1 (BD Biosciences, Franklin Lakes, NJ, USA) antibody at room temperature for 30 min in the dark. Following this, the cells were washed twice with PBS and dispersed to make a single cell suspension in 400 µl PBS. Labeled cells were assayed using a flow cytometer (BD FACSCalibur; BD Biosciences) and analyzed with FCS4 software (version 1.2.4.1; De Novo Software, Los Angeles, CA, USA). At least 10,000 events were analyzed for each sample.

**In vitro adhesion assays.** To analyze the MSC adhesion capacity, MSCs (5x10⁶cells/well) were seeded in collagen-coated 24-well plates in 250 µl complete medium and incubated for 20 min as previously described (8,24). The wells were gently washed twice with PBS to remove the non-adherent cells and the adherent cells were counted in six random fields per well under a microscope (magnification, x100). The quantity of cells adhered to the plate reflected the relative adhesion ability of the MSCs.

**Quantitative polymerase chain reaction (qPCR).** Total RNA was extracted by using the TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA); cDNA was obtained using a RevertAid First Strand cDNA Synthesis kit (Thermo Fischer Scientific) and then an amplification reaction was performed according to the manufacturer’s instructions. The gene-specific primers were designed using Primer Premier 5 software (Premier, Canada) based on cDNA sequences from Genebank and they were as follows: VCAM-1 forward, 5'-CCA GCG AGG GTC TAC CA-3' and reverse, 5'-ACA GGG CTC AGC GTC AG-3'; β-actin forward, 5'-GGT GCC GCC CCT AGG CAC CA-3' and reverse, 5'-CTC ATT GTC ACG CAC GAT-3'. A Biometra T-Gradient thermal cycler (Biometra, Göttingen, Germany) was used for PCR. The PCR conditions were as follows: denaturation at 94°C for 30 sec, annealing at 60°C (VCAM-1)/51°C (β-actin) for 30 sec and extension at 72°C for 40 sec for 35 cycles. The PCR products were electrophoresed on a 1.0% agarose gel stained with 0.5 µg/ml ethidium bromide. The electrophoresis gel containing the PCR products was scanned using the UVP gel imaging system (JD-801; Jieda, Nanjing, Jiangsu, China). The expression of VCAM-1 mRNA was normalized to the expression of β-actin mRNA.

**Western blot analysis.** Western blot analysis of cell lysates was performed as previously described (25), proteins were denatured in Laemmli sample buffer (Beyotime Institute of Biotechnology, Shanghai, China) for 5 min at 95°C, samples were separated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween-20 (TBST; Sigma, St. Louis, MO, USA) for 2 h prior to incubation with anti-VCAM-1 (1:1,000; Bioworld Technology, Inc., Minneapolis, MN, USA) overnight at 4°C and then conjugated with a secondary antibody, anti-rabbit Immunoglobulin G-horseradish peroxidase (Beyotime Institute of Biotechnology), for 1 h at room temperature. Membranes were washed three times in TBST and positive bands were detected by the enhanced chemiluminescence kit (Thermo Fischer Scientific). All the protein bands were scanned using Chemi Imager 5500 V2.03 software (Alpha Innotech, San Leandro, CA, USA). Protein band intensities were then analyzed by computerized image analysis system (Gel-Pro analyzer 4 software; Media Cybernetics, Rockville, MD, USA) and equal protein was normalized to β-actin.
Rat model of AMI and cell transplantation. Myocardial infarction was produced in male SD rats (weighing, 180-220 g) as previously described (26,27). First, a left thoracotomy was performed through the fourth intercostal space to expose the rat heart. Then, the left anterior descending coronary artery (LAD) was ligated with a 6-0 polyester suture. Successful ligation was confirmed by the typical myocardial infarction waves in electrocardiography recordings. The cells were harvested for 1 h prior to transplantation. The infarcted hearts (n=8) received intramyocardial injections of 100 µl control or treated MSCs (1x10^6 cells). The injections were performed at four different sites in the free wall of the left ventricles.

Assessment of cardiac function. Left ventricular (LV) function was assessed in anesthetized animals four weeks following transplantation of MSCs using two-dimensional echocardiography equipped with a 12-MHz probe (Philips Healthcare, Woerden, Netherlands). The animals were placed on a warming pad in the supine or lateral position. The greatest LV diameter of the internal end-diastole (LVID,d) and internal end-systole (LVID,s) was measured from the long axis view. The LV ejection fraction (LVEF,%) was calculated as: (LVID,d)^3-(LVID,s)^3)/(LVID,d)^3 x 100. All measurements were averaged on at least three consecutive cardiac cycles and analyzed by an observer blinded to the treatments received by the animals.

Histology. Animals were euthanized prior to isolation and sectioning of their hearts into two transverse slices through the infarct area. The hearts were fixed in 10% formaldehyde prior to being embedded in paraffin. Sections (3 µm) were stained with Masson’s trichrome according to the manufacturer's instructions (Maixin, Fuzhou, Fujian, China). Images of each slide were captured with an Olympus BX41 microscope (Tokyo, Japan). Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) was used to evaluate the percentage of myocardial infarction area which exhibited collagen deposition. The percentage of collagen deposition area was calculated as: (Fibrotic area/total LV area) x 100.

Statistical analysis. All values are expressed as the mean ± standard deviation (SD). Statistical analysis of the results between two groups was performed using a Student's t-test. Differences among the groups were determined by a one-way analysis of variance. The analysis was performed using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference between values.

Results

Flow cytometric analysis. Following the collection and immunostaining of the cells with antibodies, fluorescence was measured by flow cytometry. The results demonstrated the expression of cell surface markers, and that the majority of the cells were positive for CD29 and CD90, while they were negative for CD34. PE-VCAM-1 fluorescence intensities of treated MSCs were markedly increased and had statistical significance when compared with the control group (P<0.01; Fig. 1).

IL-1β and TNF-α enhance MSC adhesion in vitro. Pre-treatment with IL-1β and TNF-α significantly increased...
the MSC adhesion ability in vitro (P<0.01) with responses similar to those of the VCAM-1 expression. Incubation in 20 ng/ml cytokine moderately increased the number of adhered cells as compared with the 10 ng/ml treatment group; however, this difference was not statistically significant (P>0.05). There were only 8.4±2.3 cells adhered to the plate in the control group, but when treated with IL-1β, the number of MSCs adhered to the plate increased to 28.0±5.2 in the 10 ng/ml group and 30.4±3.4 in 20 ng/ml group. Similarly, in the TNF-α stimulation groups, the number of MSCs increased from 47.1±4.3 in the 10 ng/ml group to 49.7±6.2 in the 20 ng/ml group. By contrast, the number of adherent cells in the combined cytokine treatment group was significantly increased compared with the single cytokine groups (P<0.01). In the combined cytokine group, the number of MSCs adhered to the plates increased to 68.8±5.8 when treated with TNF-α (10 ng/ml) and IL-1β (10 ng/ml) (Fig. 2).

**IL-1β and TNF-α upregulate the gene expression of VCAM-1.** qPCR was used to detect VCAM-1 mRNA levels of MSCs.

**Table I. Effects on heart function four weeks following cell implantation.**

<table>
<thead>
<tr>
<th>Group</th>
<th>LVID,d (mm)</th>
<th>LVID,s (mm)</th>
<th>LVEF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n=5)</td>
<td>10.1±1.0</td>
<td>9.1±0.9</td>
<td>28.6±1.5*</td>
</tr>
<tr>
<td>I10 (n=6)</td>
<td>9.0±0.7</td>
<td>7.9±0.6</td>
<td>33.7±2.1**</td>
</tr>
<tr>
<td>I20 (n=7)</td>
<td>8.4±0.4</td>
<td>7.0±0.4</td>
<td>40.9±2.2**</td>
</tr>
<tr>
<td>T10 (n=7)</td>
<td>8.1±0.6</td>
<td>6.7±0.5</td>
<td>43.0±2.1**</td>
</tr>
<tr>
<td>I10+T10 (n=7)</td>
<td>8.0±0.4</td>
<td>6.3±0.4</td>
<td>49.9±2.4**</td>
</tr>
</tbody>
</table>

Echocardiographic measurement of LV at the end of the fourth week. Data are presented as the mean ± standard deviation. *P<0.01 compared with the control group; **P<0.01 compared with the combination group; #P<0.05 and $P>0.05 compared between the 10 ng/ml and 20 ng/ml groups. I10/20, treated with 10/20 ng/ml IL-1β; T10/20, treated with 10/20 ng/ml TNF-α; I10+T10, treated with 10 ng/ml IL-1β and TNF-α each; C, control; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.
Following subtraction of the background, VCAM-1 mRNA levels were compared among the different groups relative to the β-actin mRNA levels. Incubation with IL-1β (10 or 20 ng/ml) induced the mRNA expression of VCAM-1 to 0.27±0.03 and 0.29±0.03, respectively, compared with 0.09±0.01 in the untreated control group. Stimulation with TNF-α (10 or 20 ng/ml) induced the transcription of VCAM-1 to ~0.33±0.03 and 0.36±0.04, respectively. By contrast, exposure of MSCs to IL-1β (10 ng/ml) and TNF-α (10 ng/ml) resulted in a marked increase in mRNA synthesis with a value of 0.52±0.05 relative to the level of β-actin mRNA (Fig. 3).

**Protein expression of VCAM-1.** To further confirm the above results, the protein expression of VCAM-1 was quantified by measuring protein bands which were transferred to a PVDF membrane. In concordance with the flow cytometry results, adhesion experiments and mRNA data, the western blot analysis demonstrated that stimulation with IL-1β and TNF-α induced an evident increase in the VCAM-1 protein expression levels. IL-1β alone (10 ng/ml, 2.4±2.0-fold; 20 ng/ml, 2.7±3.0-fold), TNF-α alone (10 ng/ml, 3.1±0.2-fold; 20 ng/ml, 3.2±0.4-fold) and combination of the two cytokines (4.8±0.6-fold) markedly increased the protein expression of VCAM-1 in MSCs in vitro (Fig. 4).

**Measurement of heart function.** To examine the therapeutic efficacy of treated MSCs in MI in vivo, the cells were transplanted into the border region between the infarcted and normal area of rat hearts following coronary ligation. At the end of the fourth week following surgery, nine of the rats had not survived the experiment, three of them in the control group, two in the IL-1β (10 ng/ml) group and the other four were one for each group. The LVID.d and LVID.s of the heart were measured and then the LVEF was calculated (Table I). The LVEFs of the stimulation groups were evidently improved as compared with the control group (28.6±1.5%) and the specific measurement results were as follows: The LVEFs in the 10 and 20 ng/ml IL-1β groups were 33.7±2.1 and 34.8±1.7%, respectively, in the 10 and 20 ng/ml TNF-α groups they were 40.9±2.2 and 43.0±2.1%, respectively, and in the co-treatment group, the LVEF was 49.9±2.4%.

**Histological changes.** To further verify the myocardial protection effect in vivo, the cardiac slices were stained with Masson's trichrome. The non-infarcted left ventricular appeared red, while the infarcted myocardium replaced with fibroblasts and collagen appeared blue. The measurements revealed that the myocardial infarct size in both the IL-1β (10 ng/ml, 23.11±1.64; 20 ng/ml, 21.61±1.94%) and TNF-α (10 ng/ml, 17.71±1.85%; 20 ng/ml, 16.23±1.85%) groups was notably reduced compared with the control group (32.4±2.74%; P<0.01). Furthermore, the infarct size was even more reduced in the cytokine combination treatment group (8.37±1.60%). However, there was no significant difference between the infarct size in the hearts of the 20 ng/ml and 10 ng/ml cytokine stimulation groups (P>0.05; Fig. 5).

**Discussion**

In the present study, it was identified that IL-1β and TNF-α stimulation significantly elevated the VCAM-1 secretion and adhesion ability of MSCs, and the combination of these two cytokines potentiated this effect. Furthermore, intramyocardial injection with MSCs which were pretreated with IL-1β and TNF-α markedly improved the myocardial function and decreased the collagen deposition in infarcted myocardium in rats. Cytokine concentrations of 10 and 20 ng/ml were selected as the appropriate stimulation concentrations, as these concentrations were previously shown to activate paracrine signaling without changing surface makers or the viability of MSCs (28,29).

Previous studies have indicated that homing of circulating stem cells within the myocardium is possibly the first step of the myocardial regeneration process. This step requires adhesion of stem cells to the cardiac microvascular endothelium (15). Adhesion molecules are cell surface proteins that mediate the
inter-communication between cells, or between the cells and the extracellular matrix (ECM). Several investigations have demonstrated that VCAM-1 has a key role in MSC-mediated adhesion and immunosuppression (15,24). VCAM-1 is also important in the adhesion and migration of leukocytes through brain microvascular endothelial cells via binding to the α4β1 and α4β7 integrins (30,31). In previous studies, the adhesion of MSCs to endothelial cells was significantly eliminated following incubation with monoclonal blocking antibodies against VCAM-1. By contrast, it had only a weak and non-significant effect when the ICAM-1 antibody was added (15,24). In the present study, using an adhesion assay, the crucial role of VCAM-1 on MSCs adhesion was confirmed. Following the addition of cytokines, the quantity of cell adhesion to the plate markedly increased along with the upregulation of the adhesion molecule.

A number of investigators have suggested that intramyocardial MSC transplantation recruits a number of inflammatory factors which contribute to cardiac remodeling (32,33). Several studies have demonstrated that the cardioprotective effect of MSCs may be regulated by mediators which are secreted by stem cells. Together with these mediators, stem cells promote tissue repair and elicited other beneficial effects (34-36). Tsoyi et al (37) and Ward et al (38) reported that PI3K participated in the regulation of VCAM-1 expression and in intracellular signal transduction of the cell migration, which were induced by TNF-α in human endothelial cells. Other studies suggested that IL-1β induced MSC migration and adhesion through NF-kb (8). Since stem cells are consistently exposed to the inflammatory environment following implantation to ischemic areas, these inflammatory cytokines are critical for MSC behavior. Investigating the response of MSCs to an inflammatory environment will be undoubtedly valuable for improving transplantation efficiency.

Following the above rationale, MSCs were cultured in the presence of two typical inflammatory mediators, IL-1β and TNF-α, and administered to rats following experimentally-induced myocardial ischemic injury. The expression of VCAM-1 and the cardiac function of the left ventricular region were then assessed. It was identified that the expression of the adhesion molecule significantly increased following treatment with either of the cytokines, as did the cardiac function of the rats. As a number of investigations have demonstrated that the effect of inflammatory cytokines activating stem cell paracrine exhibited a dose-dependent trend (2.29), the dosage of the cytokines was doubled. Stimulation of MSCs with 20 ng/ml IL-1β or TNF-α did elevate the VCAM-1 protein expression and the quantity of plate-adhered cells compared with the 10 ng/ml-treated group; however, the difference was not statistically significant. Of note, the combination of the two cytokines induced more beneficial effects than the doubled dose alone. It is necessary to lower the dosage of the cytokines, but maintain a high level of VCAM-1 and the adhesion ability of the MSCs, as well as to avoid unwanted adversary effects to the MSCs or heart during the treatment.

There are a number limitations to be addressed in the present study. Due to the persistence of inflammatory factors in the myocardium during infarction, it remains unclear whether the complicated microenvironment would affect the treated MSCs. Furthermore, the present study did not investigate the mechanism underlying the effects of the combination of the two cytokines. Further studies are required to examine the underlying mechanisms and other biological behaviors of MSCs in the inflammatory environment, so as to fully elucidate their potential of cell-based therapies for MI.

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References


