Effects of combined mesenchymal stem cells and heme oxygenase-1 therapy on cardiac performance

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Abstract

Objective: Bone marrow mesenchymal stem cells (MSCs) have the potential to repair the infarcted myocardium and improve cardiac function. However, this approach is limited by its poor viability after transplantation, and controversy still exists over the mechanism by which MSCs contribute to the tissue repair. Methods: The human heme oxygenase-1 (hHO-1) was transfected into cultured MSCs using an adenoviral vector. Results: HO-1-MSCs survived in the infarcted myocardium, and expressed hHO-1 mRNA. The expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) was significantly enhanced in HO-1-MSCs-treated hearts. At the same time, there were significant reduction of TNF-α, IL-1-beta and IL-6 mRNA, and marked increase of IL-10 mRNA in HO-1-MSCs-treated hearts. Moreover, a further downregulation of proapoptotic protein, Bax, and a marked increase in microvessel density were observed in HO-1-MSCs-treated hearts. The infarct size and cardiac performance were also significantly improved in HO-1-MSCs-treated hearts. Conclusion: The combined approach improves MSCs survival and is superior to MSCs injection alone.

Keywords: Mesenchymal stem cells; Myocardial infarction; Heme oxygenase-1; Paracrine factors; Angiogenesis

1. Introduction

Bone marrow derived mesenchymal stem cells (MSCs) have demonstrated the potential for reducing ventricular remodeling and improving left ventricular (LV) function in various experimental myocardial infarction (MI) [1,2]. Autologous MSCs are particularly suitable for the abovementioned purposes because of their multipotency, low immunogenicity, amenability to ex vivo expansion, and genetic modification [3]. However, the exact mechanism via which MSCs transplantation improves cardiac function in the early phase post-transplantation (≤7 days) remains largely a matter of speculation. There are accumulating evidences that MSCs lack the potential to acquire a mature cardiomyocytic phenotype in the infarcted myocardium [4,5], and there is little evidence to support cellular fusion as a dominant mechanism underlying cardiac recovery in response to MSCs-based therapy [6]. Several investigators have mentioned the importance of the release of paracrine factors by MSCs [7,8]. It is known that many cytokines can directly protect native myocardium from ischemic injury, in addition to enhancing therapeutic angiogenesis. Therefore, the cytokines released by those implanted MSCs may play an important role in the process than their differentiation and fusion.

Progress in stem cell therapy is hampered by poor cell viability after transplantation. It has been indicated that transplanted cells die in the infarcted heart because of the effects of hypoxia/reoxygenation, inflammatory cytokines, and proapoptotic factors [9]. Heme oxygenase-1 (HO-1), an antiapoptotic and anti-inflammation enzyme, is a key component in inhibiting most of those factors [10]. Recently, a study emanating from Melo’s group showed that predelivery of HO-1 to myocardium led to marked functional recovery, reduced fibrosis and absence of ventricular remodeling 3 months after MI [11].

This study is intended to determine the effect of a combined strategy of MSCs transplantation with HO-1 gene therapy on cardiac function and remodeling following MI in the early post-transplanted period.
2. Materials and methods

2.1. Approval of animal experiments

All the animal experiments were conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH published No. 85-23, revised 1996). The experiments were mostly performed at Department of Cardiology, State Key Laboratory of Virology and Department of Pathology of Wuhan University, Wuhan, Hubei, China.

2.2. Preparation of recombinant adenovirus

A recombinant adenovirus containing the entire coding sequence of human heme oxygenase-1 (hHO-1) was constructed as previously described [12]. Briefly, a full-length human HO-1 gene cDNA was cloned into the adenovirus shuttle plasmid vector, pAd-CMV, which contains a cytomegalovirus promoter and a polyadenylation signal of bovine growth hormone. For construction of adenovirus containing green fluorescent protein (GFP), a shuttle vector containing human phosphoglycerate kinase gene promoter was used. The control virus lacking the hHO-1 gene (Ad) was separately produced. Recombinant adenovirus was generated by homologous recombination and propagated in 293 cells. At the stipulated time, the supernatant from the 293 cells was collected and purified on cesium chloride (CsCl) gradient centrifugation and stored in 10 mmol/l Tris—HCl (pH 7.4), 1 mmol/l MgCl2, and 10% (vol/vol) glycerol at −70 °C until used for experiments. The virus titers were determined by a plaque assay on a 293 cell monolayer.

2.3. MSCs isolation, expansion, transfection, and labeling

MSCs were isolated from bone marrow of adult Sprague-Dawley male rats and expanded according to reported protocols [1]. Briefly, bone marrow was flushed from tibias and femurs of rats using a 25-gauge needle. Whole marrow cells were cultured at a density of 1 × 107 cells/cm2 in α-minimum essential medium (α-MEM, Gibco) containing 10% fetal bovine serum (FBS, Invitrogen) and 100 u/ml penicillin—streptomycin (Sigma). The nonadherent cells were removed by changing medium at 72 h and every 3 days thereafter. After two passages, the homogeneous MSCs that were devoid of hematopoietic cells were transfected with Ad-hHO-1 or Ad-Null at multiplicity of infection (MOI) of 200. After incubation at 37 °C for 2 h, an equal volume of α-MEM containing 20% FBS was added to the medium and the cells were cultured for additional 48 h. To observe the nuclei of MSCs in vivo, sterile 4’,6’-diamidino-2’-phenylindole (DAPI) stock solution (Sigma) was added to culture medium at a final concentration of 50 μg/ml for 30 min. After labeling, the cells were washed six times using D-Hanks solution to remove unbound DAPI.

2.4. Cell implantation and quantitative analysis of cell survival

The male rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.), and mechanically ventilated. After the heart was expressed through a lateral thoracotomy, a 6-0 polypropylene thread was passed around the left coronary artery and the artery was occluded. The electrocardiogram (ECG) was recorded to confirm the presence of infarction. One hour after MI, 1 × 106 HO-1-MSCs or Null-MSCs from passage 3 in 0.1 ml of medium were injected at four sites into the infarcted border zone using a 30-gauge needle (n = 12, each group). Equivalent volume of PBS alone was injected into control animals (n = 12). Rats were killed at different time points, and the treated hearts were harvested and cryopreserved in OCT media. Serial 8 um thick sections were cut at −20 °C and placed on poly-l-lysine-coated microscopic slide. The number of DAPI-positive cells was evaluated by counting five randomly chosen fields from each of twenty sections taken from each animal [13].

2.5. Echocardiogram

The cardiac function was measured by transthoracic echocardiography at 1 week after injection by a single observer blind to the treatment regimen. The M-mode measurement was performed with a commercially available transducer system designed for cardiac ultrasound device (Phillips). To measure left ventricular (LV) function, the percent fractional shortening (FS) was calculated as follows: FS (%) = [(LVEndd – LVEsd)/LVEndd] × 100. LVEDd is LV end-diastolic dimension and LVEsd is LV end-systolic dimension. Dimensions were measured between the anterior wall and the posterior wall from the short-axis view below the level of the papillary muscle.

2.6. RT-PCR

After 1 week of transplantation, the hearts was excised, and total RNA was extracted from the infarcted border zone using TRizol (Invitrogen) reagent. The RT-PCR was performed, as previously described [14]. β-Actin was used as an internal control, and primer sequences for β-actin amplification (315 bp) were 5’-GCTGGTCGTCGACAACGGCTC-3’ (sense), 5’-CAAAACATGATCTGGGTCATCTTTTC-3’ (antisense). Primer sequences for human HO-1 amplification (555 bp) were 5’-CAGGAGAGAAGCTGTCATTTT-3’ (sense), 5’-GATGTTGAGACGGACCGAGT-3’ (antisense). Other primers used in this study were previously described [14]. The expected size of the PCR products for TNF-α, IL-1-beta, IL-6 and IL-10 was 460, 555, 295, and 346 bp, respectively.

2.7. Detection of growth factors by enzyme-linked immunosorbent assay

Myocardial vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) within peri-infarcted tissues were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit (R&D). ELISA was performed according to the manufacture’s instructions.

2.8. Western blot

After 1 week of cell transplantation, the rats’ hearts from the HO-1-MSCs group, Null-MSCs group, and PBS group were...
excised for proapoptosis assay. The hearts obtained were snap-frozen in liquid nitrogen. Western blot analysis was performed using 125 µg heart tissue extracts and electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), as previously described [8]. The monoclonal anti-Bax antibody (Santa Cruz) was used for analyzing the influence of Bax on apoptosis. Nitrocellulose membranes were then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit antibody. Blots were developed by the ECL method (Pierce), and relative protein expression was quantified by scanning densitometry.

2.9. Immunohistochemistry

After 3 weeks of injection, CD34 immunostainings were used to evaluate the microvessel formation. The different slides were prepared for immunohistochemical examination and were incubated with rabbit polyclonal anti-CD34 antibody for 30 min at room temperature. Slides were incubated with Texas Red. Twenty sections per heart were evaluated to estimate capillarity by another investigator unaware of the treatment groups. Five fields per section were randomly selected and analyzed at a magnification of 200. The number of capillaries was assessed from photomicrographs by computerized image analysis [15].

2.10. Histology

After 3 weeks of injection, the hearts were harvested, washed in PBS, and fixed in 10% formalin overnight at 4 °C. Paraffin embedded tissues were cut into 5-µm-thin sections, and Masson’s trichrome staining was performed to delineate scar tissue from viable myocardium. Twenty sections per heart were captured as digital image, and five fields per section were calculated and the collagen-delegated infarction percentage was analyzed by a blinded investigator. The calculation formula used for the infarct size was: % infarct size = infarct areas/total left ventricle area × 100% [16].

2.11. Statistics

At least three independent experiments were carried out for each analysis. Each data point was presented as mean ± SD. Statistical significance was evaluated using one-way ANOVA. A value of \( p < 0.05 \) was considered statistically significant.

3. Results

3.1. Induction of human HO-1 expression in ischemic myocardium increases MSCs survival

At day 7 after transplantation, we isolated the cardiac RNA and confirmed human HO-1 mRNA expression by RT-PCR. The human HO-1 mRNA was detected in the RNA sample extracted from cardiac tissue of HO-1-MSCs group hearts but was not detected in the cardiac RNA extracted from hearts of Null-MSCs group and PBS group (Fig. 1A). However, we were unable to detect human HO-1 mRNA expression in any of the groups at 4 weeks (data not shown).

Two days after transplantation of the cells into the infarcted border zone, the HO-1-MSCs were typically distributed in the form of clusters at the injection sites, and there were more surviving cells as evidenced by DAPI test than those of Null-MSCs groups. One week after transplantation, more DAPI-labeled HO-1-MSCs were embedded with the host myocardium than those of Null-MSCs groups. No DAPI-
labeled cells were detected in PBS group (Fig. 1B). The differences proved to be statistically significant, with 2.1-fold enhancement of cell survival on day 3 ($p < 0.01$), 1.6-fold enhancement on day 7 compared with Null-MSCs groups ($p < 0.01$) (Fig. 1C).

3.2. Paracrine function and neovascularization in response to HO-1-MSCs transplantation

At day 7 after transplantation, there was low expression level of VEGF and bFGF in the PBS-treated hearts. Their expression level was significantly higher in Null-MSCs-treated hearts than that in PBS-treated hearts, and the respective values for VEGF being $188.3 \pm 26.5$ ng/l vs $106.3 \pm 21.2$ ng/l ($p < 0.01$) and for bFGF, $108.7 \pm 18.6$ ng/l vs $66.3 \pm 9.6$ ng/l ($p < 0.01$). Their expression level was more significantly enhanced in the hearts treated with HO-1-MSCs, the values for VEGF being $264.7 \pm 23.1$ ng/l ($p < 0.01$), and for bFGF, $141.3 \pm 13.7$ ng/l ($p < 0.01$), respectively (Fig. 2A).

Quantitative analyses revealed that microvessel density of HO-1-MSCs-treated hearts was significantly higher than that of Null-MSCs-treated hearts ($p < 0.01$), and microvessel density of Null-MSCs-treated hearts was significantly higher than that of PBS-treated hearts ($p < 0.01$) (Fig. 2B and C).

3.3. HO-1-MSCs transplantation improves the inflammatory cytokines levels and reduces proapoptotic protein, Bax, levels in the infarcted border zone

We analyzed the level of inflammatory response using RT-PCR and apoptosis using Western blot. Compared with Null-MSCs-treated and PBS-treated hearts, HO-1-MSCs-treated hearts displayed a reduced trend of TNF-$
\alpha$, IL-1-beta and IL-6 mRNA expression, and an increased trend of IL-10 mRNA expression (Fig. 3A and B). As shown in Fig. 3C and 3D, 1 week after transplantation, there was a significant decrease in proapoptotic Bax expression in HO-1-MSCs-treated hearts compared with Null-MSCs-treated and PBS-treated hearts ($p < 0.01$).

3.4. Effects of HO-1-MSCs transplantation on ventricular function and remodeling

The cardiac functional parameters were evaluated by echocardiography 1 week after transplantation. The baseline body weight and echocardiography parameters were similar in each group. The M-mode echocardiography showed that both Null-MSCs-treated and HO-1-MSCs-treated hearts exhibited improved contractility and LV dilation compared with PBS-treated hearts, and both indexes were markedly improved in the hearts treated with HO-1-MSCs (Fig. 4A). In the PBS control group, a markedly decreased fractional shortening ($19.88 \pm 4.27\%$) and ejection fraction ($41.28 \pm 5.06\%$) with dilated left ventricular cavity were clearly observed. In the Null-MSCs transplantation group, a significantly higher fractional shortening ($24.56 \pm 5.32\%$), ejection function ($59.46 \pm 7.58\%$) and a smaller left ventricular cavity were observed compared with that in the PBS groups. In the HO-1-MSCs graft group, higher fractional shortening ($31.27 \pm 6.15\%$) and ejection fraction ($78.13 \pm 9.17\%$) were observed compared with that in Null-MSCs (Fig. 4B).

Three weeks after transplantation, mean infarcted size in PBS-treated hearts was $35.3 \pm 2.2\%$ of the LV. Transplantation of Null-MSCs had a modest protective effect, limiting the infarcted size to $21 \pm 2.2\%$ of the LV. In contrast, transplantation of HO-1-MSCs significantly limited infarcted size to $13.3 \pm 1.3\%$ of the LV ($p < 0.01$ compared with the other two groups) (Fig. 4C and D). Interestingly, the myocardial structure in the border zone was more intact in HO-1-MSCs and Null-MSCs graft group whereas the myocardial structure was chaotic in the control medium-treated heart (Fig. 4E).

4. Discussions

Our data showed that the paracrine factors released by MSCs combined with the HO-1 protein improved MSCs survival in ischemic myocardium, leading to efficient neovascularization, improving the mRNA levels of inflammatory cytokines,
reducing the levels of proapoptotic protein, Bax. These effects might result in a reduced remodeling and enhanced recovery of cardiac performance.

At day 7 after transplantation of HO-1-MSCs, we found human HO-1 expression in cardiac tissue. HO-1-MSCs grafting induced a higher expression level of VEGF and bFGF than that induced by Null-MSCs and PBS. VEGF is a critical angiogenic factor, which accelerates the development of microvessels and enhances regional blood flow in ischemic tissue, and VEGF can activate the myocardial PI-3K pathway and decrease
myocardial infarct size [17]. BFGF plays a vital role in cardioprotection against myocardial cell death in acute myocardial infarction, and BFGF is also an important angiogenic growth factor [18]. The higher level expression of VEGF and BFGF from HO-1-MSCs transplanted to the ischemia-damaged myocardium would provide cardioprotective effect and induce functional collateral vessels, which improve the environment for implanted cells to survive. Several reports suggest that implanted bone marrow MSCs seem to be highly sensitive to hypoxia and inflammatory environment in ischemic myocardium [19], and poor cell viability after transplantation may have a major negative impact on the efficiency of stem cells therapy [9]. Another important finding in this study is that HO-1-MSCs-transplanted hearts display a decreased trend of TNF-α, IL-1-beta and IL-6 mRNA expression, and an increased trend of IL-10 mRNA expression, compared with Null-MSCs-treated and PBS-treated hearts. In this study, we hypothesize that the protective effect may be caused by the potentially protective properties of HO-1 and by paracrine factors released by MSCs in ischemia myocardium. Our study is in agreement with previous finding from Tang’s group [20]. They injected hypoxia-regulated HO-1 plasmid modified MSCs into mouse hearts 1 h after MI, and found that the HO-1-MSCs group has a greater expression of HO-1, and HO-1 effectively increases cell survival in ischemia myocardium. However, although the purpose of Tang and our study was to increase the survival rate of MSCs using HO-1, their study showed that the hypoxia-regulated HO-1 transfected MSCs could differentiate into cardiac muscle and endothelial cells, which might result in the improvement of LV function and ameliorate LV remodeling.

It was recently reported that inflammatory process after MI peaks at 1 week, and plays a central role in initiating the process of post-infarction LV remodeling by activating fibroblast proliferation [21]. During the remodeling process, the expression of those inflammatory cytokines is activated. Many observations suggest that those cytokines are important modulators in the post-myocardial infarction remodeling process including infarct-associated inflammation, fibrosis of the myocardium, and cardiac dysfunction [11]. The improved changes of inflammation cytokine levels were accompanied by a marked decrease in infarction size and decreased collagen deposition in the infarcted myocardium treated by HO-1-MSCs, suggesting that HO-1 overexpression confers anti-inflammation and anti-fibrogenic properties to infarcted myocardium. Multiple catalytic byproducts of heme catabolism exert cytoprotective effects. It is reported that exogenous bilirubin administration reduces infarct size and enhances postischemic myocardial functional recovery in isolated hearts [22]. CO exerts powerful anti-inflammatory and anti-apoptotic effect [23]. Free iron stimulates the synthesis of the iron binding protein ferritin, which reduces iron-mediated formation of free radicals and upregulates several key cytoprotective genes [24]. In addition, HO-1 may exert direct anti-fibrogenic effects, and prevent post-infarction negative LV remodeling [11]. An improvement of cardiac performance in the heart treated by HO-1-MSCs is also accompanied by a decrease of proapoptotic protein expression. Cell apoptosis resulting from the activation of proapoptotic signal transduction pathways plays an important role in the total cell destruction during MI [25]. In this study, we observed that Bax was downregulated in myocardium treated by HO-1-MSCs compared with myocardium treated by Null-MSCs and PBS. This may be an important pathway involved in cytoprotection. Meanwhile, the secretion of cardioprotective proteins from HO-1-MSCs also contributes to the salvaging of ischemic myocardium and decreases the infarct area.

In conclusion, engrafting HO-1-MSCs into ischemic hearts has a beneficial effect, which may be attributable to paracrine action and HO-1 protein. Their interaction results in a reduced remodeling and enhanced recovery of cardiac performance. The combined approach improves MSCs survival, and is superior to MSCs injection alone.

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References

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejcts.2008.05.049.