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Thioredoxin-1 (Trx1) engineered mesenchymal stem cell therapy increased pro-angiogenic factors, reduced fibrosis and improved heart function in the infarcted rat myocardium

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ABSTRACT

Introduction: Engraftment of mesenchymal stem cells (MSCs) has emerged as a powerful candidate for mediating myocardial repair. In this study, we genetically modified MSCs with an adenovector encoding thioredoxin-1 (Ad.Trx1). Trx1 has been described as a growth regulator, a transcription factor regulator, a cofactor, and a powerful antioxidant. We explored whether engineered MSCs, when transplanted, are capable of improving cardiac function and angiogenesis in a rat model of myocardial infarction (MI).

Methods: Rat MSCs were cultured and divided into MSC, MSC + Ad.LacZ, and MSC + Ad.Trx1 groups. The cells were assayed for proliferation, and differentiation potential. In addition, rats were divided into control-sham (CS), control-MI (CMI), MSC + Ad.LacZ-MI (MLZMI), and MSC + Ad.Trx1-MI (MTrxMI) groups. MI was induced by left anterior descending coronary artery (LAD) ligation, and MSCs preconditioned with either Ad.LacZ or Ad.Trx1 were immediately administered to four sites in the peri-infarct zone.

Results: The MSC + Ad.Trx1 cells increased the proliferation capacity and maintained pluripotency, allowing them to divide into cardiomyocytes, smooth muscle, and endothelial cells. Western blot analysis, 4 days after treatment showed increased vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1), and C-X-C chemokine receptor type 4 (CXCR4). Also capillary density along with myocardial function as examined by echocardiography was found to be increased. Fibrosis was reduced in the MTrxMI group compared to MLZMI and CMI. Visualization of Connexin-43 by immunohistochemistry confirmed increased intercellular connections in the MTrxMI rats compared to MLZMI.

Conclusion: Engineering MSCs to express Trx1 may prove to be a strategic therapeutic modality in the treatment of cardiac failure.

1. Introduction

Neovascularization and regeneration of cardiac tissue *via* the administration of bone marrow stem cells (BMSCs) has emerged as an effective strategy to obviate pathophysiological remodeling following a myocardial infarction (MI). Therapeutic intervention using stem and progenitor cells is accepted to be a state-of-the-art technique for alleviating the failing heart and curing ischemic heart disease (IHD) [1–9].

Recent studies employing skeletal myoblasts (SkMs), embryonic stem cells (ESCs), BMSCs, and cardiac progenitor cells as candidates for therapeutic angiogenesis and the improvement of cardiac remodeling have yielded promising outcomes. However, some of these candidates have been associated with post-operative complications like the induction of teratomas by ESCs and the failure of SkMs to couple electromechanically with functional cardiomyocytes (CMs). Therefore, better progenitor cell therapies are necessary for reviving the weakening myocardium and alleviating the cardiomyopathic intricacies that occur in patients with diabetes, hypercholesterolemia, and subclinical atherosclerosis. Such therapies must promote CM lineage specification or trigger the proliferation of resident cardiac cells *via* paracrine stimulation to replace the apoptotic CMs.

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Studies on the use of allogenic mesenchymal stem cells (MSCs) for therapeutic angiogenesis have revealed a significant recovery in cardiac function that can be attributed to the unique abilities of MSCs to act as immunomodulators [10,11], integrate into the host myocardium to form new cardiac tissue [12], secrete paracrine effectors, and differentiate into cardiomyocyte-like-cells (CLCs) [13]. MSC-based cellular cardiomyoplasty for IHDs is already in phase II/III clinical trials [8,9,14–17]. Nevertheless, skepticism pertaining to the long-term survivability of MSCs, the optimal dosage and time of administration, and the route of delivery reveals the need for further study.

Although the reparative potential of MSCs has been well-corroborated in the literature, the survivability of engrafted MSCs in the infarcted myocardium is controversial. Decreased survival (<5%) of MCSs is mainly due to hypoxia, a paucity of survival factors, ischemia, and inflammatory cytokines resulting from oxidative stress or cardiomyocyte apoptosis. All of these factors limit the therapeutic potential of MSCs [18]. Therefore, diverse approaches involving the genetic modification of MSCs have been undertaken to increase survivability. Some studies have experimented with overexpression of pro-angiogenic factors such as Akt, vascular endothelial growth factor (VEGF), or C-X-C chemokine receptor type 4 (CXCR4) [19–21]. Other studies have investigated preconditioning with stromal cell-derived factor 1 (SDF-1) or a combination of growth factors [22–25]. Both approaches have afforded significant cardioprotective effects in the preclinical milieu. In one study, overexpression of the pro-angiogenic factor Akt in MSCs that were injected into rat myocardium after MI resulted in improved cardiac function and repair, as well as decreased remodeling [19]. Cells transfected with angiogenic genes such as VEGF, genes needed for survivability such as SDF-1 and heme oxygenase-1 (HO-1), and other pro-survival and differentiation genes such as insulin-like growth factor-1 (IGF-1) not only exhibited improved survivability, but also restored cardiac function after MI [26,27].

We have previously demonstrated that gene therapy with Trx1, a powerful antioxidant, transcription factor, and growth-factor regulator, can attenuate necrosis after MI, increase angiogenesis and vasculogenesis, and improve heart function [28]. The similar cardioprotection offered by the direct administration of adenovector encoding Trx1 (Ad.Trx1) to the risk zone in a rat MI model supports a paracrine mechanism of action *via* the administration of Trx1 to MSCs. The success of Trx1 therapy led us to develop Trx1 preconditioned MSCs to determine whether intramyocardial injection of these cells to the peri-infarct region would attenuate the pathologies that accompany MI to a greater extent than treatment with naïve MSCs.

We demonstrate for the first time that MSCs transfected with Ad.Trx1 not only maintain their capacity for survival and differentiation better than untreated cells, but also more successfully restore heart function following MI. Elevated Trx1 expression appears to promote cell survivability and proliferation *in vitro*. Additionally, rats treated by intramyocardial injection of Trx1-engineered MSCs displayed improved ejection fraction and contractility along with increased capillary density. These cardioprotective effects likely result not only from the stimulation of endogenous cardioprotective pathways by the transplanted MSCs, but also from Trx1 functioning both as a powerful antioxidant against reactive oxygen species (ROS) and as an upstream modulator of the survival factor HO-1 and the angiogenic regulators hypoxia inducible factor-1α (HIF-1α) and VEGF [28]. Thus, we demonstrate the need to investigate Trx1-engineered MSCs as a long-lasting therapeutic option for the treatment of MI.

2. Materials and methods

2.1. In vitro experimental design

2.1.1. Culture and characterization of MSCs

To characterize the MSCs (Millipore, Ballerica, MA, USA), the cells were serially passaged and maintained in MSC Expansion Medium followed by immunocytochemical characterization with a Rat MSC Characterization Kit. Antibodies against CD54 and Integrin β1 were used as positive markers for culture-expanded MSCs, whereas anti-CD14 (Millipore, Billerica, MA, USA) was used as a negative control. Rabbit IgG and mouse IgG were employed as isotype controls. Confocal images were acquired using a Zeiss LSM510 Meta confocal microscope at 400× magnification. After characterization and expansion, the MSCs were harvested at the seventh passage and used for all *in vitro* studies and for *in vivo* intramyocardial administration.

2.1.2. Transfection efficiency of Ad.Trx1 in MSCs by immunohistochemistry and Western blot analysis

2.1.2.1. Immunohistochemistry. To examine the transfection efficiency of Ad.Trx1, immunocytochemistry was performed 48 h post-transfection. Cells were assayed for Trx1 expression using rabbit anti-Trx1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:100 dilution, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG and TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, USA). The cells were imaged at 400× magnification using a Zeiss LSM510 Meta confocal microscope.

2.1.2.1.1. Western blot analysis. To examine the transfection efficiency of Ad.LacZ and Ad.Trx1, Western blot was also performed 48 h post-transfection. The cells were harvested, and whole cell lysates were prepared in 1X RIPA buffer containing 10 μL/mL of a protease inhibitor cocktail (Sigma, St. Louis, MO, USA), 0.1 M DTT, 1 mM Na_3VO_4, and 1 mM PMSF. The total protein content was measured by BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) followed by immunoblot analysis with anti-Trx-1 (Santa Cruz, Santa Cruz, CA, USA) as described previously [28]. β-actin (Mouse monoclonal antibody, Sigma, St. Louis, MO, USA) was used as a loading control.

2.2. In vitro analysis of VEGF expression by immunocytochemistry

Cells from all three groups, MSC, MSC+Ad.LacZ and MSC+Ad.Trx1 were prepared for immunocytochemistry as described above. The cells were incubated with a 1:100 dilution of a rabbit polyclonal antibody against VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by Alexa Fluor 555 goat anti-rabbit IgG. TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, USA) was applied as a nuclear stain.

2.3. In vitro analysis of dividing MSCs: α-tubulin staining

To examine whether Ad.Trx1 treatment had any effect on the number of dividing MSCs [29], cells were stained with an anti-α-tubulin mouse monoclonal antibody (clone DMIA, Abcam, Cambridge, MA, USA) to mark microtubule assembly. The cells were then imaged by confocal microscopy. Mesenchymal stem cells were treated with Ad.LacZ or Ad.Trx1 for 48 h, fixed in 10% buffered formalin, permeabilized in 0.25% TritonX-100, and blocked with 2.5% normal horse serum. Untreated MSCs were used as comparable controls. Alpha-tubulin was visualized using Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, USA) was used to stain the nuclei. Images were captured using a Zeiss LSM510 Meta at 400× magnification. At least five pictures were taken per well (n = 4).

2.4. Hypoxia treatment of cultured MSCs

The *in vitro* experiments were conducted under hypoxic conditions. For the hypoxia studies, seeded cells were placed in a Molecular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, USA). The chamber was sealed, filled with 100% N_2, and vented until an oxygen-free environment was created. The hypoxic chamber was then placed inside the CO_2 incubator at 37 °C, 5% CO_2 for 8 h before all protocols were initiated.

2.5. Estimation of Ki-67 score: an index for cell-proliferation

The Ki-67 peptide was selected as a marker of cellular proliferation in immunocytochemistry studies. Chamber slides were seeded with 2000 cells/chamber and incubated overnight. The cells were then divided into three groups: MSC, MSC + Ad.LacZ, and MSC + Ad.Trx1. The latter two groups were treated with the Ad.LacZ and Ad.Trx1 for 48 h. After aspiration of the media, each chamber was washed three times with 1 mL of 1X PBS for 5 min. The cells were then fixed with 100% methanol on ice for 10 min before being washed three additional times with PBS. Permeabilization was enhanced by incubation in a 0.25% Triton-X-100 solution in PBS for 30 min at 37 °C and then for 10 min at room temperature. The chambers were once again washed three times in PBS and blocked with 2.5% normal horse serum. The cells were then incubated overnight at 4 °C in a 1:50 dilution of a mouse monoclonal antibody against rat Ki-67 (MIB-5, Dako, Carpinteria, CA, USA) in PBS. After another series of three washes in PBS, the cells were incubated with a biotinylated anti-mouse secondary antibody and visualized with 3,3′-diaminobenzidine (DAB) using a DAB Peroxidase Substrate Kit, 3,3′-diaminobenzidine (Vector laboratories, Burlingame, CA USA). Meyer's Modified Hematoxylin (Poly Scientific, Bay Shore, NY, USA) was used to counterstain the nuclei. The Ki-67 score was calculated as the percentage of Ki-67-positive nuclei/total nuclei. Images of each chamber were taken randomly using an Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope (n = 3).

2.6. In vitro analysis of MSC differentiation

After 8 h of hypoxia, cells from all three groups were fixed in 100% methanol (as described above) before treatment with primary antibodies against lineage-specific