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TNF receptor 2, not TNF receptor 1 enhances mesenchymal stem cell-mediated cardiac protection following acute ischemia

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Abstract

BACKGROUND—Mesenchymal stem cells (MSCs) may improve myocardial function after ischemia/reperfusion (I/R) injury via paracrine effects, including the release of growth factors. Genetic modification of MSCs is an appealing method to enhance MSC paracrine action. Ablation of TNF receptor 1 (TNFR1), but not TNFR2, increases MSC growth factor production. In this study, therefore, we hypothesized that: 1. pre-ischemic infusion of MSCs derived from TNFR1 knockout (TNFR1KO) mice will further improve myocardial functional recovery; 2. TNFR2KO and TNFR1/2KO will abolish MSC-mediated protection in the heart following I/R injury.

METHODS—MSCs were harvested from adult C57BL/6J (WT1), B6129SF2 (WT2), TNFR1KO, TNFR2KO and TNFR1/2KO mice. MSCs were cultured and adopted for experiments after passage 3. Isolated hearts from adult male Sprague-Dawley rats were subjected to 25-minutes of ischemia and 40-minutes of reperfusion (Langendorff model), during which time myocardial function was continuously monitored. Before ischemia, 1 ml of vehicle or 1×10^6 MSCs/ml from WT1, WT2, TNFR1KO, TNFR2KO, or TNFR1/2KO was infused into the hearts (n=4–6/group).

RESULTS—WT1 MSC treatment prior to ischemia significantly increased cardiac function. TNFR1KO MSCs demonstrated greater cardioprotection when compared to WT MSCs following I/R as exhibited by improved LVDP and $\pm$ -dp/dt. However, infusion of MSCs from TNFR2KO and TNFR1/2KO mice either offered no benefit or decreased MSC-mediated cardiac functional recovery in response to I/R when compared with WT MSC.

CONCLUSIONS—TNFR1 signaling may damage MSC paracrine effects and decrease MSC-mediated cardioprotection, whereas TNFR2 likely mediates beneficial effects in MSCs.

Keywords

bone marrow cells; ischemia/reperfusion (I/R); paracrine effects; myocardial function

Introduction

Accumulating evidence has demonstrated that mesenchymal stem cells (MSCs) may protect the myocardium from ischemia through paracrine actions, such as the induction of growth factor production (1,2). These growth factors may mediate several beneficial effects including improvement of myocardial function, reduction of myocyte apoptosis, and conduction of positive ventricular remodeling (3,4). Among these, VEGF plays an important role in regulating MSC paracrine action. Increased VEGF has been shown to mediate MSC-induced protection in the ischemic heart (5). Overexpressing VEGF in MSCs provides greater cardiac protection (6). Therefore, genetic modification of MSCs to increase stem cell VEGF production may augment paracrine effects of stem cell cardioprotection.

After myocardial ischemia/reperfusion (I/R) injury, a substantial amount of tumor necrosis factor (TNF) is released in the heart (7,8). The locally produced TNF may exert important effects on implanted stem cell function. TNF acts by binding to two distinct receptors: a 55-kDa receptor (TNFR1) and/or a 75-kDa receptor (TNFR2) (7). Previous studies have shown that TNFR1 signaling mediates deleterious effects on TNF in cardiac cells (4,9) while the TNFR2 pathway may mediate beneficial effects in the heart (9–11). This led to the appreciation that different TNF receptors may mediate MSC function differently. Indeed, recent evidence has indicated that ablation of TNFR1 in stem cells increases VEGF secretion, whereas the absence of TNFR2 decreases MSC VEGF production (12,13). This suggests TNFR1 signaling may play a detrimental role in MSC VEGF release while TNFR2 may favor stem cell function. However, it is unknown whether genetically modified MSCs with TNFR1-deficiency (TNFR1KO) and/or TNFR2KO will affect MSC-mediated protection of myocardial function following acute I/R.

Therefore, in this study, we hypothesized that 1. pre-ischemic infusion of MSCs derived from TNFR1KO mice would further increase myocardial function compared to wild type (WT) MSCs after I/R, 2. pre-ischemic treatment with TNFR2KO or TNFR1/2 knockout (TNFR1/2KO) MSCs would neutralize MSC-improved myocardial function following I/R.

Materials and Methods

Animals

Normal adult male Sprague-Dawley rats were obtained from Harlan (250–275g, Indianapolis, IN). Both TNFR1KO and TNFR2KO mice with a background of C57BL/6J (WT1), and TNFR1/2KO mice with a background of B6129SF2 (WT2) (male, 6–8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). Receptor deficient mice are viable, normal in size and do not display any gross physical abnormalities. Animals were fed a standard diet and acclimated in a quiet quarantine room for 1 week before the experiments. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 85–23, revised 1996).

Preparation of Mouse Bone Marrow Stromal Cells

A single-step stem cell purification method using adhesion to cell culture plastic was employed as previously described (14). Briefly mouse bone marrow stromal cells were collected from bilateral femurs and tibias after sacrifice by removing the epiphyses and flushing the shaft with complete media (Isocove’s Modified Dulbecco’s Medium (GIBCO Invitrogen, Carlsbad, CA)) and 10% fetal bovine serum (GIBCO Invitrogen, Carlsbad, CA) using a syringe with a 26G needle. Cells were disaggregated by vigorous pipetting several times, and were passed through a 30- μ m nylon mesh to remove remaining clumps of tissue.

Cells were washed by adding complete media, centrifuging for 5 min at 300 g @ 24°C, and removing the supernatant. The cell pellet was then resuspended and cultured in 75 cm² culture flasks with complete media at 37°C, 5% CO₂. BMSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete media was added and replaced every three days thereafter. When the cultures reached 90% of confluence, MSCs were passaged with the addition of a solution 0.25% trypsin-EDTA (GIBCO Invitrogen, Carlsbad, CA) and replated in flasks. Cells were utilized for experimentation between passages 3–8.

Isolated heart preparation (Langendorff)

Hearts were isolated as previously described (15–17). Briefly, rats were anesthetized (sodium pentobarbital, 60 mg/kg i.p.) and heparinized (500 U i.p.), and hearts were rapidly excised via median sternotomy and placed in 4 °C Krebs Henseleit solution. The aorta was cannulated and the heart was perfused under constant pressure (mean 75 mmHg) with oxygenated (95% O₂/5% CO₂) Krebs–Henseleit solution (37 °C). A left atrial resection was performed prior to insertion of a water-filled latex balloon, which was initially adjusted to a desired mean end diastolic pressure (EDP) of 5 mmHg. Hearts were allowed to equilibrate for 15 minutes and paced at 350 bpm to ensure a standard heart rate between groups. A three-way stopcock above the aortic root was used to create warm global ischemia, during which time the heart was placed in a 37 °C degassed organ bath. After 25 minutes of ischemia, hearts were reperused for 40 minutes. The left ventricular developed pressure (LVDP), the maximal positive and negative values of the first derivative of pressure (+dp/dt and –dp/dt), and end diastolic pressure (EDP) were continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA) and an Apple G4 PowerPC computer (Apple Computer Inc., Cupertino, CA).

Experimental groups

Rat hearts were divided into the following groups: 1. vehicle control (N=6), 2. WT1 MSC infusion (N=6), 3. WT2 MSC infusion (N=5), 4. TNFR1KO (R1KO) MSC infusion (N=4), 5. TNFR2KO (R2KO) MSC infusion (N=6), and 6. TNFR1/2KO (R1/2KO) MSC infusion (N=5). Cells are collected from flasks by trypsinization and centrifuged. The cell pellet is resuspended in Krebs–Henseleit solution (37°C) and a count is performed. The appropriate amount of Krebs–Henseleit solution is then added to the cell suspension to create a solution with a final concentration of 1×10^6 cells/mL. Over the course of one minute prior to ischemia, 1 ml of this MSC solution was infused into the coronary circulation.

TNF- α , and VEGF enzyme-linked immunosorbent assay

Ventricular myocardial tissue was homogenized and TNF- α and VEGF levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA sets (R&D Systems, Inc, Minneapolis, MN). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate. Protein concentrations of samples were measured via biophotometer using the Bradford protocol. ELISA values were normalized using the protein concentrations of each sample and results are reported as pg/mg protein.

Presentation of data and statistical analysis

All reported values are mean $\pm$ SEM and $p < 0.05$ was considered statistically significant. LVDP, +dp/–dt and –dp/dt are presented as a percentage of equilibration. Myocardial functional parameters were analyzed at time 80 (end-reperfusion). Data were compared using one-way analysis of variance (ANOVA) with post-hoc Tukey test.

Results

MSCs improved cardiac function following I/R injury

Infusion of MSCs from WT1 significantly increased post-ischemic myocardial function compared to control as exhibited by improved LVDP (Figures 1A, and 4A) and +dp/dt (Figures 1B and 4B). A trend toward increased myocardial recovery compared with control following infusion of WT2 MSC was also noted in terms with respect to improved LVDP (95% CI [-28.47 to 0.1503]), +dp/dt (95% CI [-18.80 to 5.68]), and -dp/dt (95% CI [-23.77 to 2.33]). In general, infusion of WT MSCs appeared to protect cardiac contractility in hearts subjected to acute I/R, which is in line with our previous observations (18).

Absence of TNFR1 improved MSC-mediated cardioprotection

Treatment with MSCs from TNFR1KO mice prior to ischemia further improved post-ischemic cardiac function when compared to WT1 MSC group. Ablation of TNFR1 in MSCs showed an increased LVDP by 33% (Figure 2A), +dp/dt by 39% (Figure 2B), and protected -dp/dt by 43% (Figure 2C) at the end of reperfusion compared with control.

Deficiency of TNFR2 neutralized MSC-provided cardioprotection

Notably, ablation of TNFR2 in MSCs did not increase MSC-mediated myocardial function as shown by TNFR1KO MSCs. In fact, it decreased cardiac function in terms of +dp/dt compared to WT1 MSC group following I/R (Figure 3B). The absence of TNFR2 reduced MSC-mediated protection of LVDP (Figure 4A), -dp/dt (Figure 4C), and EDP (Figure 4D) to the levels seen in the control group in response to I/R.

Double knockouts of TNFR1 and TNFR2 (TNFR1/2KO) abolished MSC-mediated improvement of cardiac function

Hearts treated with WT2 MSCs exhibited a slight trend toward improved recovery compared with control (Figures 5 and 7). Hearts treated with TNFR1/2KO MSCs exhibited a slight trend toward worse recovery as compared with hearts treated with WT2 MSCs (Figure 6 and 7). No difference in recovery at end reperfusion was detected between TNFR1/2KO MSCs and control (Figure 7).

Improved functional recovery following I/R is associated with decreased levels of myocardial TNF- α —Ventricular tissue from all hearts was assayed for TNF- α and VEGF levels via ELISA. No differences in myocardial VEGF levels were detected (Figures 8B and 8D). Differences in ventricular TNF- α levels were detected with WT1, WT2 and TNFR1KO MSC-treated hearts exhibiting lower levels of TNF- α as compared with control (Figures 8A and 8C).

Discussion

In this study, by using genetically modified MSCs with ablation of TNFR1, TNFR2 or TNFR1/2, we determined the effects of TNF receptors on MSC-mediated protection of myocardial function following acute I/R. Herein, our results further confirmed that pre-ischemic infusion of MSCs improved cardiac function in response to I/R. In addition, the absence of TNFR1 increased MSC-provided cardioprotection as exhibited by further improved LVDP and +/-dp/dt in TNFR1KO MSC treated group. However, deficiency of TNFR2 or TNFR1/2 appears to have no effect or, in the case of the latter, may even negate the association of MSCs with improved myocardial recovery following I/R. Additionally, improved functional recovery correlated with reduced levels of ventricular myocardial TNF- α levels.

Stem cells have been reported to repair irreversible tissue damage via differentiation to the injured cells (19). However, accumulating evidence has questioned this mechanism due to the low numbers of stem cells engrafted into target tissue (20,21). Recently, there have been a growing number of studies indicating that cardiac protection by MSCs may be mediated through their paracrine actions, including production of protective molecules (22). Indeed, our group has previously demonstrated that treatment with MSCs prior to ischemia significantly improved myocardial function (16,23,24). Those infused MSCs were not able to differentiate into cardiomyocytes during such a brief time (25-minutes of ischemia plus 40-minutes of reperfusion), suggesting that MSC paracrine actions exert primary effects on protection of myocardial function following acute I/R. In this study, we further confirmed that paracrine effects of MSCs provide cardiac protection directly, with respect to pre-ischemic infusion of MSCs resulting in increased functional recovery following I/R.

Recently, much attention has been directed at how to optimize the therapeutic effectiveness of stem cells for clinical use. Among these, genetic modification of MSCs is an appealing method. MSCs engineered to overexpress myocardial protective factors such as VEGF and Akt have been shown to significantly augment their ability to protect the ischemic myocardium (6,25). TNF, a pro-inflammatory cytokine, is substantially produced in the heart subjected to I/R injury. TNF exerts different effects on cardiac myocytes through binding to different receptors (TNFR1 and/or TNFR2) and activating different down-stream signaling pathways (detrimental vs. beneficial) (4,9,11). Therefore, one would assume that TNF may play different roles in MSC paracrine effects depending on activation of different TNF receptors. On one hand, TNF has been reported to induce MSC apoptosis and reduce MSC survival (12,26). Conversely, the addition of TNF is able to stimulate MSC production of growth factors, including VEGF, HGF and IGF-1 in vitro (27), suggesting that TNF may benefit MSC paracrine action (28). In the present study, we found that deficiency of different TNF receptors led to different effects of MSCs on the protection of myocardial function following I/R. Ablation of TNFR1 significantly increased MSC-mediated cardioprotection, suggesting that TNFR1KO may exert a beneficial effect on MSC paracrine actions, and thus, may augment MSC-provided protection. On the other hand, the absence of TNFR2 did not improve MSC-mediated protection of myocardial function and deletion of both TNF receptors was associated with a trend toward negation of MSC-mediated protection.

In fact, our previous study has indicated that genetic deletion of TNFR1 increased MSC VEGF production (13). VEGF is an important mediator of stem cell paracrine effects. Genetically modified MSCs to overexpress VEGF have been shown to enhance MSC-mediated protection in the ischemic heart (6). In addition, adult MSCs provide greater protection of myocardial function following I/R due to their higher VEGF production when compared to neonatal MSCs (24). Furthermore, decreasing VEGF levels in MSCs by using VEGF siRNA neutralizes MSC-provided cardiac protection in the heart subjected to acute I/R injury (24). These findings suggest that modification of MSCs to increase VEGF production may augment protective paracrine effects of MSCs. Therefore, it is possible that increased VEGF production in TNFR1KO MSCs may provide greater cardioprotection following I/R. On the other hand, deficiency of the TNFR2 gene has been noted to decrease MSC VEGF production under normal cell culture conditions and stimulation with TNF or hypoxia (13). Therefore, it is not surprising that in this study, pre-ischemic infusion of TNFR2KO MSCs abolishes MSC-mediated protection of cardiac function following acute I/R.

Importantly, however, differences in myocardial VEGF levels among groups in these experiments were not detected. It is possible that the use of mouse MSCs in the rat myocardium may confound the ability to detect changes in VEGF levels particularly when

the proposed differences in VEGF are hypothesized to occur because of changes in MSC secretion of this growth factor. In this case, an antibody specific for rat VEGF was utilized for the ELISA assay. However, there is significant cross-reactivity between both the mouse and rat antibodies and the mouse and rat VEGF protein in commercially available kits and therefore changes in VEGF levels related to differences in MSC production of this growth factor may be difficult to detect via this method. Nevertheless, the previously obtained *in vitro* data described above does support a potential role for VEGF in the changes observed among the different MSCs studied with these experiments.

Ablation of TNFR1 has also been shown to decrease MSC production of TNF- α and IL-6 in response to hypoxia *in vitro* (12). This provides another possibility that decreased levels of TNF and IL-6 in TNFR1KO MSCs may alleviate MSCs' damage when they are infused into the ischemic heart. Aside from the effect of TNF- α on MSCs, it is also of interest to consider the effect that MSCs can have on TNF- α production by other cell types. Accumulating data suggests that MSCs have a unique ability to modulate the immune system and the inflammatory response (29). Recent evidence obtained from co-culture experiments suggests that MSCs can reduce TNF- α production by cells of the immune system (30). This is a possible explanation for the findings of reduced TNF- α levels in the myocardium of MSC-treated hearts observed with these experiments. It is possible that TNFR receptors differentially regulate the ability of MSCs to attenuate TNF- α production by other cell types which could provide an explanation for these findings. Additional experiments would need to be performed to confirm this.

In summary, genetic modification of MSCs is able to enhance MSC paracrine effects for widespread clinical use. This study indicates that genetically engineered MSCs with TNFR1KO increases MSC-mediated protection of myocardial function, whereas the absence of TNFR2 did not improve MSC-mediated protection of myocardial function and deletion of both TNF receptors was associated with a trend toward negation of MSC-mediated protection. Further investigations are required to determine the detailed mechanisms by which TNFR1 or TNFR2 MSCs act via paracrine actions, and to develop new methods for enhancing the therapeutic effectiveness of stem cells.

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The abbreviations used are

MSC	bone marrow mesenchymal stem cell
TNF	tumor necrosis factor α
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
TNFR1KO	TNFR1 knockout
TNFR2KO	TNFR2 knockout
TNFR1/2KO	TNFR1 and TNFR2 knockout
I/R	ischemia/reperfusion
WT1	C57BL/6J
WT2	B6129SF2

LVDP	left ventricular developed pressure
EDP	end diastolic pressure
VEGF	vascular endothelial growth factor

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Figure 1.

Effects of pretreatment of WT1 [C57BL/6J] MSCs into isolated hearts prior to ischemia on myocardial functional recovery following I/R injury. Left ventricular functional parameters over time: (A) LVDP (% of equilibration), (B) +dp/dt (% of equilibration) and (C) -dp/dt (% of equilibration), and (D) EDP (mmHg). Results are Mean $\pm$ SEM, *= p <0.05 vs. control.

Figure 2. Effects of pre-ischemic delivery of TNFR1KO MSCs into isolated hearts on myocardial functional recovery following I/R injury. Left ventricular functional parameters over time: (A) LVDP (% of equilibration), (B) +dp/dt (% of equilibration) and (C) -dp/dt (% of equilibration), and (D) EDP (mmHg). Results are Mean $\pm$ SEM, * $=p<0.05$ vs. WT1 MSC.

Figure 3.

Effects of TNFR2KO MSC infusions into isolated hearts prior to ischemia on myocardial functional recovery following I/R injury. Left ventricular functional parameters over time: (A) LVDP (% of equilibration), (B) +dp/dt (% of equilibration) and (C) -dp/dt (% of equilibration), and (D) EDP (mmHg). Results are Mean $\pm$ SEM, *= $p < 0.05$ vs. WT1 MSC.

Figure 4.

Representative results of end reperfusion comparisons of (A) LVDP (% of equilibration; absolute values are 31.13 $\pm$ 4.42 mmHg, 46.87 $\pm$ 3.99 mmHg, 70.01 $\pm$ 5.75 mmHg, and 33.31 $\pm$ 2.54 mmHg for control, WT1 MSC, R1KO MSC, and R2KO MSC, respectively), (B) +dp/dt (% of equilibration; absolute values are 913.64 $\pm$ 137.28 mmHg/s, 1573.09 $\pm$ 235.38 mmHg/s, 2145.47 $\pm$ 116.64 mmHg/s, and 899.44 $\pm$ 114.06 mmHg/s for control, WT1 MSC, R1KO MSC, and R2KO MSC, respectively), (C) -dp/dt (% of equilibration; absolute values are -527.64 $\pm$ 64.98 mmHg/s, -923.87 $\pm$ 134.81 mmHg/s, -1124.02 $\pm$ 39.27 mmHg/s, and -549.17 $\pm$ 67.16 mmHg/s for control, WT1 MSC, R1KO MSC, and R2KO MSC, respectively) and (D) EDP (mmHg) in control, WT1 MSC, TNFR1KO MSC and TNFR2KO MSC groups. Results are Mean $\pm$ SEM, *= p <0.05 vs. control, #= p <0.05 vs. WT1 MSC, &= p <0.05 vs. R1KO MSC.

Figure 5.

Effects of WT2 [B6129SF2] MSCs pre-ischemic infusion to isolated hearts on myocardial functional recovery following I/R injury. Left ventricular functional parameters over time: (A) LVDP (% of equilibration), (B) +dp/dt (% of equilibration) and (C) -dp/dt (% of equilibration), and (D) EDP (mmHg). Results are Mean $\pm$ SEM, $*=p<0.05$ vs. control.

Figure 6.

Effects of pretreatment with TNFR1/2KO MSCs to isolated hearts prior to ischemia on myocardial functional recovery following I/R injury. Left ventricular functional parameters over time: (A) LVDP (% of equilibration), (B) +dp/dt (% of equilibration) and (C) -dp/dt (% of equilibration), and (D) EDP (mmHg). Results are Mean $\pm$ SEM, $^*p<0.05$ vs. WT2 MSC.

Figure 7.

Representative results of end reperfusion comparisons of (A) LVDP (% of equilibration; absolute values are 31.13 $\pm$ 4.42 mmHg, 39.17 $\pm$ 2.95 mmHg, and 33.28 $\pm$ 3.49 mmHg for control, WT2 MSC, and R1/2KO MSC, respectively), (B) +dp/dt (% of equilibration; absolute values are 913.64 $\pm$ 137.28 mmHg/s, 982.32 $\pm$ 151.16 mmHg/s, and 1046.32 $\pm$ 93.83 mmHg/s for control, WT2 MSC, and R1/2KO MSC, respectively), (C) -dp/dt (% of equilibration; absolute values are - 527.64 $\pm$ 64.98 mmHg/s, -590.02 $\pm$ 79.96 mmHg/s, and -589.31 $\pm$ 59.61 mmHg/s for control, WT2 MSC, and R1/2KO MSC, respectively) and (D) EDP (mmHg) in control, WT2 MSC and TNFR1/2KO MSC groups. Results are Mean $\pm$ SEM, 95% confidence intervals represent WT2 MSC vs. control and R1/R2KO MSC vs. control.

Figure 8. Ventricular VEGF and TNF- α levels in hearts following I/R. Results are expressed as picograms of VEGF or TNF- α per mg of ventricular protein. *= p <0.05 vs. control, #= p <0.05 vs. WT1 MSC, &= p <0.05 vs. R1KO MSC.