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GENDER DIFFERENCES IN INJURY INDUCED MESENCHYMAL STEM CELL APOPTOSIS, EXPRESSION OF VEGF, TNF, AND IL-6 AND ABROGATION VIA TNFR1 ABLATION

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Abstract

Concomitant pro- and anti-inflammatory properties of bone marrow stem cells (BMSC) may be an important aspect of their ability to heal injured tissue. However, very few studies have examined whether gender differences exist in BMSC function. Indeed, it remains unknown whether gender differences exist in BMSC function and ability to resist apoptosis, and if so, whether TNF receptor 1 (TNFR1) plays a role in these differences. We hypothesized that TNFR1 ablation equalizes gender differences in bone marrow mesenchymal stem cell (MSC) apoptosis, as well as expression of vascular endothelial growth factor (VEGF), TNF, and interleukin (IL)-6. Mouse MSCs from male wildtype (WT), female WT, male TNFR1 knockouts (TNFR1KO), and female TNFR1KO were stressed by endotoxin 200 ng/ml or 1 hr hypoxia. MSC activation was determined by measuring VEGF, TNF, and IL-6 production (ELISA). Differences considered significant if $p < 0.05$. LPS and hypoxia resulted in significant activation in all experimental groups compared to controls. Male WT demonstrated significantly greater TNF and IL-6 and significantly less VEGF release than female WT MSCs. However, release of TNF, IL-6, and VEGF in male TNFR1 knockouts differed from male WT, but was not different from female WT MSCs. Similarly apoptosis in hypoxic male TNFR1KO differed from male WT, but it was not different from apoptosis from WT female. Female WT did not differ in TNF, IL-6, and VEGF release compared to female TNFR1KO. Gender differences exist in injury induced BMSC VEGF, TNF, and IL-6 expression. TNFR1 may autoregulate VEGF, TNF, and IL-6 expression in males more than females. MSCs are novel therapeutic agents for organ protection, but further study of the disparate expression of VEGF, TNF, and IL-6 in males and females as well as the role of TNFR1 in these gender differences is necessary to maximize this protection.

Keywords

mesenchymal stem cell; protection; endotoxin; sex; hypoxia; apoptosis

INTRODUCTION

Bone marrow stem cells (BMSCs) represent a novel treatment modality with increasing therapeutic potential [1,2]. Bone marrow hematopoietic stem cells (HSCs) [3] and

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nonhematopoietic mesenchymal stem cells (MSCs) [4] have each demonstrated positive remodeling and regeneration of viable tissues. However, recent experimental studies questioning the engraftment and transdifferentiation of BMSCs [5], HSCs [6], and MSCs [7] suggests that stem cells mediate their beneficial effects via complex paracrine actions. Indeed, we and others have previously demonstrated that BMSC differentiation is not required for cardioprotection; acute application of human BMSC into myocardium subjected to ischemia reperfusion improved functional recovery, decreased proinflammatory cytokine production, and decreased activation of proapoptotic caspases [8,9]. Thus, concomitant pro- and anti-inflammatory properties of BMSCs may be an important aspect of their ability to heal injured tissue. Several recent studies have shown that gender differences exist in monocyte proinflammatory cytokine production [10–12]. No study has addressed gender differences in the pro- and anti-inflammatory properties of BMSCs.

Proinflammatory tumor necrosis factor- α (TNF) is induced in response to various injuries [13] and plays a central role in post-injured organ dysfunction, local tissue cell apoptosis, as well as induction of proinflammatory signaling [14]. It is now recognized that TNF acts by binding to a 55-kDa receptor (TNFR1) and/or a 75-kDa receptor (TNFR2). Although these two receptors induce both distinct and overlapping responses, dysfunction and apoptosis in various tissues are initiated by binding to TNFR1 [15]. We and others have shown significantly improved myocardial function in TNFR1 knockout mice compared to wild type mice after myocardial infarction [16]. Further, the female advantage after myocardial infarction may be associated with their resistance to myocardial TNFR1 signaling [16]. Although TNFR1 exists in BMSCs [17], no study has addressed the role of TNFR1 and gender on bone marrow stem cell activation and function.

MSCs are a relatively underexplored population of BMSCs that may have advantages over the well characterized HSC population [18,19]. We hypothesized that TNFR1 ablation equalizes gender differences in bone marrow mesenchymal stem cell expression of VEGF, TNF, and IL-6. The purposes of this study were to investigate the effect of endotoxin (lipopolysaccharide (LPS)) and hypoxia on wild type and TNFR1 knockout mouse bone marrow MSC activation as measured by: 1) vascular endothelial growth factor (VEGF) expression; 2) TNF expression; 3) interleukin (IL)-6 expression; and 4) MSC apoptosis.

METHODS

Animals

C57BL/6J wild type (WT) mice and mice with targeted deletion of TNFR1 (TNFR1KO) (The Jackson Laboratory, Bar Harbor, ME) of both genders were fed a standard diet and acclimated in a quiet quarantine room for one 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 1985).

Preparation of Mouse Bone Marrow Stromal Cells

A single-step purification method using adhesion to cell culture plastic is employed as previously described[20] with the following modifications: Mouse bone marrow stromal cells were collected, after sacrifice of 8 week old mice, from bilateral femurs and tibias 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 23G needle. Cells were disaggregated by vigorous pipetting several times. Cells were passed through 30- μ m nylon mesh to remove remaining clumps of tissue. Cells were washed by adding complete media, centrifuging for 5 min at 300

rpm @ 24°C and removing supernatant. The cell pellet was then resuspended and cultured in 75 cm² culture flasks with complete media at 37°C. MSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete medium was added and replaced every three or four days thereafter. MSC cultures were maintained at 37°C in 5% CO₂ in air. When the cultures reached 90% of confluence, the MSC culture was passaged; cells were recovered by the addition of a solution 0.25% trypsin-EDTA (GIBCO Invitrogen, Carlsbad, CA) and replated in 75 cm² culture flasks.

Experimental groups

MSCs were plated in 12 well plates in a concentration of 1×10^6 cells / well / ml. MSCs were divided into four experimental groups: 1) male WT; 2) female WT; 3) male TNFR1KO; and 4) female TNFR1KO. MSCs (triplicate wells per group) were then stressed with LPS 200 ng/ml or 1 hr hypoxia. After 24 h incubation, supernatants were harvested for vascular endothelial growth factor-A (VEGF), TNF, and IL-6 assay (ELISA). The experiment was repeated on three separate occasions (n=6–11 wells / group).

TNF, IL-6, and VEGF ELISA

VEGF, TNF, and IL-6 release in the BMSC were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (R&D Systems Inc., Minneapolis, MN and BD Biosciences, San Diego, CA). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Apoptosis ELISA

Apoptosis in MSCs subjected to hypoxia was determined by ELISA using a commercially available Cell Death Detection ELISA set (Roche Applied Science, Indianapolis, IN). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Presentation of data and statistical analysis

All reported values are mean $\pm$ SEM. Data was compared using two-way analysis of variance (ANOVA) with post-hoc Bonferroni test or Student's t-test. A two-tailed probability value of less than 0.05 was considered statistically significant.

RESULTS

Effect of LPS on MSC Activation

LPS resulted in significant activation of MSCs. LPS provoked significant TNF production in male WT (657.7 ± 55.2 pg/ml), female WT (184.8 ± 82.1 pg/ml), male TNFR1KO (599.9 ± 132.2 pg/ml), and female TNFR1KO (14.51 ± 3.1 pg/ml) in comparison to controls as shown in Figure 1. In addition, LPS induced significant VEGF release in male WT (889.8 ± 44.8 pg/ml), female WT (871.1 ± 83.4 pg/ml), male TNFR1KO (736.6 ± 66.8 pg/ml), and female TNFR1KO (762.5 ± 118.5 pg/ml) in comparison to controls as shown in Figure 2. Likewise, LPS provoked a significant increase of IL-6 in male WT (157 fold), female WT (40 fold), male TNFR1KO (61 fold), and female TNFR1KO (40 fold) in comparison to controls as shown in Figure 3.

Effect of hypoxia on MSC Activation

Hypoxia resulted in significant activation of MSCs. Hypoxia provoked significant TNF production in male WT (40.5 ± 5.2 pg/ml), female WT (11.6 ± 2.2 pg/ml), male TNFR1KO (16.1 ± 3.5 pg/ml), and female TNFR1KO (5.5 ± 1.6 pg/ml) in comparison to controls as shown in Figure 1. In addition, hypoxia induced significant VEGF release in male WT (716.2 ± 1.9

pg/ml), female WT (742.5 ± 5.5 pg/ml), male TNFR1KO (500.0 ± 11.5 pg/ml), and female TNFR1KO (634.9 ± 13.8 pg/ml) in comparison to controls as shown in Figure 2. Likewise, LPS provoked a significant increase of IL-6 in male WT (9 fold), female WT (4 fold), male TNFR1KO (2 fold), and female TNFR1KO (3 fold) in comparison to controls as shown in Figure 3.

Gender differences in MSC Activation

However, both hypoxia and endotoxin demonstrated significantly greater TNF and IL-6 in WT male and significantly less VEGF expression than female WT MSCs (Figure 1–3). Interestingly, male TNFR1KO release of TNF, IL-6 and VEGF was significantly different from male WT, but not different from female WT as shown in Figure 1–3. Female WT MSCs did not significantly differ from female TNFR1KO MSC expression of VEGF.

Gender differences in MSC Apoptosis

Hypoxia induced significantly more apoptosis in WT male MSCs than in MSCs from WT female after 24 h incubation (Figure 4A). Interestingly, male TNFR1KO apoptosis was significantly lower than male WT, but not different from female WT and female TNFR1KO as shown in Figure 4B,C.

DISCUSSION

The results of this study are the first demonstration that: 1) acute MSC activation via LPS and hypoxia results in local growth factor and pro-inflammatory cytokine expression; 2) activated female MSCs exhibit decreased apoptosis as well as decreased TNF, decreased IL-6, and increased VEGF expression; and 3) ablation of TNFR1 in males equalizes the female advantage in apoptosis and TNF, IL-6, and VEGF expression.

Adult mesenchymal stem cells may mediate their acute organ-protective effects via augmented angiogenesis and other complex paracrine effects. When transplanted into a foreign, inflammatory environment, MSCs may release substances which limit local inflammation in order to enhance its survival. Acute administration into injured tissue (which precluded immediate stem cell differentiation) of murine MSCs [7,9] as well as human MSCs [8] improved function, decreased proinflammatory cytokine production, and decreased apoptosis. MSC therapy has been associated with protection via VEGF expression [21,22]. In congruence, we found that MSC stimulation with LPS and hypoxia resulted in significant release of VEGF. Determining whether BMSC release of VEGF confers protection via reduced apoptosis [23], decreased proinflammatory cytokines [24], or other mechanistic pathways [25] requires further investigation. Thus, MSCs may enhance their own survival in addition to surrounding tissue by modulating local inflammation and stimulating endogenous repair mechanisms via the release of substances such as VEGF.

Recent studies have also shown that females experience a protective advantage following several forms of acute cardiac injury [26–30]. Improved survival, less injury, and diminished inflammation are found in females when compared to males after ischemia-reperfusion (I/R) injury. [31–33]. This study further addresses the influence of gender in the injury response. Female MSCs subjected to endotoxic or hypoxic injury demonstrated significantly greater VEGF expression than male MSCs. In correlation with the proposed inflammatory modulation via VEGF, female MSCs also exhibited less TNF and IL-6 expression in comparison to males after LPS and hypoxia. Female MSCs also demonstrated significantly less apoptosis than male MSCs after hypoxic injury. Rauscher et al demonstrated that BMSCs significantly decreased serum levels of IL-6, and limited atherosclerosis in a mouse atherosclerosis progression model [24]. Whether these increased levels of VEGF and decreased levels of TNF and IL-6 mediate

protection is uncertain. Nevertheless, elevated VEGF production and decreased TNF and IL-6 expression by female MSC in the face of acute injury imply that female stem cells may have enhanced resistance to insult.

The role of TNF and the TNF receptor in stem cell function is complex and variable. It is now recognized that TNFR1 and/or TNFR2 engagement is essential for many of the biological responses of TNF. Both receptors exist in most cell types, including bone marrow stem cells [17]. In cardiac models, ablation of the TNFR1 gene blunts injury and improves survival, whereas ablation of the TNFR2 gene exacerbates injury and reduces survival [15]. This has led to the important appreciation that TNF may have beneficial or detrimental effects depending on which of its receptors is activated. In bone marrow stem cell function, this functional dichotomy in response to TNF has also been observed. While TNF may augment stem cell migration and chemoattraction [34], it also may inhibit stem cell proliferation, possibly via a TNFR1 mechanism [35]. Indeed, we observed that TNFR1 ablation significantly increases VEGF and decreases TNF and IL-6 expression in male MSCs, conferring a phenotypic MSC response in VEGF and TNF expression very similar to female MSCs. Further, TNFR1 ablation significantly reduced male MSC apoptosis after hypoxic injury. Thus, differential TNF receptor intracellular signaling may in part explain the gender differences in stem cell function described earlier. Targeting TNF signaling to increase anti-inflammatory and decrease in pro-inflammatory production may be of therapeutic value in the application of stem cell therapies.

It remains unclear what other mechanisms play a role in BMSC gender differences. Other progenitor cell populations may be differentially regulated by estrogen and progesterone, resulting in more progenitor cells in female derived primary cultures than males [36]. Interestingly, exogenous estrogen may also increase MSC function and calcium deposition [37]. Estrogen receptor alpha may be a target for estrogen and play a role in BMSC growth and differentiation [38]. Mitogen activated protein kinases and cyclin dependent kinases may mediate the proliferative effect of estrogen on mouse embryonic stem cells [39]. Thus, it appears that estrogen which has been associated with greater protection found after acute cardiac injury, may also be associated with gender differences in BMSC activity. No study has addressed whether females may derive more benefit from stem cell therapy than males. However, if estrogen indeed plays a role in mediating BMSC activity and proliferation, one can hypothesize that females may have a therapeutic advantage regarding BMSC applications.

These results demonstrate that murine mesenchymal stem cells are significant sources of local paracrine factors such as the potentially protective VEGF and inflammatory TNF. Planned ischemic events such as those that occur during cardiac surgery, angioplasty, or transplantation may allow opportunity to observe the potential clinical benefit of adult stem cell therapies. However, gender discrepancies exist in mesenchymal stem cell function after injury, and injury induced TNF signaling via TNFR1 may have disparate effects in males and females. The attractive potential for the clinical application of stem cell therapy warrants further study to elucidate the mechanistic pathways to maximize stem cell paracrine protective factor expression such as VEGF and to minimize stem cell pro-inflammatory production such as TNF.

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Figure 1.

MSC WT activation (TNF release) after injury. **A.** Male and female WT after LPS exposure, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **B.** Male and female TNFR1KO after LPS exposure, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **C.** Expression of TNF after LPS exposure. Results expressed as folds of control, mean $\pm$ SEM, *p<0.05 MWT vs MTNFR1KO. **D.** Male and female WT after hypoxia, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **E.** Male and female TNFR1KO after hypoxia, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **F.** Expression of TNF after hypoxia. Results expressed as folds of control, mean $\pm$ SEM, *p<0.05 MWT vs MTNFR1KO.

Figure 2.

MSC WT activation (VEGF release) after injury. **A.** Male and female WT after LPS exposure, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **B.** Male and female TNFR1KO after LPS exposure, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **C.** Expression of VEGF after LPS exposure. Results expressed as folds of control, mean $\pm$ SEM, *p<0.05 MWT vs MTNFR1KO. **D.** Male and female WT after hypoxia, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **E.** Male and female TNFR1KO after hypoxia, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **F.** Expression of VEGF after hypoxia. Results expressed as folds of control, mean $\pm$ SEM, *p<0.05 MWT vs MTNFR1KO.

Figure 3.

MSC WT activation (IL-6 release) after injury. **A.** Male and female WT after LPS exposure, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **B.** Male and female TNFR1KO after LPS exposure, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **C.** Expression of IL-6 after LPS exposure. Results expressed as folds of control, mean $\pm$ SEM, *p<0.05 MWT vs MTNFR1KO. **D.** Male and female WT after hypoxia, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **E.** Male and female TNFR1KO after hypoxia, results expressed as pg/ml, mean $\pm$ SEM, *p<0.05 vs control, +p<0.05 male vs female. **F.** Expression of IL-6 after hypoxia. Results expressed as folds of control, mean $\pm$ SEM, *p<0.05 MWT vs MTNFR1KO.

Figure 4.

MSC apoptosis after hypoxia. **A.** Male and female WT, results expressed as absorbance (A_{405}), mean $\pm$ SEM, $*p < 0.05$ vs control. **B.** Male and female TNFR1KO, results expressed as absorbance (A_{405}), mean $\pm$ SEM, $*p < 0.05$ vs control. **C.** Cell death all groups, mean $\pm$ SEM, $*p < 0.05$ vs MWT.