TNFR1 signaling resistance associated with female stem cell cytokine production is independent of TNFR2-mediated pathways

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Markel TA, Crisostomo PR, Wang M, Wang Y, Lahm T, Novotny NM, Tan J, Meldrum DR. TNFR1 signaling resistance associated with female stem cell cytokine production is independent of TNFR2-mediated pathways. Am J Physiol Regul Integr Comp Physiol 295: R1124–R1130, 2008. First published August 6, 2008; doi:10.1152/ajpregu.90508.2008.—End-organ ischemia is a common source of patient morbidity and mortality. Stem cell therapy represents a novel treatment modality for ischemic diseases and may aid injured tissues through the release of beneficial paracrine mediators. Female bone marrow mesenchymal stem cells (MSCs) have demonstrated a relative resistance to detrimental TNF receptor 1 (TNFR1) signaling and are thought to be superior to male stem cells in limiting inflammation. However, it is not known whether sex differences exist in TNF receptor 2 (TNFR2)-ablated MSCs. Therefore, we hypothesized that 1) sex differences would be observed in wild-type (WT) and TNFR2-ablated MSC cytokine signaling, and 2) the production of IL-6, VEGF, and IGF-1 in males, but not females, would be mediated through TNFR2. MSCs were harvested from male and female WT and TNFR2 knockout (TNFR2KO) mice and were subsequently exposed to TNF (50 ng/ml) or LPS (100 ng/ml). After 24 h, supernatants were collected and measured for cytokines. TNF and LPS stimulated WT stem cells to produce cytokines, but sex differences were only seen in IL-6 and IGF-1 after TNF stimulation. Ablation of TNFR2 increased VEGF and IGF-1 production in males compared with wild-type, but no difference was observed in females. Female MSCs from TNFR2KOs produced significantly lower levels of VEGF and IGF-1 compared with male MSCs. The absence of TNFR2 signaling appears to play a greater role in male MSC cytokine production. As a result, male, but not female stem cell cytokine production may be mediated through TNFR2 signaling cascades.

Materials and Methods

Animals. Adult male and female C57BL/6J wild-type (WT) mice, and mice with a targeted deletion of TNFR2 (TNFR2KO) (Jackson Laboratory, Bar Harbor, ME) were fed a standard diet and acclimated in a quiet quarantine room for 2 wk 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).

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Preparation of mouse bone marrow stem cells. A single-step purification method using adhesion to cell culture plastic was used as previously described (36). Following euthanasia, bone marrow stem cells were collected from male and female WT and TNFR2KO adult mice. Cells were collected from bilateral femurs and tibias by removing the epiphyses and flushing the shaft with complete media [Iscove’s Modified Dulbecco’s Medium (GIBCO Invitrogen, Carlsbad, CA) and 10% FBS (GIBCO Invitrogen)] using a syringe with a 26G needle. Cells were disaggregated by vigorous pipetting and were passed through 30-μm nylon mesh to remove remaining clumps of tissue. Cells were washed by adding complete media, centrifuging for 5 min at 300 rpm at 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. MSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete media were added and replaced every 3 or 4 days thereafter. MSC cultures were maintained at 37°C in 5% CO₂ in air. When the cultures reached 90% of confluence, the MSCs were passaged; cells were recovered by the addition of a solution of 0.25% trypsin-EDTA (GIBCO Invitrogen, Carlsbad, CA) and 10% FBS (GIBCO Invitrogen) [Iscove’s Modified Dulbecco’s Medium (GIBCO Invitrogen, Carlsbad, CA) and 10% FBS (GIBCO Invitrogen)], and replated in 75 cm² culture flasks. MSCs were negative for the hematopoietic markers CD34, CD45, and CD117, and were positive for the mesenchymal stem cell marker CD44 (27). Cells were used for experimentation between passages 3 and 9.

Experimental groups. After three passages, MSCs were plated in 12-well plates in a concentration of 1 × 10⁵ cells·well⁻¹·ml⁻¹. Male and female WT and TNFR2KO MSCs were divided into three experimental groups (triplicate wells per group) and exposed to 1) no stimulus, 2) TNF (50 ng/ml), or 3) LPS (100 ng/ml). These stimuli have previously been shown to activate MSCs and serve to increase cellular production of TNF (51). After 24 h of incubation, supernatants were collected and stored at −80°C. Experiments were performed at least three times.

Enzyme-linked immunosorbent assay. IL-6, VEGF, and IGF-1 in the MSC supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA sets (R&D Systems, 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 (n = 15–31/group).

Presentation of data and statistical analysis. All reported values are expressed as means ± SE. Data were compared using Student’s t-test. A P value of less than 0.05 was considered statistically significant.

RESULTS

TNF and LPS stimulate mesenchymal stem cells. Stimulation of wild-type (Fig. 1) or TNFR2KO (Fig. 2) MSCs with either TNF or LPS caused a significant increase in stem cell IL-6 and VEGF production from baseline values. The production of IGF-1 after stimulation was decreased compared with baseline. Male TNFR2KOs were noted to express higher basal levels of VEGF and IGF-1, indicating that TNF ablation also affected unstimulated growth factor production.

Sex differences in WT mesenchymal stem cell cytokine production. MSC production of IL-6 and IGF-1 was noted to be affected by the sex of the host from which they were derived. Female WT MSCs produced lower levels of TNF-stimulated IL-6 (male: 417 ± 60.93 pg/ml, female: 229.9 ± 58.8 pg/ml), and higher levels of TNF-stimulated IGF-1 (Male: 13.2 ± 3.4 pg/ml, Female: 26.0 ± 5.8). Sex differences were not noted in TNF-stimulated VEGF production, nor were there any observable sex differences in LPS-stimulated cytokine production (Fig. 3).

TNF2 ablation alters sex-regulated cytokine signaling in MSCs. Ablation of TNFR2 in males was noted to significantly increase the TNF and LPS-stimulated production of VEGF (TNF-stimulated, TNFR2KO: 777.7 ± 58.3 pg/ml, WT: 551.7 ± 44.8; LPS stimulated, TNFR2KO: 712.0 ± 68.8 pg/ml, WT: 519.6 ± 40.0 pg/ml) and IGF-1 (TNF stimulated, TNFR2KO: 97.9 ± 19.6 pg/ml, WT: 13.2 ± 3.4 pg/ml; LPS stimulated, TNFR2KO: 81.3 ± 18.2 pg/ml, WT: 18.5 ± 4.5 pg/ml) compared with WT. TNFR2 ablation had no effect on male stem cell IL-6 production compared with WT. Conversely, ablation of TNFR2 in females resulted in no significant differences in stimulated IL-6, VEGF, or IGF-1 production compared with wild-type values.

When sex differences were compared in TNFR2-ablated stem cells, females produced significantly lower levels of stimulated VEGF (TNF stimulated, male: 777.7 ± 58.3 pg/ml, female: 523.1 ± 52.1 pg/ml; LPS stimulated, male: 712.0 ± 68.8 pg/ml, female: 520.7 ± 49.7 pg/ml) and IGF-1 (TNF stimulated, male: 97.9 ± 19.6 pg/ml, female: 14.9 ± 4.8 pg/ml; LPS stimulated, male: 81.3 ± 18.2 pg/ml, female: 13.7 ± 4.2 pg/ml) compared with male TNFR2KO stem cells. No differ-
ences were noted in IL-6 between TNFR2-ablated female and male MSCs (Fig. 3).

DISCUSSION

Sex differences have previously been shown to alter TNF receptor signaling cascades. Studies using TNFR1-ablated mesenchymal stem cells showed that these sex disparities may be attributed to differences in TNFRI signaling (6). However, the role that TNFR2 contributed to these sex differences had not been previously defined. Herein, we confirmed that TNF and LPS stimulate MSCs to produce elevated levels of cytokines. Furthermore, we demonstrated that sex differences exist in TNF-stimulated WT MSCs, but not in those stimulated with LPS, 2) sex plays a role in TNFR1-mediated (TNFR2KO) stem cell cytokine production, 3) the production of cytokines in males, but not females, may be mediated through TNFR2 signaling cascades.

Previous animal studies have demonstrated that decreasing the bioavailability of TNF has beneficial effects after acute ischemic episodes (11). However, clinical studies have suggested that simply decreasing the bioavailability of TNF may lack benefit (26). Therefore, TNF itself may have beneficial or detrimental effects, depending on which of its receptors is activated. Earlier studies have suggested that MSCs produce vital growth factors by activation of TNF receptors (16) and downstream mediators such as p38 (47), ERK, and NF-κB (7). Therefore, unbalancing TNF signaling to diminish its deleterious effects, while enhancing its salutary properties, may be an important new approach to stem cell therapy. In an attempt to maximize stem cell paracrine effects, it is necessary to understand the mechanisms that stem cells use to produce these protective properties.

Various cytokines, including IL-6, VEGF, and IGF-1, may promote native tissue recovery after ischemic injury, and therefore, elevated local concentrations of these factors may be important. IL-6 is elevated in various pathologies, including, sepsis, ischemia, and trauma (20, 28, 43). IL-6 exhibits potent anti-inflammatory properties, including the ability to prevent apoptosis and tissue damage via the inhibition of metalloproteinases, as well as by increasing production of the IL-1 receptor antagonist (22, 42, 44). VEGF has exhibited protective properties by enhancing angiogenesis during acute inflammation and ischemia, and by improving stem cell survival during transplantation (14, 46, 50). Similarly, IGF-1 may improve organ function after ischemia by stabilizing mitochondria and by decreasing reactive oxygen species (37). IGF-1 has also been shown to decrease proapoptotic signaling and to increase cellular proliferation (15, 53).

By using a conventional TNFR2KO, we demonstrated that sex played a significant role in TNFR1-mediated stem cell paracrine factor production. We saw that TNFR2 ablation resulted in a significant increase in male MSC VEGF and IGF-1 production but had no effect on female MSC cytokine levels. Previous in vitro studies using TNFR1KO MSCs also noticed that removal of TNFR1 signaling altered cytokine levels in males but had no effect on cytokine and growth factor production in females. These data, in conjunction with ex vivo myocardial functional studies examining TNF receptor signaling, have led to the important appreciation that females may possess a naturally inherent resistance to detrimental TNFR1 signaling. However, it was not clear from our previous studies whether TNFR2 activation played a role in this TNFR1 signaling resistance. In TNFR2-ablated MSCs, the only functioning TNF receptor is TNFR1. As female wild-type MSCs exhibited similar stimulated IL-6, VEGF, and IGF-1 levels as those cells with only TNFR1 (TNFR2KO), it is likely that TNFR1 signaling resistance in female MSCs is not mediated through TNFR2 pathways.

Many theories exist that attempt to explain the sex differences associated with TNF signaling. The upregulation of estrogen receptors in females due to the presence of chronic endogenous estrogen has been suggested as one possible reason for female MSC superiority and relative TNFR1 signaling resistance (Fig. 4). It is thought that the presence of estrogen up-regulates suppressor-of-cytokine-signaling-3 expression, thereby suppressing the deleterious effects of
TNF signaling in females (29, 40). Furthermore, activation of the estrogen receptor has been associated with direct inhibition of IL-6 expression (24, 38) and upregulation of VEGF and IGF production (18, 41). Therefore, the lack of endogenous estrogen may unbalance TNF receptor signaling and predispose males to differential cytokine and growth factor production.

Other studies take an opposing stance and suggest that the presence of chronic androgens, rather than the lack of estrogens, cause the observed sex differences in TNF receptor signaling. This phenomenon has been observed in stem cells, as well as in other tissues (25, 39). On the basis of the combined results of this study and our previous work, it appears that removal of either receptor paradoxically increases growth factor production in male MSCs but has no effect in female MSCs. These observations suggest that testosterone plays an active role in male stem cell TNF-induced cytokine production. Activation of TNF receptors on male stem cells may work in an additive fashion to suppress growth factor production and decrease stem cell function. Further studies are, therefore, needed to elucidate the downstream signaling cascades associated with TNFR2-induced cytokine expression. Understanding the mechanisms that MSCs use to produce these protective factors will allow for maximal in vitro priming prior to their therapeutic use.

**Perspectives and Significance**

End-organ ischemia is quite common in the United States, and stem cell therapy may help to ameliorate the morbidity and mortality associated with this problem. As many clinical
applications have been appreciated, some centers have begun to use stem cells in human trials. Unfortunately though, there have been mixed results in the clinical setting, which may be rooted in our incomplete understanding of how stem cells function under stress. Therefore, to further understand how stem cells provide their protective properties, it is imperative that we understand not only the intracellular signaling cascades associated with stem cells, but also how these cells react and respond to individual hosts. Further in vitro and in vivo animal studies using stem cells may facilitate a better understanding of stem cell function and allow for the design of stem cells that provide maximum protection during clinical application.

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