Activation of individual tumor necrosis factor receptors differentially affects stem cell growth factor and cytokine production

Troy A. Markel,1 Paul R. Crisostomo,1 Meijing Wang,1 Christine M. Herring,1 and Daniel R. Meldrum1,2,3

Departments of 1Surgery and 2Cellular and Integrative Physiology, and 3Center for Immunobiology, Indiana University School of Medicine, Indianapolis, Indiana

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Markel TA, Crisostomo PR, Wang M, Herring CM, Meldrum DR. Activation of individual tumor necrosis factor receptors differentially affects stem cell growth factor and cytokine production. Am J Physiol Gastrointest Liver Physiol 293: G657–G662, 2007. First published July 19, 2007; doi:10.1152/ajpgi.00230.2007.—Necrotizing enterocolitis (NEC) is an emergency of the newborn that often requires surgery. Growth factors from stem cells may aid in decreasing intestinal damage while also promoting restitution. We hypothesized that 1) TNF, LPS, or hypoxia would alter bone marrow mesenchymal stem cell (BMSC) TNF, IGF-1, IL-6, and VEGF production, and 2) TNF receptor type 1 (TNFR1) or type 2 (TNFR2) ablation would result in changes to the patterns of cytokines and growth factors produced. BMSCs were harvested from female wild-type (WT), TNFR1 knockout (KO), and TNFR2KO mice. Cells were stimulated with TNF, LPS, or hypoxia. After 24 h, cell supernatants were assayed via ELISA. Production of TNF and IGF-1 was decreased in both knockouts compared with WT regardless of the stimulus utilized, whereas IL-6 and VEGF levels appeared to be cooperatively regulated by both the activated TNF receptor and the initial stimulus. IL-6 was increased compared with WT in both knockouts following TNF stimulation but was significantly decreased with LPS. Compared with WT, hypoxia increased IL-6 in TNFR1KO but not TNFR2KO cells. TNF stimulation decreased VEGF in TNFR2KO cells, whereas TNFR1 ablation resulted in no change in VEGF compared with WT. TNFR1 ablation resulted in a decrease in VEGF following LPS stimulation compared with WT; no change was noted in TNFR2KO cells. With hypoxia, TNFR1KO cells expressed more VEGF compared with WT, whereas no difference was noted between WT and TNFR2KO cells. TNF receptor ablation modifies BMSC cytokine production. Identifying the proper stimulus and signaling cascades for the production of desired growth factors may be beneficial in maximizing the therapeutic potential of stem cells.

Necrotizing enterocolitis; interleukin-6; vascular endothelial growth factor; insulin-like growth factor-1

Necrotizing enterocolitis (NEC) remains one of the most devastating intra-abdominal emergencies in newborn infants, particularly those of low birth weight (9, 16). Medical management of the more severe cases of NEC is often inadequate, thereby warranting surgical resection of the necrotic intestine. In many cases, however, surgical resection leaves the infant with a suboptimal length of small intestine that precludes adequate nutritional absorption. Therefore, these infants usually require long-term parenteral nutrition. Active research is underway to understand the mechanisms associated with the intestinal ischemia, bacterial translocation, sepsis, and organ failure often associated with NEC (2, 26). In addition, studies continue in the fields of small bowel transplantation and tissue engineering, with the hopes of finding a means to replace or supplement the native intestine (40). In this regard, bone marrow mesenchymal stem cells (BMSCs) represent a novel treatment modality with increasing therapeutic potential (11, 14, 25). The extensive proliferative and differentiation potential of BMSCs make these cells optimal for seeding tissue-engineered grafts (31). In addition, the paracrine release of protective growth factors from stem cells has been shown to benefit ischemic tissues and may possibly prove beneficial in the treatment of NEC (6, 14, 34, 43).

Tumor necrosis factor-α (TNF), a prominent member of the cytokine cascade in a number of organs, serves to elicit leukocyte migration, fever, and the acute phase response (1, 4, 8, 19–23, 24). TNF also has been shown to be a crucial member of the apoptotic pathway and has been associated with the onset of septic shock (7, 22, 38). Under physiological conditions, TNF effectively activates both of its receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (3).

During states of inflammation or ischemia, however, one TNF receptor may be preferentially activated. Traditionally, TNFR1 activation was thought to result in an elevated inflammatory response and decreased organ function after injury. This was suggested by studies in which TNFR1 ablation improved cardiac function and reduced the cardiac production of certain proinflammatory cytokines (20, 37). Furthermore, TNFR2 activation was believed to initiate protective signaling cascades, because TNFR2 ablation decreased cardiac recovery after ischemia and decreased the capacity for clinical recovery, limb perfusion, and ischemic reserve in other in vivo models (12, 15). Activation of the TNF receptors also likely affects production of other cytokines, including interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1).

Cellular expression of IL-6 is elevated in various pathologies, including sepsis, ischemia, and trauma (13, 16–18, 33). IL-6 serves to activate lymphocytes, thereby inducing antibody secretion by B cells and differentiation of cytotoxic T cells (30). Previous in vitro studies have suggested that elevated IL-6 may protect enterocytes from hypoxia- and LPS-induced apoptosis, possibly via decreased fas expression and upregulation of bcl-2 (29). Other studies have also suggested that elevated IL-6 concentrations may aid in maintaining gut mucosal integrity during stress, possibly by inhibiting metalloproteinases, which work to degrade the extracellular environment, or by increasing production of the IL-1 receptor antagonist, which has been shown to aid in suppressing the severity of colitis (5, 16, 39).
VEGF and IGF-1 also play a role in gut integrity. VEGF has been shown to chronically inhibit leukocyte/epithelial cell adherence and the effects of chronic inflammation (32). In addition, various tissue studies have demonstrated that VEGF promotes angiogenesis during acute inflammation and ischemia (35, 41). IGF-1 has been shown to improve organ function after ischemia-reperfusion injury by stabilizing mitochondria and decreasing reactive oxygen species (28). IGF-1 is also thought to protect ischemic tissue by decreasing apoptotic signaling and increasing cellular proliferation (10, 44).

In an attempt to engineer the optimal stem cell to provide maximum protective growth factor production during NEC therapy, it is essential to elucidate the signaling cascades associated with inflammation-induced growth factor production. In this regard, understanding the dual nature of the TNF receptors within BMSCs is paramount. We therefore hypothesized that 1) TNF, LPS, or hypoxic stimulation would alter stem cell cytokine and growth factor production, and 2) TNFR1 or TNFR2 ablation would result in changes to the patterns of cytokine and growth factor productions from these cells upon stimulation.

**MATERIALS AND METHODS**

**Animals.** Adult female C57BL/6J wild-type (WT) mice and mice with a targeted deletion of TNFR1 (TNFR1KO) or TNFR2 (TNFR2KO) (Jackson Laboratory, Bar Harbor, ME) were fed a standard diet and acclimated in a quiet quarantine room for 1 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” [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

**Preparation of mouse bone marrow stromal cells.** A single-step purification method using adhesion to cell culture plastic was employed as previously described (27). Briefly, BMSCs were collected from WT, TNFR1KO, and TNFR2KO female adult mice. Cells were collected from bilateral femurs and tibiae by removing the epiphyses and flushing the shaft with complete medium [Isco’s modified Dulbecco’s medium and 10% fetal bovine serum (GIBCO Invitrogen, Carlsbad, CA)] using a syringe with a 26-gauge needle. Cells were disaggregated by vigorous pipetting and passed through 30-μm nylon mesh to remove remaining clumps of tissue. Cells were washed by adding complete medium, 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 medium at 37°C. BMSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete medium was added and replaced every 3 or 4 days thereafter. BMSC cultures were maintained at 37°C in 5% CO₂ in air. When the cultures reached 90% confluence, the BMSC culture was passaged; cells were recovered by the addition of a solution of 0.25% trypsin-EDTA (GIBCO Invitrogen) and replated in 75-cm² culture flasks. Cells were used for experimentation during passages 3–10.

**Experimental groups.** After three passages, BMSCs were plated in 12-well plates at a concentration of 1 × 10⁶ cells·well⁻¹·ml⁻¹. WT, TNFR1KO, and TNFR2KO BMSCs were divided into four experimental groups (triplicate wells per group) and exposed to 1) no stimulus, 2) TNF (50 ng/ml), 3) LPS (200 ng/ml), or 4) 1 h of hypoxia via oil immersion and substrate deprivation. These stimuli have previously been shown to activate BMSCs and serve to increase cellular production of TNF, which then may activate TNFR1 or TNFR2 (42). After 24 h of incubation, supernatants were harvested and stored at −80°C. Experiments were performed at least three times (total: n = 8–10 wells/group).

**Enzyme-linked immunoassorbent assay.** TNF, IGF-1, IL-6, and VEGF content in the BMSC supernatant were determined using enzyme-linked immunosorbent assay (ELISA) by using commercially available ELISA sets (R&D Systems, Minneapolis, MN; BD Biosciences, San Diego, CA). ELISA was performed according to the manufacturers’ instructions. All samples and standards were measured in duplicate.

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

**RESULTS**

**BMSC are activated with TNF, LPS, or hypoxia.** Female BMSCs from WT, TNFR1KO, and TNFR2KO mice were activated upon stimulation with TNF, LPS, or hypoxia to produce TNF, IGF-1, IL-6, and VEGF (Figs. 1–4). Interestingly, TNFR1KO control cells maintained higher basal levels of IL-6 and VEGF, as well as lower levels of IGF-1, compared with WT cells. In addition, TNFR2KO controls maintained lower basal levels of IGF-1 and VEGF compared with WT but did not exhibit a significant difference in terms of TNF or IL-6 basal levels.

**TNF and IGF-1 production are differentially regulated by activated TNF receptors.** The production of certain cytokines and growth factors was found to differ solely dependent upon which TNF receptor was activated following cellular stimulation, and not upon the initial stimulus itself. Levels of TNF in cellular supernatants were significantly decreased in stimulated TNFR1- and TNFR2-ablated groups compared with WT. This observation was consistent regardless of whether TNF, LPS, or hypoxia was utilized to activate the cells (Fig. A–C). In addition, no statistical difference was seen between TNFR1KO and TNFR2KO groups.

**IL-6 and VEGF production are cooperatively regulated by activated TNF receptors as well as the activating stimulus.** Production of other cytokines and growth factors appeared to depend not only on the TNF receptor that was activated but also on the initial cellular stimulus. Production of IL-6 was significantly increased compared with WT regardless of the initial stimulus (Fig. 2, A–C). TNFR1 ablation, however, appeared to cause a more significant decrease in IGF-1 production compared with TNFR2 ablation. Interestingly, WT controls maintained higher amounts of basal IGF-1 production compared with knockout controls.

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**VEGF production also varied with TNF receptor activation and the type of stimulus provided.** TNFR2 ablation decreased VEGF production compared with WT following TNF stimu-
translational, whereas TNFR1 ablation resulted in no change in VEGF compared with WT (Fig. 4A). Interestingly, TNFR1 ablation resulted in a significant decrease in VEGF following LPS stimulation compared with WT, but after this stimulus, no change was noted with TNFR2 ablation (Fig. 4B). With hypoxic stimulation, TNFR1 ablation resulted in increased VEGF compared with WT, whereas no difference in VEGF production was noted between stimulated WT and TNFR2 groups (Fig. 4C).

**DISCUSSION**

Because stem cell therapy is becoming more readily applied to the treatment of ischemic diseases, the applicability of stem cells to the treatment of NEC is becoming more plausible. However, to best treat patients, it is essential to understand the molecular mechanisms of how inflammation affects stem cell growth factor production. In the present study, we confirmed that TNF, LPS, and hypoxia activate BMSCs to increase production of several cytokines and growth factors. Further-
more, we demonstrated that TNFR1 and TNFR2 play key roles in the production of these factors.

As previously stated, activation of TNFR1 traditionally has been viewed as harmful, whereas activation of TNFR2 has been viewed as modestly beneficial. However, in reality, it is more likely that both receptors play key roles in eliciting both harmful and beneficial intracellular signaling cascades that are regulated at further downstream targets. Furthermore, the data presented would suggest that regulation of certain cytokines, such as TNF and IGF-1, depends only on the TNF receptor activated, and not on the type of stimulus that activates the cell.

Production of other cytokines, however, such as IL-6 and VEGF, may depend not only on the type of TNF receptor activated but also on the stimulant used to bring about cellular activation.

TNF production, for example, was significantly decreased when either receptor was ablated, irrespective of the stimulus.
used to activate the cells. This observation would suggest that TNF production is highly dependent on the presence of both receptors and may indicate that significant TNF production is maintained by autoregulation. Disrupting either receptor, therefore, leads to decreased TNF production. Because certain in vivo studies have correlated decreased tissue levels of TNF with improved postinjury functional recovery (6, 36), TNF receptor knockout stem cells may be better suited to replace damaged native intestinal cells after an ischemic insult.

IGF-1 was also decreased with ablation of either receptor. However, TNFR1 ablation resulted in nearly nondetectable levels of IGF-1, indicating that this receptor is absolutely essential for IGF-1 production after stimulation with TNF, LPS, or hypoxia. The presence of TNFR2 also appears to be crucial to IGF-1 production but does not seem to be as essential as TNFR1. On the basis of these observations, a stem cell with functional TNF receptors would likely be required to deliver maximum amounts of IGF-1 to promote decreased apoptosis and increased cellular proliferation.

The importance of the functional TNF receptors to the production of IL-6 and VEGF was not as clear as that with TNF and IGF-1. IL-6 production was significantly elevated with TNF stimulation in both TNFR1- and TNFR2-ablated cell lines. Conversely, IL-6 was decreased compared with WT in both knockouts following LPS stimulation, whereas it was only elevated in TNFR1-ablated cells following hypoxia. TNFR2 cells had no change in IL-6 production compared with stimulated WT cells following hypoxic stimulation. Ablation of TNFR1 may therefore be beneficial if IL-6 aids in protecting intestinal cells during hypoxic and ischemic insults. However, the decreased IL-6 production in both knockouts after LPS stimulation may counteract the beneficial results seen with hypoxia, since NEC often involves systemic bacteremia in addition to intestinal ischemia.

VEGF production was significantly decreased compared with stimulated WT in TNFR1-ablated cells following LPS stimulation but was elevated in TNFR1KO cells following hypoxic stimulation. No change was observed in TNFR1-ablated cells compared with WT after TNF stimulation. TNFR2-ablated cells experienced decreased VEGF production compared with stimulated WT after TNF stimulation, but no significant change was noted following LPS or hypoxic stimulation. As with IL-6, these data would suggest that a stem cell with TNFR1 ablation would produce maximal VEGF under hypoxic and ischemic conditions but might not do so under septic conditions.

The lack of a repeatable pattern of IL-6 and VEGF production after TNF, LPS, or hypoxic stimulation would suggest that alternative intracellular signaling cascades are activated depending on the initial stimulus presented to the cell. Only one of the end results of these cascades is the production of TNF, which then stimulates either TNFR1 or TNFR2. However, a multitude of other intracellular signaling cascades could be activated, which might lead to the production of varying concentrations of other TNF-induced cytokines.

Of particular interest in this study was the observation that TNFR1-ablated cells maintained higher basal levels of both IL-6 and VEGF compared with unstimulated WT cells. This suggests that cells with a functional TNFR2 may possess the intracellular machinery capable of producing superior amounts of IL-6 and VEGF with the proper stimulation. On the other hand, TNFR2-ablated cells produced equal or lower basal levels of IL-6, VEGF, and IGF-1 compared with WT. This may suggest that TNFR1 decreases basal levels of protective growth factors.

In conclusion, BMSCs are a potent source of protective cytokines and growth factors that could potentially be harnessed for the treatment of necrotizing enterocolitis. TNFR1KO BMSCs appear to have higher basal levels of certain cytokines, including IL-6 and VEGF, whereas TNFR2KO BMSCs have equal or lower basal levels compared with WT. However, the regulation of certain cytokines after stimulation, including IL-6 and VEGF, appears to depend not only on the TNF receptor activated but also on the initial stimulus used to activate the cell. Other cytokines, such as TNF and IGF-1, appear to have a universal response to many different stimuli and depend solely on the TNF receptor activated. Further in vivo studies are needed to differentiate the cytokines and growth factors that are most important in promoting increased survival and postinjury recovery. Understanding the mechanisms that BMSCs use to produce these protective growth factors will allow for maximal in vitro priming before their therapeutic use.

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