Unexpected Macrophage Activity Alters the Inflammatory Process in Necrotizing Enterocolitis

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Keywords: Necrotizing enterocolitis; Macrophage; Cytokine; Chemokine; MIP-1 alpha; CCL3; IL-12

Introduction

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal illness primarily affecting premature infants. NEC is characterized by epithelial destruction leading to gut barrier failure, and requires surgery in 20-40% of those affected. Of infants requiring operative intervention, 20-50% will die from their disease [1-3]. Despite years of research into the cause, prevention, and treatment of NEC, the mortality rate has remained the same over the last three decades, and no definitive cause has been found [4,5].

Macrophages have been shown to play an important role in the clearance of bacteria in the initial stages of necrotizing enterocolitis (NEC). Macrophage Inflammatory Protein-1α (MIP-1α), also known as CCL3, is a chemokine produced by macrophages that enhances the immune response. It acts as both a recruiter of immune cells and a co-activator of other macrophages. Interleukin-12 (IL-12) is a pro-inflammatory chemotactant produced by several types of phagocytic cells such as dendritic cells and neutrophils, but primarily by macrophages. We hypothesized that the pro-inflammatory state associated with NEC pathophysiology would induce increased expression of MIP-1α and IL-12 that would be detectable in serum and intestinal tissue.

Materials and Methods

Approval for the study was granted by the Institutional Animal Care and Use Committee (IACUC), the Biohazard Safety Committee, and the Research and Development Committee of the Robley Rex Veterans Affairs Hospital in Louisville, KY. Timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed for one week prior to initiation of experimental protocol, and allowed water and rat chow ad lib.

Dams were then randomized to the Control or NEC group and their pups were assigned accordingly after birth. Control dams delivered their pups vaginally. Control pups were kept with their mothers and allowed to nurse on demand until the time of sacrifice for tissue and...
serum harvest. Time matched NEC dams underwent carbon dioxide anesthesia and decerebration prior to rapid cesarean delivery of their pups 12 h prematurely. NEC pups were revived and kept in a warmed incubator at 37.0 degrees centigrade.

A previously published protocol was used to induce NEC in the NEC pups [12,13]. Briefly, the pups were orally gavage fed formula consisting of 20 g Similac 60/40 (Ross Pediatrics, Columbus, OH) dissolved in 100 mL Esbilac puppy formula (Pet-Ag, New Hampshire, IL) every four hours and were given one oral dose of lipopolysaccharide (2 mg/kg Salmonella enterica serotype typhimurium; Sigma Chemical Co., St. Louis, MO) at 12 h of life. Feeds were started at 0.1 mL/feed at time 0 and advanced to 0.4 mL/ feed at 96 h for approximately 836.8 kJ/kg/day. Feeds and the oral dose of LPS were administered via a silastic 1.9 French single lumen catheter (Utah Medical Products, Inc., Midvale, UT) gently advanced into the stomach. The position was verified by the appearance of formula in the stomach during instillation of formula. NEC pups were also subjected to hypoxia (100% nitrogen gas for 60 sec) and hypothermia (4°C for ten minutes) every 12 h.

Control and NEC pups were sacrificed at 0, 12, 24, 48, 72, and 96 h of life. Both Control and NEC ileum samples were homogenized in RIPA lysis buffer (R0278 Sigma-Aldrich Chemical Co., St. Louis, MO) and a cocktail of phosphatase (P5726, Sigma-Aldrich) and protease inhibitors (P8340, Sigma-Aldrich), and protein concentrations were determined. Samples containing equal amounts of 35 μg of total protein were resolved by SDS-PAGE under reducing conditions. Electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and probed with antibodies against CCL-3 and IL-12. The slides for IHC were graded into one of four categories: (-) no significant staining, (+) slight positive, (++) moderately positive, and (+++) strongly positive.

Ileum sections for Control and NEC groups from the 48 h time point were mounted in paraffin and stained with either H&E or Immunohistochemistry (IHC). The following scale was used to grade each sample: grade 0, normal and no damage; grade 1, epithelial cell lifting or separation; grade 2, sloughing of epithelial cells to mid villus level; grade 3, necrosis of the entire villus; and grade 4, transmural necrosis [14]. These samples were selected for IHC based on our prior studies showing a significant difference in intestinal blood flow at this place in the NEC protocol timeline [15]. IHC staining was performed using rabbit anti-rodent polyclonal antibodies for MIP-1α (CCL-3) and IL-12 with a 1:1000 dilution in BSA. Each protein sample was standardized to its own β-actin level.

Serum levels of MIP-1α at 0, 12, 24, 48, 72, and 96 h are shown in Figure 1. There was no difference in the amount of MIP-1α present in NEC versus Control animals at the 0, 12, or 24 h time points. The level of MIP-1α started off somewhat high in both NEC and Control animals and experienced a significant drop between 12 and 24 h in animals in the Control group maintained Grade 0 histology throughout the course of the study.

Results

Histological signs of NEC became apparent in the NEC groups at 24 h, and were most pronounced at 48 h (Figure 1). All animals in the NEC group had at least Grade 1 histological signs of NEC by 24 h, and the majority of animals had Grade 2 changes by 48 h (Figure 2). All

Figure 1. Histopathological grading of Control and NEC animals. Histological differences became apparent and most pronounced at the 48 h time point.

Figure 2. Histopathological grading of individual animals Control and NEC. Majority of NEC animals had Grade 2 changes by the 48 h time point. All animals in the Control group maintained a Grade of 0 throughout the course of the study.

Serum levels of MIP-1α at 0, 12, 24, 48, 72, and 96 h are shown in Figure 3. There was no difference in the amount of MIP-1α present in NEC versus Control animals at the 0, 12, or 24 h time points. The level of MIP-1α started off somewhat high in both NEC and Control animals and experienced a significant drop between 12 and 24 h in

J Cytokine Biol, an open access journal
ISSN:2576-3881
Volume 2 • Issue 2 • 1000115

both groups. After 24 h, the Control animals continued to experience a decrease in MIP-1α, whereas the NEC animals showed elevated levels.

After an initial drop, Interleukin-12 levels were steady in NEC animals throughout the NEC protocol (Figure 4). Levels of IL-12 were significantly lower in NEC animals when compared to controls at 12 and 24 h. Following this, the Control animals experienced a drop in IL-12 while the NEC animals remained unchanged.

Immunohistochemistry of Control and NEC groups at the 48 h time point revealed similar results to serum levels at the same time point. The Control group (Figure 5A) showed no significant staining for MIP-1α while the NEC group stained strongly positive for MIP-1α (Figure 5B). Similarly for IL-12 the Control group displayed no significant staining (Figure 5C). The NEC group at the same time point stained strongly positive for IL-12 (Figure 5D).

Discussion

The exact pathophysiology of NEC continues to be unclear despite years of research into the cause. Many risk factors have been identified as contributing to the pathogenesis of NEC, including prematurity, enteral feeding, hypothermia, bacterial colonization, and hypoxia [16-23]. Many believe NEC to be secondary to a deranged or uncontrolled inflammatory response. Therefore, researchers have sought to elucidate the cascade of inflammatory cytokines found in NEC [24]. As a part of the innate immune system, the macrophage plays an important role in the recognition and clearance of bacteria from sites of infection. Present in tissues, macrophages are one of several cell types known as antigen presenting cells (APCs), capable of recognizing and engulfing foreign antigens for presentation to other immune cells to stimulate the clearance of infection.

Macrophages not only act as APCs and phagocytes, however. They also secrete cytokines, growth factors, and chemokines (cytokines that are chemoattractants) to recruit additional monocytes (macrophage precursors), neutrophils, and dendritic cells to the site of inflammation. Prior work by Emami et al. demonstrated the importance of macrophages in NEC caused by infection with the bacteria Cronobacter sakazakii [6]. Animals subjected to the bacteria that were depleted of macrophages and polymorphonuclear leukocytes (PMNs) were found to have higher intestinal bacterial load, higher pathologic scores for intestinal damage, and earlier mortality than those with intact macrophages and PMNs.
Macrophage inflammatory proteins-1α, 1β, and 2 (MIPs) are heparin binding proteins with inflammatory and immunoregulatory activity [8]. The MIPs were initially characterized as proteins secreted from endotoxin-stimulated mouse macrophages, but further research demonstrated that neutrophils, fibroblasts, epithelial cells, and T cells are also MIP-secreting cells. The three MIPs have distinct characteristics, but share a few overlapping roles. All MIPs act primarily in a pro-inflammatory nature and are chemotactic, acting to recruit and activate other cells at the sites of inflammation or injury. MIP-1α acts with a broader specificity than MIP-1β in that it attracts B cells, cytotoxic T cells, and CD4+ T cells, whereas MIP-1β works preferentially on CD4+ T cells [25]. The macrophage is thought to be the primary source for MIPs, but Sherry et al. demonstrated that T cells are either a major source of MIPs or that they act to markedly upregulate the production of MIPs by other cell types [7]. In that study, levels of MIP-1α were measured in mice in vitro after intraperitoneal injection of LPS, and were shown to peak at two hours, then fall to undetectable levels by eight hours.

In our study, there were similar amounts of MIP-1α at the 12 and 24 h time points in both the NEC and Control groups, and that level was trending downward. By 48 h, we saw a significantly different increase in MIP-1α in NEC animals compared to controls. This trend continued in the NEC animals and remained significant until 96 h, the end-point of our study. As one of the first responders to sites of infection and inflammation, it seems likely that the macrophage response to the induction of NEC would be nearly immediate, but we did not find that to be the case. Pathological signs of NEC were first apparent at 24 h in NEC animals, but levels of MIP-1α in those animals did not increase significantly until 48 h. Additionally, instead of peaking and then becoming undetectable, we saw a sustained increase in MIP-1α.

These findings are markedly different than those demonstrated in the prior work by Sherry et al. [7]. It is possible that the lag time between histopathological evidence of the disease compared to significantly elevated serum levels of MIP-1α in NEC animals compared to controls. A delay in macrophage activation might perpetuate further damage to the intestines, worsening NEC. The sustained increase in MIP-1α in NEC animals demonstrates the continued inflammatory response as macrophages are attempting to fight off the intestinal inflammation.

IL-12, produced by macrophages, dendritic cells, and B cells, works synergistically with IL-18 to induce production of interferon-gamma (IFN-γ) from natural killer and B cells. Together, these pro-inflammatory factors can induce intestinal inflammation in mice [26]. Prior studies have demonstrated conflicting results regarding the production of IL-12 in rats undergoing a NEC-inducing protocol when compared to dam fed controls. While Nadler et al. showed decreased IL-12 mRNA expression in NEC animals [27], Halpern et al. showed an increase in IL-12 mRNA in NEC animals and a correlation between the number of IL-12 positive cells and the degree of tissue destruction [28]. The positive correlation between the number of IL-12 positive cells and the amount of tissue damage present led many to draw the conclusion that IL-12 is involved with the initial stages of NEC and may contribute to the intestinal necrosis associated with NEC [24].

Our findings (i.e., a relatively steady level of IL-12 in NEC animals compared to a marked elevation of IL-12 at 12 and 24 h in Control animals) suggest that macrophage function in NEC animals may be diminished. One might expect a reduced inflammatory reaction, but this is not confirmed histologically. There is clear progression of inflammation and necrosis of the intestine in the NEC animals despite the decreased amount of IL-12. Therefore, although other studies have demonstrated the involvement of IL-12 in tissue inflammation in the intestine, our findings of low IL-12 with markedly inflamed intestine indicate that further investigation into the activity of IL-12 in intestine in NEC animals is warranted.

Conclusion

Macrophages play a central role in the elimination of bacteria from the gut in the initial stages of NEC. Animals undergoing a NEC-inducing protocol experienced a delayed upregulation of MIP-1α and no upregulation of IL-12. This delay in the upregulation of the macrophage-secreted pro-inflammatory cytokine MIP-1α paired with a lack of response by IL-12 in NEC animals likely contributes to the pathophysiology of NEC. Enteral supplementation of IL-12, as we have previously done with Relaxin, may offer a mechanistic explanation of IL-12 and if this cytokine is contributing positively or negatively to the pathophysiology of NEC.

Acknowledgement

The authors would like to acknowledge the technical support of Amy J. Matheson and Samuel A. Matheson in the performance of these studies. The work is also supported by Kosair Charities and the James R. Petersdorf Fund of Norton Healthcare.

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