Reduction of experimental necrotizing enterocolitis with anti-TNF-α

Melissa D. Halpern,1 Jessica A. Clark,1 Tara A. Saunders,1 Sarah M. Doelle,1 Dana Molla Hosseini,1 Anna M. Stagner,1 and Bohuslav Dvorak1,2

1Department of Pediatrics and Steele Children’s Research Center and
2Department of Cell Biology and Anatomy, University of Arizona, Tucson, Arizona

Submitted 31 August 2005; accepted in final form 31 October 2005

Am J Physiol Gastrointest Liver Physiol 290: G757–G764, 2006. First published November 3, 2005; doi:10.1152/ajpgi.00408.2005.—Necrotizing enterocolitis (NEC) is the most common gastrointestinal disease of premature infants. However, despite significant morbidity and mortality, the etiology and pathogenesis of NEC are poorly understood. Evidence suggests that ileal proinflammatory mediators such as IL-1β contribute to the pathology associated with this disease. In addition, we have previously shown that upregulation of TNF-α in the liver is correlated with ileal disease severity in a neonatal rat model of NEC. With the use of a neonatal rat model of NEC, we evaluated the incidence and severity of ileal damage along with the production of both hepatic and ileal proinflammatory cytokines in animals injected with (anti-TNF-α; n = 23) or without (NEC; n = 25) a monoclonal anti-TNF-α antibody. In addition, we assessed changes in apoptosis and ileal permeability in the NEC and anti-TNF-α groups. Ileal damage was significantly decreased, and the incidence of NEC was reduced from 80% to 17% in animals receiving anti-TNF-α. Hepatic TNF-α and hepatic and ileal IL-1β were significantly decreased in pups given anti-TNF-α compared with those sham injected. In addition, ileal luminal levels of both TNF-α and IL-1β were significantly decreased in the anti-TNF-α-injected group. Ileal paracellular permeability and the proapoptotic markers Bax and cleaved caspase-3 were significantly decreased in the anti-TNF-α group. These data show that hepatic TNF-α is an important component for the development of NEC in the neonatal rat model and suggest that anti-TNF-α could be used as a potential therapy for human NEC.

Severe ileal damage during NEC is frequently accompanied by remote organ injury resulting in pulmonary, hepatic, and/or renal failure (58). This type of remote organ injury to the liver and other organs is well documented following intestinal ischemia-reperfusion (35, 36, 88), which also has been implicated in the development of NEC (6, 81, 82, 87). Furthermore, significant pathological changes in hepatic morphology and hepatobiliary functions have been reported in patients with NEC (3, 24, 60, 61).

Evidence suggests that the risk factors for NEC induce an inflammatory cascade that results in the pathology associated with this disease (13, 64). TNF-α, a proinflammatory cytokine, has been implicated in many inflammatory diseases of the small intestine (50, 73). In infants with NEC, plasma concentrations of TNF-α were similar regardless of disease severity (59), and, in rats, NEC-induced changes in TNF-α mRNA were not reported (63). Using the neonatal rat model of NEC, we reported similarly low levels of ileal TNF-α-positive cells in animals with NEC compared with control rats (31).

The resident hepatic macrophages, Kupffer cells (KC), release a large number of inflammatory components and are considered the major source of TNF-α during endotoxemia and/or sepsis (19). It was recently demonstrated (30) that KC activation and upregulation of TNF-α in KC is correlated with ileal disease severity in the neonatal rat NEC model. This is of particular interest because our data show that the increase in hepatic TNF-α occurs when no such change was seen in the ileum. Furthermore, elevated TNF-α in the luminal intestinal contents of animals with NEC was attenuated when KC were inhibited, and inhibition of KC is associated with decreased intestinal damage (30). These results suggest the importance of the liver in the pathophysiology of NEC through the release of TNF-α into the biliary system, which can exacerbate injury in the intestine.

Anti-TNF-α therapy is currently used to treat a number of inflammatory conditions, including inflammatory bowel disease (IBD). We hypothesized that hepatic TNF-α is an important component for the development of experimental NEC, and anti-TNF-α will prevent or delay the development of disease in experimental NEC. We injected neonatal rats with developing NEC with anti-TNF-α and evaluated the incidence and severity of NEC, the production of hepatic and ileal proinflammatory cytokines, ileal apoptosis, and alterations in ileal barrier functions.

MATERIALS AND METHODS

Animal model and experimental groups. This protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801-95081). Sprague-Dawley rats (Charles River Lab-
The anti-TNF-α/H9251 vehicle alone using the same injection schedule (NEC group; medians; *P* < 0.01. Normal, undamaged tissue and published NEC scoring scale from 0 to 4, in which a score of 0 indicates normal, undamaged tissue and +4 indicates complete necrosis. Bars indicate medians. *P* ≤ 0.01.

Fig. 1. Ileal damage in necrotizing enterocolitis (NEC) is reduced with anti-TNF-α. Histological NEC scores in the NEC (n = 25) and anti-TNF-α (n = 23) groups are shown. Ileal tissue was scored using our previously published NEC scoring system (22, 23, 29–31). Blinded evaluator and graded as follows: 0 (normal), no damage; +1 (mild), slight submucosal and/or lamina propria separation; +2 (moderate), moderate separation of submucosa and/or lamina propria and/or edema in submucosal and muscular layers; +3 (severe), severe separation of submucosa and/or lamina propria and/or severe edema in submucosa and muscular layers and regional villous sloughing; and +4 (necrosis), loss of villi and necrosis. To determine the incidence of NEC, animals with histological scores of less than 2 do not have NEC. # indicates NEC.

Fig. 2. Incidence of NEC is reduced with anti-TNF-α. All animals (NEC, n = 25; anti-TNF-α, n = 23) with NEC scores of 2 or above are considered NEC positive; animals with ileal damage less than +2 do not have NEC. *P* ≤ 0.01.

Pathological changes in intestinal architecture were evaluated using our previously published NEC scoring system (22, 23, 29–31). Histological changes in the ileum were scored by a blinded evaluator and graded as follows: 0 (normal), no damage; +1 (mild), slight submucosal and/or lamina propria separation; +2 (moderate), moderate separation of submucosa and/or lamina propria and/or edema in submucosal and muscular layers; +3 (severe), severe separation of submucosa and/or lamina propria and/or severe edema in submucosa and muscular layers and regional villous sloughing; and +4 (necrosis), loss of villi and necrosis. To determine the incidence of NEC, animals with histological scores of less than +2 have not developed NEC and animals with histological scores of +2 or greater have developed NEC (22, 23, 29–31).

**Immunochemistry.** A portion of the distal ileum and liver was removed from each animal and fixed overnight in 10% buffered formalin. Paraffin-embedded sections were cut at 4-μm thickness, mounted on slides, deparaffinized and subjected to antigen retrieval using the Dako Antigen Retrieval Solution (Dako, Carpinteria, CA) for 30 minutes. The slides were washed in running water before immunostaining using mouse monoclonal antibodies against TNF-α (BD Pharmingen, San Diego, CA), ED1 (ED1, ED2, TNF-α biosource, Camarillo, CA), IL-18, mouse monoclonal antibody against TNF-α (BD Pharmingen, San Diego, CA), and anti-TNF-α (BD Pharmingen, San Diego, CA).

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performed to compare protein expression between groups with BioRad QuantityOne software.

Statistics. Statistical analyses between groups were performed using ANOVA followed by Fisher protected least-squares difference. Analysis of NEC scores was accomplished using the Mann-Whitney test for nonparametric values. The χ² test was used to analyze differences in incidence of disease. All statistical analyses were determined using the statistical program StatView (Abacus Concepts, Berkeley, CA). All numerical data are expressed as means ± SE.

RESULTS

Anti-TNF-α reduces the incidence and severity of NEC. Ileal damage was statistically significantly decreased (P ≤ 0.01) in animals receiving anti-TNF-α from a median histological NEC score of 3.0 in the NEC group to 1.5 in the anti-TNF-α group (Fig. 1). In addition, the incidence of NEC was significantly reduced from 80% (20/25) to 17% (4/23) in the anti-TNF-α group (Fig. 2). In DF pups, the median histological score was 0.5 and incidence of NEC was 0% (data not shown).

Hepatic TNF-α is decreased with anti-TNF-α. Histological evaluation revealed that an injection of anti-TNF-α antibody reduced the number of TNF-α-positive cells in the liver. TNF-α-positive cells in the NEC group were found throughout the liver tissue, with only occasional positive cells seen in the anti-TNF-α group (Fig. 3). The number of hepatic TNF-α-positive cells was statistically significantly reduced in the group given anti-TNF-α (Table 1). Elevated luminal TNF-α in the NEC group was not detectable in the anti-TNF-α group (Table 1), and serum TNF-α was not detectable in either group (data not shown). As seen previously (30), endogenous ileal TNF-α was similarly low in both groups (data not shown), and thus TNF-α found in the luminal contents likely originates in the liver.

Anti-TNF-α does not decrease numbers of KC. Prior studies from our laboratory (30) showed that the number of KC is increased during experimental NEC. There was, however, no statistically significant change in the number of either resident (ED1 positive) or newly recruited (ED2 positive) KC between the NEC and anti-TNF-α groups in this study (Table 2).

Ileal and hepatic IL-18 are decreased with anti-TNF-α. We have previously shown that IL-18 is a critical proinflammatory cytokine in experimental NEC pathogenesis (25, 27). In addition to increased hepatic TNF-α during NEC development, IL-18 is also increased in the livers of animals with NEC (30). In rats treated with anti-TNF-α, hepatic IL-18 was reduced significantly (Fig. 4 and Table 1). Whereas ileal TNF-α levels remain low in experimental NEC, ileal IL-18 levels are significantly upregulated (31). Furthermore, ileal luminal IL-18 content is much higher than ileal luminal TNF-α levels in animals with NEC, likely because of the high levels of endogenous IL-18 production in the ileum during NEC pathogenesis compared with TNF-α. Thus the majority of TNF-α found in the ileum during experimental NEC should be derived from the liver, whereas ileal IL-18 is likely a combination of both ileal and hepatic sources. Anti-TNF-α reduced ileal IL-18 (Fig. 5) and IL-18 levels in luminal flushes (Table 1). IL-18 can exist in both active (18 kDa) and nonactive precursor (24 kDa) forms and can be distinguished by the differences in their size using Western blot analysis (57, 68). Therefore, we evaluated the amount of active IL-18 in DF, NEC, and anti-TNF-α groups. Levels of active IL-18 were significantly decreased in the anti-TNF-α group compared with the NEC group (Fig. 6). There were no significant changes in either hepatic or ileal IL-12 between groups (data not shown).

Anti-TNF-α decreases markers of apoptosis and ileal permeability. TNF-α is a potent inducer of apoptosis (7, 72), and we have recently shown that apoptosis plays a strategic role in the initial stages of ileal damage in experimental NEC (20). To determine whether anti-TNF-α alters apoptosis in NEC, we evaluated the antiapoptotic marker Bcl-2 and proapoptotic marker Bax in ileal tissue from NEC and anti-TNF-α groups using Western blot analysis (Fig. 7). Bax and the Bax-to-Bcl-2 ratio were significantly decreased in the anti-TNF-α group (Table 3), indicating a shift toward cell survival. Cleavage of procaspases to active caspases is a hallmark of most apoptotic systems (27, 38), and the detection of CC3 is a sensitive indicator of apoptosis (25, 34). To confirm changes in apoptosis in the anti-TNF-α group, we stained sections of ileal tissue for CC3 (Fig. 8A). CC3-positive cells were significantly decreased in the anti-TNF-α group compared with the NEC group (Fig. 8B). Because TNF-α-induced changes in apoptosis contribute to compromised epithelial barrier functions (1, 76),

Table 1. Enumeration of KC in the liver

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<th>ED1 + Cells</th>
<th>ED2 + Cells</th>
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<tr>
<td>NEC</td>
<td>79.3 ± 9.8</td>
<td>29.5 ± 10.4</td>
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<tr>
<td>Anti-TNF-α</td>
<td>81.1 ± 19.2</td>
<td>34.5 ± 5.2</td>
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Values are expressed as means ± SE. ED1- and ED2-positive cells were counted in ten ×20 microscopic fields from stained liver tissue from each animal in the necrotizing enterocolitis (NEC, n = 23) and anti-TNF-α (n = 23) groups. KC, Kupffer cells.
we also evaluated ileal paracellular permeability between groups. Paracellular permeability was significantly decreased in animals in the anti-TNF-α group (Fig. 9).

**DISCUSSION**

Anti-TNF-α therapy is currently used to treat a variety of inflammatory disorders. Here for the first time, we show, a reduction of the incidence and severity of experimental NEC with anti-TNF-α. These data suggest that hepatic TNF-α is crucial to the development of NEC in the neonatal rat model.

In the current study, an injection of anti-TNF-α significantly decreased hepatic TNF-α and IL-18 as well as the active form ileal IL-18. It has previously been shown (30) that the gut-liver axis plays an important role in the development of experimental NEC. In our proposed model, overproduced proinflammatory mediators in the ileum enter the liver via portal circulation, leading to activation of KC. The activated KC produce inflammatory mediators in the ileum enter the liver via portal circulation, resulting in continued ileal inflammation and tissue damage. Our finding that inhibition of TNF-α reduces not only hepatic TNF-α but also other proinflammatory cytokines in both the liver and ileum adds to our proposed model for the gut-liver inflammatory loop in NEC pathogenesis.

Of the many cytokines that play important roles in inflammation, IL-18 (26, 42, 43, 57, 68), IL-12 (55, 56, 66), and TNF-α (50, 73) in particular have been implicated in inflammatory diseases of the small intestine. Furthermore, the production and regulation of these cytokines are closely intertwined. IL-18 is capable of promoting an inflammatory cascade by enhancing the release of proinflammatory TNF-α (70), and TNF-α, in turn, can stimulate production of IL-18 (17) and IL-12 (32, 74). TNF-α can also potentiate the inflammatory process via interactions with a variety of mediators such as histamine (53), eicosanoids (75), and platelet-activating factor (18, 86), some of which have been implicated in NEC pathogenesis (11, 37, 52, 84). We previously reported increased ileal IL-12 during experimental NEC (27). Interestingly, anti-TNF-α did not decrease ileal IL-12 in this study (data not shown), and significant increases in serum levels of TNF-α and IL-12 were not observed. This suggests that a more specific inflammatory response, as opposed to a systemic inflammatory response syndrome, appears responsible for the inflammatory mediators produced during the development of experimental NEC.

The intestinal environment is capable of producing an array of cytokines important in the development and control of inflammatory responses from both immune and nonimmune cells (41, 49). The mucosal immune system consists mainly of lamina propria lymphocytes, macrophages/monocytes (28), and intraepithelial lymphocytes (69), which are all capable of producing proinflammatory cytokines. It is well documented, however, that the intestinal immune system of the neonate differs significantly from more mature intestine, particularly with regard to decreased numbers of immune cells (9, 39, 51, 79). We have previously reported an increase in the numbers of mononuclear cells found in the lamina propria of animals with NEC but no correlation between these increased numbers and ileal damage (31). Because the total number of immune cells in the neonatal intestine remains quite low even during the development of NEC, the production of IL-18 by intestinal epithelial cells (85) may provide an initial proinflammatory insult during the progression of disease (29, 31). Thus the ability of anti-TNF-α to decrease ileal IL-18 may be an important component for disease reduction.

We have previously found that the numbers of KC in the liver of animals with NEC are elevated (30). However, the significant reduction of the incidence and severity of NEC by anti-TNF-α occurred without a concomitant reduction of activated KC. KC are thought to be the primary defense against bacteremia and endotoxemia via removal from portal circulation. Thus KC are chronically exposed to a variety of proinflammatory mediators from the gut (54) regardless of the state...
of inflammation. This may explain why injection of anti-
TNF-α does not affect the recruitment or activation of KC, only the levels of specific inflammatory mediators. In addition, activated KC can produce proinflammatory mediators that can activate hepatocytes via paracrine interactions (71) to produce additional proinflammatory compounds. Thus numbers of KC alone do not entirely reflect the state of hepatic response to inflammation.

TNF-α and IL-18 have been specifically implicated in hypoxia-induced inflammation in brain (33) and cardiac (17) inflammation. Suggestive evidence has shown these cytokines to be important in models of NEC where hypoxia and/or ischemia are used to induce disease (2, 89), including the neonatal rat model of NEC used in these studies (29–31, 44). Because hypoxia and/or ischemia are thought to be crucial risk factors for the development of human NEC (10, 12, 37, 62), our current finding that anti-TNF-α reduces the incidence and severity of experimental NEC adds to the current paradigm that the risk factors for NEC induce an inflammatory cascade that plays a crucial role in the development of disease.

Changes in intestinal permeability and intestinal barrier failure have been implicated in NEC pathogenesis (44–46, 67) and TNF-α has been shown to increase intestinal permeability (48, 80). TNF-α is also known as a potent inducer of apoptosis (7, 72), and apoptosis has been shown to play a strategic role in the initial stages of NEC (20, 40). Although TNF-α-induced apoptosis has been implicated in alterations in mucosal barrier function (1, 76), direct effects on tight-junction proteins may account for at least some of the mucosal disruption attributed to TNF-α (8). In this study, we have found that injection of anti-TNF-α significantly decreases ileal paracellular permeability as well as proapoptotic markers in experimental NEC. These data strongly suggest that compromised ileal barrier functions previously reported in NEC can be attributed, at least in part, to the increased TNF-α found in the ileal luminal contents.

Therapeutic strategies using monoclonal antibodies directed against TNF-α are currently being used to treat chronic IBDs such as Crohn’s disease (77, 78, 83). However, the exact mechanisms by which these biological treatments work is still unclear, and their efficacy in premature infants is unknown.
Furthermore, the onset of NEC is often rapid, and the inflammatory response is more acute than the chronic inflammation seen in IBD. However, the studies presented herein showed that TNF-α plays an important role in disease pathology in experimental NEC, and they suggest that the reduction of TNF-α may be a viable therapeutic approach for this disease in the future.

GRANTS
This work was supported by National Institute of Child Health and Human Development Grant HD-47237.

Table 3. Ileal pro- and antiapoptotic markers

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<thead>
<tr>
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<th>Bax</th>
<th>Bcl-2</th>
<th>Bax/Bcl-2</th>
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<tr>
<td>DF</td>
<td>125±24*</td>
<td>84±12</td>
<td>1.48±0.25*</td>
</tr>
<tr>
<td>NEC</td>
<td>260±38*</td>
<td>97±10</td>
<td>2.76±0.31*</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>131±18*</td>
<td>83±9</td>
<td>1.97±0.18*</td>
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Values are expressed as mean optical densities ± SE taken from Western blots; n = 5 rats in the dam-fed (DF) group, 12 rats in the NEC group, and 10 rats in the anti-TNF-α group. *P ≤ 0.01 vs. NEC.

Fig. 8. A: immunohistological staining for cleaved caspase-3 (CC3) in ileum. Representative slides from DF, NEC, and anti-TNF-α groups are shown. B: CC3-positive cells per 100 villi were determined from DF (n = 5), NEC (n = 12), and anti-TNF-α (n = 10) groups. #P ≤ 0.005 vs. DF and anti-TNF-α.

Fig. 9. Ileal paracellular permeability is decreased with anti-TNF-α. Mean counts per minute (cpm) of tritiated lactose per microliter of blood were evaluated from DF (n = 6), NEC (n = 7), and anti-TNF-α (n = 10) groups. #P = 0.05 vs. NEC.

REFERENCES


