Hepatic inflammatory mediators contribute to intestinal damage in necrotizing enterocolitis

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NECROTIZING ENTEROCOLITIS (NEC) is the most common intestinal damage in necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 284: G695–G702, 2003. First published January 15, 2003; 10.1152/ajpgi.00353.2002.—Necrotizing enterocolitis (NEC) is a common and devastating gastrointestinal disease of premature infants. Along with pathological effects in the ileum, severe NEC is often accompanied by multisystem organ failure, including liver failure. The aim of this study was to determine the changes in hepatic cytokines and inflammatory mediators in experimental NEC. The well-established neonatal rat model of NEC was used in this study, and changes in liver morphology, numbers of Kupffer cells (KC), gene expression, and histological localization of IL-18, TNF-α, and inducible nitric oxide synthase were evaluated. Intestinal luminal TNF-α levels were also measured. Production of hepatic IL-18 and TNF-α and numbers of KC were increased in rats with NEC and correlated with the progression of intestinal damage during NEC development. Furthermore, increased levels of TNF-α in the intestinal lumen of rats with NEC was significantly decreased when KC were inhibited with gadolinium chloride. These results suggest an important role of the liver and the gut-liver axis in NEC pathogenesis.

gastrointestinal system; inflammation; neonatal; gut-liver axis

Proinflammatory cytokines, including IL-18, IL-12, TNF-α, and IFN-γ, have been implicated in the pathophysiology of inflammatory bowel disease (6, 9, 26, 31, 33, 42). In addition, it has been suggested that the major risk factors for NEC promote an inflammatory cascade that results in the pathology associated with this disease (11, 39). We have recently shown that endogenous production of proinflammatory IL-18 and IL-12 in the distal ileum (site of injury) are increased and positively correlated with disease severity in the neonatal rat model of NEC. In contrast, production of proinflammatory TNF-α and IFN-γ was not elevated in the distal ileum during NEC development (20).

In addition to pathological effects in the ileum, severe NEC injury is often followed by multisystem organ failure, including liver failure (34). Although a number of studies have observed significant pathological changes in hepatic morphology and hepatobiliary function in babies with NEC (1, 36, 37), hepatic cytokine profiles during NEC development have not been examined. Transfer of ileal proinflammatory mediators into the portal circulation may stimulate the resident hepatic macrophage, Kupffer cells (KC), to produce a variety of substances that can elicit hepatic injury. These proinflammatory compounds, including cytokines and nitric oxide (NO), may also exacerbate intestinal injury via enterohepatic circulation connecting the liver and intestine. This type of remote organ injury to the liver and other organs has been well documented following intestinal ischemia-reperfusion (23, 57), which has also been implicated in the development of NEC (5, 51, 56).

Inducible NO synthase (NOS-2) can be induced by proinflammatory cytokines, and sustained upregulation can lead to pathological injury to the gut (28, 55). NO can react with superoxide to produce peroxynitrite, and peroxynitrite is responsible, in part, for the pathological effects of NO (13, 14). Ford et al. (18) observed increased NO coupled with increased nitrotyrosine in intestinal enterocytes of infants with NEC, and we have found (unpublished data) increased ileal NOS-2 mRNA expression and protein localization in the neo-

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The aim of this study was to investigate whether or not the gut-liver axis plays a role in the development and progression of NEC. NEC was induced in neonatal rats via enteral formula feeding coupled with exposure to asphyxia and cold stress. We evaluated numbers of KC, gene expression and localization of proinflammatory cytokines in the liver, histological and metabolic signs of hepatic damage, and histological localization of NOS-2 and nitrotyrosine. In addition, we examined TNF-α entering the intestinal lumen.

**METHODS**

**Animal model and diets.** This protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801–95081). Neonatal Sprague-Dawley rats (Charles River Labs, Pontage, MI) originating from 14 separate litters were utilized in 5 different experiments. Newborn rats were collected immediately after birth to prevent suckling of maternal milk. Animals were assigned to one of two experimental groups based on randomized weight and were artificially fed with rat milk substitute (RMS) or were dam fed (control). Briefly, pups from both experimental groups were stressed twice daily with asphyxia (breathing 100% nitrogen gas for 60 s) followed by cold (4°C for 10 min) to induce experimental NEC in the RMS group (4, 10). With the use of this method, 80% of the RMS-fed rats developed NEC vs. 0% of the dam-fed rats (15, 20). RMS pups were hand fed with 0.1 ml formula every 3–4 h during the first 48 h (17, 50). The hand-feeding method was replaced with mechanized artificial feeding for an additional 48 h (17). After 96 h, all surviving animals were killed via decapitation.

**NEC evaluation.** Pathological changes in intestinal architecture were evaluated by using our recently developed NEC scoring system (15, 20). Briefly, after rats were killed, the gastrointestinal tract was carefully removed. A 2-cm section of distal ileum next to the ileocecal valve from each animal was fixed overnight in 70% ethanol, paraffin embedded, microtome sectioned at 4–6 μm, and stained with hematoxylin and eosin for histological evaluation of NEC. 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, region villous sloughing; 4 (necrosis), loss of villi and necrosis (15, 20).

**Liver tissue preparation and immunohistology.** After rats were killed, livers were removed and a portion was fixed overnight in 70% ethanol, paraffin embedded, serial sectioned at 4–6 μm, and processed as previously described (20).
Brieﬂy, after being deparafﬁnized, sections were blocked with 1.5% appropriate serum (Vector Laboratories, Burlingame, CA) and then incubated with 2.0 μg/ml goat polyclonal anti-rat IL-18, NOS-2, nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA), or TNF-α (R&D Systems, Minneapolis, MN) or mouse anti-rat monoclonal ED1 or ED2 (SeroTec, Raleigh, NC), followed by biotinylated secondary antibody (Vector Laboratories), Vectastain Elite ABC reagent (Vector Laboratories), or dianminobenzidine (DAB), and were counterstained with hematoxylin. Control sections were treated with the same procedure, except they were incubated with 2.0 μg/ml goat Ig (Sigma) for IL-18, NOS-2, and TNF-α and 2.0 μg/ml mouse IgG1 (SeroTec) instead of the speciﬁc primary antibodies. No immunoaffecting was observed in the controls. Sections from both experimental groups were stained for a speciﬁc primary antibody at the same time so that comparisons between groups could be assessed. Stained slides were evaluated by a blinded observer, and enumeration of positively stained cells was accomplished by counting 10 microscopic ﬁelds at ×20 magniﬁcation.

**RNA preparation.** The remaining portion of liver was frozen in liquid nitrogen for mRNA extraction. Total RNA was isolated from liver tissue by using the RNeasy mini kit (Qiagen, Santa Clarita, CA) as described in the manufacturer’s protocol. All samples were incubated with RNase-free DNase (20 U/reaction) for 10 min at 37°C to eliminate DNA contamination. RNA concentration was quantiﬁed by UV spectrophotometry at 260 nm, and the purity was determined by the A260/A280 ratio (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). The integrity of RNA was veriﬁed by electrophoresis on a 1.2% agarose gel containing formamide (2.2 M) and ethidium bromide in 1× MOPS buffer [40 mM MOPS (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA (pH 8.0)].

**RT and real-time RT-PCR.** Real-time RT-PCR assays were performed to speciﬁcally quantify rat IL-18 and TNF-α steady-state mRNA levels. Single-stranded cDNA was reverse transcribed from 1 μg of total RNA in a 10-μl reaction mixture, as previously described in detail (16). The amounts of total RNA used in the RT reactions were calculated from the absorbency at 260 nm and veriﬁed by densitometry of the 28S ribosomal RNA band separated on denaturing agarose gels (by Gel Doc 1000 documentation system with Molecular Analyst/PC software; Bio-Rad, Hercules, CA). Real-time PCR ampliﬁcation (7, 19) was performed by using a Pre-Developed TaqMan assay reagents (Applied Biosystems, Foster City, CA) as described in the manufacturer’s protocol. Samples were subjected to 40 cycles of ampliﬁcation at 95°C for 15 s followed by 1 min at 60°C by using a GeneAmp 5700 sequence detection system (Applied Biosystems) according to the manufacturer’s instructions. Water controls were included to ensure speciﬁcity. Relative quantiﬁcation of steady-state mRNA levels between experimental groups and all mRNA measurements were calculated on the basis of total RNA concentration (8). Separate standard curves for IL-18 and TNF-α were generated from serial dilutions of nonexperimental intestinal total RNA (from 1 to 1,000 ng per RT reaction). Real-time monitoring of ﬂuorescent emission from cleavage of sequence-speciﬁc probes by the nuclease activity of Taq polymerase allowed deﬁnition of the threshold cycle during the exponential phase of ampliﬁcation. Data were expressed as multiples of induction of gene expression for animals with NEC (RMS fed) compared with that in the control group (dam fed) (20).

**Serum aspartate aminotransferase and alanine aminotransferase.** Blood was obtained from each animal before death via cardiac puncture. Serum was stored at −70°C until it was used for diagnostic testing. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined by using a Sigma Diagnostic Transaminase kit (Sigma, St. Louis, MO), procedure no. 505.

**Gadolinium chloride injection.** In two additional, independent studies, RMS-fed (NEC) and dam fed (control) rats were injected intraperitoneally once daily with either 20 mg/kg gadolinium chloride (Sigma) or an equal volume of saline (personal communication with Dr. Pablo Muriel, Cinvesta, Mexico City, Mexico). This dose of gadolinium chloride decreased the number of ED2-positive KC in the liver by 87%. As previously described, all animals were subjected to asphyxia and cold stress once daily, and after 96 h, intestinal tissue was removed and ﬂushed as described below.

**Intestinal ﬂush.** A 3-cm section of distal ileum was removed from each animal and gently ﬂushed of intestinal content with 0.5 ml sterile PBS. Flushed contents were frozen at −70°C until they were evaluated for TNF-α levels via the QuantiKine M rat TNF-α immunoassay (R&D Systems) according to the manufacturer’s protocol. All samples, standards, and controls were run in duplicate, and plates were read on a SpectraMax Plus microplate spectrophotometer system.
Statistics. Statistical analyses between control and animals with NEC were performed by using ANOVA followed by a Fisher protected least significant difference test. Correlation analyses were performed by using Spearman rank correlation. Analysis of NEC score was accomplished by using the Mann-Whitney U-test for nonparametric values. All statistical analyses were accomplished by using the statistical program StatView for Macintosh computers (Abacus Concepts, Berkeley, CA). All numerical data are expressed as means ± SE.

RESULTS

Increased KC in NEC liver. Newly recruited KC (ED1 positive) and resident KC (ED2 positive) were significantly increased in the livers of neonatal rats with NEC (fed RMS diet) compared with dam-fed animals (Fig. 1). Both ED1- and ED2-positive KC were distributed throughout the liver in both experimental groups (Fig. 2). In addition, the number of hepatic ED2-positive cells was positively correlated with severity of intestinal damage (r = 0.618; P ≤ 0.01).

Hepatic TNF-α mRNA and TNF-α- and IL-18-positive cells were increased in NEC liver. TNF-α mRNA levels in livers from animals with NEC were significantly increased compared with control pups. There was no difference in IL-18 mRNA levels between groups, however (Fig. 3A). In contrast to the mRNA data, both TNF-α- and IL-18-positive cells were significantly increased in animals with NEC compared with controls (Fig. 3B). TNF-α- and IL-18-positive cells were distributed throughout the liver in both groups (Fig. 4). The number of TNF-α- and IL-18-positive cells were positively correlated with increasing severity of intestinal damage (r = 0.568 and 0.499, respectively; P ≤ 0.05). No differences were observed for either hepatic IL-12 mRNA or localization between groups (data not shown).

Evaluation of damage in NEC liver. Evaluation of the livers of animals with NEC did not reveal histological signs of damage. Serum levels of ALT and AST were statistically, yet modestly, elevated in the NEC group (Fig. 5). However, NOS-2 was increased in NEC liver compared with controls (Fig. 6, A and B). Peroxynitrite, formed when NO reacts with superoxide, is too short lived to be easily detected. Fortunately, peroxynitrite forms nitrotyrosine residues that can be detected immunohistologically. Nitrotyrosine localization was also increased in rats with NEC (Fig. 6, C and D).

Fig. 4. Histological localization of TNF-α- and IL-18-positive cells. Representative sections from control (A and C) and NEC (B and D) rats were stained for TNF-α (top) or IL-18 (bottom). TNF-α- and IL-18-positive cells were increased in animals with NEC (B and D). Positive cells for both cytokines were observed throughout the liver. Original magnification, ×100.

Fig. 5. Serum transaminase levels in animals with and without NEC. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were statistically increased in rats with NEC (n = 6) vs. control rats (n = 5). *P ≤ 0.05.
Evidence of the gut-liver axis in NEC. Our previous studies showed extremely low levels of TNF-α mRNA expression and protein localization in the ileum (20) and jejunum (unpublished data) of animals with or without NEC. Consequently, TNF-α measured in the luminal contents of the intestine should be produced primarily by the liver and enter the intestine via the biliary system. TNF-α from intestinal flushes of animals with NEC was statistically increased compared with controls (Fig. 7). To prove that the increased TNF-α found in the intestinal luminal content of animals with NEC was derived from KC in the liver, RMS-fed (NEC) and dam-fed rats (control) were injected with gadolinium chloride, which selectively inactivates KC in vivo (24). Intestinal luminal TNF-α was significantly decreased in the NEC + gadolinium chloride group compared with the NEC group (Fig. 7). Injection of neonatal rats with gadolinium chloride also significantly decreased the number of both TNF-α- and IL-18-positive cells in the liver, confirming that KC are the primary producers of both cytokines. Furthermore, inactivation of KC significantly decreased the ileal NEC score by twofold (Table 1), verifying that hepatic TNF-α plays an important role in exacerbation of ileal damage in NEC. There were no differences in systemic blood levels of TNF-α between groups, however (data not shown).

DISCUSSION

The data presented herein show that the gut-liver axis plays an important role in NEC development. To our knowledge, this study is the first report describing dysregulation of proinflammatory cytokines and injury mediators in the liver and their association with intestinal damage during development and progression of NEC. These results suggest the importance of the liver in the pathophysiology of NEC by the release of inflammatory cytokines via the biliary system into the intestinal lumen, which can exacerbate injury in the intestine.

In this study, we demonstrate that KC activation and upregulation of both IL-18 and TNF-α in the liver of animals with NEC is correlated with severity of ileal damage. Hepatic mRNA and production of the proinflammatory cytokines IL-1α, IL-1β, IL-6, and IL-12 were also examined, but there were no alterations between groups (data not shown). This is of particular interest because it appears that only certain cytokines are upregulated in the liver during the development of NEC.

![Fig. 6. Histological localization of inducible nitric oxide synthase (NOS-2) and nitrotyrosine. Representative sections from control (A and C) and NEC (B and D) animals stained for NOS-2 (top) or nitrotyrosine (bottom) are shown. NOS-2 and nitrotyrosine were increased in animals with NEC (B and D). Original magnification, ×40.](http://ajpgi.physiology.org/)

*Fig. 7. Inactivation of KC decreases intestinal luminal TNF-α in neonatal rats with NEC. Animals were injected with either 20 mg/kg gadolinium chloride (GdCl) or an equal amount of saline once per day for 4 days. Intestinal luminal TNF-α was quantified by using sandwich ELISA from control (n = 7), control + GdCl (n = 9), NEC (n = 10), and NEC + GdCl (n = 14) groups. *P ≤ 0.001 vs. control and control + GdCl; #P ≤ 0.001 vs. NEC.*
NEC. It has been suggested that, during NEC pathogenesis, alterations in cytokine production are a result of a systemic inflammatory response syndrome (SIRS). However, in SIRS, a more generalized activation of proinflammatory cytokines occurs, including increased expression and production of IL-1 and IL-6 (12, 21, 46). Because increases in IL-1 and IL-6 are not observed, it is unlikely that a SIRS is wholly responsible for the upregulation of hepatic IL-18 and TNF-α.

In addition to ileal damage, severe NEC in infants is often accompanied by injury to the lungs, kidney, and/or liver (34). A number of studies have shown significant pathological changes in both hepatic morphology and hepatobiliary functions in infants with NEC, especially those receiving total parenteral nutrition (1, 36, 37). Moreover, hepatic injury has been documented following intestinal ischemia-reperfusion (23, 24, 57), which has been implicated in the development of NEC (5, 52, 56). However, little is known about the mechanisms initiating these changes or about proinflammatory cytokines and injury mediators during NEC development and progression. In this study, for the first time, pathological changes in the liver during NEC were evaluated. Although minimal pathological changes occurred in the liver, commonly utilized markers for assessing inflammatory-mediated damage (nitrotyrosine, ALT, and AST) were increased in animals with NEC. Although the increased ALT levels in animals with NEC did not approach a range generally observed in the liver during NEC pathogenesis. In the neonatal rat model of NEC, it is difficult to sustain the animals past 96 h. It is possible that more evidence of extensive liver damage, both biochemically and histologically, would be observed if the studies could be extended. These data also suggest, in contrast to the activation of hepatic proinflammatory mediators, actual liver damage may not be a major hallmark of the pathophysiology of experimental NEC.

NOS-2 is known to contribute to inflammatory diseases of the colon (22, 29, 30, 44), and NO has also been shown to play a role in the ileum during NEC pathogenesis (18). Furthermore, unpublished data from our laboratory has shown increased ileal NOS-2 protein and mRNA in neonatal rats with NEC. A number of studies have shown that increased production of NO, due to upregulation of NOS-2 after LPS challenge, can cause hepatic injury (35, 58) and that NO scavengers can decrease hepatocellular injury after shock (32) and endotoxin challenge (38). Here we show increased activated NOS-2 in the liver of animals with NEC. In addition, nitrotyrosine, a footprint for activation of other cytotoxic reactive nitrogen species (41, 43, 45), is markedly increased in the liver during NEC development. The presence of nitrotyrosine on its own is not conclusive evidence that NO has reacted with oxygen metabolites, and, indeed, there is no obvious structural damage in the liver during NEC. Yet significant increases in mediators of inflammation (TNF-α, IL-18, activated KC) are observed in the liver during disease development. We speculate that, after exposure to ileal IL-18 and IL-12 and/or hepatic TNF-α and IL-18 (49, 53), NF-κB is activated and translocated to the nucleus of hepatocytes, binding promoter regions of genes encoding NOS-2 and activating transcription. NO could be synthesized by NOS-2 and then could react with superoxide anion to form peroxynitrite and other cytotoxic reactive nitrogen species (2, 3, 27).

We have shown that TNF-α in the distal ileum (20) and jejunum (unpublished data) is produced at extremely low levels in animals with or without NEC. Jackson et al. (25) demonstrated that, during endotox-
emia, increased TNF-α in bile contributes to intestinal injury via the luminal route (25). Therefore, intestinal luminal TNF-α should be derived from bile. Although testing TNF-α levels in bile from neonatal rats is technically impossible, our present data reveal that increased TNF-α in the luminal contents of animals with NEC is decreased to near control levels when KC are inhibited. Furthermore, this study shows that inhibition of KC decreases the severity of NEC, providing additional evidence that the gut-liver axis plays an important role in NEC pathogenesis. It is likely that hepatic IL-18 also exits the liver and enters the intestine in bile; however, we did not examine IL-18 levels in ileal bile because it is produced constitutively in normal neonatal rats (54) and is upregulated in ileum during NEC (20).

Using our previous data and the currently presented results, we propose the following paradigm for cytokine dysregulation and the gut-liver inflammatory loop (Fig. 8). The risk factors for NEC (prematurity, formula feeding, intestinal hypoxia/ischemia, and bacterial colonization) promote the increased production of ileal IL-18 and IL-12, which contributes to tissue damage in the intestine. During the progression of NEC, ileal IL-18 and IL-12 enter the liver via the portal vein, which activates and increases the number of KC. The activated KC produce proinflammatory IL-18 and TNF-α that may induce early liver injury through activation of NOS-2 and induce the formation and release of additional proinflammatory mediators from hepatocytes. Hepatic-derived cytokines exit the liver and enter the gut to complete the inflammatory loop via bile and/or systemic circulation, resulting in enhanced intestinal inflammation and tissue damage.

The results of this study show, for the first time, concomitant activation of KC, IL-18, and TNF-α in the liver of neonatal rats with NEC and provide evidence that the gut-liver axis plays an important role in the progression of NEC. These findings suggest the importance of the liver in the pathophysiology of NEC and may provide the basis for therapeutic approaches to this disease.

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REFERENCES

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