High-mobility group box 1 protein is an inflammatory mediator in necrotizing enterocolitis: protective effect of the macrophage deactivator semapimod

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Zamora, Ruben, Anatoli Grishin, Catarina Wong, Patricia Boyle, Jin Wang, David Hackam, Jeffrey S. Upperman, Kevin J. Tracey, and Henri R. Ford. High-mobility group box 1 protein is an inflammatory mediator in necrotizing enterocolitis: protective effect of the macrophage deactivator semapimod. Am J Physiol Gastrointest Liver Physiol 289: G643–G652, 2005. First published June 9, 2005; doi:10.1152/ajpgi.00067.2005.—High-mobility group box 1 (HMG1) is a late mediator of endotoxemia known to stimulate the production of proinflammatory cytokines that are putative mediators of intestinal inflammation associated with necrotizing enterocolitis (NEC). We hypothesized that HMG1 is also involved in the pathogenesis of NEC. We examined the expression of HMG1 and the effect of the novel drug semapimod on intestinal inflammation in an experimental model of NEC in neonatal rats. Newborn rats were subjected to hypoxia and fed a conventional formula by gavage (FFH) or were breast fed (BF). Rats were killed on day 4, and the distal ileum was harvested for morphological studies and Western blot analysis. FFH newborn rats but not BF controls developed intestinal inflammation similar to the histological changes observed in human NEC. We found that the expression of HMG1 and its receptor for advanced glycation end products (RAGE) and cyclooxygenase 2 was upregulated in the ileal mucosa of FFH newborn rats compared with BF animals. Administration of the drug semapimod inhibited the upregulation of those proteins and partially protected the animals against the FFH-induced intestinal injury. Elevated levels of HMG1 were also found in ileal samples from infants undergoing intestinal resection for acute NEC. Our results implicate HMG1 and RAGE as important mediators of enterocyte cell death and hypoxia-induced injury in NEC and support the hypothesis that inhibitors such as semapimod might play a therapeutic role in chronic intestinal inflammation characterized by this animal model.

NECROTIZING ENTEROCOLITIS (NEC) is the most frequent and most lethal disease that affects the gastrointestinal tract of the premature infant (16). The overall mortality rate for patients with NEC ranges from 10% to 70% (22) and approaches 100% for patients with the most severe form of the disease, which is characterized by involvement of the entire bowel (pan-necrosis) (37). Survivors may develop short-bowel syndrome, recurrent bouts of central line-associated sepsis, malabsorption and malnutrition, and liver failure as a result of longstanding administration of total parenteral nutrition and eventually may require liver-small bowel transplantation (27, 37). Thus NEC represents one of the most important diseases seen in the neonatal population.

Although numerous risk factors including prematurity (47), hypoxia (26), formula feeding (18), bacterial infection (40), and intestinal ischemia (1) have been implicated in the pathogenesis of NEC, the exact etiology of the disease remains undefined. To understand the pathogenesis of NEC, previous authors have relied on an intravenous administration of proinflammatory cytokines or invasive surgical procedures, such as occlusion of the mesenteric vessels, to reproduce the morphological and clinical changes seen in infants with NEC (16). However, these artificial insults do not parallel the human disease. We recently revisited an animal model originally described by Barlow et al. that uses formula feeding after a brief period of hypoxia (FFH) to induce experimental NEC (5). We were able to modify this model and use it to study the expression of various cytokines in the intestine (32). Because immunological and cellular events can be easily studied in the rat, this model is also ideally suited for further investigations into the pathogenesis and treatment of NEC.

Numerous inflammatory mediators have been implicated in the cascade leading to the hemodynamic instability associated with sepsis. Elevated levels of IL-6 and TNF-α have been measured in infants with NEC, bacterial sepsis, or meningitis (21). More recently, we have shown that nitric oxide (NO) modulates intestinal changes in septic shock (17, 43). Furthermore, the end products of NO metabolism have been shown to be elevated in newborn infants as well as in adult patients with clinical sepsis (34, 41). Several studies suggest these and other mediators of sepsis are also involved in the pathogenesis of NEC. In this context, we (17) previously reported the upregulation of inducible NO synthase (iNOS) mRNA in the intestine of infants with acute NEC and its downregulation by the time of intestinal stoma closure, after the infants recovered from the acute inflammatory process (17). A similar pattern was seen for IFN-γ but not for IL-1, IL-6, transforming growth factor (TGF)-β, or TNF-α. The pattern of cytokine mRNA expression in our model of experimental NEC parallels that seen in patients with NEC (17).

More recently, Wang et al. identified and characterized high-mobility group (HMG) box 1 (HMGB1) protein (previously known as HMG-1 or amphoterin) as a potential late mediator of lethality in a mouse model (44) and a regulator of human monocye proinflammatory cytokine synthesis (3). This

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protein belongs to the HMGB group, one of the three superfamilies of HMG nuclear proteins subdivided according to their characteristic functional sequence motifs. The functional motif of this family is called the HMG box, and, according to the revised nomenclature of HMG nuclear proteins, HMGB-1 has been renamed to HMGB1 (9). The expression of HMGB1 and its role in intestinal inflammation and, in particular, in NEC are not known at present. The objective of this study was twofold. First, we examined whether the cell death-related protein HMGB1 is differentially expressed in the injured small intestine of newborn rats in experimental NEC and in the inflamed intestine of human infants with acute NEC. We then evaluated the effects of the macrophage deactivator semapimod (formerly known as CNI-1493) on the expression of proapoptotic and proinflammatory proteins and on the severity of intestinal inflammation in our experimental model of NEC as well as its effects on rat intestinal epithelial cells challenged with bacterial LPS. The results indicate that HMGB1 is an important mediator of hypoxia-induced gut injury and that suppression of proinflammatory cytokines with inhibitors such as semapimod partially protects against intestinal epithelial cell death both in vitro and in vivo.

MATERIALS AND METHODS

Reagents. LPS from Escherichia coli 0127:B8 was from Sigma (St. Louis, MO). Peroxynitrite was from Alexis Biochemicals (San Diego, CA). The drug semapimod was kindly provided by Cytokine Pharma-Sciences (King of Prussia, PA). The different antibodies used for Western blot analysis were purchased from the following sources: rabbit polyclonal anti-Bad (sc-943) and anti-Bax (P-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); cyclooxygenase (COX)-2 (murine) polyclonal antibody was from Cayman Chemical (Ann Arbor, MI); anti-HMGB1 was a polyclonal rabbit antiserum against native calf thymus HMGI/HMG1 from Upstate Biotechnology (Lake Placid, NY); rabbit polyclonal anti-iNOS antibody was from BD Transduction Laboratories (Lexington, KY); rabbit polyclonal anti-receptor for advance glycation end products (RAGE) antibody was from Affinity BioReagents (Golden, CO); and anti-p38 and anti-phospho-p38 antibodies were from Cell Signaling Technology (Beverly, MA).

Animal model of NEC. All experiments were carried out according to an animal protocol approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh. Pregnant timedated Sprague-Dawley rats (Charles River Laboratories; Wilmington, MA) were induced at term using a subcutaneous injection (1–2 U/animal) of Pitocin (Monarch Pharmaceuticals; Bristol, TN). Immediately after birth, the neonates were weighed and randomized into one of the different treatment groups. Group 1 consisted of neonates separated from their mothers, housed in a temperature- and humidity-controlled incubator (Ohio Medical Products; Madison, WI), and gavaged with a special rodent formula (0.2 ml, see below) 2 times/day and subjected to 10 min of hypoxia (5% O2-95% N2; Prax Air; Pittsburgh, PA) thrice daily in a modular chamber (Billups-Rothenberg; Del Mar, CA) as follows: pups were fed in the morning posthypoxia, exposed to a second hypoxic insult after 4 h, and then subjected to the final hypoxic insult followed by the final feed. Rats in group 3 were treated in a similar fashion to those in group 2; however, the animals received the experimental drug semapimod (0.1–1 mg/kg ip once daily, vehicle: 5% dextrose) right before hypoxia. The formula composition consisted of 15 g Similac 60/40 (Ross Pediatrics; Columbus, OH) in 75 ml of Esbilac canine milk replacer (Pet-Ag; Hampshire, IL) as described by Barlow et al. (5) and was designed to approximate the protein and caloric content of rat breast milk. The rats were killed on different days as indicated, and intestinal samples (segments of the terminal ileum for the present study) were harvested for morphological studies and Western blot analysis as described below.

Morphological evaluation of intestinal samples. Rats were killed on different days as indicated, and the distal ileum was harvested for morphological studies. For light microscopy, hematoxylin and eosin-stained slides were prepared per the standard protocol (15). A pathologist from the Children’s Hospital of Pittsburgh (Dr. R. Jaffe) blinded to the experimental groups graded the morphological changes in the intestinal epithelium. The criteria for each histological grade (0–3) were as follows: 0, normal (no pathological change, different epithelial patterns are noted: clear, vacuolar, and inclusion type); 1, mild (occasional neutrophils, separation of villus core, and mild damage to enterocytes); 2, moderate (submucosal edema, epithelial sloughing, and marked presence of neutrophils); and 3, severe (denudation of epithelium with loss of villi and transmural necrosis or perforation) (32).

Western blot analysis. Newborn rats were killed as indicated, and segments of the terminal ileum were isolated. The mucosa was gently scraped from each segment and immediately placed in cold lysis buffer containing 62.5 mM Tris (pH 6.6), 10% glycerol, 1% SDS, and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstatin, 2 μg/ml aprotonin, and 0.5 mM PMSF, all from Sigma). The samples were then homogenized and boiled for 1 min followed by centrifugation at 10,000 g for 30 min to remove the cell debris. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL) and then electroblotted onto polyvinylidene difluoride membranes (Millipore; Bedford, MA). After being blocked for 1 h with milk (5% in PBS with 0.1% Tween 20) at room temperature, the membranes were probed for 1 h at room temperature with the primary antibodies dissolved in 1% milk-PBS-Tween20 at the following dilutions: HMGB1 (1:1,000), Bad (1:1,000), Bax (1:500), iNOS (1:750), COX-2 (1:500), and RAGE (1:1,000). The membranes were then thoroughly washed and incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG; Pierce; Rockford, IL) at 1:15,000 dilution (in PBS-Tween with 1% milk) for 1 h before detection. Protein bands were visualized using a SuperSignal chemiluminescence substrate (Pierce) according to the manufacturer’s instructions.

Cell culture and Western blot analysis. The rat intestinal crypt cell line IEC-6 was purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in tissue culture medium consisting of DMEM with 4.5 g/ml glucose (Bio-Whittaker; Walkersville, MD) supplemented with 5% fetal bovine serum (Bio-Whittaker), 0.02 mM glutamine (GIBCO-BRL; Grand Island, NY), 0.1 U/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO-BRL) at 37°C and 10% CO2. Cells were pretreated with sepcamimod (0.1–10 μM solutions prepared in 5% dextrose) for 1 h before exposure to LPS (5 μg/ml) or cytomix (a combination of TNF-α [10 ng/ml], IL-β [1 ng/ml], and IFN-γ [1,000 U/ml]) for 48 h. Preparation of the ONOO- solution and cell treatment was as previously described (36). The lysates were collected for Western blot analysis as follows: proteins were extracted for 10 min at 4°C with lysis buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] supplemented with protease and phosphatase inhibitors (1 mM PMSF, 1 mM benzamidine, 50 μg/ml aprotonin, 0.5 mM Na3VO4, 20 mM NaF, and 0.5 mM phenylsulfonyl fluoride). Aliquots containing 50 μg total protein were resolved by SDS-PAGE. Gels were electroblotted onto nitrocellulose membranes. After a 1-h incubation in blocking solution (PBS, 0.1% Tween 20, and 2% fish gelatin; pH 7.5), membranes were incubated with primary (phospho-p38 and p38) and secondary horseradish peroxidase-conjugated antibodies as recommended by the man-
RESULTS

Overall mortality in the animal model of NEC. Newborn rats were fed either breast milk (BF) or a commercial formula up to 4 days with or without a 10-min hypoxic insult thrice as indicated in MATERIALS AND METHODS. As we previously reported, whereas BF animals gained weight in a normal fashion, formula-fed animals gained significantly less weight than their BF counterparts (32). Moreover, we observed a higher mortality rate (days 0–3) in formula-fed animals compared with the BF group: 34.33% for FFH rats (484/1,410) vs. 1.13% for BF controls (8/707) ($P < 0.001$, analyzed by $\chi^2$-test). The total numbers of animals are cumulative and reflect the work over the past 3 1/2 yr.

Morphological changes of intestinal samples in experimental NEC. We have previously shown that morphological analysis of ilea from 4-day-old formula-fed rats revealed various degrees of intestinal inflammation compared with ileal segments from BF controls. These inflammatory changes closely resembled the histological alterations observed in human NEC samples (32). To determine the kinetics of the development of intestinal inflammation in this model, we killed both BF and formula-fed animals on each consecutive day after birth and harvested the last 2 cm of their distal ilea for morphological analysis. The specimens for day 0 were from animals killed within 3 h after birth and showed minimal morphological changes (18.8% had a histological score of <1, $n = 48$ animals). The overall incidence of morphological and pathological changes characteristic of NEC is shown in Table 1. Intestines of BF pups were normal and rarely showed abnormal morphology. In contrast, in the FFH group, morphological evidence of NEC was detected as early as day 1 (mainly villous core separation and presence of occasional neutrophils) with moderate to severe damage at day 4 (histological scores $\geq 1$).

Analysis of ileal specimens from FFH newborn rats killed at day 5 or day 7 showed severe intestinal damage but not significantly different from FFH animals on day 4 (not shown). On the basis of this observation, we performed our subsequent studies utilizing the intestinal samples harvested from BF and FFH animals exposed to hypoxia killed on day 4 after birth.

Expression of HMGB1 protein and its receptor RAGE in the intestine. To determine whether HMGB1 is expressed in the ileum of newborn rats and whether its expression is related to intestinal damage, we analyzed the expression of this protein in ileal mucosa from both BF and FFH animals killed on different days as described in MATERIALS AND METHODS. Although the mature HMGB1 protein generally migrates as a 30-kDa band on SDS-PAGE (48), it can also appear as a doublet or two very close bands around 30 kDa, where the lower band is usually more intense (K. Tracey, unpublished observations). We found that intestines of BF pups showed a basal expression of HMGB1; however, larger protein amounts were present in the ileal mucosa of FFH animals at day 4 (Fig. 1). Interestingly, in some cases, those animals that showed histological evidence of moderate to severe intestinal damage also showed increased expression of a lower molecular mass protein (below the 14-kDa molecular mass marker and referred herein as 7- to 10-kDa bands) detected with the same anti-HMGB1 polyclonal antibody (Fig. 1).

Semapimod protects against formula-feeding/hypoxia-mediated intestinal injury and reduces ileal HMGB1 and RAGE protein expression. We hypothesized that the macrophage deactivator semapimod will exert a protective effect against formula-feeding/hypoxia-mediated intestinal injury and that it will affect the expression of HMGB1 protein. We found that an intraperitoneal administration of semapimod (0.1–1 mg/kg daily) prevented intestinal damage at doses higher than 0.5 mg/kg, as represented in Fig. 2 as a reduction in the incidence of NEC. We also examined the effect of semapimod on HMGB1 protein expression. Whereas we found a basal expression of HMGB1 protein in the ileal mucosa of BF animals, a significant increase was observed in the ileal specimens of FFH animals, which was reduced after the administration of semapimod (Fig. 3A). Administration of vehicle alone had no effect and was not different from the FFH group (not shown). This inhibition of protein expression correlated with a decrease in the severity of injury as assessed by histology (Fig. 3B).

Receptor signal transduction of HMGB1 occurs in part through RAGE in different cell types (2). We also examined the expression of RAGE in ileal mucosal scrapings in our NEC model. The anti-RAGE antibody used detects two bands in the 45-kDa range representing RAGE protein pre- and postglycosylation in mouse lung extract. In addition, this antibody detects a 25-kDa protein believed to be a proteolytic degradation product. We found that mucosal scrapings of the FFH rats had a higher expression of RAGE (both the 45- and 25-kDa bands) than the BF controls. Similar to the effect on HMGB1 in the FFH + semapimod group, expression of RAGE was reduced after the administration of semapimod (Fig. 3C).

Ileal expression of inflammatory proteins in experimental NEC: effect of semapimod. We examined the expression of three members of the Bcl-2 family known to either promote or inhibit apoptosis, namely, Bax, Bad, and Bcl-2, in the ileal mucosa of newborn rats. We found that FFH significantly upregulated the expression of the apoptotic proteins Bad and Bax in mucosal scrapings of FFH animals compared with BF controls (Fig. 4). Expression of both proteins in FFH pups was significantly decreased when the animals were administered the drug semapimod (0.75 mg/kg ip daily). Interestingly, there

Table 1. Time course effect of FFH on incidence of necrotizing enterocolitis

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Data are the percentages of rats in each group with the incidence of intestinal damage graded between 1 and 3; numbers in parentheses are the total numbers of animals per group. Newborn rats were randomized into two groups: breast-fed (BF) animals that were left with their mothers and formula-fed pups that were exposed to hypoxia (FFH) as described in MATERIALS AND METHODS. The terminal ileum of each rat was harvested on the day indicated, fixed, and stained for morphological analysis as described.
was no significant difference in the expression of Bcl-2 in BF and FFH samples (not shown). O ther proteins whose altered expression have been associated with inflammation in a number of experimental and clinical conditions are iNOS and COX-2. In our NEC model, intestinal samples from BF animals had very little or no iNOS protein, as shown by Western blot analysis (Fig. 5A). Expression of COX-2, however, was present in those samples (Fig. 5B). As expected, elevated levels of iNOS and COX-2 were found in the terminal ileum from FFH newborn rats compared with BF controls. Expression of both proteins was significantly decreased when FFH animals were administered the drug semapimod (0.75 mg/kg ip daily; Fig. 5).

Semapimod inhibits activation of p38 MAPK by bacterial LPS in rat intestinal epithelial cells. We have previously shown that LPS causes rapid but transient activation of p38 MAPK, as determined by increased phosphorylation of p38. In IEC-6 cells, activating phosphorylation of p38 increases nearly 10-fold within 5–10 min of LPS treatment and returns to basal levels 30 min after the addition of LPS (19). To examine the effect of semapimod on activation of p38 by LPS, IEC-6 cells were pretreated with different doses of the drug (0.1–10 μM) for 1 h before stimulation with 5 μg/ml LPS for 10 min. The protein lysates were then collected, and Western blot analysis for phospho-p38 and p38 (loading control) was performed as described in MATERIALS AND METHODS. We found that semapimod dose dependently inhibited the phosphorylation of p38 with a maximal effect at concentrations higher than 1 μM (Fig. 6A). We next sought to evaluate the effect of semapimod on p38 activation by inflammatory mediators other than LPS. As shown in Fig. 6B, a similar exposure of IEC-6 cells to ONOO⁻ resulted in a rapid phosphorylation of p38, which, in contrast to the LPS-induced effect, was not inhibited by 1-h pretreatment with 5 μM semapimod. Pretreatment with semapimod also failed to inhibit the stimulatory effect on p38 activation by a mixture of TNFα, IL-1β, and IFN-γ (not shown).

HMGB1 is increased in human intestinal specimens from patients with acute NEC. Intestinal specimens from seven infants with acute NEC and control intestinal specimens from seven patients who underwent intestinal resection for inflammatory conditions other than NEC were analyzed by Western blot analysis for the presence of HMGB1 protein in two independent experiments. The frozen samples (segments from the whole distal ileum) were processed as described above, and the results are shown in Fig. 7. In contrast to the intestinal specimens from the control patients, ileal specimens from NEC patients demonstrated a twofold increase in the levels of HMGB1 protein. This finding correlates with upregulation of iNOS protein (53) and higher levels of nitroso species (51) in resected ileal segments from infants with acute NEC.

Fig. 1. High-mobility group box 1 (HMGB1) is elevated in formula-fed animals exposed to hypoxia (FFH). Newborn rats were randomized into two groups: breast-fed (BF) animals (which were left with their mothers) and FFH animals, as described in MATERIALS AND METHODS. The terminal ileum of each rat was harvested on day 4, and mucosal scrapings were processed for protein isolation followed by Western blot analysis. Top: representative blot with 3 animals/group. Bottom: densitometric analysis for HMGB1 protein (30-kDa band) and low-molecular-mass products (7- to 10-kDa bands) performed as described. Results in graph are means ± SE; n = 13 animals/group. *P < 0.05 vs. BF control [analyzed by one-way ANOVA followed by Fisher’s least-significant difference (LSD) test].

Fig. 2. Administration of semapimod in vivo is protective in an experimental necrotizing enterocolitis (NEC) model. Newborn rats separated from their mothers were fed a conventional formula and exposed to hypoxia after the administration of the drug semapimod (0.1–1 mg/kg ip) (FFH + semapimod group) as described in MATERIALS AND METHODS. A segment of the terminal ileum of each rat was harvested on day 4 for morphological analysis as described. The incidence of NEC was calculated as the percentage of animals displaying pathology scores higher than 0 as assessed by histology. Results in graph are means ± SE; n = 15–45 animals/group. *P < 0.05 vs. FFH without semapimod [analyzed by one-way ANOVA followed by Fisher’s LSD test].
DISCUSSION

NEC is still the most frequent and lethal gastrointestinal disease of premature infants. Several risk factors for NEC have been identified, including low birth weight, formula feeding, bacterial colonization of the gut, and hypoxia (1–3). Several experimental animal models have been reported (4, 10, 12, 14, 20) that have intestinal hypoxia as the common denominator.

To understand the pathogenesis of this disease, we (32) developed a consistent and reproducible rat model that relies on formula feeding and hypoxia, two major risk factors for NEC. In the present study, we presented novel findings regarding the molecular mechanisms associated with the development of NEC that may shed some light on the pathogenesis of this disease.

First, we modified our experimental model (32) so that newborn rats were fed with formula twice a day and exposed to hypoxia for a longer period of time (10 min instead of 3 min). Examining a larger number of animals, we observed a higher mortality rate in the FFH animals compared with the BF group. Although malnutrition could be a contributing factor (the volume of formula consumed by the neonatal rats was less than the volume of milk consumed by BF pups), there was no apparent cause of death for most of the animals in the FFH group. The significant difference in mortality could only be attributed to aspiration during gavage. The longer exposure to hypoxia, however, exacerbated the inflammatory changes seen in the intestine of the FFH group and rendered the neonatal rats more susceptible to gut barrier failure as shown by the increased incidence of NEC starting at day 3 after birth.

In previous studies, we (32, 33) showed that NEC is characterized by the presence of a number of proinflammatory cytokines both in experimental and human NEC. Here, we...
examined the expression of the protein called HMGB1 in the small intestine of newborn rats with NEC. HMGB1 is a 30-kDa nuclear and cytosolic protein member of the HMG protein superfamily that has been widely studied as a transcription factor as well as a growth factor. HMGB1 is also defined as a cytokine.

Fig. 4. Administration of semapimod in vivo decreases the expression of Bcl-2 family members Bad and Bax in an experimental NEC model in rats. Newborn rats were randomized into three groups: BF animals, FFH animals, and FFH + semapimod animals, as described in MATERIALS AND METHODS. The terminal ileum of each rat was harvested on day 4, and mucosal scrapings were processed for protein isolation followed by Western blot analysis. A: representative blot with 3 animals/group (top) and densitometric analysis for Bad protein (bottom). Results in graph are means ± SE; n = 17–22 animals/group. *P < 0.001 vs. BF control; #P < 0.001 vs. FFH group (analyzed by one-way ANOVA followed by Tukey test).

Fig. 5. Administration of semapimod in vivo decreases the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in an experimental NEC model in rats. Newborn rats were randomized into three groups: BF animals, FFH animals, and FFH + semapimod animals, as described in MATERIALS AND METHODS. The terminal ileum of each rat was harvested on day 4, and mucosal scrapings were processed for protein isolation followed by Western blot analysis. A: representative blot with 3 animals/group (top) and densitometric analysis for iNOS protein (bottom). Results in graph are means ± SE; n = 29 animals/group. *P < 0.001 vs. BF control; #P = 0.006 vs. FFH group (analyzed by one-way ANOVA followed by Tukey test).

B: representative blot with 3 animals/group (top) and densitometric analysis for COX-2 protein (bottom). Results in graph are means ± SE; n = 16–27 animals/group. *P < 0.001 vs. BF control; #P < 0.05 vs. FFH group (analyzed by one-way ANOVA followed by Tukey test).
because it stimulates proinflammatory responses in monocytes/macrophages, is produced during systemic and local inflammatory responses, has been identified as a late mediator of lethal systemic inflammation (e.g., endotoxemia and sepsis), and is required for the full expression of inflammation in animal models of endotoxemia, sepsis, and arthritis (2, 45). However, the expression of HMGB1 in the injured intestine and its role in intestinal inflammation and, in particular, in NEC are not known at present. HMGB1 is produced by nearly all cell types, but cellular levels vary with development and age (45). As expected, we found a high expression of HMGB1 in the ileum of BF newborn rats. However, maximal expression of HMGB1 in the ileal mucosal scrapings of FFH newborn rats at day 4 coincided with the marked appearance of morphological characteristics of NEC. Interestingly, those animals that showed moderate to severe intestinal damage as assessed by histology and also showed increased expression of a lower molecular mass protein detected with the same anti-HMGB1 polyclonal antibody as a 7- to 10-kDa band. There is a large body of literature on low-molecular-mass (below 30 kDa) HMG proteins, but the role of these lower bands as cytokines is not known at present. Of course, we cannot rule out the possibility that they could also be degradation products of HMGB1. Although beyond the scope of the present work, clearly the identification and study of those bands would be useful to the understanding of the role of HMG proteins in disease.

It has been shown that HMGB1−/− necrotic cells have a greatly reduced ability to promote inflammation, which proves that the release of HMGB1 can signal the demise of a cell to its neighbors (39). On the contrary, apoptotic cells do not release HMGB1 even after undergoing secondary necrosis and partial autolysis and thus fail to promote inflammation even if not cleared promptly by phagocytic cells (39). HMGB1 is either actively secreted by monocytes/macrophages or passively released from necrotic cells from any tissue (45). Because in experimental NEC enhanced epithelial apoptosis is an initial event underlying the gross tissue necrosis of the intestinal wall (11, 25), the elevated levels of HMG1 found in the ileal mucosal scrapings of FFH newborn rats suggest that there is more synthesis of HMGB1 protein and/or more translocation from the nucleus to cytoplasm of the protein ready to get released into the extracellular milieu, where it mediates downstream inflammatory responses. Such HMGB1-related responses have been reported in endotoxemia, arthritis, and...
suggests that the upregulation of RAGE is involved in the by semapimod but also that of its receptor RAGE. This finding not only the expression of HMGB1 was significantly reduced degradation as shown by normal FFH animals. At present, we do not have an explanation for this finding, but it is unlikely to be due to total protein abundance or increased synthesis. Rather, it is likely that semapimod affects downstream signaling pathways or reduces the expression of HMGB1.

Little is known about how HMGB1 mRNA is transcriptionally regulated. Interestingly, we previously found that among the several DNA repair genes that were affected by iNOS in murine hepatocytes, there was an increased expression of the gene coding for HMG-2 protein as determined by microarray analysis (49). However, the exact relationship and whether iNOS-derived NO alters the expression or activity of HMG proteins and specifically of HMGB1 remain to be investigated. Experiments to address these questions are currently being performed in our laboratory. Furthermore, although HMGB1 has not been associated with gene transcription in vivo, it can stimulate transcription in vitro (45). Thus we cannot exclude the possibility that elevated levels of HMGB1 affect the expression of other cell-death related genes in this experimental model of NEC.

We tested the effect of semapimod (formerly known as CNI-1493), a tetravalent guanylhydrazone known to inhibit mouse macrophage arginine transport and NO production and to suppress the release of proinflammatory cytokines (7), in our experimental NEC model. In this study, we show that this macrophage-deactivating drug inhibits the expression and accumulation of HMGB1 in the ileal mucosal scrapings of FFH animals, thereby limiting the degree of intestinal injury caused by formula feeding and exposure to hypoxia. The fact that semapimod did not completely prevent the occurrence of NEC-like morphological changes in our experimental model confirms the complexity of this disease and implies that other mechanism(s) are also involved in its pathogenesis.

Outside the cell, HMGB1 binds with high affinity to RAGE, a transmembrane receptor of the immunoglobulin superfamily that is in part responsible for the receptor signal transduction of HMGB1 (24, 44). RAGE is expressed on monocytes/macrophages, endothelial cells, neurons, and smooth muscle cells (2). The accumulation of RAGE ligands leads to inflammatory disorders, and the biology of RAGE is driven by the settings in which these ligands accumulate, such as diabetes, inflammation, neurodegenerative disorders, and tumors (8). Although one study (52) has shown that a human colon adenocarcinoma cell line expresses RAGE, the expression of this protein in nontransformed intestinal epithelial cells or normal intestinal tissue has not been reported yet. In our rat model, we could hardly detect any RAGE in ileal mucosal scrapings of BF newborn rats. However, there was a higher expression of RAGE in intestinal samples from FFH animals analyzed using an anti-RAGE antibody that detects two bands in the 45-kDa range (RAGE protein pre- and postglycosylation in mouse lung extract) and a 25-kDa protein believed to be a proteolytic degradation product. Interestingly, the intensity of the 25-kDa band was significantly higher than that of the 45-kDa band in FFH animals. At present, we do not have an explanation for this finding, but it is unlikely to be due to total protein degradation as shown by normal β-actin levels. Interestingly, not only the expression of HMGB1 was significantly reduced by semapimod but also that of its receptor RAGE. This finding suggests that the upregulation of RAGE is involved in the intestinal injury caused by FFH. In this respect, a recent study (29) using novel animal models with defective or tissue-specific RAGE expression showed that deletion of RAGE provides protection from the lethal effects of septic shock caused by cecal ligation and puncture. It should be noted, however, that, although RAGE has been shown to interact with HMGB1, other putative HMGB1 receptors are known to exist but have not been characterized yet. A recent study (35) showed that, whereas RAGE played only a minor role in macrophage activation by HMGB1, the interactions of HMGB1 with Toll-like receptor (TLR)2 and TLR4 could explain the ability of HMGB1 to generate inflammatory responses that are similar to those initiated by LPS. Although the role of RAGE and other receptors in mediating inflammatory responses to HMGB1 still requires further investigation, our results suggest that RAGE accumulation and degradation accompanies the upregulation of its ligand HMGB1 associated with FFH-induced intestinal injury in experimental NEC.

As in many other cell types and tissues, intestinal apoptosis and cell death are the result of different pathways that involve a number of proinflammatory cytokines and mediators other than HMGB1. We found that expression of Cx36 and Bad, two proapoptotic members of the Bcl-2 family of proteins, were significantly upregulated by FFH in mucosal scrapings of FFH animals compared with BF controls and downregulated when the animals were administered the drug semapimod. Similarly, other proteins like iNOS and COX-2, whose altered expression has been associated with inflammation in a number of experimental and clinical conditions (for reviews, see Refs. 28, 46, and 50), were elevated in the terminal ileum of FFH newborn rats compared with BF controls and downregulated when the animals were administered the drug semapimod. Although we did not assess the integrity of the mucosal barrier in our model, it has previously been shown that HMGB1 is capable of causing derangements in intestinal barrier function in cultured Caco-2 human enterocytic monolayers and that this effect depends on the formation of NO and ONOO⁻ (38). Thus the protection conferred by semapimod may also be related to its ability to inhibit the FFH-induced increase in intestinal permeability by decreasing both HMGB1 and iNOS protein expression.

Because bacterial colonization is an important factor in the pathogenesis of NEC, it is necessary to understand the response of intestinal epithelial cells to LPS. In our model, this becomes even more important because the animals are not kept in a pathogen-free environment. Our results show that LPS activates p38 MAPK both in IEC-6 rat intestinal epithelial cells and in ileal mucosal scrapings cultured ex vivo (19). This increase in p38 activation could significantly be inhibited upon preincubation of IEC-6 cells with semapimod in a concentration-dependent fashion. More importantly, the inhibitory effect of semapimod rather than a nonspecific inhibitory effect seems to be a selective effect on the LPS signaling pathway. That is illustrated by the fact that semapimod inhibited p38 activation only in LPS-treated cells but not in cells exposed to ONOO⁻, the toxic NO-related species that also activates p38. A previous report (31) has suggested diverse effects of semapimod that are tissue specific and that confer protection against the hemodynamic and inflammatory responses to LPS. Also, semapimod has previously been shown to inhibit the expression of proin-
flammatory cytokines through a pathway involving MAPKs, specifically to inhibit the phosphorylation and activation of p38 MAPK in both human monocytes and the murine macrophage cell line RAW 264.7 (13, 30). Although inflammatory MAPKs, in particular p38 and JNK, are critically involved in the pathogenesis of Crohn’s disease (23), their involvement in the regulation of inflammatory responses in the gut needs to be conclusively demonstrated.

A recently described neural pathway, termed the “cholinergic anti-inflammatory pathway”, involves vagus nerve stimulation that inhibits the release of TNF, HMGB1, and other cytokines and protects against endotoxemia and ischemiareperfusion injury (6). By examining the effects of pharmacological and electrical stimulation of the intact vagus nerve in adult male Lewis rats subjected to endotoxin-induced shock, it was found that intact vagus nerve signaling was required for the anti-inflammatory action of semapimod (6). In that study, the intracerebroventricular administration of semapimod was 100,000-fold more effective in suppressing endotoxin-induced TNF release and shock compared with the intravenous route. In our model, intracerebroventricular administration of the drug is extremely difficult due to the small size and sensitivity of newborn pups, and thus we could not explore this route of dosing. The question of whether semapimod can be protective through the cholinergic anti-inflammatory pathway in our model and whether this novel pathway plays a role in the development of both experimental and human NEC remain to be investigated.

In summary, our study demonstrates that the proinflammatory protein HMGB1 and its receptor RAGE are elevated in the ileal mucosa of formula-fed newborn rats exposed to hypoxia. We show that the synthetic guanylhydrazone semapimod, a cytokine inhibitor and MAPK blocker, inhibits the upregulation of both HMGB1 and RAGE as well as other proinflammatory mediators, thereby limiting the intestinal injury caused by FFH in experimental NEC. In addition, this drug selectively affects the activation of p38 MAPK by LPS in IEC-6 intestinal epithelial cells, supporting the involvement of MAPKs in the protective effect of semapimod against bacterial LPS-induced intestinal injury. Our results implicate HMGB1 and RAGE as important mediators of enterocyte apoptosis/cell death and hypoxia-induced gut barrier failure associated with NEC. Sempapimod is currently being developed as a potential treatment for Crohn’s disease and other inflammatory pathologies (42). Strategies to suppress the release of proinflammatory cytokines and to inhibit the expression and activity of cytotoxic molecules such as HMGB1 with inhibitors like semapimod may offer a novel therapeutic modality to combat inflammatory conditions such as NEC.

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