Epidermal growth factor reduces intestinal apoptosis in an experimental model of necrotizing enterocolitis

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The incidence of NEC is significantly decreased in breast milk-fed compared with formula-fed infants (5, 17, 45, 59, 67). Furthermore, maternal rat milk reduces the severity of experimental NEC in the neonatal rat model of NEC (14). The components of maternal milk that are responsible for protection against NEC remain unknown, but epidermal growth factor (EGF) is one of the most promising candidates in NEC prophylaxis (12). EGF is a peptide that exhibits trophic, maturation, and healing effects on intestinal mucosa (11, 16, 53). The major sources of EGF in the developing neonate are maternal colostrum, milk, and saliva (7, 9, 13, 47, 51, 57). Diminished serum and saliva EGF levels were reported in neonates suffering NEC compared with healthy controls (28, 62). In the experimental rat model of NEC, we have previously shown that supplementation of EGF into cow’s milk-based formula reduces the incidence of NEC by 50% (15). However, the mechanisms underlying EGF-mediated reduction of NEC are still not understood.

The Bcl-2 family of cytoplasmic proteins is an important class of molecules that regulates enterocyte apoptosis (37). Bcl-2 is an anti-apoptotic protein that attenuates the effects of cytochrome c release from the mitochondria and counters the effects of the pro-apoptosis protein Bax (35, 38). Bcl-2 and Bax contribute to the signaling pathways that modulate caspase-3 activity, which is necessary for the chromatin condensation and DNA fragmentation that characterize apoptosis (31, 54). An increase in the expression of pro-apoptotic Bax relative to a reduced expression of anti-apoptotic Bcl-2 may create an environment that favors apoptosis (63).

Although end-stage NEC is characterized histopathologically as extensive necrosis, recent reports suggest that apoptosis, or programmed cell death, accounts for the initial loss of cells in the apical villi before full development of the disease (19, 33, 48). It has been suggested that activation of the EGF-receptor signaling pathway blunts apoptosis (10), thereby preserving the villus architecture. The aim of this study was to investigate if EGF-mediated reduction of NEC is connected with the alteration of the expression of pro- and anti-apoptotic genes and proteins, causing a shift in the ratio that promotes cell survival. To achieve this aim, we induced NEC in neonatal rats using formula feeding coupled with exposure to asphyxia/cold stress. We investigated the effects of enteral administration of EGF on changes in intestinal morphology and expression of the apoptotic genes Bcl-2, Bcl-xL, Bcl-w, Bax, and Bad, respectively.

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NECROTIZING ENTEROCOLITIS (NEC) is the most common gastrointestinal disease of premature infants (50). Recent reports suggest increasing occurrence of NEC with up to 9,000 cases in the United States every year, with death occurring in 20–40% of affected individuals (41, 42). Although the etiology of NEC is unknown, the major risk factors for development of the disease are prematurity, enteral feeding, intestinal hypoxia-ischemia, and bacterial colonization (6). Despite significant morbidity and mortality, there is currently no effective preventive treatment (58).
as well as protein expression of Bax and Bcl-2, in the terminal ileum (site of NEC injury). Furthermore, histological localization of cleaved caspase-3, a specific marker for apoptotic cells, was evaluated in the terminal ileum. To evaluate if EGF treatment induced epithelial cell proliferation in the terminal ileum, expression of proliferating cell nuclear antigen (PCNA) was measured.

METHODS

Animal model and diets. The protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801–95081). Neonatal Sprague-Dawley rats (Charles River Laboratories, Portage, MI) originating from 14 separate litters were used in 5 different experiments. Newborn rats were collected immediately after birth to prevent suckling of maternal milk. Animals were assigned to one of the following three experimental groups: NEC, pups artificially fed with growth factor-free rat milk substitute (RMS); NEC+EGF, pups artificially fed with RMS supplemented with 500 ng/ml rat EGF (Harlan Bioproducts, Indianapolis, IN), or dam fed (DF). Experimental NEC was induced by asphyxia and cold stress, as previously described (15, 24, 25). After 96 h, all surviving animals were killed via decapitation. Animals that died before 96 h were excluded from the study because postmortem tissue is not suitable for evaluations.

Corticosterone measurement. Blood was collected from the trunk and centrifuged for 5 min at 10,000 rpm. Plasma samples were then frozen until the assay was performed. Corticosterone was assayed in duplicate by using a double-antibody 125I RIA kit (ICN Pharmaceuticals, Costa Mesa, CA). Plasma samples were diluted 1:200 in the assay buffer before RIA.

RNA preparation. Total RNA was isolated from ileal tissue using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer’s protocol and our previous studies (15, 24). 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 ultraviolet spectrophotometry at 260 nm, and the purity was determined by the ratio of absorbance at 260 nm to that at 280 nm (SPECTRAMax PLUS; Molecular Devices, Sunnyvale, CA). The integrity of RNA was verified by electrophoresis on a 1.2% agarose gel containing formaldehyde (2.2 mol/l) and ethidium bromide. The homogenates were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected. Total protein concentration was quantified using the Bradford (4) protein assay. For protein analysis, 50 μg protein were added to an equal volume of 2X Laemmli sample buffer and boiled for 5 min. The samples were run on a 10–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) at 95 volts for 1 h. Protein was transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad) at 15 volts for 1 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma, St. Louis, MO) for 1 h at room temperature and then incubated with a rabbit polyclonal anti-Bax antibody (Pharmingen, San Diego, CA) or mouse monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After being washed, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (for Bax) or goat anti-mouse IgG (for Bcl-2; Santa Cruz Biotechnology). Proteins were visualized with a chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film. Densitometry was performed to compare protein expression between groups with Bio-Rad Quantity-One software.

Ileal morphology. From each animal, a 2-cm section of distal ileum next to the ileocecal valve was fixed overnight in 70% ethanol, washed, and stained with hematoxylin and eosin for morphometric measurements; 8–10 villi were measured for each animal, and 10–13 animals were evaluated per experimental group. Sections from animals with a NEC score of four were not included in analyses because of the lack of intact tissue to evaluate. Villi were measured from the tip to the crypt base, and the number of epithelial cells in this crypt-villus axis was enumerated. These measurements were performed using an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). Analysis of all morphological data was performed in a blind manner to prevent observer bias.

Immunohistochemistry. Expression of cleaved caspase-3 protein and PCNA were evaluated. Serial sections from ileal samples were processed as previously described (15, 24). After deparaffinization, rehydration, and incubation in hydrogen peroxide, sections were

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Table 1. Sequences of PCR primers and probes

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**Figure 1. **Western blot analysis of Bcl-2, Bax, and Bcl-xL in ileal homogenates from NEC and control rat pups. Ileal homogenates from NEC and control rat pups were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted with anti-Bax antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology). Protein bands were visualized with an enhanced chemiluminescence detection system (Pierce, Rockford, IL) and exposed to X-ray film. Densitometric analysis was performed to compare protein expression between groups with Bio-Rad Quantity-One software. (A) Western blot analysis of Bcl-2, Bax, and Bcl-xL in ileal homogenates from NEC and control rat pups. (B) Densitometric analysis of Bcl-2, Bax, and Bcl-xL protein expression in ileal homogenates from NEC and control rat pups.
blocked with 1.5% appropriate serum (Vector Laboratories, Burlingame, CA) and then incubated with the following antibodies: 2.0 μg/ml rabbit polyclonal anti-human cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) and 2.0 μg/ml goat polyclonal anti-rat PCNA (Santa Cruz Biotechnology), followed by biotinylated secondary antibody (Vector Laboratories), Vectastain Elite ABC reagent (Vector Laboratories), diaminobenzidine activated by hydrogen peroxide. Sections were then counterstained with hematoxylin. Control sections were treated with the same procedure except they were incubated with 2.0 μg/ml rabbit IgG (for caspase-3) or 2.0 μg/ml goat IgG (for PCNA; Sigma). No immunostaining was observed in the controls. Sections from both experimental groups were immunostained for a specific antigen at the same time so that comparisons between groups could be assessed. For caspase-3 measurements, stained slides were evaluated by a blind observer, and enumeration of positively stained cells was accomplished by counting 8–10 villi/animal and evaluating 5 rats/experimental group (100× magnification).

Statistics. Statistical analyses between NEC, NEC+EGF, and DF groups were performed using ANOVA followed by Fishers protected least-significant difference test. For histological NEC scores, analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Mann-Whitney U-test. For protein ratios and caspase-3 measurements, analyses were performed using the paired t-test. All statistical analyses were conducted using the statistical program Statview for Macintosh computers (Abacus Concepts, Berkeley, CA). All numerical data are expressed as means ± SE.

RESULTS

Morphometric measurements. Intestinal morphology was examined by light microscopy. The NEC group had significantly shorter villi compared with DF animals (P ≤ 0.05). Villi in the NEC+EGF group were significantly longer than those not given EGF (Fig. 1A; P ≤ 0.003). To determine whether the increase in villus height in the EGF group was a result of hypertrophy or hyperplasia, the total number of epithelial cells in each measured villus was counted, and results from these measurements were expressed as the number of epithelial cells per micrometer of villus. There were no significant differences seen between the groups, indicating that EGF caused hyperplasia of the epithelial cells and not hypertrophy (Fig. 1B).

Cell proliferation induced by EGF. To determine if EGF induces mitogenic effects on epithelial cells, the cell proliferation marker PCNA was evaluated by immunohistochemistry (Fig. 2). There were no significant differences observed between NEC and NEC+EGF groups, indicating that supplementation of EGF into formula did not induce epithelial cell proliferation in the terminal ileum.

Plasma corticosterone levels. The exposure to stress can result in increased epithelial apoptosis of the gastrointestinal tract (20, 33). To characterize animal response to repeated asphyxia/cold stress during the study, plasma corticosterone levels were measured using RIA. To assure that serum corticosterone levels were not the result of immediate asphyxia/cold stress treatment, animals were not stressed for 16 h before harvest. Rat pups artificially fed with formula (NEC) or with formula supplemented with 500 ng EGF (NEC+EGF) had significantly elevated levels of corticosterone compared with DF animals (Fig. 3; P ≤ 0.0001 and P ≤ 0.001, respectively). However, there was no statistically significant difference among NEC and NEC+EGF groups. This indicates that, although artificially fed animals are more stressed than DF animals, supplementation of EGF into formula did not reduce this effect.

Ileal mRNA levels of pro- and anti-apoptotic genes. Gene expression of pro-apoptotic Bax and Bad and anti-apoptotic Bcl-2, Bcl-w, and Bcl-xL was evaluated in the ileum using RT-PCR. Pro-apoptotic Bax mRNA levels were significantly increased in the NEC group compared with DF animals (Fig. 4A; P < 0.01). EGF supplementation markedly decreased Bax mRNA levels compared with animals fed formula alone (Fig. 4A; P < 0.05). Conversely, anti-apoptotic Bcl-2 mRNA levels were significantly decreased in the NEC group compared with DF animals (Fig. 4B; P < 0.01). Supplementation of formula with EGF significantly increased Bcl-2 mRNA levels compared with NEC (P < 0.05) but still remained markedly lower than DF animals (Fig. 4B; P < 0.01). There were no statistically significant differences in Bad, Bcl-xL, and Bcl-w expression between NEC and NEC+EGF groups (results not shown). To determine if there is a shift in the balance of
apoptotic genes that favor cell survival in EGF-supplemented animals, the ratio of Bax to Bcl-2 levels was evaluated (Table 2). The Bax-to-Bcl-2 ratio was markedly increased in the NEC group compared with DF animals. Supplementation with EGF decreased the ratio compared with animals given formula alone.

**Ileal levels of pro- and anti-apoptotic proteins.** Ileal expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins was evaluated using Western blot. Pro-apoptotic Bax protein levels were markedly increased in NEC animals compared with DF animals. Supplementation with EGF decreased expression of Bax compared with animals fed formula alone. Anti-apoptotic Bcl-2 protein levels were increased in animals supplemented with EGF compared with NEC animals (Fig. 5). The Bax-to-Bcl-2 ratio for protein expression was also evaluated and was significantly decreased in NEC+EGF animals compared with the NEC group ($n = 10$/experimental group, $P \leq 0.0001$). This indicates that EGF treatment shifts the balance of apoptotic proteins in favor of cell survival.

**Apoptosis.** Cleaved caspase-3, a specific marker for apoptotic cells, was used to detect and localize apoptotic changes in the terminal ileum of neonatal rats in all experimental groups (Fig. 6A). In the NEC group, there was a significant increase in the number of caspase-3-positive epithelial cells at the tip of the villus (Fig. 6B; $P \leq 0.001$). EGF treatment significantly decreased cleaved caspase-3 staining compared with the NEC group, with only occasional positive apoptotic cells observed in the EGF-treated rats. No cleaved caspase-3-positive cells were detected in DF controls.

**DISCUSSION**

Alterations in apoptosis may predispose the intestine to development of NEC (33). We are the first to show that EGF treatment of NEC elicits changes in expression of apoptotic genes and proteins in the terminal ileum, shifting the balance between pro- and anti-apoptotic proteins in favor of cell survival. We have previously shown that supplementation of EGF into milk formula significantly reduces the incidence of NEC in a neonatal rat model (15). These data suggest a possible mechanism of EGF-mediated reduction of the experimental NEC.

Several studies have demonstrated an important role of EGF insufficiency in the pathogenesis of NEC. Diminished serum and saliva EGF levels were reported in neonates suffering NEC compared with healthy controls (28, 62). Furthermore, a critically ill infant, diagnosed with NEC-like symptoms, was successfully treated by continuous intravenous infusion of EGF (65). In a rat model of NEC, EGF supplementation in formula reduced the incidence of experimental NEC (15), and EGF-mediated reduction of NEC was associated with downregulation of proinflammatory IL-18 and increased production of anti-inflammatory IL-10 in the site of injury (23).
ical actions of EGF are mediated through binding to EGF receptor (EGF-R); the presence of EGF-R has been reported in intestinal epithelium of infants diagnosed with NEC (18) and in a rat NEC model (15). Previous studies have shown that activation of the EGF-R may blunt apoptosis, thereby protecting intestinal structure during NEC (10).

In the normal small intestine, epithelial homeostasis is maintained by balancing the rate of cell proliferation and cell loss. It has been shown that apoptosis, and not simple exfoliation of enterocytes from the tip of villus, accounts for the majority of cell loss in the gut lumen (22, 55, 56, 61). Berseth (3) has shown that supplementation of EGF in milk formula enhances neonatal intestinal growth and intestinal cellular proliferation in suckling rats. In our NEC model, a significant reduction of villus length was measured in the intact portions of the terminal ileum of the NEC group compared with DF controls. Supplementation of formula with EGF resulted in normalization of villus size. Because the number of epithelial cells per micrometer of villus length was not different between the NEC and NEC+EGF groups, we conclude that hyperplasia rather than hypertrophy is occurring in the EGF-treated group. This conclusion is further supported by our previously published observation that ileal protein-to-DNA ratios are not different among these experimental groups (15). In addition to its mitogenic effects, EGF has multiple nonmitogenic actions within the gastrointestinal tract (see review in Ref. 66). In rat and mouse models of small bowel resection (SBR), increased intestinal morphological parameters, such as villus height or crypt depth, with exogenous EGF have been reported (8, 27, 43). The results from the present study with a NEC model indicate no changes in intestinal epithelial proliferation as a result of enteral administration of EGF. Thus the data suggest that enhanced hyperplasia of the intestinal mucosa in the NEC+EGF group results from inhibition of apoptosis of epithelial cells rather than from increased mitogenic effects of EGF.

Apoptosis is a physiological mode of cell death, distinct from necrosis, that plays an important role in many physiological and pathological processes (36, 60, 68). The intestinal epithelium has a high turnover rate that necessitates the apoptotic removal of cells at the end of their normal life cycle (2). In neonatal rats, the exposure to stress results in increased apoptosis in the gastric (20) and the intestinal epithelium (33). In vitro studies using the rat intestinal epithelial cell line IEC-6 demonstrated a dose-dependent effect of increasing corticosteroid levels on the induction of apoptosis (34). In our studies, formula feeding combined with repeated asphyxia/cold stress resulted in a significant increase of plasma levels of corticosterone in both the NEC and NEC+EGF groups (4- to 5-fold) compared with the DF group. Therefore, apoptotic changes measured between NEC and NEC+EGF groups cannot be attributed to differences in corticosterone levels. Expression of pro-apoptotic genes and proteins were significantly higher in the NEC group compared with the NEC+EGF group, and EGF treatment resulted in a shift in the balance of pro- and anti-apoptotic markers in favor of cell survival. Thus we speculate that EGF has a direct effect on blunting apoptosis at the site of NEC injury.

Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis, and the balance of their pro- and anti-apoptotic members is a critical factor for cell survival (1). The mecha-

Table 2. Ratio of Bax to Bcl-2 mRNA expression in the ileum

<table>
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<th>DF</th>
<th>NEC</th>
<th>NEC+EGF</th>
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<td>250</td>
<td>10</td>
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DF, dam fed; NEC, necrotizing enterocolitis; EGF, epidermal growth factor.

Fig. 4. Ileal mRNA levels of Bax (A) and Bcl-2 (B) were evaluated using real-time RT-PCR. The mean steady-state mRNA level for the DF group was assigned a value of 1.0, and the mean mRNA from the NEC and NEC+EGF groups was determined relative to this number. *Statistically significant vs. DF, P < 0.01. ‡Statistically significant vs. NEC, P < 0.05.

Fig. 5. Representative 21-kDa protein bands for Bax and 26 kDa protein bands for Bcl-2 by Western blot are shown for DF, NEC, and NEC+EGF groups. Expression of Bax is increased in NEC animals and decreased with supplementation of EGF in formula compared with the NEC group. Expression of Bcl-2 is increased in NEC+EGF animals compared with NEC animals. DF levels are shown as age-matched controls.
nism by which EGF protects intestinal cells against apoptosis may include the reduction of Bax expression (21), the increase of Bcl-2 (44) and Bcl-xL (49, 64) expression, and the inhibition of caspases (40). Studies using the murine model of SBR indicate that enterocyte apoptosis during intestinal adaptation is attenuated by EGF (26) and exaggerated when the EGF-R is defective (27). Similarly, Stern et al. (63) and Knott et al. (37) have shown that exogenous EGF retards rates of enterocyte apoptosis and modifies the expression of critical Bcl-2 family members. By decreasing pro-apoptotic Bax and increasing anti-apoptotic Bcl-w expression, the balance between pro- and anti-survival genes shifts in favor of cell survival. Inhibition of EGF-R signaling in the SBR model accelerates the rate of apoptosis and modifies the expression of Bcl-2-related peptides in favor of apoptosis (37). Our results using the neonatal rat model of NEC are in agreement with findings from the adult murine SBR model.

Bcl-2 and Bax are critical factors in apoptosis regulation because the molecular ratio of Bax to Bcl-2 acts as a cellular “rheostat” determining cellular flux toward or away from apoptosis (38, 52). Tissue Bax-to-Bcl-2 ratio is often used as an indicator of sensitivity to pro- or anti-apoptotic stimuli (30, 39). In this study, the development of experimental NEC in neonatal rats was associated with a huge increase in the Bax-to-Bcl-2 mRNA ratio (250-fold) in injured ileum compared with healthy DF littersmates. Bax protein expression was also significantly increased in animals with NEC compared with DF animals. These findings suggest that massive apoptosis occurred before full necrosis of ileal mucosa developed. Jilling et al. (33) recently showed that caspase-3 activity, a specific marker for apoptotic cells, is significantly increased in intestinal lysates of rats with NEC. However, it was not specified in which part of the small intestine and in which intestinal cells the increase in caspase-3 activity is occurring. In this study, the number of cleaved caspase-3-positive cells was significantly increased in the epithelial cells of the terminal ileum, the site of intestinal injury. Thus results from our study support previous finding and further clarify this observation. In conclusion, inhibition of the EGF-R signaling pathway or knockout models of NEC are necessary to further clarify the exact molecular mechanism of EGF-mediated protection. However, a recent study using waved-2 mice (defective EGF-R signaling) crossbred with Bax-null mice in a SBR model clearly indicated that impairment of EGF-R signaling augments intestinal epithelial cell apoptosis (32).

Supplementation of EGF in formula markedly decreased the Bax-to-Bcl-2 mRNA and protein ratios and dramatically reduced apoptosis. These results indicate that EGF treatment of experimental NEC alters apoptotic gene expression, thereby shifting the balance of pro- and anti-apoptotic genes toward cell survival. We speculate that EGF-mediated reduction of epithelial cell apoptosis is an important factor by which EGF reduces mucosal injury in the neonatal rat model of NEC. Better understanding of molecular processes underlying EGF-mediated reduction of experimental NEC might provide the basis for the future therapeutic strategies for the treatment of human NEC.

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EGF ALTERS APOPTOSIS IN NEC

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