Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment

Jessica A. Clark,1 Sarah M. Doelle,1 Melissa D. Halpern,1 Tara A. Saunders,1 Hana Holubec,2 Katerina Dvorak,2 Scott A. Boitano,2,3 and Bohuslav Dvorak1,2

Departments of 1Pediatrics and Steele Children’s Research Center, 2Cell Biology and Anatomy, and 3Physiology, University of Arizona, Tucson, Arizona

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EGF is a peptide that has trophic, maturational, and healing effects on the intestinal mucosa (10, 15, 42). Maternal colostrum and milk are the major sources of EGF for the developing neonate (7, 9, 12, 22). Yet, EGF is absent in all commercial infant formulas. It has been suggested that EGF insufficiency may play an important role in the pathogenesis of NEC (11). Premature neonates with NEC have diminished levels of EGF in serum and saliva compared with healthy controls (28, 51). In an experimental rat model, we have shown that supplementation of EGF into cow milk-based formula reduces the incidence of NEC (14), downregulates the overproduction of proinflammatory cytokines (24), maintains bile acid homeostasis (27), and decreases intestinal apoptosis at the site of injury (8). EGF has been shown to regulate goblet cell


Address for reprint requests and other correspondence: B. Dvorak, Dept. of Pediatrics, Univ. of Arizona, 1501 N. Campbell Ave., PO Box 245073, Tucson, AZ 85724-5073 (e-mail: dvorakb@peds.arizona.edu).

Necrotizing enterocolitis (NEC) is the most common and devastating gastrointestinal disease of premature infants (37). Despite an increasing occurrence of NEC in the United States, the etiology is unknown (49). The major risk factors associated with disease development are prematurity, enteral feeding, intestinal hypoxia-ischemia, and bacterial colonization (6). NEC is characterized pathologically by submucosal edema, mucosal ulcerations, hemorrhage, and necrosis of the distal ileum and proximal colon (30). These disturbances in epithelial integrity increase intestinal permeability in infants with NEC (41). However, it is unclear whether increased intestinal permeability is due to lesions in the mucosa or modifications to the gut barrier.

In the intestine, there are several factors that contribute to barrier integrity, such as gastric acidity, peristalsis, the mucous coat, and secreted antimicrobial factors (23, 55). Goblet cells secrete mucins, which form a semipermeable mucus layer between the lumen and intestinal epithelium (53). Several mucins have been identified; among them, mucin 2 (MUC2) is the predominant secretory mucin produced by intestinal goblet cells (16). It is speculated that in premature infants, a deficiency in the mucus layer may contribute to intestinal injury (23); however, little is known about the role of goblet cells and mucin production during NEC pathogenesis.

Tight junctions (TJs) form continuous intercellular contacts between epithelial cells and create a dynamic barrier to the paracellular movement of water, solutes, and immune cells (17). Formation of functional TJs is critical for the maintenance of gut permeability and intestinal barrier function. Several TJ proteins have been identified; among them, the transmembrane proteins occludin, claudins, and junctional adhesion molecule are considered crucial for creating the seal and regulating paracellular permeability (17). Occludin is a protein that is thought to be important for cell signaling (2) in addition to its role as a structural and functional component of the TJ (18, 20). The claudins are part of a large family (~24 members) of proteins that are thought to regulate paracellular permeability and epithelial barrier function (32, 44). Changes in the expression and localization of occludin and claudins have been implicated in barrier dysfunction during inflammatory bowel disease (IBD) in both humans (21) and animal models (1, 19). However, the role of the TJ in NEC pathogenesis is currently unknown.

EGF is a peptide that has trophic, maturational, and healing effects on the intestinal mucosa (10, 15, 42). Maternal colostrum and milk are the major sources of EGF for the developing neonate (7, 9, 12, 22). Yet, EGF is absent in all commercial infant formulas. It has been suggested that EGF insufficiency may play an important role in the pathogenesis of NEC (11). Premature neonates with NEC have diminished levels of EGF in serum and saliva compared with healthy controls (28, 51). In an experimental rat model, we have shown that supplementation of EGF into cow milk-based formula reduces the incidence of NEC (14), downregulates the overproduction of proinflammatory cytokines (24), maintains bile acid homeostasis (27), and decreases intestinal apoptosis at the site of injury (8). EGF has been shown to regulate goblet cell

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and mucin production in both the airway (54) and intestinal epithelium (10, 31). In these studies, stimulation with exogenous EGF accelerated goblet cell maturation, whereas pharmacological inhibition of the EGF receptor resulted in decreased goblet cell density. In addition, EGF prevents the disruption of TJ proteins in an injury model using Caco-2 cell monolayers (50). Currently, it is unknown whether EGF treatment is associated with changes in the intestinal barrier during NEC pathogenesis.

The aim of this study was to investigate whether the development of NEC is associated with structural and functional changes in the intestinal barrier using a rat model of NEC. In addition, we evaluated whether treatment with EGF improved intestinal barrier function and altered expression of TJ proteins in vivo. To achieve this aim, we induced NEC in neonatal rats using formula feeding coupled with exposure to asphyxia/cold stress. Intestinal permeability, goblet cell density, and MUC2 production were evaluated. Intestinal gene and protein expression and histological localization of the TJ components occludin and claudin-3 were determined. To better understand the pathology associated with NEC, all histological and biochemical evaluations were performed in both the proximal jejunum, which is often unaffected by NEC, and in the terminal ileum, the site of NEC injury. Alterations in intestinal barrier function and TJ expression may be one mechanism by which EGF prevents the development of NEC.

MATERIALS AND 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 Laboratory, Portage, MI) originating from 20 separate litters were collected by caesarian section 1 day before their scheduled birth. Animals were assigned to one of three experimental groups: NEC (n = 73), pups artificially fed with cow’s milk-based formula; NEC + EGF (n = 76), pups artificially fed with cow milk-based formula supplemented with 500 ng/ml rat EGF (Harlan Bioproducts, Indianapolis, IN); or dam fed (DF; n = 40). Artificially fed pups were hand fed 150 μl of milk formula every 5 h. All groups were stressed twice daily with asphyxia (breathing 100% nitrogen gas for 60 s) followed by cold stress (4°C for 10 min) (14, 25, 26). After 96 h, all surviving animals were killed. Animals that died before 96 h were excluded from the study because the postmortem tissue was not suitable for evaluations.

To evaluate differences between the healthy proximal jejunum and damaged terminal ileum, sections from these two regions were collected from each animal for histological and biochemical measurements. Because the amount of intestinal tissue from individual neonatal rats is limited, not all biochemical measurements were performed on every animal. Each animal was assessed histologically for ileal damage to evaluate the degree of NEC injury and randomly selected for further biochemical measurements.

NEC evaluation. Pathological changes in the intestinal architecture were evaluated using our previously published NEC scoring system (13, 14, 26, 27). Histological changes in the ileum were scored by a blinded evaluator and graded as follows: 0 (normal); 1 (mild), slight submucosal and/or lamina propria separation; +2 (moderate), moderate separation of the submucosa and/or lamina propria and/or edema in the submucosa and muscular layers; +3 (severe), severe separation of the submucosa and/or lamina propria and/or severe edema in the submucosa and muscular layers with regional villous sloughing; and +4 (necrosis), loss of villi and necrosis. Intermediate scores of +0.5, 1.5, 2.5, and 3.5 were also used to more accurately assess levels of ileal damage when necessary (27). To determine the incidence of NEC, animals with histological scores of less than +2 were considered to have not developed NEC, whereas animals with histological scores of +2 or greater were considered to have developed NEC.

RNA preparation. Total RNA was isolated from jejunal and ileal tissue using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer’s protocol and our previous studies (8, 25). All samples were incubated with RNase-free DNase (20 U/reaction) for 10 min at 37°C to eliminate DNA contamination. The RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity was determined by the 260-to-280-nm absorbance ratio (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 in 1× MOPS buffer [40 mmol/l MOPS (pH 7.0), 10 mmol/l sodium acetate, and 1 mmol/l EDTA (pH 8.0)] (8, 25).

RT and real-time PCR. Real-time PCR assays were performed to evaluate MUC2, Math1, occludin, and claudin-3 steady-state mRNA levels. cDNA was synthesized from 0.5 μg of DNase-treated total RNA. MUC2 and Math1 primers and probes were designed using Primer Express Software (Applied Biosystems, Foster, CA); the target gene probe was labeled with the fluorescent reporter dye FAM (40). The following MUC2 sequences were used (GenBank Accession No. BC036170): sense primer, 5'-actgggaatgtgactgctactg-3'; antisense primer, 5'-acccgttgtaactgtagtaaagtccat-3'; and probe, 5'-acaaggttg-ggctcctc-3'. The following Math1 sequences were used (GenBank Accession No. XM755508): sense primer, 5'-ggggcggccgcaggatt-3'; antisense primer, 5'-ttgtgaggcgcagccatc-3'; and probe, 5'-ctggcaac-cacgcctgc-3'. PreDeveloped TaqMan primers and probes were used for the detection of occludin and claudin-3 (Applied Biosystems). Reporter dye emission was detected by an automated sequencer detector combined with ABI Prism 7700 Sequence Detection System software (Applied Biosystems). Real-time PCR quantification was then performed using TaqMan 18S controls. Relative quantifications of PCR products were based upon value differences between the target and 18S control using the comparative threshold cycle method (35). Cycle parameters were 55°C × 5 min, 95°C × 10 min, and then 40 cycles of 95°C × 15 s and 58°C × 60 s. All samples were run in triplicate for each PCR.

Immunohistology of MUC2 and enumeration of goblet cells. MUC2 is produced by intestinal goblet cells and was used to specifically identify and enumerate goblet cells in the small intestine (47). A 2-cm section of the proximal jejunum and distal ileum was collected from each animal, fixed overnight in 70% ethanol, paraffin embedded, and sectioned at 4–6 μm. Serial sections were stained for MUC2. After deparaffinization and rehydration, sections were blocked with 1.5% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 30 min, incubated with 4.0 μg/ml rabbit anti-MUC2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min, washed with PBS three times, and incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) for 30 min. After sections were washed three times with PBS, Vectastain Elite ABC reagent (Vector Laboratories) was applied for 30 min, slides were washed three times with PBS, and dianisobenzene (Sigma) was utilized as the substrate. Sections were counterstained with hematxylin, dehydrated, and mounted on coverslips. Sections from all experimental groups were stained at the same time, and stained sections were evaluated by a blinded observer. MUC2-positive goblet cells were enumerated from 12 animals/group in 3 cript-villus units were counted from each animal. The total number of epithelial cells per crypt-villus unit was also enumerated. Sections from animals that exhibited full necrosis were not included in analyses because of the lack of intact tissue to evaluate.

Scanning electron microscopy. Segments of the distal ileum were fixed overnight at 4°C in 3% glutaraldehyde in 0.1 M cacodylate buffer, rinsed in 0.1 M cacodylate buffer, and incubated in 1% tannic acid in 0.15 M cacodylate buffer for 1 h. After being rinsed in cacodylate buffer, samples were postfixed in 1% osmium tetroxide in
0.1 M cacodylate buffer for 1 h and then washed in 0.1 M cacodylate buffer. Samples were dehydrated in a graded series of ethanol (30–100%), dried using hexamethyldisilazane (Electron Microscopy, Fort Washington, PA) for 3 min, and then air dried. Samples were mounted onto aluminum stubs, sputter coated with gold, and viewed using a Philips XL 30 scanning electron microscope. Digital images were acquired using FEI Microscope control software.

**Western blot analysis.** Individual frozen jejunum and ileum samples were homogenized with a hand-held homogenizer (Pellet Pestle, Kimble/Kontes, Vineland, NJ) in a 5× volume of ice-cold homogenization buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholic acid, 1% Triton X-100, 50 mM DTT, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 5 mM PMSF]. 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 protein assay (4). For protein analysis, 40 μg of protein were added to an equal volume of 2× Laemmli sample buffer and boiled for 5 min. Samples were run on a 10–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) at 95 V for 1 h. Protein was transferred to ImmunoBlot polyvinylidene difluoride membranes (Bio-Rad) at 15 V for 1 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma) for 1 h at room temperature and then incubated with primary antibody (Zymed, San Francisco, CA) overnight at 4°C. After being extensively washed, membranes were incubated for 1 h at room temperature with horse-radish peroxidase-conjugated donkey anti-rabbit IgG (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 QuantityOne software.

**Immunofluorescence microscopy of TJ proteins.** Serial sections from jejunal and ileal samples were processed as previously described (14, 25). After deparaffinization and rehydration, sections were blocked in 5% BSA to prevent nonspecific staining and incubated with one of the following antibodies: rabbit polyclonal anti-occludin (3.0 μg/ml) or anti-claudin-3 (2.0 μg/ml) (Zymed Laboratories, San Francisco, CA), followed by Alexa-conjugated secondary antibody (Molecular Probes, Eugene, OR). Nuclei were labeled using YOYO-1. Negative control sections were treated with the same procedure in the absence of primary antibody; no immunostaining was observed in the controls (not shown). Sections from each experimental group were immunostained for a specific antigen at the same time.

**Assessment of intestinal permeability.** In a separate set of experiments, intestinal permeability was evaluated using a sugar absorption test (34, 36). On day 4 of the experiment, neonatal rats from all groups (n = 22 rats/experimental group) were gavaged with 150 μl of sterile saline containing 103 ng of either [3H]lactulose (to indicate paracellular permeability) or [3H]rhamnose (to indicate transcellular permeability) (American Radiolabeled Chemicals, St. Louis, MO). All pups were fasted before the sugar absorption test to ensure equal dilution of the sugar probes. Because DF pups feed continuously and have fuller stomachs, they were fasted for 8 h; artificially fed pups were fasted for 5 h. Two hours after the administration of the sugar probe, animals were killed, and 30 μl of trunk blood were collected into scintillation vials. Kidneys were also collected and homogenized in 500 μl of sterile water before scintillation fluid was added. Radioactivity was measured using a Beckman LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

**Statistics.** Statistical analyses between the DF, NEC, and NEC + EGF groups were performed using ANOVA followed by Fisher’s protected least-significant difference test. The χ2-test was utilized to analyze differences in survival rates. Correlation analyses were performed using the Spearman rank correlation. All statistical analyses were conducted using the statistical program StatView for Macintosh computers (Abacus Concepts, Berkely, CA). All numerical data are expressed as means ± SE.

**RESULTS**

**Macroscopic appearance of the small intestine and survival rates.** The neonatal rat model of NEC is advantageous because many of the clinical and pathological changes are similar to those found in humans: the abdomen is distended, blood is detected in the stool, and the ileum and proximal colon are the most affected parts of the intestine. Furthermore, the neonatal rat model encompasses three major risk factors for human NEC: prematurity, formula feeding, and hypoxia-ischemia. Figure 1 shows the macroscopic appearance of the small intestine in DF and NEC animals. DF pups did not exhibit macroscopic damage, whereas NEC animals showed obvious injury in the distal ileum, with dilation, severe hemorrhage, and discolarization present. The proximal jejunum of NEC animals remained relatively unchanged.

The survival rates for these studies were as follows: DF, 37/40; NEC, 56/73; and NEC + EGF, 67/76. Animals with NEC had a significantly lower survival rate compared with DF animals (P < 0.05).

**Intestinal permeability.** Paracellular and transcellular intestinal permeability were assessed in vivo by orally administering the disaccharide [3H]lactulose or the monosaccharide [3H]rhamnose, respectively. Detection of [3H]lactulose in the blood was significantly increased in animals with NEC compared with DF (Fig. 2A). Interestingly, animals in the NEC + EGF group had significantly lower levels of [3H]lactulose in the blood compared with both the NEC and DF groups, indicating that EGF treatment resulted in decreased intestinal permeability via the paracellular route. Animals with NEC had significantly increased levels of [3H]lactulose in their kidneys compared with DF and NEC + EGF pups (Fig. 2B), further supporting that there is increased intestinal paracellular permeability during NEC pathogenesis. There were no significant differences in blood or kidney levels of [3H]rhamnose among groups, indicating that transcellular permeability is unaltered.
Goblet cell density and MUC2 production in the small intestine. Mucins are the primary constituents of the mucus layer, which creates a protective barrier for the epithelium. MUC2 is the predominant secreted mucin that is produced by intestinal goblet cells. MUC2 production in the jejunum and ileum, supplementation of EGF into formula resulted in a significant increase in Math1 mRNA levels compared with the DF and NEC groups (Fig. 4B). These data demonstrate that EGF can directly induce goblet cell maturation, leading to increased goblet cell density in the terminal ileum.

Scanning electron microscopy of the ileum was performed to evaluate abnormalities in goblet cell structure at the site of NEC injury (Fig. 5). Goblet cells in both DF and EGF-treated animals appeared open with visible droplets of mucin present on the epithelial surface. In animals with NEC, goblet cells appeared to be sealed with no mucin droplets present.

Alterations in occludin during NEC pathogenesis and normalization with EGF treatment. To determine whether changes in intestinal permeability were associated with alterations in the expression of TJ components, gene expression of occludin was evaluated in the intestine using real-time PCR (Fig. 6A). Jejunal occludin mRNA levels were unchanged among groups. Ileal occludin mRNA levels were significantly increased in animals with NEC compared with DF compared with DF pups. Treatment with EGF significantly reduced ileal occludin mRNA to similar levels seen in DF animals. Furthermore, there was a positive correlation ($r = 0.661$, $P \leq 0.0001$) between ileal occludin mRNA levels and the progression of ileal injury, as determined via histological NEC scores.

Histological localization of occludin in the jejunum and ileum was evaluated using immunofluorescence microscopy (Fig. 6B). In the jejunum, occludin was predominantly localized in the cytoplasm along the crypt-villus axis in all groups, with the most abundant signal detected in the DF group. In contrast, occludin expression was significantly increased in the ileum of animals with NEC compared with DF and was localized near the apical membrane in the crypts and in the cytoplasm along the villi. EGF treatment markedly reduced the expression of occludin and resulted in the redistribution to the apical and basolateral membranes of the villi. To quantify changes in protein expression, occludin levels were evaluated in the jejunum and ileum by Western blot analysis (Fig. 6C). In the jejunum, occludin protein levels were decreased in both the
NEC and NEC + EGF groups compared with DF pups. However, ileal occludin protein expression was increased in animals with NEC compared with DF. EGF treatment markedly reduced occludin protein expression to a level similar to that seen in DF animals.

**Alterations in claudin-3 during NEC pathogenesis and normalization with EGF treatment.** Preliminary studies in our laboratory using microarray revealed that among the claudin family members, expression of claudin-3 was significantly changed during NEC pathogenesis. Therefore, we further evaluated mRNA levels of claudin-3 in the jejunum and ileum using real-time PCR (Fig. 7A). Claudin-3 mRNA levels were significantly increased in both the jejunum and ileum of animals with NEC compared with DF pups. Supplementation of formula with EGF significantly decreased claudin-3 gene expression in the ileum compared with pups fed formula alone. There was a positive correlation \( r = 0.815, P = 0.0001 \) between ileal claudin-3 mRNA levels and the progression of ileal injury.

Immunofluorescence microscopy revealed that claudin-3 was localized predominantly in the crypts in both the jejunum and ileum (Fig. 7B). In animals with NEC, there was increased staining in the crypts near the apical membrane in both the jejunum and ileum compared with DF animals. EGF treatment normalized claudin-3 expression similar to DF animals in the jejunum. In the ileum, EGF treatment resulted in the redistribution of claudin-3 to the apical and basolateral membranes along the crypt-villus axis. Relative quantification of claudin-3 protein levels in the jejunum and ileum was determined by Western blot analysis (Fig. 7C). In the jejunum, claudin-3 was increased in animals with NEC compared with DF pups. EGF treatment decreased jejunal claudin-3 protein expression. Ileal claudin-3 protein levels were markedly increased in the NEC group compared with the DF group. Supplementation of EGF
DISCUSSION

Preterm neonates have increased intestinal permeability during the first 2 wk of life, which can leave the gut vulnerable to the development of intestinal complications such as NEC (48). Although the importance of intestinal barrier function in NEC pathogenesis has been suggested, it has not been previously evaluated. The present study demonstrates that significant changes occur in intestinal permeability, goblet cell function, and TJ protein expression during NEC pathogenesis. In addition, we have demonstrated that EGF treatment significantly improves intestinal barrier function.

During the early postnatal period, intestinal permeability is relatively high to allow for the absorption of immunoglobulins, growth factors, and food antigens from maternal colostrum and milk (39). In neonatal rats, transport of these macromolecules ceases after gut closure, which occurs ~21 days after birth (39). Perturbations to this immature intestinal barrier can result in damage to the mucosa, leading to barrier failure and initiation of inflammation. In a study by Piena-Spoel et al. (41), human neonates diagnosed with severe cases of NEC had increased intestinal permeability compared with healthy controls. Although in humans intestinal permeability using the sugar absorption test is evaluated by measuring the amount of lactulose or rhamnose present in the urine, in our neonatal rat model, it was not feasible to collect equal amounts of urine from each rat pup. If these nonmetabolized sugars leak out of the intestine, they will enter systemic blood via enterohepatic circulation. Consequently, these sugars will be cleared by the kidneys and concentrated in the urine. Thus, we chose to analyze [3H]lactulose and [3H]rhamnose in the blood and kidneys to determine changes in intestinal paracellular and transcellular permeability, respectively. Our results indicate that animals with NEC had significantly increased intestinal paracellular permeability compared with healthy DF pups. Transcellular permeability was unaltered during NEC pathogenesis, suggesting that the increased intestinal permeability was not due to overall gut breakdown but a reflection of alterations at the TJ barrier.

EGF plays a crucial role in the regulation of several gastrointestinal functions. A previous study (46) has shown that exogenous EGF prevents increased ileal permeability in small intestine and NEC pathogenesis, underscoring the important role of EGF in maintaining epithelial barrier integrity. In vitro studies have demonstrated that EGF plays an important role in regulating paracellular permeability. In Caco-2 cell monolayers, EGF inhibits both oxidant- and acetaldehyde-induced increases in paracellular permeability (45, 50). In the present study, sup-
plementation of EGF into formula significantly reduced intestinal paracellular permeability compared with animals with NEC and DF. We conclude that EGF treatment dramatically improves intestinal barrier function during NEC pathogenesis.

It has also been shown that EGF accelerates goblet cell maturation and mucin production in the airways and small intestine (29, 54). In the airway epithelium, both in vitro and in vivo studies have shown that EGF-R signaling regulates mucin production and goblet cell differentiation (54). In the intestine, studies (10, 29) have also shown that EGF accelerates goblet cell maturation and mucin secretion in vivo. In a model of short bowel resection, exogenous EGF increased goblet cell density, whereas pharmacological inhibition of the EGF receptor decreased goblet cell density compared with sham-operated controls (31). In the study presented herein, we found that EGF treatment significantly increased goblet cell density and MUC2 production in the ileum but had no effect on MUC2 production in the jejunum. Furthermore, ileal goblet cells in DF and EGF-treated animals appeared to be open with mucus droplets on the epithelial surface, suggesting active mucin secretion. Math1 is a transcription factor important in cell fate determination and is required for the differentiation of the secretory cell lineage in the gut (52). In Math1-null mice, the small and large intestines show normal villus architecture, lamina propria, and musculature but no goblet cells (56). Our results demonstrated that Math1 expression is significantly increased in both the jejunum and ileum of animals treated with EGF. We speculate that the mechanism by which EGF increases goblet cell density in the ileum is via the upregulation of the transcription factor Math1. The ability of EGF to accelerate goblet cell maturation and increase mucin production in the ileum allows a physical and chemical barrier to be created between the epithelial surface and intestinal lumen.

Intestinal barrier function and paracellular permeability are primarily determined by epithelial TJs. The TJ is localized on the apical membrane of polarized epithelial cells and functions to create a barrier to the paracellular movement of solutes. The TJ is a dynamic structure and can be disassembled and reorganized in response to various intracellular and extracellular stimuli (38). Several TJ proteins have been identified, and these include the transmembrane proteins occludin and claudins, which are considered to be the primary sealing and integral membrane components of the TJ. The precise role of these TJ proteins remains unclear; however, occludin has both structural and functional roles, whereas claudins appear to provide specificity for defining the paracellular permeability of various ions and solutes (44). The adult rat intestinal epithelium expresses occludin and claudins, although the expression and subcellular localization of the claudin family of proteins varies along the length of the gastrointestinal tract (44). It is important to note that TJ proteins have not been characterized in the intestinal epithelium of neonates. Consequently, this study is the first to demonstrate normal expression patterns of epithelial TJ pro-
teins in the intestine of neonatal rats as well as alterations in the TJ barrier during NEC pathogenesis.

Impaired intestinal barrier function in pathological conditions has been associated with reduced expression and changes in distribution of TJ proteins (1, 5, 21, 33, 43). While these studies were performed in adult animals or cell lines, our study is the first to report changes in expression of intestinal epithelial TJ proteins in neonatal rats. We found that the major changes in occludin and claudin-3 were in the distal ileum of animals with NEC. Interestingly, occludin expression was not increased in the jejunum of NEC pups, suggesting that occludin was being modified specifically at the site of NEC injury. Furthermore, in the ileum, occludin distribution was less organized at the plasma membrane and localized in the cytoplasm in animals with NEC. Claudin-3 expression in the small intestine showed a striking pattern of localization in the crypts. Although the exact role of claudin-3 in the small intestine remains unknown, we speculate that the increased expression of claudin-3 in the crypts of animals with NEC may contribute to increased paracellular permeability.

Fig. 6. A: jejunal and ileal mRNA levels of occludin were evaluated using real-time PCR. The mean steady-state mRNA level for the DF group was assigned a value of 1.0, and mean mRNA levels from the NEC and NEC + EGF groups were determined relative to this number. Values are means ± SE; n = 12 rats/experimental group. *P ≤ 0.0001 vs. DF and NEC + EGF. B: occludin localization was evaluated in the proximal jejunum and terminal ileum of neonatal rats. Representative slides for DF, NEC, and NEC + EGF are shown. Magnification: ×40. C: representative 65-kDa protein bands for occludin by Western blot are shown for the DF, NEC, and NEC + EGF groups in the jejunum and ileum. β-Actin (43 kDa) is shown as an internal control.
thought to be the pore-forming proteins that regulate the size selectivity of the TJ barrier. The presence of claudin-3 in the crypts may promote a “leakier” epithelium, allowing for overall increased intestinal permeability.

Although our results contradict the accepted paradigm that increased intestinal permeability is associated with decreased TJ protein expression, we hypothesize that this is a reflection of the fundamental differences between neonatal and adult intestinal epithelium. Furthermore, there is a positive correlation between histological damage and mRNA levels of occludin and claudin-3, suggesting that as the intestine becomes more damaged, the epithelial cells begin upregulating the expression of TJ components. The vast majority of studies describing TJ protein expression during intestinal injury have been performed in adult animal models of IBD. It is important to note that the pathogenesis of NEC is different from that which occurs in IBD, and sufficient literature characterizing intestinal TJs in neonates is still lacking.

Fig. 7. A: jejunal and ileal mRNA levels of claudin-3 were evaluated using real-time PCR. The mean steady-state mRNA level for the DF group was assigned a value of 1.0, and mean mRNA levels from the NEC and NEC + EGF groups were determined relative to this number. Values are means ± SE; n = 12 rats/experimental group. †P ≤ 0.01 vs. DF; *P ≤ 0.0001 vs. DF and NEC + EGF. B: claudin-3 localization was evaluated in the proximal jejunum and terminal ileum of neonatal rats. Representative slides for DF, NEC, and NEC + EGF are shown. Magnification: ×40. C: representative 22-kDa protein bands for claudin-3 by Western blot are shown for the DF, NEC, and NEC + EGF groups in the jejunum and ileum. β-Actin (43 kDa) is shown as an internal control.
Several studies have shown that EGF can influence intestinal permeability. However, there are limited studies showing the effect of EGF on the intestinal TJ barrier. Studies using human colonic mucosa (3) and Caco-2 cell monolayers (50) have shown that EGF treatment inhibits the disruption of occludin from the TJ, thereby preventing the acetaldehyde-induced increase in paracellular permeability. The present study is the first to show that EGF treatment resulted in decreased expression of occludin and claudin-3 in the ileum compared with animals fed formula alone. Importantly, occludin was redistributed in the ileum of EGF-treated animals and localized predominantly on the apical and lateral membranes of epithelial cells, indicating that EGF promotes the formation of TJs. This normalization of TJ protein expression at the site of NEC injury supports our hypothesis that EGF maintains intestinal epithelial cell integrity during disease development.

Our present work suggests the following paradigm for intestinal barrier function during NEC pathogenesis (Fig. 8). In healthy DF animals, goblet cells are present and continuously secreting low basal levels of mucins, creating a normal mucus layer. Although TJ proteins are being produced by normal neonatal epithelial cells, they are predominantly cytoplasmic and not functioning to create a tight paracellular barrier because intestinal permeability remains high to allow for absorption of milk proteins. In NEC, GCs are decreased in number, leading to a deficient mucus layer; epithelial cell apoptosis is also increased. Consequently, intestinal paracellular permeability increases. In an attempt to restore barrier function, epithelial cells upregulate expression of occludin and claudin-3. However, they are unable to form functioning TJs, and permeability remains elevated. EGF treatment accelerates the maturation of GCs via upregulation of Math1 at the site of injury, leading to an enhanced mucus layer. Occludin and claudin-3 expression are normalized after EGF treatment, and TJ proteins are redistributed to the apical and basolateral membranes. Therefore, intestinal paracellular permeability is significantly decreased compared with DF and NEC animals. These reparative mechanisms lead to restoration of intestinal barrier function and prevention of NEC.
Therefore, intestinal paracellular permeability is significantly decreased compared with DF and NEC animals. These reparative mechanisms lead to the restoration of intestinal barrier function and prevention of NEC.

In conclusion, this study shows for the first time that the intestinal barrier is significantly disrupted in the ileum during NEC pathogenesis. EGF treatment reduces intestinal permeability, increases goblet cell density and MUC2 production in the ileum, and normalizes the expression of occludin and claudin-3. We speculate that EGF is an important factor that maintains the integrity of the intestinal barrier and reduces mucosal injury in the neonatal rat model of NEC. Localized alterations in the epithelial TJ barrier may be one mechanism by which EGF protects the intestine from injury. Better understanding of the molecular processes underlying EGF-mediated reduction of experimental NEC might provide the basis for future therapeutic strategies for the treatment of human NEC.

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REFERENCES


