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Modulation of Human Intestinal Epithelial Cell IL-8 Secretion by Human Milk Factors

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ABSTRACT

Necrotizing enterocolitis (NEC) seems to result from the inflammatory response of an immature intestine. Human milk is protective against NEC *via* an unknown mechanism. We hypothesized that specific factors found in human milk would decrease stimulated IL-8 secretion in intestinal epithelial cells. HT29-cl19A and Caco2 cells were compared with the fetal human primary intestinal epithelial cell line H4 and temperature-sensitive conditionally immortalized fetal human intestinal (ts-FHI) cells. Cells were pretreated with transforming growth factor-β (TGF-β), erythropoietin (Epo), IL-10, or epidermal growth factor (EGF) at physiologic concentrations before stimulation with tumor necrosis factor-α (TNF-α) or IL-1β, and then IL-8 was measured by ELISA. The fetal cells produced significantly more IL-8 when stimulated by TNF-α or IL-1β. There were also differences in the pattern of alteration of IL-8 secretion by human milk factors. In HT29-cl19A cells, IL-10 inhibited TNF-α–stimulated IL-8 secretion by 52%, and EGF increased secretion by 144%. In H4 cells, TGF-β1 and Epo inhibited TNF-α–stimulated IL-8 secretion to control levels, and EGF increased secretion by 29%. IL-1β–stimulated IL-8 secretion was inhibited 25% by TGF-β1 in Caco2 cells and in H4 cells was inhibited by TGF-β1, Epo, and TGF-β2. TsFHI cells confirmed H4 cell results. Fetal human enterocytes have an exaggerated IL-8 secretion in response to TNF-α and IL-1β. TGF-β and Epo decrease this stimulated IL-8 secretion, which may partially explain the protective effect of human milk in NEC. **(*Pediatr Res* 53: 419–425, 2003)**

Abbreviations

DMEM, Dulbecco's modified Eagle's medium
EGF, epidermal growth factor
Epo, erythropoietin
FBS, fetal bovine serum
IEC, intestinal epithelial cell
NEC, necrotizing enterocolitis
NF-κB, nuclear factor κB
SV40, simian virus 40
TGF-β, transforming growth factor-β
TNF, tumor necrosis factor
TsFHI, temperature-sensitive fetal human intestinal cells

Evidence suggests that the intestinal epithelium is an active participant in gastrointestinal host defense and that intestinal epithelial-microbial interactions are developmentally regulated (1–4). Children are more susceptible to severe diarrhea from rotavirus and vibrio cholera toxin than are adults (5), and animal models have demonstrated prolonged rotavirus shedding and an increased secretory response to cholera toxin in neonatal compared with adult rats (6). Necrotizing enterocolitis (NEC) is another disease of intestinal maturation and microbial interaction. The primary risk factor for this disease is prematurity, and although the pathophysiology of this disease is not completely understood, an exaggerated inflammatory response to intestinal bacteria is a possible contributing mechanism (7).

Several issues make the preterm intestine increasingly susceptible to microbial interaction. Decreased intestinal blood flow regulation, gastric acid production, levels of protective mucus, proteolytic enzyme activity, intestinal motility, and levels of sIgA are contributing factors. In addition, increased intestinal permeability enhances susceptibility (8). It is known that the fetal intestine is exposed to amniotic fluid containing hormones and peptides that may have a role in intestinal maturation. Preterm infants may not have completed this maturation process when colonized by bacteria and initially fed. At this stage, the fetal intestine is normally protected in its sterile environment and may not be prepared to respond to bacterial interaction.

Human milk seems to be protective against intestinal inflammation. A study of chemical colitis in rats demonstrated decreased myeloperoxidase activity in animals fed human milk (9). A clinical study by Lucas and Cole (10) demonstrated a decreased incidence of NEC in human milk–fed preterm infants compared with formula-fed infants. Human milk contains

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several factors that may decrease the incidence of NEC. It contains factors to decrease microbial interaction with the intestinal epithelium such as lysozyme, lactoferrin, polymeric IgA, and oligosaccharides (11–13). In addition, it contains anti-inflammatory factors such as soluble tumor necrosis factor (TNF) receptor, IL-1 receptor antagonist, and acetylhydrolase (14). It also contains factors such as transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) that limit intestinal injury by stimulating intestinal repair through increased cell restitution, growth, and inhibition of apoptosis (15).

Enterocytes are active participants in host defense. In response to stimuli, enterocytes can release cytokines such as IL-6, TNF-α, and IL-8 (1). Specifically, we have previously shown that compared with adult cells, immature or undifferentiated intestinal epithelial cells (IEC) have increased IL-8 secretion in response to products associated with bacterial challenge such as lipopolysaccharide with butyrate and IL-1β (2). This exaggerated inflammatory response may have a role in NEC (16). Histopathology sections of intestine from infants with NEC reveal evidence of necrosis and inflammation (17, 18). Neutrophils in particular seem to have a central role in the intestinal injury, and it has been shown that neutrophil depletion by vinblastine eliminated intestinal injury in a rat model of NEC (19).

IL-8 is a chemokine that stimulates migration of neutrophils from intravascular to interstitial sites and can directly activate neutrophils and regulate the expression of neutrophil adhesion molecules (20–22). Thus, by recruiting and activating immune cells, IL-8 may play an important role in inflammation. Previous studies have shown that concentrations of serum IL-8 were significantly elevated in severe cases of NEC from its onset through the first 24 h (16). Surgical specimens of intestine from infants with acute NEC show up-regulation of IL-8 mRNA throughout the serosa, muscularis, and intestinal epithelium compared with those with other inflammatory conditions or those without disease (23).

On the basis of these previous observations, we studied the effect of specific factors in human milk on IL-8 secretion. We chose to test four factors—TGFβ, erythropoietin (Epo), IL-10, and EGF—that have been shown to be protective against NEC and/or have anti-inflammatory effects in other models (24–28). We hypothesized that at physiologic concentrations, these factors would decrease IL-8 secretion induced by inflammatory stimuli in IEC.

METHODS

Materials. TNF-α, IL-1β, TGF-β, Epo, EGF, IL-10, goat antihuman IL-8 antibody, and recombinant human IL-8 were obtained from R&D Systems (Minneapolis, MN, U.S.A.). Rabbit anti-human IL-8 antibody was obtained from Endogen (Woburn, MA, U.S.A.). Horseradish peroxidase goat anti-rabbit IgG antibody was obtained from Biosource (Camarillo, CA, U.S.A.). 2,2' Azino-Bis (3-Ethylbenz-thiazoline-6-sulfonic acid) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

IEC lines. Four human IEC lines were used in these studies: HT29-cl19A, Caco2, H4, and temperature-sensitive fetal human intestinal cells (tsFHI). HT29-cl19A cells originate from the subcloning of an adult colonic adenocarcinoma cell line and display a more differentiated phenotype than the original HT29 cells (29). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin. Caco2 cells also originate from an adult colonic adenocarcinoma cell line. Cells were cultured in DMEM with 10% heat-inactivated FBS, 1% glutamine, 1% sodium pyruvate, 1% amino acids, 1% HEPES, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.2 U/mL insulin. H4 cells are a human fetal nontransformed primary small IEC line that has previously been characterized in this laboratory (30). These cells do not form tight junctions or polarize. Cells were cultured in DMEM with 10% heat-inactivated FBS, 1% glutamine, 1% sodium pyruvate, 1% amino acids, 1% HEPES, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.2 U/mL insulin. These three cell lines were grown at 37°C in a 5% CO_2 atmosphere.

TsFHI cells are a human fetal small IEC line transformed with a temperature-sensitive simian virus 40 (SV40) large tumor antigen. These cells proliferate at 32°C, but when transferred to 37°C, the SV40 antigen breaks down, leading to growth arrest and acquisition of an enterocyte-like phenotype, although they remain morphologically immature and do not form tight junctions or polarize (31). These cells were provided for our studies by Dr. Andrea Quaroni (Department of Biology, Cornell University, Ithaca, NY, U.S.A.). These cells were grown at 32°C in growth medium consisting of OptiMEM I supplemented with 4% heat-inactivated FBS, 10 ng/mL EGF, 2 mM glutamine, 2 mM L-alanyl-glutamine (GlutaMAX I), 10 mM HEPES, 50 U/mL penicillin, and 50 μg/mL streptomycin. When confluent, they were transferred to 37°C and medium consisting of DMEM supplemented with 7% heat-inactivated FBS, 2 mM glutamine, 2 mM GlutaMAX I, 10 mM HEPES, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were then used after an additional 7 d.

Effect of human milk factors on IL-8 secretion. Cells were grown to confluence in 96-well plates to facilitate testing of multiple conditions. Medium was then changed, and cells were pretreated with factors in question for 24 h at physiologic concentrations found in human milk; previous dose-response experiments with these factors also showed that these doses gave the maximal effects—TGF-β 30 ng/mL (32, 33), Epo 0.1U/mL (34, 35), IL-10 10 ng/mL (36, 37), EGF 100 ng/mL (38–40). On the basis of previous dose-response and time course data, cells were then treated with either TNF-α 10 ng/mL for 6 h or IL-1β 1 ng/mL for 24 h (36). Comparable cell viability under these pretreatment and treatment conditions was confirmed in initial assays by 3-[4,5-Dimethylthyazol-2-y]-2, 5-diphenykltetrazolium bromide cytotoxicity assay. H4 cell responses to TNF-α were compared with HT29-cl19A cells, as Caco2 cells have previously been shown not to respond to TNF-α (41). H4 cell responses to IL-1β were compared with Caco2 cells as differentiated HT29 cells have been previously shown not to respond to IL-1β (42). Supernatants were collected for IL-8 determination and stored at −20°C. Cell lysates were obtained for measurement of protein concentration.

IL-8 measurement. IL-8 concentration was measured by an ELISA as described by McCormick (43). Briefly, 96-well plates (Nunc Maxisorp) were coated overnight with 8 μg/mL goat anti-human IL-8 polyclonal antibody. The plate was then washed (PBS plus 5% neonatal goat serum, 1% Tween 20 diluted 1:10) and incubated with samples at 37°C. After a second wash, the plate was incubated with 8 μg/mL rabbit antihuman IL-8 antibody. The plate was washed again and incubated with horseradish peroxidase goat anti-rabbit IgG 80 ng/mL. Finally, the plate was washed and incubated with 2,2' Azino-Bis (3-Ethylbenz-thiazoline-6-sulfonic acid). The absorbance was measured at OD 405. Samples were run in quadruplicate, and IL-8 values were normalized to measured protein concentration to allow comparison among cell types.

Protein concentration determination. For protein concentration determination, cells were lysed for 30 min on ice in the 96-well plate with 40 μL/well lysis buffer consisting of 1% Nonidet P-40, 150 mM NaCl, 1× PBS, 20 mM EDTA, 20 mM EGTA, 4 mM Na_3VO_4, and 40 mM NaF. After centrifugation, 5 μL/well lysate was used for the BioRad DC Protein Assay as per the manufacturer's protocol.

Statistical analysis. Statistical analysis was reviewed with a statistician. Results are presented as mean values of IL-8 pg/mg protein ± SEM. Statistical significance was evaluated using two-tailed t test. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of IL-8 secretion in fetal and adult IEC. The human fetal primary IEC line, H4, was compared with the adult cell lines HT29-cl19A and Caco2. Cells were treated with either TNF-α or IL-1β. Unstimulated H4 cells secreted 36 000 ± 7 000 pg/mg protein IL-8, whereas stimulated H4 cells secreted 71 000 ± 10 000 pg/mg protein IL-8 in response to TNF-α. In contrast, unstimulated HT29-cl19A cells secreted significantly less IL-8, only 336 ± 93 pg/mg protein, and 5 030 ± 485 pg/mg protein in response to TNF-α ($p < 0.05$, $n \geq 4$; Fig. 1). Similarly, stimulated H4 cells secreted 346 000 ± 49 000 pg/mg protein of IL-8 in response to IL-1β, whereas Caco2 cells secreted significantly smaller baseline IL-8 (279 ± 79 pg/mg protein) and stimulated IL-8 levels (3 350 ± 330 pg/mg protein) in response to IL-1β ($p < 0.05$, $n \geq 4$; Fig. 1).

Effect of human milk factors on IL-8 production in response to TNF-α. To determine the effect of the human milk factors, we pretreated H4 and HT29-cl19A cells with each of the factors for 24 h before the addition of TNF-α. IL-8 concentration was measured by ELISA and normalized to cell protein concentration to allow comparison among cell lines. However, for any given cell line, protein concentration was comparable for all wells, ruling out an alteration in overall protein synthesis as an explanation of the effects. Analysis without correction for protein did not change the results for any given cell line. As outlined in the methods section, concentrations comparable to physiologic levels found in human milk were used for each factor. As TGF-β2 is the predominant isoform in human milk, both TGF-β1 and TGF-β2 were tested (44).

As shown in previous studies (36), IL-10 significantly inhibited IL-8 secretion in HT29-cl19A cells by 52% from 5 030 ± 490 pg/mg protein to 2 400 ± 440 pg/mg protein ($p < 0.05$, $n \geq 4$). No other factor had an inhibitory effect in this cell line.

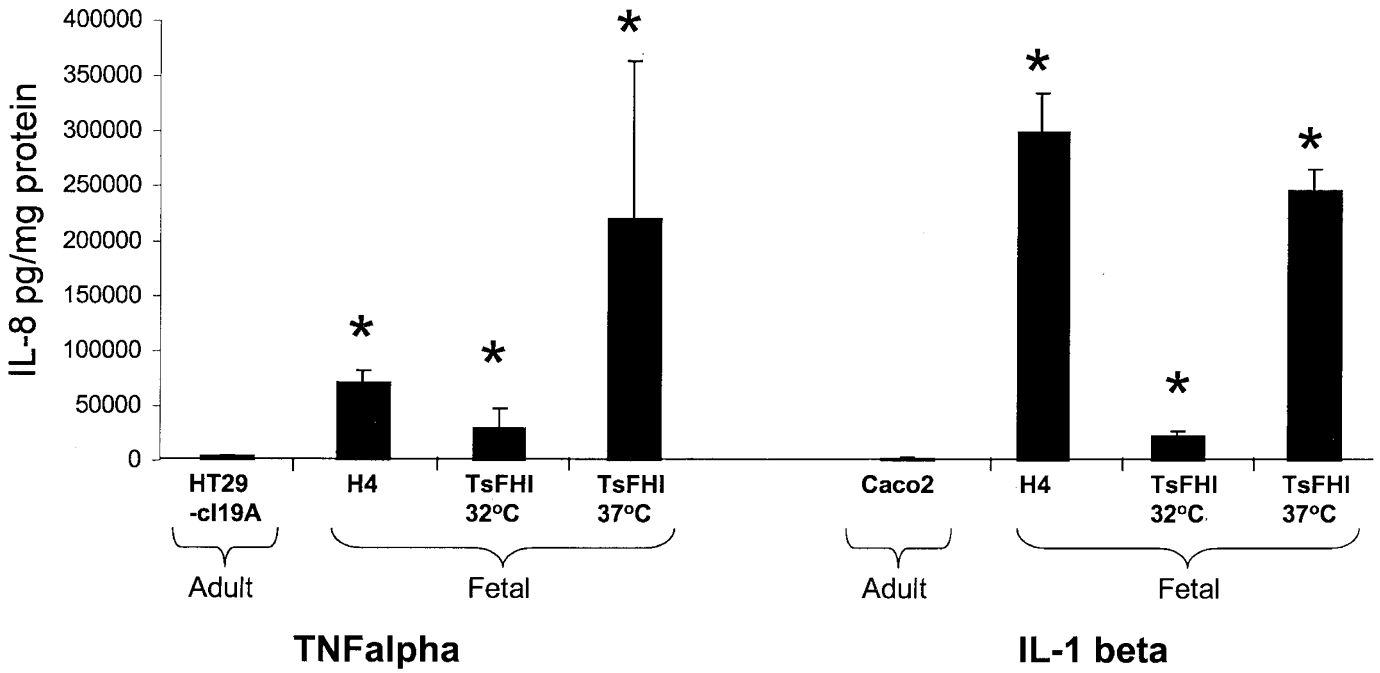

Figure 1. Increased IL-8 secretion in fetal compared with adult IEC lines when stimulated by either TNF-α or IL-1β. Human IEC were stimulated by TNF-α for 6 h or IL-1β for 24 h. IL-8 was measured by ELISA and normalized to cell lysate protein concentration. Responses in the transformed adult colonic cell lines HT29-cl19A and Caco2 were compared with the fetal small IEC lines H4 and tsFHI at 32°C (transformed) or 37°C (nontransformed). Findings are presented ± SEM; $n \geq 4$. *Statistical significance at $p < 0.05$ compared with the adult cell line.

EGF significantly increased IL-8 secretion by 144% to 12 000 ± 2 400 pg/mL ($p < 0.05$, $n \geq 4$) (Fig. 2*a*).

In contrast, TNF-α–induced IL-8 secretion in H4 cells was completely inhibited by TGF-β1 from 71 000 ± 10 000 pg/mg protein to control levels of 34 000 ± 3 500 pg/mg protein and by Epo to 41 000 ± 3 700 pg/mg protein ($p < 0.05$, $n \geq 4$). IL-10, although decreasing IL-8 production, did not have a statistically significant effect. Again, EGF significantly increased IL-8 secretion by 29% to 92 000 ± 10 000 (Fig. 2*b*).

Effect of human milk factors on IL-8 production in response to IL-1β. As above, H4 and Caco2 cells were pretreated with factors for 24 h before the addition of IL-1β. In Caco2 cells, TGF-β1 decreased IL-8 secretion by 25% from 3 350 ± 330 pg/mg protein to 2 500 ± 400 pg/mg protein ($p < 0.05$, $n \geq 4$; Fig. 3*a*). No other significant growth factor effects were observed. In H4 cells, TGF-β1 had an even greater inhibitory effect of 63% from 346 000 ± 49 000 pg/mg protein to 129 000 ± 29 000 pg/mg protein ($p < 0.05$, $n \geq 4$). In addition, Epo decreased IL-8 secretion 43% to 196 000 ± 21 000 pg/mg protein, and TGF-β2 decreased IL-8 secretion 39% to 212 000 ± 19 000 pg/mg protein ($p < 0.05$, $n \geq 4$; Fig. 3*b*).

Effect of human milk factors on IL-8 production in tsFHI cells. TsFHI cells were used to confirm results noted in H4 cells. This cell line is unique in that it is transformed by the temperature-sensitive SV40 oncogene promotor at 32°C but becomes a nontransformed cell line at 37°C when the SV40 promotor is inactivated. However, the cell line grows slowly at 32°C, limiting the number of possible observations; thus, it was used only to qualitatively confirm previous results. At 32°C, the tsFHI cells produced 7 800 ± 2 200 pg/mg protein IL-8 at baseline, 31 000 ± 17 000 pg/mg protein IL-8 when stimulated by TNF-α, and 24 000 ± 3 100 pg/mg protein IL-8 when stimulated by IL-1β. At 37°C, 92 000 ± 8 500 pg/mg protein IL-8 was secreted at baseline, 221 000 ± 143 000 pg/mg protein IL-8 in response to TNF-α, and 248 000 ± 18 000 pg/mg protein IL-8 in response to IL-1β (Fig. 1). As shown in Fig. 1, the fetal small intestinal cell lines consistently produced more IL-8 when stimulated by either TNF-α or IL-1β than the adult cell line of comparison. Interestingly, the nontransformed tsFHI cell line (37°C) secreted more IL-8 than the transformed tsFHI cell line (32°C).

Growth factor studies using the nontransformed tsFHI cells at 37°C qualitatively confirmed findings in H4 cells. TGF-β and Epo inhibited TNF-α–stimulated IL-8 secretion, and TGF-β1, Epo, and TGF-β2 inhibited IL-1β–stimulated IL-8 secretion in tsFHI cells.

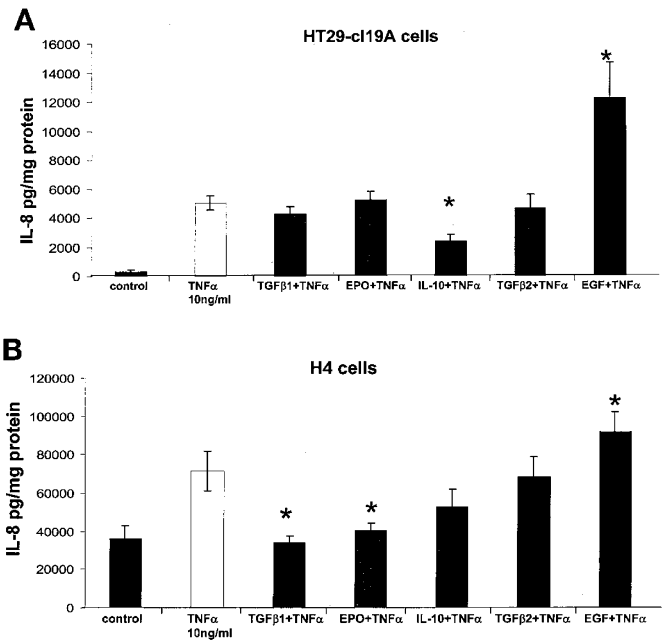

Figure 2. Effect of the human milk factors on TNF-α–stimulated IL-8 secretion by human IEC. Cells were pretreated with one of five human milk factors for 24 h before stimulation with TNF-α (10 ng/mL for 6 h). IL-8 was measured by ELISA and compared with measured protein concentrations from cell lysates. Findings are presented ± SEM; $n \geq 4$. (*a*) Results in the transformed adult colonic cell line HT29-cl19A. *Statistical significance at $p < 0.05$ for inhibition by IL-10 and accentuated stimulation by EGF of TNF-α–induced IL-8 secretion compared with stimulation with TNF-α alone, without a human milk factor pretreatment (□). (*b*) Results in the nontransformed fetal small IEC line, H4. *Statistical significance at $p < 0.05$ for inhibition by TGF-β1 and Epo and accentuated stimulation by EGF of TNF-α–induced IL-8 secretion compared with stimulation with TNF-α alone, without a human milk factor pretreatment (□).

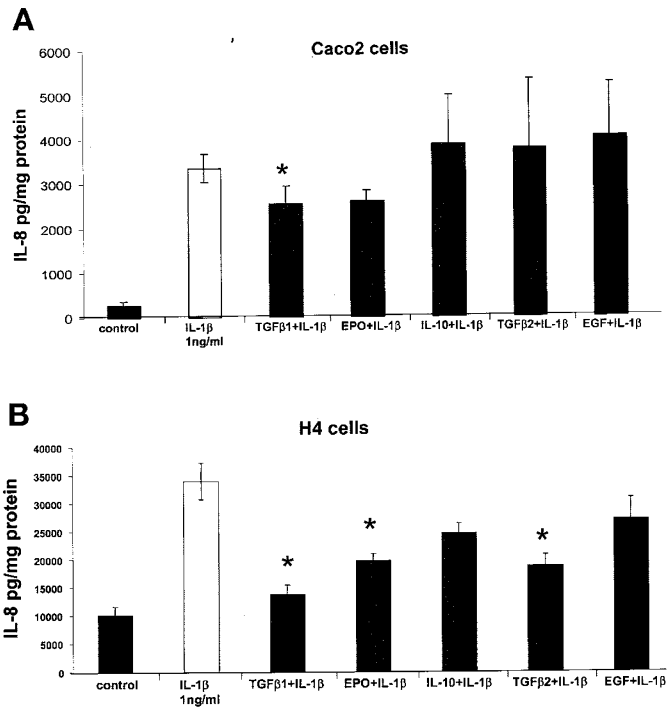

Figure 3. Effect of the human milk factors on IL-1β–stimulated IL-8 secretion by human IEC. Cells were pretreated with various factors for 24 h before stimulation with IL-1β (1 ng/mL for 24 h). IL-8 was measured by ELISA and compared with measured protein concentrations from cell lysates. Findings are presented ± SEM; $n \geq 4$. (*a*) Results in the transformed adult colonic cell line Caco2. *Statistical significance at $p < 0.05$ for inhibition by TGF-β1 of IL-1β–induced IL-8 secretion compared with stimulation with IL-1β alone, without a human milk factor pretreatment (□). (*b*) Results in the nontransformed fetal small IEC line H4. *Statistical significance at $p < 0.05$ for inhibition by TGF-β1, Epo, and TGF-β2 of IL-1β–induced IL-8 secretion compared with stimulation with IL-1β alone, without a human milk factor pretreatment (□).

DISCUSSION

These studies provide the first direct *in vitro* evidence of the anti-inflammatory effects of specific factors found in human milk on human fetal IEC. Although cell culture findings have limitations and need to be confirmed in *in vivo* models before findings are extrapolated to a disease entity, these findings in human cell culture models suggest a potentially important aspect of the protective effect of human milk against NEC. Although these factors are normally presented to the intestine in combination with the variety of factors found in human milk, these data represent an important first step toward understanding the actions *in vitro* of isolated growth factors that may potentially be useful as therapeutic agents. One difficulty in studying NEC at the IEC level has been the lack of an appropriate human model. Although animal models exist to study this disease as a whole, it is difficult to separate out effects specifically on the intestinal epithelium. IEC lines that have previously been used include rat small intestinal cell lines such as IEC-6 or human adult, colonic, transformed cell lines such as T84, Caco2, and HT29, even though NEC is a disease of the small intestine of preterm infants. H4 and tsFHI cell lines are derived from human fetal small intestine and may therefore be more representative of the susceptible preterm intestinal epithelium (30). We thus choose to compare effects in these cell lines to those of traditionally studied HT29-cl19A and Caco2 cells, which are considered to have characteristics of mature enterocytes at confluence (29, 45–47).

We have previously hypothesized that an immature gastrointestinal tract places a preterm infant at higher risk for NEC (7). Although many risk factors have been noted for NEC, including feeding patterns, amino acid profiles, and ischemia, the primary risk factor is prematurity (48–50). The pathophysiology of NEC is poorly understood and complex; thus, we have chosen to focus on the risk factor of prematurity and immaturity of the preterm intestine. One aspect of this immaturity is an exaggerated inflammatory response by IEC to microbial interaction that can produce a cascade resulting in systemic shock and death. In our studies, IL-8 secretion was induced by either TNF-α or IL-1β. Both of these mediators are known to be elevated in infants with NEC and are strong endogenous inflammatory stimulants *via* the activation of nuclear factor κB (NF-κB) leading to the increased production of many cytokines, one of which is IL-8 (16, 51–53). Our data demonstrate that fetal IEC have an increased TNF-α– and IL-1β–stimulated IL-8 production compared with adult enterocytes. As shown in Fig. 1, IL-8 secretion in the fetal enterocyte cell lines H4 and tsFHI at either 32°C (transformed) or 37°C (nontransformed) was significantly increased compared with the transformed adult colonic cell lines HT29-cl19A and Caco2. Thus, the difference is not completely explained by transformed *versus* nontransformed. This finding is consistent with that from a previous publication from our laboratory demonstrating increased lipopolysaccharide/butyrate and IL-1β–stimulated IL-8 secretion in fetal IEC and organ culture compared with adult IEC and organ culture of intestinal tissue from older children (2). Importantly, this publication demonstrated that the H4 *versus* Caco2 cell line comparison is comparable to fetal *versus* older child tissue organ culture comparison, validating use of our cell culture model for the study of development of inflammatory responses in intestinal epithelial cells. Our data also potentially suggest that the immature intestine has an elevated baseline inflammatory state.

The pathophysiology of NEC is not completely understood, and therapy is currently only supportive. Means of preventing this disease or treating it in its early stages is needed. Human milk has been shown to be protective, although the mechanism of this protection is unknown and likely multifactorial (10). Thus, we next investigated the effect of specific factors found in human milk on stimulated IL-8 secretion in IEC. Five factors found in human milk were tested—TGF-β1, TGF-β2, Epo, IL-10, and EGF. These factors were selected because they had previously been shown to have anti-inflammatory effects in other model systems and/or to have a potentially protective role in NEC (24–28).

TGF-β1 and Epo inhibited TNF-α–stimulated IL-8 secretion to near control levels in human fetal IEC. This effect was not seen in the adult cell line HT29-cl19A. Similarly, TGF-β1 and Epo significantly inhibited IL-1β–stimulated IL-8 secretion in human fetal IEC, to a greater degree than in Caco2 cells. In addition, TGF-β2, the predominant TGF-β isoform in human milk and amniotic fluid, inhibited IL-1β–stimulated IL-8 secretion exclusively in the human fetal IEC cell line H4. These potentially beneficial effects are in addition to other protective effects on the intestine demonstrated by these growth factors in other studies.

TGF-β maintains the integrity of the gastrointestinal barrier and furthermore has been shown to stimulate migration of cells across an injured epithelial monolayer, suggesting a role in restitution of the epithelium after injury (54). It is also an anti-inflammatory cytokine, as TGF-β null mice survive during milk feeding but die after weaning from an extensive inflammatory disorder (25). The role of Epo has been less well characterized in the intestine, although Epo receptors have been demonstrated on human fetal and postnatal intestine. Epo has been shown to decrease TNF-α–induced IEC apoptosis (35). Interestingly a retrospective study of preterm infants showed a decreased incidence of NEC in infants given Epo compared with infants who did not receive Epo (27). Human milk may provide necessary factors for the preterm infant in the extrauterine environment in the same way that amniotic fluid provides for the fetus in the uterine environment (55). Indeed, other preliminary unpublished observations from this laboratory have indicated that incubating H4 cells with amniotic fluid decreases TNF-α– and IL-1β–stimulated IL-8 secretion and that this effect is blocked by anti–TGF-β and anti-Epo antibodies, again suggesting that these are potentially important anti-inflammatory factors for the fetal intestine (E. Claud, unpublished observations). These studies are ongoing.

The mechanism by which IL-8 secretion was decreased is not clear. In IEC, after receptor activation, TNF-α and IL-1β can induce IL-8 secretion *via* pathways that use various adaptor molecules leading to NF-κB activation. Thus, the studied factors could alter receptor expression or induce maturation of a signal transduction pathway. Alternatively, an immature pathway may exist in the H4 cell, which accentuates IL-8 production, but is turned off with maturation of the intestinal

epithelium. Another possibility is that with maturation an alternate down-regulatory pathway that results in steps that inhibit NF-κB activation develops. Bocker *et al.* (42) demonstrated that differentiation of IEC selectively inhibits NF-κB activation in response to IL-1β, which may represent an important regulatory mechanism. Studies of receptor expression and postreceptor signaling pathways in the adult and fetal cell lines are currently under investigation in our laboratory. It is not surprising that transformed cells are less responsive to growth factors such as TGF-β, as unregulated proliferation without differentiation is often a hallmark of carcinoma cells. Furthermore, it is known that TGF-β postreceptor signaling is disrupted in many carcinoma cell lines as a result of alterations in Smad expression, the unique family of proteins that act as second messengers for the TGF-β family of receptors (56–59).

The next factor that we examined, IL-10, unexpectedly did not inhibit IL-8 secretion in H4 cells, although there was an inhibition of TNF-α–stimulated IL-8 secretion in HT29-cl19A cells consistent with findings from other studies. Because IL-10–deficient mice develop chronic enterocolitis and patients with Crohn's disease have shown improvement when treated with IL-10, we expected an anti-inflammatory effect (26, 36, 60). A study comparing preterm to term infants with lung disease showed that preterm infants had minimal IL-10 protein or mRNA despite comparable inflammation to term infants, specifically suggesting a developmental difference in the role of IL-10 and inflammation (61). The authors suggested that immature preterm infants are unable to control the inflammatory process as effectively as more mature term infants, which may explain why in the first 96 h of life, neutrophil counts and IL-8 expression decrease in term but not preterm infant bronchial lavage samples. They speculate that this may explain why preterm infants develop chronic lung disease, whereas term infants do not. We wondered whether a comparable immaturity of IL-10 production might lead to an increased inflammatory response in the preterm intestine. Our data suggest that in immature IEC, IL-10 does not directly inhibit TNF-α– or IL-1β–induced IL-8 secretion. IL-10 may affect cells other than epithelial cells, such as intestinal macrophages, or possibly affect epithelial cells indirectly *via* a two-step process. Alternatively, the absence of an IL-10 response with H4 cells may represent a developmental lack of response. A difference in receptor expression or postreceptor signaling pathways with maturation may explain why adult cells do have a decreased IL-8 production with IL-10 pretreatment but immature IEC do not.

Perhaps the most surprising finding was the increased IL-8 secretion when cells were pretreated with EGF before TNF-α stimulation. EGF has been studied extensively in the intestine. EGF receptors are present throughout the gastrointestinal tract on the basolateral membrane, which may be more accessible in the preterm intestine with increased permeability (62, 63). It promotes intestinal growth and stimulates intestinal repair (64, 65). EGF levels are decreased in the saliva, serum, and urine of patients with NEC *versus* nondiseased age-matched controls, and a recent study showed a decreased incidence and severity of NEC in a rat model treated with EGF (28, 66). There has also been a case report of an infant with enterocolitis improving after treatment with i.v. EGF (67). Thus, we hypothesized that EGF would decrease stimulated IL-8 secretion.

The increased production of IL-8 in response to EGF is not well explained. A recent paper described a pathway in IEC used by Gram-positive bacteria, which stimulates NF-κB *via* the EGF receptor (68). This pathway is separate from the toll receptor linked pathways used by Gram-negative organisms to up-regulate production of inflammatory mediators *via* NF-κB and may represent a mechanism by which EGF can up-regulate IL-8 production. Alternatively, EGF may up-regulate receptor number as described for the IL-1 receptor in other cell types (69). It is possible that other factors in human milk modulate this EGF response so that it is not proinflammatory when introduced to the intestine as part of complete human milk; however, these findings should prompt caution. Additional studies are required before using this factor as an isolated therapeutic agent to prevent NEC in humans.

CONCLUSIONS

In conclusion, we have demonstrated that H4 cells have a greater IL-8 response to both TNF-α and IL-1β than HT29-cl19A or Caco2 cells. As a fetal small intestinal nontransformed cell line, H4 may be a better model for the study of enterocyte responses as they relate to NEC. In H4 and nontransformed tsFHI cells (37°C), TGF-β1 and Epo decreased both TNF-α– and IL-1β–induced IL-8 secretion and may have therapeutic implications, whereas EGF increased TNF-α–stimulated IL-8 secretion. Outlining the mechanisms behind these effects is the focus of ongoing studies.

We have previously suggested that the injury in NEC may result from colonization of the uniquely susceptible premature intestine with pathogenic bacteria resulting in an exaggerated inflammatory response (7). TGF-β and Epo potentially decrease this exaggerated inflammatory response specifically in human fetal IEC. This effect may partially explain the protective effect of human milk in necrotizing enterocolitis.

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