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Cytokines and growth factors in the developing intestine and during necrotizing enterocolitis

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Abstract

Cytokines and growth factors play diverse roles in the uninflamed fetal/neonatal intestinal mucosa and in the development of inflammatory bowel injury during necrotizing enterocolitis (NEC).

During gestational development and the early neonatal period, the fetal/premature intestine is exposed to high levels of many “inflammatory” cytokines and growth factors, first via swallowed amniotic fluid *in utero* and then, after birth, in colostrum and mother’s milk. This article reviews the dual, seemingly counter-intuitive roles of cytokines, where these agents play a “trophic” role and promote maturation of the uninflamed mucosa, but can also cause inflammation and promote intestinal injury during NEC.

Keywords

NEC; Intestinal injury; Cytokines; Growth factors; Prevention

Introduction

Cytokines and growth factors have a complex, multifaceted role in intestinal development and the pathogenesis of NEC. The fetal/premature intestine is exposed to high levels of many growth factors and so-called “inflammatory” cytokines, first via swallowed amniotic fluid (AF) *in utero* and then, after birth, in colostrum and mother’s milk. In addition, the midgestation intestine constitutionally expresses many of the same cytokines at levels much higher than in the adult intestine. These data indicate important physiological and developmental roles of these cytokines/growth factors, and indeed, many preclinical and early clinical studies show a protective role of enterally administered cytokines against NEC. However, the onset of NEC also triggers a prominent mucosal and systemic cytokine response, and there is extensive preclinical evidence informing about the pathological

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footprint of these cytokines and supporting suppression of cytokine expression/signaling to attenuate NEC-like injury. This article reviews these dual, seemingly counter-intuitive roles of cytokines, where cytokines may play a “trophic” role and promote maturation of the uninfamed mucosa, but can also cause harm and promote intestinal injury during NEC. In the following sections, we have defined “cytokines” in a generic sense—as small, peptide extracellular-signaling molecules encompassing diverse subgroups: chemokines, colony-stimulating factors, growth factors, interleukins, interferons, and tumor necrosis factors. In addition to information from our own studies, we performed a literature search using the databases PubMed, EMBASE, and Scopus. To minimize bias, keywords from PubMed’s Medical Subject Heading thesaurus were shortlisted prior to the actual search and combined with text words likely to be used in titles and abstracts.

Cytokines in the developing intestine

The developing intestine expresses many “pro-inflammatory” cytokines at levels much higher than in the adult intestine (Fig. 1). These cytokines originate in the enterocytes and macrophages, which express high levels of several innate response receptors and transcriptional regulators such as the nuclear factor-kappa B (NF- κ B) and the activator protein 1 (AP-1) complex.^{1–7} In addition, the fetal intestine is developmentally-deficient in anti-inflammatory signaling mediators such as the single Ig interleukin-1-related receptor (SIGIRR), interleukin-1 receptor-associated kinase (IRAK)-M, tumor necrosis factor- α -induced protein 3 (TNFAIP3), and the Toll-interacting protein (TOLLIP).⁸ Some reports also show low levels of the inhibitor of κ B (I κ B).⁹

In addition to the high endogenous cytokine production, the fetal intestine is also exposed to the cytokines and growth factors present in AF. Starting at 8–11 weeks’ gestation, the fetus ingests increasing amounts of AF that may reach 500 mL/d in the 3rd trimester.¹⁰ Using a fetal sheep model, Trahair et al.^{11,12} showed that esophageal ligation (to block AF ingestion) during midgestation caused mucosal atrophy, villus blunting, and enterocyte abnormalities, such as effacement of microvilli, glycogen accumulation, cellular extrusion, and lysosomal dysgenesis. Consistent with these findings, human neonates with congenital intestinal obstruction also show villus blunting and shallow, poorly organized crypts distal to the site of obstruction.¹³ In Trahair’s model, the mucosal defects were gradually reversed following the removal of esophageal ligatures and restitution of AF ingestion but not by the infusion of Ringer’s lactate, indicating that the “trophic” effects of AF were secondary to the bioactive molecules present in AF and not merely due to the flow of fluid through the gut lumen.¹⁴

After birth, the neonatal intestine continues to be exposed to high concentrations of cytokines in colostrum and maternal milk. Colostrum and milk, which are apocrine secretions, contain a wide range of cytokines and other intracellular proteins involved in innate/adaptive immunity.¹⁵ Table 1 summarizes current evidence on cytokines expressed in the fetal/neonatal intestine, AF, and colostrum/milk, and their known functions in intestinal development.^{16–38} With an ever-increasing number of such agents, criteria have been suggested to identify factors involved in intestinal growth/development^{39,40}: (1) expression in milk or in the intestinal mucosa at the time of growth, (2) expression of cognate receptor(s) during these periods, (3) reduction of growth *in vivo* with removal of the relevant

growth factor, (4) precocious growth/maturation effects by early administration, and (5) growth/maturation effects *in vitro*.

Fetal enterocytes express the receptors for diverse cytokines, chemokines, epidermal growth factor (EGF), hepatocyte growth factor (HGF), FGF, insulin-like growth factors, chemokines, erythropoietin, and granulocyte-colony stimulating factor (G-CSF). Supporting evidence from *in vitro* and animal models suggests that many of these agents can survive digestion and increase enterocyte proliferation, migration, and differentiation, prevent apoptosis, and promote mucosal restitution in the small intestine.^{40–43}

Can enterally administered cytokines prevent NEC?

Many investigators have asked whether the premature intestine, which is deprived of the “trophic” influence of AF-borne cytokines due to preterm delivery, can be protected against NEC by enteral administration of recombinant cytokines or mother’s own AF. In these efforts, several cytokines have been tested: EGF, heparin-binding epidermal growth factor (HB-EGF), erythropoietin, G-CSF, glucagon-like peptide (GLP-2), TGF- β_2 , and HGF.^{7,42,44–47} The evidence supporting the use of each agent is summarized later.

EGF supplementation has been shown to protect rat pups against NEC-like injury. In preclinical models, EGF promotes the maturation of the intestinal barrier, reduces enterocyte apoptosis, and downregulates the inflammatory response associated with NEC-like injury.^{47–51} The physiological importance of EGF in intestinal homeostasis is supported by observations that EGF receptor (EGFR) knockout mice develop hemorrhagic necrosis of the intestine similar to neonatal NEC.⁵¹ In human studies, premature infants with low salivary EGF during the first postnatal week were noted to be at increased risk of NEC.⁵² Recently, Good et al.⁵³ demonstrated that enteral administration of AF to mouse pups reduced the severity of NEC-like injury, and showed that the protective effects of AF were due to activation of EGFR, which in turn, suppressed Toll-like receptor 4-mediated signaling in enterocytes.

HB-EGF is a member of the EGF family that is expressed as a membrane-anchored glycoprotein on macrophages and is then released in soluble form through ectodomain shedding.⁵⁴ Enterally administered HB-EGF can prevent NEC-like injury in rodent models by decreasing adhesion molecule expression and neutrophil infiltration; promoting alternative activation of macrophages (M2) to attenuate the inflammatory responses; suppressing inducible nitric oxide (NO) synthase, and the generation of NO and reactive oxygen species; improving gut microvascular perfusion; preserving intestinal stem cells; maintaining crypt cell proliferation; and protecting the intestinal barrier function.^{46,55–58}

Two hematopoietic growth factors, erythropoietin and G-CSF, have shown benefit in NEC models. Erythropoietin was first recognized for its anti-apoptotic action on erythrocyte progenitors, but it has been subsequently found to have other important non-erythropoietic functions as a neuroprotectant and as an anti-apoptotic factor in the developing intestinal mucosa.^{59,60} Enteral administration of recombinant erythropoietin was protective in the rat NEC model.^{61–65} Studies show numerous protective effects: decreased lipid peroxidation,

inhibition of NO generation, preservation of the intestinal barrier by augmenting the *zonula occludens* proteins, and decreased NEC-related autophagy and apoptosis in enterocytes.^{61–65} Similarly, G-CSF can also protect against NEC. G-CSF was first recognized for its capacity to support the clonal maturation of neutrophils, but it subsequently was found to have important developmental actions on the fetal small bowel mucosa.^{41,66} In a pilot study,⁴² preterm infants with mild NEC were assigned to receive recombinant G-CSF ($n = 18$) or placebo ($n = 10$). Encouragingly, none of the infants in the G-CSF group showed a clinical progression, whereas 5 control infants progressed to advanced NEC. G-CSF-treated infants also showed earlier resolution of clinical and radiological findings of NEC. In support of these data, G-CSF has also been shown to be protective in the rat model of NEC.⁶⁷

Christensen et al.^{41,68} combined recombinant erythropoietin and G-CSF in a sterile, crystalloid solution with electrolyte concentrations similar to amniotic fluid, for enteral feeding. They specifically used erythropoietin and G-CSF as these were the only 2 growth factors available in the US as FDA-approved medications. In this solution, erythropoietin and G-CSF were added to achieve 10-times the normal concentrations of these growth factors in AF (as the intended feeding volumes were only a tenth of the AF volumes typically ingested *in utero*—20 mL/kg/day rather than 200 mL/kg/day). Both agents have been shown to be stable in electrolyte solutions, human milk, and infant formula. So far, the feasibility of administration in premature infants and its safety has been shown in 6 clinical trials with 120 enrolled infants.⁶⁹ Early controlled studies also suggest efficacy in preventing feeding intolerance and also in treating established feeding intolerance. There is a need for larger, randomized studies to examine efficacy for prevention of NEC or the treatment of feeding intolerance.

GLP-2 is released during post-translational proteolytic cleavage of proglucagon.⁷⁰ Enteral GLP-2 promotes splanchnic perfusion, mucosal growth, and intestinal function. GLP-2 and its analogs have also been used to treat short bowel syndrome.⁷¹ In the preterm piglet model of NEC, GLP-2 did not prevent NEC despite some reduction in mucosal injury and increased trophic effect.⁷² However, a recent report shows that GLP-2 may protect against intestinal injury in the rat NEC model.⁴⁵

Jain et al.^{44,53,73} have examined whether enteral administration of mother's own AF to the neonate could protect against NEC. We showed that supplementation of formula feedings with AF prevented NEC-like injury in rat pups. Interestingly, HGF was the most abundant cytokine we measured in rat AF (median = 4892, range: 2560–7030 pg/mL), and consistent with these findings, we detected similar high concentrations of HGF in human AF (median = 8354 pg/mL, range: 108–18,654). In our study, HGF promoted enterocyte migration, proliferation, and survival. Although HGF acts mainly via ligation of its cognate receptor, *c*-met, we also detected transactivation of receptor tyrosine kinases in the EGF receptor family (ErbB2 and ErbB3), FGFRs, and VEGFR2. This cross-talk between *c*-met and other receptors could explain the observations of Good et al.,⁵³ who demonstrated that the EGFR was required for AF effects.

In our laboratory, we have shown that enteral administration of recombinant TGF- β_2 can protect murine pups against NEC-like injury by suppressing macrophage cytokine

production and inhibiting the mucosal inflammatory responses.⁷ Human milk contains biologically-important amounts of TGF- β_2 .⁷⁴ Orally-ingested TGF- β promotes gut barrier function, immune tolerance, and mucosal repair in the neonatal gastrointestinal tract.^{75–78} TGF- β_2 is the primary determinant of TGF- β bioactivity in the preterm intestine (unlike plasma, where TGF- β_1 is the dominant isoform), but is developmentally regulated and is expressed at low levels in the midgestation intestine. Orally-ingested milk-borne TGF- β_2 could possibly patch this deficiency, but there are several physiological constraints: (1) preterm human milk contains a large pool of TGF- β_2 , but most of it exists in a latent form or sequestered by chondroitin sulfate proteoglycans^{79,80} and (2) mothers who give birth to growth-restricted preterm infants may produce very low levels of TGF- β_2 in milk.⁸¹ This deficiency is further accentuated in infants receiving formula that contains no TGF- β_2 . In this context, our findings of the protective effects of enterally administered TGF- β_2 assume importance. Further study is needed to address the logistics of clinical use.

Another study showed that enterally administered TGF- β_1 can also protect rat pups against NEC-like injury.⁸² The authors showed that TGF- β_1 pretreatment of epithelial cells prevented NF- κ B activation and consequent transcription of inflammatory cytokines IL-6 and IL-8/CXCL8. These TGF- β_1 effects appeared to be mediated through the canonical Smad signaling pathway.

What is the role of cytokines in the pathogenesis of NEC?

The cytokine response during NEC has received significant scientific attention—cytokines are attractive therapeutic targets in view of well-documented effects on leukocyte recruitment/activation, barrier function, and apoptosis. In addition, the convenience of measurement in peripheral blood makes cytokines an excellent choice as candidate biomarkers of the primary disease and associated inflammation. At the tissue level, surgically-resected specimens of NEC consistently show increased mRNA expression of interleukin (IL)-1B, IL8/chemokine CXC-motif ligand (CXCL)-8, and tumor necrosis factor (TNF).^{83–85} Many existing clinical studies on tissue expression of cytokines in human NEC are constrained by the limited selection of analytes. To obtain more comprehensive information on cytokine expression in NEC, we analyzed microarray data from surgically-resected specimens of bowel affected by NEC vs. uninflamed neonatal intestine resected for intestinal obstruction/during ileostomy repair ($n = 4$ each; Fig 2). These specimens have been previously described⁸⁶ and the microarray data were stored in the public database Gene Expression Omnibus.⁸⁷ In these tissue samples, NEC increased the expression of IL1A, IL1B, IL6, IL10, TNF, CXCLs 1, 2, 3, 5, 8, and 12; CCLs 2, 3, 4, 7, 8, and 20; hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), thymic stromal lymphopoietin (TSLP), platelet factor 4, tumor necrosis factor superfamily member 9 (TNFSF9), transforming growth factor (TGF)-B1, TGFB3, and vascular endothelial growth factor (VEGF)-A. NEC downregulated TNFSF10/TNF-related apoptosis-inducing ligand (TRAIL), and the chemokine C-motif ligand 2 (XCL2). There was a trend towards decreased EGF expression that did not reach statistical significance.

The onset of NEC is also associated with increased plasma cytokine concentrations. Although some of these circulating cytokines likely originate in the inflamed bowel, plasma

cytokine concentrations may not always mirror affected tissues and may also be derived from alternative cellular sources such as circulating platelets and leukocytes.^{88,89} However, despite the lack of specificity, plasma measurements remain attractive as a diagnostic tool because of the low likelihood of harm and simpler logistics. In the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Cytokine Study, we compared serial blood spot cytokine measurements from 104 extremely-low-birth-weight infants with NEC vs. 893 who did not.⁹⁰ NEC was associated with elevated IL-6, IL-8/CXCL8, IL-10, IL-18, CCL2, CCL3, neurotrophin-4, and C-reactive protein.⁸⁹ Several other studies have also recorded elevated serum IL-6, IL-8/CXCL8, IL-10, and IL-18 concentrations during NEC.^{83,87,91–95} IL-6 has a short half-life and may be detectable only for short periods after onset of NEC,⁹⁶ whereas IL-8/CXCL8 levels show sustained elevation⁸⁷ and correlate with the extent of NEC (*NEC totalis*), the need for surgical resection of bowel, and higher 60-day mortality.^{91,94,97–99}

To determine the contribution of cytokines to the inflammatory cascades in NEC, we used a microarray-based approach.¹⁰⁰ In this study, we sought transcriptional networks likely to be activated in human NEC and in a murine NEC model, where we used 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) to induce inflammatory intestinal injury in 10-day-old murine pups.¹⁰¹ Interestingly, bacterial lipopolysaccharide (LPS) was predicted to be the top upstream transcriptional regulator in both human NEC and TNBS-mediated murine intestinal injury. LPS was predicted to induce IL-1 β , IL-6, TNF, and IFN- γ , which, in turn, activated inflammatory transcription factors nuclear factor-kappa B (NF- κ B), signal transducer and activator of transcription (STAT)-3, *c-Jun*, and the CCAAT/Enhancer Binding Protein (C/EBP)- β . In this pathophysiological model, cytokines play a central role in NEC pathogenesis. The identification of LPS as the top upstream regulator is consistent with increasing evidence that infants who develop NEC display a microbial dysbiosis (that antedates NEC) with over-representation of Gram-negative bacteria.^{102,103}

Can cytokines be used as biomarkers for prediction/early detection or therapeutic targets in NEC?

In the NICHD Cytokine Study, ELBW infants who developed NEC had lower circulating TGF- β_1 levels than controls since the time of birth.⁸⁹ The diagnostic accuracy was relatively modest – blood TGF- β_1 concentrations <1380 pg/mL on the first postnatal day predicted future onset of NEC with 64% accuracy. Still, the ability of blood TGF- β_1 to estimate the risk of NEC on day 1 was surprising because it indicated that the risk-stratification for NEC may start early, possibly *in utero*, well before the hypoxic-ischemic events of the early neonatal period, or postnatal microbial colonization and dysbiosis in at-risk infants.¹⁰⁴ The association of low blood TGF- β_1 levels with NEC was consistent with our earlier finding that preterm infants at risk of NEC show limited TGF- β bioactivity in their intestine.⁷ The reasons for low blood TGF- β_1 levels prior to the onset of NEC were unclear, although increased peripheral uptake¹⁰⁵ is a possibility in at-risk infants with an accentuated deficiency of TGF- β in the intestine due to low-grade mucosal inflammation or genetic/epigenetic factors.¹⁰⁶

In contrast to the prominent role of cytokines in NEC-related inflammatory response, cytokines may not have a major pathophysiological contribution during the period leading up to the onset of NEC. In the NICHD Cytokine Study, NEC was associated with male gender, non-Caucasian/non-African-American ethnicity, history of sepsis, low blood TGF- β_1 and IL-2, and higher IL-8/CXCL8 levels.⁸⁹ Even though infants who went on to develop NEC showed important differences in blood cytokine levels compared to controls in the early neonatal period, it was the clinical parameters, not cytokines, that accounted for most of the risk of NEC in our statistical models. These findings suggest that the association of these cytokines with NEC may be driven by the clinical variables, rather than *vice versa*.

Plasma concentrations of several cytokines, such as IL-8/CXCL8 and IL-6, rise soon after the onset of NEC and could be used for early diagnosis (*vide supra*). When compared to infants with feeding intolerance related to prematurity itself or other non-infectious causes, plasma cytokine concentrations show a high degree of specificity and correlate with disease severity.^{91,94,97–99} However, the diagnostic accuracy becomes less impressive when compared with infants who have sepsis-related ileus.^{91,94,97–99}

For treatment of NEC, anti-TNF antibodies have shown promise in the rat NEC model.^{107,108} Anti-TNF treatment was effective in reducing enterocyte apoptosis, intestinal and hepatic inflammatory responses, and tissue damage in the ileum.¹⁰⁷ These findings were confirmed in a second, independent study.¹⁰⁸

Conclusions

In above sections, we have summarized the evidence on the diverse roles of cytokines in the uninflamed neonatal intestine and during NEC. The pathophysiology of NEC is complex and poorly understood, and therefore, our understanding of cytokines in this disease also remains incomplete. Cytokines are attractive therapeutic targets and there is potential for benefit from newer anti-cytokine treatments that show great promise in inflammatory disorders in adult patients. However, because cytokines also play important developmental roles, the pros and cons of each treatment need careful evaluation before use in premature infants.

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Fig. 1.

Human fetal intestine expresses several inflammatory cytokines and transcriptional regulators at higher levels than in the adult intestine. Bar diagram (means + standard errors) show fold change in mRNA expression (normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase). Broken line represents mRNA levels in the adult intestine. De-identified human fetal intestine was obtained at medical terminations ($n = 8/\text{group}$) and the adult intestine from subjects undergoing bariatric surgery ($n = 5$). The study was deemed to be “no human subjects” research by the Institutional Review Board. Crossing-threshold cycle numbers in the 2 groups were compared by Mann–Whitney U test. $*P < 0.05$.

Fig. 2.

Cytokine expression in surgically-resected bowel specimens of NEC and uninflamed human neonatal intestine. Deidentified microarray data from NEC ($n = 4$) and uninflamed neonatal intestine (tissue resected for intestinal obstruction or during ostomy repair; $n = 4$) were downloaded from the public database Gene Expression Omnibus and analyzed using the software program Partek Genomics Suite 6.6 (Partek, St. Louis, MO). Boxplots show means $\pm$ standard errors of the expression intensity of the cytokine probe sets. Tissue samples of NEC are depicted in red squares, whereas the uninflamed neonatal intestine is shown as green triangles. Groups were compared by analysis of variance. $*P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Effect of various cytokines on intestinal mucosal development.

	Expression in fetal intestine	Presence in amniotic fluid	Presence in human colostrum and milk	Resistance to gastric digestion	Crypt enterocyte proliferation	Villus growth	Enterocyte differentiation (mucosal enzymes)	Mucosal repair	Intestinal growth	Enterocyte apoptosis	Macrophage differentiation	References
Epo	+	+	+	+	+	+		+	+	-		59-65
G-CSF	+	+	+	+								66,67
IL-2	+	±	+					+				36
IL-4	+	+	+							-		35
IL-6	±	+	+				+			-		34,40
IL-7	+	+	+							-		33
IL-11	±	±	±									32
IL-12	+	-	+									40
IL-18	+		+									17
IL-10	+	-	+						+			40
TNF	+	+	+							+		40,77
TNFSF10	+	+	+							+		29
IL-1α		+	+		+	+	+			-		30,40,77
IL-1β		+	+		+	+	+			-		30,40,77
EGF	+		+	+	+			+	+	-		47-
HB-EGF		+	+		+		+	+		-		46,54-58
TGF-α	+	+	+		+			+		-		40
IGF-I ₁	+	+	+		+			+	+			20,21
IGF-II												
HGF	+	+	+		+			+				43,44
VEGF	+	+	+								+	25
FGF-1	+	+	+		+	+	+	+	+			26
TGF-β ₁	+	+	+				+				+	7,82
TGF-β ₂	+	+	+				+				+	77-81
CXCL1	+	+	+									38,44
CXCL2	+	+	+									38,44
CXCL5	+	+	+									38,44

	Expression in fetal intestine	Presence in amniotic fluid	Presence in human colostrum and milk	Resistance to gastric digestion	Crypt enterocyte proliferation	Villus growth	Enterocyte differentiation (mucosal enzymes)	Mucosal repair	Intestinal growth	Enterocyte apoptosis	Macrophage differentiation	References
CXCL12		-	+									38,44
IL-8/ CXCL8	+	+	+	+	+					-		1,27,38,44
CCL2		+	+									44
CCL4		+										44
CCL5		+	+									44
TFF-3	+		+	+	+			+		-		28

^a + = Increased; - = decreased; ± = conflicting evidence; blank space denotes lack of information.

^b Epo = erythropoietin; G-CSF = granulocyte-colony stimulating factor; IL = interleukin; TNF = tumor necrosis factor; TNFSF10 = TNF superfamily member 10; EGF = epidermal growth factor; HB-EGF = heparin-binding EGF; TGF = transforming growth factor; IGF = insulin-like growth factor; HGF = hepatocyte growth factor; VEGF = vascular endothelial growth factor; FGF = fibroblast growth factor; CXCL = chemokine CXC-motif ligand; CCL = chemokine CC-motif ligand; IFN = interferon; TFF = trefoil factor.