Amniotic fluid-borne hepatocyte growth factor protects rat pups against experimental necrotizing enterocolitis

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NECROTIZING ENTEROCOLITIS (NEC), an inflammatory bowel necrosis of preterm infants, is a leading cause of death among neonates born before 32 wk of gestation or with a birth weight <1,500 g (20, 27). Although the etiology of NEC remains unclear, epidemiological studies show an association with diverse risk factors such as maternal chorioamnionitis, perinatal asphyxia, indomethacin therapy, viral infections, and blood transfusions (20). Current pathophysiological models suggest that NEC occurs when altered/disrupted epithelial barrier in the preterm intestine allows luminal bacteria to translocate across the epithelial barrier into the lamina propria, triggering a severe mucosal inflammatory response and tissue damage (21).

During intrauterine development, the fetus ingests progressively large volumes of amniotic fluid, which contains several cytokines and growth factors known to promote gut mucosal development (19). In the third trimester, the human fetus swallows nearly 550 ml/day amniotic fluid (29, 40). We hypothesized that the preterm intestine, which is deprived of this trophic influence of amniotic fluid and its constituent bioactive factors owing to preterm delivery, can be protected against NEC by supplementation of enteral feeds with amniotic fluid. To investigate this hypothesis, we used an established neonatal rat model of NEC-like injury (8, 11) and compared intestinal injury in formula-fed animals vs. an intervention group that received formula feeds supplemented with amniotic fluid. We further investigated the mechanistic basis of our observations using rat intestinal epithelial cells (IECs) in vitro.

METHODS

Animals

All studies were approved by Institutional Animal Care and Use Committee at University of Texas Medical Branch, Galveston. NEC-like injury was induced by a previously described method (11). Briefly, timed-pregnant Sprague-Dawley rats (Harlan, Houston, TX) were delivered prematurely on day 21.5 (term gestation = 22.5–23 days) via cesarean section under isoflurane anesthesia. Newborn pups (10–13 per litter) were reared in Styrofoam cups lined with shredded tissue paper and kept in an incubator at 35.5°C and 70% humidity.

These pups were hand fed with formula (rat milk substitute) via a 3.5 French gauge umbilical venous catheter (Vygon, Montgomeryville, PA). Feeds were started at 0.2 ml from 30 min after birth, given every 4 h, and advanced by 0.05 ml every 12 h to a maximum of 0.3 ml per feed by postnatal day 4.

Rat Amniotic Fluid

Amniotic fluid was harvested on day 17–18 of pregnancy from timed-pregnant Sprague-Dawley rats (n = 5) by laparotomy and needle aspiration from the amniotic sacs. The procedure was performed on day 17–18 of pregnancy (and not later) to obtain late-gestation amniotic fluid and yet collect adequate amounts because the volume of amniotic fluid decreases with advancing gestation in rats. Endotoxin concentrations were measured by a commercially available limulus lysate assay (Pierce, Rockford, IL). All amniotic fluid samples were kept frozen in aliquots until the time of study, with care to avoid multiple freeze-thaw cycles.

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Human Amniotic Fluid

We also collected amniotic fluid from 30 women who underwent cesarean deliveries at ≥39 wk gestation. These samples were collected at University of Texas Medical Branch, Galveston, after approval by the local Institutional Review Board. Women with multiple gestation, major fetal anomalies, diabetes mellitus, or chorioamnionitis were excluded. All amniotic fluid samples were stored in aliquots and conditions were monitored according to the Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines (25).

Amniotic Fluid Cytokine Concentrations

Cytokine concentrations were measured by a magnetic bead-based multiplex assay (Bioplex array, Bio-Rad, Hercules, CA) that included interleukin (IL)-1α, IL-6, transforming growth factor (TGF)-β1, TGF-β2, monocyte chemotactic protein-1 (MCP-1)/CC-motif ligand-2 (CCL2), macrophage inflammatory protein-1α (MIP-1α)/CCL3, MIP-3α/CCL20, and tumor necrosis factor (TNF). Hepatocyte growth factor (HGF) and epidermal growth factor were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). HGF concentrations in human amniotic fluid were measured by specific immunoassays (Luminex, Austin, TX). These analytes were short listed on the basis of known gut epithelial effects and existing data on expression in amniotic fluid (1, 15, 19, 39). We had initially planned to include insulin-like growth factor (IGF)-I and IGF-II measurements, but finally decided not to pursue these analytes because rat amniotic fluid was limited in volume and because human amniotic fluid HGF-I and IGF-II levels reported in existing literature (39) were a log-fold lower than the concentrations of HGF we detected in our amniotic fluid samples.

Experimental NEC

Amniotic fluid supplementation. We divided 80 pups (delivered from 7 dams) into a formula-fed control group and an intervention group that received formula supplemented with 30% (vol/vol) amniotic fluid (30% dilution) chosen based on in vitro studies, where amniotic fluid-induced IEC migration/proliferation reached a plateau at 30%.

HGF supplementation. We divided 58 rat pups into HGF and control groups. The two groups received a total of 18 ng recombinant HGF or an equivalent amount of bovine serum albumin divided in all feeders over a day. HGF dose was based on the estimated amount of HGF received by a term rat fetus (20–25 ml·kg⁻¹·h⁻¹) ingested amniotic fluid, average weight of 5 g, and median HGF concentration in rat amniotic fluid = 5 ng/ml.

Experimental NEC. To induce NEC-like injury, rat pups were placed in a hypoxic environment (95% nitrogen) × 60 s followed by hypothermia (4°C × 10 min), starting at 2 h after birth and then every 12 h. Pups were observed for signs of illness such as abdominal distension, respiratory distress, and lethargy. Pups were euthanized upon development of signs of intestinal injury or at 96 h and the gastrointestinal tract was visually evaluated for discoloration, hemorrage, and distension. Tissues were fixed in 10% buffered formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin for microscopic evaluation. In some experiments, tissues were frozen for RNA extraction. Histopathological changes were scored by a blinded observer (A. Maheshwari) and graded on a five-point scale for the severity of mucosal injury (11): grade 0 (normal), no damage; 1 (mild), slight submucosal and/or lamina propria separation; 2 (moderate), moderate separation of submucosa and/or lamina propria and/or edema in submucosal and muscular layer; 3 (severe), marked separation of submucosa and/or lamina propria and/or severe edema in submucosa and muscular layers, regional villous sloughing; 4 (transmural necrosis), loss of villi and transmural necrosis. NEC was defined as ≥grade 2 injury.

Cytokine Expression

We used our previously described reverse transcriptase-quantitative PCR (RT-qPCR) protocol (2, 33) to measure cytokine expression in intestinal tissue. Primers were designed by use of the Beacon Design software (Bio-Rad, Hercules, CA) and are shown in Table 1. Data were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and groupwise comparisons were made by the 2⁻ΔΔCT method. CXC-motif ligand (CXCL)-2 and CXCL5 protein expression was confirmed by Western blots (polyclonal goat antibodies from R&D Systems).

Epithelial Cells

IEC6 cells are duodenal crypt epithelial cells derived from 18- to 24-day-old rat pups (ATCC, Manassas, VA). We grew these cells at passages 20–24 in Dulbecco’s modified Eagle’s medium containing 4 mM l-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and supplemented with 10% (vol/vol) fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C and 5% CO2 (27). Media were changed 24 h after plating and then every 72 h.

Immunocytochemistry. IEC-6 cells were immunostained by using our previously described protocol (33). Briefly, IEC6 cells were grown on glass coverslips and fixed with ice-cold 4% paraformaldehyde × 5 min. After blocking (Superblock reagent, Pierce, Rockford, IL) × 30 min, cells were incubated overnight with either polyclonal rabbit anti-c-met antibody or isotype control (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary staining was performed with Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen, Grand Island, NY). Nuclear staining was obtained with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Sigma, St. Louis, MO). Images were obtained by use of a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY).

Cell proliferation. To investigate amniotic fluid or HGF effects on IEC proliferation, IEC6 cells were plated in 96-well plates at a density of 1–2 × 10⁴ per well. After 24 h, culture media were changed to phenol red-free media containing 0–30% amniotic fluid or recombinant HGF (0–10 nM). In some experiments, we added 30% amniotic fluid along with excess monoclonal anti-HGF antibody (or isotype control; 25 μg/ml; R&D Systems). After 48 h, cells were labeled overnight with bromodeoxyuridine (BrdU) and cell proliferation was measured by use of a commercially available BrdU ELISA kit (Roche, Indianapolis, IN). Briefly, cells were fixed and treated with a peroxidase-conjugated mouse monoclonal anti-BrdU antibody. BrdU incorporation was quantified by adding tetramethyl benzidine solution (substrate) and measuring absorbance at 450 and 690 nm. In this assay, absorbance is related logarithmically to cell number.

Cell migration. IEC6 cells were cultured in 60 mm dishes to 80% confluence and cell migration was measured across wound cell monolayers as described by Ciacci et al. (9). After 24 h of incubation, cell monolayers were “wounded” in triplicate with a sterile 4-mm

Table 1. Primer sequences used for real-time reverse transcriptase-PCR

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<tr>
<th>mRNA</th>
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<th>Reverse Primer</th>
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<td>TNF</td>
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<td>ATCAAGCGGATGATCAATC</td>
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<tr>
<td>GAPDH</td>
<td>CGGTTGCTGTTGCTGCTGCTG</td>
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plastic edge and washed with phosphate-buffered saline, and the media were changed. The plates were then incubated with 0–30% (vol/vol) amniotic fluid. After 8–24 h, cells were fixed with 10% formaldehyde × 20 min, and then stained lightly with hematoxylin (Sigma, St. Louis, MO). Cells that had migrated past the wound edges were counted in four high-power fields (hpf) in each of the three lines.

Cell injury. IEC-6 cells were plated into 96-well plates (1–2 × 10^5/well) and allowed to attach for 24 h. Culture media were then changed to phenol red-free medium containing 0–30% amniotic fluid. Cellular injury was induced by treatment with TNF (25 ng/ml) + cycloheximide (CHI; 2.5 μg/ml) (22). In some experiments, cells were pretreated with amniotic fluid × 24 h and then exposed to TNF/CHI, whereas both amniotic fluid and TNF/CHI were added together in other experiments. Cell viability was evaluated 24 h after TNF/CHI treatment by use of the in vitro Toxicology Assay kit XTT (Sigma). In this assay, mitochondrial dehydrogenases in viable cells act on the carboxyanilide dye XTT, releasing an orange product into the medium. After incubation × 4 h, the wells were read spectrophotometrically at 450 nm. Negative and positive controls were set up per plate with medium alone and with cells not exposed to any cytotoxic agents, respectively.

Activation of phosphoinositide 3-kinase. Phosphoinositide 3-kinase (PI3K) p85 regulatory subunit and phospho-PI3K p85 (Tyr458) were measured in IEC6 cell lysates by Western blots (both antibodies from Cell Signaling Technology, Danvers, MA) (26). We also measured PI3K activity in cell lysates using a commercially available enzyme immunoassay (EMD Millipore, Billerica, MA). In this test, the protein general receptor for phosphoinositides-1 (GRP1), which specifically binds phosphatidylinositol (3,4,5)-triphosphate (PIP3), is used to capture PIP3 generated as part of the kinase reaction or the biotinylated PIP3 tracer included in the kit. Captured biotinylated PIP3 is detected using streptavidin-horseradish peroxidase conjugate and measured as absorbance at 450 nm (A450). Because the signal intensity is inversely related to PI3K activity, PI3K activity (% control) was computed as [100 – (A450 of test sample/A450 of biotinylated PIP3) × 100].

To confirm the role of PI3K in amniotic fluid-mediated signaling, IEC6 cells were first exposed to TNF-α/CHI, TNF-α/CHI + 30% amniotic fluid, and TNF-α/CHI + 30% amniotic fluid + LY294002 (PI3K inhibitor), and cell survival was measured by XTT assay (as above).

Phospho-RTK antibody array. HGF effects on tyrosine kinase receptors other than c-met were investigated by using a phospho-receptor tyrosine kinase (RTK) antibody array (R&D Systems) per manufacturer’s instructions. The phospho-RTK antibody array includes a nitrocellulose membrane spotted with 42 different anti-RTK antibodies and includes four positive controls (phosphorylated RTKs) and five negative controls. IEC6 cells were grown to confluence in six-well plates and were then incubated with recombinant HGF (1 and 5 ng/ml) × 1 h. After cell lysis using the provided lysis buffer with added protease and phosphatase inhibitors (Pierce), cell lysates were clarified by centrifugation at 14,000 g for 5 min at 4°C and total protein was measured in the supernatants (BCA protein assay, Pierce). Array membranes were treated overnight at 4°C with lysates diluted to contain 250 μg of protein. The membranes were then washed and incubated with horseradish peroxidase-conjugated mouse anti-phosphotyrosine antibody × 2 h at room temperature. After another wash, the arrays were processed with luminol-based reagents provided in the kit. Array experiments were carried out in duplicate.

Statistical Methods

Parametric and nonparametric tests were applied by using the Sigma Stat 3.1.1 software (Systat, Point Richmond, CA). Parametric data were depicted with bar diagrams, whereas nonparametric data were shown with column scatter plots. For PCR data, crossing-threshold (ΔΔCT) values were compared across experimental groups for genes with greater than or equal to twofold change. Frequency of NEC-like injury was compared by use of the Fisher’s exact test. The Mann-Whitney U-test was used to compare two independent groups, whereas comparisons across multiple groups were made by analysis of variance or the Kruskal-Wallis H-test. In all tests, P < 0.05 was accepted as significant.
RESULTS

Amniotic Fluid Supplementation in Enteral Feeds Protects Rat Pups Against Experimental NEC

To investigate whether supplementation of enteral feeds with amniotic fluid could protect rat pups against experimental NEC, we subjected pups fed formula alone and pups fed formula supplemented with 30% (vol/vol) amniotic fluid to the experimental NEC protocol. As shown in Fig. 1, the addition of amniotic fluid to enteral feeds reduced the severity [median injury grade 2 (range 0–4) in pups fed formula alone vs. median grade 0 (range 0–4) in pups fed formula supplemented with amniotic fluid; \( P < 0.01 \)] and the incidence of NEC-like injury (63 vs. 41% in formula vs. formula with amniotic fluid groups, respectively, \( P < 0.01 \)).

Amniotic Fluid Supplementation in Enteral Feeds Reduces Tissue Expression of Inflammatory Cytokines in Experimental NEC

To investigate whether enteral administration of amniotic fluid affected the inflammatory response during NEC, we used RT-qPCR to measure mRNA expression of selected inflammatory mediators associated with NEC (\( n = 5 \) animals/group) (24). As shown in Fig. 2A, NEC was associated with increased expression of TNF, CXCL1, CXCL2/macrophage inflammatory protein-2α, CXCL5, CCL2/MCP-1, CCL4/MIP-1β, CCL5/RANTES (regulated and normal T cell expressed and secreted), interferon (IFN)-γ, and the enzyme inflammatory-nitric oxide synthase (iNOS). In the supplemental amniotic fluid group, NEC was associated with a significantly smaller rise in CXCL2, CXCL5, CCL2, CCL5, IFN-γ, and iNOS expression compared with NEC in the formula-alone group.

Interestingly, there was a tendency (statistically not significant) toward increased tissue expression of CXCL2, CCL4, and iNOS in pups that received amniotic fluid supplementation but did not develop NEC. In support of these data, NEC in pups receiving formula alone was associated with increased protein expression of CXCL2 and CXCL5, but intestinal injury in pups receiving formula supplemented with amniotic fluid was not associated with a change in these proteins (Fig. 2B).

Fig. 2. Amniotic fluid (AF) supplementation in enteral feeds reduces tissue expression of inflammatory cytokines in rat pups with experimental NEC. A: bar diagram (means ± SE) shows fold change in cytokine mRNA expression in ileal tissue from formula-fed rat pups without NEC, formula-fed rat pups with NEC, pups who received formula supplemented with amniotic fluid, and pups in the group receiving formula supplemented with amniotic fluid that developed NEC. *\( P < 0.05 \) vs. formula-fed pups without NEC; #\( P < 0.05 \) vs. formula-fed pups with NEC. B: representative Western blots show CXCL2 and CXCL5 expression in intestinal tissue.

Fig. 3. Amniotic fluid contains large amounts of hepatocyte growth factor. Column scatter plots show the concentrations of key cytokines and growth factors in rat amniotic fluid. ***\( P < 0.001 \). Inset: concentrations of some of the same analytes in human amniotic fluid.
HGF Is Expressed in High Concentrations in Amniotic Fluid

We next measured the amniotic fluid concentrations of cytokines/growth factors known to play a developmental role in the intestine. We detected IL-1β (median 4.9, range 1.2–8.4 pg/ml), IL-6 (median 134.2, range 95.4–164.5 pg/ml), CCL2/MCP-1 (median 1,208, range 895.6–2,006 pg/ml), CCL3/MIP1α (median 11, range 6–15.1 pg/ml), CCL20/MIP3α (median 200, range 43–732.1 pg/ml), TGF-β1 (median 1,043, range 754.2–1,302 pg/ml), TGF-β2 (median 256, range 11–502 pg/ml), EGF (median 76, range 28–141 pg/ml), and HGF (median 4,892, range 2,560–7,030 pg/ml). HGF was the most abundant of the cytokines we measured in rat amniotic fluid (Fig. 3). Consistent with these findings, we detected similar high concentrations of HGF in human amniotic fluid (median 8,354 pg/ml, range 108–18,654; inset). Similar to rat amniotic fluid, HGF concentrations in human amniotic fluid were several log-folds higher than some of the other analytes.

Amniotic Fluid Effects on IEC Proliferation, Migration, and Protection Against Apoptogenic Injury Are Mediated by HGF

We hypothesized that the protective effects of amniotic fluid against NEC were mediated, at least in part, via the “trophic”

Fig. 4. Amniotic fluid effects on intestinal epithelial cells (IEC) proliferation, migration, and protection against apoptogenic injury are mediated by hepatocyte growth factor (HGF). A: fluorescence photomicrographs show c-met immunoreactivity in IEC6 cells. Left: isotype control. B: bar diagram (means ± SE) show that amniotic fluid and recombinant HGF (rHGF) increased cell migration across wounded monolayers. Representative photomicrographs (×50) show cell migration at 8, 16, and 24 h. C: bar diagram (means ± SE) shows bromodeoxyuridine (BrdU) incorporation in IEC6 cells. D: bar diagram (means ± SE) show optical density (450 nm) in a XTT cell survival assay in the presence of TNF and cycloheximide. Gray bars depict data when IEC6 cells were pretreated with amniotic fluid or recombinant HGF, whereas black bars show the effects of simultaneous treatment with TNF and cycloheximide plus amniotic fluid or HGF. The effects of amniotic fluid on cell proliferation, migration, and cell survival were blocked in the presence of excess neutralizing anti-HGF antibody. *P < 0.05, **P < 0.01 vs. measurements in media alone. In D, #P < 0.05 vs. the effects of 30% amniotic fluid.
effects of amniotic fluid-borne HGF on gut epithelium. To investigate this hypothesis, we first confirmed that IEC6 cells expressed c-met, the cognate receptor for HGF (Fig. 4A). We next treated IEC6 cells with amniotic fluid or recombinant HGF in vitro and measured cell proliferation, migration, and survival, cellular changes that would be needed to promote healing at sites of mucosal injury. To measure cell survival, we treated IEC6 cells with TNF/CHI, a well-characterized apoptotic stimulus where CHI, a nonspecific inhibitor of eukaryotic protein synthesis, potentiates TNF cytotoxicity by presumably inhibiting the generation of cell survival factors (22). Amniotic fluid increased cell migration, proliferation, and survival in IEC6 cells in a dose-dependent fashion, and these changes were reproduced by recombinant HGF (Fig. 4, B–D). Amniotic fluid effects on cell proliferation, migration, and survival were blocked by neutralizing anti-HGF antibody, indicating that HGF was a major contributor to these trophic effects of amniotic fluid.

Amniotic Fluid and HGF Effects on IEC Cell Survival Are Mediated via Activation of Phosphoinositide 3-Kinase

Because HGF is known to signal via PI3K, we next asked whether amniotic fluid effects on cultured IEC6 cells were also mediated via PI3K. Both amniotic fluid and recombinant HGF induced PI3K phosphorylation in IEC6 cells (Fig. 5, A and B). Amniotic fluid-induced PI3K phosphorylation was partially blocked by anti-HGF antibody, emphasizing the key role of HGF in amniotic fluid signaling in IECs. In support of these data, the protective effect of amniotic fluid against TNF and cycloheximide-induced cell death was blocked by the PI3K inhibitor LY294002 (Fig. 5C).

HGF Treatment Results in the Transactivation of Several Receptor Tyrosine Kinases

To investigate how a single mediator, HGF, could reproduce the effects of amniotic fluid, which is a complex biological system with a large number of bioactive factors, we asked whether HGF effects in IEC6 cells extended to more than one signaling pathway. To investigate HGF effects, we used a phospho-RTK antibody array that detected phosphorylation of 42 different RTKs. As shown in Fig. 6, HGF treatment of IEC6 cells resulted in the activation of several RTKs other than c-met, notable ones being ErbB2 (erythroblastic leukemia viral oncogene homolog 2), ErbB3, fibroblast growth factor receptor (FGFR)2, FGFR3, IGF-I receptor, vascular endothelial growth factor receptor (VEGFR)2, and Ryk (related to receptor tyrosine kinase).

Enterally Administered Recombinant HGF Protects Rat Pups Against Experimental NEC

Finally, we sought to confirm whether the protective effects of amniotic fluid against experimental NEC could be reproduced by enteral supplementation with recombinant HGF. Indeed, as depicted in Fig. 7, rat pups receiving formula with supplemental HGF showed a significant reduction in the incidence and severity of NEC-like injury.

DISCUSSION

We show that the addition of amniotic fluid to formula feeds can protect premature rat pups against experimental NEC.
esophagus or the small intestine caused ultrastructural abnormalities in IECs such as delayed disappearance of the apical endocytic network, disrupted or absent microvilli, glycogen accumulation, and inappropriate cell extrusion. Similarly, we have previously shown that human neonates with congenital intestinal obstruction have histopathological abnormalities such as villus blunting and loss of crypt depth with abnormal crypt organization (10). In undifferentiated 15-day murine fetal duodenal explants, rat amniotic fluid extracts have been shown to induce IEC proliferation and promote crypt and villus formation (23). The effects of swallowed amniotic fluid on the fetal intestine are mediated via cytokines and growth factors in amniotic fluid and cannot be explained merely on the basis of the flow of fluid through the gut lumen (4); fetal/neonatal IECs express the cognate receptors for amniotic fluid-borne growth factors such as HGF, EGF, TGF-β, insulin-like growth factors, erythropoietin, and granulocyte-colony stimulating factor and, as shown in Fig. 4, show increased proliferation, migration, and survival in the presence of amniotic fluid (5, 6, 14, 19).

Our findings on the protective effects of amniotic fluid against NEC are consistent with recent observations of Good et al. (13), who demonstrated that enteral administration of amniotic fluid reduced the severity of intestinal injury and iNOS expression in a murine neonatal model of NEC, restored IEC proliferation, and resulted in better-preserved mucosal architecture. Good et al. showed that amniotic fluid protected against NEC-like injury through activation of the EGF receptor (EGFR), which, in turn, suppressed Toll-like receptor 4-mediated signaling in IECs. Although the concentrations of EGF we detected in rat amniotic fluid in our study were much lower (range 28–141 pg/ml) than the nanogram concentrations tested by Good et al. in their in vitro studies, the anti-inflammatory effects of amniotic fluid were similar in the two models. In another study, Siggers et al. (34) showed that enteral administration of amniotic fluid in preterm pigs can reduce bacterial colonization, mucosal inflammatory responses, and NEC. In the present study, we show for the first time that amniotic fluid may promote epithelial restitution and prevent NEC-like injury and that these effects are mediated by HGF.

We identified HGF as an abundant cytokine/growth factor in amniotic fluid. HGF expression has been previously noted in both rodent and human amniotic fluid and is believed to originate in the allantois (14, 17, 31). HGF plays a critical role in trophoblast development; homozygous HGF-null embryos have severely impaired placentas with fewer labyrinthine trophoblast cells and die before birth (38). HGF also promotes mesenchymal-epithelial interaction (35) and has been reported to affect hepatocytes and epithelial cells in several organ systems. Our findings on HGF effects on IECs are consistent with previous observations of Hirai et al. (14), who showed...
that HGF was an important contributor to amniotic fluid-induced cell proliferation in FHs-74-Int human fetal IECs. Similar findings were reported by Setoyama et al. (32), who showed that HGF enemas can promote colonocyte proliferation at sites of mucosal injury in a rat model of inflammatory bowel disease. In rats with surgical short bowel syndrome, HGF can increase mucosal protein and DNA content during intestinal adaptation (16). In the kidney, HGF has been shown to induce cytoskeletal remodeling and promote cell motility, proliferation, and specific differentiation in epithelial cells, leading to the formation of branched tubules (3, 30).

HGF signaling is mediated via ligation of its cognate receptor, c-met, a tyrosine kinase that transmits the HGF signal into the cytoplasm to promote autophosphorylation and recruitment of signal transduction proteins such as the c-Src kinase, phospholipase C-γ, the Grb2-SoS complex, and PI3K (7, 31). HGF also transactivated several other RTKs in the EGFR receptor family (ErbB2, ErbB3), FGFRs, and VEGFR2. ErbB2 is a member of the EGFR receptor family and is known to heterodimerize with the EGF-ligated EGFR to promote EGF signaling (18). These data are consistent with emerging evidence that EGFR-activated signaling can be activated not only by its own ligands, but also by other growth factors such as several ligands of the G protein-coupled receptors, TGF-β, the insulin-like growth factors, and HGF (12, 28, 37). Because both c-met and the ErbB receptors share some of the intracellular signaling pathways, HGF-mediated transactivation of ErbB2 and ErbB3 may also enhance HGF signaling by increasing the intensity or the duration of these receptor-mediated signals (41), leading to synergistic effects on IECs. The cross talk between c-met and these receptors can possibly explain the differences between our neonatal rat model vs. the findings reported by Good et al. (13), who demonstrated that the EGFR was required for amniotic fluid effects in their murine model.

In conclusion, we have shown that supplementation of formula feeds with amniotic fluid or recombinant HGF can reduce the risk of NEC in the premature intestine. Our study has important limitations such as the lack of information on microbiota and, similar to most small animal studies, paucity of information on clinical/demographic characteristics of study animals. The strengths are in the use of a well-characterized information on clinical/demographic characteristics of study animals. The strengths are in the use of a well-characterized model of NEC and the identification of the mechanistic basis of the protective effects of amniotic fluid against NEC. Although further study is needed to ascertain the risk of transmission of infectious agents before amniotic fluid could be adopted for clinical use, the identification of recombinant HGF and downstream signaling mediators carries new possibilities for the development of protective strategies against NEC.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


