Glucocorticoid responsiveness in developing human intestine: possible role in prevention of necrotizing enterocolitis

N. Nanda Nanthakumar,1 Cheryl Young,2 Jae Sung Ko,1 Di Meng,1 Ji Chen,1 Timothy Buie,1 and W. Allan Walker1

1Developmental Gastroenterology Laboratory, Combined Program in Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts; and 2DePauw University, Greencastle, Indiana

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Nanthakumar, N. Nanda, Cheryl Young, Jae Sung Ko, Di Meng, Ji Chen, Timothy Buie, and W. Allan Walker. Glucocorticoid responsiveness in developing human intestine: possible role in prevention of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 288: G85–G92, 2005; doi:10.1152/ajpgi.00169.2004.—Necrotizing enterocolitis (NEC) is a major inflammatory disease of the premature human intestine that can be prevented by glucocorticoids if given prenatally before the 34th wk of gestation. This observation suggests that a finite period of steroid responsiveness exists as has been demonstrated in animal models. Human intestinal xenografts were used to determine whether a glucocorticoid responsive period exists in the developing human intestine. Developmental responsiveness was measured by lactase activity and inflammatory responsiveness by IL-8, IL-6, and monocyte chemotactic protein-1 (MCP-1) induction after an endogenous (IL-1) or exogenous (LPS) proinflammatory stimulus, respectively. Functional development of ileal xenografts were monitored for 30 wk posttransplantation, and the lactase activity recapitulated that predicted by in utero development. Cortisone acetate accelerated the ontogeny of lactase at 20 wk (immature) but the effect was lost by 30 wk (mature) posttransplant. Concomitant with accelerated maturation, the IL-8 response to both IL-1b and LPS was significantly dampened (from 6- to 3-fold) by glucocorticoid pretreatment in the immature but not mature xenografts. The induction of IL-8 was reflected at the level of IL-8 mRNA, suggesting transcriptional regulation. The excessive activation of IL-8 in the immature gut was mediated by a prolonged activation of ERK and p38 kinases and nuclear translocation of NF-kB due to low levels of I kB. Steroid pretreatment in immature intestine dampens activation of all three signaling pathways in response to proinflammatory stimuli. Therefore, accelerating intestinal maturation by glucocorticoids within the responsive period by accelerating functional and inflammatory maturation may provide an effective preventive therapy for NEC.

cortisone acetate; interleukin-8; human ileum; intestinal inflammation; signal transduction

NECROTIZING ENTEROCOLITIS (NEC) is a devastating gastrointestinal inflammatory disease, predominantly occurring in premature infants in neonatal intensive care units worldwide (10, 17, 20). Ninety percent of infants with NEC are < 1,500 g body wt, and the frequency and severity of the disease increases with the degree of prematurity. The pathology of NEC includes extensive intestinal inflammation, ulceration, epithelial necrosis, and pseudomembranous intestinal, and, in advanced cases, the inflammation leads to gastrointestinal perforation and sepsis (5). NEC is an acquired inflammatory disease and is believed to be caused by an inappropriate excessive inflammatory response by the immature gut of the newborn to bacterial colonization after the introduction of enteral feeding (5, 10). A coincidental decrease in NEC was observed when premature infants were treated either prenatally or postnatally with glucocorticoids in an attempt to prevent respiratory distress syndrome (3, 14). However, the degree of prevention was much greater with prenatal treatment. Glucocorticoids are an important trophic factor responsible for the normal development of the gut (13, 16, 21). With the use of animal models of NEC, exogenous and endogenous glucocorticoids have been shown to prevent the onset by accelerating the maturation of gut defenses (18, 20, 26). Because NEC is typically associated with an underdeveloped gut (5, 17), exogenous glucocorticoids in premature infants may prevent the disease by accelerating the maturation process. However, not all studies using glucocorticoids have shown a decreased incidence of NEC (6). This discrepancy could be attributed to the timing of the intervention because sensitivity to glucocorticoids has been shown to be restricted to a finite period during development (24, 31).

In rodents, glucocorticoid sensitivity is restricted to ∼2 wk postpartum, a period of time between the establishment of the first and second phase of functional development (16, 24). Both exogenous and endogenous glucocorticoids can accelerate the maturation of digestive function, can strengthen the intestinal barrier to macromolecules, and can induce an adult type of epithelial proliferation and differentiation (16, 28). This period of rodent development corresponds to the second and third trimesters in human gestation (13, 21). If a similar period of glucocorticoid sensitivity existed in the developing human intestine as in the rodent gut, then steroid treatment to prevent NEC may only be effective during this period of sensitivity. This may explain why fetuses given steroids in utero have a greater degree of NEC prevention than do infants treated postpartum. To date, absence of a suitable in vivo model system for studying the developing human intestine has precluded studies to determine whether a similar period of glucocorticoid sensitivity exists in the developing human gut comparable with that of the neonatal rodent.

We recently established a human xenograft model that can be used to study in vivo development of the human gut in late gestation (25, 29). We have demonstrated the long-term via-
ability of this model as well as the potential role of xenografts in recapitulating in utero development. This model can also be used to evaluate the effect of trophic factors on gut maturation (25). Because animal models suggest a restrictive period of corticosteroid sensitivity, we have hypothesized that glucocorticoids can mediate its beneficial effect against NEC only if administered at an appropriate period in the development of the fetal gut. Previously, we (23) showed that IL-8 is a good marker for the inflammatory response in developing human gut mucosa and is produced in large part by the epithelium in response to proinflammatory stimuli, such as IL-1β and LPS (endotoxin). We have also shown that the inflammatory response in fetal gut to these stimuli is excessive compared with the response by intestinal epithelium from the gut of newborns and children. In this report, we have begun to use the human xenograft model to determine whether an age-related period of glucocorticoid sensitivity exists in the developing human intestine, and if so, what its effect is during that period on the intestinal response to proinflammatory stimuli. Specifically, the response to endogenous (IL-1β) or exogenous (LPS) proinflammatory stimulus was determined after developing human xenografts were pretreated with steroids. These effects were compared with that of the infant small intestinal response to the same proinflammatory stimuli. We herein provide strong evidence that the pathophysiological inflammatory response in premature infants with NEC is, in part, related to gut immaturity and that maturation of inflammation in response to steroids occurs during a finite period in utero in a manner similar to that described in animal models.

MATERIALS AND METHODS

Chemicals. Ultrapure D-glucose, sucrose, lactose, LPS, and BSA were obtained from Sigma (St. Louis, MO). Protein concentrations were measured by using a Bicinchoninic Acid Based protein assay kit (Pierce) against BSA standards in a calorimetric assay according to the manufacturer’s protocol. Cell culture medium-RL (CMRL1066), FBS, nonessential amino acids, glutamine, penicillin, and gentamicin were obtained from GIBCO-BRL (Rockville, MD). Tissue culture plastics were obtained from Fisher Scientific (Lincoln Park, NY). IL-1β and components of the IL-8 ELISA were obtained from R&D Systems (Minneapolis, MN). All other chemicals were either of reagent or molecular grade. Stock solutions of cortisone acetate (Cortone; Merck, Sharp & Dohme, West Point, PA) were purchased from the hospital pharmacy. Glucose was measured by using the Trinder 100 kit from Sigma as described previously (24).

Animals and human xenograft transplantation. Four-week-old homozygous severe combined immunodeficient (SCID) mice were housed in a specific pathogen-free facility and maintained on rodent laboratory chow 5001 (Ralston Purina, St. Louis, MO) and water ad libitum (22). The animals were raised in air-conditioned quarters at 1°C on a 12:12-h light-dark cycle with lights on at 0600. Sterilized food (rodent laboratory chow 5001) and deionized water were provided ad libitum from the day of arrival until the completion of the experiments. To avoid circadian influences, all animals were killed between 1100 and 1300. Surgery and postoperative care were monitored according to an approved animal protocol of the research animal care committee of the Massachusetts General Hospital (MGH) as well as the guidelines published by the American Physiological Society (1).

Eighteen- to twenty-week-old human small intestine was obtained from prostaglandin/saline-induced aborted fetuses with informed consent according to the policies of the Committee for the Protection of Human Subjects from Research Risks at the Brigham and Women’s Hospital and the Human Investigation Committee at MGH. To maintain sterility, tissues were collected only from fetuses in which the abdomen had not been previously opened. These tissues were then transported to the laboratory in ice-cold fresh CMRL 1066 medium containing glutamine, nonessential amino acids, penicillin, and gentamicin as described previously (23). Tissues were processed as described below.

Sterile fetal ileum in 2-cm segments stripped of its mesentery was implanted subcutaneously into homozygous SCID mice. A detailed description of the xenograft technique has been reported previously (25, 29). Briefly, on the day of xenotransplantation mice were anesthetized, and, under aseptic conditions, a small incision was made in the skin in the back of each recipient mouse. A segment of fresh fetal intestine was inserted, the skin was closed with wound clips, and the animals were then returned to their cages. Subsequently, mice were assessed daily for the next 3 days and once weekly thereafter. Successful xenografts that would develop into intestinal tissues were used in these experiments.

Ileal xenografts were harvested at 9, 20, and 30 wk posttransplantation and analyzed. Histology was performed at the time of harvest, and tissues were assayed for sucrase and lactase activities. At 9 wk posttransplantation, sucrase and lactase activities in xenografts were not significantly different from that of the 20-week-old aborted fetal ileal tissue that was used for transplantation, and the level of sucrase activity was not significantly altered up to 30 wk. An increase of lactase activity was paralleled by increasing steady-state levels of lactase mRNA in ileal grafts, as described previously in jejunal xenografts (25).

Small intestinal mucosal biopsies from infants and older children were obtained in the Pediatric Endoscopy Suite at MGH with informed consent when these children were endoscoped and biopsied for diagnostic purposes. Only biopsies from patients without any histological abnormalities were used in these studies. Tissues were transported to the laboratory in ice-cold CMRL 1066 medium containing 40 μg/ml penicillin and gentamicin and processed (23). Samples of intestine from all specimens tested were assayed for sucrase enzymatic activity before and after treatment with endotoxin and IL-1β to assess the viability and of the tissue. Samples of tissues before and after treatment were also obtained for histological examination.

Intestinal organ culture. To confirm any differences in proinflammatory stimuli, we compared the endotoxin/IL-1β response in human small intestinal biopsies and in developing ileal xenografts. Organ culture was performed in a Falcon organ culture dish maintained at 37°C with 95% O2-5% CO2 and saturated water vapor as described previously (19, 23). Culture medium used was CMRL 1066 supplemented with glucose (5 g/l), tricine buffer (20 mol, pH 7.4), hydrocortisone hemisuccinate (0.5 μg/l), b-retinyl acetate (1 mg/ml), glutamine (3 mM), and 5% FBS (Hyclone, Logan, UT), penicillin G (100 U/l), and gentamicin (50 mg/l).

To induce developmental changes in the human intestinal xenograft, a single injection of cortisone acetate was used as an exogenous source of glucocorticoid, rather than daily injections of dexamethasone to reduce the amount of stress to SCID mice. A number of xenografts were treated with a single subcutaneous injection of vehicle (saline; control) or cortisone acetate suspension at a dose of 50 mg/100 g body wt as described previously (25). A week after the treatment 20- (immature) and 30-wk-old (mature) steroid- and saline-treated xenografts were harvested and used for organ culture experiments (at least 10 explants from each xenograft) in addition to assaying for disaccharidases. The human intestinal xenografts were then cultured as described above. The culture experiments were allowed to equilibrate for at least 6 h before half of them were treated with either 50 μg/ml of LPS, IL-1β (1 ng/ml) in medium, or PBS (control). For organ cultures, at least 6–8 biopsies were used for each treatment. The cultures were treated with LPS, IL-1β, or control medium for 18 h; medium and tissue were collected and assayed. The
capacity of LPS and IL-1β to induce IL-8, IL-6, and MCP-1 were quantified by an ELISA assay and expressed as picograms per milligram of total tissue protein. The tissue was homogenized in 9 volumes of 0.15 M KCl and assayed for total protein using a BCL kit against BSA standards (24). Sucrase activity was determined to assess functional viability of the organ culture after each experiment, and histology sections were obtained to assess structural integrity.

**Assay for disaccharidase activities.** The level of sucrase and lactase were determined as described previously (24). Briefly, frozen tissues were homogenized in 9 volumes of 0.15 M KCl using a Potter-Elvehjem Teflon-glass homogenizer. The tissue homogenate was then assayed in duplicate using ultrapure sucrose and lactose as substrates. The amount of glucose liberated by these disaccharides was quantitated by using a glucose-oxidase method (Trinder 100 kit; Sigma) as described previously (24). Protein concentration was measured by using a BCL kit (Pierce, Rockford, IL) against BSA standards according to the manufacturer’s protocol. Enzyme activity was expressed as micromoles of substrate hydrolyzed per hour per milligram of total protein.

**RNA isolation, RT-PCR, and Northern blot analysis.** Total cellular RNA from the jejunum was isolated by using guanidine isothiocyanate extraction and pelleted through a cesium chloride cushion as previously described (23). RT-PCR analysis was carried out with 1–2 μg of total RNA using oligo(dT)random primers and specific cDNA products amplified for 28 cycles using specific pairs of primers and conditions for demonstration of IL-8, lactase, sucrase, and GAPDH as described previously (11, 23). After the RT-PCR reaction, products were separated by electrophoresis in 1.5 to 2% agarose gels and visualized by ethidium bromide staining. Relative amounts of the PCR products were measured by densitometry, and the ratio of IL-8 to GAPDH was calculated as described previously (11, 23). Signals were quantified by phosphoimaging. These results were confirmed by Northern blots generated according to a standard protocol (24) using 10 μg total RNA per lane. Probes used for Northern blot analysis were rat sucrase-isomaltase cDNA (24), rat lactase cDNA (25), human IL-8, and GAPDH cDNA (11, 23). The cDNA inserts were 32P labeled by using the random primer method and prehybridization, and hybridization was performed as described previously (24). After initially being probed with IL-8 cDNA, the blots were stripped in 0.15 M NaCl/0.15 M sodium citrate and 0.5% SDS at 90°C twice and then washed with prehybridization solution at 55°C before being reprobed for GAPDH. To correct for loading variations, these data were expressed as a ratio of the hybridization signal of the band of interest (e.g., IL-8 and lactase) to that of GAPDH, the constitutive marker.

**Western blot analysis.** At the end of the experiment, tissues were lysed with extraction buffer [10 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% (octylphenoxy)polyethoxethanol (Igepal CA-630)] containing protease and phosphatase inhibitor cocktail (Sigma). Lysates were clarified by centrifugation at 4°C for 15 min at 16,000 g. Denatured 50 μg of lysate was fractionated in SDS-PAGE gel and then transferred onto nitrocellulose membranes (11). These membranes were then blocked in blocking buffer (5% nonfat milk in Tris-buffered saline plus 0.1% Igepal CA-630) before being probed with primary antibodies for 1 h at specific dilutions according to the manufacturer’s protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Membrane was then washed twice in fresh blocking buffer before being probed with a species-specific secondary antibody linked to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). The membranes were developed with an enhanced chemiluminescence detection kit (Pierce), and the intensity of the bands was quantitated by densitometry (Applied Biosystems, San Diego, CA) using unsaturated films with NIH Image software as described previously (11, 23).

**Statistics.** Results are presented as the means ± SE. Effects of age and treatment on chemokine secretion were analyzed by a two-way ANOVA. After overall significance was determined, post hoc tests for individual variables were performed by a two-tailed, unpaired t-test, and, when necessary, Bonferroni correction was used with the post hoc t-tests. Differences with a P value of <0.05 were considered significant.

**RESULTS**

**Effects of cortisone acetate on the maturation of disaccharidases in ileal xenografts.** Glucocorticoid responsiveness in immature (20-wk-old) and mature (30-wk-old) human xenografts was determined by measuring disaccharidase activities after treatment with cortisone acetate. In these experiments, SCID mice with ileal grafts were treated either with cortisone acetate or saline injection a week before being harvested at 20 wk and 30 wk posttransplantation for histology as well as for lactase and sucrase activities (Fig. 1). The results indicate that the mature levels of disaccharides in ileal xenografts are much lower than the levels observed in the jejunal xenografts as reported earlier (25). Saline-treated immature grafts showed high sucrase and low lactase activities, and pretreatment with cortisone acetate significantly induced lactase (P < 0.035) but had no effect on sucrase (P > 0.47) activity. In contrast, cortisone acetate treatment had no effect on either lactase (P > 0.78) or sucrase (P > 0.58) activities in the mature xenografts. These results correspond to previous observations made in jejunal xenografts (25).

**Effect of cortisone acetate on ileal xenograft responsiveness to IL-1β.** Responsiveness to IL-1β was determined by using cortisone acetate or control pretreated immature (20-wk-old)
and mature (30-wk-old) ileal xenografts. In these experiments, SCID mice with immature and mature xenografts were injected either with cortisone acetate or saline a week before grafts were harvested at 20 wk and 30 wk posttransplantation, grafts were then studied as organ cultures and stimulated either with IL-1β or control medium. The tissue response was measured by assaying for IL-8, IL-6, and MCP-1 (Fig. 2) after 18 h.

There was an age-dependent decreased effect after cortisone acetate treatment on the IL-1β-induced expression of IL-8, IL-6, and MCP-1 (P < 0.005, P < 0.001, and P < 0.01, by ANOVA, respectively). As expected, there was an overall significant induction of IL-8, IL-6, and MCP-1 (P < 0.01, P < 0.005, and P < 0.05, by ANOVA, respectively) in response to IL-1β in both immature (20-wk-old) and mature (30-wk-old) ileal grafts, regardless of steroid treatment. IL-1β was able to stimulate an increased IL-8 by 6-fold, IL-6 by 7.5-fold, and MCP-1 by 4-fold in immature grafts. However, a prior cortisone acetate treatment led to a significant reduction in these responses resulting in only 2.5-fold for IL-8, 3.5-fold for IL-6, and 2.4-fold for MCP-1 induction. In contrast, in the 30-wk-old mature grafts, IL-1β stimulated 3-fold for IL-8, 4-fold for IL-6, and 2.3-fold for MCP-1, and no additional effects were noted with cortisone acetate treatment. Experiments were also performed by using organ cultures of infant intestinal biopsies. In response to IL-1β, there was an induction of 3.2-fold for IL-8, 3.75-fold for IL-6, and 2.5-fold for MCP-1. Levels of these chemokines were comparable with mature xenografts (Table 1). IL-1β-mediated induction of IL-8 in both immature and mature xenografts was reflected at the level of IL-8 mRNA with an overall significance noted with cortisone acetate treatment (P < 0.005, by ANOVA) (Fig. 3). In immature xenografts, IL-1β treatment resulted in a 6.5-fold induction of IL-8 mRNA over controls (P < 0.005), but with cortisone acetate pretreatment, the level of induction was reduced to 2.4-fold over controls (P < 0.01). In contrast, in mature ileal grafts, IL-1β

Table 1. Comparative levels of chemokine induction in mature xenografts and infant intestinal biopsies after IL-1β stimulation in organ culture

<table>
<thead>
<tr>
<th>IL-8*</th>
<th>IL-6*</th>
<th>MCP-1*</th>
<th>Relative fold IL-8 mRNA†</th>
<th>Sucrase activity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant biopsies</td>
<td>308±38</td>
<td>324±18</td>
<td>68±9</td>
<td>2.3±0.8</td>
</tr>
<tr>
<td>Mature xenograft</td>
<td>270±29</td>
<td>380±29</td>
<td>87±12</td>
<td>2.7±1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE in *picogram per milligram protein, †fold difference in IL-1β-simulated vs. control, and ‡micromole per hour per microgram protein. MCP-1, monocyte chemotactic-protein-1.
induced only a two- to threefold increase compared with controls and had no additional effect with cortisone acetate treatment ($P > 0.63$). Levels of mRNA induction mirror the levels of IL-8 chemokine secretion, suggesting that the steroid effect was at the level of transcription, although all tissues showed a low basal IL-8 mRNA. Experiments with infant biopsies in culture showed a twofold induction of IL-8 mRNA in response to IL-1β comparable with the induction observed in the mature xenograft (Table 1).

The level of sucrase activity in organ cultures was not significantly different in either immature or mature ileal grafts, regardless of treatment ($P > 0.7$), and morphology was intact before and after treatment (data not shown), suggesting that the integrity of both the xenografts and infant biopsies in organ culture was not altered during the experiments.

**Effect of cortisone acetate on ileal xenograft responsiveness to LPS.** Responsiveness to LPS was determined by using cortisone acetate- or saline-treated immature (20-wk-old) and mature (30-wk-old) ileal xenografts. In these experiments, SCID mice with immature and mature xenografts were injected either with cortisone acetate or saline a week before grafts were harvested. At 20 wk and 30 wk posttransplantation, grafts were then studied as organ cultures, stimulated either with LPS or control medium and assayed for IL-8 induction (Fig. 4). There was a significant (3-fold) reduction in LPS-induced IL-8 secretion in the immature grafts ($P < 0.05$) pretreated with cortisone acetate, whereas no difference was noted in mature grafts ($P > 0.69$). As expected, an overall significance of IL-8 induction was noted in response to LPS in both immature and mature ileal grafts, regardless of cortisone acetate treatment ($P < 0.01$, by ANOVA). The level of IL-8 mRNA induced by LPS in these cultures was comparable with that of secreted IL-8 in both mature and immature xenografts (data not shown). All tissues expressed a low basal IL-8 protein and mRNA. In addition, the morphology and sucrase activity was comparable in all tissues, regardless of age or treatment, suggesting no loss of tissue integrity during the experiments (data not shown).

**Effect of cortisone acetate on signaling pathways in developing ileal xenografts after IL-1β stimulation.** These experiments were performed according to the conditions described in previous experiments except that the tissues were harvested 15, 30, 60, and 120 min after IL-1β stimulation. Fifty micrograms of cytosolic protein were fractionated in a 10% SDS-PAGE gradient gel, transferred onto a membrane, and probed for IκBα, ERK, and phosphorylated (phospho)-ERK, as well as p38, phospho-p38, and villin according to the manufacturer’s recommendation (Santa Cruz Biotechnology). Villin expression was determined to assess the quality of the epithelial isolation, control of constitutive markers, and loading control. The epithelial villin expression in ileal xenografts was unchanged, regardless of treatment or age. Expression of IκBα protein was quantitated by densitometry and normalized to expression levels of villin (Fig. 5A). The rate of activation of ERK kinase (Fig. 5B) and p38 kinase (Fig. 5C) on stimulation by IL-1β in immature (20-wk-old) ileal xenograft pretreated either with cortisone acetate or saline (control) alone was quantitated and expressed in densitometric units. A prolonged decrease in the IκBα level was observed in untreated immature grafts. However, with prior cortisone acetate treatment, IκBα protein recovered rapidly to mature levels. In contrast, no difference in response was noted with cortisone acetate- and saline-pretreated mature ileal grafts (data not shown). A prolonged activation of phospho-ERK and phospho-p38 signaling pathways was also observed in immature ileal grafts, but its activation was rapidly lost with cortisone acetate pretreatment. However, the levels of total ERK and p38 protein were unchanged in either mature or immature ileal grafts, regardless of cortisone acetate treatment. The rate of activation of JNK-1 kinase was determined, but there was no difference, regardless of age or pretreatment with cortisone acetate (data not shown).

In the immature xenografts, these data suggest the existence of insufficient IκBα to inhibit nuclear translocation of NF-κB, an extended activation of ERK and p38 kinases in response to proinflammatory stimuli. In contrast, cortisone acetate pretreatment accelerated the development of the immature xenografts, leading rapidly to a mature rate of increase in IκB expression, presumably to lessen the nuclear translocation of NF-κB and to inactivate ERK and p38 kinases in response to proinflammatory stimuli, attaining kinetics similar to that observed in mature (30-wk-old) xenografts. The most profound difference of these changes was observed 60 min after IL-1β stimulation, and a representative Western blot is shown in Fig. 5D.

**DISCUSSION**

Functional development of the human gut during the second and third trimesters of gestation is largely undefined because of the lack of access to tissue and an available model that can recapitulate ontogeny (13, 16). Previously, we (25) showed that the developing fetal human intestinal xenograft model may recapitulate the predicted in utero development of the human small intestine. Twenty-week-old fetal intestine is capable of regenerating as a xenograft into an appropriate spatial and...
we have been able to identify a finite period of glucocorticoid intestine. However, by using the xenograft model in this study, lung. We have no comparable data in the developing small existence of a limited period of glucocorticoid sensitivity in the steroid effect is lost by 34 wk of gestation, indicating the tory distress syndrome in preterm infants (6) suggested that the controlled trials of corticosteroid treatment to prevent respira-

chonical ventilation (2, 14, 27). A meta-analysis of randomized and lung development, permitting earlier weaning from me-

number of beneficial effects in preterm infants in brain, gut, also reduce the incidence of NEC. Steroid treatment provides a tory distress syndrome (33). Fortuitously, it was reported (3,

premature infants in neonatal intensive care units for respira-

mothers at risk for complications of premature delivery and third trimesters.

period of sensitivity should be effective during the second to temporal distribution of crypt-villus architecture and an expres-

ion of disaccharidases recapitulating in utero development (25, 29). Although stimulation by glucocorticoids can acceler-

ate the ontogeny of the xenograph, data presented in this report suggest that its effect is restricted to a brief period of time in utero. A similar loss of steroid responsiveness has previously been documented in the developing rodent small intestine (24, 31). In the rat small intestine, glucocorticoid responsiveness was observed to occur between the first and second phases of the functional maturation (e.g., the first 2 wk of postnatal development) (24). In rodents, the second phase of development is characterized by the ontology of sucrase, but in humans it is characterized by the ontology of lactase (reviewed in Refs. 13 and 16). If the human intestine’s developmental response to steroids is analogous to that of the rodent, the period of sensitivity should be effective during the second to third trimesters.

Glucocorticoids have been indispensable in the treatment of mothers at risk for complications of premature delivery and premature infants in neonatal intensive care units for respira-

tory distress syndrome (33). Fortuitously, it was reported (3, 14) that the use of steroids under these treatment conditions can also reduce the incidence of NEC. Steroid treatment provides a number of beneficial effects in preterm infants in brain, gut, and lung development, permitting earlier weaning from me-

chonical ventilation (2, 14, 27). A meta-analysis of randomized controlled trials of corticosteroid treatment to prevent respira-

tory distress syndrome in preterm infants (6) suggested that the steroid effect is lost by 34 wk of gestation, indicating the existence of a limited period of glucocorticoid sensitivity in the lung. We have no comparable data in the developing small intestine. However, by using the xenograft model in this study, we have been able to identify a finite period of glucocorticoid responsiveness using disaccharidases as a marker of general intestinal development and IL-8, IL-6, and MCP-1 as markers of immune responsiveness to inflammatory stimuli. However, the steroid-induced inflammatory response may also lead to detrimental effects, especially in very-low-birth-weight infants, resulting in increased bronchopulmonary dysplasia and intes-

nal perforations (12, 32, 33, 35, 36). This suggests that it is important to identify the critical period as well as the dose and duration of treatment that could benefit premature gut without causing inflammatory damage in other tissues. By elucidating the mechanism(s) of glucocorticoid effect using human intesti-

nal xenographs, more targeted therapy can be developed to prevent NEC while avoiding the detrimental effects on other tissues.

NEC is typically associated with gut immaturity (5, 10, 28). There is also strong evidence to suggest that initial bacterial colonization of the developing intestine plays an important role in the onset of NEC (5, 7, 21). Commensal bacteria that become established after birth (9), particularly gram-negative bacteria (15), communicate with the intestinal epithelium via glycoprotein or glycolipid receptors located on the apical surface of the intestinal epithelium (7). Through this commun-

ication, microbes elicit effector responses in enterocyte structure and/or function for their own survival. For example, bacterial cell wall components, also known as pathogen-assoc-

iated molecular patterns (PAMP), of gram-negative and -posi-
tive bacteria [e.g., endotoxin (LPS) and peptidoglycan, respec-
tively] are able to communicate with enterocytes via pattern-

recognition receptors [e.g., Toll-like receptor (TLR)-4 and -2, respectively] (4, 21, 34). Previously, we (11, 23) have shown that these two pattern-recognition receptors, crucial to the recognition of PAMPs to elicit an innate immune response by enterocytes, are expressed on immature fetal enterocytes and
are responsive to LPS. This interaction leads to specific activation of transcription factors, such as NF-κB, leading to production of proinflammatory cytokines (TNF-α, IL-6, and IL-8) (8, 23, 28). IL-8 is a neutrophil-attracting chemokine known to be produced by enterocytes in response to inflammatory stimuli and plays an important role in recruiting neutrophils from the intravascular space to interstitial and luminal sites (4, 19). NEC is characterized by a hemorrhagic inflammatory necrosis of the distal small bowel and proximal colon with extensive infiltration by neutrophils (5, 17). We have previously reported (4, 23) that the pathogenesis of NEC may be, in part, due to an immature (inappropriate) intestinal epithelial immunological response to luminal bacterial stimuli by excessive activation of IL-8. Because the disease develops principally in premature infants at the time of initial bacterial colonization (5, 10, 17, 20), we choose for this study to measure the activation of proinflammatory chemokines, such as IL-8, IL-6, and MCP-1 as effector responses to endogenous (IL-1β) and exogenous (LPS) proinflammatory stimuli.

The levels of IL-8, IL-6, and MCP-1 response to proinflammatory stimuli and after treatment by glucocorticoids suggests that steroids may be regulating excessive response of all these chemokines and cytokines by one or more mechanisms of attenuation. Therefore, in an attempt to define a mechanism(s) for this excessive IL-8 response, we have begun to investigate the likely signaling mechanisms for NF-κB, ERK, p38, and JNK-1 pathways. We observed a prolonged activation of all the pathways except JNK-1 in the immature intestine (20-wk-old postxenograft transplants), suggesting that these pathways are inappropriately developed and may contribute to the excessive chemokine response in the immature intestine. In recent studies (4), we have also shown that activation of the TLR-5, another pattern-recognition receptor for flagella, by a commensal *Escherichia coli* can lead to an excessive activation of the NF-κB pathway in immature intestinal epithelial cells. As part of that study of TLR-5 receptor activation, Claud et al. (4) showed that a decreased expression of β2 integrin was observed in the immature intestinal epithelium in the absence of sufficient β2 integrin to bind and NF-κB to prevent its nuclear translocation for transcriptional activation, presumably leading to a greater stimulation of IL-8 transcription in the immature enterocytes. This observation that commensal bacteria can cause inflammation in immature but not in mature enterocytes (4) may help to explain the increased incidence of intestinal inflammation in premature babies leading to NEC after initial bacterial colonization. Therefore, to help determine the mechanism of the steroid effects in NEC, we examined in this study the signal pathways before and after use of cortisone acetate in immature and mature xenografts. We showed that the steroid effect appears to stimulate the upregulation of IkBα (Fig. 5), thus interfering with the excessive nuclear translocation of NF-κB to transcriptionally activate genes, such as IL-8. Interestingly, steroid pretreatment was able to accelerate the maturation of the tissue as well as able to attenuate three distinct but important signaling pathways (NF-κB, ERK, p38) by an as yet incompletely understood mechanism. An alteration in the kinetics of NF-κB, ERK, and p38 signaling pathways may explain the specific developmental regulation of these mediators in the immature gut. This may be the basis for excessive inflammation. Additional studies are currently underway to elucidate the relative importance of the ontogeny of these pathways of inflammation and the presumed mechanisms by which glucocorticoids exert an anti-inflammatory effect in the immature gut.

In previous studies comparing 20-wk-old fetal (immature) grafts directly with biopsies from infant (mature) intestine, we have presented direct evidence that the immature gut responds excessively to proinflammatory stimuli, such as LPS and IL-1β (23). Using the xenograft model in this report, we have systematically analyzed the role of the developing intestine and its response to these stimuli by measuring IL-8, IL-6, and MCP-1 in developing intestinal mucosa. Furthermore, glucocorticoids have been shown to reduce the degree of these inflammatory responses by twofold (for IL-8 and MCP-1) and threefold (for IL-6), and, at the same time, accelerate the maturation of the gut. Although it is not known whether maturational effects are an integral part of this reduced response to proinflammatory stimulation, these experiments suggest that during in utero development, there exists a finite period of intestinal responsiveness to glucocorticoids, and this observation may explain, in part, the preferential effect of steroid treatment in preventing the onset of NEC in premature infants when given in utero (6). It will be of interest to determine whether other components of intestinal defense, such as the mucosal barrier to macromolecular uptake, tight junction proteins, expression of antimicrobial peptides (e.g., defensins) in Paneth cells, and bile acid reabsorption are also modulated by glucocorticoids during this same responsive period or whether it is only confined to specific aspects of ontogeny. Therefore, the xenograft model may provide an important simulated in vivo model to delineate periods and markers for glucocorticoid responsiveness in the developing human gut during the second and third trimester of in utero development and may help in devising therapeutic strategies in preventing the onset of age-related diseases specific to premature infants.

Using the xenograft model, we are currently in the process of identifying additional markers that clearly define the various phases of in utero development and the period of glucocorticoid sensitivity as well as determining the development of mucosal immune defenses, macromolecular uptake, and/or barrier property of the epithelium. Furthermore, the formation of a lumen in the xenografts will enable us to use this model to determine the role of luminal compared with systemic stimulus in ontogeny and their contribution to age-related pathophysiological conditions, such as toxigenic diarrhea (10, 28). Premature infants given their mothers’ breast milk have no, or a much less severe, incidence of NEC (10, 14, 17). However, it is not known which component of colostrum and/or mature human breast milk prevents the onset of NEC in premature infants. The xenograft model may provide an opportunity to elucidate the role of various factors in mother’s milk in attenuating the excessive inflammatory and other immature responses of the immature intestine that initiates the onset of NEC.

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