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TGF-β_2 Suppresses Macrophage Cytokine Production and Mucosal Inflammatory Responses in the Developing Intestine

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BACKGROUND & AIMS: Premature neonates are predisposed to necrotizing enterocolitis (NEC), an idiopathic, inflammatory bowel necrosis. We investigated whether NEC occurs in the preterm intestine due to incomplete noninflammatory differentiation of intestinal macrophages, which increases the risk of a severe mucosal inflammatory response to bacterial products. **METHODS:** We compared inflammatory properties of human/murine fetal, neonatal, and adult intestinal macrophages. To investigate gut-specific macrophage differentiation, we next treated monocyte-derived macrophages with conditioned media from explanted human fetal and adult intestinal tissues. Transforming growth factor-β (TGF-β) expression and bioactivity were measured in fetal/adult intestine and in NEC. Finally, we used wild-type and transgenic mice to investigate the effects of deficient TGF-β signaling on NEC-like inflammatory mucosal injury. **RESULTS:** Intestinal macrophages in the human preterm intestine (fetus/premature neonate), but not in full-term neonates and adults, expressed inflammatory cytokines. Macrophage cytokine production was suppressed in the developing intestine by TGF-β, particularly the TGF-β_2 isoform. NEC was associated with decreased tissue expression of TGF-β_2 and decreased TGF-β bioactivity. In mice, disruption of TGF-β signaling worsened NEC-like inflammatory mucosal injury, whereas enteral supplementation with recombinant TGF-β_2 was protective. **CONCLUSIONS: Intestinal macrophages progressively acquire a noninflammatory profile during gestational development. TGF-β, particularly the TGF-β_2 isoform, suppresses macrophage inflammatory responses in the developing intestine and protects against inflammatory mucosal injury. Enterally administered TGF-β_2 protected mice from experimental NEC-like injury.**

Keywords: Necrotizing Enterocolitis; Macrophage; Newborn; Inflammation; TGF-β.

Premature neonates born before 32 weeks of gestation or with a birth weight <1500 g are predisposed to necrotizing enterocolitis (NEC), an idiopathic, inflammatory bowel necrosis characterized by *pneumatosis intestinalis* (accumulation of gaseous products of bacterial fermentation within the bowel wall), inflammation, and tissue necrosis.[1,2] Existing data indicate that bacterial flora normally present in the gut lumen, not specific bacterial pathogens, play a major role in the pathogenesis of NEC.[1,2] The pathophysiological importance of bacteria in NEC is underscored by the frequent detection of bacteria and *pneumatosis* in intestinal tissue, occurrence of NEC only after postnatal bacterial colonization and never in the sterile intrauterine microenvironment before birth, inability to induce NEC-like lesions in germ-free experimental animals, and by observations that enteral antibiotics may reduce the incidence of NEC in preterm infants.[2,3] Based on current evidence, NEC is believed to occur when mucosal injury or altered permeability in the preterm intestine permits the translocation of luminal bacteria across the epithelial barrier, which in turn triggers a severe inflammatory response.[2,4]

Gut mucosal injury and bacterial translocation are frequent events in critically ill patients of all ages, but unlike in premature infants, these invading bacteria do not evoke an NEC-like inflammatory response in the mature intestinal mucosa. Bacteria that breach the gut epithelial barrier are normally eliminated by resident macrophages in the lamina propria, the first phagocytic cells of the innate immune system to encounter these micro-organisms.[5,6] Unlike macrophages in other organ systems that release cytokines/chemokines on phagocytosis of bacteria to stimulate a local inflammatory response, intestinal macrophages are profoundly suppressed for cytokine production.[6] This unique dichotomy of phagocytic vs inflammatory properties in intestinal macrophages plays an important role in maintaining the

Abbreviations used in this paper: ECM, extracellular matrix; IEC, intestinal epithelial cell; IL, interleukin; LPS, lipopolysaccharide; NEC, necrotizing enterocolitis; PAF, platelet-activating factor; T-CM, tissue-conditioned media; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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normal absence of inflammation in the gut mucosa (despite close proximity to immunostimulatory bacteria), as illustrated by spontaneous onset of enterocolitis in genetically modified mice with defects in differentiation of intestinal macrophages.[6–9] In this context, we hypothesized that the risk of NEC in the premature intestine is related to the state of differentiation of intestinal macrophages. We postulated that the noninflammatory differentiation of intestinal macrophages is a function of gestational maturation and is therefore incomplete in the preterm intestine, increasing the risk of a severe mucosal inflammatory response to bacterial products. In this study, we investigate maturational changes in gut macrophage differentiation in the context of NEC using a variety of in vitro and in vivo models.

Methods

Human Intestinal Tissue Samples

Human intestinal tissues were collected after approval by local Institutional Review Boards. Fetal intestinal tissue (11−24 weeks, n = 25) was obtained at elective terminations of pregnancy. Tissue samples of advanced NEC (n = 8) were compared with healthy tissue margins obtained during resection for indications other than NEC (premature neonates: repair of ostomy, n = 5; gestational age 27, 27.5, 28.5, 30.5, and 32 weeks; full-term neonates: atresia/obstruction; n = 3). Adult tissues were obtained during bariatric surgery (n = 5).

Immunohistochemistry

Tissue sections (and cells) were stained for macrophage markers (HAM56 or F4/80), tumor necrosis factor-α (TNF-α), interleukin (IL)-8, TGF-β_2, and TGF-β receptors using our previously described fluorescence protocol[6,10–12] (included in Supplementary Material).

Murine Intestinal Macrophages

Murine studies were approved by the local Institutional Animal Care and Use Committee. Murine intestinal macrophages were isolated by standard methods including density centrifugation and adherence to polystyrene,[8,13] as described in the Supplementary Material.

Real-Time Polymerase Chain Reaction

Inflammatory cytokines and TGF-β isoforms were measured by our previously described quantitative polymerase chain reaction method using SYBR green.[14,15]

Tissue-Conditioned Media (T-CMs)

We prepared T-CMs from fresh human and murine intestinal tissue by using a previously reported protocol.[16] Briefly, fresh intestinal tissue was cleaned, intestinal epithelial cells (IECs) were removed by dispase/EDTA to expose the lamina propria, and the remaining tissue was incubated overnight in serum-free RPMI. The exfoliated epithelial cells were incubated overnight in a separate plate to prepare epithelial-conditioned media. In some experiments, we used intact tissue (with epithelium) to prepare the T-CMs. A detailed protocol is included in the Supplementary Material.

Treatment of Monocyte-Derived Macrophages With T-CMs

Blood monocytes from healthy adult volunteers were isolated by Ficoll-Hypaque centrifugation and immunoselection with CD14 microbeads (Miltenyi, Biotec, Auburn, CA).[10] Monocyte-derived macrophages (20,000/well) were incubated in 96-well plates with T-CM (250, 500, and 1000 μg total protein/mL) $\times$ 2 to 24 hours and then stimulated with 500 ng/mL *Escherichia coli* lipopolysaccharide (LPS) (predetermined optimum; Sigma, St Louis, MO) for up to 18 hours.

Measurement of Inflammatory Cytokines

TNF-α (human and murine), IL-6, IL-1β, and IL-8 were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN).

Neutrophil Chemotaxis

Culture supernatants from monocyte-derived macrophages were tested for neutrophil chemotactic activity using our fluorescence-based protocol[17] described in the Supplementary Material.

Nuclear Factor−κB Activation

Macrophages were treated with T-CMs and LPS as described. Nuclear factor−κB p65 phosphorylation was measured by ELISA (SuperArray Biosciences, Frederick, MD).

TGF-β Bioactivity

TGF-β bioactivity was measured by a quantitative luciferase assay; T-CMs were added for 16 hours to mink lung epithelial cells transfected with a luciferase plasmid containing the TGF-β−responsive promoter of the platelet activator inhibitor−1 gene[18]; Smad2/3 phosphorylation (ser423, ser425) was measured by Western blots using polyclonal antibodies against total and phosphorylated Smad 2 (Santa Cruz Biotechnology, Santa Cruz, CA) and appropriate secondary reagents.[19] TGF-β activity was neutralized in vitro by addition of excess (15 or 50 μg/mL) neutralizing anti-human TGF-β antibody.

Assays for TGF-β Isoforms

Total and active TGF-β_1, TGF-β_2, and TGF-β_3 were measured by ELISA (R&D). T-CMs containing only 1 unique TGF-β isoform were derived from 20- to 24-week T-CMs (n = 3) by removing 2 of the 3 TGF-β isoforms by immunoprecipitation;[19] the presence/absence of TGF-β isoforms was confirmed by ELISA. Macrophages were treated with parent or derivative T-CM before LPS stimulation. In some experiments, we treated

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