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Alterations in platelet-derived growth factor expression in the pathophysiology of necrotizing enterocolitis

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ABSTRACT

Background: Necrotizing enterocolitis (NEC) involves impaired ileal blood flow due to alterations in vascular tone control and intestinal angiogenesis. Platelet-derived growth factor (PDGF) is a mediator of normal angiogenesis in intestinal epithelium. We hypothesized that gene dysregulation during experimental NEC results in altered PDGF expression.

Methods: Sprague–Dawley rats were randomized to groups by litter. Controls were delivered vaginally and dam-fed. NEC groups were delivered prematurely by cesarean section and subjected to an established NEC protocol. Ileum was obtained at 0, 12, 24, 48, 72, and 96 h of life from all animals ($N = 108$ animals). Western blot analysis was carried out for every time point, and samples were evaluated by immunohistochemistry. Antibodies against PDGF-A, PDGF-B, and their receptors, PDGFR-α and PDGFR-β, were used. Statistical analysis was performed using two-way analysis of variance with *a priori* $P < 0.05$.

Results: Ileal PDGF-A concentration was higher in controls *versus* NEC from 24–96 h of life. Its receptor, PDGFR-α, was low in concentration in both groups at all time points. PDGF-B concentration was increased in controls at 24 and 72 h of life but decreased at the 48-h mark. Its receptor, PDGFR-β, was also low in both groups at 12 and 24 h but increased in controls at 48 and 72 h.

Conclusions: These data support our hypothesis that PDGF and PDGF receptor expression are altered in experimental NEC. Dysregulation of PDGF during intestinal maturation could contribute to the development of NEC. Further investigation into this pathway could yield new therapeutic targets for this devastating disease.

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1. Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal disease among neonates and a major cause of mortality in premature infants [1]. Studies have shown a mortality rate of 15%–40% in infants with NEC [2]. The pathogenesis of NEC involves a combination of deranged function of the intestinal microvasculature and angiogenesis that leads to altered blood flow in the intestine.

Vasoconstriction of the microvasculature of the small intestine causes hypoperfusion, hypoxia, intestinal necrosis, and death. Recent studies have shown that an imbalance of inflammatory mediators in an NEC animal model result in a compromise of blood flow of the intestine, which leads to ischemic injury [3]. Still, due to the prematurity associated with NEC, impaired angiogenesis is also a potential contributing factor to be considered when determining the cause of altered intestinal blood flow and function.

Platelet-derived growth factor (PDGF) ligands and their receptors play a major role during general embryonic development [4]. Knockout murine studies have verified that many cellular responses to PDGF are essential during gestation [5]. These studies have shown that PDGF-B and its receptor PDGFR-β are essential for the development of support cells in the vasculature, whereas PDGF-A and its receptor PDGFR-α are broadly required during embryogenesis.

We hypothesized that protein expression of angiogenic growth factors PDGF-A and PDGF-B and their receptors, PDGFR-α and PDGFR-β, are diminished in NEC-affected intestine such that microvessel growth is severely impaired or functionally altered in an animal model of NEC.

2. Methods

The Institutional Animal Care and Use Committee, Biohazard Safety Committee, and Research and Development Committee at the Robley Rex VA Medical Center in Louisville, KY, approved this research protocol before study initiation. Timed pregnant Sprague–Dawley dams (Harlan, Indianapolis, IN) were maintained in an Association for Assessment and Accreditation of Lab Animal Care approved Veterinary Medical Unit for at least 1 wk before delivery of pups. Dams were acclimated on a 12 h light–dark cycle and were allowed rat chow and water *ad libitum*. The dams were then randomized to the control or experimental NEC protocol, and their pups were assigned to groups by litter.

The control groups were vaginally delivered and kept with the dam until the day of the experiments. Control animals were fed on demand by the dam, and their hour of birth was recorded so that the pups could be time-matched to the protocol of the NEC groups. The NEC groups were delivered by cesarean section 12 h prematurely and then entered into the experimental NEC protocol. For the NEC groups on the day of the cesarean section, the dam was weighed and anesthetized with carbon dioxide; the pups were delivered quickly after the dam underwent decerebration. The NEC group pups were immediately revived and warmed in a humidity-controlled incubator at 37.0°C.

As reported in previous studies, NEC was induced by formula feeds via gastric gavage every 4–5 h, intermittent hypoxia (100% nitrogen gas for 60 s), and hypothermia (4°C for 10 min) every 12 h and a single gastric dose of lipopolysaccharide at 12 h of life [3,6,7]. Formula feeds consisted of 20-g Similac 60/40 (Ross Pediatrics, Columbus, OH) dissolved in 100 mL Esbilac (PetAg, New Hampshire, IL) puppy formula. These feeds were calculated to supply approximately 836.8 kJ/kg/d.

Rat pups were sacrificed at 0, 12, 24, 48, 72, and 96-h time points after delivery. Both control and NEC ileum samples were homogenized in RIPA lysis buffer (R0278 Sigma–Aldrich Chemical Co, St. Louis, MO) and a cocktail of phosphatase (P5726, Sigma–Aldrich) and protease inhibitors (P8340, Sigma–Aldrich), and protein concentrations were determined. Samples containing equal amounts of 35 μg of total protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and probed with antibodies PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β all with a 1:1000 dilutions in bovine serum albumin. Each protein sample was standardized to its own β-actin level.

NEC was graded as follows: grade 0, normal and no damage; grade 1, epithelial cell lifting or separation; grade 2, sloughing of epithelial cells to mid villus level; grade 3, necrosis of the entire villus; and grade 4, transmural necrosis [8]. Mucosal and submucosal ileum cross sections for control and NEC groups from the 48-h time point were mounted in paraffin and stained with either hematoxylin and eosin (H&E) or immunohistochemistry (IHC). These samples were selected for IHC based on our prior studies showing a significant difference in intestinal blood flow at this place in the NEC protocol timeline [9]. IHC staining was performed using rabbit anti-rodent polyclonal antibodies for PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β. The slides for IHC were graded into one of four different categories: (-) no significant staining, (+) slight positive, (++) moderately positive, and (+++) strongly positive.

All data are expressed as mean ± standard error of the mean. Differences between groups and time points were determined by two-way analysis of variance using SigmaPlot for Windows 11.1.0.102 (Systat Software, Inc, San Jose, CA). The null hypothesis was rejected with *a priori* at $P < 0.05$. When differences were found using analysis of variance, the *post hoc* Tukey–Kramer honestly significant difference test was applied.

3. Results

Nine control and nine NEC animal samples were obtained for each group, at each time point ($N = 108$ animals). Figure 1 shows PDGF-A protein levels in the ileum at 0, 12, 24, 48, 72, and 96 h of life in the NEC and control groups. NEC levels of PDGF-A were not different compared with the control group at 12 h of life but were significantly decreased at 24, 48, 72, and 96 h of life when compared with the controls. Table describes the intensity of IHC staining in mucosal epithelial cells and the histologic severity of NEC in the ileum. At the 48-h time point, PDGF-A staining by IHC is more prominent in controls compared with NEC, which supports the Western blot data.