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Review

Necrotizing enterocolitis (NEC): what's going on

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Abstract

Necrotizing enterocolitis (NEC) is among the most common and devastating diseases in neonates and, despite the significant advances in neonatal care and clinical and basic science investigations, its etiology remains incompletely understood, specific treatment strategies are lacking, and morbidity and mortality from this disease remain high. Recent improvements in the pathophysiology of NEC may have therapeutic consequences. Toll-like receptors and intestinal microflora play an increasing role in the pathogenesis of NEC. Pharmacologic inhibition of TLR signaling, the use of novel nutritional strategies, and microflora modulation may represent novel promising approaches to the prevention and treatment of NEC. This review focuses on current and future therapeutic perspectives, starting from the recent acquisitions in the pathogenic mechanisms of NEC.

Keywords

Toll-like receptor, microflora, nutrition, innate immunity, newborn, sepsis.

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Introduction

Necrotizing enterocolitis (NEC) is an inflammatory disease of the intestine, often associated with sepsis, and frequently complicated by perforation, peritonitis, and death. NEC is among the most common and devastating diseases affecting neonates and despite the significant advances in neonatal care and in clinical and basic science investigation, its etiology remains incompletely understood, specific treatment strategies are lacking, and morbidity and mortality from this disease remain high. It has thus become a priority for research. The excessive inflammatory process that starts in the intestine in necrotizing enterocolitis extends systemically, to affect even distant organs such as the brain, thus exposing infants with NEC at increased risk for neurodevelopmental delay (nearly a 25% chance of microcephaly) [1, 2]. The total annual estimated cost of caring for affected infants with NEC in the United States ranges between $500 million and $1 billion [3]. Despite a critical need to develop strategies for disease prevention and treatment, efforts to improve outcomes have been hampered by an incomplete understanding of its pathogenesis. Our review focuses on the recent advances made in the pathophysiology of NEC, which may translate into future therapeutic developments.

Definition and clinical presentation

The term "necrotizing enterocolitis" (NEC) often reflects a spectrum of intestinal conditions that differ with respect to the pathogenesis and the strategies required for prevention and treatment [1]. There are different forms of intestinal injury that occur most often: term infants present with spontaneous intestinal perforation and classic necrotizing enterocolitis; in term and late preterm infants the disease usually occurs in the first week after birth and is more frequently associated with other problems such as maternal illicit drug use, intestinal anomalies (e.g. aganglionosis or atresias), congenital heart disease and perinatal stress that may affect mesenteric blood flow. Spontaneous intestinal perforation, often diagnosed as necrotizing enterocolitis, probably represents a different disease entity with a different pathogenesis; it usually occurs in several days after birth and is not associated with enteral feeding. This disorder is characterized by only minimal intestinal inflammation and necrosis, as evidenced by low levels of serum inflammatory cytokines [3, 4].

Understanding pathogenesis to develop new therapeutic strategies

The pathogenesis of NEC is multifactorial and the condition may have different presentations. Alterations of the innate immunity response or in intestinal microbiota, and microcirculatory dysfunctions, associated with infections are the basis for the development of all cases of NEC (**Fig. 1**).

Recently, the link between different pathogenic aspects of NEC has become clearer. The intestinal mucosa of the premature infant presents a state of constant injury and repair. Injury to the intestinal mucosa may depend on a variety of conditions typical of prematurity, including hypoxia [5, 6], infection [7], and inappropriate enteral nutrition [8]. Healing begins immediately after the epithelial injury through the process of enterocyte migration, which consists in the movement of healthy enterocytes into the wounded area [9]. Subsequently, the proliferation of new enterocytes within the crypts of Luberkuhn complete the repair process [10]. It has been recently suggested that NEC is associated with a marked inhibition in both enterocyte migration and proliferation, making the host uniquely susceptible to further injury and bacterial translocation [11]. Structured factors located on the epithelial surface, namely the toll-like receptor (TLR) play a major role in tissue repair. TLR are a series of conserved receptors that recognize pathogens and initiate immune responses. Among the known human TLR, TLR4 seems to have a crucial role in NEC development. The activation of the innate immune receptor TLR4 inhibits enterocyte migration and leads to enterocyte apoptosis, whereas the inhibition of TLR4 signaling in the newborn intestinal epithelium prevents NEC development and attenuates the degree of enterocyte apoptosis, as demonstrated both *in vitro* and *in vivo* [11-13]. However, the observation that most premature infants do not develop NEC, despite the seemingly tonic activation of TLR4, suggests that TLR4 signaling is somehow curtailed within the newborn intestinal epithelium, thus limiting the propensity for NEC development. Developing fetuses express elevated levels of TLR4 until the end of the gestation. This over-expression could be due to the role of TLR4 in regulating proliferation and differentiation of the intestinal epithelium [14]. The persistently elevated expression of TLR4 during intrauterine life not increases the risk of NEC for the fetus, probably because it lives in a sterile environment. At the end of the gestation, the

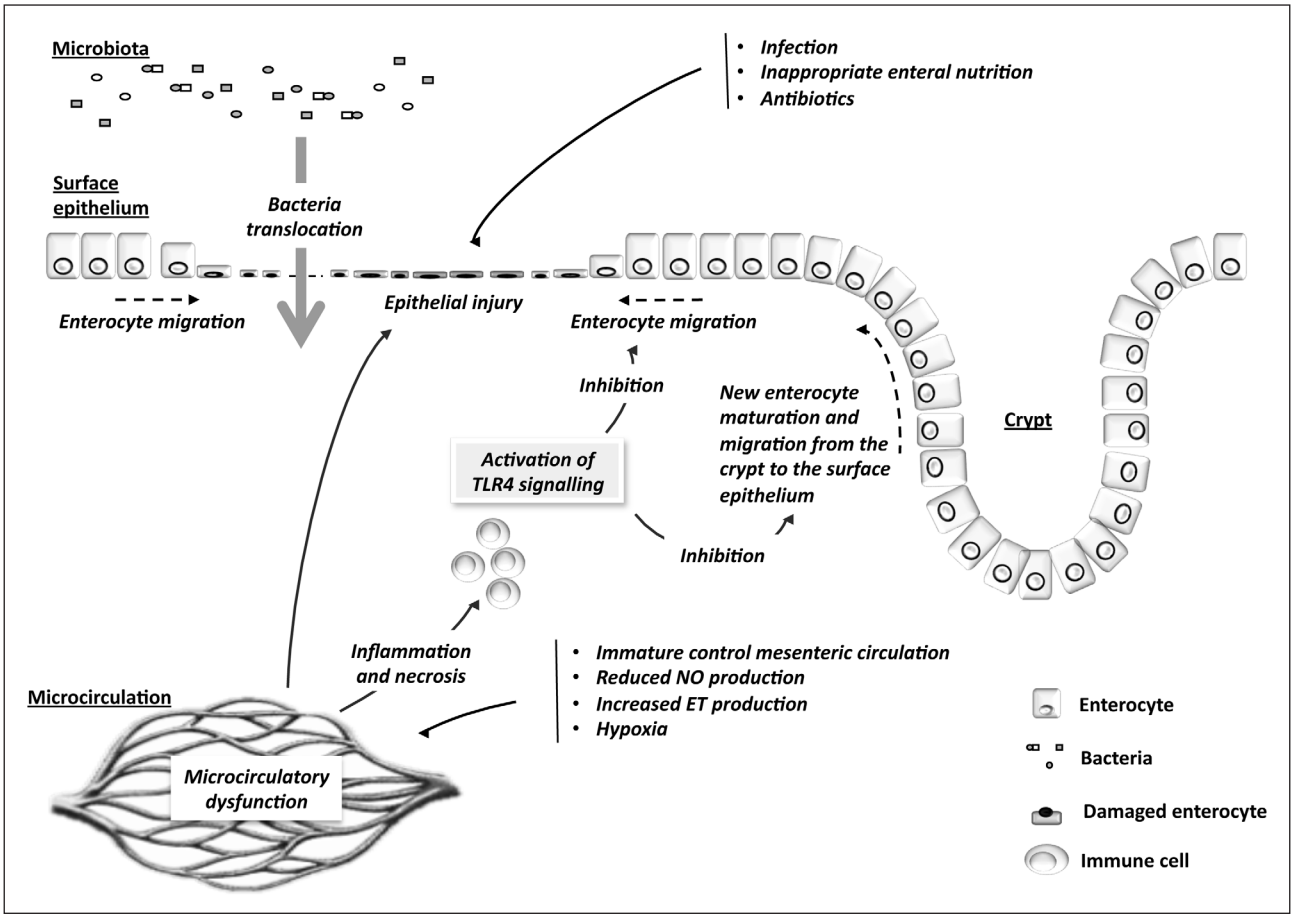

Figure 1. Many factors typical of prenatal birth such as infections, inappropriate enteral nutrition, antibiotics use, microcirculatory dysfunction and hypoxia induce epithelial injury. Hyperactivation of toll-like receptor-4 (TLR4) signaling affects the healing process favoring pathological bacteria translocation across epithelial barrier. NO: nitric oxide; ET: endothelin.

neonate expresses low levels of TLR4 and, in the presence of a normal intestinal microflora, it does not develop a pathologic inflammatory response. By contrast, the expression of TLR4 in preterm babies is high, and when the premature intestine is colonized by pathogenic flora, TLR4 signaling is over-activated, leading to increased mucosal injury and decreased mucosal repair. The final effect of these TLR4-mediated responses leads to gut barrier failure, bacterial translocation, intestinal inflammation and development of systemic sepsis [11]. On the other hand, not all premature neonates exposed to the same environmental factors develop NEC. It seems, therefore, that the consequences of exaggerated TLR4 signaling in premature neonates can be confined by counter regulatory mechanisms that limit TLR4 activation. These mechanisms include intra- and extracellular factors and, possibly, microflora composition. Heat shock proteins, of which Hsp70 is a predominant member, are a family of intracellular proteins activated by a variety of stressors, which contribute to the delivery of target proteins to the ubiquitin-proteosome system for degradation through cochaperone molecules such as CHIP [15]. Hsp70 has a protective role in the intestine probably limiting TLR4 signaling in enterocytes [16]. The mechanism involves an increase in CHIP-mediated ubiquitination, and the degradation of TLR4 via the ubiquitin-proteosomal pathway [16]. TLR4 activation itself significantly increased Hsp70 expression in enterocytes, which provides a mechanism of autoinhibition of TLR4 signaling in enterocytes. Reduced activity of Hsp70 or hyper-activation of TLR disrupts this balance and induces NEC. On the contrary, up-regulation of Hsp70 leads to a reduction in TLR4 signaling and a decrease in enterocyte apoptosis [16]. Interestingly, an extracellular factor possibly affecting TLR4 signaling is the epidermal growth factor (EGF). The fetus continuously swallows amniotic fluid; the amniotic fluid limits the extent of TLR4 signaling in the fetal intestinal mucosa and in cultured enterocytes exposed to bacterial products [17], thus markedly reducing the degree of pro-inflammatory

cytokine release. Amniotic fluid is extremely rich in EGF. This extracellular factor inhibits TLR4 signaling via peroxisome proliferator-activated receptor gamma (PPARγ) [17]. Other important factors, at least in part related to TLR signaling, have been recently explored in the pathogenesis of NEC. TLR4 signaling can upregulate Platelet-activating factor (PAF) expression thus increasing injury in experimental models of NEC [18, 19]. Accumulation of ileal bile acids causes significant injury in the small intestine, and acts in concert with TLR4 pathway [20]. Intestinal integrity restitution requires intercellular connectivity, mediated through small channels, namely gap junctions, rich in connexin protein [21]. Proinflammatory cytokines (i.e. INFγ) cause the internalization of connexin 43, thereby impairing intercellular connectivity and reducing the extent of intestinal restitution [21, 22].

Nearly all the studies on NEC associate infections with the disease; however, rather surprisingly, the specific mechanisms by which infections contribute to NEC remain unknown and no microbe has been identified as determinant etiologic factor. The use of new molecular biology techniques have provided opportunities to re-examine this unresolved problem. Recent studies have identified an abundance of Proteobacteria (including many commonly observed Gram-negative pathogens) in fecal samples of babies with NEC [23-25]. Additional findings included a loss of gut microbial diversity and depletion of enterococcal populations in NEC [25]. More recently, a correlation was made between the clinical finding of *Pneumatosis intestinalis* and the presence of clostridial species (*Clostridium butyricum* and *Clostridium paraputrificum*) [26]. All these results suggests that NEC may not result from a single causative species but more likely from a currently undefined dysbiosis.

Altered microcirculatory control in the intestinal mucosa is a major etiologic factor for intestinal epithelial injury [27]. Nitric oxide is the main regulatory mediator of mucosal microcirculation. Premature neonates showed reduced levels of nitric oxide at the mucosal level. Nutrition may modulate mucosal microcirculation by affecting nitric oxide production.

What is going on in the prevention and treatment of necrotizing enterocolitis

The mainstay in the prevention and treatment of NEC remain a correct management of fluid intake, nutrition, prevention of infections and adequate antibiotic therapy [1-4]. On the basis of the new evidence in the understanding of the pathogenic mechanisms, new therapeutic approaches can be hypothesized. Recent advances in the pathogenic mechanism of NEC suggest that future treatment may involve nonspecific immunological approaches, such as pharmacologic inhibition of TLR signaling, administration of specific feeding, or manipulation of the intestinal environment.

In a mice model, the pharmacological up-regulation by Celastrol of Hsp70 within the intestinal mucosa led to a reduction in TLR4 signaling and a decrease in enterocyte apoptosis, and finally to an attenuation of NEC severity [28, 29]. Alternative methods may be modulation of other mediators of TLR signaling such as EGF, PPARγ, connexin 43 or INFγ [17, 21, 22, 30]. Further studies are required to establish a real applicability of these novel therapeutic options to the prevention and treatment of NEC.

A relatively well studied approach to manipulate gut microbiome is the administration of probiotics. Although its efficacy in the prevention and treatment of NEC has been demonstrated in some articles [31], more work is critically needed to determine well-tolerated and effective dosing strategies and to identify the long-term effects of microbial manipulation on health and development. The extrapolation of evidence available for probiotics to neonates should take into account the characteristics of the products utilized and of the population included in the studies. A specific strain used in a population from a developed country may not be effective in neonates from other countries, who may have different environmental and genetic conditions. The debate as to whether to give probiotics systematically to preterm infants is still ongoing. The American Pediatric Surgical Association Outcomes and Clinical Trials Committee systematic review concluded in 2012, was in support of the recent Cochrane reviews regarding the use of prophylactic probiotics in preterm infants weighing less than 2,500 g to reduce the incidence of NEC [32]. However, there is insufficient evidence to recommend the routine use of probiotics to decrease NEC [33]. Many aspects may have influenced the different results obtained in relation to the efficacy of probiotics in preterm neonates. In particular, the baseline NEC rate was a major factor affecting the potential benefit of probiotic supplementation in a population. Specifically, the effect of probiotics decreases dramatically in areas where the occurrence of NEC is low; hence further studies are needed before a benefit in these areas can be established.

Limitations to future trials may be large sample sizes required to demonstrate the benefit of probiotics. For example, Mihatsch et al. [33] calculated that with a 5% incidence of NEC, at least 714 infants per group would be required to demonstrate a 50% reduction rate ($\alpha = 0.05$, $\beta = 0.2$). Despite the larger clinical trials currently underway, there is no ongoing trial targeting a sample size that large.

A novel perspective is represented by fecal transplantation. This techniques has proven effective in the treatment of refractory *Clostridium difficile* colitis and in some cases of inflammatory bowel disease [34-36]. Fecal transplantation involves direct transfer of fecal material from a healthy donor to a recipient's upper or lower intestinal tract [34-36]. No data are currently available for neonates with NEC, and important limitations should be considered to hypothesize this indication for neonates at risk of this condition (i.e. modalities of transfer of fecal material from donor to recipient).

The adoption of new feeding strategies and formula composition may have a positive impact on NEC development. Endothelial nitric oxide is an important regulator of vascular perfusion and is synthetized from the amino acid L-arginine. Hypoargininemia is frequently observed in preterm neonates and may predispose them to NEC. Recently, Policarpou et al. have demonstrated that enteral L-arginine supplementation can be safely administered in VLBW neonates and appears to reduce the incidence of stage III NEC [37]. Larger studies are needed to further evaluate the effect of L-arginine supplementation in preventing NEC in VLBW infants. Enteral glutamine supplementation decreased gastrointestinal dysfunction, number of days when feeding was withheld, and serious infectious episodes [38]. Moreover, experimental studies have shown that glutamine plays an important role in maintaining the functional integrity of the gut by serving as fuel for enterocytes, stimulating mucosal cell proliferation and differentiation, improving mucus quality, and maintaining the integrity of tight junctions [38]. Improved intestinal integrity, in turn, leads to decreased bacterial translocation, decreased systemic spread of bacteria, and consequently may lead to decreased infectious morbidity [39, 40]. However, so far only animal studies have provided direct evidence for this hypothesis.

Conclusions

Based upon these findings, we believe that a better understanding of the early mechanisms at the basis of NEC development will offer new and innovative approaches to this severe condition. Considering the role of the epithelium, it is essential to explore how the gut environment contributes to the immune response, in order to improve the clinical management and limit the complication associated with NEC. Further well designed trials in specific populations are advocated to verify the therapeutic approaches hypothesized above.

Declaration of interest

The Authors declare that there is no conflict of interest.

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