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Jay and Margie Grosfeld Lecture

Roles of nitric oxide and intestinal microbiota in the pathogenesis of necrotizing enterocolitis☆

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ABSTRACT

Necrotizing enterocolitis remains one of the most vexing problems in the neonatal intensive care unit. Risk factors for NEC include prematurity, formula feeding, and inappropriate microbial colonization of the GI tract. The pathogenesis of NEC is believed to involve weakening of the intestinal barrier by perinatal insults, translocation of luminal bacteria across the weakened barrier, an exuberant inflammatory response, and exacerbation of the barrier damage by inflammatory factors, leading to a vicious cycle of inflammation-inflicted epithelial damage. Nitric oxide (NO), produced by inducible NO synthase (iNOS) and reactive NO oxidation intermediates play a prominent role in the intestinal barrier damage by inducing enterocyte apoptosis and inhibiting the epithelial restitution processes, namely enterocyte proliferation and migration. The factors that govern iNOS upregulation in the intestine are not well understood, which hampers efforts in developing NO/iNOS-targeted therapies. Similarly, efforts to identify bacteria or bacterial colonization patterns associated with NEC have met with limited success, because the same bacterial species can be found in NEC and in non-NEC subjects. However, microbiome studies have identified the three important characteristics of early bacterial populations of the GI tract: high diversity, low complexity, and fluidity. Whether NEC is caused by specific bacteria remains a matter of debate, but data from hospital outbreaks of NEC strongly argue in favor of the infectious nature of this disease. Studies in *Cronobacter muytjensii* have established that the ability to induce NEC is the property of specific strains rather than the species as a whole. Progress in our understanding of the roles of bacteria in NEC will require microbiological experiments and genome-wide analysis of virulence factors.

Necrotizing enterocolitis (NEC), a severe inflammation of the small intestine, is the most common and most lethal disease affecting the GI tract of the premature infant. Despite aggressive medical and surgical treatment, the mortality rate for NEC is typically 15%–30% in the very low birth weight neonates and approaches 100% in cases involving pan-necrosis [1,2]. Unfortunately, the incidence of NEC continues to rise because recent advances in neonatology have resulted in the survival of infants born at 23 weeks gestation [3].

1. Pathogenesis of NEC

According to the currently accepted pathogenetic scenario, perinatal insults such as hypoxia, hypothermia, and enteral feeding with formula compromise the barrier function of the intestinal epithelium [4]. At the same time, bacterial colonization of the neonate's GI tract occurs [5–7]. Some luminal bacteria and their components, such as lipopolysaccharide (LPS), CpG DNA, or flagellin, translocate across the compromised barrier and engage immunocytes, eliciting the production of inflammatory factors including proinflammatory cytokines, NO and peroxynitrite, and inflammatory prostanoids [8–12]. These proinflammatory stimuli damage the barrier by their effects on the epithelial tight junctions [13]. They also increase the rate of enterocyte cell death and decrease the rates of enterocyte proliferation and migration [14]. Increased barrier damage and decreased epithelial restitution further compromise the mucosal barrier, leading to more bacterial translocation, more inflammation, and more epithelial injury, which, if unchecked, may culminate

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in intestinal perforation, fulminant sepsis and death (Fig. 1). Although this scenario is broadly accepted, the specific details at each step remain largely unknown. In this review we will examine the roles of inflammatory NO and opportunistic pathogens in the pathogenesis of NEC.

2. Role of nitric oxide

Nitric oxide is known to play a paradoxical role in intestinal physiology. NO is produced from arginine in a reaction catalyzed in the intestine mainly by two NO synthases (NOS), endothelial NOS or eNOS, and inducible NOS, or iNOS. eNOS is constitutively expressed in the intestinal microcapillaries at low levels and is responsible for the low background levels of NO. Low levels of NO regulate vascular tone and mucosal blood flow in a cyclic GMP- and neuron-dependent manner. Constitutively low production of NO is also required for the maintenance of mucosal capillaries [15] and mucosal homeostasis [16]. NO may protect from oxidative stress by scavenging oxygen radicals [17]. eNOS-derived NO promotes leukocyte adhesion to the endothelium, facilitating leukocyte recruitment [18]. These are all beneficial effects. iNOS is upregulated during inflammation and is responsible for high levels of NO, which dramatically increase blood flow by dilating the capillaries. Our lab was the first to demonstrate that sustained upregulation of iNOS in the intestine caused by LPS, can lead to gut barrier failure [12]. Indeed, high levels of NO seen during inflammation exert detrimental effects on the gut barrier leading to increased bacterial translocation [19,20], impaired mitochondrial function [21] and epithelial restitution [22], as well as decreased leukocyte recruitment by the endothelium [23,24]. NO readily reacts with the superoxide ion to form peroxynitrite, a reactive oxygen and nitrogen species that is highly toxic to epithelial cells [25]. Peroxynitrite may damage the epithelium in multiple ways. It may induce enterocyte apoptosis and inhibit epithelial restitution processes including enterocyte proliferation and migration [26–28]. We were the first to demonstrate increased expression of iNOS mRNA and protein in the intestinal mucosa during NEC [29]. iNOS upregulation is accompanied by increased rate of enterocyte apoptosis. The latter colocalizes with iNOS expression and nitrotyrosine immunoreactivity, a molecular footprint of peroxynitrite, in enterocytes. iNOS expression decreased at the time of stoma closure when the acute inflammation had subsided [25]. Thus, on the one hand NO plays an important role in intestinal homeostasis, but on the other hand, high levels of NO contribute to the epithelial damage seen in NEC.

3. Regulation of iNOS expression by bacteria

Regulation of the iNOS gene in response to bacteria and their pathogen-associated molecular patterns has been most extensively studied in macrophages and neutrophils, the specialized cells of the innate immune system, in the C57Bl/6 strain of mice. In these cells, iNOS is induced by pathogenic bacteria such as *Listeria monocytogenes*, or bacterial components such as LPS. This induction involves signaling via pattern recognition Toll-like receptors, activation of the transcription factor nuclear factor kappa B (NF-kB), binding of NF-kB to the iNOS promoter, and transcriptional induction of the iNOS gene [30,31]. Toll-like receptor ligands alone cause only moderate induction of iNOS in macrophages; full-scale induction requires costimulation with type I or type II interferons [32]. These interferons are produced by natural killer cells and T cells in response to stimulation with Toll-like receptor ligands. Interferons act via their cognate receptors on macrophages to activate signal transduction activators of transcription (STATs) and interferon response factors (IRFs), which form transcription activation complexes on the iNOS promoter at distinct locations from NF-kB-binding sites [32]. Thus, efficient induction of iNOS in mouse macrophages requires synergy between macrophages and T cells, between Toll-like receptor ligands and interferons, and between distinct transcription activation complexes acting on the iNOS promoter.

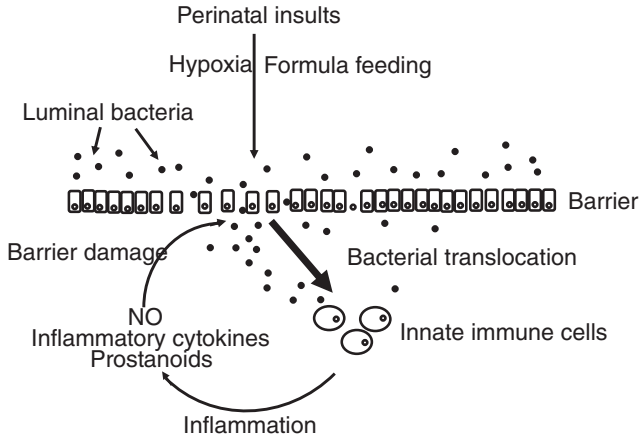

Fig. 1. Pathogenesis of NEC. Perinatal insults of prematurity including hypoxia, formula feeding, and colonization with opportunistic pathogens compromise the gut barrier, leading to bacterial translocation. After crossing the barrier, bacteria engage the innate immune cells of the lamina propria and elicit an inflammatory response by stimulating production of nitric oxide, inflammatory cytokines, and inflammatory prostanoids. These inflammatory factors further compromise the gut barrier, increasing bacterial translocation and exacerbating inflammation. A vicious circle of inflammation-inflicted barrier damage and bacterial translocation culminates in intestinal necrosis.

Findings in C57Bl/6 mice may not be universally applicable. Macrophages from BALB/c mice produce much less iNOS upon stimulation with LPS and interferon compared to those from C57Bl/6 mice [33]. Numerous studies failed to detect iNOS induction in human macrophages/monocytes of various tissue origin upon stimulation ex vivo, although expression of iNOS was sometimes detected in macrophages isolated from patients with a variety of inflammatory disorders [34]. Similarly, little or no iNOS induction was observed in rat, bovine, or porcine macrophages [35,36]. It has been suggested that stimuli other than LPS/interferon are required to induce iNOS in macrophages of species other than mice [37]. Indeed, induction of iNOS by *Leischmania* is strongly potentiated by IL-1β [38]. Differences in organization of the iNOS promoter between mice and other vertebrates [39] may reflect the roles of alternative transcription factors and signaling pathways.

Another possibility is that cell types other than macrophages could be sources of NO during NEC. iNOS expression is not confined to macrophages, it has been detected in a broad array of tissues and cell types [40]. Of interest to mucosal pathophysiology, iNOS is expressed in intestinal smooth muscle cells [41,42], in endothelial cells of the intestinal capillaries [23,24], and in enterocytes [43,44]. Moreover, the epithelium is the major site of iNOS expression and NO production during intestinal inflammatory disorders [45,46]. We have shown that experimental NEC induced by the opportunistic pathogen *Cronobacter muytjensii* in rats depends on the upregulation of intestinal iNOS [47]. Although the intestinal epithelium may be a major source of NO in the pathogenesis of NEC, the molecular mechanisms of mucosal iNOS induction in this disease remain unclear.

4. Microbiota in NEC

Current evidence suggests that bacterial colonization of the gut is a key prerequisite for the pathogenesis of NEC. Indeed, germ-free rats [48] or mice [49] do not develop NEC. Over the last 20 years there have been numerous attempts to identify specific bacteria that contribute to the development of NEC. These studies compared bacterial populations found in patients with NEC to those found in healthy individuals. Although some studies reported an association between the prevalence of proteobacteria [50–55], clostridia [56], staphylococci [57], or decreased bacterial species diversity [53,55] with the development of NEC, other studies failed to corroborate these findings. The same bacterial species that were found in NEC patients were also found in healthy