

The development of animal models for the study of necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is the leading cause of death and long-term disability from gastrointestinal disease in preterm infants, and is characterized by acute and chronic intestinal inflammation that may lead to systemic sepsis and multi-system organ failure. NEC typically develops in the preterm infant after the administration of tube feeds, although it may occasionally be seen in full-term babies. Despite extensive clinical experience in the management of patients with NEC, the underlying cellular and molecular mechanisms leading to its development remain incompletely understood. Several animal models have therefore been developed in a variety of species in order to study the pathogenesis of NEC and to develop more effective treatment strategies. This review seeks to examine the pros and cons of animal models that have been developed in the study of NEC over the past 30 years. It will highlight the various strengths and weaknesses of experimental approaches that have been used, and discuss potential directions for the development of such models for the future.

Necrotizing enterocolitis: clinical concepts and pathogenetic mechanisms

Necrotizing enterocolitis (NEC) is an acute inflammatory disease that affects the intestine of neonates resulting in intestinal necrosis, systemic sepsis and multi-system organ failure (Lin and Stoll, 2006). It is the leading cause of death and long-term disability from gastrointestinal disease in preterm infants (Blakely et al., 2005). NEC affects approximately 20% of preterm infants and is gradually increasing in frequency, owing in part to the increased number of preterm infants that are born each year (Feng et al., 2005; Henry and Moss, 2005; Hsueh et al., 2003; Stoll, 1994; Warner and Warner, 2005). NEC is not only one of the most serious clinical problems to affect neonates, but also one of the most challenging to treat. Up to one-third of all babies with NEC in North America die. Challenges in the treatment of NEC are compounded by the fact that the mechanisms leading to NEC development are incompletely understood. Whereas treatment of early stage NEC may be managed by a com-

bination of antibiotics and cessation of oral feeds, advanced cases, in which intestinal necrosis is present, require intestinal resection or abdominal drainage and can be a risky undertaking in these sick, fragile patients (Henry and Moss, 2008). Clearly, a more complete understanding of the causes of NEC is required to more effectively design therapeutic strategies.

NEC typically develops after the onset of enteral feeds and when the intestinal tract has become colonized; several lines of evidence indicate that the interaction between indigenous bacteria and the newborn intestine have a crucial role in NEC pathogenesis (Anand et al., 2007; Grave et al., 2007). Although a more complete discussion of the current thinking regarding the pathogenesis of NEC is beyond the scope of the current work, several recent reviews have explored the pathogenesis of NEC in detail (Anand et al., 2007; Frost et al., 2008; Lin et al., 2008). A conclusion shared by many investigators in the field is that, despite several decades of work into the pathogenesis of NEC, the overall mortality rate remains

high and our overall understanding of its causes remains correspondingly low. As such, the development of reliable and reproducible animal models for the study of NEC remains a crucial component in our attempt to determine its underlying causes. This review seeks to examine the animal models that have been developed in the study of NEC over the past 30 years, and will highlight the various strengths and weaknesses of each experimental approach while discussing potential directions for the development of models for the future.

Challenges in the development of animal models for the study of NEC

Several unique features of NEC pose challenges to the development of adequate animal models. For instance, NEC develops in premature infants shortly after they are born, necessitating models in which NEC develops in the preterm or early post-term period. This is particularly challenging in mice given their small size as pups. Further, NEC is characterized by a complex combination of pathological events, including intestinal inflammation and systemic sepsis, which distinguishes it from other intestinal inflammatory conditions such as ulcerative colitis and Crohn's disease. In addition, the inflammation of NEC within the human intestine is not consistently localized, instead it is characteristically patchy affecting the small bowel, the large bowel or a combination of both (Gribar et al., 2008), a finding that further distinguishes NEC from other intestinal inflammation diseases that may predominantly affect the colon, such as infection. Finally, there are no experimental models of 'spontaneous NEC', or any genetic deletions or mutations that are known to give rise to NEC in mice or other species. Therefore, the induction of NEC requires manipulation of the gastrointestinal tract and immune system of the host, which is particularly challenging in very young mice. Despite these potential challenges, developing accurate models of NEC is a crucial step towards deciphering the

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Case study

Baby 'GH' was born at 27 weeks' gestation weighing 1100 grams. Other than prematurity, he had no congenital anomalies and was initially placed on an infant ventilator to support his premature lungs. By 1 week of age GH was weaning from the ventilator; he was started on feeds through a nasogastric feeding tube, initially at 1 cubic centimeter (cc)/hour. After 36 hours of feeding the child suddenly became ill – his abdomen became acutely distended, his stools became bloody and he began to appear mottled and underperfused. An abdominal X-ray revealed the presence of *Pneumotosis intestinalis* in the terminal ileum and ascending colon, and his blood work revealed combined leukocytosis and thrombocytopenia. Feeds were stopped and the patient began receiving broad-spectrum antibiotics. After 24 hours the child became more ill, with increased abdominal distention, progressive hypotension and the development of septic shock. Inotropic support was initiated, and blood and platelets were transfused. The X-ray revealed persistent pneumatosis, increased ascites and a generalized paucity of intraluminal gas. Given how progressively ill the child was becoming, he was prepared for exploratory laparotomy, which encountered a moderate amount of turbid ascites and revealed that approximately 30% of the small intestine was necrotic. The remainder of the intestine was viable. The necrotic intestine was resected, and an ileostomy and mucus fistula were created. The child recovered over the ensuing 2-3 weeks and gradually re-started on tube feeds. Subsequently, the child began to grow and develop, so that by 4 months of age he was feeding from a bottle, interacting appropriately and growing normally. He underwent an additional operation at 5 months of age to reverse his stoma, and by 6 months of age he was receiving fortified formula and was starting to meet his developmental milestones.

newborn rats that were stressed by formula feeding, asphyxia and/or exogenous bacterial colonization (Caplan et al., 1994). The extent of intestinal injury was determined by gross examination as well as histopathology. These authors determined that the combination of asphyxia, formula feeding and bacteria was required for the development of NEC. Asphyxia was a crucial instigating factor, because formula and bacterial exposure without asphyxia resulted in normal intestine and minimal mortality (Caplan et al., 1994). Caplan et al. used this model to identify the crucial role of platelet activating factor (PAF) in the pathogenesis of NEC (Caplan et al., 1997), and modified the model by adding a period of cold exposure. Using this approach, they showed that administration of the probiotic bifidobacteria might reduce NEC severity in newborn rats (Caplan et al., 1999). Nadler et al. made further adaptations to the model by standardizing the insult, using a hypoxia chamber to quantify the precise concentration of inspired oxygen to which the rats would be exposed, and by providing a measure of the biological features of the animal's intestines (Nadler et al., 2000). Specifically, they demonstrated that NEC was associated with the upregulation of inducible nitric oxide synthase (iNOS) mRNA, enterocyte apoptosis, and decreased interleukin 12 (IL-12) production in the intestinal epithelium (Nadler et al., 2000). Dvorak et al. used a model of experimental NEC in rats, exposed to a combination of asphyxia and cold stress over 4 days, and evaluated the extent of NEC by gross and histological scoring of ileum damage (Dvorak et al., 2003). Using this model, they identified a significant decrease in NEC severity in the presence of exogenously administered epidermal growth factor (EGF) and maternal milk, by a mechanism thought to involve IL-10 (Dvorak et al., 2002; Dvorak et al., 2003). Working with rat pups delivered on day 21 of gestation by Caesarean section, Besner et al. modified this model slightly to include the administration of lipopolysaccharide (LPS, 2 mg/kg) in addition to gavage feeds, hypoxia and hypothermia, and showed that the exogenous delivery of heparin-binding EGF (HB-EGF) had a protective effect (Feng et al., 2006). Additional laboratories have adapted the rat model of NEC in various experimental settings, and have made significant advances in our understanding of NEC. Selected contributions include the

complexity of this disease. Fortunately, many laboratories including our own have achieved success in the development of experimental NEC in a variety of species. The different NEC animal models are reviewed below. In each case, the relevant methodology involved with developing the model is highlighted, along with the key steps undertaken to validate it. While a review such as this will inevitably omit certain references, we have attempted to be as thorough as possible, so as to provide the reader with a current overview of advances in the development of animal models of NEC.

When is an experimental model of NEC truly NEC?

As described above, NEC occurs in newborn infants, typically during prematurity and after the onset of enteral feeds, and involves patches of the small and/or large intestines. Therefore, animal models of NEC need to mimic this pattern of disease and demonstrate histopathology resembling the human condition. It is noteworthy that several investigators have used animal models of NEC that rely on the induction of intermittent ischemia and reperfusion of the intestine, typically through temporary occlusion of the blood supply to the small intestine (Carrasco et al., 2004; Ceylan et al., 2005; Cintra et al., 2008; Dimmitt et al., 2003; Langer et al., 1995). Although such models may provide valuable information regarding the biology of intestinal ischemia-reperfusion injury (a distinct and important

clinical entity in its own right), they only partially share the pathogenetic mechanisms involved with human NEC and infrequently cause the typical pattern of pathology that characterizes NEC. As a result, one should be cautioned when applying these results to the study of NEC. For the purposes of the current discussion, experimental NEC models are considered to be applicable when they rely on pathogenetic mechanisms that are similar to those known to be associated with NEC – specifically the administration of enteral feeds in the preterm or early post-term period, and when they cause similar histopathological and systemic changes that characterize the human disease.

The development of experimental NEC in rat pups

In 1975, Barlow and Santulli developed one of the earliest descriptions of a suitable NEC model, in which the disease was induced through the combined treatment of formula gavage with intermittent episodes of either cold or hypoxic stress (Barlow and Santulli, 1975). NEC induction was validated by histopathology. This paper, which is one of the most frequently cited papers throughout the pediatric surgical literature, included an interventional aspect through its demonstration that the administration of breast milk protected animals from the development of NEC. Several other investigators used this model and refined it. For example, Caplan et al. adapted this model to work with both full-term and premature

roles of pro-inflammatory cytokines in the development of NEC (Seitz et al., 2005), the roles of enterocyte migration in the resolution of NEC (Cetin et al., 2004), and the protective effects of various agents including: maternal cortisol (Israel et al., 1990), intestinal trefoil factor (Guthmann and Kluthe, 2007; Shi et al., 2007), vitamin E (Cadir et al., 2008), captopril (Zani et al., 2008), polyunsaturated fatty acid supplementation (Lu et al., 2007), the inhibition of NF- κ B (De Plaen et al., 2007), administration of pentoxifylline (Travadi et al., 2006), granulocyte colony stimulating factor (Canpolat et al., 2006), arginine (Shah and Shah, 2007) and anti-TNF- α (Halpern et al., 2006). Taken together, these studies illustrate the important role that the rat model has played in advancing our understanding of NEC pathogenesis and in devising novel therapeutic approaches for this disease.

Development of NEC mouse models

The tractability of mouse genetics has enabled many mouse models of clinical diseases to be developed. However, until recently, the development of experimental NEC in mice had not been achieved, primarily owing to the technical challenges presented by the need to gavage feed neonatal mice, which are very small compared with rats. However, several groups have overcome these technical challenges and have developed mouse models of NEC. Caplan et al. induced NEC in mice that were delivered by Cesarean section and then subjected to formula feeding and cold asphyxia stress (Jilling et al., 2006). In this model, the incidence of NEC was determined and validated by a combination of histological scoring and gene expression using quantitative real-time PCR of intestinal samples (Jilling et al., 2006). This study identified a crucial role for Gram-negative bacteria in the pathogenesis of NEC and demonstrated that Toll-like receptor 4 (TLR4)-mutant mice were protected from NEC development (Jilling et al., 2006). Baregamian et al. induced NEC in Swiss Webster mice using a combination of gavage feeds and hypoxia, and demonstrated a role for phosphoinositide 3-kinase (PI3K) signaling in NEC pathogenesis (Baregamian et al., 2007). Halpern et al. induced NEC in wild-type and IL-18-deficient mice by administration of cow's-milk-based formula, cold stress and hypoxia, and showed a crucial role for IL-18 in its pathogenesis (Halpern et al.,

2008). The development of NEC was validated by assessing the presence of histological changes in the ileum (Halpern et al., 2008). Our laboratory has developed a model of experimental NEC in several strains of genetically modified mice, using a combination of twice-daily hypoxia and formula gavage, to establish the potential role of various signaling molecules in the pathogenesis of this disease. In particular, we have induced NEC in interferon γ (IFN- γ) knockout mice (Leaphart et al., 2007b), iNOS-knockout mice (Cetin et al., 2007) and TLR4-mutant mice (Leaphart et al., 2007a) to demonstrate the importance of these signaling molecules in the pathogenesis of NEC. The mechanisms involved probably include signaling molecules that regulate intestinal gap junction communication, and mucosal injury and repair. As with previous models, the ability of these animals to recapitulate the human disease was assessed using a combination of screening for intestinal morphology and characteristic NEC cytokine profiles. Of note, we find that it is not possible to induce NEC in mice beyond 14-21 days of age, probably because they are too mature. Because of growing interest in determining the molecular pathways and inflammatory process important to NEC pathogenesis, there is likely to be increased interest in developing mouse NEC models to exploit the powerful genetic tools available in this species.

Piglet models of NEC

Piglet models to study NEC have been popular for many years, since the gastrointestinal tract of the piglet resembles the human intestine more closely than the rodent tract does, particularly around birth and at weaning (Sangild, 2006). Cohen et al. described a model in which newborn piglets were subjected to a hypoxic insult [50% reduction in baseline partial pressure in arterial oxygen (PaO₂) for 30 minutes] and hypothermic stress (core temperature reduced to 35°C for 30 minutes). After 3-4 days, approximately half of the animals developed histological changes that resembled NEC (Cohen et al., 1991). Crissinger et al. developed an NEC model in 1-day-old piglets using a combination of cow formula and ischemia/reperfusion, and showed an important role for lipids in its pathogenesis (Crissinger et al., 1994). Di Lorenzo et al. administered isosmolar acidified casein solution into intestinal seg-

Clinical terms

- Ascites** – abnormal accumulation of serous fluid inside the intraperitoneal space, such as between organs
- Congenital anomaly** – an unusual feature that is present at birth
- Feeds** – feeding tubes, which pass nutrients directly to the patient at sites along the gastrointestinal tract; oral feeds are administered through the mouth and enteral feeds deposit nutrients directly into the stomach, duodenum or jejunum
- Ileostomy** – creation of an opening into the ileum (part of the small intestine) to bypass all or part of the large intestine
- Inotropic support** – induction of muscle contraction, usually performed to increase the contractility of the heart
- Laparotomy** – open surgical examination of the abdomen
- Leukocytosis** – elevated number of circulating white blood cells
- Mucus fistula** – a permanent abnormal passageway between two organs, in this case between the ileum and large intestine
- Necrosis** – pathological cell death
- Necrotizing enterocolitis (NEC)** – death of intestinal tissue, usually affecting infants or neonates, with unknown cause
- Sepsis** – the presence of infectious organisms (usually bacteria) and/or their toxins in the bloodstream. This systemic illness can lead to a drop in blood pressure known as septic shock that can cause multiple organ failure and death
- Thrombocytopenia** – deficiency of circulating platelets

ments of 1-day-old piglets, and demonstrated the development of intestinal inflammation after 3 hours (Di Lorenzo et al., 1995a). They subsequently demonstrated the protective effects of enterally administered L-arginine on the severity of NEC in this model (Di Lorenzo et al., 1995b). Interestingly, Sangild et al. showed that a large proportion of preterm pigs that were reared in infant incubators and fed a human infant formula, spontaneously developed fatal NEC-like symptoms, and administration of oral colostrum reduced the severity of NEC (Sangild et al., 2006). This study was particularly noteworthy in showing that the development of NEC-like intestinal inflammation occurred after administration of oral colostrum in preterm piglets, without the need to expose to hypoxia or hypothermia. In subsequent studies, these authors further validated their model by evaluation of IL-6 release (Siggers et al., 2008). The newborn piglet model of NEC has been used by oth-

ers who have shown, for instance, that T-cell-mediated mucosal immunity is attenuated in NEC (Anttila et al., 2003), and the importance of reactive oxygen species (Koivusalo et al., 2002), rectosigmoid intramural pH (Koivusalo et al., 2000) and platelet activating factor (Ewer et al., 2004) in NEC development. Taken together, these findings indicate that the piglet model of NEC has important relevance in understanding the pathogenesis of NEC.

Limitations of current animal models

The fact that the underlying causes of NEC remain obscure makes it challenging to develop clinically relevant animal models that perfectly replicate the clinical condition. Therefore, each of the current models suffers from certain limitations. For example, the murine models that use full-term mice replicate the histopathology and cytokine profile of clinical NEC, but suffer from the fact that NEC occurs most commonly in premature infants, and yet this model relies on a full-term animal. Our group and others who favor this model point out that 10-day-old mice are still relatively preterm, i.e. eyes remain closed, skin remains transparent, minimal mobility. Other scientists induce NEC in mice that have been delivered 1 or 2 days early by caesarean section, to more closely model the preterm system. However, these premature mice are usually used to model NEC immediately after they are born and before the intestine has been colonized with bacteria. This poses an additional limitation in terms of the applicability to clinical NEC, which occurs after approximately 7-10 days of age in humans after the intestine has been fully colonized with microbial flora. The rat model of NEC suffers from similar limitations to that of mice, but has the added drawback of being less suitable to the study of specific genes. The piglet model, in which administration of formula to preterm piglets leads to the induction of NEC, is very attractive in terms of simplicity, yet is limited because it lacks the hypoxic insult that is thought to occur with the development of clinical NEC. Thus, a 'perfect' model to understand NEC pathogenesis requires further development.

The future of animal models for NEC research

The utility of animal models of NEC is largely dependent upon their validity with

Clinical and basic research opportunities

- To determine the molecular mechanisms that lead to the development of NEC
- The identification of predictive indicators for disease onset, progression or localization in a particular individual
- The development and characterization of models that are a workable size and that physiologically recapitulate the gastrointestinal environment of a preterm human infant
- Creation of genetic models for NEC – no known mutations or deletions have been identified that cause NEC in model animals and there is no animal model available to study spontaneous NEC

respect to the clinical condition. To this end, the induction of experimental NEC should be associated with a cytokine profile and histopathologic findings that mirror the human disease. Given that this may be achieved in the mouse, the rat and the piglet, each of these systems may provide a platform for studying NEC. Knockout and transgenic systems are being used to understand, in greater depth, the molecular mechanisms that lead to NEC development, leading to a significant advantage of mouse models because these systems are more widely available in the mouse relative to other species. In all cases, careful validation of the model, assessment of its compatibility with the human condition, ease of administration, and a focus on limiting animal suffering whenever possible are considerations that should be entertained when selecting an individual model to use. Through these efforts, it is hoped that progress will be made not only in advancing our understanding of the pathogenesis of this devastating disease, but also in bringing relief to those patients afflicted by it, as well as to their families.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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