Current research on epidemiology, pathogenesis

and management of necrotizing enterocolitis

Simon Eaton[1], Clare M. Rees[1] and Nigel J Hall[2]

[1]UCL Institute of Child Health and Great Ormond Street Hospital for Children,

London, UK, [2]Faculty of Medicine, University of Southampton, Southampton, UK

Corresponding author:

Simon Eaton, PhD

Department of Paediatric Surgery,

UCL Institute of Child Health,

30 Guilford Street,

London,

WC1N 1EH

UK

Telephone: +44 (0)20 7905 2158

Fax: +44 (0)20 7404 6181

Email: s.eaton@ucl.ac.uk

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Abstract

Despite decades of research on necrotizing enterocolitis, we still do not fully understand the pathogenesis of the disease, how to prevent or how to treat the disease. However, as a result of recent significant advances in the microbiology, molecular biology, and cell biology of the intestine of premature infants and infants with necrotizing enterocolitis, there is some hope that research into this devastating disease will yield some important translation into effective prevention, more rapid diagnosis, and novel therapies for the disease.

Introduction

Although necrotizing enterocolitis (NEC) is one of the most common life-threatening surgical diseases affecting neonates, we still do not completely understand the pathogenesis or how to prevent or treat the disease [1]. Probiotics have not eradicated the disease [2] and the surgical outcomes do not appear to have improved over twenty years [3]. The high mortality (around 30% for surgical NEC [3] and long-term morbidity [4,5] of survivors mandates urgent research into the pathogenesis, diagnosis, prevention and treatment of this devastating disease.

In this review article, it is not our aim to comprehensively describe all new advances in NEC, but instead to describe some areas of progress, focusing wherever possible on clinical studies.

Epidemiology

Comprehensive data on NEC incidence and/or surgical outcomes is available from some counties, areas, or healthcare systems, including population-based data from Sweden [6] and the Vermont-Oxford Network [7]. Until recently, data from the UK has been limited to a survey study of 158 NICUs [8], but following the UK National Confidential Enquiry into Patient Outcome and Death report in which gaps in our knowledge about NEC in the UK were highlighted [9], two national prospective studies were initiated, one focused on neonatal aspects, the other on surgical. In the neonatal study, data were prospectively acquired from 163 English neonatal units over 2 years; thus including 118 073 infants, of whom 531 (0·4%) developed severe NEC with a mortality of 48% [10]. Interestingly, given the well-known protective effect of breast milk, in these infants the absolute risk reduction from human milk compared with bovine products was modest. Routinely acquired examination and radiological data from this study has also been used to generate a gestational age specific case definition, which has a

sensitivity of 66%, a specificity of 94% which the authors suggest should be widely adopted [11]. The surgical study, from the British Association of Paediatric Surgeons Congenital Anomalies Surveillance System, collected data from 28 neonatal surgical centres over one year and showed that 236 infants required surgery for NEC in the UK over one year, and highlighted the variation in surgical practices; we eagerly await publication of these data. In both epidemiological studies, infants were identified who died without having surgery. In addition, there are those infants who have a laparotomy in which the whole intestine is necrotic prompting a decision to withdraw active care. This then leads to the question of whether paediatric surgeons should be intervening earlier, and if so, what should be the indications for surgery? A large multi-institutional study from the US has attempted to prognosticate NEC infants into low, intermediate and high risk on the basis of routinely acquired clinical data including radiological and laboratory findings. Their machine-learning model performs well, and the authors suggest that dynamic risk stratification will assist in determining the need for additional diagnostic testing and guide potential therapies [12].

Improved diagnostic methods

Diagnosis of NEC currently relies on a combination of clinical symptoms and signs, and radiological features, which are encapsulated in the Bell's stages of disease, which although frequently criticized, are still widely used to describe populations of infants with NEC. For many years, researchers have sought plasma, urine or stool biomarkers that would be diagnostic for NEC [13]. One of these biomarkers is intestinal fatty acid binding protein (I-FABP), which is released into the circulation from damaged enterocytes, and is excreted in urine. It has been shown in many studies that I-FABP is increased around the time of development of NEC [14]. However, I-FABP concentrations in healthy premature infants are somewhat variable and the half-life of I-FABP in plasma is short, so that use of I-FABP concentration in plasma or urine to diagnose early NEC is limited. Similarly, although I-FABP can usefully distinguish those infants that have more extensive disease and thus require more extensive resection [15], the short half-life means that unless there is ongoing enterocyte damage, some infants with very extensive disease can have paradoxically low I-FABP levels (as all the enterocytes have been lost).

Recently, biomarker discovery studies of the urinary proteome [16] and peptidome [17] have identified proteins and peptides associated with poor outcome (i.e. disease progression). Importantly, the biomarkers alone were not able to completely distinguish progressors from non-

progressors, but were able to when clinical factors were incorporated into the prediction model too. This raises the question of the clinical utility of these markers – neither an ELISA (for I-FABP) nor mass-spectrometry are currently anywhere near point-of-care testing, so whether they can be usefully incorporated into medical and surgical decision making, particularly when clinical information also has to be taken into account, is uncertain. Circulating cytokines are markers of systemic immune status, and pro-inflammatory cytokines have long been known to be elevated in NEC. There has been limited information, however, on whether cytokine measurements can be useful in diagnosis or risk assessment in NEC. Using a cytokine array only IL-10, IL-8 and IL-6 were shown to be significantly higher in surgical NEC than in matched controls [18], similar findings on IL-6 and IL-8 being reported by others. The same researchers then focussed in on IL-8, which is a chemoattractant for neutrophils and macrophages whose intestinal expression has been linked with NEC [19], and showed that IL-8 is a suitable marker for preoperative risk assessment, that IL-8 levels obtained within 6 hours prior to surgery correlate with the actual intraoperative disease extent [20] and that high levels correlate with 60-day mortality [21]. Interestingly, although IL-8 is in theory non-specific, whereas I-FABP discussed above is specific for the gut, IL-8 may in fact be a better marker than I-FABP [22]. IL-8 is a routine test in some hospitals, so unlike many laboratory-based biomarkers, might prove to be clinically useful, although precise cutoffs and criteria for intervention must be developed.

Another type of biomarker that has also seen recent interest is the use of analysis of volatile organic compounds (VOCs) in stool samples as markers of bacterial metabolism. In one pilot study, it was shown that a difference in VOC profile in stool samples between control infants and infants with NEC could be used to predict NEC before clinical symptoms [23], and a larger study aimed to corroborate these findings is currently underway. Another recent study used an 'electronic nose' system, in which VOCs are not quantitatively measured, but differences in intensity across an array of sensors are used to discriminate samples. The study showed that fecal VOC profiles of infants with NEC could be discriminated from controls, from 2-3 days predating the onset of clinical symptoms [24]. Again, there may be problems in utilizing these interesting biomarkers in a clinically useful way,

Given that most premature infants will have some form of continual physiological monitoring it would be useful to incorporate these measures into early warning tools to predict NEC. Some neonatal units are now incorporating sophisticated analysis algorithms into their

bedside monitoring, and ultimately these may provide just such a prediction tool. Abnormal heart rate characteristics have been suggested as a useful clinical tool to predict sepsis [25], and have shown initial promise to predict NEC before clinical diagnosis in a prospective trial setting [26]. However, a more recent study in which the same system was used in routine clinical practice suggested that abnormal heart rate characteristics have limited ability to detect sepsis [27].

Whilst plain radiology remains the primary imaging modality for diagnostic purposes it is clear that ultrasound has promise for imaging infants with NEC. Pneumatosis intestinalis and portal venous gas are both readily seen during abdominal ultrasound and in fact PVG may be detected earlier by US than by plain radiography. However it is the ability of ultrasound to detect infants with more advanced disease and those who may benefit from surgery that has attracted greatest attention. Ultrasound permits examination of the intestinal wall in a much more detailed way than is possible with plain radiographs and in particular allows an assessment of the thickness and perfusion of the intestinal wall and peristalsis. Characteristic perfusion patterns have been associated with inflammation, ischaemia and necrosis [28]. Further information that can be obtained includes the presence of free intra-abdominal gas and the presence and nature of any free intra-abdominal fluid that may be indicative of intestinal perforation. The real challenge is to determine whether US can be used to accurately identify infants with NEC who would benefit from surgery [28,29]). Further prospective evaluation is undoubtedly needed.

Near infra-red spectroscopy (NIRS) provides a non-invasive measurement of tissue oxygen saturation, which potentially allows a real-time window into intestinal perfusion, which is measured as tissue fractional oxygen saturation after taking into account arterial saturation concomitantly measured by pulse oximetry. Although one might assume that in NEC infants, intestinal perfusion would be lower in infants predisposed to NEC, in fact that cerebral oxygenation, rather than intestinal oxygenation, is impaired in the first days of life in infants that go on to get NEC, whereas intestinal perfusion is impaired immediately preceding clinical NEC [30]. In a further study, although NIRS could not distinguish between definite NEC and absence of NEC, it could differentiate between complicated from uncomplicated NEC [31]. Encouragingly, these differences in NIRS readings also correlated well with I-FABP levels, thus validating the use of NIRS to examine intestinal impairment [32].

Pathogenesis

The main factors thought to be involved in the pathogenesis of NEC are: intestinal immaturity, enteral feeds, the intestinal microbiome, inflammation and local ischaemia and/or reperfusion injury. We will briefly discuss recent research in each of these areas below.

Intestinal immaturity

The fetal gut develops in an environment where exposure to microbes is limited. Therefore, premature infants are exposed to a much greater diversity and quantity of bacteria, viruses and fungi. The premature infant gut displays an excessive inflammatory response [19], and toll-like receptor 4 seems to play a key role in this inflammatory response ([33] see below). However, a difference in inflammatory response is not the only aspect of the premature infant intestine that might be affected. The neonatal gut also seems to be more susceptible to intestinal ischaemia/reperfusion injury than adult gut [34], and the activity of carbohydrate digestive enzymes is significantly lower in preterm intestine than term intestine [35]. Gut motility is also different in preterm and term infants [36]. Another potentially important difference between premature and term infants is that of maternal separation. Partial separation of mouse pups from the mother is enough to induce changes in colonic histology and permeability [37], although the relevance of these observations to NEC is unknown.

Enteral feeding

Although it has long been known that firstly, NEC predominantly occurs in premature infants that have been enterally fed, and secondly that human breast milk is protective towards NEC, we do not completely understand how the type of feed interacts with other risk factors. Interestingly, the protective effect of breast milk appears to be dose related [38] although as discussed in the epidemiology section above, the absolute risk reduction may be modest [10]. A huge array of protective factors present in breast milk has been suggested and some of these have been suggested as potential preventative measures or treatments (see below). One mechanism by which feed components could influence intestinal gene expression is epigenetics, defined as '*relating to or arising from non-genetic influences on gene expression'*. As epigenetic changes frequently involve methylation, they are potentially influenced by diet. A current area of research interest is the potential epigenetic effects of breast milk and other enteral feeds [39]. Marked epigenetic changes have been observed in the intestine of premature infants [40], and in a pig model, enteral feeding has been linked with epigenetic changes causing upregulation of pro-

inflammatory genes [41]. In addition, the type of enteral feed can also interact with other risk factors described below such as the gut microbiome [42] and intestinal blood flow [43].

Intestinal microbiome

While the precise role of bacterial agents in the development of NEC is unclear, several factors implicate their involvement. Occasionally NEC is observed to occur in clusters in which identical organisms are responsible [44]. However, different organisms are grown from separate outbreaks so it cannot be claimed that a single organism is involved in development of NEC. Bacterial involvement in the pathogenesis of NEC is also implicated by association; endotoxaemia [45,46] and positive blood cultures are common in infants with NEC and the gastrointestinal pneumatosis found in NEC contains 30% hydrogen [47], a gas produced solely by bacterial metabolism. As long ago as 1975, it was hypothesized that a dysbiosis (imbalance between protective microflora and harmful microflora) was involved in the pathogenesis of NEC [48]. The recent explosion of interest in the intestinal microbiome, and the availability of high throughput pyrosequencing techniques, has led to several relevant research studies in NEC. However, such data are very complex, and analysis of these data in a very heterogeneous disease like NEC is extremely challenging, especially where both the nosocomial microbiota and their measurement methods vary between neonatal units [49]. Nevertheless, studies suggested a loss in microbial diversity to occur immediately before NEC onset [50,51]. These preliminary findings have been recently corroborated by a large multicentre study [52].

Inflammation

Histologically, there is a massive intestinal inflammatory response in NEC. Some authors have even suggested that there may be antenatal precedents to this exaggerated inflammatory response, such as chorioamnionitis. A recent systematic review and meta-analysis of available studies concluded that chorioamnionitis increased the risk of NEC [53]. Differences in the immune response to mucosal damage and the microbiota may also be responsible for the exaggerated inflammatory response in NEC (reviewed [54]). Recent studies have highlighted such differences, such as those showing that intraepithelial T cell receptor γδ lymphocytes are decreased in surgical NEC specimens compared with appropriate controls [55] as are lamina propria T regulatory cells [56]. A key player in intestinal inflammation and the response to pathogens is TLR4, and recent work has shown that TLR4 signalling is important in the

development of NEC [33,57-59]. Intriguingly, TLR4 signalling also links to other factors involved in the pathogenesis of NEC, such as the microcirculation ([59] see below).

Ischaemic injury

From early descriptions, ischaemia/reperfusion injury due to relative splanchnic hypoperfusion was though to play a part in the pathogenesis of NEC. A primary role for intestinal ischaemic damage long fell out of favour, but recently some evidence from both animal models and clinical studies has resurrected the potential role of intestinal ischaemia, although probably not as the sole initiating factor. Experimental studies have suggested that in NEC, there is an impairment in intestinal microcirculation [60-62] which can be improved by direct peritoneal resuscitation [63-66]. A potential role for splanchnic hypoperfusion in NEC has been suggested from a variety of clinical studies: firstly, there is a decline in mesenteric oxygenation when preterm infants are fed during red blood cell transfusion [67]. Secondly, there is increasing recognition that an important subset of infants with NEC have congenital cardiac disease that may predispose to splanchnic hypoperfusion [68]. Thirdly, several clinical studies have suggested that arginine and/or citrulline, amino acids which are important in production of nitric oxide and regulation of intestinal blood flow, are decreased in NEC and that supplementation of infants with arginine may prevent NEC [69-73]. Finally, a recent histological study of resection tissue from infants with NEC has shown that the hypoxia markers hypoxia inducible factor 1alpha (HIF-1alpha) and glucose transporter 1 (GLUT1) were elevated in NEC, but not in spontaneous intestinal perforation or where only a short segment of bowel was affected [74].

Prevention

Dietary / pharmacological approaches

There are several suggested dietary and/or pharmacological approaches to the prevention of NEC. Many of these agents are found in human breast milk, for example lactoferrin is a glycoprotein that forms part of the innate immune response. A small randomised study in VLBW infants found that episodes of sepsis were reduced in infants given oral human lactoferrin but none in the treatment or placebo group developed NEC [75]. A randomised controlled trial of bovine lactoferrin showed a reduced incidence of NEC compared to controls, and a reduced risk of death or progression to stage 3 NEC [76]. Oligosaccharides are complex carbohydrates that are components present at much higher concentration in human breast milk than formula. They

have been shown to prevent NEC in a rodent model [77], have shown promise in preventing NEC in a limited human trial [78] and seem to have generated a recent flurry of activity amongst patent lawyers. Another series of compounds that have potential use in preventing NEC is a new class of TLR4 inhibitors, which by preventing TLR4 activation may inhibit the inflammatory cascade in NEC [79,80]. Of course, probiotics have polarized the neonatal community regarding their ability (or not) to prevent NEC [81]. An extensive discussion is beyond the scope of this review, and unfortunately we seem no closer to a definitive answer despite large-scale trials such as ProPrems [82] and PIPS [83]. Hopefully mechanistic studies (e.g. [84,85] [86,87]), together with more detailed understanding of the microbiome and its influence of development of NEC (referred to above) may help us to find a way out of this impasse.

Therapy

Recent evidence from three different research groups has suggested that stem cells are able to influence the course of the disease in experimental NEC (reviewed [88]): amniotic fluid stem cells [89], mesenchymal stem cells [90,91] and enteric neural stem system cells [92]. In addition to the beneficial effects of AFS cells and the conditioned medium from AFS cells, other recent evidence highlights the potential role of factors in amniotic fluid in the prevention and/or treatment of NEC. Pig and human amniotic fluid, centrifuged so presumed to be largely cell-free, have been shown to stimulate proliferation and migration of rat intestinal epithelial cells *in vitro* [93]. In a well-established, clinically relevant model of spontaneous NEC in preterm piglets, postnatal minimal enteral feeding with porcine amniotic fluid decreased inflammation together with incidence and severity of NEC[94], but had no effect when given later, with full enteral feeding, although the beneficial effect during minimal enteral feeding was not consistently observed [93] [94]. Another group has shown that enteral delivery of amniotic fluid decreases severity of NEC in a mouse model [95]. This effect was shown to be mediated via epidermal growth factor (EGF) present in amniotic fluid, and its receptor, epidermal growth factor rector (EGFR). In additional experiments, both *in vitro* and *in vivo* it was shown that amniotic fluid modulates the ability of lipopolysaccharide to trigger toll-like 4- receptor (TLR4) –dependent inflammatory signalling, and these effects were mediated via EGF/EGFR[95]. It has also been suggested that these protective effects of amniotic fluid are mediated via hepatocyte growth factor [96].

Conclusions

Despite understanding the cardinal features involved in the pathogenesis of NEC, and decades of research, we still do not fully understand this devastating disease, how to prevent it or treat it. Nevertheless, the broad range and depth of research on the topic does lead to a degree of optimism that clinicians and scientists will hopefully be able to deliver novel preventative, diagnostic and therapeutic strategies that will translate into improved outcomes.

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