See ARTICLE page 1166

Are EGF and TLR-4 crucial to understanding the link between milk and NEC ?

JPM Derikx[1], BW Kramer[2,3,4] and TGAM Wolfs[2,3]

No milk today, NEC would not go away (inspired by a Herman's Hesmits song)

Premature neonates are at risk for compromised postnatal outcomes such as poor nutritional uptake, failure to thrive, and subsequent postnatal growth restriction, which are all conditions with potential irreversible, lifelong consequences.[1,2] Moreover, prematurity is the most important independent risk factor for necrotizing enterocolitis (NEC), a severe gastrointestinal disease with a mortality of 20–40%, affecting 10% of very low birth weight infants.[3] NEC is a multifactorial disease and predisposing or underlying disorders might have a prenatal transitional and postnatal origin.[3,4] A derailed inflammatory response against commensal bacteria by the immature intestine following mucosal injury is the leading hypothesis in the pathophysiology of NEC.[3,5] Since human breast milk protects infants from NEC,[6,7] a variety of studies focussed on unraveling the protective component(s) of breast milk and their mode of action.

Breast milk contains numerous components that have immunomodulatory and gut-maturating properties, including human milk oligosaccharides, caseins, and whey proteins, such as growth factors (insulin-like growth factor, heparin-binding epidermal growth factor-like growth factor (HB-EGF), transforming growth factor-β 2, and epidermal growth factor (EGF)).[8] The first evidence for a protective role of EGF against NEC was suggested from a case, reporting the recovery of necrotic bowel disease in a critically ill 8-month-old girl after the infusion of EGF systemically.[9] This was followed by the observation that infants with NEC had significantly diminished levels of salivary and serum EGF compared with postnatal age- and birth weight-matched babies.[10] Furthermore, EGF is highest in early breast milk with $\sim 100\ \mathrm{ng\ ml^{-1}}$ and decreases over lactation.[11,12] Interestingly, EGF levels in breast milk of mothers with extremely preterm babies are much higher than in term infants.[11] Numerous experimental studies in rat and mice NEC models confirmed the protective effect of EGF,[13,14] supporting the hypothesis that EGF in breast milk may be, at least in part, responsible for the protection against NEC. However, the exact mechanism for its beneficial effects needed to be clarified.

This problem has now been experimentally addressed in a rodent NEC model and *in vitro* by Good *et al.* in today's issue.[15] First, both *in vitro* (in IEC-6 enterocytes, expressing both Toll-like receptor-4 (TLR-4) and epidermal growth factor receptor (EGFR)) and *in vivo* (wild-type and transgenic neonatal mice) experiments demonstrated that breast milk inhibited TLR-4 signaling via EGF-mediated EGFR activation. Next, using IEC-6 cells, it was shown that the downstream target GSK3β, a critical component of the Wnt/β–catenin signaling pathway, mediates this EGFR-dependent TLR-4-driven immune activation, thereby improving enterocyte proliferation and inhibition of enterocyte apoptosis. Finally, newborn mice receiving breast milk were protected to develop experimental NEC via the inhibition of TLR-4, requiring EGF. For these experiments, they both used mice lacking intestinal EGFR, EGF-depleted milk and supplementation of recombinant EGF in milk. These observations provide a novel mechanistic link between EGF from breast milk and various innate immune receptors.

However, it is not clear whether EGF is the sole compound from breast milk that is of importance in the protection for NEC. Besner *et al.*[16,17] have shown that HB-EGF is protective for experimental NEC in a newborn rat model, *in vitro* and *ex vivo* organoid cultures. Reduction of NEC symptoms was dependent upon EGFR activation and mediated via the MEK 1/2 and PI3K signaling pathways, protecting intestinal stem cells from injury. Furthermore, HB-EGF was shown to preserve intestinal microvascular blood flow in rat pups with experimental NEC[18] along with increasing enterocyte migration and

[1]Department of Surgery, Maastricht University Medical Center and Nutrition and Toxicology Research Institute, Maastricht, The Netherlands. [2]Department of Pediatrics/Neonatology, Maastricht University Medical Center, Maastricht, The Netherlands. [3]School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands and [4]School of Mental Health and Neuroscience, Maastricht University, Maastricht, The Netherlands. Correspondence: BW Kramer, (b.kramer@maastrichtuniversity.nl)

published online 3 June 2015. doi:10.1038/mi.2015.37

proliferation.[19] The human milk oligosaccharides (HMO) were also found to improve the survival and reduce the severity in rat pups with experimental NEC. In particular, the HMO disiallacto-N-tetraose was identified to be beneficial.[20] Unfortunately, both EGF and HB-EGF are absent from all commercially available infant formulas. Consequently, no studies have been performed in babies using EGF or HB-EGF.

Furthermore, recent studies identified more pathways that are activated by EGF or via its receptor, including the involvement of EGFR in lipopolysaccharide-induced cyclooxygenase-2 expression in enterocytes.[21] Among these pathways is autophagy, a homeostatic process including the sequestration of cytoplasmic components in autophagosomes, which was reported to be activated in babies with NEC and in a rat model of NEC, which appeared to be also EGF dependent.[22] EGF also alters the balance between regulators of apoptosis thereby reducing intestinal epithelial cell apoptosis and maintaining epithelial homeostasis.[13] Another important role of EGF is its direct effect on neonatal gut development, as was shown by the exogenous infusion of EGF *in utero* in rabbits, leading to stimulated intestinal growth and increased maturation of intestinal enzyme activity.[23] Using transgenic mice and cultured enteroids, the group of Hackam[24] recently demonstrated that TLR-4 activation induces endoplasmic reticulum-stress mediated apoptotic cell death of intestinal stem cells and subsequent NEC development. At present, it remains unclear whether the protective effects of EGF rely on improved epithelial integrity, on anti-inflammatory aspects, or the combination of both phenomena.

The results of Good *et al.* need to be considered in the context that the development of the murine immune system does not resemble the situation in humans. This might explain the observed differences in the expression of pathogen recognition receptors in mice/rats on one hand and preclinical animal models and humans on the other hand and direct the subsequent discussion whether NEC development results from decreased or exaggerated immune activation. From preclinical large models and data in humans, it appears that the development of innate immune components is gestation dependent. In particular, TLR-4 mRNA levels are lower in preterm than in term pigs and highest at a developmental stage when NEC sensitivity is minimal.[25] In sheep, we showed relatively low intestinal TLR-4 and MD-2 mRNA levels at early gestational ages, which were further downregulated during endotoxin-induced chorioamnionitis.[26] In humans, increased TLR-2 and TLR-4 mRNA levels were shown in the intestine of immature babies when compared with children of older age, whereas its negative regulators (e.g., A-20 and TOLLIP) were downregulated in immature fetal intestine and NEC patients when compared with tissue from children.[27] Premature infants and neonates with NEC did not express MD-2 in their intestinal epithelium, whereas MD-2 protein was expressed in the healthy term neonatal and adult intestine. Moreover, NEC patients also lacked MD-2 staining in the abundantly recruited inflammatory cells.[28] In line with these data, Jenke *et al.* showed that human β-defensin-2 protein and mRNA levels and MD-2 and TLR-4 mRNA were almost absent in intestinal specimens from infants with severe NEC.[29] Collectively, these data from large animal models and humans suggest that the immune system of the immature gut inadequately responses to microflora which colonize the gut directly after birth.

The next step is to translate the new mechanistic insights by which breast milk, a multipotent drug, mediates protection against the development of NEC into the clinic for babies that are fed with formula. This translational approach can be conducted in large animal models including pigs, sheep, and primates since organ development, the developmental biology, and the physiology of such large animals closely resembles the human situation.[30,31] In addition, studies in babies were till recent hampered by lack of (noninvasive) markers for intestinal inflammation, damage, and maturation. Importantly, we recently reported on the availability of such human gut-specific biomarkers, which enable us to measure gut maturation and to early diagnose systemic and intestinal inflammation in NEC patients, which may be of help for the implementation of these findings into clinical practice.[32–36]

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Andrews, W.W., Goldenberg, R.L., Faye-Petersen, O., Cliver, S., Goepfert, A.R. & Hauth, J.C. The Alabama Preterm Birth study: polymorphonuclear and mononuclear cell placental infiltrations, other markers of inflammation, and outcomes in 23- to 32-week preterm newborn infants. *Am. J. Obstet. Gynecol.* **195**, 803–808 (2006).
2. Graham, P.L. 3rd, Begg, M.D., Larson, E., Della-Latta, P., Allen, A. & Saiman, L. Risk factors for late onset gram-negative sepsis in low birth weight infants hospitalized in the neonatal intensive care unit. *Pediatr. Infect. Dis. J.* **25**, 113–117 (2006).
3. Neu, J. & Walker, W.A. Necrotizing enterocolitis. *N. Engl. J. Med.* **364**, 255–264 (2011).
4. Been, J.V., Lievense, S., Zimmermann, L.J., Kramer, B.W. & Wolfs, T.G. Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. *J. Pediatr.* **162**, 236–242 (2013).
5. Gordon, P.V. & Swanson, J.R. Necrotizing enterocolitis is one disease with many origins and potential means of prevention. *Pathophysiology* **21**, 13–19 (2014).
6. Lucas, A. & Cole, T.J. Breast milk and neonatal necrotising enterocolitis. *Lancet* **336**, 1519–1523 (1990).
7. Quigley, M.A., Henderson, G., Anthony, M.Y. & McGuire, W. Formula milk versus donor breast milk for feeding preterm or low birth weight infants. *Cochrane Database Syst. Rev.*, CD002971 (2007).
8. Chatterton, D.E., Nguyen, D.N., Bering, S.B. & Sangild, P.T. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int. J. Biochem. Cell Biol.* **45**, 1730–1747 (2013).
9. Sullivan, P.B., Brueton, M.J., Tabara, Z.B., Goodlad, R.A., Lee, C.Y. & Wright, N.A. Epidermal growth factor in necrotising enteritis. *Lancet* **338**, 53–54 (1991).
10. Helmrath, M.A., Shin, C.E., Fox, J.W., Erwin, C.R. & Warner, B.W. Epidermal growth factor in saliva and serum of infants with necrotising enterocolitis. *Lancet* **351**, 266–267 (1998).
11. Dvorak, B., Fituch, C.C., Williams, C.S., Hurst, N.M. & Schanler, R.J. Increased epidermal growth factor levels in human milk of mothers with extremely premature infants. *Pediatr. Res.* **54**, 15–19 (2003).

12. Moran, J.R., Courtney, M.E. & Orth, D.N. *et al.* Epidermal growth factor in human milk: daily production and diurnal variation during early lactation in mothers delivering at term and at premature gestation. *J. Pediatr.* **103**, 402–405 (1983).
13. Dvorak, B. Milk epidermal growth factor and gut protection. *J. Pediatr.* **156**, S31–S35 (2010).
14. Coursodon, C.F. & Dvorak, B. Epidermal growth factor and necrotizing enterocolitis. *Curr. Opin. Pediatr.* **24**, 160–164 (2012).
15. Good, M. *et al.* Breast milk protects against the development of necrotizing enterocolitis through inhibition of Toll Like Receptor 4 in the intestinal epithelium via activation of the epidermal growth factor receptor. *Mucosal Immunol.* (2015) (this issue).
16. Feng, J., El-Assal, O.N. & Besner, G.E. Heparin-binding EGF-like growth factor (HB-EGF) and necrotizing enterocolitis. *Semin. Pediatr. Surg.* **14**, 167–174 (2005).
17. Chen, C.L. *et al.* Heparin-binding EGF-like growth factor protects intestinal stem cells from injury in a rat model of necrotizing enterocolitis. *Lab. Invest.* **92**, 331–344 (2012).
18. Yu, X., Radulescu, A., Zorko, N. & Besner, G.E. Heparin-binding EGF-like growth factor increases intestinal microvascular blood flow in necrotizing enterocolitis. *Gastroenterology* **137**, 221–230 (2009).
19. Feng, J. & Besner, G.E. Heparin-binding epidermal growth factor-like growth factor promotes enterocyte migration and proliferation in neonatal rats with necrotizing enterocolitis. *J. Pediatr. Surg.* **42**, 214–220 (2007).
20. Jantscher-Krenn, E. *et al.* The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats. *Gut* **61**, 1417–1425 (2012).
21. McElroy, S.J. *et al.* Transactivation of EGFR by LPS induces COX-2 expression in enterocytes. *PLoS One* **7**, e38373 (2012).
22. Maynard, A.A. *et al.* Epidermal growth factor reduces autophagy in intestinal epithelium and in the rat model of necrotizing enterocolitis. *Am. J. Physiol.* **299**, G614–G622 (2010).
23. Buchmiller, T.L. *et al.* Effect of transamniotic administration of epidermal growth factor on fetal rabbit small intestinal nutrient transport and disaccharidase development. *J. Pediatr. Surg.* **28**, 1239–1244 (1993).
24. Afrazi, A. *et al.* Toll-like receptor 4-mediated endoplasmic reticulum stress in intestinal crypts induces necrotizing enterocolitis. *J. Biol. Chem.* **289**, 9584–9599 (2014).
25. Bering, S.B., Bai, S., Zhang, K. & Sangild, P.T. Prematurity does not markedly affect intestinal sensitivity to endotoxins and feeding in pigs. *Br. J. Nutr.* **108**, 672–681 (2012).
26. Wolfs, T.G. *et al.* Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. *PLoS One* **4**, e5837 (2009).
27. Nanthakumar, N. *et al.* The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS One* **6**, e17776 (2011).
28. Wolfs, T.G. *et al.* Localization of the lipopolysaccharide recognition complex in the human healthy and inflamed premature and adult gut. *Inflamm. Bowel Dis.* **16**, 68–75 (2010).
29. Jenke, A.C., Zilbauer, M., Postberg, J. & Wirth, S. Human beta-defensin 2 expression in ELBW infants with severe necrotizing enterocolitis. *Pediatr. Res.* **72**, 513–520 (2012).
30. Wolfs, T.G., Jellema, R.K., Turrisi, G., Becucci, E., Buonocore, G. & Kramer, B.W. Inflammation-induced immune suppression of the fetus: a potential link between chorioamnionitis and postnatal early onset sepsis. *J. Matern. Fetal Neonatal Med.* **25**, 8–11 (2012).
31. Sangild, P.T., Thymann, T., Schmidt, M., Stoll, B., Burrin, D.G. & Buddington, R.K. Invited review: the preterm pig as a model in pediatric gastroenterology. *J. Anim. Sci.* **91**, 4713–4729 (2013).
32. Derikx, J.P., Luyer, M.D., Heineman, E. & Buurman, W.A. Non-invasive markers of gut wall integrity in health and disease. *World J. Gastroenterol.* **16**, 5272–5279 (2010).
33. Reisinger, K.W. *et al.* Intestinal fatty acid-binding protein: a possible marker for gut maturation. *Pediatr. Res.* **76**, 261–268 (2014).
34. Reisinger, K.W., de Vaan, L., Kramer, B.W., Wolfs, T.G., van Heurn, L.W. & Derikx, J.P. Breast-feeding improves gut maturation compared with formula feeding in preterm babies. *J. Pediatr. Gastroenterol. Nutr.* **59**, 720–724 (2014).
35. Reisinger, K.W. *et al.* Noninvasive measurement of fecal calprotectin and serum amyloid A combined with intestinal fatty acid-binding protein in necrotizing enterocolitis. *J. Pediatr. Surg.* **47**, 1640–1645 (2012).
36. Reisinger, K.W. *et al.* Non-invasive serum amyloid A (SAA) measurement and plasma platelets for accurate prediction of surgical intervention in severe necrotizing enterocolitis (NEC). *PLoS One* **9**, e90834 (2014).