Gut at Risk

Epithelial nutrient digestion and absorption, protection and defence

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Chapter 1

General introduction

GENERAL INTRODUCTION

The newborn's transition from intrauterine to extrauterine life is marked by a chain of rapid and complex physiological changes. For example, this adaptive process is characterised by the transition from foetal 'respiratory' activity to normal ventilation.[1] Onset of respiration stimulates a series of cardiopulmonary changes – e.g. increase in pulmonary blood flow and closure of the foramen ovale and ductus arteriosus – as the newborn makes the transition from foetal to neonatal circulation. Furthermore, in utero, the foetus is dependent on the placenta and swallowed amniotic fluid for its nutrition. Pivotal, too, is the physiological transition that occurs as the foetus, once born, replaces the placenta with his gastrointestinal (GI) tract to obtain nutrition. Enteral feeding, preferably breast milk, is vital for optimal growth and development of the newborn.[2] Therefore, adequate gut development and maturation is indispensable in maintaining neonatal health.

Development of the intestinal tract

Human gut development starts during the fourth week of gestation with the creation of an endodermal gut tube distinguishable into blind-ended cranial foregut, blind-ended caudal hindgut and midgut.[3, 4] The midgut differentiates into distal duodenum, jejunum, ileum, coecum, ascending colon, and two-thirds of the transverse colon. By 13 weeks of gestation, intestinal organogenesis is complete. Ontogeny of the intestine during the second and third trimesters of gestation is characterised by continued growth with characteristic rotations, formation of a crypt-villus axis, and gut-specific cellular and functional epithelial differentiation. Several molecular pathways – e.g. hedgehog, bone morphogenetic protein and Notch signalling pathways, the Hox and Sox transcription factors, and the Wnt/β-catenin signalling pathway – regulate these processes.[5-8] These developmentally critical pathways are important for intestinal morphology, epithelial homeostasis (i.e. proliferation and differentiation) and apoptosis of the intestinal epithelium, implying that intestinal morphology and epithelial differentiation are genetically imprinted. The intestinal epithelium develops from a monolayer of undifferentiated, later differentiated, columnar epithelial cells appearing from the stem cell compartments in the crypts. The stem cells produce a transit cell population that, after additional divisions, migrates and differentiates upwards the villi or downwards to the bottom of the crypts (Figure 1). The epithelial cells form a barrier that separates the external environment, i.e. the gut lumen, from the protected internal milieu. The small intestinal epithelium is composed of four different cell types: absorptive enterocytes, mucus-secreting goblet cells, Paneth cells and a variety of enteroendocrine cells. While enterocytes, goblet cells and enteroendocrine cells are located along the villus and in the upper part of the crypt, Paneth cells reside at the crypt base (Figure 1). The colonic epithelium lacks Paneth cells; its enterocytes, goblet cells and enteroendocrine cells are organised into crypts and surface cuffs. The intestinal epithelium exerts many physiological functions by expressing cell-specific proteins of which several are present during foetal life.

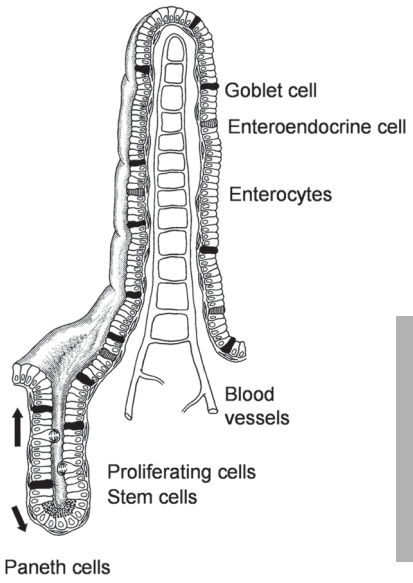

Figure 1
Schematic representation of the small intestinal epithelium (as published by Dekker *et al.* 2002[9]). The small intestinal epithelium contains four different cell types: enterocytes, goblet cells, enteroendocrine cells and Paneth cells.

Small intestinal enterocytes enable digestion, uptake and transport of dietary nutrients by expressing substances such as the sugar-degrading enzyme sucrase-isomaltase (SI), the lactose-degrading enzyme lactase, hexose transporters and fatty acid binding proteins.[10-12] Brush border enzymes start developing early in gestation. For example, SI protein reaches substantial levels between gestational weeks 10 and 22, then gradually increases in level prior to birth.[11] After birth, levels rapidly decline to levels found early in gestation. Lactase is expressed in human foetal intestine at a time in gestation just after the onset of SI expression.[11] The expression remains relatively low till 24 weeks of gestation, after which a gradual increase in activity is observed. At 32 weeks of gestation a marked increase in lactase activity is found, continuing through birth and early infancy. Furthermore, enterocytes of the small intestine are responsible for hexose transport across the epithelium. The major hexose transporters are the apical Na^+-coupled glucose transporter SGLT-1, the glucose transporter Glut2, located on the basal membrane, and the fructose transporter Glut5, located on the apical membrane of enterocytes.[13] In foetal small intestine, SGLT-1 mRNA is detected at 17 weeks of gestation and Glut2 and Glut5 mRNA are already observed at 11 weeks post-conception.[13-15] Moreover, Glut5 protein has been identified on the apical membrane of human foetal small intestine at 16-25 weeks of gestation.[15] Colonic enterocytes are specialised in electrolyte and water absorption by expressing, e.g. carbonic anhydrases (CAs) detectable in the foetal GI tract from the second trimester.[16-18] Enterocytes also synthesise membrane-bound mucins (MUC), like MUC1, MUC3A, MUC3B and MUC4.[19, 20] These mucins are involved in epithelial cell-signalling, adhesion, growth, and modulation of the immune system, and more recent studies have also implicated a role for them in cellular behaviour, i.e. during proliferation and differentiation as reviewed by Hollingsworth *et al.*[20] Goblet cells synthesise the secretory mucin 2 (MUC2), the structural component of the intestinal mucus layer that serves as a barrier protecting the epithelium from mechanical stress, bacteria, viruses and other pathogens.[21-23] During foetal intestinal development mucin mRNAs are

detected as early as 6.5 weeks of gestation (MUC3) and MUC2 mRNA is present after 9 weeks of gestation.[24] Goblet cells are also known to synthesise and secrete trefoil factor 3 (TFF3), a bioactive peptide involved in epithelial protection and repair.[25] Trefoil factor 3 is clearly expressed in the intestinal mucosa as early as 11–12 weeks of gestation.[26] Furthermore, both Paneth cells and enterocytes are instrumental in the epithelial defence by synthesising antimicrobial peptides (e.g. lysozyme and defensins 5 (HD5) and 6 (HD6)) and alkaline phosphatase (AP), respectively.[27] These peptides appear early in human foetal intestine; lysozyme is detectable in foetal Paneth cells at 20 weeks, HD5 and HD6 mRNA expression is detected at 13.5 weeks and AP activity is detectable in foetal duodenum as early as 7 weeks of gestation.[28–30] Enteroendocrine cells are specialised in the mucosal secretion of hormonal peptides such as glucagon-like-peptide 2 (GLP-2) and peptide YY (PYY), already present during early foetal life.[13]

Thus, by developing a highly organised functional intestinal epithelium, term newborns are able to tolerate enteral feeding essential for adequate growth and development. However, in the foetal and/or neonatal period, disturbance of normal intestinal development or intestinal diseases may alter epithelial cell functions – and hence intestinal functions.

Foetal and neonatal intestinal disorders

Advances in prenatal, neonatal and surgical care have increased survival rates of premature infants (born before 37 weeks of gestation) and (pre)term infants born with a congenital anomaly.[31–39] Consequently, neonatologists, paediatricians and paediatric surgeons are facing a constellation of congenital and acquired anomalies, often involving the GI tract.

Several congenital anomalies of the intestinal tract may occur during embryonic or foetal life, i.e. Meckel's diverticulum, intestinal atresia, volvulus and malrotations. Normal intestinal development in utero might be disrupted in these circumstances. Ontogeny of the GI tract is thought to be governed by complex interactions between genetic factors and extrinsic or environmental factors. Of the latter, particularly the role of the contents of the intestinal lumen, i.e. swallowed amniotic fluid, in the development of the GI tract has been studied. For example, animal studies found that interruption of amniotic fluid ingestion results in severe GI tract growth retardation.[40–42] However, studies describing intestinal development in human infants born with congenital anomalies of the GI tract are largely lacking.

Necrotising enterocolitis (NEC) is the most devastating acquired intestinal disease in the neonatal period.[43] It most likely represents the clinical culmination of various risk factors (e.g. prematurity, enteral feeding, hypoxia and pathogenic bacterial colonisation) interacting with each other to produce severe intestinal injury through an inflammatory pathway.[44] NEC predominantly affects premature low birth weight infants and often necessitates extensive bowel resection and the creation of a temporary entero- or colostomy. Even if intestinal surgery is successful and food intake is adequate, the infant may still show prolonged GI dysfunction characterised by impaired motility and malabsorption of dietary nutrients, micronutrients, water and electrolytes, post-operatively. The critical state of malabsorption

can be defined as short bowel syndrome (SBS).[45] The literature provides various definitions of SBS, based either on anatomical loss of bowel length or on prolonged periods of parenteral nutrition, so-called functional SBS.[46] SBS presents with a variety of clinical symptoms ranging from gastric acid hypersecretion, functional pancreatic insufficiency, biliary lithiasis and liver injury, bone disease, and vascular and liver complications related to prolonged use of total parenteral nutrition. Small bowel transplantation, sometimes combined with a liver transplant, may be necessary in some cases.[47]

Various methods, such as the sugar absorption test[48], gut hormone profiles[49, 50] and enterostomy fluid analysis[51], have been introduced to monitor the condition of the residual bowel in human neonates after intestinal surgery. However, specific data on human intestinal epithelial functions such as nutrient digestion and absorption, protection, defence, and proliferation, are still scarce.

Mucus as a physical barrier

Given the key role of the intestine in maintaining the neonate's health, it is essential that intestinal integrity and epithelial barrier functions adequately protect against pathogenic bacterial insults and noxious substances. Apart from its immunologic barrier function (sIgA, sIgM), the intestine has several non-specific barrier functions, as provided by its continuous mucus layer, epithelial protein expression (e.g. lysozyme and defensins), peristalsis and colonisation of commensal bacteria. All of these processes closely interact to form a solid intestinal barrier.[52] Infants born prematurely, however, may show immature mucus layer and thus impaired intestinal barrier function, which might be conducive to the development of intestinal disease (e.g. NEC). In this context, the necessity of having an intact mucus layer as a substantial component of the non-specific barrier function is obvious.

The mucus layer forms a physical barrier between the underlying epithelium and the lumen of the GI tract.[21] As mentioned above, it mainly consists of the secretory mucin MUC2, which gives mucus its high density and viscoelasticity.[53] The peptide backbone of MUC2 is characterised by tandem repeat sequences rich in threonine, serine and proline.[23] Both thickness and composition of the mucus layer – and thus barrier function – depend on the dynamic equilibrium between anabolic processes (expression, synthesis and secretion from goblet cells) and catabolic processes (degradation).[53] Furthermore, mucus layer integrity has been associated with the whole-body nutritional state. For example, protein-energy malnutrition[54], starvation[55] and total parenteral feeding[56, 57] will alter the mucus layer by decreasing the mucus content. Besides, dietary restriction of the essential amino acid threonine was found to impair intestinal mucin synthesis, at least in rats.[58]

Among other essential amino acids, threonine seems particularly important in maintaining gut barrier function. Studies in neonatal pigs showed that dietary threonine is utilised in large amounts by the portal-drained viscera (intestine, pancreas, spleen and stomach).[59-61] Furthermore, in human neonates, the splanchnic tissues utilise 70-80% of dietary threonine intake, irrespective of the amount of enteral threonine intake (unpublished data Van der

Schoor *et al.*). Because MUC2 is particularly rich in threonine, the high visceral threonine requirement presumably reflects the synthesis rate of MUC2. Threonine therefore might be of critical nutritional importance in maintaining the protective mucus layer, and thus intestinal barrier function.

As mentioned earlier, adequate gut development and maturation is indispensable in maintaining neonatal health. Especially, preterm infants and infants with a congenital intestinal anomaly are at more risk to develop a compromised gut. Several authors have shown that these are prone to develop diseases in later life.[62, 63] Furthermore, intolerance to enteral feeding and subsequent malnutrition in the first weeks of life might have a sustained effect on later outcome. For example, Lucas *et al*. demonstrated that suboptimal neonatal nutrition influences neurodevelopment and has major effects on later cognitive function in preterm infants.[64, 65] Overall, these effects may be consequences of 'programming', whereby a stimulus at a critical, sensitive period of early life results in long-term changes in physiology or metabolism. Neonatal care, therefore, must focus on reaching excellent condition of the newborn's gut, i.e. providing adequate nutrient absorption, intestinal defence, and intestinal barrier function to optimise growth and development. The studies described in this thesis aimed to gain more insight into gut epithelial functions in newborns with various intestinal diseases and into the intestinal fate of threonine, specifically in maintaining epithelial barrier function.

THESIS OUTLINE AND AIMS

The overall aims of the work presented in this thesis are:

1. To evaluate intestinal epithelial functioning and epithelial differentiation in human neonates after intestinal surgical intervention.
2. To study intestinal barrier function by means of MUC2 synthesis and precursor metabolism in experimental animal models and human neonates.

Part I: Intestinal epithelial functions

The first part of this thesis describes various intestinal epithelial functions. **Chapter 2** describes a study evaluating net weekly dietary protein absorption capacity of the small intestine in human neonates with an enterostomy created at intestinal surgical intervention. The next two chapters focus on the intestinal cell-type-specific protein expressions of enterocytes, goblet cells and Paneth cells as parameters for intestinal epithelial differentiation, and thus epithelial functioning. Aiming to demonstrate the relative involvements of the contents of the intestinal lumen and the genetic programme in intestinal epithelial development, **chapter 3** describes intestinal epithelial differentiation proximal and distal to a jejunal or ileal atresia in early human postnatal life. Epithelial cell-type-specific protein expression in the residual bowel of human neonates who underwent bowel resection for NEC is evaluated in **chapter 4**.

Part II: Gut barrier function

As the neonatal pig is considered to be an appropriate model for the human infant, **chapter 5** describes the predominant metabolic fates of dietary threonine for different protein intakes in piglets. Then, we used Muc2-deficient mice to directly address the question whether dietary threonine is mainly utilised for intestinal Muc2 synthesis **(Chapter 6)**. **Chapter 7** describes a novel tracer method to determine the role of dietary threonine in intestinal MUC2 synthesis and to calculate the Fractional Synthetic Rate (FSR) of small intestinal MUC2 in human neonates as a parameter for intestinal barrier function *in vivo*. **Chapter 8** finally presents the results of a study investigating systemic threonine incorporation into small intestinal MUC2.

A general discussion in which we also give our recommendations for future, relevant research completes this thesis **(Chapter 9)**. The results of the studies are summarised in **chapter 10**.

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Part I

Intestinal epithelial functions

Chapter 2

Dietary protein absorption of the small intestine in human neonates

Maaike W. Schaart, Adrianus C.J.M. de Bruijn, Dick Tibboel, Ingrid B. Renes and Johannes B. van Goudoever

Under review

ABSTRACT

Background: The intestine plays a key role in the absorption of dietary proteins, which determines growth of human neonates. Bowel resection in the neonatal period brings loss of absorptive and protective surface and may consequently lead to malabsorption of dietary nutrients. However, there are no data on net dietary protein absorption of the small intestine in the period following intestinal surgery in human neonates. We therefore evaluated dietary feeding tolerance and quantified net dietary protein absorption capacity of the small intestine in human neonates in whom a temporary jejunostomy or ileostomy was created.

Methods: Seventeen patients were included in the study. We collected small intestinal outflow fluid at the level of the enterostomy weekly for 24–48 hours during weeks 3 through 6 post-operatively. Protein levels in the intestinal outflow fluid were determined by Bicinchoninic Acid (BCA) assay.

Results: In 14 patients an enteral intake of more than 100 ml/(kg·d) was reached at a median of 17 (8–32) days post-operatively. Three patients did not reach this level within the study period. Overall, the net dietary protein absorption capacity was 70–90% of the total enteral protein intake.

Conclusions: This study demonstrates that the dietary protein absorption capacity of the small intestine is intact in human neonates following intestinal surgery in a very critical period of their lives. Furthermore, our results do not support the use of hydrolysed or elemental formula feeding in newborns with an enterostomy to improve amino acid uptake.

Abstract

INTRODUCTION

The major goal of infant feeding is to provide an optimal balance of dietary nutrients for growth and development. Adequate dietary protein intake is a key component in this balance.[1, 2] Then, a well-functioning small intestine is indispensable in maintaining optimal digestion and absorption of dietary nutrients. Several (congenital) gastrointestinal anomalies, such as intestinal atresia, volvulus, necrotising enterocolitis (NEC), gastroschisis and extensive Hirschsprung's disease, may however reduce absorptive capacity during foetal life and/or may require extensive small bowel resection with creation of a temporary enterostomy in the neonatal period.[3] The prominent clinical consequence of small bowel resection is loss of absorptive and protective surface, which in turn may lead to malabsorption of dietary nutrients.[4] Neonates experiencing intestinal failure require adapted nutrition, often a combination of parenteral nutrition and (minimal) enteral feeding. Present treatment strategies primarily support the process of intestinal adaptation, aiming at improving enteral feeding tolerance and absorption capacity. Over the last decades, several authors found the intestinal adaptation process to be characterised by epithelial morphological and functional changes compensating for the functional loss of resected bowel.[5-9] Dietary fibre, free fatty acids, short chain triglycerides, and glutamine have been proposed to facilitate adaptive responses.[10-14] Therefore, a semi-elemental diet is common practice after excessive bowel resection.[4] Randomised clinical trials supporting this practice have nevertheless not been accomplished to date. By contrast, there is also a tendency to use breast milk under these circumstances, at least in term neonates not suffering from NEC.[4, 15] The optimal feeding strategy for infants following (extensive) bowel resection remains controversial therefore.[4] Type of enteral feeding may vary with age, extent of resection and underlying general and bowel health. Oral high-fat, low-carbohydrate diets are currently used in infants following bowel resection.[16] Yet the irreversible losses of proteins, fat, carbohydrates and free amino acids via the intestinal outflow fluid in patients with an enterostomy constitute sources of nutritional inefficiency. The few available studies investigating ileal losses of amino acids and nitrogen in human adults report substantial losses, the extent of which depends on type of dietary protein ingested.[17, 18] Such losses may become critical in neonates with an enterostomy following intestinal surgery, because they need adequate intake and absorption of dietary proteins and amino acids to maintain maximal protein synthesis and tissue accretion. We therefore performed a study primarily aimed at determining net dietary protein absorption capacity of the small intestine in human neonates with a temporary jejunostomy or ileostomy a few weeks (wk) after the operation. As intestinal adaptation is best reflected by progress in tolerance for enteral nutrition, we also determined time needed to reach an enteral intake of more than 100 ml/(kg·d) as a parameter for enteral feeding tolerance.

MATERIALS AND METHODS

Patients

Eligible for this study were preterm neonates admitted to the Neonatal Intensive Care Unit (NICU) or the Paediatric Surgical Intensive Care Unit (PSICU) at the Erasmus Medical Centre (MC) –Sophia Children's Hospital (Rotterdam, the Netherlands) from November 2003 till December 2005 after bowel resection for necrotising enterocolitis, intestinal atresia or isolated bowel perforation and in whom a temporary jejunostomy or ileostomy was created. Patients with cystic fibrosis were excluded. The Erasmus MC Institutional Review Board approved the study protocol. Written, informed consent was obtained from the parents. Table 1 shows relevant patient characteristics.

Feeding regimens adhered to our feeding protocol for newborns; breast milk and/or formula (Nenatal®, Nutricia, Zoetermeer, the Netherlands; Nenatal Breast Milk Fortifier® (BMF), Nutricia, Zoetermeer, the Netherlands; Enfamil AR®, Mead Johnson, Woerden, the Netherlands) and parenteral nutrition containing glucose, amino acids (Primene® 10%, Clintec Benelux NV, Brussels, Belgium), and lipids (Intralipid® 20%, Fresenius Kabi, Den Bosch, the Netherlands). Patients with NEC received full parenteral feeding during the first 10 post-operative days (d). Next, minimal enteral feeding was added during 4 d, after which enteral feeding was gradually introduced by continuous nasogastric gavage feeding or bottle-feeding under simultaneous diminishing of the parenteral nutrition. Other patients were started on full parenteral feeding with introduction of enteral feeding within several days.

Study protocol

During post-operative weeks 3 through 6, we collected small intestinal outflow fluid at the level of the enterostomy once a week for 24-48 hours. Samples were collected in adhesive bags and stored at −80°C for further analysis. Each patient's total dietary protein intake was weekly determined during each study period.

Analysis of small intestinal outflow fluid

Samples of each study period were weighed and pooled per patient, and next homogenised in homogenisation buffer, 50 mmol/l Tris(hydroxymethyl)aminomethane (Tris, Gibco), 5 mmol/l ethylene-diamino-tetra-acetate (EDTA, Merck). The homogenate was centrifuged (3.500 *g*, 10 minutes, 4°C) to remove the fat layer. Aliquots of 50 µl were used to determine the protein concentrations (mg/ml) in the small intestinal outflow fluid by Bicinchoninic Acid (BCA) protein assay at 58°C during 30 minutes.[19]

Calculations

Net absorption of dietary proteins was calculated by subtracting the protein amount excreted in the outflow fluid from the protein amount enterally ingested, and expressed as percentage of total dietary protein intake:

Net dietary protein absorption capacity (%) = ($Protein_{in}$ − $Protein_{out}$)/ $Protein_{in}$ * 100%

Table 1 Patient characteristics

Patients	GA *(wk)*	BW *(g)*	Reason of small intestinal resection	Resected small intestine
1* †	36	3140	Multiple small intestinal atretic segments	Jejunum and ileum Residual bowel length: 35 cm jejunum and 25 cm ileum including the ileocaecal valve #
2*	25	755	NEC small intestine	12 cm jejunum
3* †	33	1450	NEC small intestine	Jejunum and ileum Residual bowel length: 24 cm jejunum and 25 cm ileum excluding the ileocaecal valve #
4	25	940	NEC small intestine	Jejunum and ileum Residual bowel length: 50 cm small intestine including the ileocaecal valve #
5	28	1190	NEC ileum	Minimal ileal resection excluding the ileocaecal valve
6	32	1515	Ileal perforation	
7	26	830	NEC ileum	
8	28	695	NEC ileum	
9	27	1040	Ileocaecal perforation	
10†	26	950	NEC small intestine	
11	32	995	NEC small intestine	
12	32	2190	NEC colon	Minimal ileal resection including the ileocaecal valve
13	34	1845	NEC colon	
14	32	1570	NEC colon	
15	30	885	NEC small intestine	
16	34	2310	NEC colon	Extensive colonic resection
17	34	1905	NEC colon	Extensive colonic resection
Mean ± SD	30 ± 4	1424 ± 672		

GA: gestational age; **BW:** birth weight; **NEC:** necrotising enterocolitis
* neonates with a jejunostomy
† enteral feeding tolerance was <100 ml/(kg·d)
# <35% small bowel length is remained as compared to normal age-adjusted small intestinal length

Enteral feeding tolerance was defined as the time span (d) between the day of surgery and the day when total enteral intake exceeded 100 ml/(kg·d).

As we were not aware of any reference values for small intestinal absorption capacity, we used a reference value of >85% for the dietary protein absorption capacity of the whole intestine measured in preterm infants as described by Sulkers *et al*. and Schanler *et al*.[20, 21]

Statistics

Patient characteristics are presented as the mean values plus or minus the standard deviation (SD) or as the median (minimum–maximum). Absorption capacities are expressed as the mean (95% confidence interval). Statistical differences were evaluated using the Mixed Model ANOVA. A value of $p<0.05$ was considered to indicate statistical significance.

RESULTS

Patients

Of 20 eligible patients, three were excluded due to problems with stoma fluid collection. The 17 studied patients (jejunostomy: 3; ileostomy: 14) had mean gestational age of 30 ± 4 wk and mean birth weight of 1424 ± 672 g (Table 1). Median postnatal age at surgery was 7 (1–44) d. In 11 patients, minimal length of the small intestine was resected (in 4 including ileocaecal valve), two patients underwent extensive colonic resection and one patient had 12 cm of jejunum resected (Table 1). Furthermore, 3 patients had less than 35% small intestinal length remaining, which was based on normal intestinal length measurements corrected for gestational age as determined by Touloukian *et al*.[22] Mean enteral protein intakes for patients with a jejunostomy and those with an ileostomy are shown in Table 2. As expected, enteral protein intakes had significantly increased over the study period ($p=0.032$). Overall, mean dietary protein intakes significantly differed between patients with a jejunostomy and those with an ileostomy ($p=0.013$). According to the preference of the attending consultant, seven infants were fed Nenatal®, three were fed a combination of breast milk enriched with BMF® and Nenatal®, three were fed breast milk enriched with BMF® and one was fed Enfamil AR 1® solely. One infant was fed breast milk only, and two infants were initially fed breast milk but were switched to a combination of breast milk enriched with BMF® later.

Feeding tolerance

Three infants (Table 1) did not reach the limiting value of 100 ml/(kg·d) enteral nutrition: further increase in enteral nutrition was withheld because of an upsurge in stoma fluid production. One had undergone minimal ileal resection, the other two had lost major parts of the small intestine. Re-establishment of intestinal continuity was hastened to obtain normal body growth. The remaining 14 patients reached enteral intake exceeding 100 ml/(kg·d) at a median of 17 (8–32) d following bowel resection.

Table 2 Weekly dietary protein intake *(g/(kg·d))* and absolute dietary protein absorption *(g/(kg·d))* during the 4-wk study period in human neonates with a temporary jejunostomy or ileostomy, respectively.

	Dietary protein intake *(g/(kg·d))*		**Absolute dietary protein absorption *(g/(kg·d))***	
Weeks	Jejunostomy	Ileostomy	Jejunostomy	Ileostomy
3	0.27 (0.06-1.29)	1.33 (0.73-2.43)	0.19 (0.03-1.18)	1.02 (0.50-2.07)
4	0.47 (0.15-1.52)	2.59 (1.56-4.30)	0.40 (0.10-1.59)	2.30 (1.26-4.20)
5	0.75 (0.31-3.02)	3.53 (1.88-6.63)	0.76 (0.20-2.91)	3.14 (1.49-6.64)
6	1.43 (0.46-4.44)	3.52 (1.61-7.70)	1.13 (0.30-4.34)	3.22 (1.27-8.13)

Values are expressed as the mean (95% confidence interval).

Net dietary protein absorption capacity

Mean absolute dietary protein absorption for patients with a jejunostomy and those with an ileostomy, is shown in Table 2.

Mean absolute dietary protein absorption is dependent on the level of the enterostomy (p=0.020). Overall, mean absolute dietary protein absorption increased during the total 4-wk study period (p=0.037). Figure 1 shows net absorption capacity of dietary proteins weekly measured for patients with a jejunostomy and those with an ileostomy.

Overall, the post-operative weeks in which the net absorption capacities were measured, did not influence the net dietary protein absorption capacity (p=0.076). Furthermore, the level of the enterostomy did not influence the net absorption capacity of dietary proteins (p=0.198). For all patients, the small intestine was capable of absorbing 70-90% of the total dietary protein intake.

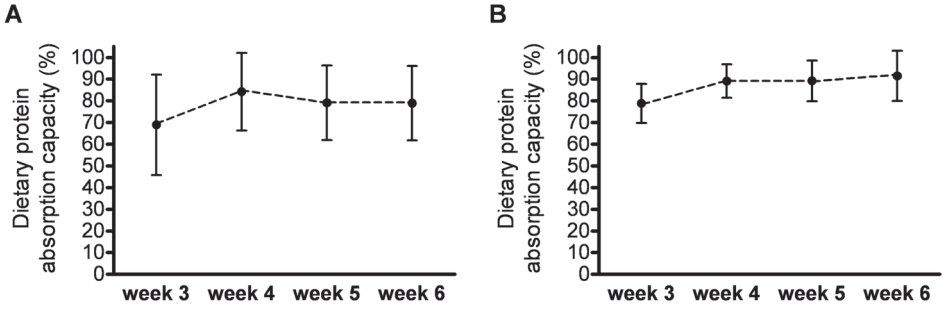

Figure 1 Net dietary protein absorption capacity in human neonates with a jejunostomy (A) or ileostomy (B). Values are expressed as percentage (%) of total dietary protein intake (mean (95% confidence interval)).

DISCUSSION

Irreversible protein losses at the level of enterostomy may considerably influence daily protein requirements. We set out to determine the net dietary protein absorption capacity of the intestine in human neonates following intestinal surgery with concomitant creation of a temporary enterostomy in the neonatal period. We demonstrated that the small intestine is responsible for 70–90% of the dietary protein absorption in these infants, in line with findings in preterm infants without an enterostomy.[20, 21] Since the neonates did not receive a hydrolysed or elemental formula, we conclude that our results do not support the use of such formula to improve amino acid uptake in infants with an enterostomy.

To our knowledge, this is the first study evaluating dietary protein absorption in human neonates with either a jejunostomy or ileostomy. As patients with a jejunostomy showed mean absorptive capacity around 80% over the 4-wk post-operative period, it seems that dietary proteins are absorbed far before the terminal ileum. The high absorptive capacity of the jejunum in these patients could be a result of early intestinal adaptation (<6 weeks). There is evidence, however, that most of the dietary nutrients are absorbed between the stomach and the proximal part of the jejunum. For example, nearly 70% of the pea-protein N is absorbed between the stomach and 200 mm after Treitz's ligament in humans.[23] The question is whether neonates with jejunostomy would retain around 80% absorption capacity if their absolute dietary protein intake should reach the much higher levels seen in neonates with an ileostomy. Intestinal length might be far too short to process these amounts of proteins.

Our approach of measuring small intestinal absorption capacity was based on several assumptions, which we shall discuss here. First, we assumed that the total protein amount measured in jejunal or ileal outflow fluid was not partially of endogenous origin, but only derived from the dietary protein intake. In general, the outflow fluid of enterostomies contains endogenous proteins and amino acids derived from the upper gastrointestinal tract – e.g. digestive enzymes, glycoproteins derived from the mucus layer, epithelial cells, microbial proteins, free amino acids, and peptides. In the present study, however, we did not correct net dietary protein absorption for this endogenous component, and the net dietary protein absorption might therefore be underestimated. Recent studies found endogenous ileal amino acids excretions both in human adults with ileostomies who were on a protein-free diet and those who ingested protein-containing meals.[17, 18, 24] The endogenous losses of amino acids were not negligible, but rather constituted a substantial proportion of daily requirements. Regrettably, there are still no data on the contribution of small intestinal endogenous protein losses in human neonates. Second, our method of determining protein concentration in the intestinal outflow fluid has a slight limitation. The BCA assay is not able to detect certain single amino acids and dipeptides. The net dietary protein absorption measured in the current study might therefore be overestimated due to non-detectable amounts of these single amino acids and dipeptides. Previous work involving the ingestion of [^{15}N]-labelled dietary proteins in adults showed that the true ileal digestibility of a number

of protein sources is >90% – with only minimal variation among the common sources of dietary protein.[25-28] We still feel, therefore, that the BCA assay is a reliable indicator of dietary protein absorption capacity of the small intestine in human neonates with a temporary enterostomy following intestinal surgery.

As mentioned earlier, amino acid losses in jejunal or ileal outflow fluid deserve further investigations. In human adults with ileostomies, total gastrointestinal losses of indispensable amino acids were found to account for 14-61% of the daily requirement of these essential amino acids.[24] Interestingly, threonine loss (61%) was particularly high compared with the other indispensable amino acids. This may be related to the high threonine content in gastrointestinal mucins, especially MUC2, which may constitute one of the major forms of amino acid loss. It may be worthwhile to determine the exact nature of the jejunal and ileal amino acid losses in human neonates in order to improve nutritional management in neonates with an enterostomy.

Furthermore, an important issue is whether the protein amounts collected from jejunal and ileal outflow fluid equal the irreversible protein losses. The colon is generally thought to be incapable of absorbing free amino acids or proteins. However, an amino acid transporter is expressed on the apical side of colonic enterocytes in mice.[29] A study in humans suggests that the colon contains peptide transporters that might be crucial in colonic amino acid absorption.[30] Further studies need to determine whether these transporters are functionally expressed in human colonic epithelium. Thus, the presumption that dietary proteins are irreversible lost at the ileum must be reconsidered in view of the possibility that colonic epithelium might be involved in intestinal protein absorption.

In summary, this study demonstrates that the dietary protein absorption capacity is intact in human neonates with an enterostomy. From the high percentage of protein absorption observed in patients with a jejunostomy, it would appear that large amounts of dietary milk or formula proteins are completely digested and absorbed before the last part of the small intestine.

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Chapter 3

Does small intestinal atresia affect epithelial protein expression in human newborns?

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ABSTRACT

Background: Bowel segments distal to a congenital intestinal obstruction have been suggested to be immature. In other words, luminal components such as amniotic fluid (before birth) and/or enteral nutrition (after birth) may be required to activate intestinal epithelial protein expression, thereby influencing epithelial differentiation. We investigated cell type-specific protein expression proximal and distal to jejunal and ileal atresias in human newborns.

Methods: We immunohistochemically studied intestinal tissue specimens of 16 newborns who had undergone surgery for jejunal or ileal atresia. Sections were taken from both the proximal and distal sides of the atresias.

Results: For all patients, the enterocyte-specific markers: lactase, sucrase-isomaltase, sodium glucose cotransporter 1, glucose transporters 2 and 5, intestinal fatty acid binding protein and alkaline phosphatase were expressed at a mean 3 ± 1 days after birth, both proximal and distal to jejunal and ileal atresias. Expression of goblet cell-specific markers mucin 2 and trefoil factor 3 and that of the Paneth cell marker lysozyme was maintained at either side of the atretic segment.

Conclusions: With respect to the markers used, the human small intestinal epithelium is already differentiated shortly after birth. The absence of intestinal continuity in case of a jejunal or ileal atresia does not affect epithelial protein expression. This would seem to indicate that the developing small intestinal epithelium matures independently of luminal components.

Abstract

INTRODUCTION

At an estimated overall incidence of 1 in 1500 to 5000 live births, small intestinal atresia is a well-known cause of neonatal intestinal obstruction. It is believed to have its genesis as an embryopathy at the level of the duodenum in the first trimester of pregnancy or as an anomaly of ischaemic origin in the second or third trimester of pregnancy.[1]

The small intestine is one of the most metabolically active tissues in the body. Its best-regulated functions are epithelial proliferation, differentiation and apoptosis. Four specialised cell types in the epithelium play key roles in maintaining intestinal functions: enterocytes, goblet cells, Paneth cells and enteroendocrine cells. Enterocytes facilitate digestion, uptake and transport of nutrients by expressing (e.g. the sugar-degrading enzyme sucrase-isomaltase (SI), the glucose and fructose transporters 2 and 5 (Glut2 and Glut5), and the intestinal fatty acid binding protein (i-FABP).[2, 3] Enterocytes also express alkaline phosphatase (AP), which aids intestinal defence by detoxifying endotoxins.[4] Goblet cells synthesise the secretory mucin 2 (MUC2), the structural component of the intestinal mucus layer that protects the epithelium from mechanical stress, bacteria, viruses and other pathogens.[5] Goblet cells also synthesise and secrete trefoil factor 3 (TFF3), a bioactive peptide involved in epithelial protection and repair.[6] Synthesising antimicrobial peptides, Paneth cells are crucial to epithelial defence.[7, 8] Enteroendocrine cells are specialised in the mucosal secretion of hormonal peptides.

Evaluation of mucosal morphology in chick embryos[9] and foetal lambs[10, 11] after experimental intestinal atresia, and in human neonates[12] with jejunal or ileal intestinal atresia revealed an abundance of shorter, flattened villi in the proximal, dilated segment. On the other hand, the distal, narrowed segment had tall, hypertrophic villi, often obliterating the intestinal lumen. The obstruction would prevent access of luminal components that may stimulate intestinal development, such as amniotic fluid (before birth) and/or enteral nutrition (after birth). Absence of these components may lead to an immature bowel distal to the obstruction. In the current study, we therefore hypothesised that human newborns show mature epithelial differentiation proximal to a jejunal or ileal atresia, but immature epithelial differentation distally. We used a variety of relevant markers to immunohistochemically investigate enterocyte differentiation: lactase, SI, sodium glucose cotransporter 1 (SGLT-1), Glut2, Glut5, i-FABP and AP. Goblet cell differentiation was determined by the expression of MUC2 and TFF3 and Paneth cell differentiation by the expression of the antibacterial enzyme lysozyme. Thus, by studying all these parameters in conjunction, we aimed to gain more insight into intestinal epithelial protein expression and thus epithelial differentiation in human newborns, proximal and distal to jejunal and ileal atresias.

MATERIALS AND METHODS

Patients

Patients eligible for this study were newborns who had undergone surgery for jejunal or ileal atresia in the Department of Paediatric Surgery, Erasmus Medical Centre (MC) – Sophia Children's Hospital (Rotterdam, the Netherlands). The diagnosis had been made by plain x-ray or ultrasound and was confirmed at surgery. We excluded cases of duodenal atresia, atresias complicating meconium ileus and/or meconium peritonitis, and atresias associated with gastroschisis. Table 1 shows main clinical characteristics of the 16 patients studied.

Ten of the 16 newborns had received standard nutrition for 1 or 2 days (d) after birth, before being transferred to our hospital. Upon onset of clinical symptoms for intestinal obstruction, they had been given adequate intravenous fluid resuscitation to achieve hemodynamic stabilisation. Antenatal maternal ultrasonography showing polyhydramnion or dilated intestines had pointed at intestinal obstruction in the other 6 patients. They received no enteral feeding after birth but intravenous glucose infusion.

Experimental design

We studied intestinal tissue sections of these patients provided by the Erasmus MC Tissue Bank, with permission of the local medical ethics committee and according to the Code Proper Secondary Use of Human Tissue, thus in compliance with Dutch law and ethics regulations. Expression of the epithelial enzymes was studied in sections taken at surgery, proximal and/or distal to the jejunal or ileal atresia in close vicinity to the resection areas. For patients with multiple atresias we preferentially used sections close to the first atretic segment. If these were not available, we designated a random intestinal segment proximal or distal to one of the other atretic segments as the distal part and studied this. In addition we studied small intestinal tissue samples of 8 control patients. For the 5 patients, undergoing stoma formation or bowel resection in the neonatal period for intestinal perforation (n=1), bowel necrosis (n=1), volvulus (n=1) and bowel stenosis (n=2), these had been obtained during surgery. For the 3 patients, these had been obtained at autopsy after death from complications of congenital diaphragmatic hernia (n=2) or persistent pulmonary hypertension of the newborn (n=1). All intestinal biopsies were immediately fixed in 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS) and prepared for light microscopy. Two investigators, blinded for the proximal and distal resection areas, independently assessed the sections on histology and epithelial protein expression.

Histology

Sections of 5-µm thickness were routinely stained with hematoxylin and eosin to study histological changes (i.e. distortion of crypt/villus epithelium and lymphoid aggregates close to the jejunal or ileal atresia). Atrophy was defined as a crypt/villus ratio of 1:3 or less, and hypertrophy was defined as a crypt/villus ratio of more than 1:4.

Table 1 Patient characteristics

Patients	Gender	GA *(wk)*	BW *(g)*	Age at surgery *(d)*	Diagnosis	Prenatal US	Associated anomalies
1	M	36	2780	2	Jejunal atresia	NA	None
2	F	38	2000	2	Ileal atresia	NA	Arthrogryposis multiplex congenita
3	F	35	1975	5	Ileal atresia	NA	None
4	M	36	2750	2	Multiple jejunal atresia	Polyhydramnion, dilated intestines	None
5	F	40	3550	1	Jejunal atresia	NA	None
6	F	36	2350	3	Multiple small intestinal atresias	Polyhydramnion	None
7	M	42	2910	3	Ileal atresia	NA	None
8	M	37	3100	2	Jejunal atresia	Polyhydramnion	None
9	M	43	3100	4	Ileal atresia	NA	None
10	F	34	2960	4	Apple peel atresia	NA	Volvulus
11	M	40	3385	2	Ileal atresia	NA	Volvulus
12	M	35	1725	1	Multiple jejunal atresias	Dilated intestines	None
13	M	36	3280	2	Jejunal atresia	NA	None
14	M	37	2755	0	Multiple small intestinal atresias	Dilated intestines	Left ventricle dilatation
15	F	38	3360	1	Multiple jejunal atresias	Dilated intestines, malrotation	Situs inversus, asplenia, cardiac problems
16	F	37	2335	5	Jejunal atresia	NA	TTTS
Mean ± SD		37 ± 2	2770 ± 551	3±1			

GA: gestational age; **BW:** birth weight; **US:** ultrasound; **F:** female; **M:** male; **NA:** not available; **TTTS:** twin-to-twin transfusion syndrome

Immunohistochemistry

Five-micrometer-thick paraffin sections were cut and deparaffinised through a graded series of xylol-ethanol as described previously.[13] Briefly, endogenous peroxidase activity was inactivated with 3% (vol/vol) hydrogen peroxide in PBS for 30 minutes (min), followed by rinsing in PBS for 15 min. The sections were boiled in 0.01 mol/l citrate buffer (pH 6.0) or ethylene-diamino-tetra-acetate (EDTA, 5 mmol/l, pH 8.0) for 10 min. To reduce non-specific binding, the sections were then incubated with TENG-T (10 mmol/l Tris-HCl, 5 mmol/l EDTA, 150 mmol/l NaCl, 0.25% (wt/vol) gelatin, 0.05% (wt/vol) Tween-20) for 30 min. This was followed by overnight incubation with primary antibodies. To determine enterocyte-specific protein expression we used anti-human lactase (DR BB 2/33; 1:2000; A.Quaroni), anti-rat SI (1:6000)[14], anti-rabbit SGLT-1 (1:2000)[15], anti-human Glut2 (1:2000; B.Thorens), anti-rat Glut5 (1:1500; D.R.Yver) and anti-human i-FABP (1:4000).[16] As a marker for goblet cell-specific protein expression, we used a human MUC2-specific antibody (We9, 1:200)[17] and anti-human TFF3 (1:2000, see following paragraph for antibody preparation). Anti-human lysozyme (1:25, Dako, Glostrup, Denmark) was used to detect Paneth cell-specific protein expression. Sections were then incubated for 1 hour (h) with biotinylated horse anti-mouse IgG (diluted 1:1000, Vector Laboratories, England) or with biotinylated goat anti-rabbit IgG (diluted 1:2000, Vector Laboratories, England) followed by 1-h incubation with ABC/PO complex (Vectastain Elite Kit, Vector Laboratories) diluted 1:400. After incubation, binding was visualised in 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 0.02% (vol/vol) H_2O_2 in 30 mmol/l imidazole, 1 mmol/l EDTA (pH 7.0).

TFF3 cloning, expression and antibody preparation

The coding sequence for human (h)TFF3 was amplified from the HITF plasmid using primers: 5'-TACGTAGAGGAGTACGTCGGCCTG-3' containing the *Sna*BI restriction site and 5'-TCAATGATGATGATGATGATGGAAGGTGCATTCTGCTTCCT-3'.[18] The resulting polymerase chain reaction product was cloned into pCR2.1 and verified by sequence analysis. Subsequently, the coding sequence of hTFF3 was subcloned into the yeast expression vector pPic9K using *Sna*BI and *Eco*RI. The vector was transformed into, and expressed by, the *Pichia pastoris* strains KM71 and GS115 by using the *Pichia* Multi-Copy Expression kit (Invitrogen) according to the manufacturer's instructions. Recombinant hTFF3/HIS-tag fusion proteins were isolated using a Nickel column (Pharmacia, Diegem, Belgium) according to the manufacturer's instructions. The eluted recombinant hTFF3 protein was dialysed and concentrated. Subsequently, New Zealand White rabbits (Broekman, Utrecht, the Netherlands) were immunised with recombinant hTFF3 diluted in Gerbu Adjuvant (Instruchemie, Hilversum, the Netherlands) according to the manufacturer's instructions. The polyclonal TFF3 antibody preparation was performed with approval of the Erasmus MC Animal Studies Ethics Committee.

Histochemistry

Enterocyte-specific alkaline phosphatase activity was assessed by a 1-step assay. The deparaffinised and rehydrated tissues sections were incubated with a Tris-buffer (pH 9.5) containing 50 µl 4-nitroblue tetrazolium chloride (Vector Laboratories) and 37.5 µl 5-bromo-4-chloro-3-indolyl-phosphate (Vector Laboratories) according to the

manufacturer's protocol. The colour reaction was performed for 1 h in the dark and was stopped with distilled water; slides were mounted with aquamount improved (Gurr, Brunschwig, Amsterdam, the Netherlands).

Statistics

Patient characteristics are presented as the mean ± SD.

RESULTS

Patients

The 16 patients with a jejunal or ileal atresia had mean gestational age of 37 ± 2 weeks (wk) and mean birth weight of 2770 ± 551 g (Table 1). Surgery had been performed at 3 ± 1 d after birth. In 4 patients, intestinal anastomosis had not been possible during the initial surgery, and they underwent re-anastomosis after 54 ± 58 d. One infant had died 38 d after birth from complications of antenatal ischaemia caused by twin-to-twin transfusion syndrome.

The 8 control patients had mean gestational age of 37 ± 2 wk, and intestinal surgery or autopsy had been performed 4 ± 4 d after birth.

Morphology

Sections, both proximal and distal to jejunal and ileal atresias, showed a patchy pattern of morphological changes. Changes observed were villus atrophy, flattening of crypt and villus cells, flattening of the surface epithelium and villus hypertrophy (Figure 1). The lamina propriae contained hemorrhages and some lymphoid aggregates, plasma cells, individual lymphocytes, and eosinophilic granulations comparable with the normal situation in the lamina propriae.

Enterocyte-specific protein expression

Enterocyte-specific protein expression was studied immunohistochemically, using antibodies against lactase, SI, Glut2, Glut5, i-FABP and SGLT-1. All enterocyte markers were normally expressed in control sections (data not shown). Lactase, SI, SGLT-1 and Glut5 protein expression is normally confined to the brush border of villus enterocytes.[19, 20] In the atresia patients, these proteins were expressed both proximal and distal to the atretic segment in jejunum as well as ileum (Figures 2A and B: SI; Figures 2C and D: lactase; Glut5 and SGLT-1: data not shown). Glucose and fructose transporter 2 was expressed at the basolateral membrane of villus enterocytes. Similar to the proteins expressed at the brush border, Glut2 was normally expressed proximal and distal to jejunal and ileal atresias (Figures 2E and F).

Intestinal-FABP protein is normally found in the cytosol of the jejunal and ileal villus enterocytes.[21] As with the membrane-bound proteins, expression of i-FABP was normal (Figures 2G and H). The *in situ* AP activity was observed in the brush border of the surface enterocytes both in proximal and distal segments of jejunal and ileal atresias (data not shown). Expression of all described enterocyte-specific proteins did not differ between segments characterised by atrophy and segments characterised by hypertrophy (Figures 3A and B: SI; other markers: data not shown). Thus, all enterocyte-specific proteins investigated were present in the small intestine (jejunum and ileum) at 3 ± 1 d after birth. Enterocyte-specific protein expression in the small intestine was unaffected by the presence of a jejunal or ileal atresia.

Goblet cell- and Paneth cell-specific protein expression

Goblet cell-specific protein expression patterns were studied using MUC2 and TFF3 specific antibodies. Similar to enterocyte-specific protein expression, control sections showed normal expression of MUC2 and TFF3 (data not shown).

Trefoil factor 3 specificity was determined on adult human stomach tissue obtained during gastroscopy and control small intestinal sections. Figure 4 shows the negative and positive controls for TFF3.

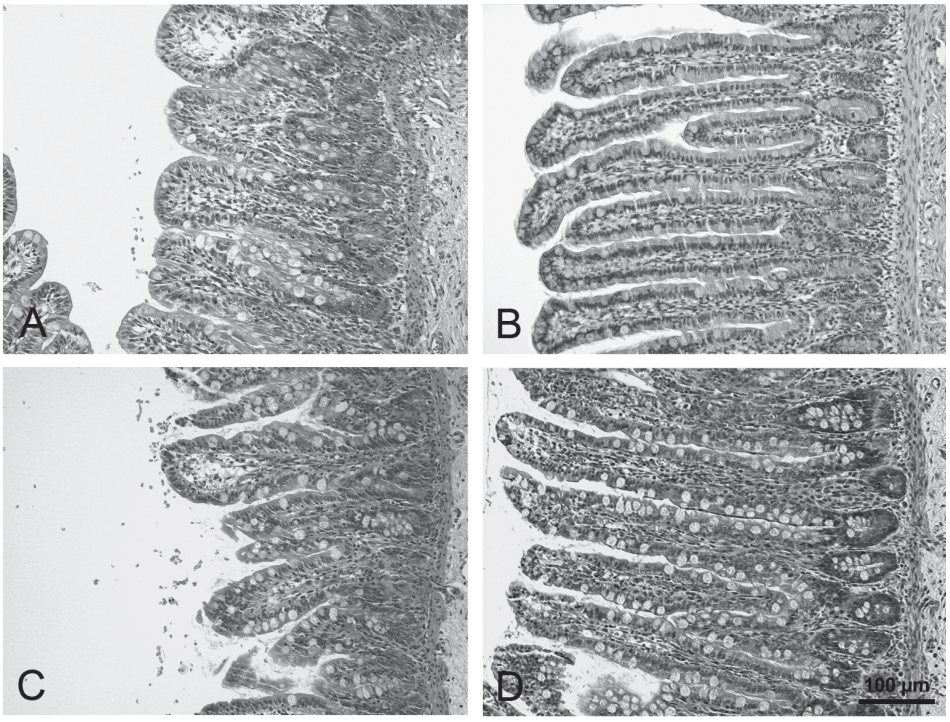

Figure 1 Epithelial morphology. Hematoxylin and eosin staining. Atrophic (A and C) and hypertrophic (B and D) segments were observed both proximal (A and B) and distal (C and D) to jejunal and ileal atresias. Colour figure, page 182.

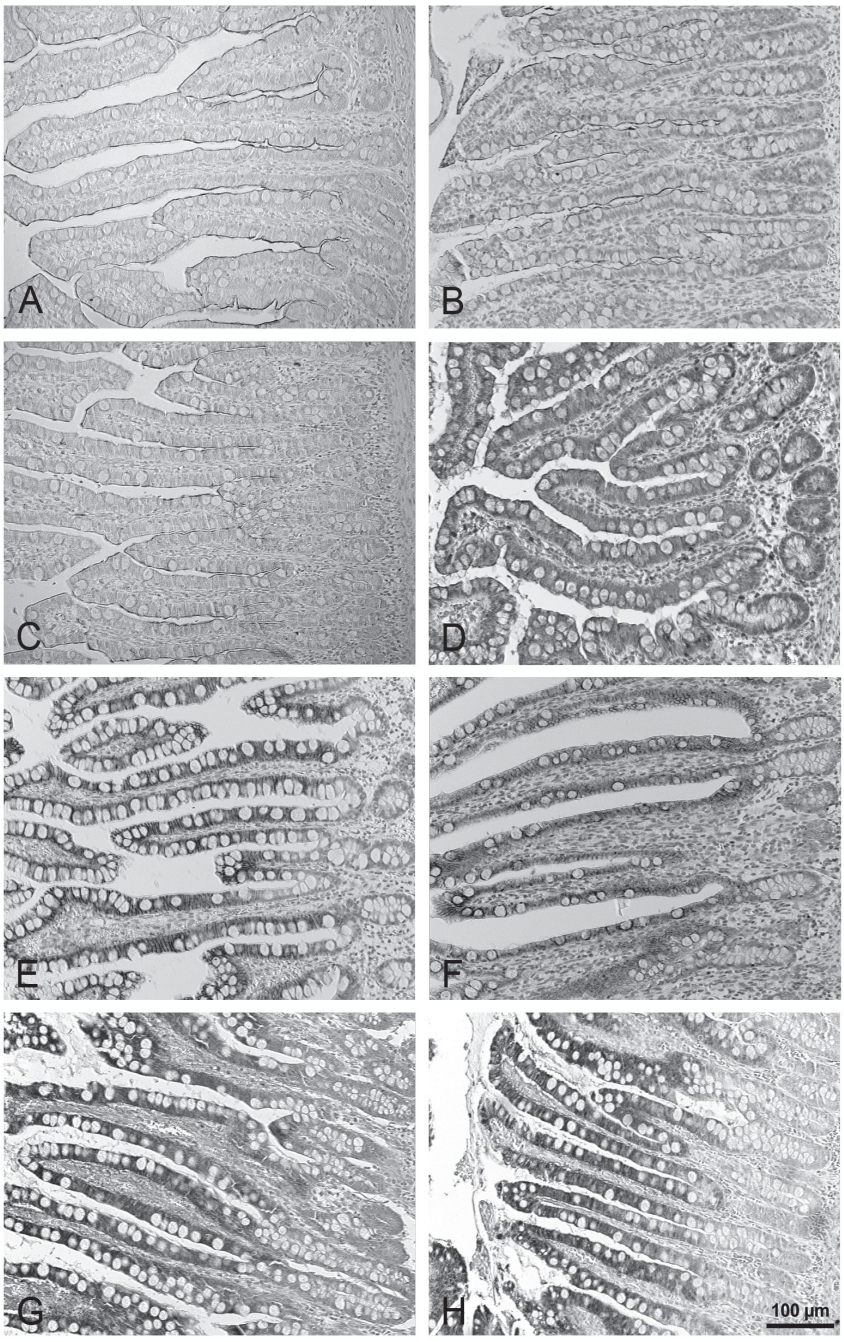

Figure 2 Enterocyte-specific protein expression. Sucrase-isomaltase (A and B) and lactase (C and D) were both expressed proximal (A and C) and distal (B and D) to jejunal and ileal atresias. Glucose and fructose transporter 2 was expressed at the basolateral membrane of jejunal and ileal villus enterocytes, both proximal (E) and distal (F) to the atretic segment. Intestinal fatty acid binding protein was expressed in the cytosol of jejunal and ileal villus enterocytes both proximal (G) and distal (H) to the atresia. Colour figure, page 183.

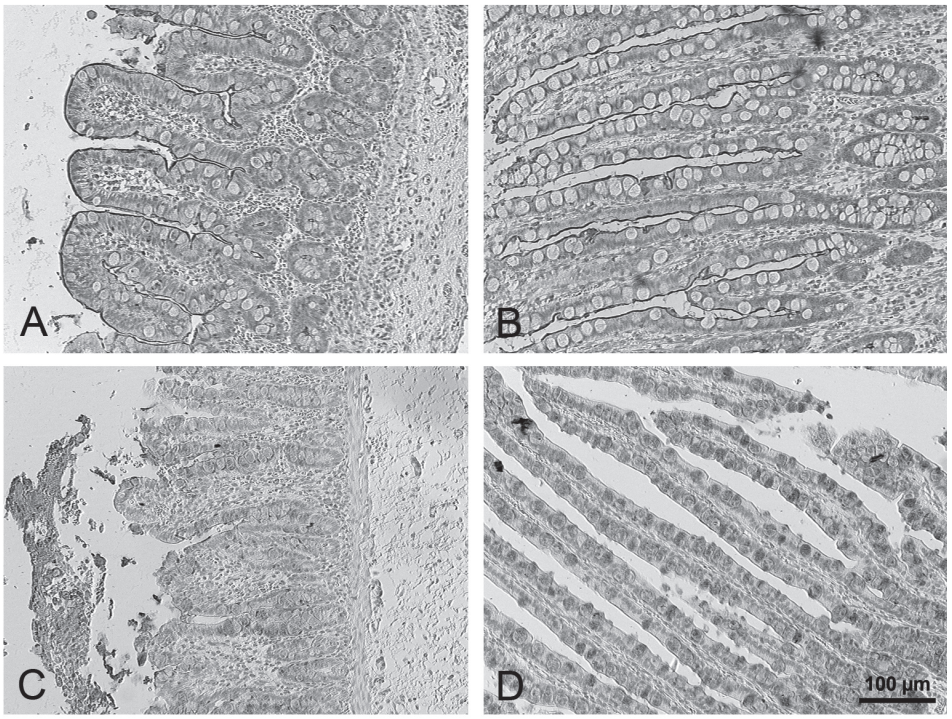

Figure 3 Enterocyte-specific SI and goblet cell-specific MUC2 expression in areas characterised by atrophy or hypertrophy. Enterocyte-specific SI expression (atrophy (A) and hypertrophy (B)) and goblet cell-specific MUC2 expression (atrophy (C) and hypertrophy (D)). Colour figure, page 184.

No differences were found in MUC2 and TFF3 protein expression on either side of the jejunal or ileal atresia independent of atrophy or hypertrophy (Figures 3C and D: MUC2; TFF3: data not shown). Goblet cell-specific MUC2 and TFF3 expression is shown in Figures 5A-D.

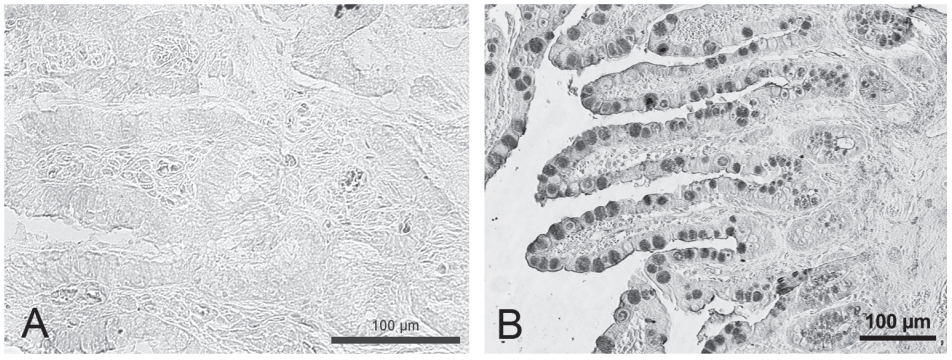

Figure 4 Trefoil factor factor 3 specificity. Trefoil factor 3 specificity was determined on adult human stomach tissue (A; negative control) and human neonatal small intestinal tissue (B; positive control). Colour figure, page 184.

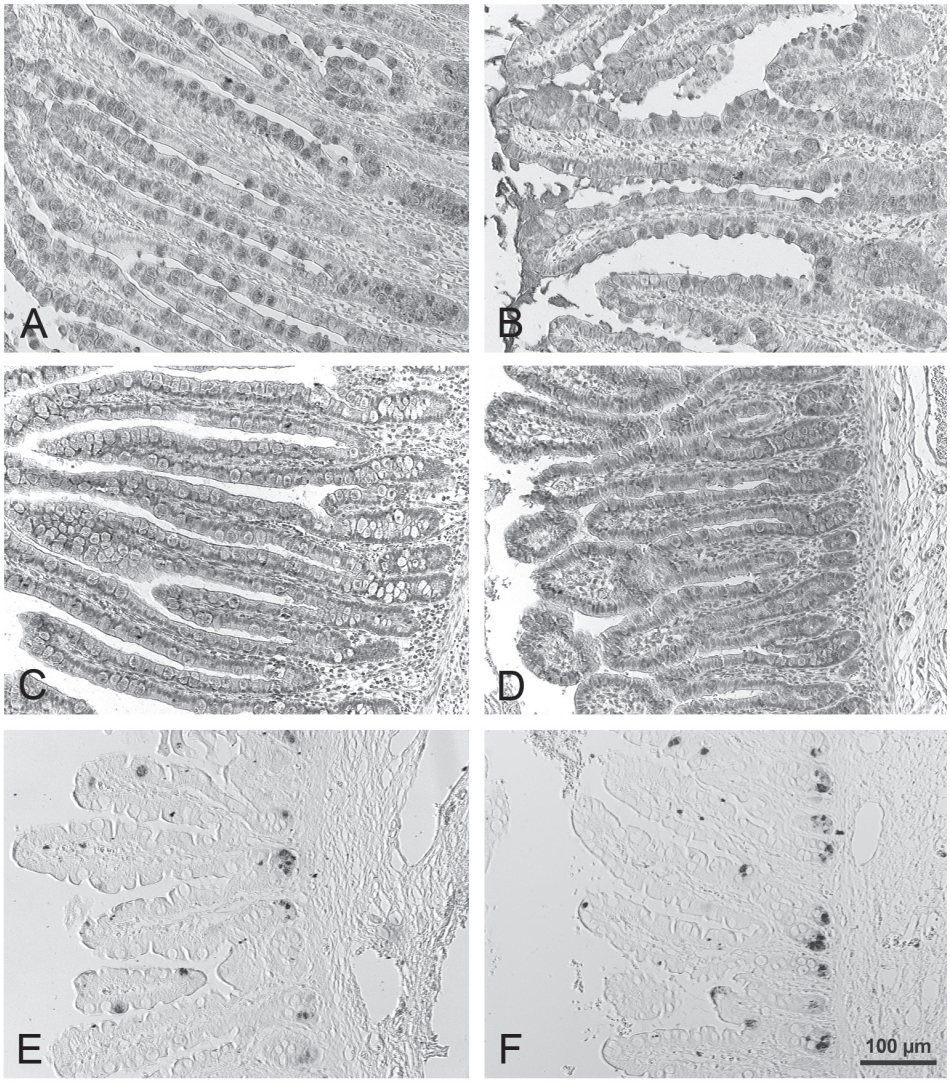

Figure 5 Goblet cell-specific protein expression and Paneth cell-specific protein expression. Mucin 2 (A and B) and TFF3 (C and D) were expressed on either side of jejunal or ileal atresias (proximal: A and C; distal B and D). Lysozyme was expressed in Paneth cells at the crypt base in jejunum and ileum, both proximal (E) and distal (F) to the atresias. Colour figure, page 185.

Lysozyme was used as a marker for Paneth cell-specific cell function. It showed a normal expression pattern in control neonatal small bowel (data not shown). In the biopsies taken from our patients, lysozyme was expressed in Paneth cells at the crypt base in jejunum and ileum. Lysozyme expression in patients' sections did not differ between proximal and distal segments (Figures 5E and F) and neither between atrophic and hypertrophic areas (data not shown). Also, localisation of Paneth cells at the bottom of the crypt did not differ between proximal and distal segments. Thus, similar to the enterocyte markers, goblet cell-

and Paneth cell-specific protein expression was observed at a mean age of 3 d after birth. Expression did not differ either proximal or distal to jejunal and ileal atresias.

DISCUSSION

We investigated the effect of jejunal and ileal atresia on epithelial morphology and epithelial protein expression in human newborns, proximal and distal to the atretic segment, shortly after birth. Morphological analysis demonstrated structural alterations of villi and crypts at both sides of the atretic segment. We observed areas with hypertrophic villi at either side of the jejunal and ileal atresia. Our findings are in line with studies by Touloukian and Wright[12] and Tilson[22] showing significant villus hypertrophy, most marked in segments distal to the small intestinal obstruction. However, next to hypertrophic villi we found villus atrophy both proximal and distal to jejunal and ileal atresias, which was not previously reported. The natural history of the defect during the prenatal period of life (i.e. vasculary insufficiency resulting in the atresia followed by patchy repair) may explain our histological findings.

In agreement with our findings in normal neonatal intestinal tissue, several studies have clearly demonstrated expression of lactase, SI, SGLT-1, Glut2, Glut5 and AP; the goblet cell markers MUC2 and TFF3 and the Paneth cell marker lysozyme already at messenger RNA (mRNA) and/or protein level in healthy foetal small intestine and persisting after birth.[6, 23, 24] More specifically, mRNAs encoding the glucose transporter proteins Glut2 and Glut5 are detectable in human foetal intestine as early as 11 weeks post-conception.[23, 24] Sodium glucose cotransporter 1 mRNA is detectable at 17 weeks of gestation and sucrose-isomaltase mRNA at 13 weeks of gestation.[23] Lactase is expressed early in gestation, but its activity increases markedly during the third trimester, probably to meet the needs of full-term newborns.[24] Dahlqvist and Lindberg detected jejunal AP activity in 11-wk-old foetuses.[25] Intestinal fatty acid binding protein has not been studied in foetal human tissue, but is expressed in adult intestinal tissue.[26] Lin and colleagues detected the goblet cell marker TFF3 by immunohistochemistry in human intestine as early as 12 weeks of gestation.[6] Goblet cell-specific MUC2 mRNA is expressed throughout the intestinal epithelium at 9 weeks post-conception, therefore concomitantly with endodermal cytodifferentiation associated with the formation of premature crypts and villi.[27] Paneth cell-specific lysozyme specifically stains at about 20 weeks of gestation.[28] In the present study we found for each patient enterocyte-, goblet cell-, and Paneth cell markers expression proximal to the jejunal or ileal atresia. Taken together these data indicate that the epithelial-specific proteins are expressed in early neonatal life (within 3 d following birth) suggesting that enterocytes, goblet cells, and Paneth cells can express a wide variety of proteins very early in life. More importantly, the expression of these proteins is not affected by the jejunal or ileal atresia itself.

The presence of luminal components such as amniotic fluid and biliary-pancreatic secretion during foetal intestinal development may be crucial to epithelial protein induction in utero and after birth. Surana and Puri, for example, showed that amniotic fluid has a nutritive

role for the foetus and that intestinal obstruction blocking this pathway causes intrauterine growth retardation.[29] Furthermore, animal studies found experimental elimination of foetal ingestion to result in retarded growth of the gastrointestinal tract and changes in the intestinal epithelial architecture.[30, 31] Our present findings in human material nevertheless suggest that absence of luminal factors does not affect induction and/or maintenance of epithelial-specific protein expression. Furthermore, these data imply that an intrinsic program encoded in the epithelial cells determines enterocyte-, goblet cell- and Paneth cell-specific protein expression. Our findings are in line with isograft studies demonstrating normal induction of intestinal liver-FABP in foetal intestinal tissues implanted subcutaneously into nude mice. Rubin and colleagues therefore suggest that the gut stem cell is multipotent, has great capacity for self-renewal and can to be programmed/imprinted with positional information.[32] Recent mouse studies show that mesenchymal proteins like Wnt and bone morphogenetic proteins can direct intestinal epithelial differentiation and thus epithial-specific gene expression[33-35], implying a pivotal role for these factors in the epithelial differentiation program. On the other hand, studies in pigs and sheep found systemic and luminal factors (e.g. hormones and growth factors) to influence intestinal development and differentiation.[36]

Although we detected no differences in SI and lactase protein expression proximal and distal to jejunal and ileal atresias, we cannot rule out differences in enterocyte-specific enzyme activity as we did not quantify disaccharidase activity. Serrano *et al*. did, however, and measured lower activity of SI and lactase proximal to a small intestinal obstruction. Distally, only lactase activity was significantly reduced.[37] Reduced lactase activity might affect lactose digestion and thus might indirectly be responsible for these patients' failure to thrive. Surgery for small intestinal atresia regularly is associated with symptoms of malabsorption and growth retardation.[38] Even if intestinal surgery is successful and food intake is adequate, these symptoms may persist for several months.[39] However, we still feel that factors such as villus morphology, gut caliber, motility and remaining intestinal length have greater impact on delayed post-operative recovery than the reduced protein expression levels observed by Serrano *et al*.[37]

Like the enterocyte markers, the goblet cell markers MUC2 and TFF3 and the Paneth cell marker lysozyme were also expressed at both sides of the jejunal and ileal atresia. Thus, our findings suggest that defence and repair functions of the small intestinal mucosa are maintained by goblet cell and Paneth cell expression of MUC2, TFF3 and lysozyme, respectively, close to the atretic segment.

In conclusion, in contrast to observations in animals the present study demonstrates that the small intestinal epithelium in humans is mature at birth. Luminal components, such as amniotic fluid (before birth) and enteral nutrition (after birth), are not essential for the epithelial maturation of the intestine. Taken together, the results highlight that epithelial protein expression, which is crucial to nutrient absorption, epithelial defence and repair in the small intestine, is genetically imprinted and that indicates the presence of indispensable ontogenetic factors.

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Chapter 4

Epithelial functions of the residual bowel after surgery for necrotising enterocolitis in human infants

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Submitted

ABSTRACT

Background: Information on epithelial functions of the residual bowel after resection for necrotising enterocolitis (NEC) in human infants is scarce.

Aim: To evaluate epithelial functions in the intestinal resection margins of tissue obtained at bowel resection for acute NEC and consecutive stoma closure.

Methods: Epithelial morphology, proliferation and protein expression were (immuno)histochemically studied.

Results: Acute NEC was associated with severe and mild epithelial damage varying from epithelial loss to fairly unaffected epithelium. Epithelial proliferation was increased both at acute NEC and at stoma closure. In acute NEC, lactase, glucose transporter 2 and 5 expression was down-regulated in severely affected epithelium, whereas sucrase-isomaltase and intestinal fatty acid binding protein expression was maintained. Furthermore, goblet cells continued to express mucin 2 and trefoil factor 3, however, their numbers were decreased. Moreover, in acute NEC, Paneth cells were weakly lysozyme-positive and were reduced in number. At stoma closure, expression of the above cell type-specific markers had completely been re-established.

Conclusions: Residual bowel after resection for acute NEC shows a disturbed epithelial proliferation/differentiation balance. Acute NEC was associated with down-regulation of distinct enterocyte-specific proteins. Due to goblet cell- and Paneth cell loss in acute NEC, mucosal barrier and defence functions might be impaired.

Abstract

INTRODUCTION

Necrotising enterocolitis (NEC) is the most common gastrointestinal disease of premature infants and a major cause of morbidity and mortality in neonatal intensive care units.[1] More specifically, it might be a disease of the immature mucosal barrier.[2] Several risk factors for its aetiology have been proposed, including prematurity, hypoxia, enteral feeding and bacterial colonisation, but its pathophysiology is hardly understood.[1] NEC is characterised by severe intestinal necrosis and, therefore, often requires surgical intervention sometimes complicated by short-bowel syndrome.[3, 4]

Several groups have examined the pathogenesis of NEC in animal models and in human infants.[5-10] Increasing evidence suggests that the risk factors mentioned above stimulate proinflammatory mediators, such as tumour necrosis factor (TNFα), platelet activating factor (PAF), interleukin (IL)-1, IL-8, and macrophage migration inhibitory factor (MIF). These in turn initiate an inflammatory cascade leading to intestinal damage and necrosis.[11, 12] Therefore, an intact intestinal epithelial barrier function is indispensable to prevent such damage, and thus to maintain neonatal health.

Intestinal epithelium consists of several specialised cell types of which enterocytes, goblet cells and Paneth cells are of special interest in this study. Small intestinal enterocytes facilitate digestion, uptake and transport of nutrients by expressing, e.g. sucrase-isomaltase (SI) and lactase.[13] Colonic enterocytes express specific proteins along the apical membrane, such as carbonic anhydrases (CAs) and Na^+/H^+ exchangers, involved in colonic CO_2 excretion, intracellular pH regulation, Na^+ and Cl^- absorption and indirectly water transport.[14] The intestinal fatty acid binding protein (i-FABP) involved in fatty acid uptake and cellular transport of fatty acids is expressed both in enterocytes of the small intestine and proximal colon.[15] Goblet cells synthesise and secrete mucin 2 (MUC2), the structural component of the protective mucus layer, and trefoil factor 3 (TFF3), a bioactive peptide involved in epithelial protection and repair.[16-19] Paneth cells contribute to epithelial defence by synthesising antimicrobial peptides such as lysozyme, human defensin 5 and 6, and $sPLA_2$.[20] In healthy intestine, the enterocyte-, goblet cell- and Paneth cell functions are tightly regulated. Severe intestinal necrosis and inflammation in neonatal NEC might affect the cell type-specific protein expression, and thus epithelial cell type-specific functions. Several methods are used to evaluate intestinal functions of the remaining bowel in human infants following bowel resection for NEC such as the sugar absorption test[21], gut hormone profile[22] and enterostomy fluid analysis.[23] Yet, specific data on epithelial functions of the residual bowel are scarce. In the present study, therefore, we investigated enterocyte-, goblet cell-, and Paneth cell-specific protein expression in bowel samples of neonates who underwent bowel resection and/or stoma formation/closure either for NEC or for other conditions (controls). These protein expressions served as parameters for epithelial functions of the residual bowel.

MATERIALS AND METHODS

Patients

Eligible for this study were infants admitted to the Neonatal Intensive Care Unit (NICU) or the department of Paediatric Surgery of the Erasmus Medical Centre (MC) – Sophia Children's Hospital (Rotterdam, the Netherlands) who had undergone bowel resection for NEC in the neonatal period. The diagnosis had been made by clinical symptoms and characteristic features on an abdominal x-ray. NEC was confirmed at surgery and by histological features characteristic for NEC identified in resected intestinal tissue sent for routine histopathology. Table 1 shows main clinical characteristics of the 21 infants studied. In all cases an enterostomy or colostomy had been created during surgery. Bowel samples of the resection margins were taken at the initial surgery and at the time of stoma closure (recovery phase). The paediatric surgeons determined resection margins on macroscopic most healthy tissue. The resection margins were considered as representative for the neonate's entire remaining bowel. As bowel biopsies from healthy children cannot be obtained, we used as control samples small intestinal (n=5) and colonic (n=5) tissue from neonates who underwent stoma formation, stoma closure or bowel resection for small intestinal atresia, Hirschsprung's disease, meconium peritonitis or milk curd syndrome. No additional tissue was removed for research purposes alone. The Erasmus MC Institutional Review Board approved the study protocol. Written, informed consent was obtained from the parents. In addition, foetal bowel samples served to specifically investigate epithelial-specific MUC2, carbonic anhydrase IV (CA IV), and i-FABP protein expression during human intestinal development. The Erasmus MC Tissue Bank provided foetal intestinal tissue (n=10; gestational age (GA) of 20 (16-25) weeks (wk)) collected from foetuses after terminations of pregnancy or after premature birth unrelated to intestinal problems. In all cases autopsy had been performed shortly after death, and intestinal biopsies investigated had minimal post-mortem changes due to autolysis. Tissue collection adhered to the Code Proper Secondary Use of Human Tissue, and thus complied with Dutch law and ethics regulations. All bowel samples were immediately fixed in 4% (wt/vol) paraformaldehyde in phospate buffered saline (PBS) and prepared for light microscopy. Two investigators independently assessed the sections on histology and epithelial protein expression.

Histology

Sections of 5 µm thickness were routinely stained with Alcian Blue/Nuclear Fast Red to study morphological and histological changes. Atrophy was defined as a crypt/villus ratio of 1:3 or less.

Table 1 Patient characteristics

Patients	Gender	GA *(wk)*	BW *(g)*	Postnatal age at surgery *(d)*	Postnatal age at stoma closure *(d)*	Location of NEC
1	F	33	1450	6	57	Jejunum/ileum
2	F	28	1130	12	-	Jejunum
3	F	34	1845	7	115	Ileum/colon
4	M	34	2310	3	84	Colon
5	M	34	1905	6	45	Colon
6	F	32	1240	4	- *	Colon
7	M	38	2790	7	160	Colon
8	M	32	2190	7	101	Ileum/colon
9	F	39	2130	6	134	Colon
10	M	27	760	15	-	Jejunum/ileum
11	F	25	755	24	36	Jejunum
12	M	34	2020	8	-	Jejunum/colon
13	F	32	1570	13	78	Ileum/colon
14	M	28	1190	10	103	Ileum
15	M	26	830	11	125	Ileum
16	F	37	4060	17	44	Jejunum/ileum
17	F	38	3150	3	74	Colon
18	M	30	930	33	109	Jejunum/ileum
19	M	25	940	28	142	Ileum
20	M	30	1525	12	40	Ileum
21	M	30	885	30	74	Ileum
Median		32	1525	11	82	
Min-max		25-39	755-4060	3-33	36-160	

GA: gestational age; **BW:** birth weight; **NEC:** necrotising enterocolitis; **F:** female; **M:** male
* stoma closure in another hospital; no bowel samples collected

Immunohistochemistry

Five-micrometer-thick paraffin sections were cut and deparaffinised through a graded series of xylol-ethanol following a previously described procedure.[24] Briefly, the endogenous peroxidase activity was inactivated with 3% (vol/vol) hydrogen peroxide in PBS for 30 minutes (min), followed by rinsing in PBS for 15 min. Sections were boiled in 0.01 mol/l citrate buffer (pH 6.0) or ethylene-diamino-tetra-acetate (EDTA, 5 mmol/l, pH 8.0) for 10 min. To reduce non-specific binding, sections were then incubated with TENG-T (10 mmol/l Tris-HCl, 5 mmol/l EDTA, 150 mmol/l NaCl, 0.25% (wt/vol) gelatin, 0.05% (wt/vol) Tween-20) for 30 min. This was followed by overnight incubation with primary antibodies. To determine enterocyte-specific protein expression of the small intestine we used anti-human lactase (DR BB 2/33; 1:2000; A.Quaroni), anti-human SI88 (1:1000)[25], anti-human Glut2 (1:200; B.Thorens), anti-rat Glut5 (1:1000; D.R.Yver) and anti-human i-FABP (1:100).[26] Enterocyte-specific protein expression of the colon was determined with anti-human CA IV (1:16000; W.S.Sly). As a marker for goblet cell specific protein expression, we used a human MUC2-specific antibody (We9, 1:200)[27] and anti-human TFF3 (1:1000).[28] Anti-human lysozyme (1:25, Dako, Glostrup, Denmark) was used to detect Paneth cell specific protein expression. To study epithelial proliferation, we used anti-human MIB-1 (Ki-67; 1:1000).[29] Sections were then incubated for 1 hour (h) with biotinylated horse anti-mouse IgG (diluted 1:1000, Vector Laboratories, England) or with biotinylated goat anti-rabbit IgG (diluted 1:2000, Vector Laboratories, England), followed by 1-h incubation with ABC/PO complex (Vectastain Elite Kit, Vector Laboratories) diluted 1:400. After incubation, binding was visualised in 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 0.02% (vol/vol) H_2O_2 in 30 mmol/l imidazole, 1 mmol/l EDTA (pH 7.0).

Statistics

Patient characteristics are presented as median (minimum-maximum).

RESULTS

Patients

The 21 infants with acute NEC had median GA of 32 (25-39) wk and median birth weight of 1525 (755-4060) g, Table 1. Before onset of NEC, all infants were fed breast milk or formula feeding according to our standard neonatal feeding protocol. Eleven infants had NEC in the small intestine, 4 both in the small intestine and in the colon, and 6 showed only damage of the colon. Median postnatal age at initial surgery was 11 (3-33) days (d). Due to complications (e.g. septic shock) caused by NEC, 3 infants died within 24 h after the initial surgery. One infant underwent stoma closure elsewhere and tissue samples were not obtained. Stoma closure in the remaining 17 infants was accomplished at median postnatal age of 82 (36-160) d. Control bowel samples were obtained from 10 infants with median GA of 38 (30-40) wk and median birth weight of 3018 (1510-4630) g.

Morphology

In 10 patients with acute NEC, the intestinal mucosa (n=3 out of 15 (small intestine); n=7 out of 10 (colon)) was completely denuded. Epithelium of the remaining small and large bowel showed severe or mild mucosal damage in acute NEC. Severe epithelial damage implied massive crypt/villus loss and almost complete villus atrophy (crypt/villus ratio approximately 1:1) in the small intestine (Figure 1A). Furthermore, severely affected areas in the colon showed crypt loss as well as loss of surface epithelium (Figure 1D). Mild damage was characterised by regions with villus atrophy in the small intestine (crypt/villus ratio approximately 1:2) and flattening of crypt and villus epithelial cells (Figure 1B). In colon, mild damage was defined by flattening and loss of only a few surface epithelial cells (Figure 1E). Additionally, the lamina propriae contained hemorrhages, erosions and inflammatory infiltrates in the small intestine as well as in the colon.

At stoma closure, epithelial morphology had almost completely restored, both in small and large intestinal tissue samples (Figures 1C and F). However, slight epithelial villus atrophy with a crypt/villus ratio of ≤ 1:3 was still present in several small intestinal tissue samples.

Epithelial proliferation

Epithelial proliferation in the small intestine and colon was studied by immunohistochemical staining of the Ki-67 antigen. The corresponding control samples showed proliferative cells at the bases of the crypts (not shown).

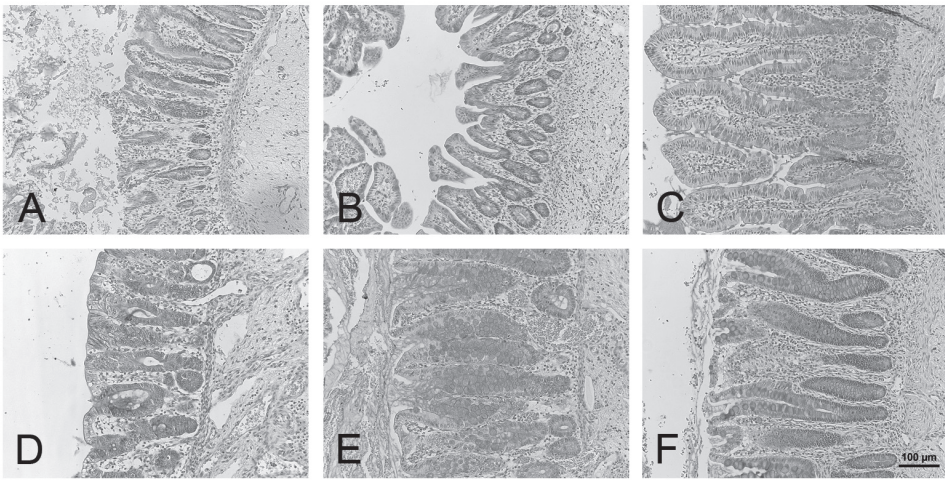

Figure 1 Epithelial morphology of the intestine in acute NEC and at stoma closure. Alcian Blue/Nuclear Fast Red staining of the small intestine (A-C) and colon (D-F). Morphology of acute NEC is characterised by severe (A and D) and mild (B and E) epithelial damage both in small and large intestines. At stoma closure, epithelial morphology has almost completely been restored (C and F). Colour figure, page 186.

In acute NEC, an increase in proliferative cells was observed in the small intestine and colon, both in areas with severe (Figures 2A and D) and with mild epithelial damage (Figures 2B and E). Specifically, the proliferative zone was not limited to the small intestinal crypts, but extended along the villi. In large intestinal tissue the proliferative cells extended from crypt base to crypt upper half in acute NEC. At stoma closure, proliferation in the residual bowel was still increased in crypts (till crypt upper half) of both the small intestine and colon (Figures 2C and F).

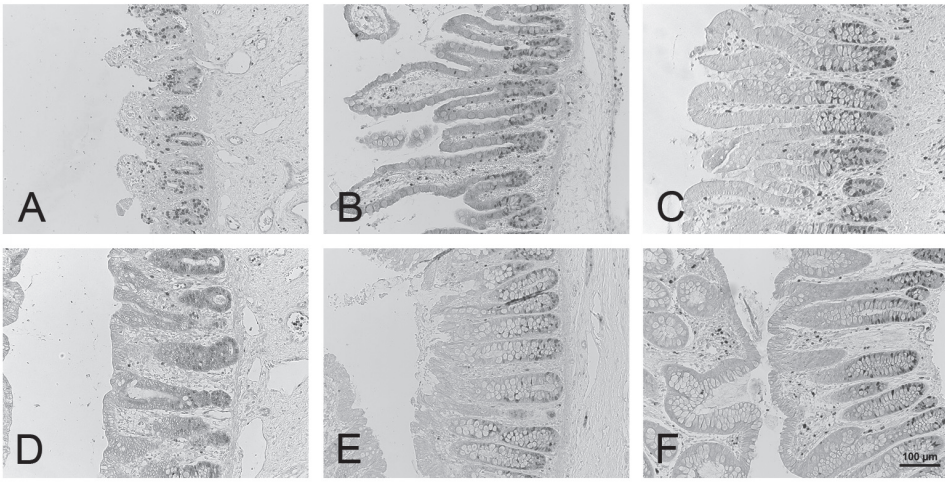

Figure 2 Proliferation in the small intestine and colon of infants with NEC. A and D: severe epithelial damage; B and E: mild epithelial damage. Proliferative activity was increased in acute NEC (small intestine: A and B; colon: D and E) and increased in crypts (till upper half of the crypt) of the small intestine and colon at the recovery phase (C and F, respectively). Colour figure, page 186.

Enterocyte-specific protein expression

Enterocyte-specific protein expression of the small intestine was immunohistochemically studied using antibodies against SI, Glut2, Glut5, lactase and i-FABP. Specifically, SI and i-FABP proteins were expressed in enterocytes of foetal bowel (Figures 3A and C). In control tissue samples, all proteins studied were expressed by villus enterocytes (Figures 4A-C, Glut5 and lactase not shown).

In acute NEC, SI protein expression in the remaining small bowel was confined to the brush border of villus enterocytes, both in areas with severe and mild epithelial damage (Figures 5A and B). Similarly, cytoplasmatic i-FABP protein expression of the small intestine in acute NEC, both in areas with severe and mild epithelial damage (Figures 5D and E), did not differ from that in control small intestinal tissue sections (Figure 4C).

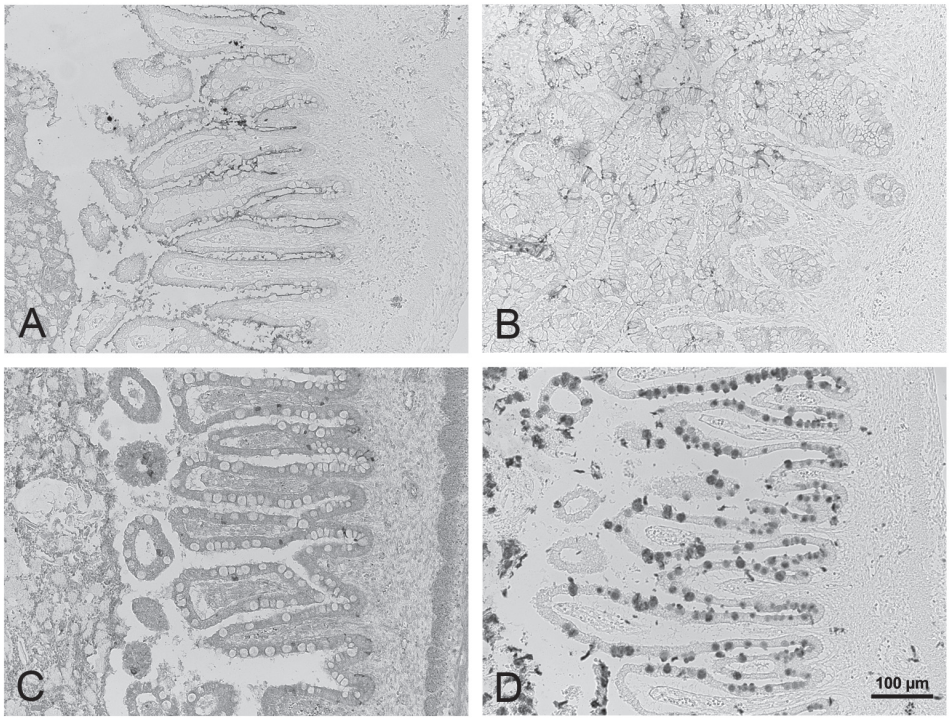

Figure 3 Foetal protein expressions. Sucrase-isomaltase (A), CA IV (B), i-FABP (C) and MUC2 (D) protein expression in foetal intestinal tissue. Colour figure, page 187.

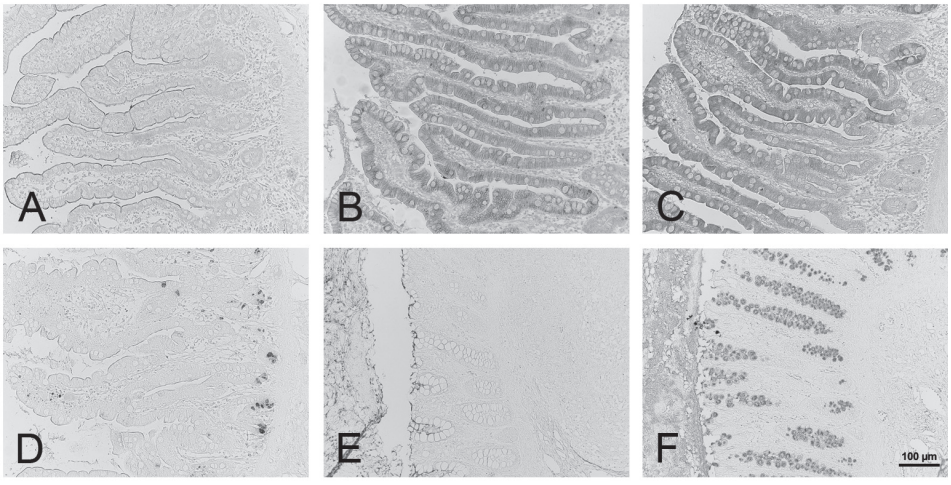

Figure 4 Neonatal protein expressions. Sucrase-isomaltase (A), Glut2 (B), i-FABP (C) and lysozyme (D) were expressed in the small intestine and CA IV (E) and MUC2 (F) were expressed in the colon of neonatal tissue. Colour figure, page 188.

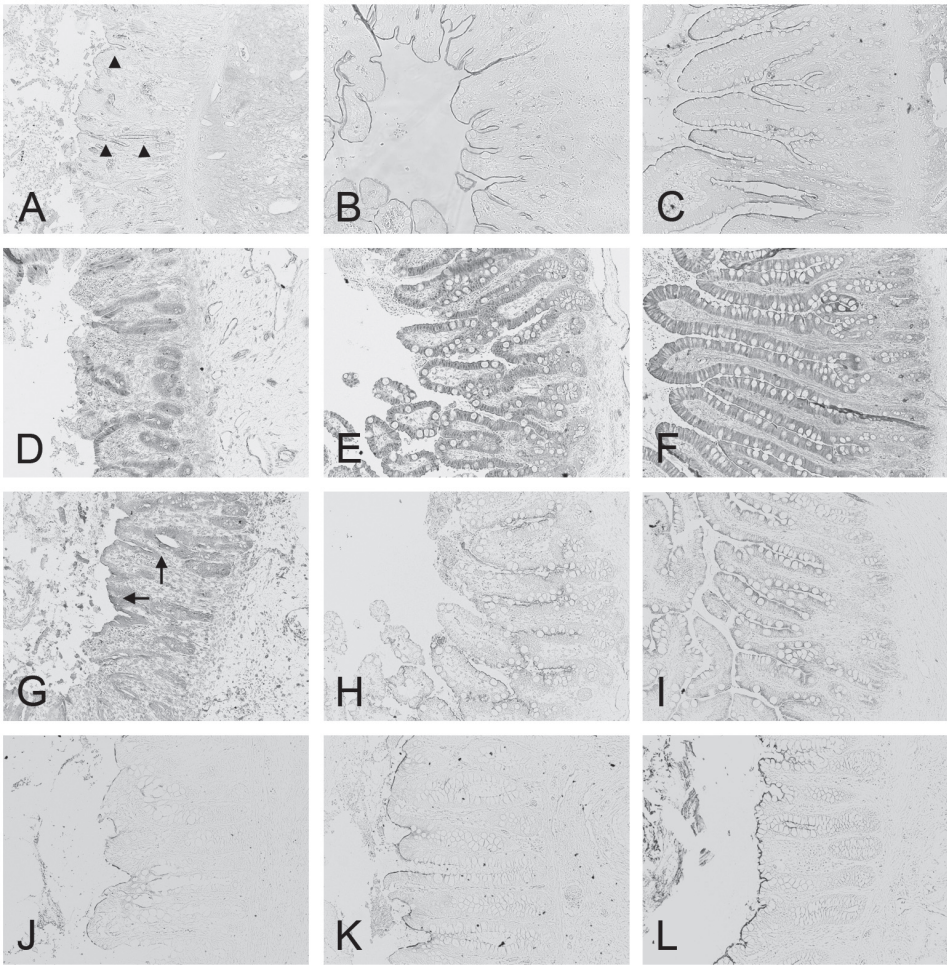

Figure 5 Epithelial enterocyte-specific protein expression of the intestine in acute NEC and at stoma closure (recovery phase). A, D, G and J: severe epithelial damage; B, E, H and K: mild epithelial damage. Enterocyte-specific SI expression (acute NEC (A (arrowheads) and B) and recovery phase (C)). Intestinal fatty acid binding protein was expressed in the cytosol of villus enterocytes in acute NEC (D and E) and at stoma closure (F). Enterocyte-specific lactase was expressed both in the acute phase (G (arrows) and H) and recovery phase (I). Carbonic anhydrase IV was expressed by the surface enterocytes of the colon both in the acute phase (J and K) and recovery phase (L). Colour figure, page 189.

Glut5 (not shown) and lactase (Figure 5H) were continuously expressed in small bowel samples of acute NEC patients with mild epithelial damage. In contrast, weak or no Glut 5 (not shown) and lactase expression was observed in severely damaged small intestinal tissue in acute NEC (Figure 5G). Similarly, Glut2 protein was moderately expressed at the basolateral membrane in relatively unaffected small bowel in acute NEC, but protein expression was weakly positive in the residual small bowel with severe epithelial injury (not shown). At stoma closure, all of the above-mentioned enterocyte-specific proteins were normally expressed by villus

enterocytes of the surface epithelium (Figures 5C, F and I) of the remaining small bowel.
CA IV is expressed by colonic surface enterocytes, and was used as a marker for colonic enterocyte function. It was normally expressed in foetal and control neonatal bowel (Figures 3B and 4E, respectively). In acute NEC, CA IV expression appeared unaltered in resection margins with severely damaged epithelium as well as in resection margins with mild epithelial damage (Figures 5J and K). It was maintained by all surface enterocytes of the remaining colon at stoma closure (Figure 5L).

Goblet cell-specific protein expression

The goblet cell-specific protein expression was studied using MUC2 and TFF3 specific antibodies both for small intestinal and colonic epithelium. Foetal goblet cells showed MUC2 protein expression similar to that in mature control intestinal tissue (Figures 3D and 4F (colon)). In control samples, TFF3 protein was normally expressed by goblet cells in villi and crypts of jejunum, ileum and colon (not shown). In acute NEC, severely damaged small and large intestinal tissues showed fewer goblet cells due to loss of crypt and surface epithelium. In contrast, areas characterised by mild mucosal injury showed similar or even increased numbers of goblet cells compared with control samples. Furthermore, the goblet cells present demonstrated normal MUC2 (Figures 6A, B, G and H) and TFF3 protein expression (Figures 6J and K) in both the small intestine and colon. At stoma closure, MUC2 and TFF3 proteins were strongly expressed by goblet cells of renewed epithelium in accordance with control samples (Figures 6C, I and L).

Paneth cell-specific protein expression

Lysozyme was used as a marker for Paneth cell-specific cell function. Paneth cells are normally present at the crypt bases of jejunum and ileum. Strong positive staining for lysozyme was found in cytoplasmatic granules of Paneth cells in all control small intestinal tissue samples (Figure 4D). Lysozyme-positive cells, probably macrophages, were also detected in the lamina propriae of controls. In acute NEC, lysozyme-positive Paneth cells were still present in the resection margins characterised by mild epithelial damage (Figure 6E). However, in these areas, lysozyme expression in the Paneth cells was often weak compared to control samples. Severe mucosal damage was associated with reduced numbers of Paneth cells due to epithelial loss. Similar to areas with mild epithelial damage, lysozyme was weakly expressed by the Paneth cells in severely damaged epithelium (Figure 6D). At stoma closure, Paneth cells in patients with acute NEC demonstrated increased strong expression of the antibacterial protein lysozyme (Figure 6F). Additionally, the localisation of Paneth cells at the crypt bases was similar between control small bowel and the residual small intestine of NEC patients both in acute NEC and at stoma closure.

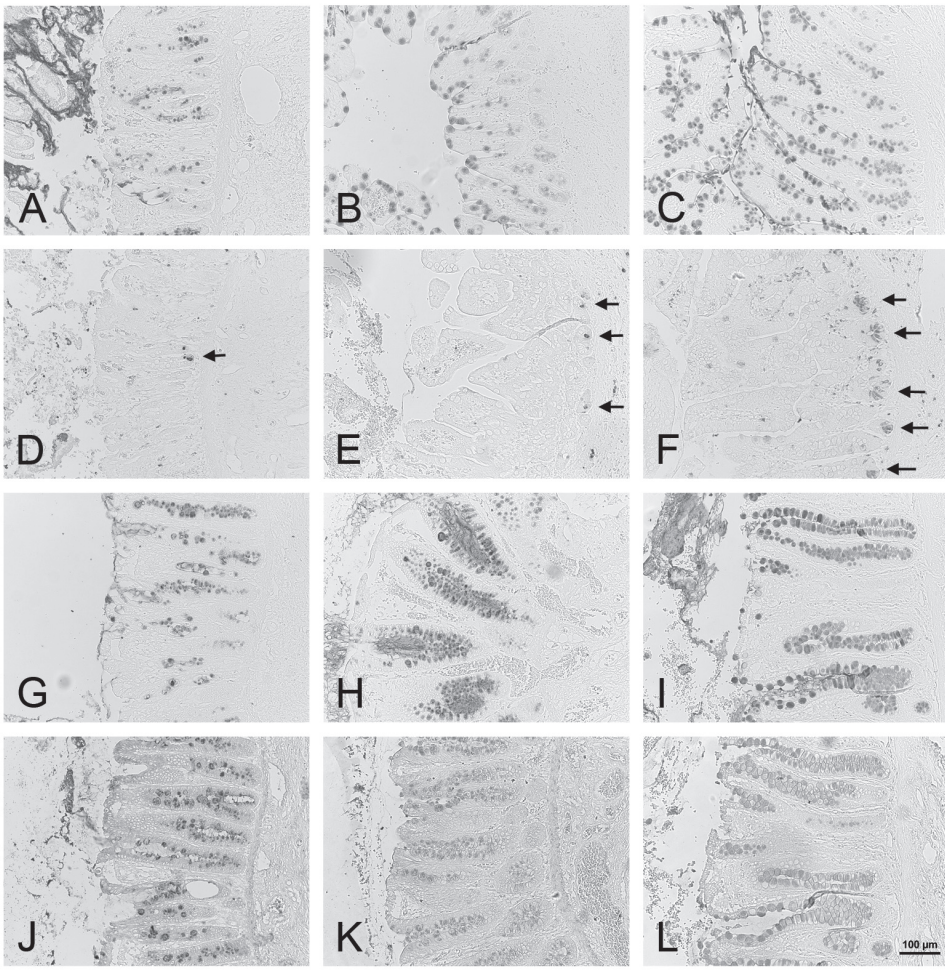

Figure 6 Goblet cell-specific protein expression and Paneth cell-specific protein expression. A, D, G and J: severe epithelial damage; B, E, H and K: mild epithelial damage. Expressions of MUC2 protein in the small intestine and colon in the acute (small intestine: A and B; colon: G and H) and recovery phase (small intestine: C; colon: I) of NEC. Trefoil factor 3 protein was expressed in the colon in acute NEC (J and K) and at recovery (L). Lysozyme was expressed by Paneth cells in the crypts of the small intestine both in acute NEC (D and E) and at stoma closure (F). Arrows indicated lysozyme-positive Paneth cells. Colour figure, page 190.

DISCUSSION

We evaluated the epithelial morphology, epithelial proliferation and enterocyte-, goblet cell-, and Paneth cell-specific protein expression as parameters for epithelial functioning of the residual bowel in human infants who underwent bowel resection for acute NEC and at the time of stoma closure (recovery phase).

Several studies clearly demonstrate that the enterocyte-specific proteins lactase, SI, Glut2

and Glut5, the goblet cell-specific protein TFF3, and Paneth cell marker lysozyme are already expressed in normal foetal small and/or large intestine and remain expressed after birth.[30-34] However, data on the expression of the enterocyte-specific proteins i-FABP and CA IV and goblet cell-specific MUC2 protein during foetal life are lacking. Before studying the protein expression of i-FABP, CA IV and MUC2 in NEC patients we first had to determine whether these cell type-specific markers are already expressed in normal foetal and neonatal intestine. Human foetal intestinal tissue (median GA of 20 (16-25) wk) showed MUC2, i-FABP and CA IV protein expression in goblet cells and enterocytes, respectively. Additionally, the markers were also expressed in neonatal intestinal tissue. These data, together with previous findings, indicate that both enterocytes and goblet cells express a wide variety of proteins in foetal life, continuing after birth.

At surgery for acute NEC, the paediatric surgeon based the location of the resection margins on macroscopically healthy intestinal tissue. Thereby, the resection margins were assumed representative for the remaining bowel. However, microscopic analysis of the resection margins still revealed mucosal damage, from complete epithelial loss to fairly unaffected epithelium. Consequently, in response to NEC-induced epithelial injury present in the residual intestine after resection, a repair process must immediately be initiated to maintain intestinal integrity, protecting the host from transfer of pathogenic bacteria across the intestinal mucosa, and thus preventing further epithelial damage. Therefore, increased epithelial proliferation and migration of epithelial cells to the sites of injury is essential for adequate epithelial repair. We found proliferative cells extendeding along the villus in the small intestine and in colon from crypt base to crypt upper half, implying increased epithelial proliferation in the remaining bowel of infants with acute NEC. This increased proliferative activity appears to restore epithelial morphology, as seen at stoma closure (recovery phase). Previous studies in human infants with NEC confirm the pattern of severe epithelial destruction followed by an increase in proliferative activity of the residual bowel.[35] However, increased epithelial cell proliferation might be associated with a decrease in epithelial cell differentiation, suggesting an altered epithelial protein expression pattern. We observed gradations in protein expression of several enterocyte-specific markers in the small intestine. Namely, in the remaining small bowel SI and i-FABP expression was maintained in areas with severe epithelial damage, whereas lactase, Glut2 and 5 were only weakly or not at all expressed in these areas. Thus, distinct enterocyte-specific proteins were down-regulated during the process of severe epithelial damage, whereas others were maintained. This specific down-regulation might result from altered protein stability in acute NEC. Similarly, the intestinal epithelium perhaps actively down-regulates the protein expression of lactase and glucose transporters to save energy for re-establishment of the impaired mucosal barrier. Epithelial-specific protein down-regulation in the remaining bowel after resection for acute NEC may have clinical consequences in that the enterocytes loose their capacity to synthesise proteins essential for efficient nutrient digestion and absorption. Speculating, down-regulation of these proteins might contribute to a temporarily decreased absorption capacity of dietary nutrients. However, we found enterocyte-specific protein expression to have fully recovered at the time of stoma closure.

Rapid repair of the mucosal barrier following even minor epithelial injury is essential to preserve intestinal metabolism. Trefoil peptides and mucins are vital components in this process. Despite the fewer numbers observed in severely damaged intestinal epithelium of infants with acute NEC, goblet cells continued to express MUC2 and TFF3 protein. Those patients recovering from NEC maintained MUC2 and TFF3 protein expression. However, Vieten *et al*. demonstrated a down-regulation of TFF3 expression both at protein and mRNA level in acute NEC.[36] They also found continued down-regulation of TFF3 protein expression in the recovery phase. Furthermore, in the current study, Paneth cells in the residual small intestine were weakly lysozyme-positive in acute NEC, both in mild and severely damaged tissue, compared to the recovery phase. At least two interpretations of this observation are possible. First, Coutinho *et al*. suggest delayed maturation of lysozyme-expressing Paneth cells in these patients.[37] This explanation is unlikely, however, because lysozyme is already expressed by the Paneth cells at 20 weeks of gestation.[34] Second, the Paneth cells might rapidly secrete lysozyme in response to epithelial injury. However, this innate defence mechanism might not be sufficient to prevent further epithelial damage finally leading to NEC. Taken together, this data suggests that MUC2, TFF3 and lysozyme expressions in the residual bowel of infants with acute NEC might be insufficient to protect the epithelium and to repair epithelial injury.

In summary, we found a disturbed epithelial proliferation/differentiation balance of the residual bowel in infants with acute NEC. Furthermore, distinct enterocyte-specific functions were down-regulated. In the acute phase, NEC may be associated with reduced MUC2, TFF3 and lysozyme expression levels leading to impaired epithelial barrier function and defence. However, infants recovering from NEC showed fully re-established epithelial functions, such as nutrient digestion and absorption, intestinal barrier function, and innate defence, at least at the time of stoma closure (approximately 10 weeks since the initial surgery). Whether large individual differences in recovery exists between patients cannot be extracted from our present data. Longitudinal, prospective and repeated bowel samples are required, however, for ethical reasons not feasible in these vulnerable newborns.

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Part II

Gut barrier function

Chapter 5

Threonine utilisation is high in the intestine of piglets

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ABSTRACT

Background: The whole-body threonine requirement in parenterally fed piglets is substantially lower than that in enterally fed piglets, indicating that enteral nutrition induces intestinal processes in demand of threonine. We hypothesised that the percentage of threonine utilisation for oxidation and intestinal protein synthesis by the portal-drained viscera (PDV) increases when dietary protein intake is reduced.

Methods: Piglets (n=18) received isocaloric normal or protein- restricted diets. After 7 hours of enteral feeding, total threonine utilisation, incorporation into intestinal tissue and oxidation by the PDV, were determined with stable isotope methodology ([U-^{13}C]threonine infusion).

Results: Although the absolute amount of systemic and dietary threonine utilised by the PDV was reduced in protein-restricted piglets, the percentage of dietary threonine intake utilised by the PDV did not differ between both groups (normal protein 91% vs. low protein 85%). The incorporation of dietary threonine into the proximal jejunum was significantly different compared with the other intestinal segments. Dietary, rather than systemic threonine was preferentially utilised for protein synthesis in the small intestinal mucosa in piglets that consumed the normal protein diet ($p<0.05$). Threonine oxidation by the PDV was limited during normal protein feeding. In protein restricted pigs, half of the total whole-body oxidation occurred in the PDV.

Conclusions: We conclude that, *in vivo*, the PDV have a high obligatory visceral requirement for threonine. The high rate of intestinal threonine utilisation is due mainly to incorporation into mucosal proteins.

Abstract

INTRODUCTION

The small intestine is one of the most metabolically active tissues in the body. For example, the portal-drained viscera (PDV), i.e. the intestine, pancreas, spleen and stomach, in neonatal pigs account for only 4 to 6% of the whole-body mass, but are responsible for ~25% of the total whole-body CO_2 production, and for 20-50% of the total protein turnover.[1-5]

Studies in pigs showed that >70% of the first-pass metabolism of some essential amino acids by the splanchnic tissues occurs in the intestine.[6-9] In humans, the splanchnic tissues retain between 20 and 50% of the dietary intake of specific essential amino acids.[5, 10, 11] For some dispensable amino acids, notably glutamate, the first-pass splanchnic extraction exceeds 90% of the dietary intake, in both humans and in pigs.[12, 13] Together, these studies indicate that the small intestine has a substantial amino acid metabolism.

In this context, threonine is of critical nutritional importance, because it is the single most used indispensable amino acid by the metabolism of the PDV. The retention of threonine by the PDV in first-pass ranges from 60 to 80% of the dietary intake under normal feeding circumstances, whereas the first-pass metabolism of other indispensable amino acids such as lysine and leucine accounts for roughly one-third of the dietary intake.[9, 14, 15] Consistent with the finding that so much threonine is utilised by the PDV, the whole-body threonine requirement is reduced by 60% in piglets receiving total parenteral nutrition compared with enteral nutrition.[16] A key unresolved question is the metabolic fate of threonine used by the gut. A major metabolic fate of threonine is likely the incorporation into mucosal proteins because some of these proteins contain a high amount of threonine in their peptide backbone (e.g. mucin).[17, 18] However, another metabolic fate of threonine could be oxidation because previous studies showed that some indispensable amino acids including lysine and leucine are oxidised within the gut.[14, 19] If the predominant metabolic fate of dietary threonine in the intestine is oxidation, then the first-pass metabolism by the intestine is a source of nutritional inefficiency. Therefore, determining the rate of intestinal oxidation and incorporation into mucosal proteins was the first objective of our study.

Mucosal cells receive substrates directly from both the diet and the mesenteric circulation; previous studies showed a compartmentalisation of amino acids by the intestinal tissues.[8, 13, 14, 20, 21] The proportions of visceral threonine metabolism derived from the luminal and the arterial sites are not known. Thus, the second objective was to determine the relative rate of the systemic threonine metabolism by the PDV.

The degree to which the first-pass utilisation of amino acids is dependent on the nutrient composition and dietary protein intake is an important question. An obligatory high utilisation rate of indispensable amino acids, which is independent of dietary protein intake, would result in a very low systemic availability of dietary amino acids at a low protein intake with subsequently impaired growth. Although there have been few investigations on this issue, the available data are contradictory. In a previous study with growing pigs, we showed

that during protein restriction, intestinal growth is preserved, apparently at the expense of skeletal muscle growth.[22, 23] In addition, we found that a prolonged period of protein restriction lowers the fractional synthesis rate of total mucosal protein by only 25–40%.[24, 25] Determination of total threonine utilisation by the PDV in piglets fed a low-protein diet was the last objective of our study.

We studied the utilisation of systemic threonine by the PDV in 4-week-old piglets fed isocaloric diets having either a normal protein (NP) or low-protein (LP) content. By using the stable isotope methodology, we were able to measure: *1)* the intestinal incorporation of threonine, *2)* systemic threonine uptake by the PDV, *3)* the systemic oxidation of threonine by the PDV, and *4)* the response in intestinal and whole-body threonine metabolism to protein restriction.

MATERIALS AND METHODS

Piglets

The Baylor College of Medicine Animal Protocol Review Committee approved the study. Housing and care of the piglets conformed to the USDA guidelines. The study involved 4-week-old female crossbred piglets (n=18; Large White X Hampshire X Duroc) purchased from the Texas Department of Criminal Justice. The piglets were received at the CNRC when they were 2 weeks old and were fed a liquid milk replacer (Litterlife, Merrick) at a rate of 50 g/(kg body weight·d). The composition (/kg dry matter) of the milk replacer was 500 g lactose, 100 g fat and 250 g protein. The calculated energy density was 18 MJ gross energy/kg dry matter.

Study design

The study design was described previously.[14, 19, 21, 26] At a postnatal age of 3 weeks, piglets (n=11) were surgically implanted with catheters after overnight food deprivation. The surgery entailed the placement of catheters into the carotid artery, portal vein and jugular vein. Eight piglets received a catheter into the duodenum for enteral tracer administration. One piglet received the threonine tracer enterally as well as intravenously. In addition, an ultrasonic flow probe (Transonic Systems) was placed around the portal vein. After surgery, the piglets were administered complete intravenous (i.v.) nutrition for 24–36 hours (h). They then were fed either regular Litterlife [NP diet: 12.7 g protein, 5.1 g lipid and 25.5 g carbohydrates/(kg·d)] or a diet that contained only 40% of the protein content in Litterlife [LP diet: 5.1 g protein, 7.5 g lipid and 30.4 g carbohydrates/(kg·d)]. Protein intake during LP feeding was deliberately set at a rate to provide enough protein to compensate for obligatory amino acid oxidation. The diets were made isocaloric by adding lactose (Sigma Chemical) and corn oil in the same ratio as in the control (NP) diet. The piglets were given ~4 times as much energy and protein compared with human infants to provide enough to maintain an adequate growth rate, which is 4 times as high in piglets as in human neonates. At postnatal day (d) 28, whole-body CO_2 production was measured with an infusion of [1-^{13}C]bicarbonate. The

[U-^{13}C]threonine infusion protocols were conducted on postnatal d 30 and 32, when the piglets had received full enteral feeding of the same diet (either NP or LP) for at least 8 d.

Isotope tracer protocol

After overnight food deprivation, the piglets consumed a meal that supplied one-seventh of the preceding daily intake to restore intestinal motility. Immediately thereafter, a continuous gastric infusion of diet was started at a rate that provided one-fourteenth of the preceding daily intake each hour. On postnatal d 28, [1-^{13}C]bicarbonate (99%, Cambridge Isotope Laboratories) was infused into the jugular catheter at a rate of 10 μmol/(kg·h). Arterial and portal blood samples (1 ml) were taken at 15-minutes (min) intervals from minute 75 to 120 of infusion. A methodological study in 4 piglets infused with $^{13}NaH^{13}CO_3$ for 7 h at d 28, 30 and 32 showed that bicarbonate kinetics per kilogram body weight remained the same although they were growing in that period. A plateau in enrichment was reached after 60 min. On postnatal d 30-32, [U-^{13}C]threonine (98%, Cambridge Isotope Laboratories) was infused via either the duodenal or the jugular catheter at a rate of 10.8 μmol/(kg·h) for 5 h. During the last h of the tracer infusion, 4 arterial and portal blood samples were drawn at 15-min intervals. The piglets were killed with an arterial injection of sodium pentobarbital (50 mg/kg) and sodium phenytoin (5 mg/kg) (Beutanasia-D; Schering-Plough Animal Health). The abdomen was opened and the entire small intestine distal to the ligament of Treitz was immediately flushed with ice-cold saline. After flushing, the intestine was divided into 2 equal parts; the proximal half was designated the jejunum and the distal part, the ileum. These 2 parts were divided in half, resulting in a total of 4 segments: proximal jejunum, distal jejunum, proximal ileum and distal ileum. The 4 segments were weighed and then the tissue samples were snap-frozen in liquid nitrogen and stored at −70°C until analysis for tracer enrichment, protein and DNA. The first part of each intestinal segment was taken for analysis applying the same procedure.

Sample preparation

Blood samples: Small aliquots (0.2 ml) were taken for direct determination of the concentrations of blood gases (Chiron Diagnostics), glucose and lactate (YSI analyser). The isotopic and concentration measurements of the amino acids and $^{13}CO_2$ were made on whole blood as described in detail in a previous publication.[14]

Intestinal tissue samples: Each intestinal tissue sample (200 mg) was homogenised in water and aliquots removed for analysis of protein and DNA as described previously.[25] The protein fraction was isolated by adding 1 ml of 2.0 mol/l perchloric acid. The intestinal tissue/perchloric acid mixture was centrifuged at 1800 *g* for 20 min. Pellets were washed 3 times with 3 ml of 0.2 mol/l perchloric acid to remove the remaining free amino acids. The washed pellets were hydrolysed by adding 0.5 ml of 6 mol/l hydroxychloride and incubating at 110°C in sealed tubes for 24 h. The protein hydrolysates were dried under nitrogen at 55°C and the residue was dissolved in 0.5 ml water. Amino acids were isolated from protein hydrolysates by cation exchange separation as described previously for the blood amino acid fraction.

Derivatisation: Threonine was converted to its N-ethoxycarbonylethylester derivative according to a modified method of Husek.[27]

Mass spectrometry

Blood samples: Whole-blood samples were prepared for amino acid and CO_2 analysis as described previously.[13, 14] Isotopic enrichment of threonine and CO_2 was measured by isotopic ratio MS.[26] The atom percent enrichment was converted to mole percent threonine enrichment (MPE), after accounting for the 2.25-fold dilution of carbon in the derivative and the measured [^{13}C]-abundance (98%) of the threonine tracer.

Intestinal tissues: A Thermo Finnigan Delta-XP isotope ratio MS coupled online with a trace GC (Thermo Electron) and a combustion interface type 3 (Thermo Finnigan) was used for the [^{13}C/^{12}C]-ratio measurement of threonine. Aliquots of 1 µl of the chloroform suspension containing the amino acid derivatives were introduced to the GC system by a CTC PAL autosampler (CTC Switzerland). The flow was set at a constant rate of 1 ml/min and samples were introduced in splitless mode. A DB-225ms (Agilent) capillary column 30 m in length with an i.d. of 0.25 mm was used for the chromatographic separations. The injector temperature was 250°C and the oven temperature was programmed starting at 160°C for 1 min, then increased from 160°C to 230°C at a rate of 5°C/min and held at 230°C for 5 min.

After separation using capillary GC, amino acids were combusted online at 940°C and introduced as CO_2 into the isotope ratio MS, where the [^{13}C/^{12}C]-ratio was measured.

Calculations

Previous studies from our laboratory and those of others used a steady state, whole-body model of amino acid metabolism that was developed as described by Waterlow *et al*.[28] This model assumes a common metabolic amino acid pool through which all amino acids move, either as dietary or systemic amino acids or from protein breakdown, or to exit for protein synthesis or oxidation. This movement through the metabolic pool is called flux. From the measurements of arterial and portal enrichments of isotopically labelled tracers, arterial and portal amino acid concentrations, and portal blood flow, the total and systemic uptake of substrates across the PDV can be calculated.[14, 19, 21, 26]

The equations used for calculating the threonine metabolic fate were described previously.[14, 26] For the calculations of intestinal threonine incorporation, we used a value of 3.39 g threonine/100 g total amino acids measured in neonatal pigs as done by Wu *et al*.[29]

Statistics

Results concerning weight gain, CO_2 production, oxidation, and data for samples taken over the last h of the tracer study are expressed as the mean ± the inter-animal SEM. Other data are presented as the median (minimum-maximum). Differences between the balances of the piglets fed the NP or LP diet were tested by an independent samples *t* test or Mann-Whitney test (two-tailed). Significance between the incorporation of dietary threonine into different segments of the intestine was assessed using ANOVA and a paired *t* test (two-tailed). Differences between the incorporation of dietary threonine in different feeding groups were assessed using an independent samples *t* test (two-tailed). Differences with $p<0.05$ were considered to be significant.

RESULTS

Before surgery, the piglets were gaining weight at a rate of 55 ± 2.3 g/(kg·d). Body weights of the 2 groups did not differ on the day of surgery (5.35 ± 0.09 kg). After surgery, weight gain in the NP piglets (n=9) were 45 ± 2.6 g/(kg·d) and in LP piglets 21 ± 2.2 g/(kg·d) (n=9) ($p<0.0001$), a difference that affected the body weight at postnatal d 30 and 32 when the piglets were killed (NP: 8.53 ± 0.29 vs. LP 7.07 ± 0.15 kg, $p<0.0001$).

Neither the whole-body CO_2 production [57.8 ± 3.0 mmol/(kg·h), (n=11)], nor the CO_2 production by the PDV [9.4 ± 1.4 mmol/(kg·h), (n=11)], was affected by protein restriction. Overall, the PDV accounted for 16% of the total whole-body CO_2 production.

Table 1 summarises the results obtained during the i.v. [U-^{13}C]threonine tracer infusions in both feeding groups. At 7 h after the start of feeding, the arterial and portal threonine concentrations were significantly lower in piglets fed LP than in those fed NP. The portal mass balance of threonine [(dietary intake + systemic intake) – portal outflow] tended to be lower ($p=1.00$) in the protein-restricted group [median NP: 150 (-223 to 245) µmol/(kg·h) vs. median LP: 100 (-211 to 235) µmol/(kg·h)]. During the i.v. tracer administration, the portal isotopic enrichment of threonine was lower than the arterial isotopic enrichment, indicating that there was net intestinal uptake of systemic threonine during both feeding periods.

Table 1 Portal blood flow, arterial and portal enrichments of threonine and CO_2, arterial and portal concentrations of threonine and CO_2, flux, tracer balances and portal mass balances in piglets fed a NP or a LP diet in combination with an i.v. infusion of [U-^{13}C]threonine [1,2]

	NP (n=6)	LP (n=5)	p-value
Portal blood flow, *l/(kg·h)*	4.8 ± 0.8	4.9 ± 0.4	NS
Infusion tracer rate, *µmol/(kg·h)*	11.04 ± 0.42	11.54 ± 0.41	NS
Arterial [Threonine], *µmol/l*	1181 ± 69	698 ± 144	0.010
Portal [Threonine], *µmol/l*	1203 ± 68	710 ± 158	0.012
Arterial Threonine IE, *MPE*	0.776 ± 0.051	0.899 ± 0.049	NS
Portal Threonine IE, *MPE*	0.681 ± 0.041	0.863 ± 0.048	0.022
Tracer balance, *µmol/(kg·h)*	-4.87 ± 1.70	-0.68 ± 0.64	NS
Portal mass balance,[3] *µmol/(kg·h)*	150 (-223 to 245)	100 (-211 to 235)	NS
Flux (Q), *µmol/(kg·h)*	1434 ± 150	1243 ± 57	NS
Arterial [CO_2], *mmol/l*	25.57 ± 0.65	28.05 ± 0.38	0.021
Portal [CO_2], *mmol/l*	27.80 ± 0.46	29.63 ± 0.40	0.024
Arterial CO_2 IE, *MPE*	0.005 ± 0.001	0.001 ± 0.001	0.006
Portal CO_2 IE, *MPE*	0.004 ± 0.001	0.001 ± 0.000	NS

[1]Values are means ± SEM unless otherwise noted.
[2]Abbreviations: **IE:** isotopic enrichment; **MPE:** mole percent excess; **NS:** not significant ($p>0.05$).
[3]Values are medians (minimum-maximum)

The percentage of arterial threonine that was taken up by the PDV was 10% in piglets fed NP, whereas it decreased to 3% in piglets fed LP.

In piglets fed NP, two-thirds of the utilised threonine was sequestered by the PDV from the systemic circulation (Table 2). Of even more interest, the systemic threonine utilisation was significantly affected by a lower protein intake. The total threonine utilisation by the PDV was significantly lower in LP piglets [274 (139–585) µmol/(kg·h)] than in NP piglets [784 (689–1157) µmol/(kg·h)]. The equivalent of 85% of the total threonine intake was utilised in the PDV in piglets fed LP, whereas 91% of the threonine intake was utilised in piglets fed NP ($p>0.05$).

The intestinal [U-^{13}C]threonine enrichment after 5 h of tracer infusion in each of the 4 intestinal segments was expressed as mole percent excess (MPE) (Figure 1). After the i.v. infusion, the values did not differ among the 4 intestinal segments and or between piglets fed NP or LP. During enteral tracer administration, the threonine enrichment was significantly higher in the proximal jejunum than in the other segments, indicating that the majority of the dietary threonine was taken up in the proximal part of the small intestine.

The utilised threonine can be used for oxidation and protein synthesis. By measuring the isotopic enrichment of threonine in the intestinal mucosa, we were able to quantify the incorporation of threonine into mucosal proteins (Table 2). The incorporation rate of dietary threonine into intestinal protein was higher in piglets fed NP [439 (275–779) µmol/(kg·h)] than in those fed LP [250 (232–362) µmol/(kg·h)].

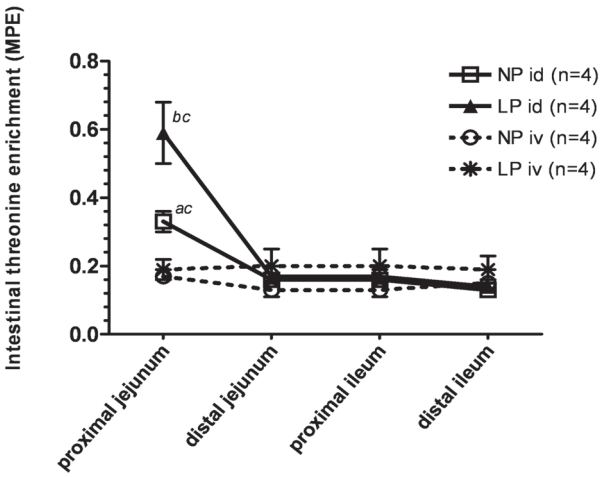

Figure 1 Intestinal threonine enrichment in piglets fed a NP or LP diet in combination with an intravenous (i.v.) or intraduodenal (i.d.) infusion of [U-^{13}C]threonine measured in the 4 segments of the small intestine. Values are means ± SEM. [a]Different from the other intestinal segments in the NP i.d. group ($p<0.01$); [b]Different from the other intestinal segments in the LP i.d. group ($p<0.02$); [c]NP i.d. differed from LP i.d. ($p<0.05$).

These amounts represent 57% (NP-fed) and 86% (LP-fed) of the total PDV threonine utilisation. In both NP- and LP-fed piglets, the intestine utilised both dietary and systemic threonine for intestinal protein synthesis, but dietary threonine was used predominately. Dietary and systemic threonine incorporation did not differ between piglets fed NP or LP diet, although dietary threonine incorporation tended to differ (p=0.057) between the NP and LP groups.

Table 2 Threonine intake, systemic and total utilisation of threonine by the portal-drained viscera (PDV), the systemic oxidation by the PDV, and the dietary and systemic intestinal incorporation of threonine in piglets fed either a NP or a LP diet[1]

	NP (n=6)	LP (n=5)
Intake, *µmol/(kg·h)*	934	374
Systemic utilisation, *µmol/(kg·h)*	467 (60-1334)	80(0-235)*
Total utilisation, *µmol/(kg·h)*	784 (689-1157)	274 (139-585)**
% of the intake	91	85
Threonine incorporation, *µmol/(kg·h)*		
Dietary (n=8)	439 (275-779)	250 (232-362)
Systemic (n=8)	131 (91-136)#	83 (52-132)

[1]Values are medians (minimum-maximum) unless stated otherwise.
Asterisks indicate different from NP: * p<0.05, ** p<0.01. # Different from NP dietary, p<0.05.

The intestinal threonine oxidation did not differ between piglets fed NP [15 ± 7 µmol/(kg·h)] or LP [28 ± 10 µmol/(kg·h)]. Direct oxidation is therefore not a major metabolic pathway of threonine in the intestine. We measured [^{13}C]-enrichment of glycine in arterial and portal blood samples to account for threonine dehydrogenase degradation pathway, but [^{13}C]glycine enrichment did not differ from baseline values. The whole-body threonine oxidation as measured by the systemically infused threonine was 117 ± 18 µmol/(kg·h) during NP feeding. The systemic visceral threonine oxidation represented 13% of the whole-body threonine oxidation. Whole-body threonine oxidation was affected in piglets fed LP [61 ± 6 µmol/(kg·h) (p<0.05)] and systemic visceral oxidation accounted for approximately half of the total oxidation rate.

DISCUSSION

Threonine is an important limiting amino acid for growth and maintenance in diets for pigs.[30] In addition, threonine is of critical importance for the intestinal function because it is essential to the structural protein mucus layer, which lines the gastrointestinal tract. Several studies showed that the utilisation of a specific amino acid is not constant over a wide range of intakes.[31–33] The aim in the present study was to investigate the effect of protein restriction on intestinal and whole-body threonine metabolism in piglets. We found that, irrespective of the dietary protein intake, the PDV extracted a very large amount of systemic threonine, which was incorporated mainly into intestinal mucosal proteins. Our results showed no effect of the LP diet in intestinal weight (data not shown). However, there was significant difference in body weight gain between the 2 feeding groups, with NP-fed piglets weighing more. These results suggest that during protein restriction, the intestine is spared in favour of other tissues.

In recent years, there has been growing recognition that a very large proportion of certain nonessential amino acids (e.g. aspartate, glutamate and glutamine) from the diet are utilised by the intestine and do not appear in the systemic circulation.[12, 13] Similarly, we showed previously in piglets that the net portal balance of lysine is significantly less than the dietary intake, indicating that the PDV tissues utilise a considerable amount of dietary lysine.[14] Moreover, we showed that in piglets fed high protein, most of the lysine used by the PDV is derived from the systemic circulation, but this shifts to an increased first-pass use during protein restriction. A similar phenomenon occurs for threonine. Two-thirds of the threonine utilisation is derived from the systemic circulation in piglets fed NP, whereas the majority of the utilised threonine was derived from the intestinal lumen in piglets fed LP.

In a previous study, we showed that intestinal recycling of amino acids contributes significantly to their systemic availability.[26] In the present study, we postulate that a small amount of threonine was recycled by the PDV because we only infused for 5 h. However, as discussed above, the amount of recycled threonine might be underestimated because the degradation of secreted proteins and subsequent absorption take more time. We therefore assume that the threonine utilised for intestinal protein synthesis eventually becomes at least partially available for the peripheral tissues through an efficient reabsorption process. The recycling of threonine might also explain the growth of the piglets during the study. We think it is likely that our results reflect the first process immediately after feeding was started because we measured for 5 h; thus, the intestinal net uptake of threonine might be an overestimation.

Previous studies showed that threonine is one of the most important amino acids in (glyco-) proteins (e.g. mucins). Mucins contain large amounts of proline, threonine and serine in their peptide backbone, together comprising 20–55% of the amino acid composition.[17, 18, 34] These glycoproteins are the main constituents of the mucus layer. It appears that most threonine used by the intestine is for mucosal and secretory protein synthesis because

threonine oxidation represents only 2-9% of the total threonine utilised. Total intestinal threonine incorporation contributed 71% to total threonine utilisation. This incorporation can be within mucins and in goblet cells that are not yet secreted or into constitutive proteins in the intestine. We realise that this number is somewhat uncertain because we had to use a different set of piglets for the intraduodenal study. Variability among the piglets results in variability in the incorporation data. Because only 15 µmol/(kg·h) was oxidised in piglets fed NP (2%), 27% of the threonine utilised is unaccounted for. This is probably a reflection of the amount of threonine secreted (probably as mucins) into the lumen. Loss of apical cells (with proteins) also contributes. In piglets fed LP, slightly more than the total amount of utilised threonine was found incorporated within the intestine. This is probably due to variability among the different pigs. However, we are certain that the vast majority of the utilised threonine is used for protein synthesis within the intestine.

The last observation in this study that requires comment is the systemic and whole-body threonine oxidation. Our results indicate that the visceral oxidation of threonine under normal feeding conditions accounts for one-eighth of the whole-body threonine oxidation, and that this visceral threonine oxidation is not suppressed during protein restriction. In contrast to our previous findings regarding intestinal lysine oxidation, the PDV oxidises threonine that is taken up from the mesenteric artery.[14] However, we measured the visceral threonine oxidation, which included the oxidation of threonine by the pancreas, spleen and stomach. These findings challenge the traditional concept that threonine is not catabolised by the intestinal mucosa, and further enzymological work is therefore required to establish biochemical origins for intestinal catabolism of dietary threonine.

In examining whole-body threonine oxidation, Chu and Hegsted[33, 35] observed no changes in threonine dehydratase activity in rats fed protein-free or threonine-free diets, and concluded that no adaptation occurs in whole-body protein metabolism when threonine is specifically lacking. In contrast to their findings, we found a substantially decreased whole-body threonine oxidation during protein restriction. According to Ballevre *et al*.[36] who developed and validated a multitracer method in pigs allowing the calculation of the partition of both threonine pathways, the measurement of [^{13}C]-labelled CO_2 probably underestimates threonine oxidation. In addition, we did not find a significant enrichment in glycine during the administration of labelled threonine. Nevertheless, whole-body threonine oxidation was significantly lowered by protein restriction, suggesting a protein-sparing adaptive mechanism.

For effective nutritional support for neonates to achieve normal growth, it is necessary to evaluate the amount of amino acids that is actually available from the diet for absorption. This is not an easy task because the flow of amino acids delivered to the small intestines is comprised of microbial proteins and proteins secreted by the intestinal mucosal cells. The neonatal piglet model is considered to be an appropriate model for the human infant due to similarities in gastrointestinal physiology and functions, and metabolism. The high intestinal threonine utilisation might have important nutritional value, especially in children

with impaired gut function. These infants probably need large amounts of dietary threonine to maintain the mucus layer that protects the whole gastrointestinal tract.

In conclusion, the present study demonstrates that during protein restriction the PDV maintain a high rate of metabolism and continue to utilise a very high amount of dietary threonine during the first period of feeding. Threonine is incorporated mainly into intestinal mucosal proteins, and the level of protein intake affects the site of threonine utilisation by the PDV, as it switches from dual threonine use (i.e. dietary and systemic) to predominantly luminal utilisation of threonine when dietary protein becomes limiting.

Taken together, the results highlight the important role of the intestine in modulating dietary amino acid availability to the body and point to the obligatory requirement of threonine for maintaining intestinal integrity.

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Chapter 6

Dietary threonine metabolism in the intestine of wild type and mucin 2- deficient mice

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Under review

ABSTRACT

Background: Mucin 2 (Muc2) is the major secretory mucin of the mucus layer that lines the intestinal epithelium. Muc2 is characterised by tandem repeats rich in threonine-proline-serine.

Methods: Since previous studies in piglets showed that the intestine utilises 60–90% of dietary threonine intake, we compared threonine utilisation in the intestines of Muc2 knockout ($Muc2^{-/-}$) and wild type ($Muc2^{+/+}$) mice to investigate whether dietary threonine is mainly used for intestinal Muc2 synthesis. Concentrations and isotopic enrichment of threonine were measured by GC(IR)MS in the small intestine, colon and colonic content of mice given a [U-^{13}C]threonine bolus enterally.

Results: In the intestinal tract, 40.1% of dietary [^{13}C]threonine was recovered after 6 hours in $Muc2^{+/+}$ mice – i.e. 32.4% as [^{13}C]threonine and 8.3% as [^{13}C]glycine vs. 43.6% in $Muc2^{-/-}$ mice – i.e. 33.1% as [^{13}C]threonine and 9.5% as [^{13}C]glycine. Most of the dietary [^{13}C]threonine recovered from the intestinal tract was derived from the colonic content in both types of mice. Interestingly, 27.4% of the recovered [^{13}C]threonine in the colonic content of $Muc2^{+/+}$ mice was incorporated into Muc2. Overall, $Muc2^{-/-}$ mice showed higher amounts of incorporated [^{13}C]threonine into mucosal proteins. Furthermore the entire intestine of $Muc2^{-/-}$ mice showed a significantly higher oxidation rate compared to $Muc2^{+/+}$ mice.

Conclusions: This is the first study demonstrating threonine utilisation in mice. In absence of Muc2, dietary threonine is used for constitutive protein synthesis or is metabolically oxidised as shown in $Muc2^{-/-}$ mice. However, one of the pivotal metabolic fates of dietary threonine utilised by the intestine is incorporation into Muc2.

Abstract

INTRODUCTION

The gastrointestinal tract is lined by a mucus layer – a protective barrier between the epithelium and the environment.[1] Mucus production, and thus the mucus layer, is alerted by intestinal stress, i.e. inflammatory bowel disease[2] and its integrity has been associated with nutritional state.[3-6] Mucins represent the principal protein constituent of mucus, and Muc2 is the predominant gastrointestinal mucin.[7] Mucins have a central backbone rich in threonine, proline and serine residues that account for 20–55% of total amino acid composition.[8] A characteristic feature is the high density of oligosaccharides *O*-linked to threonine and/or serine residues in the central protein backbone resulting in a high resistance to proteolysis.[9]

Animal studies have indicated substantial amino acid metabolism in the intestine.[10] Amino acids are quantitatively important nutrients for growth and development, essential in protein synthesis and obligatory for maintaining intestinal mass and integrity. Studies in piglets showed high intestinal utilisation of essential amino acids in first-pass: for example, first-pass metabolism of lysine and leucine accounts for approximately one-third of the total dietary intake.[11, 12] The human intestine retains between 20–50% of the dietary intake of specific essential amino acids.[13-16] Recently, we demonstrated that in piglets between 80–90% of dietary threonine, one of the indispensable amino acids, is utilised by the intestine.[17] Interestingly, additional data demonstrated a 60% reduction in whole-body threonine requirements in piglets receiving total parenteral nutrition, compared to orally fed control animals.[18] In this context, intestinal demand for dietary threonine probably results from its incorporation into secretory mucins rich in threonine residues. Further support for this hypothesis is a study showing that specific restriction of dietary threonine impaired intestinal mucin synthesis.[19] We therefore hypothesised that the major metabolic fate of dietary threonine is incorporation into intestinal mucins, specifically Muc2. To investigate whether dietary threonine is utilised in Muc2 synthesis, we compared [^{13}C]threonine enrichment in the small intestine, colon and faecal proteins of Muc2-deficient mice (Muc2$^{-/-}$), which do not synthesise the mucin Muc2[20, 21] to that of wild type (Muc2$^{+/+}$) mice. Threonine enrichment was also measured in stomach content and in serum to evaluate the whole gastrointestinal tract and systemic availability of dietary threonine.

Once taken up by the mucosal cells, threonine may have different metabolic fates, including oxidation. Threonine is catabolised either by threonine dehydratase (TDH) to NH_4^+ and 2-ketobutyrate, which is irreversibly converted to CO_2, or by threonine dehydrogenase (TDG) to form 2-amino-3-ketobutyrate, which is mainly converted to glycine and acetyl-CoA.[22] In piglets and rats, the TDG pathway accounts for 80% of threonine oxidation.[22, 23] If absorbed dietary threonine is not used for intestinal protein synthesis, its metabolic fate might be oxidation. Therefore, we measured intestinal [^{13}C]glycine amounts in Muc2$^{-/-}$ and Muc2$^{+/+}$ mice.

Thus, by studying threonine metabolism in Muc2$^{-/-}$ mice and Muc2$^{+/+}$ mice, we gained insight into intestinal threonine metabolism, and specifically into the role of dietary threonine in intestinal Muc2 synthesis.

MATERIALS AND METHODS

Animals

Eight-week-old, female, previously described Muc2$^{-/-}$ mice[21] and corresponding wild type littermates were housed in the same specific pathogen-free environment; animal care and procedures were in compliance with Erasmus Medical Centre (MC) – Animal Ethics Committee guidelines.

Experimental setup

Mice (n=17, for both groups) were weighed and fasted 2 hours (h) prior to receiving a threonine gavage, but had free access to drinking water. They received 2.1 µmol [U-^{13}C]threonine/g body weight (Sigma, St. Louis) orally, based on i) average normal daily food intake (3 g/d) for female inbred strains[24], ii) threonine composition of mouse chow (0.69% (wt/wt) threonine; 0.50% was used to calculate the definitive threonine amount, as not to overload the gastrointestinal tract) provided by Special Diets Services (Witham, Essex, UK), and iii) corrected for the total 6 h time span of the experiment.[25] Similarly, control mice (n=2, for both Muc2$^{-/-}$ and Muc2$^{+/+}$ mice) received an oral gavage of PBS. After the threonine gavage the mice had free access to standard rodent pellets (Special Diets Services). However, the experiment was initiated in the early morning to simulate normal feeding routine (normal daily intake during the night, and little to no food intake during the day as to minimise dilution of the [U-^{13}C]threonine). The mice were sacrificed by CO_2 inhalation 1, 3 or 6 h (n=5, per genotype, at each time point) after threonine administration. Blood was collected via heart puncture. Plasma was separated from whole blood by centrifugation (3000 *g* for 5 minutes (min)) and kept at -80°C until further analysis. Subsequently, the stomach, small intestine and colon were rapidly removed. The small intestine was halved into duodenum-jejunum and jejunum-ileum segments, referred to as proximal small intestine and distal small intestine, respectively. Both segments were opened longitudinally, thoroughly washed in PBS, weighed, snap frozen in liquid nitrogen, and stored at -80°C until protein concentration and tracer enrichment analysis. Both the colon and stomach were opened longitudinally, the contents were removed, weighed, snap frozen in liquid nitrogen, and stored at -80°C. The same procedure as described for the small intestine was applied to the colon.

Mass spectrometry

Tissue sample preparation

Intestinal tissue samples were homogenised in water (100 mg/ml) and the faecal samples were homogenised by adding water, ratio 2:1. Total protein concentrations were measured using the Bicinchoninic Acid Protein Assay Reagent (Pierce, Rockford, IL). The protein fraction

was precipitated by adding 0.5 ml of 2.0 mol/l perchloric acid (PCA) to an aliquot (500 µl) of homogenised tissue sample and subsequent centrifugation at 2500 *g* and 4°C for 20 min. The supernatant was collected for each individual sample. Pellets were washed three times by adding 4 ml of 0.2 mol/l PCA followed by centrifugation. The washing fluids from each sample were collected and combined with the corresponding supernatant. Excess PCA was neutralised by adding KOH (4 mol/l), followed by brief centrifugation. To be able to determine the concentrations of either free [^{13}C]threonine and [^{13}C]glycine, 20 µl of internal standard (1 mg/ml norvaline) was added to each sample. Also a standard sample of norvaline and a known amount of threonine or glycine was used to determine the response factor for threonine or glycine, respectively. Subsequently, this factor was used for determination of threonine and glycine concentrations in the samples. These samples were then dried under a nitrogen stream and stored. Twenty microlitre internal standard (1 mg/ml norvaline) was added to each washed pellet to be able to determine the concentrations of incorporated [^{13}C]threonine and [^{13}C]glycine. Subsequently, the pellets were hydrolysed by adding 0.5 ml of 6 mol/l HCl and incubating at 110°C in sealed tubes for 24 h. The protein hydrolysates were dried under nitrogen at 55°C and the residue was dissolved in 0.5 ml water. Amino acids were isolated from protein hydrolysates and supernatant by cation exchange separation as described previously.[17] Amino acids were eluted with 3 ml 6 mol/l NH_4OH and dried under nitrogen at 30°C. Samples were derivatised with ethyl-chloroformiate and threonine was converted to its N-ethoxycarbonylethylester derivative by a method described in our previous work.[17]

Analysis of tissue samples, stomach and colonic content

[^{13}C]Threonine and [^{13}C]glycine enrichments in the different organs were measured by a Thermo Electron Delta-XP isotope ratio MS (Bremen, Germany) coupled online with a trace GC (Thermo Electron) and a combustion interface type 3 (Thermo Electron) which was used for [^{13}C/^{12}C]-ratio measurement of both threonine and glycine. Aliquots of 1 µl of the chloroform suspension containing the amino acid derivatives were introduced into the GC system by a CTC PAL autosampler (CTC Switzerland). Chromatographic conditions were as described previously.[17] After separation using capillary GC, amino acids were combusted online at 940°C and introduced as CO_2 into the isotope ratio MS, where the [^{13}C/^{12}C]-ratio was measured for both threonine and glycine. The atom percent enrichment was converted to mole percent threonine or glycine enrichment, after accounting for the 2.75-fold or 3.5-fold dilution of carbon in the derivative, respectively, and the measured [^{13}C]abundance (98%) of the threonine tracer. The enrichment was expressed as mole percent excess (MPE).

Preparation and analysis of blood samples

Small aliquots of plasma (20 µl) were prepared to determine threonine enrichment and concentration by gas chromatography-mass spectrometry (GC-MS). Briefly, 20 µl of internal standard (1 mg/ml norvaline) was added to 20 µl plasma and deproteinised with sulfocalisylicacid 6%. After centrifugation for 10 min at 4°C and 3000 rpm, the amino acids in the supernatant were isolated by cation exchange separation as described above. The eluate was dried overnight at 30°C under nitrogen. Residues were resuspended by adding 200 µl dichloromethane, and subsequently dried under nitrogen for 1 h at 35°C.

Finally, t-butyldimethylsilyl derivates were formed by adding 25 µl of dimethyl-formamide and 25 µl of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide to the dried residue and heating at 60°C for 1 h. Standard curves were prepared by mixing aqueous solutions of natural and labelled threonine for both enrichment and concentration determination. GC-MS analyses were performed on a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD-800 mass spectrometer (Interscience BV, Breda, the Netherlands). One microliter of the derivative was analysed in split mode (1:20) on a DB-17, 30 m x 0.25 mm capillary column (Agilent). Selective ion monitoring was carried out at m/z 404.3, 405.3 and 408.3. The enrichment was expressed as mole percent excess (MPE).

Calculations

Basal [^{13}C]threonine and [^{13}C]glycine enrichments were determined in each tissue of the control (PBS) mice. These enrichments were subtracted from the [^{13}C]enrichments measured in the experimental animals. Total [^{13}C]threonine amount in each tissue (µmol) was calculated as follows:

(Threonine enrichment (MPE)/100) * Conc. Threonine $_{tissue}$ (µmol/g) * (weight) $_{tissue}$

Subsequently, for each individual mouse threonine amount was expressed as percentage of total threonine administered enterally. An equivalent calculation was used to determine total [^{13}C]glycine in each tissue sample.

Statistics

Data are presented as the mean ± the inter-animal standard error of mean (SEM). Differences in [^{13}C]threonine (free and incorporated into proteins) for each animal group over time were assessed using ANOVA. Differences in protein levels and [^{13}C]threonine and [^{13}C]glycine (free and incorporated) between the Muc2$^{-/-}$ and Muc2$^{+/+}$ mice at each time point were evaluated using the Mann-Whitney test (two-tailed). $P<0.05$ was considered to indicate statistical significance.

RESULTS

Dietary [^{13}C]threonine distribution

The total [^{13}C]amount recovered consisting of [^{13}C]threonine and [^{13}C]glycine was expressed as percentage of total dietary [^{13}C]threonine intake. The total [^{13}C]amount recovered in the gastrointestinal organs and serum increased with time after threonine administration in both mice types (Figure 1A). Figure 1B shows that total amounts of free [^{13}C]threonine recovered increased significantly from 4.7% at 1 h to 10.1% at 6 h after administration in Muc2$^{-/-}$ mice, but not in Muc2$^{+/+}$ mice. Total amounts of recovered bound [^{13}C]threonine significantly increased in both mice types. Figure 1C shows the distribution of dietary [^{13}C]threonine 6 h after [^{13}C]threonine administration. The free amounts of [^{13}C]threonine in the stomach content could not be included in our analysis since they exceeded the maximum measurable amount (41% MPE).

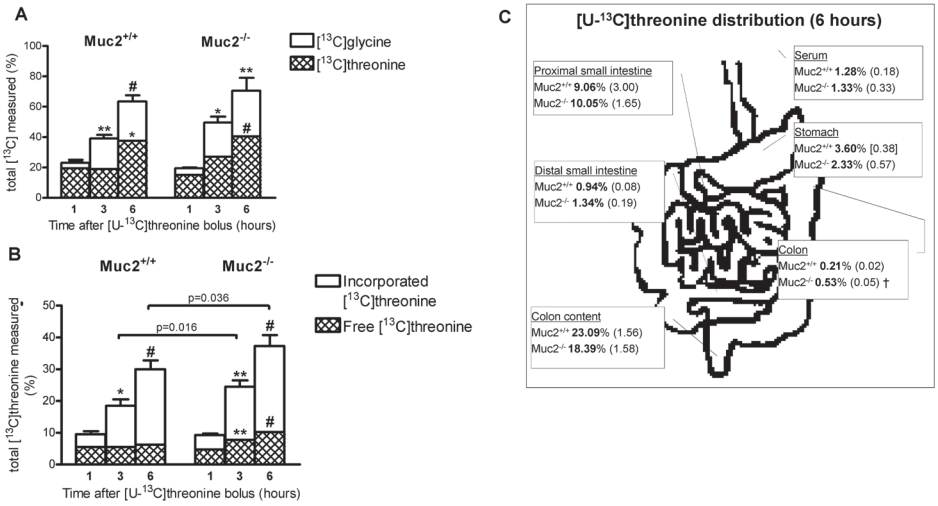

Figure 1 Dietary threonine distribution and recovery.
(A) Total [^{13}C] (threonine+glycine) recovered in the tissues sampled (free and incorporated threonine and glycine in serum, proximal and distal small intestine, colon and colonic content and incorporated threonine and glycine in the stomach) expressed as percentage of threonine administered during time (▭) [^{13}C]glycine; (▨) [^{13}C]threonine; (B) The total amount of free and incorporated [^{13}C]threonine in the tissues sampled. The amounts of free [^{13}C]threonine recovered, increased significantly at 1 hour to 6 hours after administration in the Muc2$^{-/-}$ mice and was significantly higher at 3 and 6 hours compared to the Muc2$^{+/+}$ mice. The total amount of recovered bound [^{13}C]threonine, significantly increased in both types of mice. (C) The distribution of dietary threonine at 6 hours expressed as percentage of threonine administered. The values represent the sum of free and incorporated threonine in the tissues samples, with exception of the stomach (free [^{13}C] dietary threonine not determinable). Error bars indicate the standard error of mean. All significancies 1 hour vs. 3 or 6 hours * p<0.05; †p=0.016; **p<0.01; # p=0.001.

Stomach content

Incorporated [^{13}C]threonine levels in the stomach content decreased with time in both mice types ((Muc2$^{+/+}$: 13.9% at 1 h vs. 3.6% at 6 h, not significant (NS)) and (Muc2$^{-/-}$: 6.4% at 1 h vs. 2.3% at 6 h, NS)). Threonine incorporation did not differ between mice types.

Small intestine

Total protein amounts in the distal small intestine significantly exceeded those in the proximal small intestine (p<0.001, Muc2$^{-/-}$ and Muc2$^{+/+}$ mice, Figures 2A and D), and did not differ between mice types.

Free [^{13}C]threonine in the proximal small intestine decreased significantly between 1 and 6 h after threonine gavage in Muc2$^{+/+}$ mice, but not in Muc2$^{-/-}$ mice, which showed only a transient decrease of free [^{13}C]threonine at 3 h post-threonine delivery (Figure 2B). Incorporated [^{13}C]threonine in the proximal small intestine significantly increased with time in both mice types, but did not significantly differ between types at any time point analysed (Figure 2E).

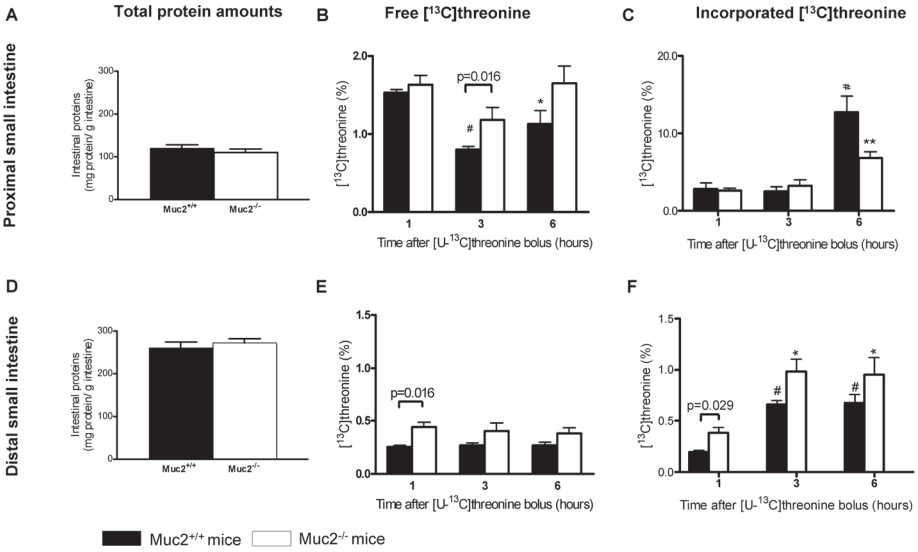

Figure 2 The protein content and threonine metabolism in the small intestine.
(A, D) Total protein amounts in the proximal and distal small intestine in $Muc2^{+/+}$ and $Muc2^{-/-}$ mice. (B, E) The free [^{13}C]threonine in the proximal small intestine decreased during time in the $Muc2^{+/+}$ mice and remained stable in the $Muc2^{-/-}$ and was significantly higher 3 hours after the threonine gavage. The free [^{13}C]threonine in the distal small intestine remained stable during the experimental period and was significantly higher in the $Muc2^{-/-}$ mice at 1 hour after threonine administration [p=0.016 1 hour, $Muc2^{-/-}$ mice vs $Muc2^{+/+}$]. (C, F) The incorporated [^{13}C]threonine in proximal small intestine increased during time. The incorporated [^{13}C]threonine in the distal small intestine also increased during time. The $Muc2^{-/-}$ mice showed a trend towards higher [^{13}C]threonine incorporation which was significantly higher after 1 hour of threonine administration compared to the $Muc2^{+/+}$ mice. Error bars indicate the standard error of mean. All significancies 1 hour vs. 3 or 6 hours *p<0.05; **p<0.01, #p<0.001.

At all time points, free [^{13}C]threonine percentage in the distal small intestine was significantly lower (p<0.03) than that in the proximal small intestine in both mice types. In contrast to the proximal part (Figure 2C), a lower amount of [^{13}C]threonine was incorporated into proteins of the distal small intestine (Figure 2F). However, threonine incorporation into intestinal proteins significantly increased after the threonine gavage. One hour after enteral threonine, the $Muc2^{-/-}$ mice showed significantly higher threonine incorporation into intestinal proteins compared to $Muc2^{+/+}$ mice. This difference levelled off at later time points.

Colon

Total protein concentration in the colon of $Muc2^{-/-}$ mice was modestly but consistently higher than in $Muc2^{+/+}$ mice (Figure 3A). The pool of free [^{13}C]threonine in colonic tissue was significantly higher in $Muc2^{-/-}$ mice throughout the experiment and its levels significantly declined over time in both mice types. However, kinetics in $Muc2^{+/+}$ mice were faster compared to $Muc2^{-/-}$ mice. Incorporated [^{13}C]threonine levels in the colon increased significantly over time and were significantly lower in $Muc2^{+/+}$ mice at all time points.

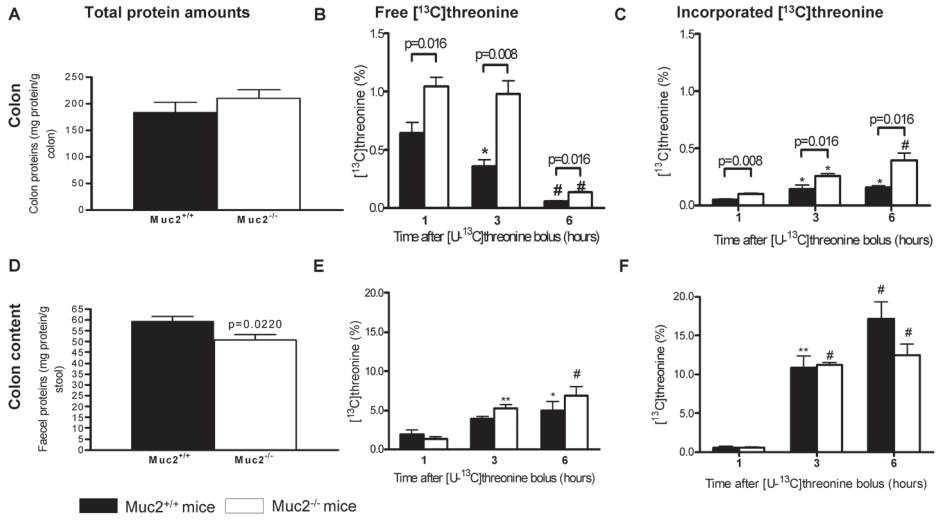

Figure 3 The protein content and threonine metabolism in the colon.
(A, D) total protein amounts in the colon and colonic content in Muc2$^{+/+}$ and Muc2$^{-/-}$ mice. (B, E) The free [^{13}C]threonine in the colon declined during the experimental period in contrast to the colonic content in which the free [^{13}C]threonine increased during the experiment (1 hour vs. 3 and 6 hours, *p<0.05; **p<0.01; #p<0.001 Muc2$^{-/-}$ mice, Muc2$^{+/+}$ mice, NS all time points). (C, F) The incorporated [^{13}C]threonine in the colon increased during time. The incorporated [^{13}C]threonine in the colonic content also increased during time. The Muc2$^{+/+}$ mice showed a trend towards higher [^{13}C]threonine incorporation at 6 hours. Error bars indicate the standard error of mean. All significancies 1 hour vs. 3 or 6 hours *p<0.05; **p<0.01, #p<0.001.

Colonic content

Muc2$^{+/+}$ mice faeces had a significantly higher protein content than those of Muc2$^{-/-}$ mice (Muc2$^{+/+}$: 59.2 mg/g and Muc2$^{-/-}$: 50.8 mg/g, p=0.022), as shown in Figure 3D. Free [^{13}C]threonine in colonic content increased with time in both mice types without significant differences between types. Furthermore, levels of incorporated [^{13}C]threonine into the proteins of the colonic content increased significantly over time in both mice types. [^{13}C]Threonine incorporation in proteins of colonic content of Muc2$^{+/+}$ mice at 6 h was consistently higher, yet these differences were not statistically significant. Interestingly, taking into consideration that Muc2 is the principal constituent of the intestinal mucus, we suggest that the difference (27.4%) in [^{13}C]threonine incorporated into proteins of the colonic content of Muc2$^{+/+}$ and Muc2$^{-/-}$ mice is attributable to the presence of the mucin Muc2 in the faeces of Muc2$^{+/+}$ mice at 6 h after threonine intake.

Serum

Free [^{13}C]threonine levels in serum significantly decreased over time ((Muc2$^{+/+}$: 0.7% at 1 h vs. 0.02% at 6 h, p<0.001) and (Muc2$^{-/-}$: 0.4% at 1 h vs. 0.02% at 6 h, p<0.001)). Levels of incorporated [^{13}C]threonine into proteins in serum increased over time in both groups (0.3% at 1 h vs. 1.3% at 6 h, p<0.01).

Intestinal and whole-body threonine oxidation

Total [^{13}C]glycine levels reflected metabolic oxidation of threonine in the small intestine of both mice types. Figure 4A shows that the initial peak of total glycine accumulation at 3 h reverted to basal levels in both mice types by 6 hours post threonine gavage. However, Muc2$^{-/-}$ mice had persistently higher oxidation rates of dietary threonine, a difference more pronounced in the colon where total [^{13}C]glycine amounts also increased over time in both mice types (Figure 4B).

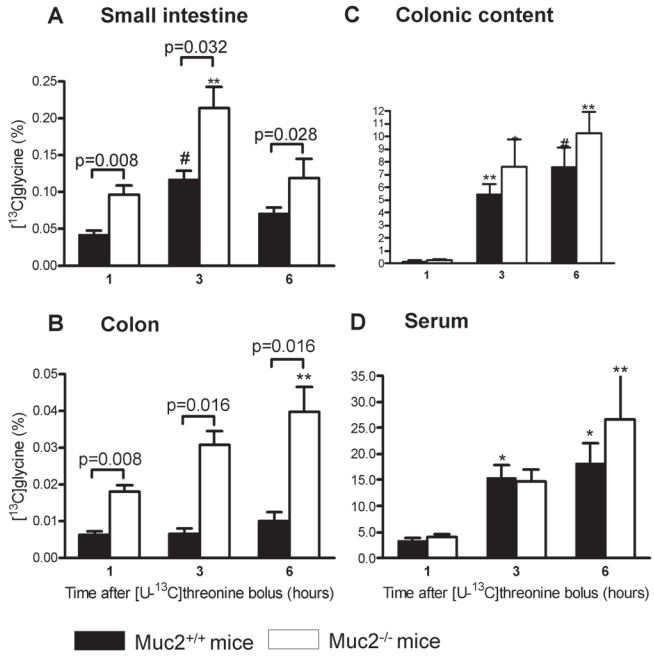

Figure 4 Intestinal and whole-body threonine oxidation
(A) The total [^{13}C]glycine amounts in the small intestine and colon (B) of Muc2$^{+/+}$ and Muc2$^{-/-}$ mice increased during time. Overall, the Muc2$^{-/-}$ mice showed a higher oxidation of dietary threonine compared to Muc2$^{+/+}$ mice, which was significantly higher in the small intestine and colon at each time point. (C) The total [^{13}C]glycine amounts in the colonic content and serum (D) of Muc2$^{+/+}$ and Muc2$^{-/-}$ mice significantly increased during time. There were no significant differences in [^{13}C]glycine levels in the serum between the Muc2$^{-/-}$ and Muc2$^{+/+}$ mice throughout the experiment. Error bars indicate the standard error of mean. All significancies 1 hour vs. 3 or 6 hours *p<0.05; **p<0.01, #p<0.001.

Total [^{13}C]glycine amounts in the colonic content of both mice types significantly increased over time without any appreciable difference between types (Figure 4C). In serum total [^{13}C]glycine amounts in both mice types also significantly increased over time, without significant differences between types (Figure 4D).

In summary, Muc2$^{+/+}$ mice showed 40.1% dietary [^{13}C]threonine recovered in the intestinal tract after 6 h vs. 43.6% in Muc2$^{-/-}$ mice. In the whole intestine, 26.2% of administered [^{13}C]threonine in Muc2$^{+/+}$ mice and 24.1% in Muc2$^{-/-}$ mice, respectively, was incorporated into proteins. Most dietary [^{13}C]threonine recovered from the intestinal tract was detected in the colonic content in both mice types. In all other tissues and blood, except gastric content, [^{13}C]threonine recovered was two to tenfold lower than in colonic content. Interestingly, 27.4% of recovered [^{13}C]threonine in the colonic content of Muc2$^{+/+}$ mice was incorporated into Muc2. Finally, the entire intestine of the Muc2$^{-/-}$ mice showed a significantly higher oxidation rate compared to Muc2$^{+/+}$ mice.

DISCUSSION

Previous studies showed that there is substantial amino acid metabolism in the intestine.[10] Specifically, the essential amino acid threonine is thought to be of utmost importance for intestinal Muc2 synthesis.[11, 17, 18] In the current study we aimed to gain more insight into intestinal threonine metabolism, and more specifically the role of dietary threonine in intestinal Muc2 synthesis, by studying threonine utilisation in Muc2$^{-/-}$ and Muc2$^{+/+}$ mice.

In line with previous studies, we found that the murine gastrointestinal tract takes up dietary threonine.[11, 17, 18] We retrieved 40.1% and 43.7% of dietary threonine in Muc2$^{+/+}$ and Muc2$^{-/-}$ mice, respectively, either as free or incorporated threonine (Figure 1B). Interestingly, we detected threonine incorporated into proteins of the stomach content after 1 h. To our knowledge these results are the first to indicate absorption of dietary nutrients from the lumen of the stomach. As previous studies demonstrated that the stomach wall secretes Muc5ac and Muc6[26], threonine might be incorporated into these proteins in the stomach. However, this remains to be investigated. In the present study, threonine distribution in the gastrointestinal tract and in serum was similar for both mice types studied. Muc2 deficiency therefore seems to have no initial impact on the intestinal threonine absorption capacity and the systemic availability of dietary threonine. However, [^{13}C]threonine was given as dietary free threonine. The molecular form of ingested threonine (free, peptide bound or protein bound) offered to the epithelium of the gastrointestinal tract might affect whole-body threonine kinetics. This phenomenon was earlier described by Daenzer *et al.*, demonstrating that protein-bound leucine was used more efficiently for liver protein synthesis than dietary free leucine.[27] More specifically, in the gastrointestinal tract we recovered 41.5% of dietary [^{13}C]threonine after 6 h in Muc2$^{+/+}$ mice vs. 44.8% in Muc2$^{-/-}$ mice, either as free or incorporated threonine or glycine. Thus, while dietary threonine utilisation seems similar between Muc2$^{-/-}$ and Muc2$^{+/+}$ mice, the primary metabolic fate, such as protein synthesis or

oxidation, may differ. For this reason we investigated threonine metabolism in different intestinal segments.

Overall, the pool of free threonine in the total small intestine remained constant in concentration and amount during the 6 h experiment with no significant differences between mice types. In contrast, free threonine in colonic tissue decreased over time in both groups. Remarkably, $Muc2^{-/-}$ mice showed significantly higher amounts of free threonine in colonic tissue, suggesting a dietary threonine excess due to Muc2 deficiency. In contrast to the tissue samples, the colonic content showed a similar increase of free threonine over time in both mice types. This might be due to breakdown of *de novo* synthesised proteins other than Muc2. Our results are supported by findings from previous studies showing that the Muc2 central peptide backbone is highly resistant to proteolytic breakdown by bacteria and enzymes.[9] Additionally, the increase of free [^{13}C]threonine in the colonic content might be caused by saturation of the small intestinal absorption capacity due to excess of [^{13}C]threonine given by gavage.

Incorporation of dietary threonine into intestinal proteins was found to be higher in the proximal small intestine compared to other further distal intestinal segments. The proximal small intestine therefore seems to play a major role in the utilisation of dietary threonine, in line with other studies suggesting that most of dietary proteins and amino acids are absorbed by the proximal jejunum.[28] Incorporation of dietary threonine increased in each intestinal segment sampled for both mice types. The higher amounts of [^{13}C]threonine incorporated into proteins of the proximal small intestine – as compared to the distal small intestine and colon – can be explained by i) higher availability of [^{13}C]threonine in the proximal small intestine or ii) higher turnover of secreted proteins along the intestinal proximal–distal axis. The latter hypothesis is supported by a previous study showing that total protein synthesis, and more specifically Muc2 secretion, is higher in the distal colon compared to the proximal colon[29] and is hardly detectable in the small intestine (our unpublished data). In agreement, Atuma *et al*. showed that mucus thickness increases over the intestinal proximal–distal axis.[30] Moreover, in our study total protein amount in colonic content was higher in $Muc2^{+/+}$ mice, demonstrating that Muc2 is mainly secreted by the colon. Furthermore, most dietary [^{13}C]threonine recovered from the intestinal tract was derived from the colonic content, 27.4% of which was incorporated into the protein Muc2 in $Muc2^{+/+}$ mice. In addition, a previous study in $Muc2^{-/-}$ mice showed no evidence of up-regulation of other known secretory mucins of the intestinal tract to compensate for the lack of Muc2.[21] Assuming that this does occur however, 27.4% incorporation of threonine into Muc2 in the $Muc2^{+/+}$ mice would be an underestimation. Altogether, these data strengthen our hypothesis that dietary threonine is incorporated into the secretory mucin Muc2, with increased secretion rate along the intestinal proximal–distal axis. Furthermore, our data in $Muc2^{-/-}$ mice demonstrate that dietary threonine is also used for intestinal constitutive protein synthesis and secretion.

Regarding the issue of intestinal and whole-body threonine oxidation, our data suggest that a large proportion of absorbed threonine is incorporated into Muc2, although not in Muc2-deficient mice. This is why a larger proportion of dietary threonine may have been oxidised. However, there are no data on the presence of catabolic enzymes in murine intestinal mucosa. Previous studies reported absence or only negligible activity of threonine catabolic enzymes in piglet intestinal mucosa.[31] However, in the present study [^{13}C]glycine was detectable in the intestine of both mice types, strongly suggesting that the catabolic enzyme TDG is present and active in murine intestinal mucosa. Overall, Muc2$^{-/-}$ mice oxidised more dietary threonine than Muc2$^{+/+}$ mice, indicating that Muc2 deficiency results in more inefficient utilisation of dietary threonine.

In summary, to our knowledge this is the first study demonstrating threonine utilisation in mice. One of the pivotal metabolic fates of dietary threonine utilised by the intestine is its incorporation into Muc2. In the absence of Muc2, dietary threonine is mainly used for constitutive protein synthesis or becomes a substrate for metabolic oxidation.

ACKNOWLEDGEMENTS

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Chapter 7

A novel method to determine small intestinal barrier function in human neonates *in vivo*

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Based on a publication by Gut 55 (9): 1366–1367, 2006 (see Appendix, page 180)

ABSTRACT

Background: The mucin 2 (MUC2) is the structural component of the protective mucus layer, which contains high amounts of threonine, serine and proline in their peptide backbone. The aim was to develop a tracer method to study intestinal MUC2 synthesis and to calculate the fractional synthetic rate (FSR) of small intestinal MUC2 in human neonates as a parameter for intestinal barrier function.

Methods: Intragastric infusion of [U-^{13}C]threonine was used to determine the incorporation of threonine into secreted MUC2 in the outflow fluid of 5 premature neonates with an enterostomy following bowel resection for necrotising enterocolitis. Small intestinal MUC2 was isolated using CsCl gradient ultracentrifugation. Fractions of these samples were analysed using SDS-PAGE gels. Mucins in gel were stained with Periodic Acid/Schiff's staining. The isotopic enrichment of threonine was measured in MUC2 using Gas Chromatography Isotopic Ratio Mass Spectrometry. The threonine enrichment was used to calculate the FSR of MUC2.

Results: Dietary threonine was incorporated into MUC2 of the small intestine. The total time from ingestion of threonine to incorporation into MUC2 and MUC2 secretion ranged from 6-10 hours in patients with an ileostomy. The FSR of MUC2 ranged from 12.1-89.7% per day.

Conclusions: Dietary threonine is used for MUC2 synthesis. This new method gives the opportunity to investigate small intestinal MUC2 synthesis. The FSR of MUC2 might be used as a tool to study intestinal barrier function.

Abstract

INTRODUCTION

The small intestine is a large and metabolically active organ system in the body.[1-3] Several studies in neonatal pigs demonstrated that amino acids play critical and specific roles in intestinal metabolism. In this context, threonine is of vital nutritional importance, because it is an indispensable amino acid utilised in a large amount by the portal-drained viscera.[4] Recently, we showed that the equivalent of almost 90% of dietary threonine is utilised by the intestine of piglets.[5] A consistent finding is a 60% reduction in whole-body threonine requirement in piglets receiving parenteral nutrition as compared with enteral nutrition.[6] These findings indicate that enteral nutrition induces intestinal processes in demand of threonine. A major metabolic fate of threonine might be incorporation into secretory mucins, the structural component of the mucus layer of the gastrointestinal tract.

The mucus layer forms a physical barrier between the underlying epithelium and the lumen of the gastrointestinal tract.[7, 8] Mucus mainly consists of the secretory mucin MUC2, which is secreted by the goblet cells of the intestine and gives intestinal mucus a high density and viscoelasticity. MUC2 is a glycoprotein rich in oligosaccharides, *O*-linked to threonine and serine residues in the peptide backbone.[9] MUC2 contains high amounts of threonine, proline and serine residues, which together constitute 20-55% of total amino acid composition.[10] The high visceral need for threonine presumably reflects the high synthesis rate of MUC2. Thus threonine might be one of the amino acids essential for maintaining the protective mucus layer, and thus intestinal barrier function.

The most devastating intestinal disease in preterm infants is necrotising enterocolitis (NEC).[11] Bowel resection is sometimes necessary and may lead to a drastic loss of absorptive and protective surface. This in turn may result in malabsorption of nutrients or even in the so-called short bowel syndrome.[12] Infants with impaired gut function following massive bowel resection require adequate gut adaptation and recovery of intestinal function to avoid the impact of malabsorption and pathogenic bacterial insults. As suggested above, threonine might be indispensable in maintaining the protective mucus layer. We therefore hypothesised that one of the metabolic fates of dietary threonine is incorporation into intestinal MUC2. In this study we developed a novel tracer method to investigate MUC2 synthesis in the small intestine of human neonates following bowel resection for NEC. We measured the [U-^{13}C]threonine enrichment in MUC2 isolated from small intestinal outflow fluid. Secondly, we calculated the fractional synthetic rate (FSR) of small intestinal MUC2 from the linear increase of [U-^{13}C]threonine enrichment in MUC2. This rate might be used as a parameter for the small intestine's capacity to synthesise and secrete MUC2 and thus maintain gut barrier integrity.

MATERIALS AND METHODS

Patients

Patients eligible for this study were neonates admitted to the Neonatal Intensive Care Unit (NICU) or the department of Paediatric Surgery of the Erasmus MC-Sophia Children's Hospital (Rotterdam, the Netherlands) after bowel resection for NEC. The diagnosis was made on the basis of clinical symptoms and characteristic features on an abdominal x-ray. NEC was confirmed at surgery and from histological features characteristic for NEC on resected intestinal tissue sent for routine histopathology. All neonates received a temporary enterostomy during surgery. The Erasmus MC Institutional Review Board approved the study protocol. Written, informed consent was obtained from the parents.

Five neonates were included in the study. Table 1 lists their main clinical characteristics. Full parenteral feeding (glucose, amino acids (Primene® 10%, Clintec Benelux NV, Brussels, Belgium), and lipids (Intralipid® 20%, Fresenius Kabi, Den Bosch, the Netherlands)) was given during the first 10 post-operative days. Next, minimal enteral feeding was added during 4 days, after which enteral feeding was gradually introduced by continuous nasogastric gavage feeding or bottle-feeding under simultaneous diminishing of the parenteral nutrition. Enteral nutrition consisted of a combination of breast feeding and formula feeding (Nenatal®, Nutricia, Zoetermeer, the Netherlands; Pepti Junior®, Nutricia, Zoetermeer, the Netherlands).

Protocol

The study was accomplished approximately 4 weeks (wk) (4 ± 1 wk) following bowel resection. Then, a continuous 12-hour (h) infusion [7.43 μmol/kg and 7.43 μmol/kg·h] of [U-^{13}C]threonine (99.47%, Cambridge Isotopes Laboratories, Andover, MA) was administered enterally by feeding tube. All isotopes were tested and found sterile and pyrogen-free before use in our studies. The intestinal outflow fluid samples were collected in adhesive bags at 3-h intervals during 2 days (d) and stored at −80°C for further analysis. Figure 1 outlines the tracer-infusion study described.

Analytical procedure

Isolation of MUC2

MUC2 was isolated from the intestinal outflow fluid according to the method described by Tytgat *et al*. with slight modifications.[8] Briefly, samples of each time period were homogenised in homogenisation buffer, pH 7.5 (6 mol/l guanidinium hydrochloride (Sigma), 50 mmol/l Tris(hydroxymethyl)aminomethane (Tris, Gibco), 5 mmol/l ethylene-diamino-tetra-acetate (EDTA, Merck) and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF, Sigma)). The disulfide bonds of mucins were chemically reduced by adding 100 mmol/l dithiotreitol (DTT, Sigma) and stirring overnight at 4°C in the dark. The homogenate was centrifuged to remove the fat layer and to isolate the supernatant (23.700 *g*, 10 minutes (min), 4°C (Mutifuge 3 S-R, Heraeus)). Sulfhydryl groups were carboxymethylated through addition of 250 mmol/l iodoacetamide (Sigma) in the supernatant and stirring for another 24 h at 4°C.

Table 1 Patient characteristics and kinetic parameters of small intestinal MUC2

Patients	Gender	GA *(wk)*	BW *(g)*	AS *(d)*	Cause	Enterostomy	Residual small intestine	MUC2 secretion time *(h)*	FSR *(%/d)*
1	F	34	1845	7	NEC (colon)	End-ileostomy	small intestine without 1.5 cm of the ileum and the ileocaecal valve	< 6	19.6
2	M	34	2310	5	NEC (colon)	End-ileostomy	total small intestine	< 7	77.8
3	M	34	1905	6	NEC (colon)	End-ileostomy	small intestine without 10 cm of the ileum and the ileocaecal valve	< 7	89.7
4	M	32	2190	7	NEC (colon)	End-ileostomy	small intestine without 10 cm of the ileum and the ileocaecal valve	< 10	31.1
5	F	33	1450	6	NEC (small intestine)	Jejunostomy	24 cm jejunum and 25 cm ileum without the ileocaecal valve	< 3	12.1
Median		34	1905	6					31.1
Min-Max		32-34	1450-2310	5-7					12.1-89.7

GA: gestational age; **BW:** birth weight; **AS:** age at surgery; **FSR:** fractional synthetic rate; **F:** female; **M:** male; **NEC:** necrotising enterocolitis

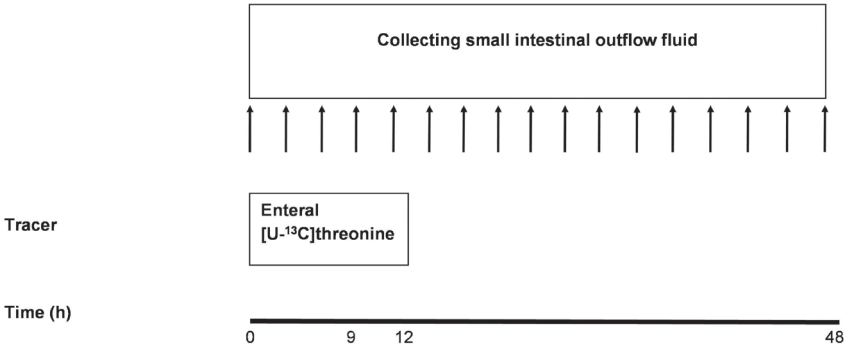

Figure 1 Study protocol. Enteral [U-^{13}C]threonine administration lasted 12 h. During 48 h, small intestinal outflow fluid was collected at 3-h intervals.

Mucins were purified by equilibrium ultracentrifugation on a CsCl (Roche) density gradient. The first and second gradients had a guanidinium HCl concentration of 4 mol/l with CsCl added to a density of 1.40 g/ml. In the third gradient CsCl was added to a density of 1.50 g/ml, and guanidinium HCl concentration was reduced to 0.2 mol/l. Isopycnic density gradient centrifugation was performed in a Beckmann ultracentrifuge, 70 Ti rotor at 250.000 *g* for 72-88 h at 4°C. One ml fractions were collected and extensively dialysed against distilled water at 4°C and stored at -20°C for further analysis.

Analysis of MUC2

Samples of dialysed fractions were analysed on reducing polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulfate (SDS-PAGE).[13] All gels were composed of 3% stacking gel and 4% running gel using a stock solution containing 30% acrylamide and 2% bis-acrylamide. Prestained Precision Plus Protein standards were purchased from Bio-Rad (ranging from 10 kDa to 250 kDa). Prior to SDS-PAGE analysis, samples were boiled for 5 min in buffer containing 1% (vol/vol) 2-mercaptoethanol (Bio-Rad) and 2% (wt/vol) SDS. Gels were stained with periodic acid-Schiff's reagent (PAS, Sigma) according to Konat *et al*.[14], with Phast Gel™ Blue R (Coomassie, Amersham), and with Silver Staining according to the manufacturer's protocol. As mucins have a characteristic buoyant density of approximately 1.45 g/ml, the density of CsCl gradient fractions was measured by weighing 1 ml of each fraction using a calibrated pipette. Hexose assay was performed using orcinol (Sigma) according to François *et al*.[15] with D-galactose:fucose (3:2 (wt/wt)) as standard. Quantity of hexose was expressed as the optical density (OD) at 540 nm. Mucin-containing fractions, i.e. fractions that contained a high molecular weight PAS-positive band at approximately 550 kDa after SDS-PAGE had a buoyant density of ± 1.45 g/ml and contained relatively high hexose levels, were pooled and stored at −20°C for further analysis.

Western blot analysis

The mucin-containing fractions were incubated with neuraminidase (Sigma) for 3 h at 37°C. Neuraminidase treatment was used before incubation with the mouse monoclonal antibody PMH1. The pooled fractions (16 µl) were loaded and run on a SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membranes (Protan BA 83, 0.2 µm) and blocked for 1 h at room temperature in blocking buffer containing 50 mmol/l Tris, pH 7.8, 5% (wt/vol) non-fat dry milk powder (Campina Melkunie Eindhoven, the Netherlands) 2 mmol/l $CaCl_2$, 0.05% (vol/vol) Triton X-100 and 0.01% Antifoam B (Sigma-Aldrich). Blots were incubated overnight at 4°C with mouse monoclonal antibody PMH1[16] (1:10 in blocking buffer) or rabbit polyclonal antibody HCM-1[8] (1:2500 in blocking buffer). After washing with PBS containing 0.2% Tween-20, blots bound antibodies were detected using HRP conjugated goat anti-mouse secondary antibody (PMH1) and HRP conjugated goat anti-rabbit secondary antibody (HCM-1) (1:1000 in blocking buffer) and SuperSignal® West Femto Luminol Enhancer kit (Pierce).

Mass spectrometry

Analysis of [U-^{13}C]threonine

After the third CsCl gradient run, aliquots of 1800 µl of the pooled MUC2 fractions were dialysed, freeze dried and prepared for mass spectrometric analysis. The dried samples were hydrolysed by adding 1 ml of 6 mol/l hydroxychloride and incubating at 110°C in sealed tubes for 24 h. The protein hydrolysates (1000 µl) were dried under a nitrogen stream at 55°C and the residue was dissolved in 0.5 ml water. The amino acids in the residue were bound to a 1 ml AG50 W-X8, H^+ cation exchange column (Biorad, Richmond, Virginia, USA). Amino acids were eluted with 3 ml 6 mol/l NH_4OH and dried under nitrogen at 30°C. Threonine was converted to its N-ethoxycarbonylethylester derivative according to the method used in our previous work.[5] The [^{13}C/^{12}C]-ratio of threonine in isolated MUC2 was measured by a Thermo Electron Delta-XP isotope ratio MS linked online with a trace GC (Thermo Electron, Bremen, Germany) and a combustion interface type 3 (Thermo Electron, Bremen, Germany). Aliquots of 1 µl of the chloroform suspension containing the amino acid derivatives were introduced into the GC system by a CTC PAL autosampler (CTC Switzerland). Chromatographic conditions used were those described previously. After separation using capillary GC, amino acids were combusted online at 940°C and introduced as CO_2 into the isotope ratio MS, where the [^{13}C/^{12}C]-ratio was measured. The enrichment was expressed in mole percent excess (MPE).

Calculations

MUC2 secretion time was defined as the time interval between start of enteral [U-^{13}C]threonine administration and the appearance of enriched MUC2 in small intestinal outflow fluid.

Fractional synthetic rate (FSR) of MUC2 is expressed as percentage of the total MUC2 pool synthesised per day.

FSR was calculated by:

$$\frac{\text{slope of the linear increase of [U-}^{13}\text{C]threonine enrichment of MUC2}}{\dfrac{\text{([U-}^{13}\text{C]threonine (µmol/kg·h))}}{\text{([U-}^{13}\text{C]threonine (µmol/kg·h) + nutritional threonine intake (µmol/kg·h))}}} * 24$$

The threonine amount (µmol/kg·h) in total enteral nutrition was used as a substrate for true precursor pool enrichment in the intestinal lumen. For the threonine amount of breast milk (one patient was breast-fed), we used a value of 4850 µmol/l threonine as measured in term infants by Hanning *et al*.[17]

Statistics

Data are presented as mean plus or minus the standard deviation (SD) or as median (minimum-maximum).

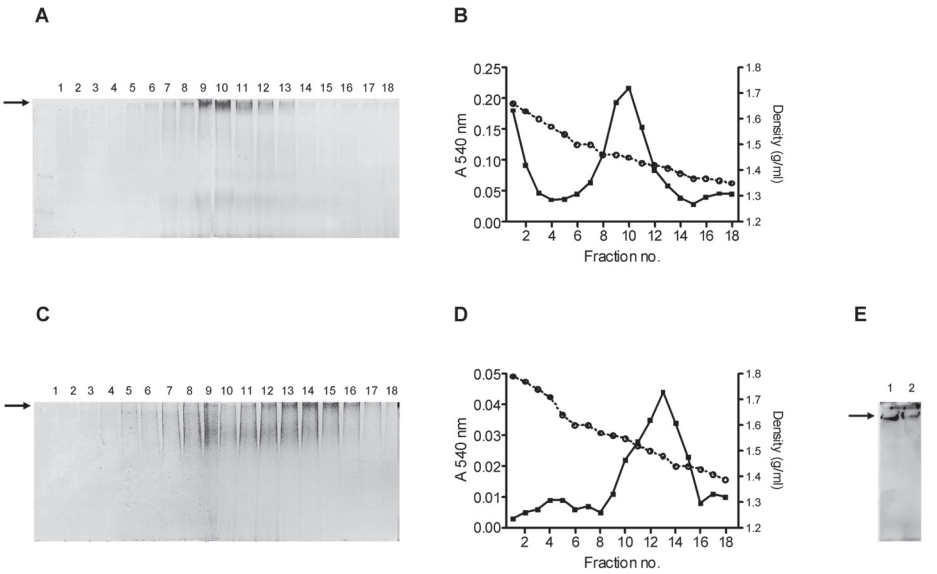

Figure 2 Isolation and characterisation of human MUC2 in the intestinal outflow fluid. A and C represent reducing 4% stacking and 3% running SDS-PAGE gel containing dialysed fraction samples from the CsCl density gradients. PAS-positive fractions 7-12 after the first ultracentrifugation (A) and PAS-positive fractions 12-15 after the third ultracentrifugation (C) were pooled and subjected to Western blot analysis (E; the first (lane 1) and the third (lane 2) ultracentrifugation). B and D represent the density (o) and hexose content (■) of the same samples as represented in the gel.

RESULTS

Patients

Five neonates who underwent bowel resection for NEC were studied (median gestational age 34 (32–34) wk and median birth weight 1905 (1450–2310) g, Table 1). Surgery had been performed at 6 (5–7) d after birth, with ileostomy in four patients and jejunostomy in one. At the time of the study (4 ± 1 wk following bowel resection) all were clinically stable and enteral protein intake was 2.8 ± 1.4 g/(kg·d).

Isolation and characterisation of MUC2

Mucins were isolated from the small intestinal outflow fluid according to their characteristic buoyant density of about 1.45 g/ml. The first gradient fractions 7 to 12 showed densities between 1.40 and 1.50 g/ml. In these fractions a high molecular weight glycoprotein could be stained with PAS after SDS-PAGE (Figure 2A), but was not stained by Coomassie Blue (data not shown). Moreover, the presence of this high molecular weight band of approximately 550 kDa, visible after PAS-staining, corresponds with a peak in hexose assay (Figure 2B). These fractions were regarded as the mucin-containing fractions. Smaller proteins stained by Coomassie Blue were mainly present in fractions with a density around 1.30 g/ml (data not shown). The mucin-containing fractions were pooled and run on a second CsCl gradient. Results of PAS staining after SDS-PAGE in fractions 7 to 11 of the second gradient were similar to the first gradient results (data not shown). To remove the nucleic acids, mucin-containing fractions of the second ultracentrifugation were pooled and run on a third CsCl gradient. The fractions 12–15 contained the purified mucin MUC2. These fractions had a buoyant density of 1.45 g/ml and contained a high molecular mass band of ± 550 kDa, which was PAS-positive on SDS-PAGE (Figure 2C). Moreover, this high molecular weight band corresponded with a peak in hexose assay (Figure 2D). Each mucin fraction was free of contaminating proteins detected by Coomassie Blue staining as well as silver staining (data not shown). The mucin-containing fractions were pooled and prepared for amino acid analysis and threonine enrichment measurements on the GC-IRMS. Moreover, Western blot analysis using PMH1 (Figure 2E) and HCM-1 (data not shown), antibodies specific for MUC2, revealed that the pooled fractions 7–12 after running the first CsCl gradient and fractions 12–15 after running the third CsCl gradient contained MUC2.

Threonine enrichment

GC-IRMS analysis demonstrated the presence of the dietary threonine tracer in MUC2 isolated from the small intestinal outflow fluid, indicating that dietary threonine was incorporated into MUC2. Figure 3 shows the values of MUC2-bound threonine enrichment and the time curve of tracer incorporation into MUC2 for a representative patient. Threonine enrichment rose almost linearly, starting within 6–10 h after commencement of the threonine administration. Maximal threonine enrichment in isolated MUC2 was reached at 21 ± 2.4 h in patients with an ileostomy. The time needed to absorb dietary threonine, and to synthesise and secrete MUC2 into the intestinal lumen was ranged from approximately 6–10 h in patients with an ileostomy (Table 1).

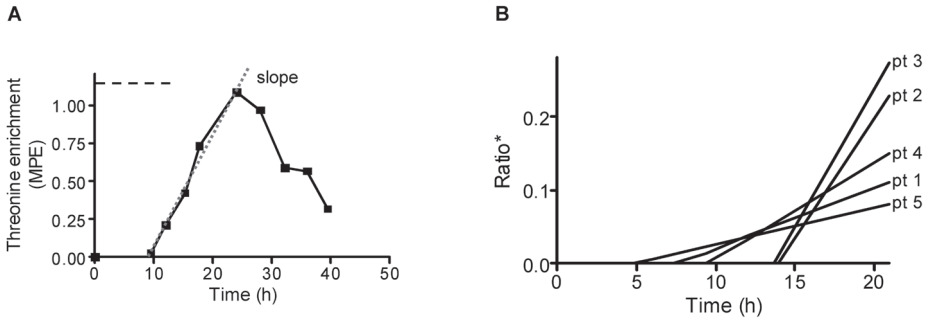

Figure 3 Threonine enrichment in small intestinal MUC2. Threonine enrichment in purified small intestinal MUC2 in one representative patient with an ileostomy (A). Ratio (*) of the slope of the linear increase in [U-^{13}C]threonine enrichment of purified MUC2 versus luminal [U-^{13}C]threonine precursor enrichment during the experiment for all patients (B). Fractional synthetic rate of MUC2 can be determined by multiplying the slope (ratio/Δt) by 24 hours and 100%. (━ ━ infusion time).

In contrast, the corresponding time for the single patient with a jejunostomy was less than 3 h. The calculated luminal [U-^{13}C]threonine precursor enrichment was 9.0 ± 3.1%. The linear increase of [U-^{13}C]threonine enrichment in MUC2 and the luminal [U-^{13}C]threonine precursor enrichment were used to calculate the FSR. It ranged from 19.6 - 89.7%/d (patients with an ileostomy) and was 12.1%/d in the patient with a jejunostomy (Table 1).

DISCUSSION

The main purpose of our study was to develop a tracer method to study small intestinal MUC2 synthesis. We also calculated the FSR of MUC2 in order to determine its feasibility as a parameter of intestinal barrier function. To our knowledge, this is the first report that determines the FSR of small intestinal MUC2 in human neonates.

A major problem in studying mucins is the difficulty in isolating and purifying the glycoproteins, due to their size and complexity.[9] Several methods of isolating intracellular and extracellular intestinal mucin MUC2 – from colonic mucosal scrapings in rats as well as human colonic biopsies – are described in the literature.[8, 18] However, human studies on intestinal mucin synthesis *in vivo* are lacking because of methodological restrictions. Apart from ethical concerns, it would be hard to obtain intestinal biopsies from preterm neonates, in view of the immaturity and fragility of their gastrointestinal tract. Developing a stable isotope technique, which is not harmful for (preterm) neonates, we were able to examine intestinal MUC2 synthesis and one of the metabolic fates of dietary threonine in the small intestine *in vivo*.

Previously, we demonstrated that the intestine of piglets utilised both dietary and systemic threonine for intestinal protein synthesis, but that dietary threonine was used predominately.[5] As mentioned earlier, the peptide backbone of intestinal mucins is rich in

threonine, which probably reflects the high utilisation rates of this essential amino acid. We therefore hypothesised that dietary threonine is incorporated into the gel-forming mucin MUC2. Indeed, our results showed that dietary threonine is used for MUC2 synthesis within 10 h after administration, and that this MUC2 is secreted into the small intestinal lumen. Recently, Faure *et al*. demonstrated that mucin synthesis in rats was sensitive to dietary threonine restriction, suggesting that threonine might be the limiting factor for intestinal mucin MUC2 synthesis.[19] Together, these data suggest that threonine restriction in enteral nutrition lead to impaired protective function of the mucus layer. Therefore, it might be essential to investigate the minimal threonine amount that is required to synthesise sufficient intestinal mucins for maintaining intact protective barrier function.

There are only few studies that make use of the FSR (%/d) to assess the capacity of synthesising intestinal proteins *in vivo*. Many different physiological and pathological situations were found to affect intestinal mucosal protein turnover.[20-22] However, the wide range in FSR of intestinal MUC2 measured in our study cannot easily be explained by different pathological situations. The values found in the patients with an ileostomy (19.6 to 89.7%/d) imply renewal of the small intestinal mucus layer every 2-10 d. Nakshabendi *et al*. found an intestinal mucosal protein FSR of 51%/d and 29%/d in human jejunum and ileum, respectively.[23] Rittler *et al*. reported an ileal mucosal protein FSR of 15%/d in control adult patients and a significant doubling in post-surgical adult patients (27%/d).[24] Thus, we confirm that the small intestinal mucosa has a high rate of MUC2 synthesis.

In spite of its wide range encountered in our study, the FSR of MUC2 might be valuable as a tool for intestinal functions, especially barrier function. As such, it may be of great importance in monitoring patients with impaired gut function, especially those with short bowel syndrome. The more extensive the resection of the small bowel, the higher the loss of absorptive and defensive surface area is that transports nutrients and protects against pathogenic bacterial colonisation. Serial measurements of the FSR of MUC2 may be valuable to assess intestinal adaptation, and to consider different treatment and nutritional support options in patients who underwent (massive) bowel resection. Yet more detailed studies are needed, investigating the impact of different pathological situations and several interventions, like medication and nutrition, on the FSR of MUC2 and to establish the FSR as a tool to improve medical management of patients with an impaired gut function.

Taken together, this study introduces a novel tracer method to investigate MUC2 synthesis and specifically one of the metabolic fates of dietary threonine *in vivo*. Besides, it suggests that the FSR of MUC2 might serve as a tool for assessing intestinal barrier function.

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Chapter 8

Small intestinal MUC2 synthesis in human preterm infants

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Submitted

ABSTRACT

Background: Mucin 2 (MUC2) is the structural component of the intestinal protective mucus layer, which contains high amounts of threonine in its peptide backbone. MUC2 synthesis rate might be a potential parameter for intestinal barrier function.

Aims: To determine whether systemic threonine was used for small intestinal MUC2 synthesis and to calculate the MUC2 fractional synthetic rate (FSR) in human preterm infants.

Methods: Seven preterm infants with an enterostomy following bowel resection for necrotising enterocolitis received intravenous infusion of [U-^{13}C]threonine to determine incorporation of systemic threonine into secreted MUC2 in intestinal outflow fluid. Small intestinal MUC2 was isolated using CsCl gradient ultracentrifugation and gravity gel filtration chromatography. MUC2-containing fractions were identified by SDS-PAGE/Periodic Acid-Schiff's staining and Western blot analysis and were subsequently pooled. Isotopic enrichment of threonine, measured in MUC2 using Gas Chromatography Isotopic Ratio Mass Spectrometry, was used to calculate the FSR of MUC2.

Results: Systemically derived threonine was indeed incorporated into small intestinal MUC2. Median FSR of small intestinal MUC2 was 67.2 (44.3-103.9) % per day.

Conclusions: Systemic threonine is rapidly incorporated into MUC2 in the small intestine of preterm infants, and thereby, MUC2 has a very high synthesis rate.

Abstract

INTRODUCTION

The mucus layer forms a physical barrier between the underlying epithelium and the lumen of the gastrointestinal tract.[1] It protects the epithelium against noxious agents, viruses and pathogenic bacteria. The structural component of this mucus layer is secretory mucin 2 (MUC2), which is secreted by goblet cells of the intestine and gives intestinal mucus a high density and viscoelasticity.[2] MUC2 is a glycoprotein rich in oligosaccharides, *O*-linked to threonine and serine residues in the peptide backbone.[3] MUC2 contains high amounts of threonine and proline tandem repeat sequences, which together constitute 20-55% of total amino acid composition.[4] Threonine is an indispensable amino acid utilised in a large amount by the portal-drained viscera in first-pass.[5] Recently, we showed that the equivalent of almost 90% of dietary threonine is utilised by the intestine of piglets.[6] The high visceral need for threonine presumably reflects the high synthesis rate of secretory mucins, e.g. MUC2. Therefore, threonine might be one of the amino acids essential for maintaining the protective mucus layer, and thus intestinal barrier function.

Disturbances in intestinal barrier function, characterised by inappropriate initial bacterial colonisation of the intestine and immature epithelial responses to bacteria and their toxins are thought to be crucial in the development of necrotising enterocolitis (NEC) in preterm infants.[7] NEC is the most devastating intestinal disease in premature infants and a regular indication for neonatal bowel resection.[8] Post-operatively, adequate gut adaptation and recovery of the intestinal barrier function are required to avoid malabsorption and pathogenic bacterial insults. Therefore, NEC patients receive total parenteral nutrition for a variable period of time after surgical intervention. However, lack of enteral nutrition can induce intestinal atrophy and may reduce digestive, absorptive and protective capacity.[9-11] In these circumstances, the usage of systemically derived substrates such as threonine might be of vital nutritional importance to maintain and restore an intact mucus layer, and thus intestinal barrier function.

We recently developed a tracer method to study small intestinal MUC2 synthesis and to determine the fractional synthetic rate (FSR) of MUC2 in human neonates as a potential parameter for intestinal barrier function.[12] We found that dietary threonine is incorporated into small intestinal MUC2. However, as the tracer was administered enterally, we could not verify whether threonine used for MUC2 synthesis was derived directly from the diet, and/or after intestinal absorption, and thus from the systemic circulation. As already known, the intestine utilises amino acids in first-pass and/or from the systemic site.[5, 13-15] Hence, threonine incorporated into small intestinal MUC2 may be derived from either the lumen or the basolateral site (systemic circulation). Moreover, our study in piglets demonstrated that two-thirds of threonine utilised by the portal-drained viscera was extracted from the systemic circulation during normal protein feeding.[6]

Thus, in the current study, our main objective was to determine whether a functional small intestine of preterm human infants after bowel resection for NEC was able to incorporate systemically derived threonine into MUC2. Furthermore, the FSR of MUC2 was calculated to determine the small intestine's capacity to synthesise and secrete MUC2 from systemically derived threonine.

MATERIALS AND METHODS

Patients

Patients eligible for this study were infants admitted to the Neonatal Intensive Care Unit (NICU) or the department of Paediatric Surgery of the Erasmus Medical Centre (MC)-Sophia Children's Hospital (Rotterdam, the Netherlands) after bowel resection for NEC. The diagnosis NEC was made on specific clinical symptoms (e.g. abdominal distension, bloody stools and gastric residuals) and characteristic features on an abdominal x-ray. NEC was confirmed at surgery and from histological features on resected intestinal tissue sent for routine histopathology. All infants received a temporary enterostomy during surgery. The Erasmus MC Institutional Review Board approved the study protocol. Written, informed consent was obtained from the parents.

Seven infants were included in the study. Table 1 lists their main clinical characteristics. Full parenteral feeding (glucose, amino acids (Primene® 10%, Clintec Benelux NV, Brussels, Belgium), and lipids (Intralipid® 20%, Fresenius Kabi, Den Bosch, the Netherlands)) was given during the first 10 post-operative days (d). Next, minimal enteral feeding was added during 4 d, after which enteral feeding was gradually introduced by continuous nasogastric gavage feeding or bottle-feeding under simultaneous diminishing of the parenteral nutrition. Enteral nutrition consisted of a combination of breast feeding and formula feeding (Nenatal®, Nutricia, Zoetermeer, the Netherlands; Pepti Junior®, Nutricia, Zoetermeer, the Netherlands).

Protocol

The study was performed when the infant was clinically stable, i.e. about 4 weeks (4 ± 1 wk) following bowel resection. Then, a continuous 12-hour (h) infusion [7.43 μmol/kg and 7.43 μmol/kg·h] of [U-^{13}C]threonine (99.47%, Cambridge Isotopes Laboratories, Andover, MA) was administered intravenously. All isotopes were tested and found sterile and pyrogen-free. The small intestinal outflow fluid samples were collected in adhesive bags at 3-h intervals during 2 d and stored at −80°C for further analysis. At time zero (baseline value) and after 9 h and 12 h of tracer administration, blood samples were collected by heelstick. Blood was centrifuged immediately to separate plasma and cells. The plasma was stored at −80°C until further analysis. Figure 1 outlines the tracer-infusion study described.

Table 1 Patient characteristics and kinetic parameters of small intestinal MUC2

Patients	Gender	GA *(wk)*	BW *(g)*	AS *(d)*	Cause	Enterostomy	Residual small intestine	MUC2 secretion time *(h)*	FSR *(%/d)*
1	M	28	695	11	NEC (small bowel)	Ileostomy	Small bowel excluding 7 cm ileum	3-6	103.9
2	M	30	930	33	NEC (small bowel)	Jejunostomy	Small bowel excluding 5 cm jejunum and 15 cm ileum	<3	85.7
3	M	25	940	15	NEC (small bowel)	Ileostomy	Small bowel excluding 24 cm ileum and the ileocaecal valve	9-12	44.3
4	M	30	885	30	NEC (small bowel)	Ileostomy	Small bowel excluding 10 cm ileum and the ileocaecal valve	*	55.5
5	M	26	950	44	Intestinal obstruction after conservative treatment for NEC	Ileostomy	Total small bowel	<3	67.2
6	M	32	995	11	NEC (small bowel)	Ileostomy	Small bowel excluding 10 cm ileum	<3	54.7
7	M	33	1445	5	NEC (colon)	End-Ileostomy	Total small bowel	<3	69.3
Median		30	940	15					67.2
Min-Max		25-33	695-1445	5-44					44.3-103.9

GA: gestational age; **BW:** birth weight; **AS:** age at surgery; **NEC:** necrotising enterocolitis; **FSR:** fractional synthetic rate; **M:** male
* not determined

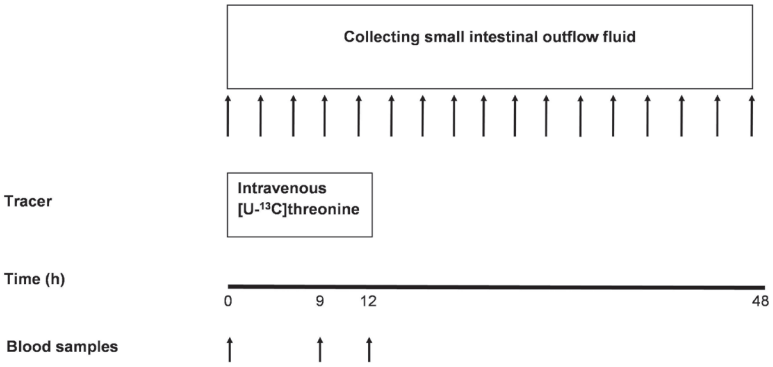

Figure 1 Outline of the study. [U-^{13}C]threonine administration lasted 12 h. During 48 h, intestinal outflow fluid was collected at 3-h intervals. At three time points, blood samples were collected by heelstick.

Analytical procedure

Isolation of MUC2

MUC2 was isolated from the small intestinal outflow fluid using CsCl density gradient ultracentrifugation (UC) according to the method previously described combined with gravity gel filtration chromatography.[2, 12] Briefly, samples were homogenised in homogenisation buffer, pH 7.5 (6 mol/l guanidinium hydrochloride (guanidinium HCl, Sigma), 50 mmol/l Tris(hydroxymethyl)aminomethane (Tris, Gibco), 5 mmol/l ethylene-diamino-tetra-acetate (EDTA, Merck) and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF, Sigma)). The disulfide bonds of mucins were chemically reduced by adding 100 mmol/l dithiotreitol (DTT, Sigma) and stirring overnight at 4°C in the dark. The homogenate was centrifuged to remove the fat layer and to isolate the supernatant (23.700 *g*, 10 minutes (min), 4°C (Mutifuge 3 S-R, Heraeus)). Sulfhydryl groups were carboxymethylated through addition of 250 mmol/l iodoacetamide (Sigma) in the supernatant and stirring for another 24 h at 4°C. Mucins were purified by equilibrium ultracentrifugation on a CsCl (Roche) density gradient. The gradient had a guanidinium HCl concentration of 4 mol/l with CsCl added to a density of 1.40 g/ml. Isopycnic density gradient centrifugation was performed in a Beckmann ultracentrifuge, 70 Ti rotor at 250.000 *g* for 72-88 h at 4°C. One ml fractions were collected and the mucin containing fractions were analysed as described below. Mucin-containing fractions were pooled and further purified by gravity gel filtration chromatography using columns (Econo-column, Biorad, Veenendaal, the Netherlands) filled with 20 ml of Sepharose CL-2B resin. The resin was equilibrated with 50 mmol/l Tris, 5 mmol/l EDTA and 1 mmol/l PMSF, pH 7.5 containing 4 mol/l guanidinium HCl. One ml of the mucin-containing fractions of each time point was loaded onto the columns. Mucins were eluted in the void volume of the column with 50 mmol/l Tris, 5 mmol/l EDTA and 1 mmol/l PMSF, pH 7.5 containing 4 M guanidinium HCl.

The mucin-containing fractions were extensively dialysed against distilled water at 4°C and stored at -20°C until further analysis.

Analysis of MUC2

Samples of dialysed fractions were analysed on reducing polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulfate (SDS-PAGE).[16] Gels were composed of 3% stacking gel and 4% running gel using a stock solution containing 30% acrylamide and 2% bis-acrylamide. Prestained Precision Plus Protein standards were purchased from Bio-Rad (ranging from 10 kDa to 250 kDa). Prior to SDS-PAGE analysis, samples were boiled for 5 min in buffer containing 1% (vol/vol) 2-mercaptoethanol (Bio-Rad) and 2% (wt/vol) SDS. Gels were stained with periodic acid-Schiff's reagent (PAS, Sigma) according to Konat *et al*.[17], with Phast Gel™ Blue R (Coomassie, Amersham), and with Silver Staining according to the manufacturer's protocol. As mucins have a characteristic buoyant density of approximately 1.45 g/ml, the density of CsCl gradient fractions was measured by weighing 1 ml of each fraction using a calibrated pipette. Hexose assay was performed using orcinol (Sigma) according to François *et al*.[18] with D-galactose:fucose (3:2 (wt/wt)) as standard. Quantity of hexose was expressed as the optical density (OD) at 540 nm. Mucin-containing fractions, i.e. fractions that contained a high molecular weight PAS-positive band at approximately 550 kDa after SDS-PAGE had a buoyant density of ± 1.45 g/ml and contained relatively high hexose levels, were pooled and stored at −20°C for further analysis.

Western blot analysis

The mucin-containing pooled fractions (16 µl) were loaded and run on a SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membranes (Protan BA 83, 0.2 µm) and blocked for 1 h at room temperature in blocking buffer containing 50 mmol/l Tris, pH 7.8, 5% (wt/vol) non-fat dry milk powder (Campina Melkunie Eindhoven, the Netherlands) 2 mmol/l $CaCl_2$, 0.05% (vol/vol) Triton X-100 and 0.01% Antifoam B (Sigma-Aldrich). Blots were incubated overnight at 4°C with rabbit polyclonal antibody HCM-1 (1:2500 in blocking buffer).[2] After washing with PBS containing 0.2% Tween-20, blots bound antibodies were detected using HRP conjugated goat anti-rabbit secondary antibody (HCM-1; 1:1000 in blocking buffer) and SuperSignal® West Femto Luminol Enhancer kit (Pierce).

Mass spectrometry

Analysis of [U-^{13}C]threonine

After gravity gel filtration chromatography, aliquots of 3-4 ml of the pooled MUC2 fractions were dialysed, freeze dried and prepared for mass spectrometric analysis. The dried samples were hydrolysed by adding 1 ml of 6 mol/l HCl and incubating at 110°C in sealed tubes for 24 h. The protein hydrolysates (1000 µl) were dried under a nitrogen stream at 55°C and the residue was dissolved in 0.5 ml water. The amino acids in the residue were bound to a 1 ml AG50 W-X8, H^+ cation exchange column (Biorad, Richmond, Virginia, USA). Amino acids were eluted with 3 ml 6 mol/l NH_4OH and dried under nitrogen at 30°C. Threonine was converted to its N-ethoxycarbonylethylester derivative according to the method used in our previous work.[6] The [^{13}C/^{12}C]-ratio of threonine in isolated MUC2 was measured by a Thermo Electron

Delta-XP isotope ratio MS linked online with a trace GC (Thermo Electron, Bremen, Germany) and a combustion interface type 3 (Thermo Electron, Bremen, Germany). Aliquots of 1 µl of the ethyl acetate suspension containing the amino acid derivatives were introduced into the GC system by a CTC PAL autosampler (CTC Switzerland). Chromatographic conditions were like those described previously. After separation using capillary GC, amino acids were combusted online at 940°C and introduced as CO_2 into the isotope ratio MS, where the [^{13}C /^{12}C]-ratio was measured. Enrichment was expressed in mole percent excess (MPE).

Analysis of [U-^{13}C]threonine in blood samples

Small aliquots of plasma (50 µl) were prepared to determine threonine enrichment and concentration by gaschromatography - mass spectrometry (GC-MS). Briefly, 20 µl of internal standard (1µmol/ml [^{15}N]threonine) was added to 50 µl plasma and deproteinised with sulfo-salicylic acid (SSA) 6% (wt/vol). After centrifugation for 10 min at 4°C and 20.000 *g*, the amino acids in the supernatant were isolated by cation exchange separation as described above. The eluate was dried overnight at 30°C under a stream of nitrogen. Finally, t-butyldimethylsilyl derivates were formed by adding 25 µl of dimethyl-formamide and 25 µl of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide to the dried residue and heating at 60°C for 1 h. Standard curves were prepared by mixing aqueous solutions of natural and labelled threonine for both enrichment and concentration determination. GC-MS analyses were performed in selective ion monitoring mode (SIM) on a Carlo Erba GC8000 gas chromatograph connected to a Fisons MD-800 mass spectrometer (Interscience BV, Breda, the Netherlands). One microliter of the derivative was injected in split mode (1:20) on a DB-17, 30 m x 0.25 mm capillary column (Agilent). Selective ion monitoring was carried out at m/z 404.3, 405.3 and 408.3. Enrichment was expressed in MPE.

Calculations

MUC2 secretion time was defined as the time interval between start of intravenous [U-^{13}C]threonine administration and the appearance of enriched MUC2 in small intestinal outflow fluid.

Fractional synthetic rate (FSR) of MUC2 is expressed as percentage of the total MUC2 pool synthesised per day. Plasma threonine enrichment is used as precursor.

FSR was calculated by:

$$\frac{\text{slope of the linear increase of [U-}^{13}\text{C]threonine enrichment of MUC2}}{\text{[U-}^{13}\text{C]threonine enrichment in plasma}} * 24$$

Statistics

Data are presented as the mean values plus or minus the standard deviation (SD) or as median (minimum-maximum).

RESULTS

Patients

The seven infants studied had median gestational age of 30 (25-33) wk and median birth weight of 940 (695-1445) g (Table 1). Surgery was performed at median 15 (5-44) d after birth, with creation of a jejunostomy (n=1) or ileostomy (n=6). At the time of the study (mean 4 ± 1 wk following bowel resection) patients had mean enteral protein intake of 2.6 ± 1.1 g/(kg·d). Moreover, 5 infants had a glucose infusion and one infant received both enteral and parenteral nutrition during the study.

Isolation and characterisation of mucins

MUC2 was isolated from the intestinal outflow fluid using CsCl density gradient UC and was characterised by PAS-staining, hexose assay, buoyant density and Western blot analysis as described previously.[2, 12] The mucin-containing fractions were pooled and further purified by gravity gel filtration chromatography. The fractions 5-8 contained the purified mucin MUC2. These fractions contained a high molecular mass band of ± 550 kDa, which was PAS-positive on SDS-PAGE (Figure 2A). Moreover, this high molecular weight band corresponded with a peak in hexose assay (Figure 2C). Each mucin fraction was free of contaminating proteins detected by Coomassie Blue staining as well as silver staining (data not shown).

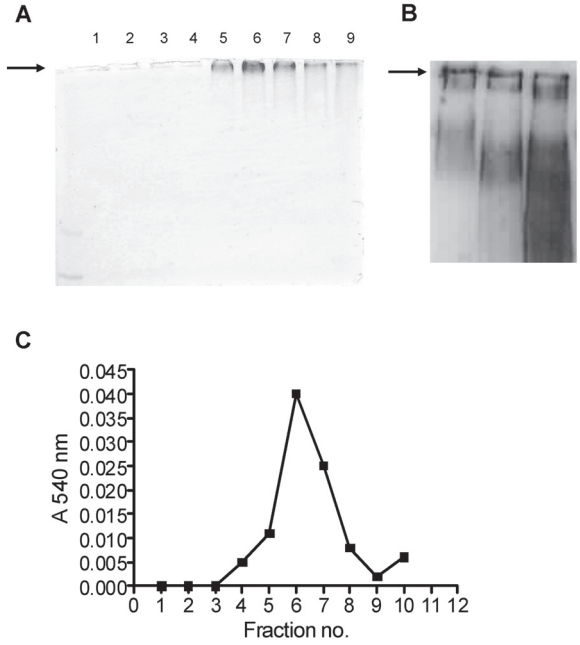

Figure 2 Isolation and characterisation of human MUC2 in the intestinal outflow fluid. A represents reducing 4% stacking and 3% running SDS-PAGE gel containing dialysed mucin fractions purified by gradient density ultracentrifugation followed by Sepharose CL-2B gravity gel filtration. PAS-positive fractions 5-8 after the gravity gel filtration chromatography (A) were pooled and subjected to Western blot analysis (B; 3 time points of different representative patients). C represents the hexose content (■) of the same samples as represented in the gel.

Furthermore, Western blot analysis using anti-HCM-1, an antibody specific for MUC2, revealed that the pooled fractions 5-8 after Sepharose CL-2B gravity gel filtration contained MUC2 (Figure 2B).

Threonine enrichment

The [U-^{13}C]enrichment of plasma threonine reached a steady state of 3.63 ± 0.56 MPE in all patients between t=9 and t=12 h, seeing that slopes of enrichment between these time points did not significantly differ from zero. Figure 3 shows the values of MUC2-bound threonine enrichment and the time curve of systemic tracer incorporation into MUC2 for two representative patients. Threonine enrichment rose almost linearly in isolated MUC2 (maximal threonine enrichment (1.06 ± 0.50 MPE)) and gradually decreased after mean 21 ± 2 h. Time needed to absorb systemic threonine, and to synthesise and secrete MUC2 into the small intestinal lumen was within 3 h in 4 patients, between 3-6 h and between 9-12 hours in the other two patients (Table 1). Median FSR of small intestinal MUC2 was 67.2 (44.3 - 103.9) % per day (Table 1).

DISCUSSION

Tracer studies in mammals showed that amino acids in the intestine derive from both dietary and arterial sources and are used for mucosal protein synthesis and energy generation.[5, 13-15, 19-21] As MUC2 is the predominant secretory mucin in the intestine and its peptide backbone is rich in threonine, we speculated that absorbed threonine is mainly used for MUC2 synthesis in order to maintain the protective mucus layer, and thus gut barrier function.

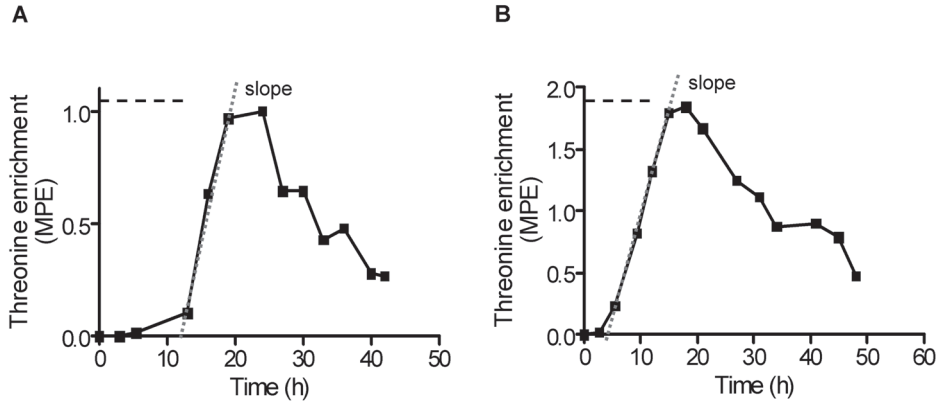

Figure 3 Threonine enrichment in MUC2 in two representative patients. Threonine enrichment in purified MUC2 in one representative patient with an ileostomy (A) and the single patient with a jejunostomy (B). These data allow us to determine the secretion time and to calculate the fractional synthetic rate of MUC2. (▬ ▬ infusion time)

As threonine cannot be synthesised *de novo*, the epithelial cells are offered high quantities of threonine from both the intestinal lumen (i.e. the diet or proteolysis (recycling of threonine)) and the systemic circulation during enteral nutrition. The question arises whether threonine is utilised differently when presented to epithelial cells via the lumen or via the circulation. In piglets, we previously showed that two-thirds of threonine utilised by the intestine derive from the systemic circulation during normal protein feeding.[6] However, dietary, rather than systemic threonine was preferentially utilised for constitutive protein synthesis in the small intestinal mucosa of these piglets. The current study demonstrated that even during enteral feeding the intestine utilises systemically derived threonine for MUC2 synthesis. We therefore suggest that dietary threonine is preferentially taken up by enterocytes and is used for constitutive protein synthesis, whereas goblet cells might extract threonine from the systemic site and use it for intestinal MUC2 synthesis. Hence, we assumed that goblet cells, which secrete MUC2 and trefoil peptides, are not capable to absorb threonine directly from the intestinal lumen in contrast to enterocytes.[22] That intestinal channelling of amino acids to specific metabolic products occurs dependent on the site of absorption during enteral feeding was also suggested by Reeds *et al*. for the mucosal glutamate pool in enterally fed piglets.[23] Our previous study showed that dietary threonine was incorporated into small intestinal MUC2, which seems in contrast to the present results. However, the enteral tracer administered in that study might have been absorbed into the portal vein and subsequently in the systemic circulation: thereafter, it might have been utilised by the intestine via the systemic site.

The gastrointestinal tract plays a significant role in protein metabolism of the whole body. Its tissues represent only 5% of the total body weight, but their high rates of metabolism make them responsible for approximately 15–50% of the whole-body oxygen consumption and protein turnover.[24–27] Specifically, the intestinal mucosa is a very dynamic, metabolically active tissue that utilises substantial amounts of dietary amino acids for intestinal protein synthesis and its catabolism.[5, 6, 19] Faure *et al*. showed diminishing FSR of intestinal proteins along rat gut, i.e. from 122.2%/d for the duodenum to 43.6%/d for the colon.[28] The pattern was different for mucins, however, with FSR 116.2%/d for duodenum, 135.6%/d and 137.9%/d for jejunum and ileum, respectively, and 112.1%/d for the colon. In human adults, the FSR of small intestinal mucosal proteins varies between 15–51%/d along the small intestine.[29, 30] In the present study, the FSR of MUC2 measured in human preterm infants varied between 44.3–103.9%/d and between 12.1–89.7%/d in our previous study. Moreover, the FSR of other tissue proteins like albumin and skeletal muscle proteins is much lower, approximately 6%/d and 2%/d in human adults, respectively.[31–33] In this context, the small intestinal MUC2 synthesis rate is notably high (median 67.2%/d) in preterm infants recovering from NEC and is among the highest so far determined in humans.

The importance of enteral intake of nutrients in maintaining gut morphology and integrity has been reported by several authors.[10, 11, 34] However, after bowel resection for i.e. NEC, most infants are fed parenteral nutrition solely for a certain period of time. Although parenteral nutrition is known to induce gut atrophy and thereby loss of absorptive capacity, knowledge

on its effect on mucin synthesis in humans is lacking. Thus far, only studies in rats showed that parenteral nutrition is associated with compromised intestinal barrier function due to diminished mucin production.[35] Furthermore, the intestinal threonine requirement of parenterally fed piglets is significantly lower than that of piglets fed enterally, which might suggest diminished MUC2 synthesis during parenteral feeding.[36] Our present study showed that systemic threonine is utilised for intestinal MUC2 synthesis in preterm infants who were predominantly fed enteral nutrition. However, it is yet unknown whether systemic threonine is utilised similarly in infants fed full parenteral nutrition, since other factors such as bowel movements, enteric nerve stimulation or hormone production related to the presence of nutrients within the gastrointestinal tract might be important.

In conclusion, systemically derived threonine is rapidly incorporated into small intestinal MUC2, and thereby, MUC2 synthesis rate of the small intestine is high.

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Chapter 9

General discussion

GENERAL DISCUSSION

In human newborns, the neonatal period is characterised by rapid growth and development; therefore, a well-functioning gastrointestinal (GI) tract is indispensable. Specifically, the assignment of the small intestine is to efficiently digest and absorb dietary nutrients to meet the growing infant's whole-body nutrient and fluid requirements. Advances in perinatal-neonatal care and therapeutic regimens have raised survival rates of premature infants and neonates born with congenital anomalies, albeit with sometimes significant long-term morbidity. These vulnerable infants are at more risk to develop a compromised gut, mainly as a consequence of physiologic GI tract immaturity and/or specific GI diseases and anomalies, but also because these conditions often require bowel resection. Hence, alterations in nutrient demands and feeding intolerance are frequently observed, which make supplying the newborn's nutritional requirements difficult.

Many clinical conditions in the (premature) neonate may necessitate small intestinal resection; however, the most common indications are congenital anomalies such as intestinal atresia and volvulus, and acquired diseases such as necrotising enterocolitis (NEC).[1] The functional consequences of small bowel resection depend on underlying cause, age-adjusted small bowel length, anatomical site of the resected small intestine, and remaining small intestinal surface area. Length of the small intestine varies markedly with gestational age and doubles during the last trimester of pregnancy.[2] More specifically, at 27 weeks of gestation the length of the small intestine is approximately 115 cm, but in term infants it reaches 250 cm. After birth, the highest increase in bowel length is during the first year of life.[3] A preterm infant's remaining small intestine after bowel resection may therefore have a potential larger capacity for additional growth and functional adaptation than is the case for term infants.

Survival after neonatal small bowel resection has improved considerably over the last 30 years. Survival rates after extensive resections of the small intestine increased from 56% in 1972 to around 90% in children born after 1980.[4-6] Mortality after bowel resection for necrotising enterocolitis in small preterm infants is reduced as well; however, it is still round 35%.[7] This improvement may be linked to several factors including sophisticated surgical techniques, liver disease prevention, better treatment and reduction of catheter-related sepsis, and safer and efficacious parenteral nutrition.[6] Despite these advances, the state of malabsorption, short bowel syndrome (SBS), is regularly observed. The literature provides various definitions of SBS, based either on anatomical loss of bowel length or on prolonged periods of parenteral nutrition, so-called functional SBS.[8] Excessive intestinal resection diminishes the absorptive and protective surface area such that sufficient dietary nutrient uptake is severely compromised. SBS is a common problem in paediatric surgery and a significant cause of infant mortality and morbidity, largely dependent on the complications of parenteral nutrition (cholestasis) and the remaining small bowel length.[9] Moreover, it may require long-term hospitalisation and prolonged treatment with parenteral nutrition, which could have a remarkable psychosocial impact on these infants and their parents.

The key to infants' survival and clinical recovery following small bowel resection is the ability of the residual gut to adapt to its modified condition. Numerous research groups over the past decades have tackled the issue of post-resection intestinal adaptation. Several studies in animals have been performed to investigate the morphological and functional aspects of gut adaptation, whereas only a few studies have been implemented in humans.[10, 11] These studies primarily focus on the compensatory intestinal response to the acute loss of mucosal surface area in an attempt to restore normal digestive and absorptive capacity. This adaptive mechanism and its mediators are not completely understood, but a variety of trophic factors (e.g. glutamine, growth hormone and glucagon-like peptide-2) are thought to be involved in enhancing the intestinal adaptive response.[12-14] To date, evidence points at enteral nutrition as a pivotal trophic factor in stimulating intestinal adaptation after bowel resection.[15, 16] Furthermore, (enteral) nutrition must aim at improving the neonate's nutritional status to sustain normal growth and development; however, optimal nutritional support in infants following bowel resection remains controversial. Due to the lack of properly designed randomised clinical trials and the fact that almost all trials are underpowered, the medical and nutritional management of these paediatric patients is still based on 'trial and error'. Therefore, understanding of residual bowel functioning is crucial to develop new medical treatment modalities and optimal nutritional strategies for these infants. To this aim we need to establish objective parameters of bowel function that may serve to evaluate possible effects of therapeutic interventions on the remaining intestine.

In the following section we discuss the major findings of this thesis in relation to other studies. Furthermore, we deal with the methodological limitations encountered in performing a clinical study in infants who had had neonatal bowel resection. Finally, we recommend future research using the knowledge obtained from our studies.

MAJOR FINDINGS

The studies described in this thesis highlight three pivotal functions of the intestinal epithelium: 1) nutrient digestion, absorption and utilisation, 2) protection and repair, and 3) defence. Based on these epithelial functions, the condition of the residual bowel and a novel potential parameter to evaluate intestinal functioning after neonatal bowel resection in human infants are discussed below.

Nutrient digestion, absorption and utilisation

Epithelial protein expression

As mentioned earlier, good nutritional strategies are extremely important for the many more infants who now survive after (extensive) small bowel resection in the neonatal period. However, a major impediment to the progress of research in the area of intestinal adaptation after neonatal bowel resection is the lack of knowledge concerning the enterocyte-specific expression of proteins indispensable for optimal nutrient digestion and absorption. In general, expression patterns of many enterocyte-specific proteins have been studied during

ontogeny and after birth. Although their developmental expression patterns are very different, our study showed that the intestinal epithelium expresses a wide variety of enterocyte-specific proteins very early in life (Chapter 3). Furthermore, the presence of a congenital small intestinal atresia had no substantial effect on enterocyte-specific protein expression. The literature suggests that luminal components are essential for adequate enterocyte-specific protein induction of the intestine both in utero and after birth.[17-19] However, we found that the enterocyte-specific protein expression distal to a congenital atresia of the small intestine in human infants was genetically programmed, despite the absence of enteral substrate during foetal intestinal development (Chapter 3). In particular, this indicates that luminal components are not crucial for the induction of enterocyte-specific functions during development, and thereby, it implies the presence of specific ontogenetic factors. Thus, the enterocyte-specific proteins essential for digestion and absorption of dietary nutrients – e.g. sodium glucose cotransporter 1 (SGLT-1), glucose and fructose transporters 2 and 5 (Glut2 and Glut5), lactase and sucrase-isomaltase (SI) – are expressed in the entire residual small bowel after surgical correction of small intestinal atresia The question remains, however, whether quantities and activities of these proteins are sufficient for adequate nutrient digestion and absorption prenatally and postnatally, seeing that the lack of luminal components could down-regulate these proteins at transcriptional and/or (post-)translational level. Thus far, no reports are available on these regulatory (cellular and/or molecular) mechanisms of enterocyte-specific protein expression and the response to different substrates, for instance breast milk versus formula feeding in human infants.

The next question is whether NEC, as an acquired disease, affects enterocyte-specific functions such as nutrient digestion and absorption, fatty acid uptake and transport, and electrolyte and water absorption. Acute NEC was associated with diminished enterocyte-specific expression of lactase and Glut2 and 5 in the residual bowel – particularly in areas with severe epithelial damage (Chapter 4). In contrast, other enterocyte-specific markers such as SI and intestinal fatty acid binding protein (i-FABP) were maintained at protein level independent of the degree of epithelial damage. Furthermore, infants with acute NEC showed increased proliferation in the remaining bowel. The down-regulation of distinct enterocyte functions might result from altered protein stability in acute NEC. Alternatively, the intestinal epithelium might actively down-regulate the expression of lactase and glucose transporters to conserve sufficient energy in favour of other epithelial functions like proliferation, protection, repair and defence. This response – selective down-regulation of enterocyte-specific functions with maintenance of other cell type-specific functions – has been shown in an experimental model of methotrexate (MTX)-induced epithelial damage in rats: while enterocyte-specific proteins were distinctly down-regulated, the goblet cell- and Paneth cell functions were spared.[20] Recently, de Koning *et al*. demonstrated that during epithelial damage after MTX-treatment in mice, the intestinal epithelium was preferentially involved with proliferation rather than differentiation.[21] All this, seemingly aimed at restoring barrier function, would result in compromised absorptive function of the small intestinal epithelium. Whether the above-mentioned observation in animals is a general phenomenon in response to epithelial damage, and thus apposite to our results in preterm infants with

acute NEC, might only be speculated. At the time of stoma closure in infants recovering from NEC, epithelial proliferation was still increased and the enterocyte-specific protein expression was completely restored. This indicates that epithelial damage in preterm infants with NEC is reversible and thus, enterocyte-specific functions have recuperated at the time of restoration of intestinal continuity. Unfortunately, just like in infants with a congenital small intestinal atresia, we cannot expound on quantities and activities of enterocyte-specific proteins crucial for adequate nutrient digestion and absorption in infants with NEC, because these have not been investigated so far. The timing of (re)introduction of enteral feeding in infants recovering from NEC remains therefore difficult. Piena-Spoel *et al*. evaluated the condition of the bowel in infants with NEC using the sugar-absorption test (lactulose/rhamnose ratio).[22] They showed an individual variability in the recovery of intestinal permeability in those infants, and therefore, an individual approach in (re)introducing enteral feeding seems justified. In particular, care should be taken to ensure that intestinal permeability is not increased at the time of reintroducing enteral feeding. Combining more parameters of intestinal functioning, such as the sugar-absorption test and the above-described determination of enterocyte-specific proteins in repeated bowel biopsies could enable to set a more accurate timepoint of enteral refeeding in infants recovering from NEC.

Dietary protein absorption and enteral feeding

In line with the restored enterocyte-specific functions of the remaining bowel, dietary protein absorption capacity in preterm infants recovering from NEC is intact. In general, optimal protein nutrition is necessary to maximise synthesis and accretion of tissue proteins and to substitute unavoidable protein losses. Our study in human infants who underwent bowel resection in the neonatal period demonstrated that dietary protein absorption capacity of the small intestine amounted to 70-90% of the total enteral protein intake (Chapter 2). Then, as the absorption capacity in infants with a jejunostomy was 70-80%, we concluded that dietary protein was primarily absorbed in the jejunum, thus far before the end of the small intestine. A similar observation was made in piglets: we showed that most dietary threonine was taken up in the proximal jejunum (Chapter 5). These data are consistent with the findings observed by Gausseres *et al*. in human adults and Stoll *et al*. in neonatal pigs.[23, 24] Both studies demonstrate absorption of dietary proteins or amino acids to vary along the length of the small intestine. It is conceivable, therefore, that the duodenum and jejunum preferentially absorbs nutrients directly from the diet. As the small intestine, especially the jejunum, efficiently digests and absorbs complex dietary proteins, only little protein reaches the stoma outflow fluid or the colon when intestinal continuity is preserved. Therefore, if tolerated, enteral feeding is the first choice of nutritional support in the post-resection period in human infants. Apart from its nutritional benefit, enteral feeding – provided it is not given by feeding tube – promotes oral feeding skills and physiological gut adaptation processes to reach intestinal autonomy (weaning off parenteral nutrition). Although all medical centres now recommend early use of enteral feedings, there is incomplete agreement about its optimal composition; an elemental, semi-elemental or polymeric diet. In view of the small intestine's accurate dietary protein absorption, human infants after bowel resection will probably not need hydrolysed or elemental formula. Many centres prescribe a semi-

elemental diet including high-fat and low-carbohydrate components to infants whose gut is compromised after small bowel resection, although randomised clinical trials supporting this practice are lacking.[25] Furthermore, Vanderhoof and Young suggested that paediatric patients with a short small bowel and colon in continuity may benefit from a high-carbohydrate and high-caloric diet because of colonic absorption of carbohydrates.[25] A novel trend is breast milk as the first choice of enteral feeding after neonatal bowel resection, even though it contains lactose and complex fats and proteins.[1] Breast milk has the benefit of containing non-nutritive factors such as prostaglandins, immunoglobulin A (IgA), leukocytes, nucleotides and trophic factors. A study by Andorsky *et al*. provides indeed further evidence that breast milk enhances outcomes in infants with SBS, and might therefore be an attractive nutritional alternative to infant formulas.[26] Nevertheless, we suggest that upcoming research programs should pay more attention to resolving the persistent controversy of optimal nutritional management after (massive) bowel resection in infants.

Intestinal amino acid absorption and utilisation

Previous studies in enterally fed animals have shown that a substantial proportion (30-60%) of dietary amino acids are sequestered by the intestine in first-pass and that the utilisation rates are higher in preterm infants than in adults.[27-30] Amino acids taken up by the intestine may have various metabolic fates such as protein synthesis, biosynthetic pathways and irreversible oxidation to CO_2. Noteworthy from a metabolic perspective, therefore, was the disproportionate high threonine utilisation by the intestinal tissues in neonatal pigs (Chapter 5). In line with this finding, Van der Schoor *et al*. observed that the splanchnic tissues in human preterm infants extract comparable large quantities of dietary threonine (unpublished data). This high need of dietary threonine might reflect intestinal mucin 2 (MUC2) synthesis, the structural component of the mucus layer whose peptide backbone is rich in threonine, proline and serine. However, dietary threonine in piglets was mainly incorporated into proteins of the intestinal wall when measured after 5 hours of labelled threonine infusion (Chapter 5). In mice, nevertheless, much dietary threonine absorbed by the intestine was indeed used for MUC2 synthesis (Chapter 6). In chapters 7 and 8, ultimately, we showed that dietary threonine is incorporated into small intestinal MUC2 of human preterm infants. Thus, we indisputably proved that one of the intestinal metabolic fates of dietary threonine is incorporation into MUC2. However, minimal intestinal threonine requirements to maintain a competent protective mucus layer for different clinical conditions in humans are still unknown.

While intestinal amino acid metabolism has been widely studied both in animals and humans, little research has been done on the site of amino acid absorption by the intestine. In the early seventies, Alpers reported that intestinal crypt cells predominantly use systemically derived amino acids, whereas villus cells mainly use amino acids from the luminal site.[31] Moreover, Windmueller and Spaeth observed the lumen-derived and blood-derived utilisation of two closely related amino acids, glutamate and glutamine, and demonstrated that the metabolism of luminal glutamate was even more extensive than that of arterial glutamine.[32] In piglets, dietary, rather than systemic threonine was preferentially

utilised for protein synthesis in the small intestine (Chapter 5). In addition, dietary and not systemic glutamate was also used for mucosal gluthatione synthesis.[33] This indicates that the route of amino acid uptake by the intestine depends on its epithelial cell type-specific metabolic fate. Therefore, we might speculate that enterocytes preferentially absorb threonine from the apical site for constitutive protein synthesis, whereas goblet cells extract threonine from the basolateral site and use it for intestinal MUC2 synthesis. The latter was confirmed by determining threonine enrichment in isolated small intestinal MUC2 in human preterm infants through intravenous administration of the threonine tracer (Chapter 8). However, the relative contributions of dietary and systemically derived threonine to MUC2 synthesis remain unresolved.

On the other hand, dietary threonine can be used for other purposes than intestinal protein synthesis, e.g. oxidation. The most important substrates for intestinal energy generation in neonatal pigs and humans are glucose, glutamate and glutamine.[34-36] Threonine oxidised by the portal-drained viscera in piglets contributes for approximately 13% to the whole-body threonine oxidation (Chapter 5). Dietary threonine oxidation was also observed in mice; however, the oxidative rate of dietary threonine in Muc2-deficient mice was significantly higher than in their wild type littermates, indicating that Muc2 deficiency results in inefficient utilisation of dietary threonine (Chapter 6). Furthermore, in neonates fed full enteral nutrition, threonine oxidation was completely absent (Van der Schoor *et al*. unpublished data). Taken together, intestinal oxidation is a minor metabolic fate of dietary threonine, which implies nutritional efficacy of threonine in early neonatal life.

Protection, repair and defence

Epithelial protein expression

The intestinal epithelium consists of a complex protection, repair and defence system to prevent pathogenic bacteria, viruses, and other noxious agents from infiltrating the body and to restore potential epithelial damage. It is generally accepted that MUC2 and trefoil factor 3 (TFF3) – both secreted by goblet cells – play a fundamental role in epithelial protection and repair of the intestine. Moreover, antibiotic peptides expressed by Paneth cells of the small intestine contribute to local defence. Consistent with the enterocyte-specific protein expression, goblet cells and Paneth cells express cell type-specific proteins in utero and after birth as described in chapter 3. However, to date, these goblet cell- and Paneth cell-specific proteins have not been investigated in the residual bowel of infants after neonatal bowel resection. Contrary, there is an overwhelming amount of literature investigating the consequences of neonatal massive bowel loss (80%) in rats. Nevertheless, this post-resection intestinal adaptation research has mainly focussed on the physiological and morphological processes of the residual bowel related to nutrient digestion and absorption rather than on its ability to protect against epithelial damage. We showed that the remaining goblet cells in the residual bowel of infants operated on for acute NEC continued to express MUC2 and TFF3 proteins (Chapter 4). However, Vieten *et al*. recently demonstrated that TFF3 expression is down-regulated in infants with acute NEC at protein as well as mRNA level, implying an insufficient repair function of the intestinal epithelium during damage.[37] Furthermore,

in acute NEC, lysozyme expression in the residual bowel was only weakly positive. Thus, goblet cell- and Paneth cell functions were maintained in human infants with NEC-induced epithelial damage. However, it is still unknown whether their secreted proteins, essential for epithelial protection, repair and defence, are sufficient to restitute and regenerate injured intestinal epithelium. According to Vieten *et al.*, down-regulation of TFF3 gene expression was sustained in those infants recovering from NEC, indicating a prolonged period of diminished epithelial repair.[37] Whether this apparent down-regulation is also observed for MUC2, is not yet known. However, our immunohistochemical data suggest that the goblet cell- and Paneth cell-specific protein expressions are completely restored at the time of stoma closure (during post-resection intestinal recovery), implying a well-adapted intestinal epithelium. In particular, the anti-bacterial lysozyme showed an increased expression in infants recovering from NEC. A possible interpretation of this finding is protection against the bacterial overgrowth that often complicates bowel resection.[38] Bacterial overgrowth, which further exacerbates intestinal failure[39], is likely to occur when the ileocaecal valve is absent, when a tight anastomosis or a partial obstruction is present, or when a dilated segment of bowel with poor motility exists. Although we did not systematically investigate specific clinical symptoms of bacterial overgrowth in our study population, lysozyme expression may increase to strengthen the epithelial defence in order to eliminate excessive growth of commensal flora and potential pathogens. To date, there is no evidence that lysozyme production and secretion is elevated in the post-operative period after bowel resection for NEC. However, the possible associations between NEC and lysozyme expression offer support for future studies addressing the roles of lysozyme and other endogenous host defence factors in the pathophysiology of this disease.

Gut barrier function

The mucosal barrier of the intestine comprises numerous factors contributing to maintain barrier integrity, including e.g. intestinal peristalsis, the mucus layer, and secretion of IgA and trefoil peptides. Mucus is a highly hydrated gel layer and consists of water (95%) and the secretory mucin MUC2 (5%). Using Muc2-deficient mice, van der Sluis *et al*. recently showed that MUC2 is crucial for epithelial protection, and thus in maintaining intestinal barrier function.[40] Therefore, optimal regulation of MUC2 synthesis and its secretion is indispensable to reach intestinal autonomy after bowel resection in humans.

The peptide backbone of MUC2 is known to be rich in the amino acids threonine, proline and serine. The application of threonine as a tracer to gain more insight in intestinal MUC2 synthesis, and thus barrier function *in vivo,* would therefore seem a reasonable approach. Intestinal barrier function might be defined more precisely by calculating the Fractional Synthetic Rate (FSR) of MUC2. The FSR of small intestinal MUC2 is relatively high (12.1-103.9%/d) compared with protein synthesis rates of other tissues in human adults. However, its wide range among all infants studied (Chapters 7 and 8) raises concerns about the validity to use it as a parameter of intestinal barrier function. Due to the impossibility of quantifying small intestinal MUC2 and the lack of reference values, we could not establish a possible relationship between the clinical condition of the infants studied and their capacity to secrete

MUC2 and to maintain an adequate mucus layer. Nevertheless, studying larger groups of infants with various intestinal problems and the inclusion of a control group may be means to optimise acceptable values for the FSR of MUC2 and thereby, its reliability as an objective parameter for intestinal barrier function. Furthermore, FSR measurements of MUC2 *in vivo* might be worthwhile to investigate gut barrier function in reponse to various stimuli such as feeding, fasting, sepsis and disease. For example, Faure *et al*. already reported that the FSR of intestinal mucins was reduced during dietary threonine restriction in rats.[41] This might indicate that in clinical situations associated with increased intestinal threonine utilisation or reduced threonine intake, gut barrier function is impaired. Accordingly, they found that an increase in dietary threonine supply promotes intestinal mucin synthesis during mucosal inflammatory damage in rats.[42] Both studies imply that the FSR of mucins might be a reliable potential parameter in order to study intestinal barrier function.

METHODOLOGICAL CONSIDERATIONS

Study population

Critical factors in interpreting the results in chapters 2, 7 and 8 of this thesis are the small number of infants included and the remarkable heterogeneity among the infants studied. As neonatal bowel resection is inevitable for various intestinal problems, a heterogeneous group of neonates were eligible for inclusion in the three-year study period. As they also showed widely ranging gestational age, birth weight, illness severity and co-morbidities, it is difficult to draw general conclusions. Furthermore, a limiting factor for inclusion was the creation of an enterostomy during the initial surgery, which was required for the clinical studies described in chapters 2, 7 and 8 of this thesis. Consequently, protein absorption capacities of the small intestine were determined in a very heterogeneous group of infants of whom only three had a jejunostomy (Chapter 2). We recommend continuing this study by including more infants with an enterostomy, but preferably those with a jejunostomy. However, despite the availability of a nation-wide SBS-registry, the number of paediatric surgical centres in the Netherlands seems too small to meet this prerequisite. Overall, a small and heterogeneous group of infants remains available for inclusion; however, the parenteral informed consent rate for inclusion in this group is exclusively high.

To partially exclude heterogeneity, we studied a relatively small and selected group of preterm neonates – i.e. those recovering from NEC – to determine the FSR of small intestinal MUC2 (Chapters 7 and 8). However, these infants might have an intrinsic different FSR of MUC2 compared to control neonates. Additionally, the wide range of FSR of MUC2 encountered makes it hard to draw conclusions. We therefore recommend studies in larger patient groups together with the inclusion of a control group to correlate calculated FSR values. Nevertheless, the inclusion of an age-matched control group is very complicated due to the necessity of surgical intervention to create an enterostomy and the possible influence of the underlying disease on MUC2 synthesis; the latter may result in abnormal quantities of MUC2 or in abnormal MUC2 structure. For example, Heys *et al*. and Rittler *et al*. demonstrated that

intestinal protein synthesis rates rise in various pathological conditions in human adults, e.g. benign and malign colorectal tumours, inflammatory bowel disease, and following surgical intervention.[43-45] Taken these factors as much as possible into account, we assume that infants with a congenital intestinal atresia who underwent bowel resection with the creation of an enterostomy are the best available controls.

Study design

Intestinal biopsies and blood samples

For several studies addressed in this thesis an adjustment of the current study design would be imperative. For instance, to evaluate the process of intestinal adaptation following neonatal bowel resection (Chapter 4) more appropriate, repeated intestinal biopsies are needed. However, for obvious ethical reasons and the vulnerability of the intestine after surgical intervention, we could not sample additional intestinal tissue. This limitation certainly affected the determination of the true precursor pool enrichment (aminoacyl-tRNA-bound amino acid enrichment) indispensable for the calculation of the FSR of MUC2. We therefore used the calculated luminal threonine enrichment (Chapter 7) or the plasma threonine enrichment (Chapter 8) as reliable substitutes for the true intracellular threonine enrichment of goblet cells in human infants. As enteral and intravenous administration of threonine tracer did not coincide, evaluation of the relative contributions of dietary and systemic threonine to incorporation into small intestinal MUC2 was not feasible.

Composition of enteral feeding

Another confounding factor in studying the absorption capacities and MUC2 synthesis rates might be varying composition of enteral feeding, because it was not standardised among the infants studied. Amino acid absorption is more rapid and efficient when the infant is given short peptides rather than free amino acids.[46, 47] In addition to the quality of the protein, digestion and absorption vary according to the type of ingested dietary protein. As early as the early 1980s it was shown that alpha-lactalbumin (whey) is better absorbed than casein.[48] Furthermore, Boirie *et al*. found that amino acids derived from a high-casein diet are slowly released by the gut compared to amino acids derived from whey diets.[49] In our study we only used formula feeding with a whey-casein ratio of 60:40, a ratio deduced from human breast milk.[50] However, whey from human milk contains vital substances such as alpha-lactalbumin, lactoferrin, immunoglobulins, albumin, defensins (e.g. lysozyme), growth factors, and hormones, which are not added to infant formulas. These factors might influence epithelial functions such as nutrient absorption and mucosal protection and defence; therefore, we recommend delineating the possible effects of different infant formulas and human milk on specific epithelial functions.

FUTURE PERSPECTIVES

Although the studies described in this thesis undeniably gave more insight into intestinal (epithelial) functions mainly in infants after neonatal bowel resection, there are still several issues for future studies, which are discussed below.

Intervention studies

Although many studies investigating intestinal adaptation have evaluated the ability of the intestine to adapt after small bowel resection, there are still only few objective parameters monitoring the effect of novel therapeutic regimens on intestinal functions. We therefore introduced the FSR of MUC2 as a potential parameter in studying intestinal barrier function. After establishing normal values, various nutritional intervention studies may be performed to evaluate nutritional effects on gut barrier function by determining MUC2 synthesis rates. First, adding non-digestible carbohydrates, usually oligosaccharides (prebiotics) or live microbial (bacteria or yeast) food supplement (probiotics) to infant formulas to improve intestinal flora of formula-fed infants can be considered as a major innovation. In human premature infants, reduced incidence and severity of NEC was associated with the enteral administration of probiotics.[51] Although there is evidence that indigestible substrates can increase small intestinal and colonic mucin secretion to a variable extent *in vivo*[52] and that certain probiotic bacteria stimulate mucin secretion by intestinal epithelial cells *in vitro*[53], their possible effects on mucin synthesis in human infants are not known. Furthermore, several questions still need to be addressed to understand when and how to use prebiotics and/or probiotics, the optimal strain(s), timing, dosage and duration of their supplementation in relation to specific intestinal diseases. Second, nutritional intervention studies may be performed using diets supplemented with different concentrations of a specific nutrient. As threonine is the prominent indispensable amino acid in the peptide backbone of MUC2, it might be worthwhile to monitor the MUC2 synthesis rate, and thus gut barrier function, as a function of dietary threonine supply in human infants. Other examples for future research related to nutritional support are the influences of different feeding modes (formula versus breast milk), routes of feeding, colonisation of pathogenic bacteria, and medications on gut barrier function measured by MUC2 synthesis rates.

Optimal nutritional support

Limited research concerning the nutritional requirements after surgical intervention in human infants has been performed to date. Studies in adult surgical patients have shown that operative stress causes an overall increase of energy expenditure and catabolism.[54, 55] In contrast, Powis *et al*. showed no significant differences in whole-body protein flux, amino acid oxidation, and protein degradation between the pre-operative and post-operative period in infants and young children who had undergone major abdominal operations.[56] A metabolic pilot study in septic infants with NEC also demonstrated no increase in whole-body protein turnover, synthesis and catabolism.[57] Moreover, a state of relative amino acid deficiency has been postulated in infants with NEC.[58] It is therefore speculated that infants divert their available amino acids preferably to (local) wound healing and repair rather

than whole-body growth.[57] This might explain the lack of growth commonly observed in infants with critical illnesses or sepsis. As the metabolic response to stress-related clinical conditions in infants differs from that in adults, it is not possible to translate adult nutritional recommendations to neonates. Therefore, further studies are needed to investigate this metabolic response in infants in order to design appropriate nutritional support.

More specifically, the intestinal demand of specific amino acids might be increased after intestinal surgical intervention in neonates. For example, intestinal threonine utilisation rates could be expected to be higher after bowel resection to restore its barrier function by synthesising more MUC2. Special roles for glutamine, glutamate and aspartate have also been suggested, because they are utilised in high amounts by the intestine and are the major energy sources for the small intestine.[34-36] Furthermore, other studies have attempted to support the concept that glutamine supplementation should be beneficial to human adults and infants with GI diseases and their often associated surgical interventions; however, no conclusive evidence has been obtained thus far.[12, 59] Another nutritional important amino acid is arginine, which is essential for nitric oxide synthesis. Nitric oxide is an important regulator of vasomotor function in the intestine[60], it acts as a neurotransmitter to regulate peristalsis[61] and it is critical for intestinal defence[62]. Therefore, it has been suggested that arginine supplementation in preterm infants may help in the prevention of NEC by promoting nitric oxide synthesis; nevertheless, the current data are insufficient to support a firm recommendation at this time.[63] Moreover, it is known that preterm infants have a relative arginine deficiency, which results in hyperammonaemia and cardiovascular, neurological, and intestinal dysfunction. The recommended minimum and maximum concentrations of arginine in formulas for preterm infant are known[64]; however, arginine requirements for stressed infants are lacking.

Thus, overall, more knowledge of intestinal amino acid requirements is needed to develop new enteral amino acid solutions to optimise neonates' nutrition and health, especially those with a compromised gut.

Nutritional assessment

Although we did not aim to study the nutritional status of infants who underwent bowel resection in the neonatal period, nutritional status is a pivotal topic in the follow-up of these infants. Extensive bowel resection is associated with a spectrum of malnutrition resulting from inadequate bowel length. More specifically, protein-energy malnutrition in infants is associated with poor growth and reduced or delayed mental and psychomotor development.[65-68] The study described in chapter 2 showed that after bowel resection the residual small intestine is still capable to absorb dietary proteins. However, the question remains whether infants who underwent neonatal bowel resection with the creation of a temporary entero- or colostomy are adequately fed. Therefore, indirect calorimetry measurements might be necessary to establish the individual minimal energy

requirements in those infants. Furthermore, it is of utmost importance to carefully monitor long-term nutritional status and growth of these infants. Therefore, two years ago, an interdisciplinary bowel failure outpatient clinic was instituted guiding 10 patients with a different level of parenteral nutrition at home. Most children show normal growth pattern, experience normal pubertal development and achieve normal adult size.[6] However, some may experience growth failure after weaning off parenteral nutrition and need resumption of parenteral nutritional support, especially during puberty.[6] Several non-invasive methods, e.g. anthropometry, bio-electrical impedance analysis (BIA) and dual-energy x-ray absorptiometry (DEXA) may be worthwhile in assessing an infant's nutritional status following bowel resection. Additionally, these methods might be related to parameters of intestinal functioning, such as the biological marker citrulline.[69, 70]

CONCLUDING REMARKS

The results from our studies emphasise that the intestinal epithelium is relatively differentiated shortly after birth. However, the residual bowel of neonates after bowel resection for NEC showed a disturbed proliferation/differentiation balance resulting in epithelial dysfunction, especially with regard to those functions indispensable for digestion and absorption of dietary nutrients. During post-resection intestinal adaptation of the residual bowel, these epithelial functions are restored. Nevertheless, until more information on the quantities and activities of these epithelial functions becomes available, nutritional support after neonatal bowel resection should primarily focus on the preservation of growth. Additionally, by using stable, isotopically labelled amino acid tracers, we were able to investigate amino acid intermediate metabolism in infants with a compromised gut. Moreover, threonine tracer was used to study intestinal barrier function and to calculate the FSR of MUC2, which may serve as a parameter to evaluate possible effects of novel medical and nutritional treatments in humans after bowel resection.

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Chapter 10

Summary
Samenvatting

SUMMARY

Adequate gut development and maturation is indispensable in maintaining neonatal health. Especially, preterm infants as well as infants with a congenital intestinal anomaly are at more risk to develop a compromised gut, mainly as a consequence of physiologic gastrointestinal tract immaturity and/or specific gastrointestinal diseases and anomalies, but also because these conditions often require bowel resection. Neonatal bowel resection leads to loss of absorptive and protective surface area, often resulting in malabsorption of dietary nutrients. This in turn may lead to altered nutrient demands and feeding intolerance, which complicate meeting neonates' nutritional requirements. Neonatal care, therefore, must focus on reaching excellent condition of the newborn's, sometimes compromised, gut, i.e. providing adequate nutrient absorption, intestinal defence and intestinal barrier function to optimise growth and development.

The first part of this thesis explores different gut epithelial functions in human newborns with congenital or acquired intestinal diseases after neonatal surgical intervention. Part two next describes the intestinal fate of dietary threonine, specifically in maintaining intestinal barrier function.

In **chapter 1** we provide the background and aims of the studies presented in this thesis. We generally introduce the development of the gut and the various functions of the intestinal epithelium, such as nutrient digestion and absorption, protection, defence, and proliferation, in humans. Furthermore, influences of normal intestinal development disturbance or intestinal diseases in the foetal and/or neonatal period, which may alter epithelial cell functions, and hence intestinal functions, are discussed.

In **chapter 2** we describe the dietary feeding tolerance (>100 ml/(kg·d)) and the dietary protein absorption capacity of the small intestine in human neonates who underwent intestinal surgery in the neonatal period. Infants (n=14) reached enteral intake exceeding 100 ml/(kg·d) at a median of 17 (8–32) days following bowel resection. In 17 infants, we determined protein levels in the small intestinal outflow fluid collected at the level of the enterostomy weekly for 24–48 hours during weeks 3 through 6 post-operatively. Overall, the study showed that the small intestine is responsible for 70–90% of the dietary protein absorption in these infants. As neonates with a jejunostomy showed mean protein absorptive capacity around 80% over the 4-wk post-operative period, it seems that large amounts of dietary milk or formula proteins are completely digested and absorbed before food intake reaches the last segment of the small intestine. As the neonates did not receive a hydrolysed or elemental formula, our results do not support the use of such formula to improve amino acid uptake in infants with an enterostomy.

In **chapter 3**, we retrospectively investigated the epithelial cell type-specific protein expression, proximal and distal to jejunal and ileal atresias, in human newborns (n=16). As the congenital obstruction would prevent access of luminal components (e.g. amniotic fluid

(before birth) and/or enteral nutrition (after birth)) that may stimulate intestinal development, the bowel distal to the atresia may be immature. We found that the enterocyte-specific markers: lactase, sucrase-isomaltase, sodium glucose cotransporter 1, glucose transporters 2 and 5 (Glut2 and 5), intestinal fatty acid binding protein (i-FABP) and alkaline phosphatase, the goblet cell-specific markers: mucin 2 (MUC2) and trefoil factor 3 (TFF3), and Paneth cell marker lysozyme were expressed at a mean 3 ± 1 days after birth, both proximal and distal to jejunal and ileal atresias. This indicates that the human small intestinal epithelium is already differentiated shortly after birth and that the absence of intestinal continuity does not affect epithelial protein expression. Thus in humans the developing small intestinal epithelium seems to mature independently of luminal components. These results highlight that epithelial protein expression, which is crucial to nutrient absorption, epithelial defence and repair in the small intestine, is genetically imprinted, implying the presence of indispensable ontogenetic factors.

In line with the previous study, **chapter 4** evaluates epithelial cell-type-specific functions of the residual bowel after resection and consecutive stoma closure for the most devastating acquired intestinal disease in human preterm infants, necrotising enterocolitis (NEC). The residual small bowel or colon of human neonates (n=21) operated on for acute NEC was characterised by severe and mild epithelial damage, varying from epithelial loss to fairly unaffected epithelium. We found that the proliferation/differentiation balance of the residual bowel in infants with acute NEC was disturbed. To be precise, epithelial proliferation was increased in acute NEC and distinct enterocyte-specific functions (lactase and Glut2 and 5 expression) were down-regulated, whereas SI and i-FABP expressions were maintained in severely affected areas. In contrast, in areas with mild epithelial damage, lactase and Glut2 and 5 protein expression was not or only slightly affected. Furthermore, acute NEC may be associated with reduced MUC2, TFF3 and lysozyme expression levels leading to impaired epithelial barrier function and defence. Nevertheless, infants recovering from NEC showed completely re-established epithelial functions, such as nutrient digestion and absorption, intestinal barrier function, and innate defence, at least at the time of stoma closure (approximately 10 weeks since the initial surgery).

After studying various intestinal epithelial functions by analysing cell type-specific protein expression, the second part of this thesis was focussed on the absorption capacity and intestinal barrier function by studying first-pass utilisation and metabolism of the indispensable amino acid threonine in animals and human neonates.

In **chapter 5**, we describe the utilisation of systemic threonine by the portal-drained viscera (PDV) in 4-week-old neonatal pigs during a normal (NP) and a low protein (LP) intake. First, the study demonstrated that during protein restriction the PDV maintains a high rate of metabolism and continues to utilise a very high amount of dietary threonine during the first period of feeding. For example, two-thirds of the utilised threonine was sequestered by the PDV from the systemic circulation in NP-fed piglets and moreover, systemic threonine utilisation was significantly lowered during protein restriction. Notably, the equivalent of

85% of the total threonine intake was utilised in the PDV in piglets fed a LP diet, whereas 91% of the threonine intake was utilised in piglets fed a NP diet ($p>0.05$). Second, most dietary threonine was taken up in the proximal jejunum under either feeding regimens. Both dietary and systemic threonine were incorporated into intestinal proteins, however, the level of protein intake affects the site of threonine utilisation by the PDV, as it switches from dual threonine use (i.e. dietary and systemic) to predominantly luminal utilisation of threonine during protein restriction. Finally, catabolism is a minor metabolic fate of threonine in the intestine, because threonine oxidation represents only 2–9% (NP and LP diet, respectively) of the total threonine utilised by the intestine. Furthermore, systemic threonine oxidation by the PDV represents 13% of the whole-body threonine oxidation. The findings from this chapter indicate that the intestine has a pivotal role in modulating dietary amino acid availability to the body and point to the obligatory requirement of threonine for maintaining intestinal integrity.

As the intestinal mucin Muc2 is characterised by tandem repeats in its peptide backbone, which is rich in the amino acids threonine, proline and serine, the high intestinal threonine incorporation (**Chapter 5**) may reflect Muc2 synthesis in intestinal goblet cells. Therefore, in **chapter 6**, we used Muc2 knockout ($Muc2^{-/-}$) and wild type ($Muc2^{+/+}$) mice to directly address the question whether dietary threonine is mainly utilised for intestinal Muc2 synthesis. The study revealed that one-fourth of total administered dietary threonine was incorporated into proteins along the whole intestine, in both $Muc2^{+/+}$- and $Muc2^{-/-}$ mice. Most dietary threonine recovered from the intestinal tract was detected in the colonic content in both mice types. However, 27.4% of recovered dietary threonine in the colonic content of $Muc2^{+/+}$ mice was incorporated into Muc2. Overall, $Muc2^{-/-}$ mice showed higher amounts of incorporated threonine into mucosal proteins. Finally, the entire intestine of the $Muc2^{-/-}$ mice showed a significantly higher threonine oxidation rate compared to $Muc2^{+/+}$ mice. Thus, the findings of this study confirm that one of the metabolic fates of dietary threonine utilised by the intestine is incorporation into Muc2, whereas intestinal threonine oxidation plays a minor role.

In **chapter 7**, we describe a novel tracer method to determine the role of dietary threonine in small intestinal MUC2 synthesis in human neonates following bowel resection. The study was performed approximately 4 weeks (4 ± 1 wk) after bowel resection. Then, a continuous 12-hour (h) infusion of threonine tracer was administered enterally by feeding tube. The incorporation of dietary threonine was determined in secreted MUC2 isolated from the outflow fluid (collected during 24–48 h) of 5 premature neonates with an enterostomy following bowel resection for NEC. Dietary threonine was incorporated into small intestinal MUC2. Furthermore, the Fractional Synthetic Rate (FSR) of MUC2 ranged from 12.1–89.7% per day (%/d). Despite its wide range, we suggest that the FSR of MUC2 might be a potential parameter for monitoring intestinal barrier function *in vivo*.

As in **chapter 7** the threonine tracer was administered enterally, we could not verify whether threonine used for small intestinal MUC2 synthesis in human neonates was derived directly

from the diet, or after intestinal absorption, and thus from the systemic circulation. Therefore, **chapter 8** reports the incorporation of systemically derived threonine into small intestinal MUC2. The study – in 7 human preterm neonates following bowel resection for NEC – showed that even during enteral feeding the intestine utilises systemically derived threonine for MUC2 synthesis in the small intestine. It seems therefore likely that dietary threonine is preferentially taken up by enterocytes and is used for constitutive protein synthesis, whereas goblet cells might extract threonine from the systemic site and use it for intestinal MUC2 synthesis. Median FSR of small intestinal MUC2 was 67.2%/d (ranging from 44.3 to 103.9%/d). Compared to the synthesis rates of other proteins in human adults, such as albumin and skeletal muscle protein (6%/d and 25%/d, respectively), the small intestinal MUC2 synthesis rate is notably high in preterm infants and is among the highest so far determined in humans.

In **chapter 9**, we discuss our findings and possible limitations of the chosen study design and study population. Furthermore, we make recommendations for future studies.

The main conclusions obtained from the studies described in this thesis are the following:

- The dietary protein absorption capacity of the remaining small intestine in human neonates after intestinal surgical intervention is intact, which does not support the use of hydrolysed or elemental feeding in infants with an enterostomy.

- Epithelial proteins involved in nutrient digestion and absorption, epithelial protection, defence, and repair are expressed in the human small intestine shortly after birth and are genetically programmed.

- Epithelial protein expression – crucial for nutrient digestion and absorption, epithelial protection, defence, and repair – is completely restored in the residual bowel of human infants with NEC-induced epithelial damage at the time of stoma closure (± 10 weeks post-initial surgery).

- High amounts of the essential amino acid threonine are utilised by the intestine, and this reflects one of its metabolic fates; incorporation into intestinal MUC2.

- Dietary threonine is used for intestinal MUC2 synthesis, and its small intestinal synthesis rate is very high in human preterm infants with an enterostomy.

- The FSR of small intestinal MUC2 may be used as an objective parameter in monitoring intestinal barrier function, however, more studies are needed to justify its usefulness.

SAMENVATTING

Adequate ontwikkeling van de darm is van essentieel belang voor de gezondheid van de pasgeborene. Prematuur geboren kinderen en kinderen met een aangeboren darmafwijking hebben echter een groter risico op een verminderde darmfunctie, bijvoorbeeld ten gevolge van de fysiologische onrijpheid van het maagdarmkanaal, de aanwezigheid van specifieke ziekten en/of afwijkingen van het maagdarmkanaal en de eventueel daaropvolgende darmresectie (= verwijderen van het zieke gedeelte van de darm). Neonatale darmresectie leidt tot het verlies van absorberend oppervlak. Dit kan resulteren in malabsorptie van voedingsstoffen, voedingsintolerantie en een veranderde voedingsbehoefte. De zorg voor deze zieke pasgeborenen moet zich daarom richten op het bereiken van een goede conditie van de darm, dat wil zeggen een adequate absorptie van voedingsstoffen, goede afweer en barrièrefunctie van de darm om de groei en ontwikkeling van de pasgeborene te optimaliseren.

Het eerste gedeelte van dit proefschrift beschrijft verschillende functies van het darmepitheel in pasgeborenen met een aangeboren of een verworven darmziekte, die zijn geopereerd in de neonatale periode. Deel 2 beschrijft de functie van het essentiële aminozuur threonine in de darm en in het bijzonder met betrekking tot het onderhouden van de barrièrefunctie van de darm.

Hoofdstuk 1 schetst de achtergrond van het onderzoek en zet de doelen van de in dit proefschrift beschreven studies nader uiteen. De ontwikkeling van de darm en diverse functies van het darmepitheel, zoals digestie en absorptie van voedingsstoffen, bescherming, afweer en proliferatie, worden beschreven. Vervolgens worden de mogelijke invloeden beschreven van een verstoorde darmontwikkeling of van darmziekten gedurende de foetale en neonatale periode, die veranderde celfuncties van het darmepitheel tot gevolg kunnen hebben en dus een veranderde functie van de darm.

Hoofdstuk 2 beschrijft de voedingstolerantie (>100 ml/(kg·d)) van enterale voeding en de eiwitabsorptiecapaciteit van de dunne darm in pasgeborenen die in de neonatale periode een darmoperatie met aansluitend de aanleg van een (tijdelijk) dunnedarmstoma hebben ondergaan. De onderzochte kinderen (n=14) bereikten een enterale voedingsinname van meer dan 100 ml/(kg·d) op mediaan dag 17 (8-32) na de darmresectie. Om de eiwitabsorptiecapaciteit van de dunne darm te bepalen, werd in 17 kinderen de totale hoeveelheid uitgescheiden eiwit in de ontlasting bepaald. Ontlasting werd verzameld op het niveau van het dunnedarmstoma gedurende 24-48 uur in week 3 tot en met week 6 na de darmoperatie. Uit het onderzoek is gebleken dat de dunne darm verantwoordelijk is voor 70-90% van de totale eiwitabsorptie. De neonaten met een jejunostoma hadden tevens een gemiddelde eiwitabsorptiecapaciteit van rond de 80%, gemeten over de post-operatieve periode (week 3 tot en met week 6). Dit betekent dat grote hoeveelheden moedermelk of eiwitten afkomstig van formula-voeding volledig worden verteerd en opgenomen door de darm voordat het laatste segment van de dunne darm is bereikt. Omdat de onderzochte

kinderen geen gehydrolyseerde of elementaire voeding kregen, suggereren onze resultaten dat dergelijke voeding niet nodig is om de opname van aminozuren bij kinderen met een dunnedarmstoma te bevorderen.

In **hoofdstuk 3** is retrospectief onderzoek verricht naar de celspecifieke eiwitexpressie van het darmepitheel, proximaal en distaal van een jejunum- of een ileumatresie (= aangeboren obstructie van de darm) in 16 pasgeboren kinderen. Omdat een dergelijke aangeboren darmobstructie de toevoer van luminale componenten zoals vruchtwater (voor de geboorte) en/of enterale voeding (na de geboorte), die mogelijk de darmontwikkeling stimuleren, voorkomt, zou de darm distaal van de obstructie onrijp kunnen zijn. Onze studie toonde aan dat de enterocyt-specifieke markers: lactase, sucrase-isomaltase, natrium glucose cotransporter 1, glucose transporters 2 and 5 (Glut2 en Glut5), vetzuur bindend eiwit (i-FABP) en alkalische fosfatase; de gobletcel-specifieke markers: mucin 2 (MUC2) en trefoil factor 3 (TFF3) en de Paneth cel marker lysozym tot expressie kwamen gemiddeld 3 ± 1 dagen na de geboorte, zowel proximaal als distaal van jejunum- en ileumatresieën. Dit betekent dat het epitheel van de dunne darm al is gedifferentieerd kort na de geboorte en dat de afwezigheid van de darmcontinuïteit geen effect heeft op de eiwitexpressie van het darmepitheel. Dus, humaan dunne darmepitheel ontwikkelt zich onafhankelijk van luminale componenten. Deze resultaten benadrukken dat de eiwitexpressie van het darmepitheel, belangrijk voor de absorptie van voedingsstoffen, epitheliale afweer en herstel van de dunne darm, genetisch is vastgelegd. Dit impliceert de aanwezigheid van essentiële ontogenetische factoren.

In overeenstemming met de vorige studie, beschrijft **hoofdstuk 4** de epitheliale celspecifieke functies van de resterende darm na resectie en opeenvolgende sluiting van het stoma bij pasgeboren kinderen met een ernstige verworven darmziekte, namelijk necrotiserende enterocolitis (NEC). De resterende dunne en dikke darm van 21 kinderen die geopereerd werden in de acute fase van NEC wordt gekenmerkt door ernstige tot milde epitheliale schade. De proliferatie/differentiatie balans van het epitheel in de resterende darm is verstoord gedurende de acute fase van NEC. De epitheliale proliferatie is toegenomen en enkele enterocyt-specifieke functies, bijvoorbeeld de expressie van de eiwitten lactase, Glut2 en Glut5, is in de acute fase verminderd. De expressie van de eiwitten SI en i-FABP blijft echter gehandhaafd, zelfs in zeer ernstig beschadigd darmweefsel. In darmweefsel met milde epitheliale schade blijft de expressie van lactase en Glut2 en Glut5 redelijk behouden. Tevens wordt de acute fase van NEC geassocieerd met een verminderde expressie van MUC2, TFF3 en lysozym. Dit zou kunnen leiden tot een verminderde epitheliale barrièrefunctie en afweer. Desalniettemin zijn de epitheliale functies zoals de expressie van eiwitten belangrijk voor vertering en absorptie van voedingsstoffen, de barrièrefunctie en afweer van de darm volledig hersteld ten tijde van de sluiting van het stoma (ongeveer 10 weken na de eerste darmoperatie).

Na het bestuderen van diverse epitheliale functies van de darm door de analyse van celspecifieke eiwitexpressie, richt deel 2 van dit proefschrift zich op de absorptiecapaciteit en de barrièrefunctie van de darm door het bestuderen van het first-pass verbruik en metabolisme van het essentiële aminozuur threonine in zowel dieren als pasgeboren kinderen.

In **hoofdstuk 5** beschrijven we het verbruik van het essentiële aminozuur threonine, afkomstig uit de bloedcirculatie, door de 'portal drained viscera' (PDV = maag, darmen, milt en alvleesklier) in pasgeboren biggen met een normale of beperkte eiwitinname. Ten eerste toont de studie dat gedurende een eiwitbeperkt dieet de PDV een hoog metabolisme behoudt en dat het grote verbruik van threonine wordt gecontinueerd. Tweederde van de threonine, opgenomen door de PDV, is afkomstig uit de bloedcirculatie in neonatale biggen die gevoed worden met een normaal eiwithoudende voeding. De opname van threonine uit de bloedcirculatie is echter significant verlaagd gedurende een eiwitbeperkt dieet. Vijfentachtig procent van de totale threonine inname wordt gebruikt in de PDV in biggen die een eiwitbeperkt dieet krijgen, terwijl 91% van de threonine inname wordt gebruikt in biggen met een normaal eiwithoudende voeding ($p>0.05$). Ten tweede wordt threonine uit het dieet voornamelijk opgenomen in het proximale jejunum onder beide voedingsomstandigheden. Zowel threonine opgenomen vanuit de bloedcirculatie als threonine direct afkomstig uit het dieet wordt ingebouwd in eiwitten van de darm. Tijdens een normale eiwitinname wordt threonine echter zowel vanuit de bloedcirculatie als vanuit het dieet opgenomen, terwijl dit verandert naar overwegend threonine uit het dieet gedurende een eiwitbeperkte voeding. Ten slotte bedraagt de oxidatie (=verbranding) van threonine in de darm slechts 2-9% van de totaal verbruikte hoeveelheid threonine in de darm. De resultaten van dit hoofdstuk impliceren dat de darm een belangrijke rol speelt in de uiteindelijke beschikbaarheid van aminozuren voor de rest van het lichaam. Tevens laat dit zien dat threonine belangrijk is om de integriteit van de darm te behouden.

Het eiwit Muc2 in de darm wordt gekenmerkt door tandem repeats van de aminozuren threonine, proline en serine. De grote hoeveelheden threonine die worden ingebouwd in eiwitten van de darm (**hoofdstuk 5**) kunnen de Muc2 synthese in de gobletcellen van de darm weerspiegelen. In **hoofdstuk 6** wordt gebruik gemaakt van de Muc2 knock out ($Muc2^{-/-}$) en wild type ($Muc2^{+/+}$) muizen om te bestuderen of threonine uit het dieet direct wordt gebruikt voor Muc2 synthese in de darm. De studie laat zien dat een kwart van de totale enterale threonine inname wordt ingebouwd in eiwitten van de darm, zowel in $Muc2^{-/-}$ als in $Muc2^{+/+}$ muizen. De grootste hoeveelheid threonine wordt teruggevonden in de ontlasting in beide typen muizen, verzamelt vanuit het lumen van het colon. Van deze teruggevonden threonine in $Muc2^{+/+}$ muizen wordt echter, 27.4% gebruikt voor de inbouw in Muc2. In $Muc2^{-/-}$ muizen wordt threonine voornamelijk ingebouwd in de mucosale eiwitten van de darm. Tevens laat de gehele darm van de $Muc2^{-/-}$ muizen een significant hogere threonine oxidatie zien vergeleken met de $Muc2^{+/+}$ muizen. Concluderend, de resultaten van dit onderzoek bevestigen dat threonine uit het dieet wordt gebruikt voor Muc2 synthese in de darm en dat oxidatie van threonine een ondergeschiktere rol speelt.

In **hoofdstuk 7** wordt een nieuwe tracermethode beschreven om te bepalen welke rol threonine uit het dieet heeft in de MUC2 synthese in de dunne darm bij kinderen die een darmresectie hebben ondergaan. De studie werd uitgevoerd ongeveer 4 weken (gemiddeld 4 ± 1 week) nadat de darmresectie had plaatsgevonden. Vervolgens werd gedurende 12 uur een infuus met threoninetracer gegeven via de maagsonde. De ontlasting uit het stoma werd gedurende 24-48 uur verzameld van 5 prematuur geboren kinderen met een dunnedarmstoma, die een darmresectie hadden ondergaan voor NEC. De Fractionele Synthesesnelheid (FSR) van MUC2 varieert van 12.1-89.7% per dag (%/d). Ondanks deze variatie zou de FSR een potentiële parameter kunnen zijn om de barrière functie van de darm in vivo te vervolgen.

In de studie in **hoofdstuk 7** werd de threoninetracer enteraal gegeven. In die studie kon dus niet worden bepaald of threonine, noodzakelijk voor de MUC2 synthese in de dunne darm, direct afkomstig is uit de voeding of dat het eerst wordt opgenomen door de darm om vervolgens vanuit de bloedcirculatie weer te worden heropgenomen. **Hoofdstuk 8** beschrijft de inbouw van threonine, opgenomen vanuit de bloedcirculatie, in MUC2 van de dunne darm. Deze studie werd uitgevoerd in 7 prematuur geboren kinderen nadat zij werden geopereerd voor NEC. Dit onderzoek toont aan dat threonine, opgenomen vanuit de bloedcirculatie, wordt gebruikt voor MUC2 synthese in de dunne darm van neonaten met enterale voeding. Samen met de eerder beschreven resultaten in biggen, lijkt het er op dat threonine uit het dieet bij voorkeur direct wordt opgenomen door enterocyten en wordt gebruikt voor eiwitsynthese in de darmwand. De gobletcellen zullen echter waarschijnlijk threonine bij voorkeur vanuit de bloedcirculatie opnemen en het vervolgens gebruiken voor MUC2 synthese. De mediane FSR van MUC2 gesynthetiseerd in de dunne darm is 67.2%/d (variërend van 44.3 tot 103.9%/d). Vergeleken met de synthesesnelheden van andere lichaamseiwitten in volwassenen, zoals albumine en skeletspiereiwitten (6%/d versus 25%/d), is de synthesesnelheid van MUC2 in de dunne darm zeer hoog.

In **hoofdstuk 9** worden de resultaten en de mogelijke beperkingen van de gekozen studieopzet en -populatie besproken. Tevens worden aanbevelingen voor vervolgonderzoek gegeven.

De belangrijkste conclusies die uit de studies beschreven in dit proefschrift kunnen worden getrokken, zijn:

- De eiwitabsorptiecapaciteit van de resterende dunne darm in pasgeborenen nadat zij een darmoperatie hebben ondergaan, is intact. Het gebruik van gehydrolyseerde of elementaire formula-voeding in kinderen met een dunnedarmstoma is daarom niet vereist.

- Epitheliale eiwitten die betrokken zijn bij de digestie en absorptie van voedingsstoffen, epitheliale bescherming, afweer en herstel komen kort na de geboorte al tot expressie in de dunne darm en zijn dus genetisch bepaald.

- Ten tijde van het sluiten van het stoma is de epitheliale eiwitexpressie, cruciaal voor digestie en absorptie van voedingsstoffen, epitheliale bescherming, afweer en herstel, volledig hersteld in de resterende darm van kinderen die necrotiserende enterocolitis hebben doorgemaakt.

- Grote hoeveelheden van het essentiële aminozuur threonine worden door de darm gebruikt, onder andere voor de inbouw van threonine in MUC2 in de dunne darm.

- Threonine uit de enterale voeding wordt gebruikt voor MUC2 synthese in de darm. De synthesesnelheid van MUC2 is erg hoog in prematuur geboren kinderen met een dunnedarmstoma.

- De FSR van MUC2 bepaald in de dunne darm zou een objectieve parameter kunnen zijn om de barrièrefunctie van de darm te vervolgen. Meer studies zijn echter nodig om het gebruik van deze bepaling te rechtvaardigen.

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DANKWOORD

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Curriculum Vitae

CURRICULUM VITAE

Maaike Schaart was born on October 6th, 1978 in Dordrecht, the Netherlands. She passed her secondary school exam (VWO) at the Prins Willem Alexander College in Leerdam in 1996. In the same year she started with studying Biomedical Sciences at the University of Leiden. In 1997 she started her medical training at the Faculty of Medicine at the University of Leiden. In 2001 she participated in a project investigating the Newborn Individual Developmental Care and Assessment Program (NIDCAP) at the department of Neonatology, University of Leiden. After obtaining her medical degree in 2003, she worked as a research fellow at the department of Neonatology and department of Paediatric Surgery of the Erasmus MC – Sophia Children's Hospital in Rotterdam (supervised by Prof.dr. J.B. van Goudoever, Prof. dr. D. Tibboel and dr. I.B. Renes) working on the research presented in this thesis. From July 2004 till January 2007 she worked as a doctor at the Sanquin Blood Bank South West Region. In January 2007 she will start her clinical paediatric residency in training (AIOS) at the Reinier de Graaf Gasthuis in Delft (head: Dr. N. van der Lely) as part of her training at the Leiden University Medical Centre (LUMC) in Leiden (head: Dr. R.N. Sukhai and Prof.dr. F.J. Walther). She lives together with Jaap Teunissen.

List of publications

LIST OF PUBLICATIONS

Schaart MW, Schierbeek H, van der Schoor SR, Stoll B, Burrin DG, Reeds PJ, van Goudoever JB. Threonine utilization is high in the intestine of piglets. J Nutr. 2005 Apr;135(4):765-70.

van Goudoever JB, van der Schoor SR, Stoll B, Burrin DG, Wattimena D, Schierbeek H, **Schaart MW**, Riedijk MA, van der Lugt J. Intestinal amino acid metabolism in neonates. Nestle Nutr Workshop Ser Pediatr Program. 2006;(58):95-108.

Schaart MW, Schierbeek H, de Bruijn AC, Tibboel D, van Goudoever JB, Renes IB. A novel method to determine small intestinal barrier function in human neonates *in vivo*. Gut. 2006 Sep;55(9):1366-7.

Schaart MW, Yamanouchi T, van Nispen DJ, Raatgeep HC, van Goudoever JB de Krijger RR, Tibboel D, Einerhand AWC, Renes IB. Does small intestinal atresia affect epithelial protein expression in human newborns? J Ped Gastroenterol Nutr., 2006, in press.

Schaart MW, van der Sluis M, de Koning BAE, Schierbeek H, Velcich A, Renes IB, van Goudoever JB. Dietary threonine metabolism in the intestine of wild type and mucin 2- deficient mice. Under review.

Schaart MW, de Bruijn AC, Tibboel D, Renes IB, van Goudoever JB. Dietary protein absorption of the small intestine in human neonates. Under review.

Schaart MW, de Bruijn AC, Bouwman DM, de Krijger RR, van Goudoever JB, Tibboel D, Renes IB. Epithelial functions of the residual bowel after surgery for necrotising enterocolitis in human infants. Submitted.

Schaart MW, de Bruijn AC, Schierbeek H, Tibboel D, van Goudoever JB, Renes IB. Small intestinal MUC2 synthesis in human preterm infants. Submitted.

Appendix

A novel method to determine small intestinal barrier function in human neonates *in vivo*

(Gut 55 (9): 1366–1367, 2006)

Mucin 2 (MUC2) is the structural component of the protective mucus layer of the gastrointestinal tract, and is secreted by goblet cells.[1] MUC2 is a glycoprotein that contains high amounts of threonine and proline residues.[2, 3] Recently, we showed that almost 90% of dietary threonine is utilised by the intestine of piglets in first-pass.[4] This high visceral threonine requirement presumably reflects the high synthesis rate of MUC2. In this context, threonine might be of critical nutritional importance in maintaining good intestinal barrier function. Neonates with impaired gut function following bowel resection require adequate gut adaptation and recovery of intestinal barrier function to avoid the consequences of malabsorption of dietary nutrients and pathogenic bacterial insults. We therefore used a tracer method to study the role of dietary threonine in intestinal MUC2 synthesis and to calculate the fractional synthetic rate (FSR) of small intestinal MUC2 in human neonates as a parameter for intestinal barrier function.

Five neonates with bowel resection for necrotising enterocolitis were studied (gestational age 33 ± 1 weeks; four had an ileostomy and one had a jejunostomy). Four weeks post-operatively a continuous [U-^{13}C]threonine infusion was administered enterally by feeding tube over 12 hours (materials and methods are available as supporting material online on the Gut website at http://www.gutjnl.com/supplemental). Using triple CsCl density gradients, mucins were isolated from intestinal outflow fluid collected at three hour intervals over two days.[5] Mucin-containing fractions had a buoyant density between 1.40 and 1.55 g/ml, were stained with periodic acid/Schiff's (PAS) reagent (Figures 2A and C, page 112), had an apparent molecular weight of 550 kD, and corresponded to a peak in the hexose assay (Figures 2B and D, page 112). Western-blot analysis using PMH1, a monoclonal antibody specific for MUC2, revealed that pooled PAS-positive fractions (that is, fractions 13–15) contained MUC2 (Figure 2E, page 112).[6] GC-IRMS analysis demonstrated the presence of the threonine tracer in MUC2 isolated from the intestinal outflow fluid (Figure 3A, page 114), indicating that dietary threonine was incorporated into MUC2. Threonine enrichment rose linearly during threonine administration and gradually decreased after administration was stopped. Time to absorb threonine and incorporate threonine into MUC2, and subsequently to secrete threonine as part of MUC2 into the intestinal lumen, ranged from approximately six to 10 hours in patients with an ileostomy (Table 1). In contrast, MUC2 secretion time for the single patient with a jejunostomy was less than three hours. The linear increase of [U-^{13}C]-enrichment in MUC2 and the luminal [U-^{13}C]threonine precursor enrichment were used to calculate the FSR (see Gut website at http://www.gutjnl.com/supplemental). FSR was 12.1 – 89.7% per day (Table 1, page 109).

This is the first study which has determined small intestinal MUC2 synthesis in human neonates. FSR of mucosal proteins is 51%/day and 15–29%/day for human jejunum and ileum, respectively.[7, 8] As the FSR of MUC2 in the present study ranged from 12.1 to 89.7%/day, we conclude that the small intestinal epithelium has a high rate of MUC2 synthesis. Despite its wide range, the FSR of MUC2 might be valuable as a tool to study intestinal barrier function. Serial measurements of the FSR of MUC2 may be used to monitor intestinal adaptation, and to assess treatment and feeding options in patients following bowel resection. We consider our data as a starting point to investigate the impact of different pathological situations and interventions, such as medication and nutrition, on the FSR of MUC2, and it could be implemented as a tool to improve medical management of patients with impaired gut function.

In conclusion, the development of a tracer method to determine MUC2 synthesis and the FSR of MUC2 in the small intestine in vivo provides the opportunity to study the determinants of intestinal barrier function in a detailed manner.

Acknowledgement
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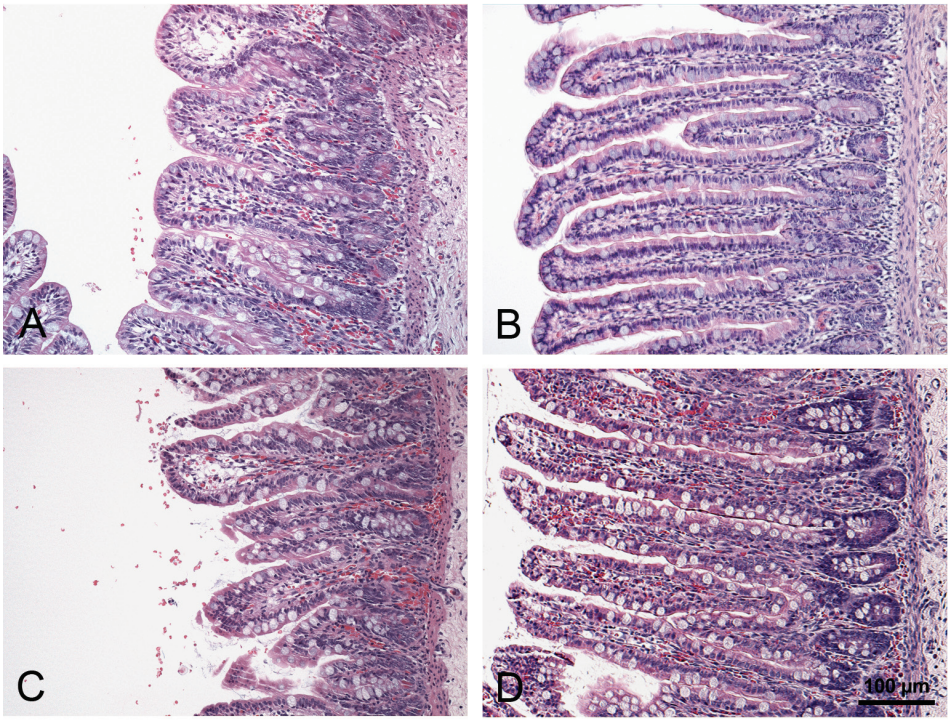

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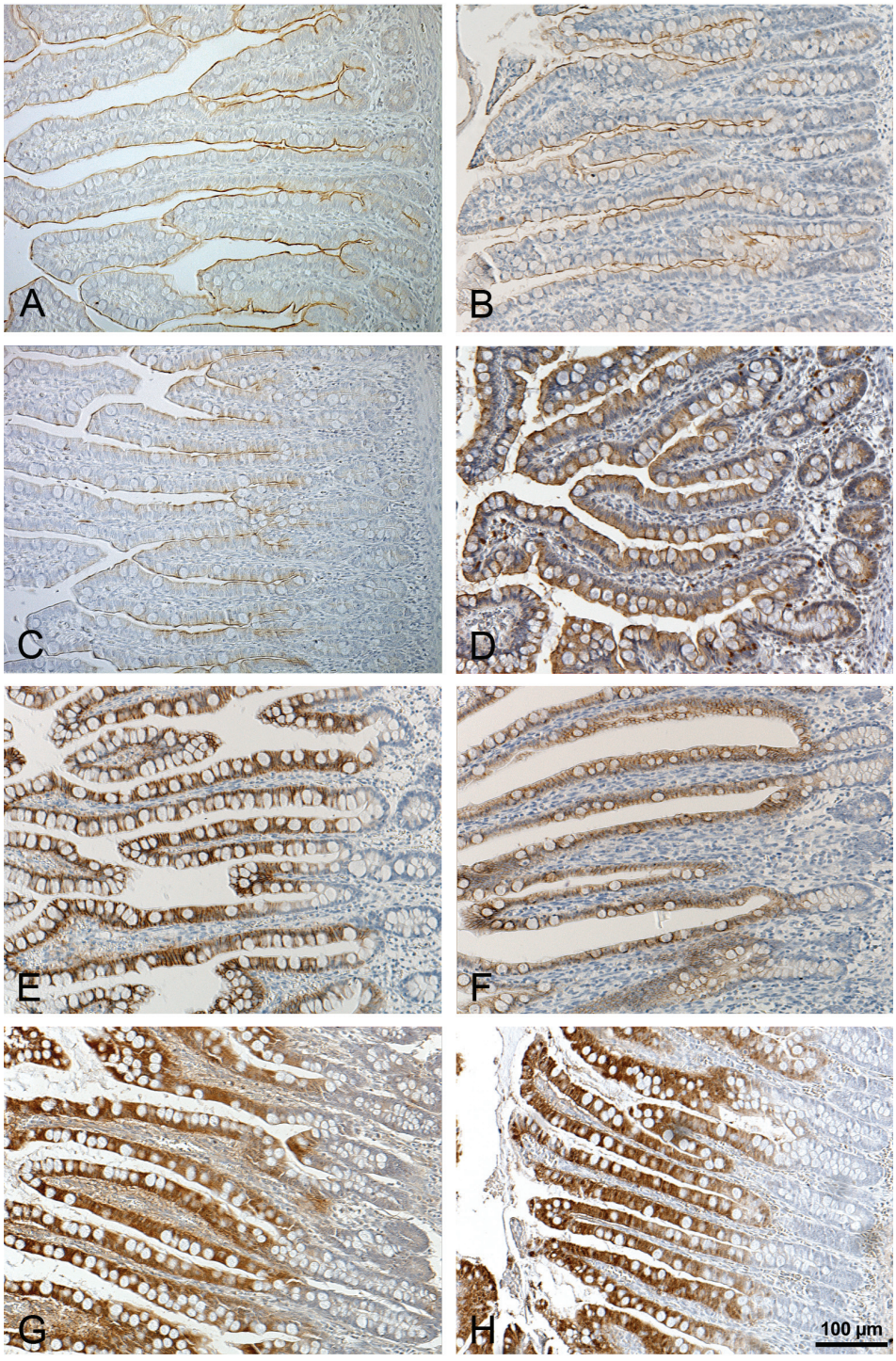

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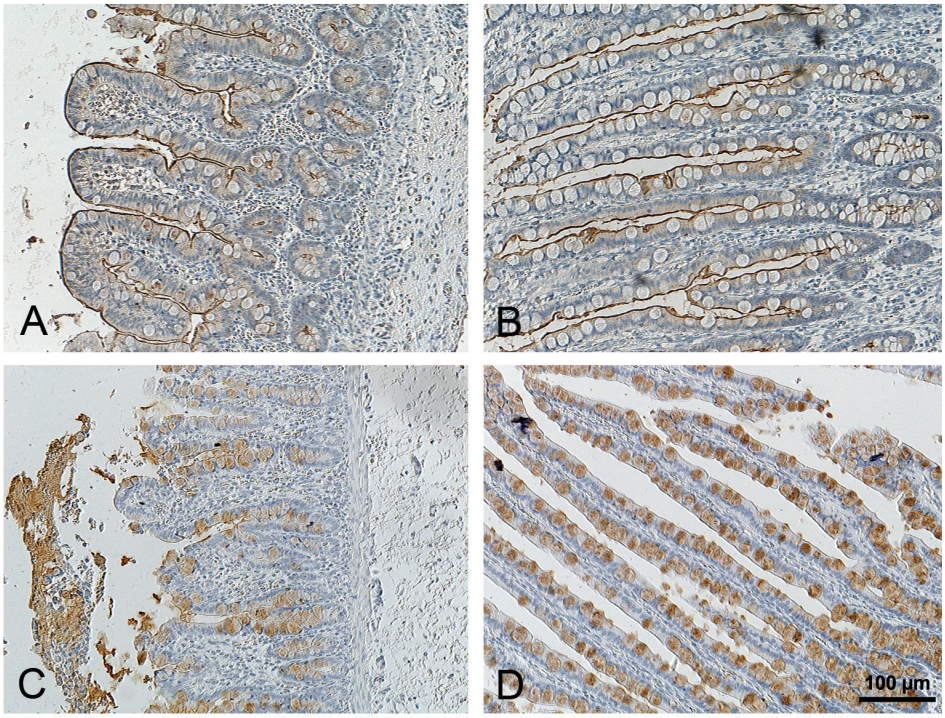

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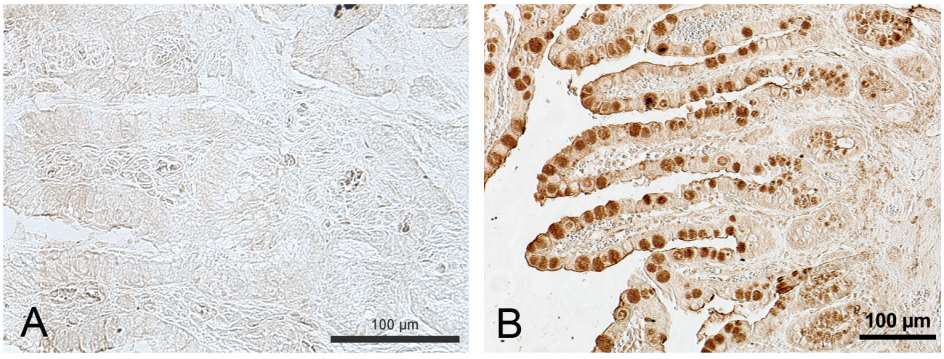

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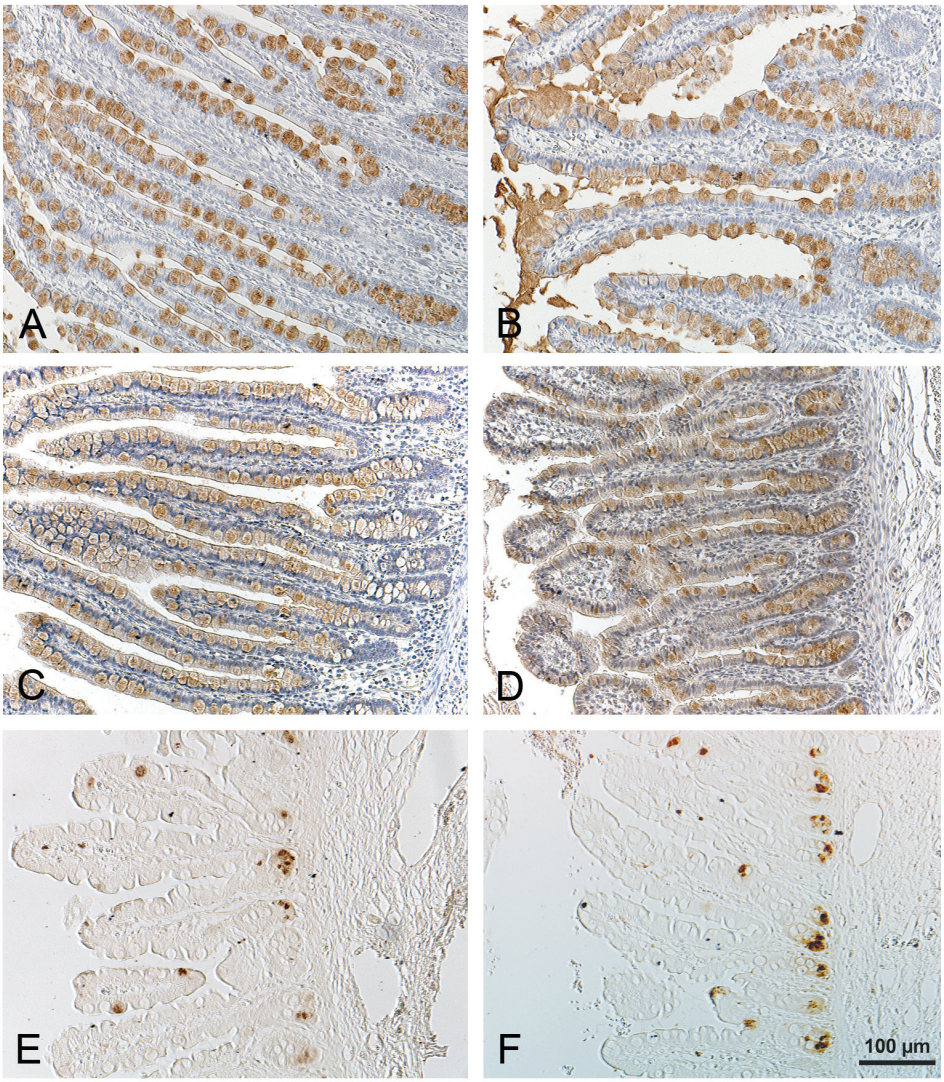

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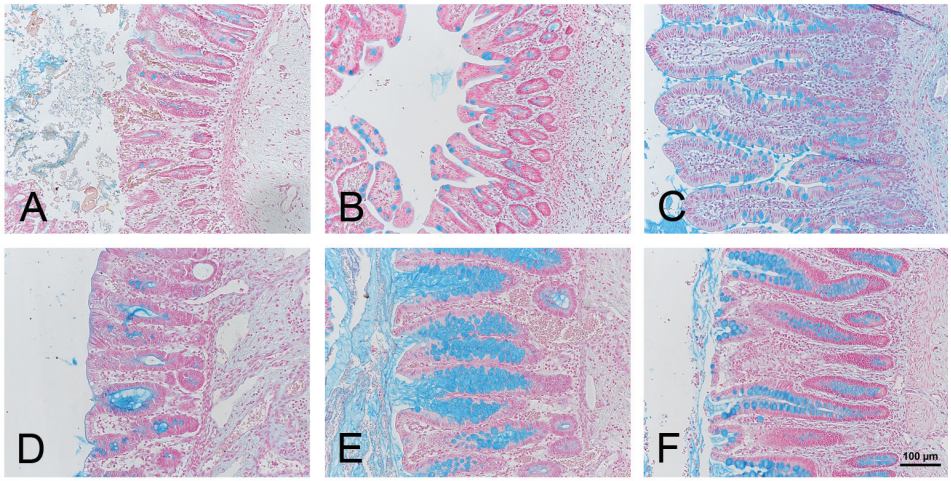

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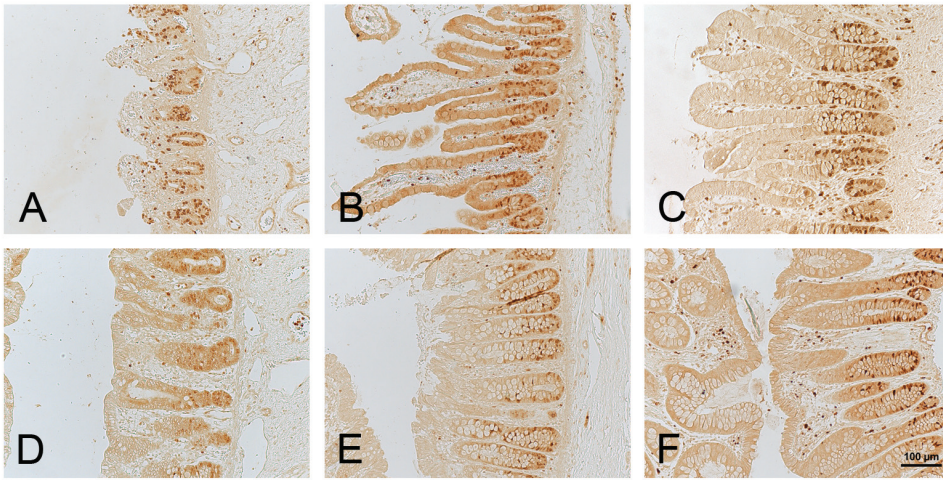

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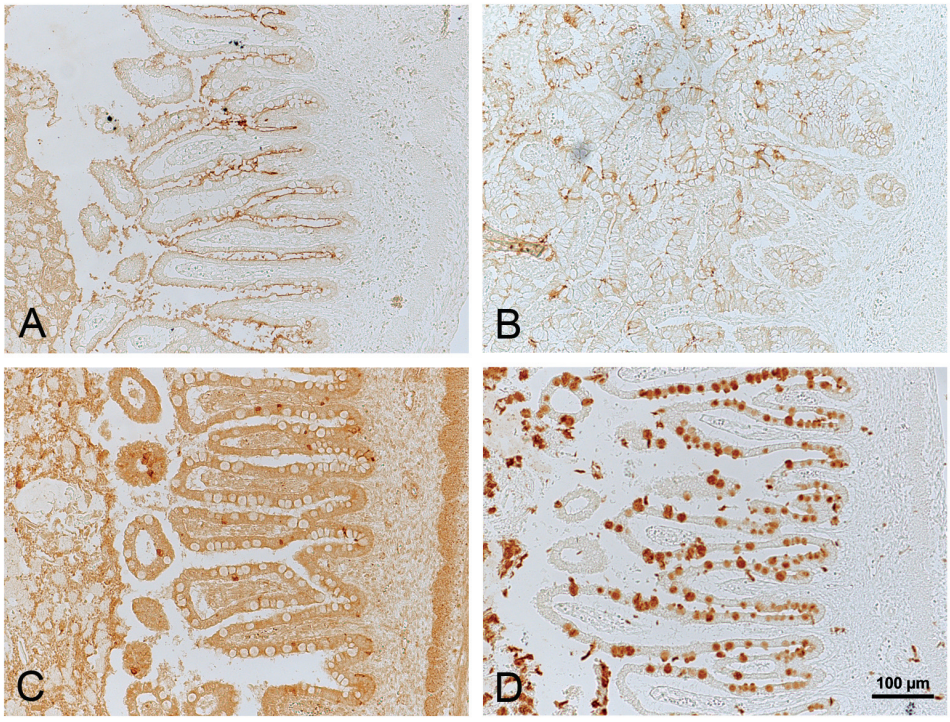

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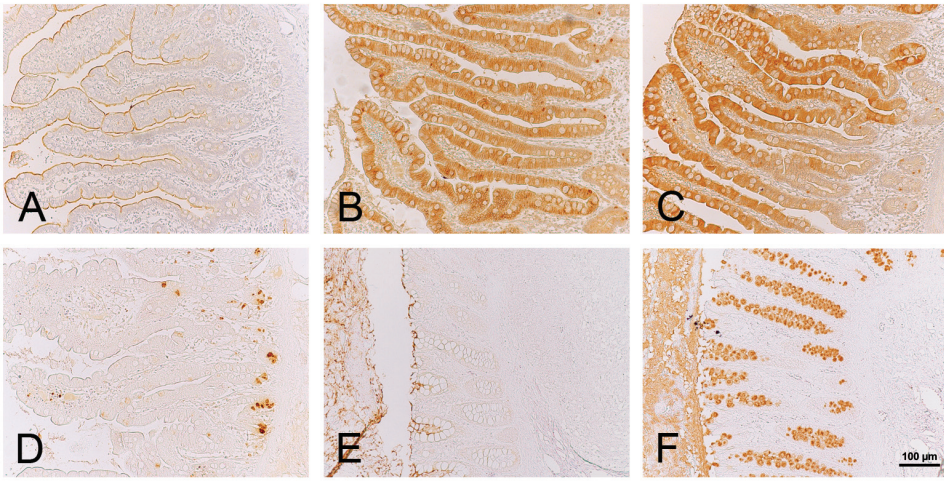

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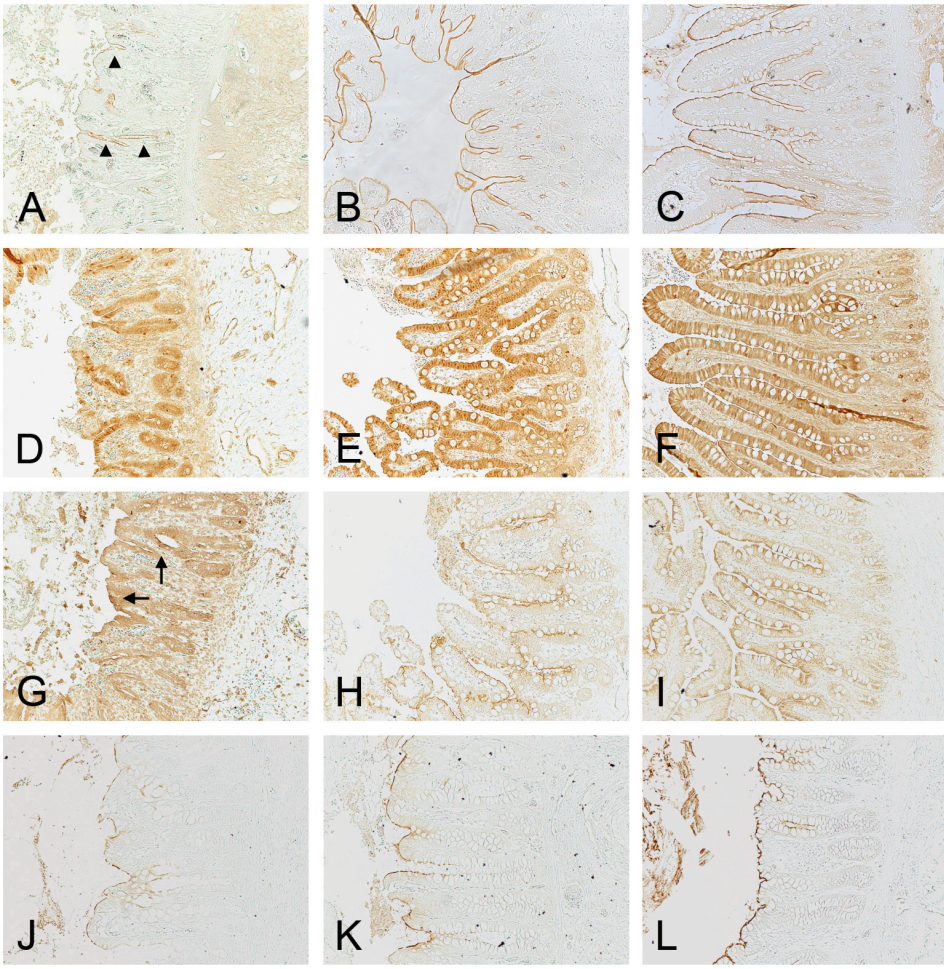

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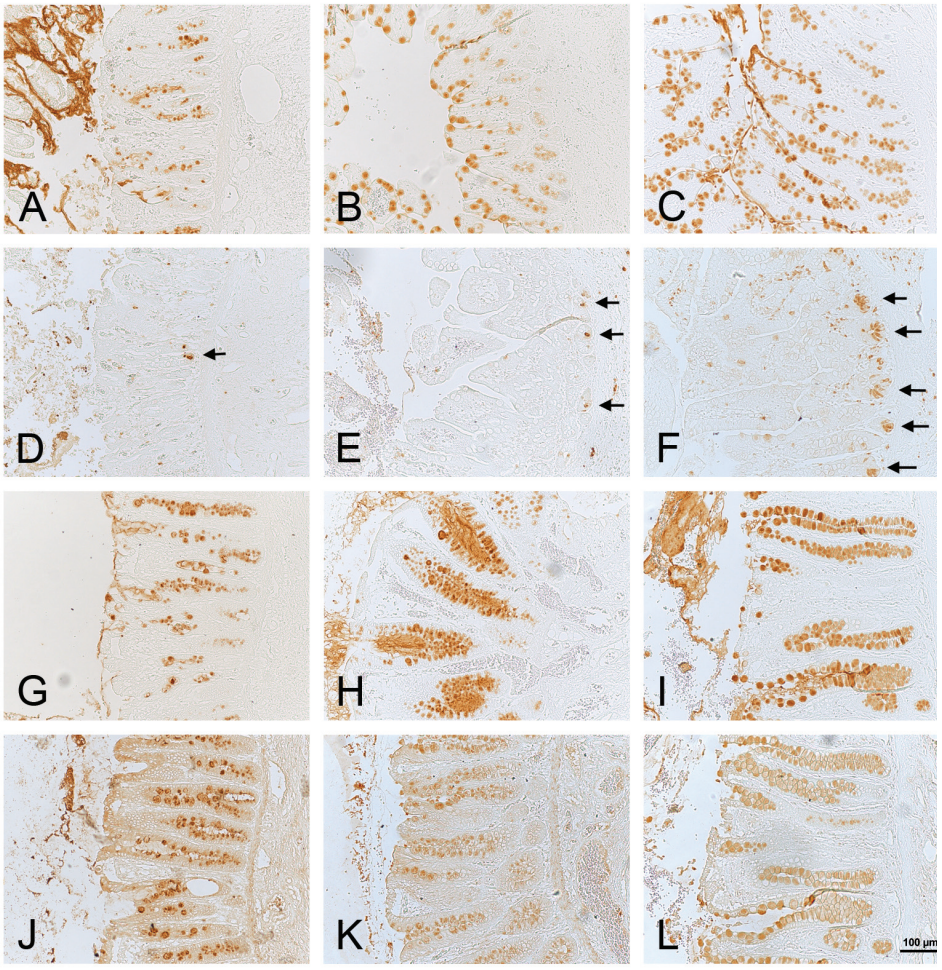

Figure 6, chapter 4