Mucin gene expression in human embryonic and fetal intestine

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Abstract

Background—The intestinal epithelium is covered by a continuous layer of mucus which is secreted by well differentiated epithelial cells. Disregulation of the expression of mucins has been reported to have possible implications in the neoplastic process which affects intestinal mucosae. It is well known that preneoplastic and neoplastic tissues can express fetal phenotypic characteristics.
Aims—To assess whether the expression of mucin genes in the intestinal tract is linked to the stage of cellular differentiation and tissue development, by studying the expression of six mucin genes in human fetal small intestine and colon, and also adult tissues.
Methods—In situ hybridisation was used to study mRNA expression of MUC2, MUC3, MUC4, MUC5B, MUC5AC, and MUC6 in 32 human embryos and fetuses (6.5–27 weeks gestation). Normal adult mucosae were used as controls.
Results—Three mucin genes, MUC2, MUC4, and MUC5AC, were differently expressed in fetal intestine compared with expression in normal adults.
Conclusion—These differences in mucin gene expression suggest a possible regulatory role for these products in intestinal epithelial cell differentiation.
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The intestinal epithelium is covered by a continuous layer of mucus, which is primarily secreted by epithelial goblet cells. This mucus layer has both protective and lubricative functions, protecting the mucosa from dehydration and mechanical damage during the motile process of digestion. Mucus also provides a physical barrier between the underlying epithelium and luminal contents, such as pathogenic bacteria and toxins, viruses, and parasites.[1][2] It also protects the epithelium from attack by pancreatic digestive enzymes and bacterial proteases. Nevertheless, mucus is permeable to low molecular weight components and this property is important for the intestinal absorption of nutrients. In fetal intestine, mucus secretion begins as early as 10 weeks after gestation.[3] This affords protection against bile and swallowed amniotic fluid, and contributes towards meconium formation.

Mucins are the major macromolecular components of mucus. Initial histochemical studies showed that changes in the biochemical characteristics of mucins occur in intestinal disorders.[4][5] The use of other conventional tools, such as lectins and antibodies, has subsequently confirmed these findings.[6–11] More recently, techniques of molecular cloning allowed the identification of several mucin genes. To date, eight different mucin genes (MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7) have been identified,[12–21] and an additional partial cDNA has also been reported.[22] Each of these mucin genes contains tandemly repeated sequences that encode peptides rich in serine and threonine residues, which support the numerous O-glycan chains. The use of northern blotting and in situ hybridisation techniques utilising specific nucleotide probes has shown a tissue and cell specific distribution of apomucin mRNAs.[23][24] Qualitative and/or quantitative alterations in mucin gene expression in both preneoplastic and neoplastic lesions have been previously reported.[25–27] mRNA levels for MUC2 and MUC3 are increased in colloid colon cancer compared with normal colon. However, in moderately and well differentiated colon cancer, the expression of MUC2 and MUC3 is reduced.[25][26] We have recently shown that MUC2 is overexpressed and MUC5AC is aberrantly expressed in rectosigmoid villous adenoma.[27] Furthermore, the level of expression of MUC5AC in villous adenoma is linked to the degree of cellular dysplasia.[27]

It is well known that preneoplastic and neoplastic tissues can express fetal-like morphological and phenotypic characteristics. Thus, to assess whether expression of mucin genes is linked to the stage of epithelial cytodifferentiation, we studied the expression of six mucin genes in the small and large intestine of 32 human embryos and fetuses aged between 6.5 and 27 weeks of gestation.

Methods

TISSUES
Tissues from five human embryos and 27 human fetuses were obtained after spontaneous or therapeutic abortion with local ethical committee approval. The specimens ranged in age from 6.5 to 27 weeks of gestation, dated from the last menstruation (data obtained from clinical records and confirmed by the foot and crown-rump length). There was no evidence of congenital anomalies in the digestive tract of any of the specimens. Tissue samples of normal jejunum, ileum, and colon were taken less than 30 minutes after removal. Five fetuses, aged 18 to 27 weeks, provided several colon samples (ascending colon, transverse colon, descending colon, and rectum).

Table 1 Mucin gene expression in the primitive gut

Age (weeks)	Number	Probes					
		MUC2	MUC3	MUC4	MUC5AC	MUC5B	MUC6
6.5	1	−	+	+	−	−	−
8	1	−	+	+	+	−	−
9	1	+	+	+	−	−	−

Specimens of normal adult intestinal mucosae (three jejunum; six ileum; 10 colon) were obtained from patients without evidence of neoplastic or inflammatory bowel disease, and were used as controls.

Each specimen was immediately immersed either in fresh 4% paraformaldehyde or 10% formalin in phosphate buffer for in situ hybridisation, and further embedded in paraffin wax. Sections, 3 μm thick, were cut, mounted on gelatin covered slides, and stored at 4°C until used. Adjacent sections from the same blocks were systematically stained with haematoxylin-eosin-safran and Astra blue for a histological control.

PROBES

In situ hybridisation was performed using six different ^{35}S-labelled oligonucleotide probes corresponding to each tandem repeat domain of MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 as described in earlier studies.[23 27] MUC1 and MUC7 were not tested in this study as MUC1 is considered to be a mucin-like encoding "episialin" (also called PEM or PUM) which is a membrane bound O-glycoprotein[12–14] and MUC7 mRNA expression is restricted to sublingual and submandibular glands.[21]

IN SITU HYBRIDISATION

The hybridisation steps were as described in detail by Audié *et al.*[23] Briefly, tissue sections were deparaffinised, rehydrated, incubated with 2 μg/ml proteinase K (Boehringer Mannheim, Meylan) for 15 minutes, and then fixed again in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes. Sections were then immersed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 minutes and prehybridised in 4× SSPE (standard sodium phosphate EDTA), 1× Denhardt's buffer for 45 minutes. Sections were hybridised overnight at 42°C in 20–60 μl of a 4× SSPE solution containing 50% formamide (vol/vol), 0.1% N-lauroylsarcosine (wt/vol), 1.2 M sodium phosphate, pH 7.2, 1× Denhardt's buffer, 3 mg/ml yeast tRNA, 20 mM dithiothreitol, and 7.5×10^3 dpm/μl ^{35}S-labelled oligonucleotide. After post-hybridisation washes, slides were dipped in LM-1 emulsion (Amersham, Les Ulis), developed at 4°C for two to three weeks, and finally counterstained with methyl green pyronin.

The following controls were performed: fetal tissue sections were treated with 50 μg/ml RNAse A (Boehringer Mannheim, Meylan); fetal tissue sections were treated with a large excess of unlabelled oligonucleotide identical to or distinct from the radiolabelled probe; and adult and fetal tissue sections were tested in parallel under the same conditions.

Results

EMBRYOS AND FETUSES

MUC2—Tables 1 and 2 summarise hybridisation data. The MUC2 probe gave the strongest signal in all specimens, irrespective of gestational age. In the small intestine, MUC2 mRNAs were detected as early as nine weeks after gestation, and were located between the primordial villi, in regions of epithelium that eventually give rise to the crypts of Lieberkühn (fig 1a). After 10 weeks the signal was located predominantly within immature crypts of Lieberkühn which persisted until 23 weeks. Very few goblet cells at the villous surface showed any signal (fig 1b). At 23 weeks of gestation, the signal remained stronger in the crypts, but more villous goblet cells were labelled (fig 1c,d). The signal was located in the perinuclear zone within goblet cells. From week 26 of gestation, the signal was located in the majority of goblet cells, both on villi and in crypts (fig 1e). In the large intestine, MUC2 mRNAs were detected within the crypts from week 12 of gestation and remained similar up to 27 weeks irrespective of the region (ascending colon, transverse colon, descending colon, and rectum).

MUC3—mRNAs for MUC3 were detected as early as 6.5 weeks of gestation (table 1). In embryonic specimens, the MUC3 probe gave continuous and homogeneous labelling throughout the whole epithelium. After nine weeks, the signal was restricted to the primitive villi in both goblet and absorptive cells. In absorptive cells, the signal was diffuse throughout the cytoplasm. A weak signal was occasionally detected within crypt cells (fig 1f,g). This distribution of MUC3 was common to both small and large intestine.

MUC4—mRNAs for MUC4 were detected in the anterior portion of the primitive gut as early as 6.5 weeks after gestation. The labelling was continuous and homogeneous throughout the epithelium until eight weeks (table 1, fig 1h). In the small intestine, the MUC4 probe gave weak labelling between nine and 15 weeks of gestation where the signal was limited to

Table 2 MUC2 mRNA distribution in embryonic, fetal and adult intestine

Age (weeks)	Number	Small intestine	Colon
10	2	S−/C+ CP	S−
10.5	3	S−/C+ CP	S−
12	2	S−/C+ CP	S−
12.6	1	S+/C+ CP	ND
13	1	S+/C+ CP	S−/C+ CP
13.3	1	S+/C+ CP	ND
14.1	3	S+/C+ CP	ND
14.3	1	S+/C+ CP	ND
14.6	2	S+/C+ CP	ND
14.7	1	S+/C+ CP	ND
14.8	1	S+/C+ CP	ND
14.9	2	S+/C+ CP	ND
15.0	2	S+/C+ CP	ND
15.1	1	S+/C+ CP	ND
15.2	1	S+/C+ CP	ND
18	1	S+/C+ CP	S+/C+ CP
23	1	S+/C+ CP	S+/C+ CP
26	2	S+/C+	S+/C+ CP
27	1	S+/C+	S+/C+ CP
Adults	19*	S+/C+	S+/C+

*Three jejunums, six ileums, 10 colons.
S, surface epithelium; C, crypt epithelium; CP, crypt predominant; ND, not determined.
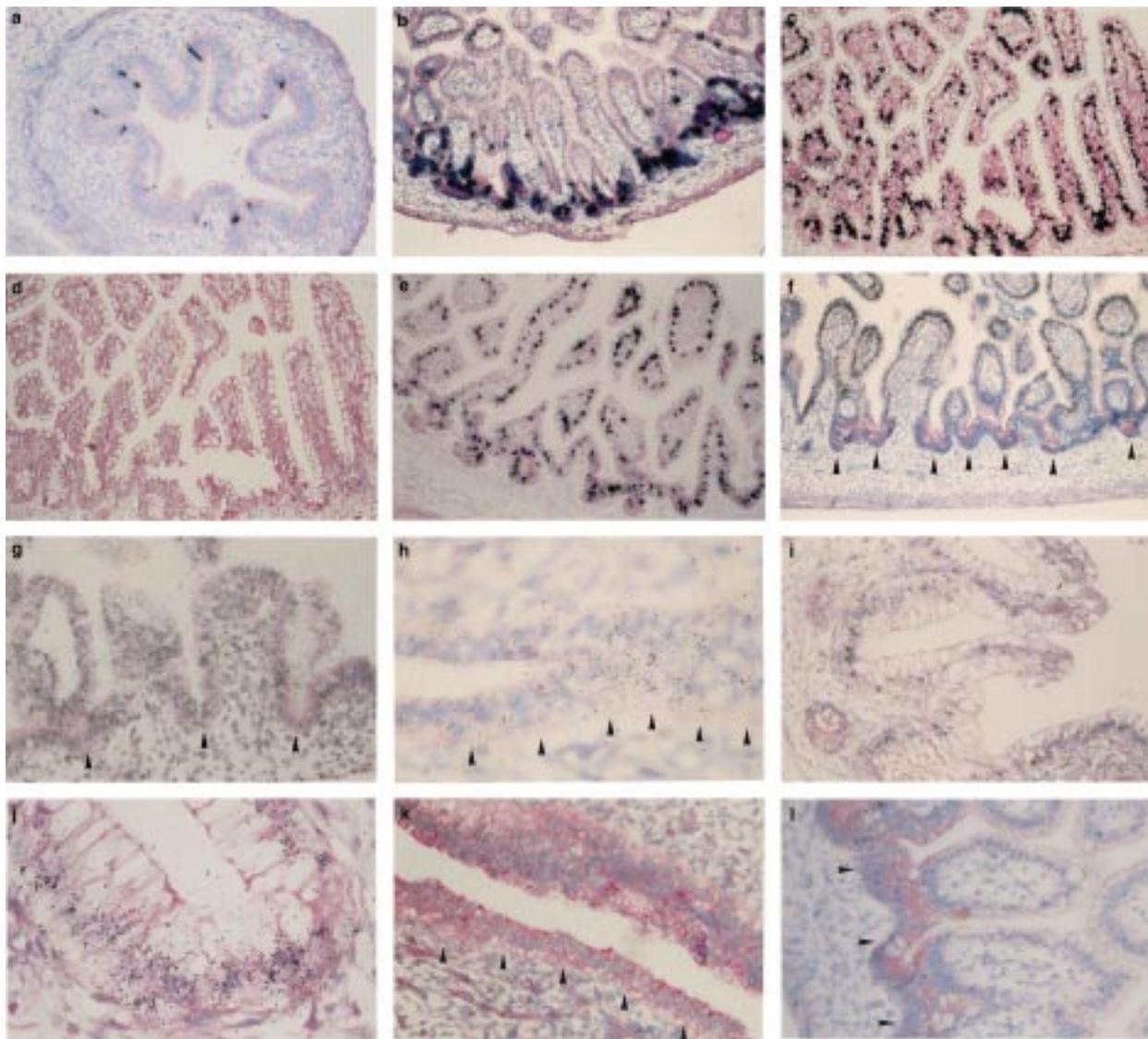
Figure 1   In situ hybridisation for mucin gene mRNAs in embryonic and fetal intestine. (a) Small intestine at 10 weeks gestation with MUC2 probe: the signal is located predominantly between the primordial villi (methyl green pyronin counterstain; original magnification ×200). (b) Small intestine at 12 weeks' gestation with MUC2 probe: the signal is located predominantly within immature crypts of Lieberkühn (methyl green pyronin counterstain; original magnification ×200). (c, d) Ileum at 23 weeks' gestation: (c) with MUC2 probe: signal is stronger in the crypts, but the majority of villous goblet cells are labelled; (d) with MUC2 probe and an excess of cold unlabelled MUC2 oligonucleotide (negative control): hybridisation is negative (methyl green pyronin counterstain; original magnification ×200). (e) Ileum at 26 weeks' gestation with MUC2 probe: signal is distributed in goblet cells both on villi and in crypts (methyl green pyronin counterstain; original magnification ×200). (f) Small intestine at 12 weeks' gestation with MUC3 probe: continuous and homogeneous labelling along the villous epithelium, both in goblet and absorptive cells; no labelling present in crypts (arrows) (methyl green pyronin counterstain; original magnification ×400). (g) Small intestine at 14.1 weeks' gestation with MUC3 probe: continuous and homogeneous labelling along the villous epithelium, both in goblet and absorptive cells; weak labelling is also present in crypts (arrows) (methyl green pyronin counterstain; original magnification ×500). (h) Anterior portion of primitive gut at 6.5 weeks' gestation with MUC4 probe: continuous labelling along epithelium (methyl green pyronin counterstain; original magnification ×1000). (i, j) Colon at 23 weeks' gestation with MUC4 probe: signal is located in the perinuclear region of goblet cells (methyl green pyronin counterstain; original magnification: (i) ×400; (j) ×1000). (k) Middle portion of primitive gut at 8 weeks' gestation with MUC5AC probe: continuous labelling along the undifferentiated epithelium (methyl green pyronin counterstain; original magnification ×500). (l) Ileum at 12 weeks' gestation with MUC5AC probe: labelling is limited to clusters of epithelial cells both on villi and in crypts (methyl green pyronin counterstain; original magnification ×500).

occasional crypt and villous cells (tables 1 and 3). MUC4 mRNAs were not detectable at the other stages of fetal development. In the colon, MUC4 mRNAs were detected after 10 weeks throughout the undifferentiated epithelium. From week 12 of gestation, epithelial cells on villi and in crypts were labelled. Although the signal was weak and located in the perinuclear region in the majority of goblet cells, some cells expressed intense labelling (fig 1i,j). This hybridisation pattern remained constant until 27 weeks of gestation irrespective of the colonic region examined.

MUC5AC—The MUC5AC probe showed a moderate signal in the primitive intestine at eight weeks of gestation. After eight weeks, MUC5AC mRNAs were inconsistently found (tables 1 and 4). The labelling was limited to clusters of epithelial cells, both on villi and in crypts (fig 1k). At 12 weeks, MUC5AC mRNAs were observed in the ileum, but not in the colon (fig 1l). After 12 weeks, MUC5AC was not detected in any region of the intestine.

MUC5B and MUC6 were not observed in either embryonic or fetal intestine by in situ hybridisation.

Table 3   MUC4 mRNA distribution in embryonic, fetal and adult intestine

Age (weeks)	Number	Small intestine	Colon
10	2	–	+
10.5	3	2+/1–	+
12	2	+	1–/1+
12.6	1	+	ND
13	1	+	+
13.3	1	+	ND
14.1	3	1+/2–	ND
14.3	1	+	ND
14.6	2	+	ND
14.7	1	+	ND
14.8	1	–	ND
14.9	2	1+/1–	ND
15.0	2	–	ND
15.1	1	–	ND
15.2	1	–	ND
18	1	–	+
23	1	–	+
26	2	–	+
27	1	–	+
Adults	19★	J:–; I:4+/2–	+

★Three jejunums, J; six ileums, I; 10 colons.
ND, not determined.

Controls for in situ hybridisation were negative when tissues had been pretreated by ribonuclease A prior to hybridisation, or when sections were hybridised with an excess of cold unlabelled specific oligonucleotide (fig 1d). Hybridisation with an excess of unlabelled non-specific oligonucleotide did not alter the signal.

NORMAL ADULT CONTROLS
MUC2—The MUC2 probe hybridised strongly to the perinuclear region of goblet cells of villi and crypts in both small and large intestinal tissues (fig 2a). All absorptive cells and a minority of goblet cells remained unlabelled.

MUC3—The MUC3 probe labelled all superficial epithelial cells homogeneously. In the small intestine, the signal was present in goblet and absorptive cells throughout the villous epithelium and was generally absent in crypts (fig 2b). In colonic tissues, the signal was localised to goblet and absorptive cells of the superficial epithelium and in the upper region of the crypts.

MUC4—The MUC4 probe gave a moderate signal in the perinuclear region of all epithelial

Table 4   MUC5AC mRNA distribution in embryonic, fetal and adult intestine

Age (weeks)	Number	Small intestine	Colon
10	2	–	–
10.5	3	1+/2–	–
12	2	1+/1–	–
12.6	1	–	ND
13	1	–	–
13.3	1	–	ND
14.1	3	–	ND
14.3	1	–	ND
14.6	2	–	ND
14.7	1	–	ND
14.8	1	–	ND
14.9	2	–	ND
15.0	2	–	ND
15.1	1	–	ND
15.2	1	–	ND
18	1	–	–
23	1	–	–
26	2	–	–
27	1	–	–
Adults	19★	–	–

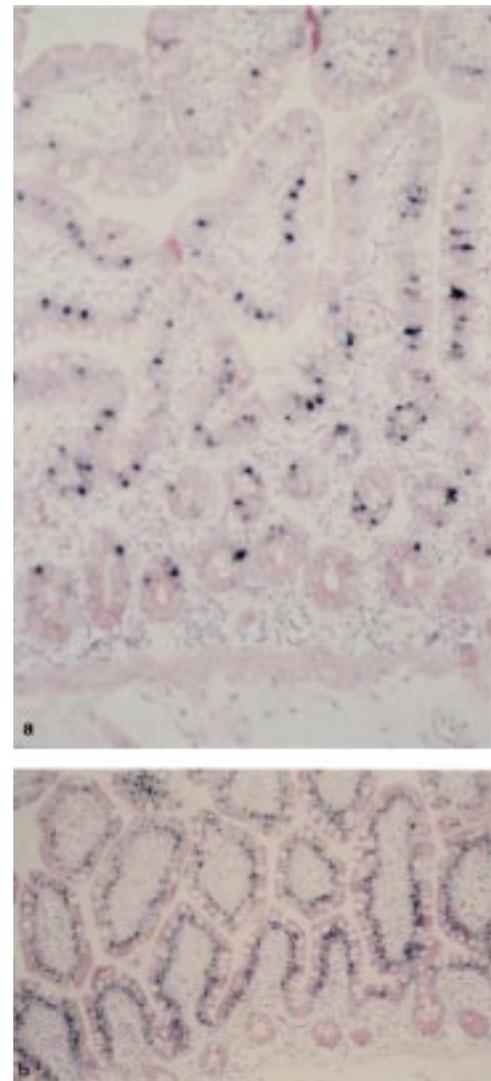★ Three jejunums, six ileums, 10 colons.
ND, not determined.

Figure 2   In situ hybridisation for mucin gene mRNAs in adult intestine. (a) Jejunum with MUC2 probe: signal is located in the perinuclear region of goblet cells both on villi and in crypts (methyl green pyronin counterstain; original magnification ×200). (b) Jejunum with MUC3 probe: continuous and homogeneous labelling along the villous epithelium, both in goblet and absorptive cells (methyl green pyronin counterstain; original magnification ×200).

cells in the colon, although scattered cells were occasionally more intensely labelled. In the small intestine, MUC4 mRNAs were detected in only four out of the six ileum specimens examined, where the intensity and the pattern of the labelling also varied. No hybridisation was observed to the jejunum in any of the specimens examined.

MUC5B, MUC5AC, and MUC6 were not detected in the small or large intestine by in situ hybridisation.

Discussion
We have studied the expression of MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 in 32 human embryos and fetuses at different gestational ages, in order to establish their developmental pattern of gene expression and to compare this with adult tissues. To our knowledge, the only other study describing developmental expression of intestinal mucin

genes was performed by Chambers *et al*,[28] who analysed MUC1 and MUC2 gene expression in four fetuses aged between 12 and 19 weeks' gestation.

Similar changes in mucosubstance composition have been shown for colonic carcinomas/adenomas and fetal intestine using conventional methods such as lectin chemistry, immunochemistry, and histochemistry.[5 29–33] However, these alterations are not precisely defined due to the limited knowledge of the lectin and antibody specificities and of the relation between oligosaccharide structures and apomucins. Therefore, in this study, we utilised in situ hybridisation, adopting specific oligonucleotides corresponding to each tandem repeat domain of MUC2, MUC3, MUC4, MUC5B, MUC5AC, and MUC6 as a more accurate measure of the developmental regulation of intestinal mucin gene expression. Importantly, this approach also enabled us to distinguish differences in mucin core expression versus differential glycosylation recognised by lectins/antibodies.

Our results show that MUC2 is expressed throughout the intestinal epithelium as early as nine weeks of gestation and is therefore concomitant with endodermal cytodifferentiation associated with the formation of premature crypts and villi.[34 35] In this respect, the pattern of MUC2 expression in embryos and fetuses is quite different from that observed in normal adult intestinal mucosae, where it is restricted to villous goblet cells and immature crypt cells.[23 26 27] At nine and 10 weeks' gestation, MUC2 is expressed between the primordial villi, and then predominantly within the developing crypts until week 23 of gestation in the small intestine, and week 27 in the colon. These findings are in accordance with those reported by Chambers *et al*.[28] Furthermore, we have shown that the developmental switch from a fetal to an adult pattern of MUC2 gene expression occurs at around 23 weeks in the small intestine and 27 weeks for the colon. At this time, fetal intestinal epithelial cells are fully differentiated as part of the precocious developmental pattern observed in utero.[34–36] All intestinal epithelial cells originate from pluripotent stem cells situated at the crypt base. The majority of daughter cells migrate up towards the villous tip while they undergo terminal differentiation.[37 38] In situ hybridisation showed that MUC2 is first expressed within undifferentiated crypt epithelial cells at earlier stages of development, whereas it is expressed in both crypt cells and mature villous goblet cells at later stages.

In fetuses older than 23 weeks, as in adults, most goblet cells of the small intestine express MUC2 irrespective of their degree of cytodifferentiation. Within the mature goblet cell population, occasional cells do not express MUC2. Distinct subpopulations of goblet cells have been defined by cell kinetic studies, ultrastructural features, and mucin specific monoclonal antibodies.[37 39 40] The heterogeneity in MUC2 gene expression might be related to one of these distinct subpopulations of goblet cells, as it is not linked to the cell cycle since mature goblet cells do not divide.[39]

Notably, MUC2 is the major fetal and adult colonic mucin. Therefore, this implies that the cancer like mucin glycosylation apparent in the fetus is unlikely to be mediated via a difference in mucin core expression.[5 29–33]

We have shown that MUC3 and MUC4 are expressed at a very early gestational age in humans (as early as 6.5 weeks). At this developmental stage, the intestinal epithelium is stratified and undifferentiated.[34–36] Thus, MUC3 and MUC4, unlike MUC2, are expressed in the primitive gut endoderm prior to epithelial cytodifferentiation. In adults, MUC3 and MUC4 differ from MUC2, MUC5AC, MUC5B, and MUC6 as they are expressed both in mucus secreting and non-mucus secreting cells—for example, intestinal absorptive cells or bronchial ciliated cells. Thus, in addition to providing epithelial protection, MUC3 and MUC4 mucins may possibly play additional physiological roles—for example in regulating epithelial cytodifferentiation or housekeeping function.

In normal adults, MUC5AC is the major mucin gene expressed in the gastric and respiratory mucosae, and is not normally expressed in the intestinal tract.[23] Interestingly, we have shown that MUC5AC is expressed in embryonic intestine up to eight weeks of gestation. However, after 12 weeks, expression is significantly reduced as mRNAs were not detected in any intestinal region from week 13 of gestation. Thus, MUC5AC is developmentally expressed during intestinal ontogenesis. Recently, MUC5AC was shown to be expressed in rectosigmoid villous adenomas. A negative correlation could be established between MUC5AC gene expression and the grade of cellular atypia. The expression of MUC5AC is more intense in villous adenomas with low grade of dysplasia compared with villous adenomas with high grade dysplasia, and disappears in invasive carcinomas.[27] Our present results show that MUC5AC gene expression in villous adenomas is reminiscent of the MUC5AC expression in fetal intestine. It is noteworthy that MUC5AC was also shown to be expressed in a population of mucus secreting HT-29 cells selected by adaptation to methotrexate.[41 42] These cells form a monolayer of polarised goblet cells that exhibit a discrete apical brush border and secrete mucins of gastric immunoreactivity, two characteristics that are reminiscent of the differentiation of the human fetal colonic epithelium.[41 42] These data, associated with the selective expression of MUC5AC in early fetal life, might imply a role for this mucin in intestinal epithelial cell differentiation, both in fetal development and in tumorigenesis.

In summary, three mucin genes, MUC2, MUC4, and MUC5AC, are differently expressed (both spatially and temporally) in the intestine of embryos and fetuses, compared with normal adults, suggesting a role for these genes in the normal development of fetal intestine. We have confirmed that MUC3 gene expression varies along the crypt villous axis.

Furthermore, we have shown that MUC4 expression seems to be linked to the epithelial cell position along the jejunum to colon axis. MUC2 expression is cell lineage dependent since it is expressed only in mature goblet cells. MUC5AC expression is stage dependent as it closely relates to intestinal development.

Taken together, these results show a complex regulation of the mucin genes. To date, very little information is available concerning promoters, transcription factors, and the regulation of mucin genes, except for MUC1. In the future, isolation of the promoters, functional mapping studies, and experiments in transgenic mice as well as in isografts or xenografts of fetal intestine, should provide new insights defining factors which direct the correct cell lineage and spatiotemporal patterns of mucin gene expression. Knowledge of the sequential activation of the different mucin genes in embryos and fetuses might allow a better understanding of intestinal lesions where alterations in mucosecretion are apparent, in particular premalignant and malignant lesions and inflammatory bowel disease.

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