Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium

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Abstract

Background—In normal gastric epithelium, MUC5AC is detected in superficial epithelium associated with Lewis type 1 antigens and MUC6 is detected in antral glands with Lewis type 2. Therefore, the stomach constitutes an excellent model to examine the role of glycosyltransferases in determining the specificity of apomucin glycosylation.

Aims—To determine the molecular basis of this association and to examine changes in expression of gastric and intestinal apomucins and their association with Lewis antigens during the gastric carcinogenesis process.

Methods—Fucosyltransferase (FUT1, FUT2, FUT3) and mucin (MUC5AC, MUC6) transcripts were detected using reverse transcription-polymerase chain reaction. Apomucin (MUC2, MUC4, MUC5AC, MUC6) and Lewis antigen (types 1 and 2) expression were analysed using single and double immunohistochemistry and in situ hybridisation.

Results—In the normal stomach, FUT1 is exclusively detected associated with MUC6; FUT2 is only detected when MUC5AC is present. This co-regulation is lost in gastric tumours, as is differential expression of MUC5AC and MUC6 in normal gastric epithelial cells. In gastric tumours, especially those with the intestinal phenotype, MUC2 and MUC4 genes are upregulated, and gastric-type and intestinal-type mucins are coexpressed. These changes are early events in the gastric carcinogenesis process, as they are detected in intestinal metaplasia.

Conclusions—The glycosylation pattern found in normal gastric epithelium is dictated by the specific set of fucosyltransferases expressed by the cells rather than by the apomucin sequence. The development of intestinal metaplasia and gastric cancer is associated with the appearance of cellular phenotypes that are absent from normal epithelium.

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tissues. Their molecular heterogeneity resides in both the apomucin and carbohydrate chains which are linked by an O-glycosidic bond. Until now, cDNAs from nine different mucin genes have been cloned (MUC1–MUC4, MUC5B, MUC5AC, MUC6–MUC8).^{1–11} Each apomucin gene has a characteristic pattern of tissue distribution and none of the apomucins is restricted to a single epithelium.^{12–15} To date, there are only indirect data concerning the relationship between the mucin gene expression pattern and specific function of mucus in a given tissue.

Mucus covering the gastric epithelium plays a major role in protection from acid pH and mechanical aggression. The antral epithelium displays two well characterised populations of mucous secreting cells: the superficial epithelium and the deep glands. Cells in the superficial epithelium express MUC5AC in association with type 1 Lewis antigens (Le^a and Le^b) and produce neutral mucins. In contrast, deep gland cells express MUC6 in association with type 2 Lewis antigens (Le^x and Le^y) and secrete acid mucins.¹⁴ Therefore, the gastric epithelium constitutes an adequate model system to study the relationship between apomucin expression and glycosylation. It is currently not known if this specific pattern of glycosylation is determined by the primary amino acid sequence of the apomucins or by the fucosyltransferases expressed in each cell type. Seven human fucosyltransferase genes have been cloned. Among them, FUT1, FUT2, and FUT3 code for the enzymes responsible for Lewis antigen synthesis in epithelial cells.^{15–16} FUT1 and FUT2 are the α -1,2-fucosyltransferases responsible for the synthesis of Lewis type 2 (Gal β 1,4-GlcNAc) and type 1 (Gal β 1,3-GlcNAc) antigen precursors.^{15–17} The α -1,3/4 fucosyltransferase, FUT3, is involved in the synthesis of all Lewis antigens by adding an α -fucose to the type 1 and type 2 precursor structures.¹⁶

Alterations in expression of Lewis structures have been described in several gastric pathologies. Among them are ectopic expression of Le^a in intestinal metaplasia from individuals with the secretor phenotype,^{18–19} which has been proposed as a marker of increased cancer

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Mucins are highly glycosylated proteins and the principal components of mucus, a viscoelastic gel that covers the surface of epithelial

Abbreviations used in this paper: RT-PCR, reverse transcription-polymerase chain reaction; MoAb, monoclonal antibody; AP, alkaline phosphatase; PBS, phosphate buffered saline; HRP, horseradish peroxidase; DTT, dithiothreitol; PFA, paraformaldehyde; DIG, digoxigenin.

Table 1 Characteristics of antibodies used in this study

Antibody	Specificity	Type	Reference
T-174	Lewis a	MoAb	23
T-218	Lewis b	MoAb	23
77/180	Lewis y	MoAb	15
LDQ10	MUC2 TR	MoAb	24
Anti-MUC4	MUC4 TR	Rabbit polyclonal	*
CLH2	MUC5AC TR	MoAb	22
LUM5-1	MUC5AC, non TR	Rabbit polyclonal	25
Anti-MUC6	MUC6 TR	Rabbit polyclonal	14

*De Bolós, unpublished.
MoAb, monoclonal antibody.

risk, and upregulation of sialyl-Le^x related to an increased risk of metastasis and poor prognosis in gastric cancer patients.²⁰ In addition, up-regulation of mucin genes that are normally absent from a healthy stomach (that is, MUC2 and MUC3),²¹ as well as downregulation of gastric mucin genes associated with tumour type and stage (that is, MUC5AC) have been described.²²

Based on these findings, we attempted to elucidate the molecular basis of the association between apomucins and Lewis antigens in the normal stomach, and changes in apomucin and Lewis antigens in gastric neoplastic and preneoplastic lesions. Firstly, we analysed mucin and FUT transcripts in the normal stomach. We also examined expression of gastric (MUC5AC and MUC6) and intestinal (MUC2 and MUC4) mucin genes in gastric tumours, and their association with Lewis antigens and expression of FUT1–FUT3 mRNA. In addition, we analysed mucin gene expression and coexpression of apomucins and Lewis antigens in lesions representative of the sequential carcinogenesis process.

Methods

TISSUE SAMPLES

Normal and neoplastic stomach tissue samples were obtained at surgery performed at Hospital Ntra Sra Aránzazu (San Sebastian, Spain) and Hospital del Mar (Barcelona), snap frozen in isopentane cooled to -80°C , and embedded in OCT (optimal cutting temperature) for cryostat sections. Normal stomach samples were obtained from individuals who underwent gastrectomy for duodenal ulcer or from macroscopically normal areas at a distance >10 cm from a gastric cancer. Biopsies from gastric endoscopies were fixed in 10% formaldehyde and embedded in paraffin. A diagnosis was made on the basis of haematoxylin/eosin staining. Intestinal metaplasia was defined as follows: complete type when absorptive, Paneth, and goblet cells were identified; and incomplete type when columnar and goblet cells were present. For RNA extraction, normal and tumour samples were collected from patients undergoing surgery, snap frozen as soon as possible, and stored at -80°C . In some specimens, superficial and deep epithelium were separated by scraping.

ANTIBODIES AND IMMUNOHISTOCHEMICAL ASSAYS
Characteristics of the antibodies used in this study are shown in table 1. Monoclonal antibodies (MoAb) T-174,²³ T-218,²³ B12 (Dr

R Castro, Barcelona, Spain), and CLH2²² were used as undiluted hybridoma supernatant; MoAbs 77/180¹⁴ and LDQ10²⁴ were purified and used at 25 $\mu\text{g}/\text{ml}$. Rabbit polyclonal serum LUM5-1, raised against a synthetic peptide from the COOH terminal domain of MUC5AC, was used at 1/1000–1/2000.²⁵ MUC5AC was always detected with LUM5-1 except for the MUC5AC/MUC6 double labelling experiments which were performed with CLH2 MoAb. Rabbit polyclonal anti-MUC4 antibodies were raised against a KLH conjugate of a synthetic peptide corresponding to the tandem repeat sequence of MUC4 (TSSASTGHATPLPVTG) following procedures reported elsewhere.¹⁴ Polyclonal anti-MUC4 and anti-MUC6 antibodies were purified by affinity chromatography on synthetic peptides as previously described¹⁴ and were used at 1/10–1/50 dilutions. The specificity of all antibodies has been reported previously (see table 1), except for that of MUC4. Specificity of the anti-MUC4 affinity purified antibodies was determined by ELISA and by peptide inhibition assays using immunohistochemistry, as previously described.¹⁴ No reactivity was detected for any of the peptides encompassing the tandem repeat sequences of MUC1–MUC8, except for that of MUC4 (De Bolós, unpublished data). Pre-immune rabbit serum was used as a negative control.

The indirect immunoperoxidase technique was performed as described previously.¹² For double labelling immunoassays, paraffin sections were rehydrated and fixed with cold acetone for 10 minutes. Endogenous peroxidase and alkaline phosphatase (AP) were blocked with 4% H_2O_2 and 3 mM levamisole in phosphate buffered saline (PBS) for 10 and 15 minutes, respectively. Non-specific binding sites were blocked by incubating the sections with 5% skim milk in PBS/0.04% Tween 20. The two primary antibodies from different species were diluted in PBS with 1% bovine serum albumin, and incubated together for 1.5 hours. After four washes with PBS, sections were incubated with a mix of the two secondary antibodies, one labelled with horseradish peroxidase (HRP) and the other with AP (Dako, Denmark) for one hour. Sections were washed with PBS and then with 0.2 M Tris, pH 8.2. AP was developed with 0.1% Fast BlueBB, 0.02% Naphtol AS-MX phosphate in 0.2 M Tris, pH 9.1, and HRP was developed with 0.5% diaminobenzidine in PBS with 0.1% H_2O_2 . After washing in distilled water, sections were mounted with an aqueous mounting medium (Aquatex, Merck, Germany). Scoring of reactions was performed as described previously.¹² Percentage of reactive cells was an estimate of the whole tissue section. Double labelling immunofluorescence assays were performed as described previously.¹⁴ Confocal microscopy analysis was performed using a Leica TCS NT instrument.

For immunoelectron microscopy, indirect double labelling techniques with CLH2 and anti-MUC6.1 antibodies were performed on tissues embedded in Lowicryl 4KM, as described elsewhere.²⁶ The anti-MUC6.1 antibodies were visualised with Protein A-gold

complexes (5 nm Ø) (Department of Cell Biology, School of Medicine, Utrecht University, the Netherlands); CLH2 antibody was detected with goat antimouse Ig (Dako) and Protein A-gold complexes (15 nm Ø) (Utrecht University). Reactivity was visualised using a Philips CM100 electron microscope.

WESTERN BLOTTING

Briefly, tumour samples were homogenised in 50 mM Tris, pH 7.5, 5 mM EDTA, 6 M guanidinium hydrochloride, 1 mM phenylmethyl sulphonyl fluoride, reduced with 100 mM dithiothreitol (DTT) (Sigma, Missouri, USA), and incubated in the dark for 24 hours. Sulphydryl groups were stabilised by adding 250 mM iodoacetamide (Sigma) and stirring in the dark for an additional 24 hours.²⁷ Proteins (50 µg/well) were separated using a 6% sodium dodecyl sulphate-polyacrylamide gel, and western blotting was performed as described previously¹⁴ except that membranes were deglycosylated with 10 mM sodium periodate in 50 mM sodium acetate buffer (pH 4.5–5) for one hour in the dark.

IN SITU HYBRIDISATION

Synthetic oligonucleotides (48mers) corresponding to the tandem repeat sequences of mucin genes were used. Sense oligonucleotides encompassing the same sequences were used as negative controls in selected experiments. Oligonucleotides were labelled with digoxigenin (DIG) following the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Samples were immediately fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and stored at 4°C. Sections were deparaffinised and rehydrated. Proteinase K digestion (1 µg/ml in 0.1 M Tris HCl, pH 7, 0.5 M EDTA) was performed for 15 minutes at 37°C. Samples were fixed with 4% PFA for 15 minutes and treated with 0.1 M triethanolamine, pH 8, and 0.25% acetic anhydride for 10 minutes. After prehybridisation in 4×SSPE with 1% Denhardt's at 42°C for one hour, sections were dehydrated and hybridised overnight at 40°C with DIG labelled oligonucleotides (200–400 µg/ml) diluted in 4×SSPE, 1% Denhardt's, 50% formamide, 20 mM DTT, 0.1 M sodium phosphate, pH 7.2, 1% sarkosyl, and tRNA (250 µg/ml). Post-hybridisation washes were done stepwise from 4×SSPE at 20°C to a final wash with 0.1×SSPE at 42°C. Sections were rehydrated, dried, and incubated with 5% normal horse serum. AP conjugated sheep anti-DIG antibodies (Boehringer Mannheim) were incubated overnight at 4°C. AP was developed overnight at 4°C using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Promega, Wisconsin, USA) in 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris HCl, pH 9.5. Slides were rinsed and mounted in Aquatex.

The antisense oligonucleotide sequences used were: MUC2 (5' GGT CTG TGT GCC GGT GGG TGT TGG GGT TGG GGT CAC CGT GGT GGT GGT 3'), MUC4 (5' GTC GGT GAC AGG AAG AGG GGT GGC GTG ACC TGT GGA TGC TGA

GGA AGT 3'), MUC5AC (5' AGG GGC AGA AGT TGT GCT CGT TGT GGG AGC AGA GGT TGT GCT GGT TGT 3'), and MUC6 (5' CAT CTG TGC GTG GGT AGG GGT GAT GAC TGT GTG AGT ACT TGG AGT CAC 3').

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated using the guanidinium thiocyanate/phenol/chloroform extraction method.²⁸ cDNA was synthesised using 5 µg of DNase I treated RNA with 200 U of M-MLV reverse transcriptase. FUT1 and FUT2 transcripts were amplified by PCR using primers described by Kelly and colleagues.^{15, 17} cDNA was amplified under the following conditions: 1.5 minutes at 94°C, annealing/extension at 72°C for 2.5 minutes (40 cycles), and final elongation at 72°C for 10 minutes. For FUT3 mRNA detection, primers published by Cameron and colleagues¹⁶ were used. PCR conditions were: 1.5 minutes at 94°C, 1.5 minutes at 70°C, two minutes at 72°C (40 cycles), and final extension at 72°C for 10 minutes. K-ras and β-actin primers were used to control for mRNA quality.^{29, 30} The size of the PCR products was 510 bp for FUT1, 198 bp for FUT2, 582 bp for FUT3, 265 bp for K-ras, and 349 bp for β-actin.

Mucin transcripts were amplified using the following primers: MUC5AC sense GTA CCA GAA CAG TCG ACC T, MUC5AC antisense CTC TTC CAC CTC GGT GTA GC, MUC6 sense GTC CAC TTC TGC TTC GAT CCA CTC, and MUC6 antisense GGA CCT GTG GAA GAG ACA TGA CTG; oligonucleotides were selected based on published sequences.^{9, 31}

The conditions for amplification were, for MUC5AC, one minute at 94°C, 0.5 minutes at 58°C, and 0.5 minutes at 72°C (35 cycles) and for MUC6, one minute at 94°C, 0.5 minutes at 70°C, and 0.5 minutes at 72°C (30 cycles). The size of the PCR products was 257 bp for MUC5AC and 359 bp for MUC6. PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide.

Results

MUCIN AND FUCOSYLTRANSFERASE GENE EXPRESSION IN NORMAL GASTRIC MUCOSA

In normal stomach, gastric mucin genes were expressed in two different cell populations: MUC5AC was detected in superficial epithelium and MUC6 in deep glands. In these cell populations, MUC5AC and MUC6 are associated with Lewis type 1 and type 2 antigens, respectively.¹⁴ For intestinal mucin genes, MUC2 was detected in a variable proportion of cells in 50% of fundic and antral samples, as described previously.¹² MUC4 specific affinity purified polyclonal antibodies were used to determine MUC4 expression in normal stomach: this apomucin was detected in the superficial epithelium of 2/10 fundic and 3/14 antral samples. Similar results were obtained by in situ hybridisation: MUC4 was found in 1/2 and 2/4 fundic and antral tissue samples, respectively (data not shown). In some cases, slight

Figure 1 Mucin and fucosyltransferase mRNA expression in gastric mucosa scrapings detected by RT-PCR (S, superficial epithelium; G, deep glands). MUC6 and FUT1 were detected only in the deep gland scrapings. MUC5AC and FUT2 were detected in both superficial epithelium and deep glands, suggesting that cells from the superficial epithelium contaminated the deep gland fraction.

gastritis was observed. Therefore, MUC4 (in common with MUC2) is expressed mainly in the intestine but not in the stomach.

Association between expression of MUC5AC and MUC6, and FUT1, FUT2, and FUT3 genes was analysed by RT-PCR using RNA from superficial epithelium and deep gland scrapings of normal stomach. Results are shown in fig 1. In normal stomach, transcripts of FUT1, the α 1,2-fucosyltransferase that catalyses the transfer of fucose to type 2 precursor structures, were detected exclusively in samples from deep glands in association with MUC6 transcripts (n=8). In contrast, transcripts of FUT2, the transferase involved in the synthesis of Lewis type 1 antigens, were found in normal samples from both superficial epithelium and glands, as was the case for MUC5AC transcripts, suggesting that scrapings did not contain a pure population of deep epithelial cells (fig 1). Transcripts of FUT3, the α -1,3/4 fucosyltransferase involved in the synthesis of all Lewis antigens, were detected in all samples from normal tissues.

EXPRESSION OF GASTRIC AND INTESTINAL APOMUCINS IN GASTRIC CANCER: COEXPRESSION WITH LEWIS ANTIGENS

Antibodies detecting gastric and intestinal apomucins were first used to analyse their expression in a panel of 35 fresh frozen stomach tumours obtained at surgery. MUC5AC was detected, using the LUM5-1 polyclonal antiserum, in 3/19 intestinal-type and in 3/15 diffuse-type tumours. In contrast, MUC6 was detected in the majority of intestinal (18/18) and diffuse (15/16) tumours. MUC2 and MUC4 were more commonly detected in intestinal (9/15 and 13/16, respectively) than in diffuse-type tumours (5/17 and 6/15, respectively). A cytoplasmic diffuse staining was observed for all antiapomucin antibodies. Differences in staining rate between intestinal and diffuse tumours were statistically significant for both MUC2 and MUC4 ($p=0.039$ and 0.016 , respectively). The highest percentage of positive cells was detected with the anti-MUC6 antibodies: >50% in intestinal-type tumours and 30–100% in the diffuse type. MUC5AC apomucin was always expressed in <50% of cells. For MUC2 and MUC4, a high degree of heterogeneity regarding the number of positive cells was found in both types of gastric tumours (10–100%). These results were confirmed by in situ hybridisation: MUC5AC was detected in 4/7 tumours, the proportion of positive cells ranging from 10 to 60%, whereas MUC6 was expressed in 7/7 samples. Representative results of MUC4 expression using immunohistochemical and in situ hybridisation assays are shown in fig 2A and 2B. To confirm that several apomucin genes are expressed in the same tumour, reduced and carboxymethylated tumour homogenates were analysed by western blotting on sodium periodate treated membranes. Figure 2C shows detection of MUC2, MUC4, MUC5AC (detected with the CLH2 MoAb), and MUC6 in tumour samples.

To determine if the differential distribution of MUC5AC and MUC6, and their association with Lewis antigens, was maintained in gastric

Figure 2 Mucin expression in gastric tumours. (A) MUC4 detected by immunohistochemistry in an intestinal-type tumour. (B) MUC4 expression by in situ hybridisation in a diffuse-type tumour (original magnification: A $\times 200$; B $\times 100$). (C) Coexpression of several apomucin genes in gastric tumours: western blot detection of MUC2, MUC4, MUC5AC, and MUC6 apomucins in lysates of intestinal (i) (1, 2, 4) and diffuse (d) (3) gastric tumours. Nitrocellulose membranes were treated with 100 mM sodium periodate before immunodetection. Several apomucins are detected in the same tumour sample. Markers (-) indicate the borders between running and stacking gels.

tumours, double labelling immunohistochemical techniques were used. Three patterns of gastric apomucin expression were found: MUC5AC+/MUC6+, MUC5AC-/MUC6+, and MUC5AC+/MUC6-. Cells coexpressing MUC5AC and MUC6, a phenotype absent from the normal gastric epithelium, were identified in 9/10 intestinal (fig 3A) and 3/7 diffuse gastric carcinomas. Figure 3B shows colocalisation of the two apomucins in mucus droplets of an intestinal-type tumour cell using double labelling immunoelectron microscopy. Regardless of the apomucins expressed in the tumour, both types of Lewis antigens were detected, indicating that the association between apomucin and Lewis antigens is lost on neoplastic transformation. MUC5AC/Lewis type 2+ cells were detected in 3/9 intestinal and 3/6 diffuse tumours, whereas MUC6/Lewis type 1+ cells were found in 7/9 intestinal and 4/6 diffuse carcinomas (fig 3C, D). Similarly, coexpression of MUC2 and MUC4 apomucins with Lewis type 1 and type 2 antigens was observed (data not shown). These patterns of apomucin/Lewis antigens were not detected in normal gastric epithelium and represent the appearance of an aberrant cell phenotype.

RELATION BETWEEN EXPRESSION OF MUC5AC/MUC6 AND FUCOSYLTRANSFERASE GENES IN GASTRIC TUMOURS

The lack of association between apomucin and Lewis antigens in gastric tumours suggests alterations in the pattern of FUT gene expression. We analysed the pattern of mucin and fucosyltransferase transcripts in tumour samples. In most cases (10/14), MUC5AC, MUC6, FUT1, and FUT2 were simultaneously

Figure 3 Gastric cancers contain cells displaying abnormal patterns of apomucin/Lewis antigen coexpression. (A) Double labelling immunohistochemical detection of MUC5AC (blue) and MUC6 expression (light brown) cells. Doubly labelled cells display a dark brown colour (original magnification $\times 400$). (B) Double labelling immunoelectron microscopy using antibodies against MUC5AC (CLH2) and MUC6 (anti-MUC6.1) and sections from Lowicryl 4KM embedded tissues. Mouse antibodies were detected using 15 nm gold particles (arrowheads) and rabbit antibodies using 5 nm gold particles (arrows) (original magnification $\times 63\,000$). (C) Double labelling immunohistochemical detection of MUC5AC (brown) and Lewis y (blue). (D) Double labelling immunohistochemical detection of MUC6 (brown) and Lewis b (blue). Arrowheads in (A), (C), and (D) indicate coexpression in tumour cells (original magnification $\times 400$).

Table 2 Mucin and fucosyltransferase mRNA expression in gastric tumours detected using RT-PCR

	MUC5AC	MUC6	FUT1	FUT2
n=10	+	+	+	+
n=2	+	-	+	+
n=1	-	+	+	+
n=1	+	-	-	+

detected; in two cases, MUC5AC was detected in association with FUT1 and FUT2 in the absence of MUC6 transcripts. In one of these cases, immunohistochemical analysis confirmed expression of MUC5AC, Lewis type 1, and Lewis type 2. This finding supports the conclusion that MUC5AC apomucin glycosylation is dictated by fucosyltransferases. In two additional cases, mucin and FUT transcripts showed other expression patterns (table 2, fig 4). FUT3 transcripts were detected in 12/14 of the tumour samples tested.

DETECTION OF APOMUCINS AND LEWIS ANTIGENS IN PRENEOPLASTIC LESIONS
Because gastric cancers display abnormal patterns of apomucin and Lewis antigen expression, we analysed at which stage in the stomach carcinogenesis process these changes occur using paraffin embedded samples obtained at gastroscopy (table 3). In chronic gastritis (n=10), the pattern of gastric and intesti-

Figure 4 RT-PCR analysis of fucosyltransferase (FUT1 and FUT2) and mucin (MUC5AC and MUC6) transcripts in two gastric tumours (A and B). There was no association between the presence of MUC5AC and MUC6, and FUT2 and FUT1 transcripts, respectively.

nal apomucin expression was similar to that of normal tissues. In one case, MUC6 was detected in both the deep glands and the superficial epithelium. MUC4 was detected in 6/8 cases in the superficial epithelium whereas MUC2 was generally undetectable (1/10). Antral glands were always unreactive with the antibodies against the intestinal mucins. *Helicobacter pylori* was detected in 3/9 samples with gastritis with no correlation with expression of MUC2 and MUC4.

In incomplete and complete intestinal metaplasia, expression of MUC5AC and MUC6 was downregulated in some cases. In contrast, MUC2 and MUC4 expression was activated in most cases of intestinal metaplasia, whether incomplete (n=10) or complete (n=4). The proportion of MUC2 expressing cells was generally higher (80–100%) than that of MUC4 (20–70%). Perinuclear staining was observed with the antibodies detecting mucin tandem repeat sequences whereas vacuolar staining was observed with the LUM5-1 antiserum. Figure 5A shows expression of gastric (MUC6) and intestinal (MUC2) apomucin genes in the same metaplastic cell.

Samples with dysplasia, from patients with intestinal metaplasia and/or neoplasia, showed a pattern of apomucin expression similar to that observed in incomplete intestinal metaplasia: all four apomucin genes were commonly detected. Tumour biopsies obtained at gastroscopy (n=13) were also analysed (table 3). Regardless of the histological subtype, MUC5AC was detected in a higher proportion of tumour biopsies than in surgical specimens. This discrepancy may be due to the fact that some gastric tumours contain cells with gastric features in the superficial areas whereas invasive areas are less differentiated.²² Results obtained with anti-MUC5AC antibodies were comparable when samples from the same patient were analysed using cryostat or paraffin embedded sections.

The pattern of Lewis/apomucin association detected in normal gastric epithelium was not

Table 3 Apomucin expression in gastric lesions obtained at gastroscopy detected by immunohistochemistry using specific antibodies

	Gastritis	Metaplasia		Dysplasia	Carcinoma	
		Incomplete	Complete		Intestinal	Diffuse
MUC2	1/10	10/10	4/4	6/10	6/6	3/7
MUC4	6/8	5/6	4/4	7/8	6/6	1/7
MUC5AC	10/10	8/13	1/3	7/10	5/6	7/7
MUC6	10/10	9/13	1/4	5/9	5/6	3/7

observed in metaplastic and dysplastic lesions, and there was no correlation between expression of apomucin genes and Lewis antigens. These changes, detected in incomplete metaplasia, appear to be early events in the stomach carcinogenesis process. Examples are shown in fig 5B and 5C.

Discussion

The stomach constitutes an excellent model to study the contribution of different mucin genes to mucus function and the relationship between apomucin expression and glycosylation. In our study, we examined: (1) the association of mucin mRNA, FUT mRNA, and Lewis antigen expression in normal and neoplastic stomach samples and (2) changes in apomucin/Lewis antigen expression in lesions representative of the stomach carcinogenesis process, including data on MUC4 apomucin.

Gastric carcinomas are histologically well defined into two main types: intestinal and diffuse. Intestinal tumours are thought to evolve from intestinal metaplasia although the precise sequence of molecular and histological events is not known, whereas diffuse tumours lack recognisable precursor lesions.^{32, 33} Mucin gene expression is altered in both types of gastric tumours: downregulation of MUC5AC and MUC6 associated with the ectopic expression of MUC2 has been reported in stomach tumours using specific antibodies.²¹ Here, we have confirmed and extended previous findings on MUC2, MUC5AC, and MUC6 and have provided evidence that MUC4, an apomucin expressed in the normal intestine but not in the normal stomach, is upregulated in gastric cancers. Abnormal expression of mucin genes in gastric tumours is associated with loss of the coordinated expression of apomucins and

Lewis antigens observed in the normal stomach. Our results using normal gastric mucosal scrapings support the hypothesis that such an association results from co-regulated expression of mucin and FUT genes in a specific tissue pattern rather than from instructive signals provided by the primary sequence of the apomucin, although this idea requires formal demonstration. In stomach tumour cells, this co-regulation is lost at the level of the mucin glycosylation patterns (MUC5AC/Lewis type 1 and MUC6/Lewis type 2) and at the level of mucin and FUT transcripts. Loss of the normal glycosylation patterns detected in tumours is already present in incomplete and complete intestinal metaplastic and dysplastic cells. As previously described, mucin^{12, 13} and glycosyltransferase^{16, 34} genes display characteristic patterns of tissue distribution suggesting that these genes may be co-regulated. Little is known of the genetic elements that control tissue specific expression of mucin and FUT genes. Regarding the latter, they may include the alternative use of multiple promoters as has been described for FUT1.^{35, 36} Regarding the mucin genes, a preliminary analysis of the MUC2 promoter has not disclosed until now sequences for binding of tissue specific transcription factors.³⁷ As for MUC5AC, the amino terminal and 5' flanking region of the MUC5AC gene has been cloned and the 4 kb fragment immediately upstream of the transcription start site contains the elements responsible for transcriptional activation of the reporter in response to *Pseudomonas aeruginosa* exoproducts.³⁸ There are no published data on MUC4 and MUC6 promoters. The availability of cell lines expressing high levels of MUC5AC or MUC2, such as subpopulations of HT-29 cells,^{31, 39} should facilitate analysis of the coordinate, or non-coordinate, expression of mucin and FUT genes.

In intestinal metaplasia, alterations in expression of gastric mucin genes and ectopic detection of MUC2 and MUC3 have been reported.²¹ Recently, two types of intestinal metaplasia have been described with regard to the apomucins detected: in complete metaplasia, upregulation of MUC2 is associated with a decrease in expression of MUC1, MUC5AC,

Figure 5 Expression of apomucins and Lewis antigens in intestinal metaplasia. Double labelling immunofluorescence detection of: (A) MUC2 (green) and MUC6 (red); (B) MUC4 (green) and Lewis b (red); (C) MUC5AC (green) and Lewis y (red). In incomplete intestinal metaplasia, coexpression of gastric and intestinal apomucins at the single cell level is demonstrated. In addition, the association between apomucin and Lewis antigens present in normal stomach is lost (original magnification: A $\times 630$; B, C $\times 400$).

and MUC6; in incomplete metaplasia, co-expression of high levels of MUC2, MUC1, MUC5AC, and MUC6 takes place.⁴⁰ Our studies, including MUC4, confirm these previous data and indicate that MUC4 is also expressed in complete and incomplete intestinal metaplastic and in dysplastic cells. Taken together, these results demonstrate that complete intestinal metaplasia is associated with coordinated activation of expression of MUC2 and MUC4, two mucin genes expressed in the normal intestine, and downregulation of MUC5AC and MUC6, the main mucin genes expressed in the stomach. Therefore, an apparently normal intestinal differentiation programme is established. In contrast, incomplete intestinal metaplasia is associated with expression of both intestinal and gastric mucin genes, indicating concomitant activation of genes associated with two distinct cell lineages in the normal gut. Because incomplete intestinal metaplasia appears to be associated with a high risk for the development of gastric cancer, these studies support the idea that this precursor lesion already harbours genetic or epigenetic alterations that may be related to establishment of an aberrant differentiation programme. Molecular studies should help to establish the genetic events that lead to intestinal gastric cancer as there is evidence that genetically unstable cell clones are detected in both types of intestinal metaplasia and in dysplasia.^{41 42}

Changes in apomucin and Lewis antigen expression in incomplete metaplasia are of potential relevance to the development of cancer as they involve modifications of the gastric ecological niche: carbohydrate epitopes may be important for adhesion of microorganisms to epithelial cells. For example, binding of *Helicobacter pylori* to the normal gastric epithelium has been shown to be mediated by Lewis b antigen⁴³ and the presence of this pathogen has been associated with specific ABO structures.⁴⁴ Abnormal expression of MUC6 and Lewis x in the superficial epithelium of the stomach from *H. pylori* infected patients has been described⁴⁵; however, in our study, MUC6 was almost exclusively detected in the deep glands, regardless of the presence of this pathogen (data not shown). More work is necessary to clarify how changes in expression of mucins and Lewis antigens can affect colonisation of the stomach epithelium by this pathogen. The presence of fucosylated structures has also been reported to be related to colonisation of the gastrointestinal epithelium by bacteria in experimental models. In mice, activation of the α -1,2-fucosyltransferase gene and expression of the corresponding fucosylated products in the small intestinal epithelium require the presence of the normal microflora.⁴⁶ Establishment of experimental models for *H. pylori* infection, such as the FUT3 transgenic mouse,⁴⁷ will be useful for studies on the role of mucin associated carbohydrate epitopes in modulating colonisation of the gastric mucosa and in the development of preneoplastic and neoplastic gastric lesions.

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