Pergamon

European Journal of Cancer Vol. 32A, No. 10, pp. 1788–1796, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00168-2

Original Paper

Alteration in Mucin Gene Expression and Biological Properties of HT29 Colon Cancer Cell Subpopulations

H. Kitamura,[1] M. Cho,[2] B.H. Lee,[2] J.R. Gum,[2] B.B. Siddiki,[2] S.B. Ho,[3] N.W. Toribara,[2] T. Lesuffleur,[4] A. Zweibaum,[4] Y. Kitamura,[1] S. Yonezawa[1] and Y.S. Kim[2]

[1]Kagoshima University School of Medicine, Kagoshima City, Japan; [2]Gastrointestinal Research Laboratory (151M2), VA Medical Center, 4150 Clement St, San Francisco, California 94121-1598, and the University of California, San Francisco, California 94121, U.S.A.; [3]University of Minnesota, Minneapolis, Minnesota, U.S.A.; and [4]INSERM U178, Villejuif, France

Previous studies from our laboratory have shown that HT29 cells selected by adaptation to methotrexate (HT29-MTX) express mature mucins that differ in their immunoreactivity to antibodies against gastric mucin and in the level of one of two major gastric mucin *MUC5AC* (*MUC5*) mRNA compared with parental HT29 cells. In this study, we examined the expression of another major gastric mucin, *MUC6* mRNA, as well as that of *MUC2*, *-3* and *-5* mRNAs in HT29-MTX cells. We also exmained their relationship to mucin-related antigen expression and biological properties of the cells such as adhesion to matrigel and E-selectin and *in vitro* invasiveness, liver colonising activity and degree of differentiation of nude mouse xenograft. Slot blot and Northern analysis revealed markedly increased levels of *MUC5* mRNA but no change in *MUC6* mRNA level in HT29-MTX cells compared with parental HT29 cells which express barely detectable levels of *MUC6* mRNA. A nuclear run-on study showed that *MUC5* mRNA was up-regulated at the transcriptional level. The marked increase in *MUC5* mRNA was associated with a significant increase in the expression of human gastric mucin and apomucin antigens in HT29-MTX cells. When the adhesive capacity of two cell lines was compared, HT29-MTX cells showed significantly lower adhesion to E-selectin consistent with their lower expression of sialyl Le^x and sialyl Le^a antigens compared with HT29 cells. HT29-MTX cells also showed lower adhesive capacity to matrigel than HT29 cells. Interestingly, HT29-MTX cells exhibited significantly decreased liver colonisation capacity in nude mice following splenic vein injection. Furthermore, nude mouse xenograft tumours produced by HT29-MTX cells exhibited a significantly greater degree of differentiation, consisting of mucin-secreting glands than those produced by HT29 cells. In conclusion, these results indicate a shift of predominantly colonic-type mucins to the gastric type, specifically the surface epithelial cell type (MUC5) but not the mucous neck cell or antral gland type (MUC6) in HT29-MTX cells and strongly suggest that altered regulation of mucin genes and the degree of differentiation in cancer cells may be responsible for the altered biological behaviour of these cells.

Key words: mucin genes, colon cancer cell, methotrexate, differentiation
Eur J Cancer, Vol. 32A, No. 10, pp. 1788–1796, 1996

INTRODUCTION

MUCINS ARE very large glycoproteins which provide the framework for the secreted mucus gels and are associated with the epithelial membranes of gastrointestinal, respiratory and genitourinary organs [1, 2]. Although the heterogeneity in the structure of the carbohydrate side chains of mucins has long been recognised [3, 4], the protein backbone (apomucin) structure of mucins has only recently begun to be elucidated [5–19].

Through genomic and cDNA sequencing, at least nine different human mucin genes have been identified to date [7–19]. Interestingly, these mucin genes are expressed in a tissue-specific manner by the highly specialised cell types. For example, *MUC2* and *MUC3* are highly expressed in the small intesine and colon [20], while *MUC5AC* and *MUC6* are highly

Correspondence to Y.S. Kim.
Received 23 Oct. 1995; revised 26 Feb. 1996; accepted 28 Feb. 1996.

expressed in stomach [21–24]. However, these mucin genes are also expressed to some degree in other tissues. With malignant transformation, the tissue-specific mucin gene expression breaks down [20, 21, 25–29].

Recently, we have observed in human cancer cell lines that mucin genes are expressed differentially depending on the growth condition and differentiation states [30–32]. For example, several colon cancer cell lines have been found to express higher levels of mRNA and synthesise and secrete more mucin during their preconfluent state than post-confluency [28, 29]. In other studies with parental HT29 colon cancer cells and two populations of mucus-secreting HT29 cells, selected by adaptation to methotrexate (HT29-MTX)[3] or 5-fluorouracil (HT29-FU), altered levels of expression of various mucin genes and altered immunoreactivity to antibodies against gastric or colonic mucins have been observed [31, 32].

In the present study, we sought to examine further the possible mechanisms of the differential expression of two predominantly colon-associated mucin genes (*MUC2* and *MUC3*) and two predominantly stomach-associated mucin genes (*MUC5AC* and *MUC6*) in parental HT29 cells and HT29-MTX cells. The expression of various apomucins and cancer associated mucin-related carbohydrate antigens were examined. In addition, the capacity to bind to E-selectin and matrigel and *in vitro* invasive capacity and liver colonising (metastatic) properties of both types of cells were also investigated to evaluate the immunological and biological consequences of altered mucin gene expression.

MATERIALS AND METHODS

Cell culture

The parental cell line was obtained from the late Jorgen Fogh [33]. A mucus-secreting subpopulation resistant to 10^{-6} M MTX was obtained as previously reported [34] and is referred to as HT29-MTX. Cells were grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (56°C, 30 min), 100 unit/ml penicillin, and 100 μg/ml streptomycin sulphate. All experiments and maintenance of cells were carried out in 75 cm^2 T flasks (Corning Glassworks, Corning, New York, U.S.A.) at 37°C in a 7% CO_2 93% air atmosphere. Cells were seeded at 1.5×10^6 flask. All cells were harvested 7 or 17 days after confluency.

Antibodies

Monoclonal antibody (MAb) ZE4, a gift from D.M. Swallow (University College London) was raised against crude preparation of human intestinal brush border membranes. This antibody recognises mucins histologically and stains colonic goblet cells but not stomach (unpublished results). MAb M1, which specifically reacts with gastric mucins, was a gift from J. Bara [35]. Rabbit polyclonal antibodies, anti-MRP (produced against MUC2 human intestinal mucin repeat peptide), and anti-M3P (produced against MUC3 human intestinal mucin repeat peptide), were used for studying the extent of the exposure of the unique tandem repeat peptide of each mucin [12, 13]. Rabbit polyclonal antihuman hydrogen fluoride-treated fundic (Apo-F) and antrum (Apo-A) gastric mucin antibodies were prepared as reported [17]. For studying the nature of mucin oligosaccharide, the following lectins and MAbs were used: *Arachis hypogae*, specific for T antigen (Galβ3-GalNAc), 91S8 specific for Tn antigen (GalNAc) [36], JT10e specific for STn antigen (Sialyl Tn) [37], SNH3 specific for SLe^x [Neu5Ac α2-3Galβ1-4 (Fucα1-3) GlcNAc-R] [38] and 19-9H specific for SLe^a [Neu5Acα2-3Galβ1-4(Fucα-1-4)GlcNAc-R] [39].

cDNA probes

MUC2 was detected using SMUC41 cDNA [12], *MUC3* with SIB124 cDNA [13], *MUC5* with a 600 bp tandem repeat cDNA [22] and *MUC6* with NT44 cDNA [17].

Northern blot analysis

Total RNA was extracted from each cell line using guanidinium isothiocyanate and centrifugation through a CsCl cushion [40]. Ten micrograms of each of total RNA sample were fractionated by electrophoresis in 1.5% agarose gels containing 6.6% formaldehyde. A buffer system consisting of 10 mM disodium phosphate (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA was used for electrophoresis. Fractionated samples were transferred to nitrocellulose membranes (Bio-RAD) using 20 × SSC and incubated for 30 min at 80°C in a vacuum oven. Hybridisation was conducted as for prehybridisation except that 1×10^6 cpm/ml of dCTP [^{32}P]-labelled probe and 10% dextran sulphate was added. Membranes were washed with 2 × SSC containing 0.1% SDS 1.5 h at room temperature. Final wash was for 1 h at 55°C in 0.1 × SSC 0.1% SDS, after which autoradiography was performed at −70°C.

RNA slot blots

For slot blot analysis, 10 μg of total RNA was transferred to Zeta probe blotting membrane (Bio-Rad) using ice cold 10 mM NaOH, 1 mM EDTA. After transfer, membranes were rinsed in 2 × SSC, 0.1% SDS and incubated for 30 min at 80°C. Hybridisation and autoradiography were carried out as for Northern blot analysis.

Nuclear run-on assay

The procedure used was performed as described previously [41]. Nuclei were isolated by differential centrifugation and stored frozen at −70°C. Transcription reactions were carried out by the addition of 60 μl of 5 × run-on buffer (25 mM Tris HCl, pH 8.0, 1.25 mM $MgCl_2$, 750 mM KCl, 2 mM levels of ATP, GTP and CTP, 0.5 mM EDTA, 2.5 mM DTT, 35% glycerol) and 30 μl of [^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml) to 210 μl of nuclei storage buffer containing 2×10^7 nuclei. Incubation was for 30 min at 26°C. Transcription was terminated by addition of 200 U/ml of RNase-free DNase I, 150 U/ml RNasin (RNase inhibitor), and 60 mg/ml of yeast t-RNA in buffer containing 10 mM Tris–HCl, 1 mM EDTA, 0.1 M NaCl (pH 7.4) and incubated for 15 min at 26°C. Proteinase and SDS (750 mg/ml and 1% final concentration, respectively) were added to the reaction mixture and incubated for 30 min at 37°C. Labelled nuclear RNA was isolated as described [41]. The final RNA pellet was suspended in 10 mM Tris–HCl containing, 0.3 M NaCl, 0.15% SDS, 1 mM EDTA, pH 8.0.

Plasmid (3 μg) containing probe cDNA (0.5 μg) of *MUC5* and *MUC6* were denatured in 0.3 M NaOH and 10 mM EDTA for 30 min at 65°C and then neutralised with the same amount of ice cold 2 M ammonium acetate. The pBluescript and GADPH-containing plasmids were used for negative and positive controls. Plasmid cDNAs and probe cDNAs were applied to a nylon membrane (ICN, California, U.S.A.) using

a slot blot apparatus (Bio-RAD), and the membrane was incubated for 1 h at 80°C. The membrane was then prehybridised for 18 h at 65°C in 10 mM TES (pH 7.4) containing 0.2% SDS, 0.3 M NaCl, 10 mM EDTA, 5 mg/ml yeast t-RNA, 50 mg/ml salmon sperm DNA, 0.05% sodium pyrophosphate, and 2 × Denhardt's solution.

Hybridisation was conducted for 48 h at 65°C using 1×10^6 cpm/ml of ^{32}P-labelled nuclear RNA in 10 mM TES, 0.3 M NaCl, 10 mM EDTA and 0.2% SDS. Then the membrane was washed, rinsed and air dried, and autoradiography was carried out at −70°C [40].

Southern blot analysis

DNA was prepared using proteinase K and RNase digestion and phenol-chloroform extraction. For blotting, genomic DNAs were digested with restriction enzymes HinfI for *MUC2*, BamHI for *MUC3* and *MUC5*, and TaqI for *MUC6*.

Immunoblot

For protein slot blot, 7.5 μg cell homogenates were applied to nitrocellulose membranes using a Bio-Dot-Apparatus (BioRad Labs, Richmond, California, U.S.A.). The nitrocellulose membranes were blocked overnight in blocking buffer (5% bovine serum albumin in PBS). The membranes were incubated for 1 h first with antibody and then washed with PBS containing 0.05% Nonidet P-40 six times. The membranes were then incubated with secondary antibody and washed again. Then membranes were finally incubated with ^{125}I-labelled protein A (1×10^6 cpm/ml) in blocking buffer for 1 h and then the membranes were washed as above. Autoradiography was carried out using X-OMat AR-2 film (Kodak) exposed to the nitrocellulose membranes at −70°C. Individual bands were quantified using gel analysis software and a computer.

E-selectin (ELAM-1) binding assay

Adhesion of cells to a soluble chimeric protein consisting of the extracellular portion of E-selectin (ELAM-1) and a fragment of human IgG1 (ELAM-Rg) was measured on 96-well plates. ELAM-Rg (0.25 μg/well) and chimeric protein CD7 (a gift from A. Aruffo, Bristol Myers-Squibb, Seattle, Washington, U.S.A.) and IgG1 as control was adsorbed to the plate at 4°C overnight. The wells were blocked with 1% bovine serum albumin (BSA), 1×10^5 cells were added and the plate was incubated for 1 h at RT with rotation. After washing with PBS, the attached cells were quantitated with the MTT colorimetric method.

Cell adhesion assay

Basement membrane matrigel (BD, Bedford, Massachusetts, U.S.A.), an extract of Englebreth-Halm Swarm tumour, was used to coat the wells (8 μg/well) of 96 plates at 37°C for 2 h. The coated wells were washed with serum-free medium, and cells added at the concentration of 1×10^5 cells/well in the serum-free medium. After incubation, the plate was gently washed and attached cells were incubated with 3-(4,5-dimethylthiazole-2y1,-2,5 diphenyl-2,4 tetrazolium bromide (MTT, Sigma) for 1 h at room temperature followed by removal of supernatant. The cells were washed three times with PBS, and the dye bound by attached cells was dissolved in DMSO and the absorbance was measured at 540 nm. The percentage of attached cells was calculated. For cell adhesion and E-selectin binding assays and *in vitro* assays, 7-day post-confluent cells were used.

In vitro *invasion assay*

For evaluating the ability of tumour cells to penetrate basement membrane matrigel, transwell cell culture chambers (Costar, Cambridge, Massachusetts, U.S.A.) were used. Matrigel, 100 μl diluted 1:10 in serum-free medium (120 μg/well), was applied to the 8 μm pore polycarbonate filter. Cells (2×10^5) were added and incubated for 48 h. The percentage of invasive cells was determined by the MTT colorimetric method.

Liver colonisation assay

The ability of the tumour cells to colonise the liver after intrasplenic injection was tested as previously described [42, 43]. Tumour cells were grown in 75 cm^2 tissue flasks, harvested, and resuspended in serum-free DMEM at a concentration of 10^7 cells/ml. Athymic nude mice were anaesthetised, prepared in a sterile environment, and the spleen exteriorised through a flank incision. One million cells in 0.1 ml were slowly injected into the splenic pulp through a 30 gauge needle over 1 min followed by splenectomy 2 min later. The nude mice were sacrificed 4 weeks later. The tumour burden of the nude mouse liver after injection of HT29 or HT29-MTX cells was assessed by liver weight and the number of tumour nodules in the liver.

Xenograft of athymic nude mice

Cells in 100 μl of serum-free medium were injected (10^6) subcutaneously into the shoulder of the nude mice, and the xenograft tumours were removed when they were approximately 5 mm in diameter for histological study. The sections were stained with Alcian blue periodic acid-Schiff (AB-PAS) stains [44].

RESULTS

Expression of mRNA

Slot blot analysis of total RNA extracted from HT29 cells and HT29-MTX cells showed that *MUC2* mRNA was strongly expressed in HT29 cells at 7-day postconfluency but its expression was markedly lower (25%) in HT29-MTX cells at the same stage of confluency (Figure 1). Interestingly, *MUC2* mRNA was expressed at much lower levels (20%) in HT29 cells at 17-day confluency compared with 7 days post-confluency, but no significant difference in the level of *MUC2* mRNA expression was observed between the two cell types at 17-day postconfluency. *MUC3* mRNA levels were similar between the two cell types at 7-day post-confluency and both types of cells showed increased (2-fold) mRNA levels at

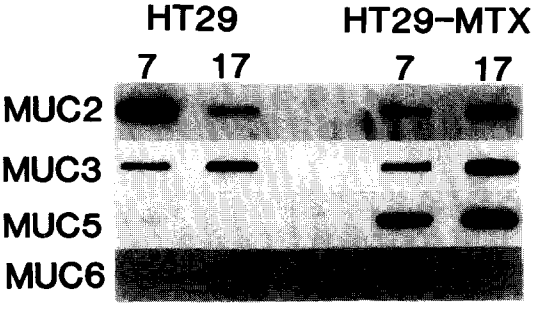

Figure 1. Slot blot analysis of mucin mRNA. Slot blots containing total cellular RNA (10 μg) were hybridised with various mucin cDNA probes. HT29 cells and HT29-MTX cells at 7- and 17-day postconfluency were examined.

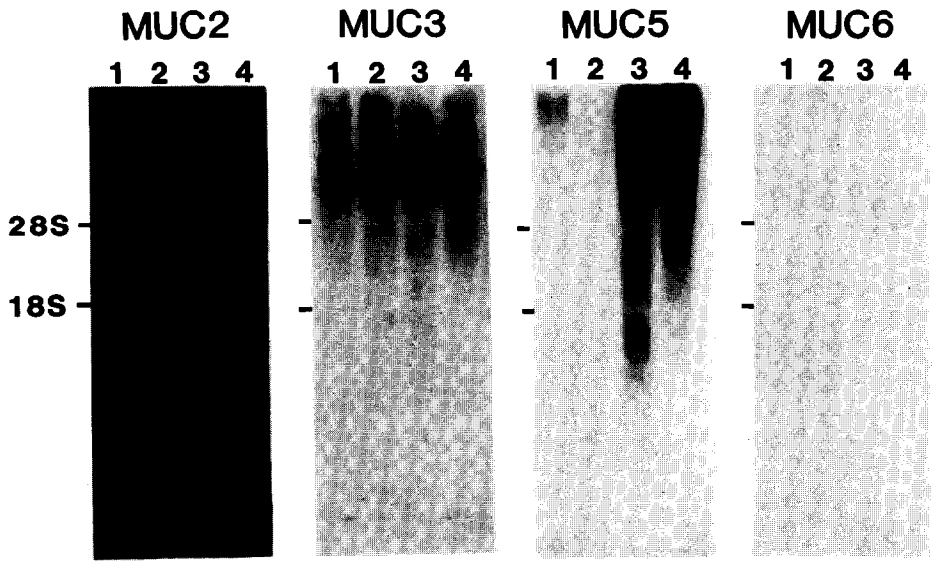

Figure 2. Northern blot analysis. Total cellular RNA (10 μg) was hybridised with *MUC2*, *-3*, *-5* and *-6* cDNA probes. Lane 1, HT29 7 day; lane 2, HT29 17 day; lane 3, HT29-MTX 7 day; lane 4, HT29-MTX 17 day. Day indicates days of postconfluency.

17-day postconfluency, although the degree of increase was greater for HT29-MTX cells. However, the most striking observation was the marked higher levels of the *MUC5* mRNA in HT29-MTX cells at both time periods compared with HT29 cells. *MUC6* message was barely detectable in both lines at both time periods. The results of the Nothern blot analysis were consistent with the slot blot data (Figure 2).

Nuclear run-on assay

The results of nuclear run-on assays are shown in Figure 3 where transcription rates of the *MUC5* and *-6* genes are compared in the two cell lines at days 7 and 17 postconfluency. *MUC5* transcription rates were clearly higher in HT29-MTX cells for both time periods compared with HT29 cells. The transcription rates appeared to be lower in HT29 cells for *MUC5* and *-6* on day 17 postconfluency compared with day 7 postconfluency. However, the bands were too faint to determine if a difference was present in HT29-MTX cells between the two time periods.

Southern blot analysis

Figure 4 shows Southern blot analysis of genomic DNA samples from each cell line on days 7 and 17 postconfluency. *MUC2* cDNA was hybridised with genomic DNA samples digested with the restriction enzyme HinfI (Figure 4). One major band at 7.0 kb is observed in all lanes. In addition, one higher molecular weight band was observed in HT29-MTX cells at 7-day confluency. When *MUC3* cDNA was hybridised with genomic DNAs digested with BamH1, there were multiple bands of similar sizes in all lanes (Figure 4). When the DNA fragment digested with BamH1 was hybridised with *MUC5* cDNA, all blots had a strong band at around 21 kb (Figure 4). When the DNA fragment digested with TaqI was hybridised with *MUC6* cDNA, all samples had a strong band at about 16 kb and another band at approximately 21 kb (Figure 4). Thus, in this Southern blot analysis, DNA samples from both HT29 cells and HT29-MTX cells at both time periods showed similar patterns and intensity for all mucin cDNA probes employed.

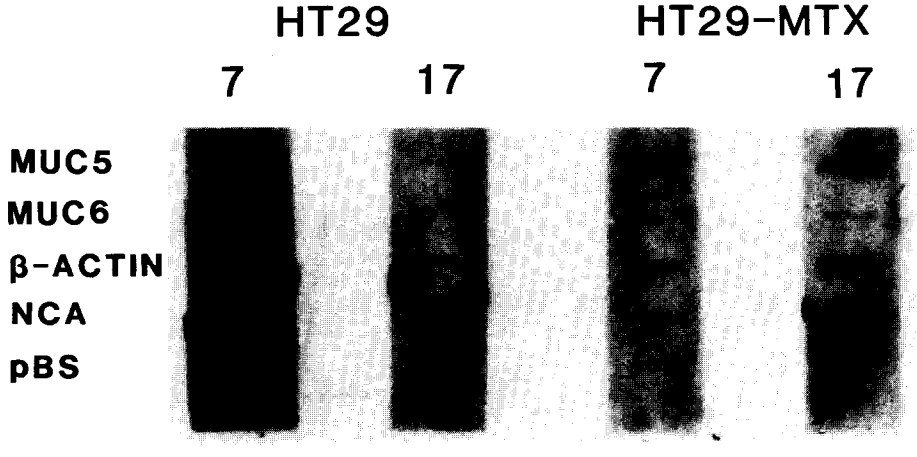

Figure 3. Nuclear run-on assay. Nuclei was isolated by differential centrifugation. 300 μCi of [^{32}P]UTP and 2 × 10^7 cell nuclei were used per reaction and 10 μg of probe was applied per lane. pBS, plasmid Bluescript, is a negative control and NCA is a non-specific cross-reacting antigen.

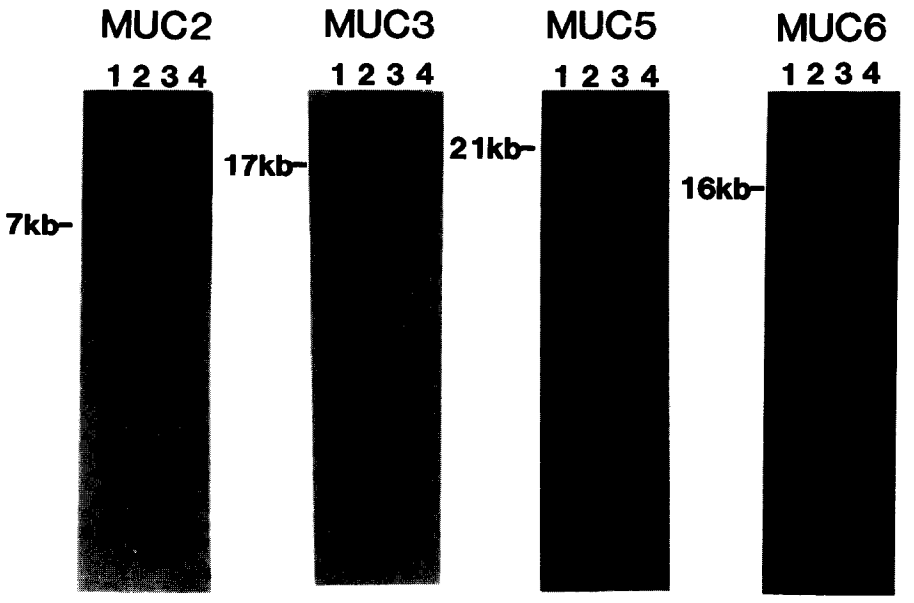

Figure 4. Southern blot analysis. DNA samples from HT29 and HT29-MTX cells after electrophoresis were hybridised as described in Northern analysis. Lane 1, HT29 7 day; lane 2, HT29 17 day; lane 3, HT29-MTX 7 day; lane 4, HT29-MTX 17 day.

Immunoblots

Three antibodies specific to gastric mucins (MAb M1 and polyclonal antibody anti-apo A and anti-apo F) were used. MAb M1, which recognises gastric mucin peptide and/or saccharide [35], showed a marked higher level (at least 25-fold) in HT29-MTX cells compared with HT29 cells at both time periods (Figure 5). With anti-apo A and anti-apo F, antibodies which recognise apomucin (apoprotein) epitope, a 3–6-fold higher level was observed in HT29-MTX cells compared with HT29 cells. In contrast, no difference between the two cell types was observed when three antibodies to colonic mucins (MAb, ZE4, which recognises colonic mucin peptide and/or carbohydrate (personal communication, D.M. Swallow) and polyclonal antibodies, anti-MRP and anti-M3P which recognise synthetic tandem repeat peptides of MUC2 and MUC3 were used.

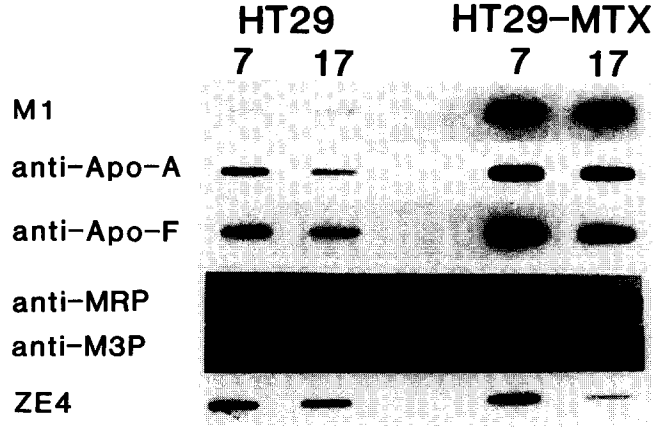

Figure 5. Immunoblots. Slot blot analysis using MAb M1 (gastric mucin M1 epitope), or rabbit polyclonal antibodies, anti-Apo-A (antrum gastric apomucin), anti-Apo-F (fundic gastric apomucin), anti-MRP (tandem repeat peptide of MUC2), anti-M3P (tandem repeat peptide of MUC3), and MAb ZE4 (normal human small intestinal mucosa).

The nature of mucin oligosaccharides was also studied by slot blot analysis using antibodies. When the blots were incubated with *Arachis hypogaea* lectin, T antigen expression was higher (by 4–5-fold) in HT29-MTX cells at both time periods compared with HT29 cells. With MAb 91S8 (specific for Tn antigen), a 3-fold higher level was observed in HT29-MTX cells compared with HT29 cells. However, there was a lower intensity of bands with JT10e (specific for STn antigen) and 19-9H (specific for SLe^a) in HT29-MTX cells compared with HT29 cells, as was the case with SNH_3 MAb (specific for SLe^x) (Figure 6).

Binding to matrigel and E-selectin

HT29-MTX cells adhered less well to matrigel than HT29 cells (Figure 7). Similarly, HT29-MTX cells showed less binding to E-selectin compared with HT29 cells (Figure 7). This latter observation is consistent with the lower expression of SLe^a and LSe^x antigens in HT29-MTX cells which have been reported to be ligands for E-selectin on activated endothelial cells.

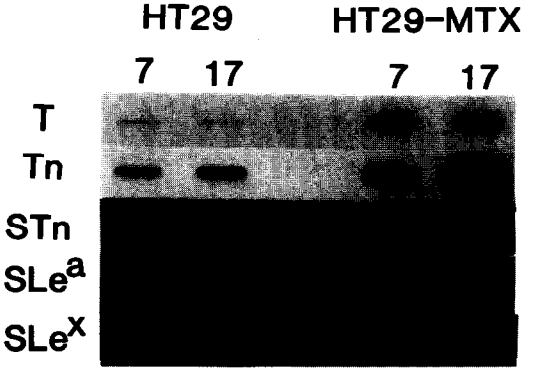

Figure 6. Immunoblots. Slot blot analysis using a lectin, *Arachis hypogae* (T antigen), and MAb 91S8 (Tn antigen), MAb JT10e (Sialyl Tn), and MAb 19-9H (SLe^a), MAb SNH3 (SLe^x).

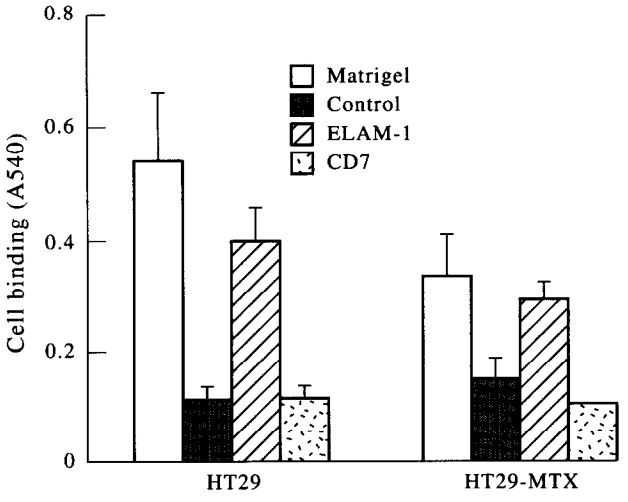

Figure 7. Adhesion and E-selectin binding assays. Adhesion of cells to matrigel was determined by MTT colorimetric method ($P < 0.005$). 0.02% BSA was used as control. Columns represent means of 18 experiments (±S.D.). Binding of cells to chimeric protein of extracellular region of E-selectin and human IgG (ELAM-1) were determined by MTT colorimetric methods ($P < 0.005$). The chimeric protein of CD7 and IgG1 was used as control. Columns represent means of 12 experiments; bars (±S.D.).

In vitro *invasion*

Although HT29-MTX cells tended to have slightly lower *in vitro* invasion activity than HT29 cells, this difference did not reach a statistical significance using Student's *t*-test ($P \geq 0.05$) (Figure 8).

Liver colonisation

Liver colonisation after splenic injection of tumour cells in the nude mouse provides a method for evaluating the last phases of metastasis [42, 43]. When liver tumour burden was examined 4 weeks after the intrasplenic injection of 7-day postconfluent HT29 or HT29-MTX cells, there was a marked difference between the two groups. Nude mice injected with HT29 cells showed liver colonisation in 6 of 6 mice while only 1 of 6 nude mice injected with HT29-MTX cells showed liver colonisation. Moreover, the number of nodules and the weight of the liver of the nude mice injected with HT29 cells was much greater than those injected with HT29-MTX cells (Table 1).

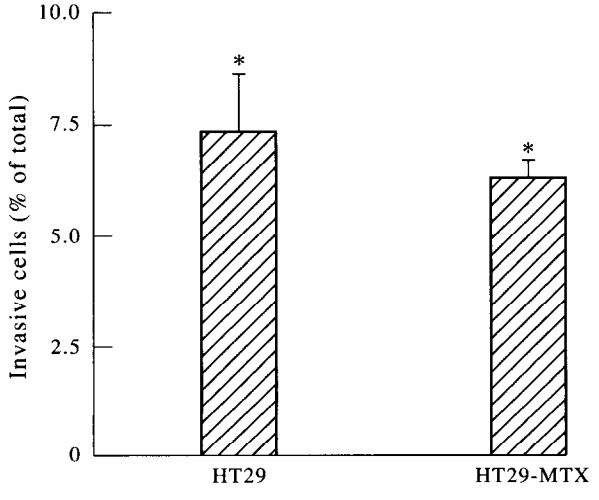

Figure 8. *In vitro* invasion assay. Matrigel was applied to trans-well cell culture chambers. Cells (2×10^5) were applied and incubated for 48 h. The percentage of invasive cells was determined by MTT colorimetric method (see Materials and Methods for details). Columns represent means of six experiments (±S.D.). *, not significant.

Xenograft morphology

AB-PAS staining of the nude mouse xenograft tumours injected with HT29-MTX cells showed a marked increase in the numbers of differentiated mucin secreting glands compared with those injected with HT29 cells (Figure 9).

DISCUSSION

Recently, we reported that HT29-MTX cells in 7-, 14- and 21-day postconfluency showed marked increases in steady state levels of both *MUC3* and *MUC5* mRNAs with predominant gastric mucin cell type immunoreactivity, whereas 7- and 14-day postconfluent HT29-FU cells expressed markedly increased steady state mRNA levels of *MUC2* with predominant colonic cell type mucin immunoreactivity [32]. In the present study, we sought to examine in more detail the possible mechanisms involved in altered mucin gene expression, and its relationship to mucin related antigen expression and biological characteristics such as adhesion to the extracellular matrix, E-selectin, *in vitro* invasiveness and liver colonising activity of HT29-MTX cells. The present study confirmed our previous observation that marked reduction in steady state mRNA levels of *MUC2* and a slight to moderate increase in *MUC3* mRNA levels occur in HT29-MTX cells [32]. Since *MUC2* mRNA is highly expressed in the goblet cells of intestine while *MUC3* is expressed in both the goblet cells and absorptive cells [20], this result suggests differential regulation of two intestinal mucin genes.

Interestingly, of two major gastric mucin related genes, *MUC5* and *MUC6* [21–24], only *MUC5* mRNA was found to be markedly elevated. *MUC6* mRNA could not be detected in either HT29 or HT29-MTX cells. Thus, HT29-MTX cells upregulate *MUC5* mRNA, but not *MUC6* mRNA, indicating a shift of predominantly colonic type mucins in parental cell line HT29 to a gastric type, specifically the surface epithelial cell type (*MUC5*) but not the mucous neck cell or antral gland type (*MUC6*) in HT29-MTX cells. The nuclear run-on data showed marked increases in *MUC5* gene transcription indicating that an increase in *MUC5* gene expression in HT29-MTX cells is regulated at the transcriptional level. The amplification of the dihydrofolate reductase gene is a well-known mechanism of resistance to MTX and the changes in phenotype of MTX resistance at various concentrations of MTX has been

Table 1. Liver colonisation after intrasplenic injection of HT29 and HT29-MTX cell lines

Cell line	Number of metastasis-bearing mice	Liver (weight* in grams)	Number of tumour nodules
HT29	6/6	2.44 ± 0.85†	>14
HT29-MTX	1/6	1.04 ± 0.13	3

Hepatic tumour burden 4 weeks after intrasplenic injection of 10^6 tumour cells. *Mean ± S.D., †$P < 0.01$.

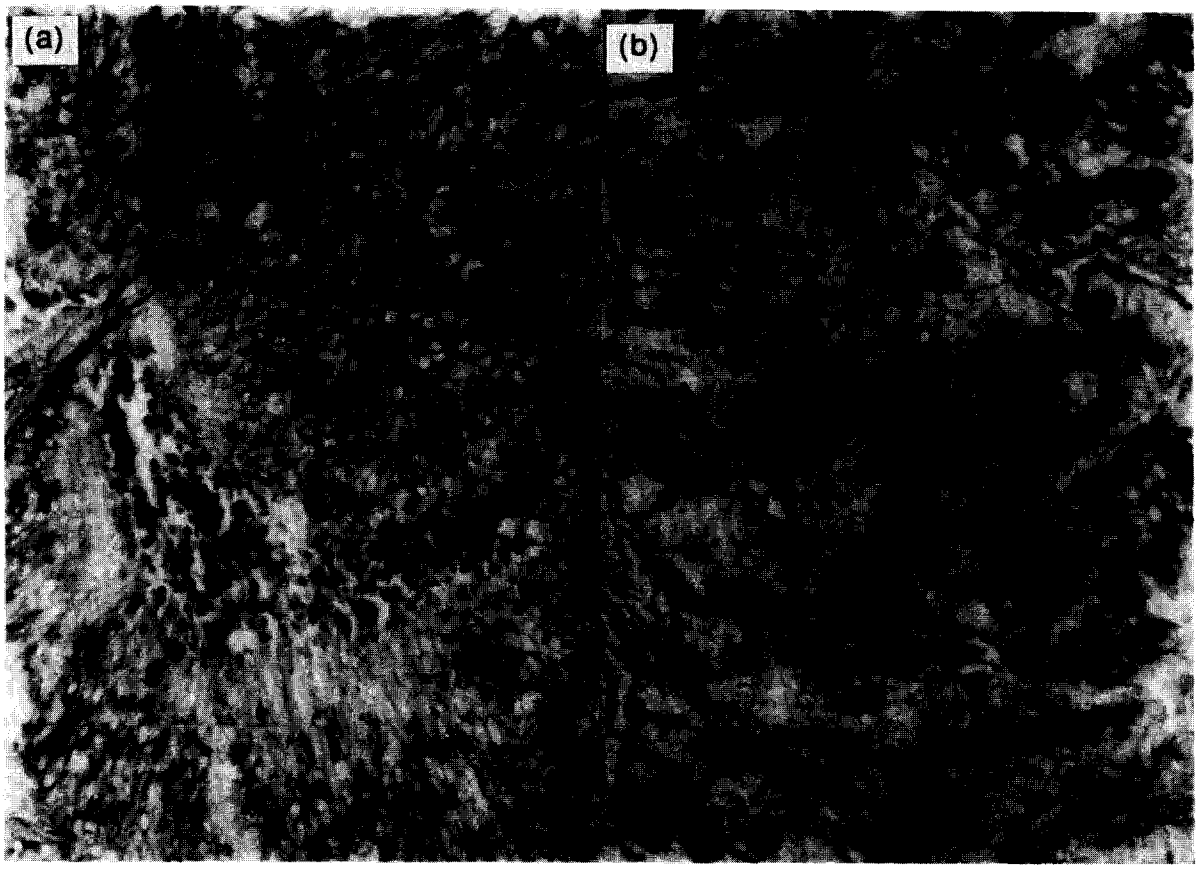

Figure 9. Xenograft tumours. Xenograft tumours from the athymic nude mice injected with HT29 (a) and HT29-MTX (b), respectively are stained with Alcian blue-periodic acid Schiff (AB/PAS).

shown to be correlated with the amplification of this enzyme [45]. However, there was no obvious amplification or rearrangement of the *MUC5* mucin gene or other mucin genes examined as indicated by our Southern analysis data. The markedly increased expression of gastric mucin (M1) and gastric apomucin (A-HG and F-HF) in HT29-MTX cells compared with HT29 cells is consistent with an observed increase in message. Although the mechanisms responsible for this change are not clear, the following possibilities may be considered: (1) MTX may induce gastric surface mucous cell type metaplasia in HT29 cells; (2) MTX may exert selective pressure on a subpopulation of cells exhibiting a gastric surface mucous cell phenotype; or (3) alternatively, MTX may up-regulate transcription of *MUC5* by interacting directly or indirectly with the control elements of the gene. Clearly, further studies are necessary to elucidate the mechanism of this important phenomenon.

Differences in the sialylation of cell surface glycoproteins and glycolipids between cells of low and high metastatic potential have been reported [46–48]. Recent studies indicate that when primary and metastatic colon cancer tissues are compared, the pattern of expression of tumour-associated mucin-related carbohydrate antigens is different. In metastatic colon cancer cells, the level of expression of Tn and T antigens are reduced while those of sialyl Tn and sialyl T antigens are increased, as was the sialyl Le^x antigen [49–41]. These data suggest that increased sialylation of mucin type glycoproteins are correlated with increased metastatic properties of cancer cells. Although the precise mechanisms involved in this association are not well understood, sialyl Le^x and sialyl Le^a antigens have been demonstrated to bind to vascular endothelium through E-selectin, which may result in increased colonisation of liver by the metastatic cancer cells [52–54].

While these antigens are examined, HT29-MTX cells show markedly increased T and Tn antigen expression concomitant with reduction in the expression of sialylated antigens such as sialyl Tn, sialyl Le^a and sialyl Le^x antigens. Thus, one might speculate that HT29-MTX cells may have less liver colonising activity than HT29 cells due, in part, to their reduced ability to adhere to endothelial cells in the liver. Indeed, our studies clearly showed that HT29-MTX cells have markedly reduced E-selectin binding activity and liver colonising activity compared with HT29 cells. However, it is not known whether the increased expression of *MUC5* is responsible for these properties in HT29-MTX cells.

There was a trend for slightly less invasive activity of HT29-MTX cells. However, no statistical significance was achieved. This result may be due in part to the long time (48 h) that is required for the *in vitro* invasion assay which may lead to some reversion of the time and density dependent phenotypes of the cells. Although the precise mechanism is not clear, the reduced binding of HT29-MTX cells to matrigel may in part reflect the decreased sialylation of mucin and/or increased expression of T and Tn antigens in these cells. A marked increase in differentiated mucin secreting glands in nude mouse xenograft tumours produced by HT29-MTX cells compared with those produced by HT29 cells indicates that HT29-MTX cells selected by adaptation to MTX represents differentiated

mature mucous cells of the gastric surface epithelial type. Acquisition of differentiated phenotypes by HT29 cells may in part be responsible for MTX resistance of HT29 cells selected by adaptation to MTX.

Further studies are necessary to clarify the mechanisms by which MTX resistant HT29 cells up-regulate the *MUC5* gene, express reduced levels of sialylated carbohydrate antigens and acquire reduced liver colonising activity and differentiated morphology. However, these data support the hypothesis that the expression of distinct mucin species and cellular differentiation may play an important role in the biological behaviour of normal and cancer cells.

1. Neutra MR, Forstner JF. Gastrointestinal mucus: synthesis, secretion, and function. In Johnson LR, ed. *Physiology of the Digestive Tract.* Raven Press, 1987, 975–1009.
2. Rose MC. Mucins: structure, function and role in pulmonary diseases. *Am J Physiol* 1992, **263**, L413–L429.
3. Podolsky DK. Oligosaccharide structures of isolated human colonic mucin species. *J Biol Chem* 1985, **260**, 15510–15515.
4. Capon C, Laboisse CL, Wieruszeki JM, Maoret JJ, Augeron C, Fournet B. Oligosaccharide structures of mucins secreted by the human colonic cancer cell line CL.16E. *J Biol Chem* 1992, **267**, 19248–19257.
5. Kim YS, Gum JR, Byrd JC, Toribara NW. The structure of human intestinal apomucins. *Am Rev Res Dis* 1991, **144**, S10–S14.
6. Timpte CS, Eckhardt AE, Abernethy JL, Hill RL. Porcine submaxillary gland apomucin contains tandemly repeated identical sequences of 81 residues. *J Biol Chem* 1988, **263**, 1081–1088.
7. Siddiqui J, Abe M, Hayes D, Shani E, Yunis E, Kufe D. Isolation and sequencing of a cDNA coding for the human DF3 breast carcinoma-associated antigen. *Proc Natl Acad Sci USA* 1988, **85**, 2320–2323.
8. Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, *et al.* Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem* 1990, **265**, 15286–15293.
9. Wreschner DH, Hareuveni M, Tsarfaty H, *et al.* Human epithelial tumor antigen cDNA sequences. Differential splicing may generate multiple protein forms. *Eur J Biochem* 1990, **189**, 463–473.
10. Ligtenberg MJL, Vos HL, Gennissen AMC, Hilkens J. Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. *J Biol Chem* 1990, **265**, 5573–5578.
11. Lan MS, Batra SK, Qi W, Metzgar RS, Hollingsworth MA. Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J Biol Chem* 1990, **265**, 15294–15299.
12. Gum JR, Byrd JC, Hicks JW, Horibara NW, Lamport DTA, Kim YS. Molecular cloning of human intestinal mucin cDNAs: sequence analysis and evidence for genetic polymorphism. *J Biol Chem* 1989, **264**, 6480–6487.
13. Gum JR, Hicks JW, Swallow DM, *et al.* Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. *Biochem Biophys Res Commun* 1990, **171**, 407–415.
14. Aubert JP, Porchet N, Crepin M, *et al.* Evidence for different human tracheobronchial mucin peptides deduced from nucleotide cDNA sequences. *Am J Respir Cell Mol Biol* 1991, **5**, 178–185.
15. Porchet N, Van Cong N, Dufosse J, *et al.* Molecular cloning and chromosomal localization of a novel human tracheo-bronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. *Biochem Biophys Res Commun* 1991, **175**, 414–422.
16. Meerzaman D, Charles P, Daskal E, Polymerophoulos MH. Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (MUC5). *J Biol Chem* 1994, **269**, 12932–12939.
17. Toribara NW, Roberton AM, Ho SB, *et al.* Human gastric mucin: identification of a unique species by expression cloning. *J Biol Chem* 1993, **268**, 5879–5885.
18. Bobek LA, Tsai H, Biesbrock AR, Levine MJ. Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J Biol Chem* 1993, **268**, 20563–20569.
19. Shankar V, Gilmore MS, Elkins RC, Sachdev GP. A novel human airway mucin cDNA encodes a protein with unique tandem repeat organization. *Biochem J* 1994, **300**, 295–298.
20. Chang S-K, Dohrman AF, Basbaum CB, *et al.* Localization of intestinal mucin (MUC2 and MUC3) mRNA and apomucin in human normal intestine and colon cancer tissues by *in situ* hybridization and immunohistochemistry. *Gastroenterology* 1994, **107**, 28–36.
21. Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP. Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by *in situ* hybridization. *J Histochem Cytochem* 1993, **41**, 1479–1485.
22. Ho SB, Roberton AM, Shekels L, Lyftogt CT, Niehans GA, Toribara NW. Expression of cloning of gastric mucin complementary DNA and localization of mucin gene expression. *Gastroenterology* 1995, **109**, 735–747.
23. de Bolós C, Garrido M, Real FX. MUC6 apomucin shows a distinct normal tissue distribution that correlates with Lewis antigen expression in the human stomach. *Gastroenterology* 1995, **109**, 723–734.
24. Kim YS, Gum JR. Diversity of mucin genes, structure, function and expression (editorial). *Gastroenterology* 1995, **109**, 999–1013.
25. Byrd JC, Ho JJL, Lamport DTA, *et al.* Relationship of pancreatic cancer apomucin to mammary and intestinal apomucins. *Cancer Res* 1991, **51**, 1026–1033.
26. Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. *Cancer Res* 1992, **52**, 5971–5978.
27. Ho SB, Niehans GA, Lyftogt CT, *et al.* Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res* 1993, **53**, 641–651.
28. Balagé C, Gambús G, Carrato C, *et al.* Altered expression of MUC2, MUC4, and MUC5 mucin genes in pancreas cancer cell lines and tissues. *Gastroenterology* 1994, **106**, 1054–1061.
29. Yan PS, Ho SB, Itzkowitz SH, Byrd JC, Siddiqui B, Kim YS. Expression of native and deglycosylated colon cancer mucin antigens in normal and malignant epithelial tissues. *Lab Invest* 1990, **62**, 698–706.
30. Niv Y, Byrd JC, Ho SB, Dahiya R, Kim YS. Mucin synthesis and secretion in relation to spontaneous differentiation of colon cancer cells *in vitro*. *Int J Cancer* 1992, **50**, 147–152.
31. Dahiya R, Lesuffleur T, Kwak KS, Zweibaum A, Kim YS. Methotrexate resistance of HT29 human colonic adenoma cells is associated with increased mucin synthesis and secretion. *Cancer Res* 1992, **52**, 4655–4662.
32. Lesuffleur T, Porchet N, Aubert J-P, *et al.* Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. *J Cell Science* 1993, **106**, 771–783.
33. Fogh J, Trempo G. Hew human tumor cell lines. *Human Tumor Cells In Vitro.* Plenum, 1975, 115–141.
34. Lesuffleur T, Barbat A, Dussalulx E, Zweibaum A. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. *Cancer Res* 1990, **50**, 6334–6343.
35. Bara J, Gautier R, Daher N, Zaghouani H, Burtin P. Monoclonal antibodies against oncofetal mucin M1 antigen associated with precancerous colonic mucosae. *Cancer Res* 1986, **46**, 3983–3989.
36. Huang J, Siddiki B, Byrd JC, Kim YS. Monoclonal antibodies against partially deglycosylated colon cancer mucin that recognize Tn antigen. *Disease Markers* 1992, **10**, 81–94.
37. Siddiki BB, Huang J, Ho JJL, *et al.* Monoclonal antibody directed against colon cancer mucin has high specificity for malignancy. *Int J Cancer* 1993, **54**, 467–474.
38. Muroi K, Suda T, Nojiri H, *et al.* Reactivity profiles of leukemic myeloblasts with monoclonal antibodies directed to sialosyl Le^x and other lacto series type 2 chain antigens: absence of reactivity with normal hematopoietic progenitor cells. *Blood* 1992, **79**, 713–719.
39. Magnani JL, Steplewski Z, Koprowski H, Ginsburg V. Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19–9 in the sera of patients as a mucin. *Cancer Res* 1983, **43**, 5489–5492.
40. Chirgwin JM, Przybyla AF, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. *Biochemistry* 1978, **18**, 5294–5299.

41. Celano P, Baylin SB, Casero RA. Polyamines differentially modulate the transcription of growth associated genes in human colon carcinoma cells. *J Biol Chem* 1989, **264**, 8922–8927.
42. Bresalier RS, Boland CR, Kim YS. Characteristics of colorectal carcinoma cells with high metastatic potential. *Gastroenterology* 1984, **87**, 115–122.
43. Bresalier RS, Raper SE, Hujanen ES, Kim YS. A new animal model for human colon cancer metastasis. *Int J Cancer* 1987, **39**, 625–630.
44. Fillipe MI. Mucins in the human gastrointestinal epithelium: a review. *Invest Cell Pathol* 1979, **2**, 295–316.
45. Lesuffleur T, Barbat A, Luccioni C, *et al.* Dihydrofolate reductase gene amplification-associated shift of differentiation in methotrexate-adapted HT29 cells. *J Cell Biol* 1991, **115**, 1409–1418.
46. Bresalier RS, Rockwell RW, Dahiya R, Kim YS. Cell surface sialoprotein alterations in metastatic murine colon cancer cell lines selected in an animal model for colon cancer metastasis. *Cancer Res* 1990, **50**, 1299–1307.
47. Passaniti A, Hart GW. Cell surface sialylation and tumor metastasis. *J Biol Chem* 1988, **263**, 7591–7603.
48. Dennis JW, Leferte S. Tumor cell surface carbohydrate and the metastatic phenotype. *Cancer Metast Rev* 1987, **5**, 185–204.
49. Bresalier RS, Ho SB, Kim YS. Alterations in mucin type glycoproteins in tumor colorectal cancer metastases. *Gastroenterology* 1990, **98**, A274.
50. Itzkowitz SH, Bloom EJ, Kokal WA, *et al.* Sialosyl-Tn: a novel mucin antigen associated with poor prognosis in colorectal cancer patients. *Cancer* 1990, **66**, 1960–1966.
51. Matsushita Y, Clearly KR, Ota DM, Hoff SD, Irimura T. Sialyldimeric Le^x antigen expressed on mucin-like glycoproteins in colorectal cancer metastasis. *Lab Invest* 1990, **63**, 780–791.
52. Lauri D, Needham L, Martin-Padura T, Dejana E. Tumor cell adhesion to endothelial cells: endothelial leucoctye adhesion moleule-1 as an inducible adhesive receptor specific for colon carcinoma cells. *J Natl Cancer Inst* 1991, **83**, 1321–1324.
53. Kojima N, Handa K, Newman W, Hakomori S. Inhibition of selectin dependent tumor cell adhesion to endothelial cells and platelets by blocking O-glycosylation of these cells. *Biochem Biophys Res Commun* 1992, **182**, 1288–1295.
54. Irimura T, Nakamori S, Matsushita Y, *et al.* Colorectal cancer metastasis determined by carbohydrate-mediated cell adhesion: role of sialyl Le^x antigens. *Cancer Biol* 1993, **4**, 319–324.

Acknowledgements—We thank Ms Rita Burns for her expert assistance in the preparation of this manuscript. This study was supported by Grants AM17938 and CA45967 from NIH and by the Department of Veterans Affairs Medical Research Service.