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Original Paper

Mucins Secreted by Cell Lines Derived from Colorectal Mucinous Carcinoma and Adenocarcinoma

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Mucinous (colloid) carcinoma and well- to moderately-differentiated adenocarcinoma of the colon differ in the pattern and the amount of mucin secretion and perhaps in their behaviour and clinical outcome. To ascertain why these differences exist and to elucidate the mechanisms of tumour progression, we examined two model human cell lines derived from colorectal mucinous carcinoma (C1a) and moderately differentiated adenocarcinoma (HM3) which show typical pathological and mucin staining patterns of the respective type of carcinomas to nude mouse tumour xenografts. Specifically, we sought to determine if there were quantitative and qualitative differences in mucin synthesis, in mucin gene expression and in biological properties between the two model cell lines. Northern blot analysis showed that *MUC2* mRNA levels were significantly higher in C1a cells compared with HM3 cells, while those of *MUC3*, *-5* and *-6* mRNA were lower. C1a cells secreted approximately five times more radiolabelled apomucin and 1.5 times more glycosylated apomucin than HM3 cells. When the carbohydrate side-chain length of secreted mucins by these cell lines were examined by β-elimination followed by P4 column chromatography, C1a mucins had mostly short carbohydrate side-chains, while HM3 cells had predominantly longer side-chains. Western blot analysis of the cell homogenate showed higher expression of MUC2 apomucin and mucin-associated carbohydrate antigens, such as T, Tn and sialyl Tn, with decreased sialyl Le^x expression in C1a cells compared with HM3. Immunohistochemical analysis of 35 colorectal adenocarcinoma and 25 mucinous colorectal carcinoma tissues also demonstrated increased MUC2 apomucin, T, Tn and sialyl Tn antigens in the mucinous cancer specimens. Examination of the biological properties of these cell lines showed that C1a cells had significantly higher *in vitro* invasive activity in assays of invasion and collagenase activity and significantly lower E-selectin binding and liver colonisation activities in nude mice. These results indicate that colorectal mucinous carcinoma cells differ considerably from colorectal adenocarcinoma cells, both qualitatively and quantitatively, in the pattern of mucin gene expression and in the synthesis and secretion of mucin. In addition, biological studies showed that mucinous carcinoma cells have a greater degree of invasiveness, but less liver colonising activity. These results suggest that the biological and mucin characteristics of mucinous carcinoma cells contribute to extensive local invasion through tissue stroma as the predominant mechanism of tumour progression, while the biological and mucin characteristics of well- to moderately-differentiated colorectal adenocarcinoma contribute to progression via distant metastasis formation. © **1997 Elsevier Science Ltd.**

Key words: mucins, mucinous (colloid) carcinoma, colorectal adenocarcinoma, adhesion, invasion, metastasis

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INTRODUCTION

ALTHOUGH MUCINOUS (colloid) carcinoma of the colon has been reported to have a worse prognosis than adenocarcinoma of the colon [1–3], it is now thought that mucin production is not an independent prognostic factor, yet mucinous cancers are more likely to present at an advanced stage [4, 5] and have an increased recurrence rate after resection [6, 7]. Although both forms of colorectal carcinoma are known to synthesise and secrete mucin, histochemical studies of mucinous carcinoma are characterised by the copious secretion of mucin into surrounding stromal tissues [2, 8], while well- to moderately-differentiated colorectal carcinoma secretes mucin into the glandular lumen [1].

Alterations in mucin have been reported to occur both quantitatively and qualitatively in colon cancer cells [9–11]. These alterations have been associated with changes in the biological properties of these cells in areas such as cell–cell and cell–substratum interactions [12–15], growth regulation [12], immune recognition [16], invasive properties [17, 18] and progression to metastasis [17–19]. Although the pattern of mucin secretion appears to be the major difference between mucinous carcinoma and adenocarcinoma, little is known about the characteristics of mucin and its possible role in determining the biological properties of mucinous colorectal carcinoma cells. Mucinous carcinoma has been difficult to evaluate biochemically and biologically because it is usually found in varying amounts in tumours that already have areas of moderate- to well-differentiated adenocarcinoma [1].

One solution to these difficulties is to develop experimental models of mucinous colorectal carcinoma and colorectal adenocarcinoma using model cell lines. For our series of experiments, we had two appropriate human cell lines available: the C1a cell line, which is an adherent cell line derived from a typical histologic mucinous (colloid) type human colon carcinoma [20], and the HM3 cell line, which is a mucin-secreting variant of a moderately well-differentiated colorectal carcinoma cell line LS174T [21]. We hypothesised that quantitative and qualitative differences in mucin synthesis and secretion may occur in cells derived from mucinous (colloid) cancers compared with moderately-differentiated colorectal adenocarcinomas, and that these differences in mucin synthesis and secretion may be associated with different biological properties. To our knowledge, this study represents the first to demonstrate the differences in biochemical and molecular properties of mucins and in biological properties of colorectal mucinous (colloid) carcinoma and adenocarcinomas.

MATERIALS AND METHODS

Cell lines and cell culture

The HM3 cell line is a mucin-secreting variant selected from the human colon cancer cell line LS174T in this laboratory [21, 22]. The C1a cell line is an adherent variant selected from the mucinous (colloid) colon cancer cell line 5583s (kindly provided by Dr F.T. Bosman) [20] and grown as a monolayer. The cells were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% heat-inactivated fetal calf serum (56°C, 30 min), 100 units/ml penicillin, and 100 μg/ml streptomycin sulphate. They were incubated at 37°C in a 7% CO_2 atmosphere. All experiments described in this study were carried out at 90% confluency.

Xenograft of nude mice

Cells were grown to confluency, harvested by scraping, and resuspended in serum-free medium. Athymic mice (BALBc nu/nu) obtained from Simonsen (Gilroy, California, U.S.A.) were injected in the shoulder subcutaneously (s.c.) with 1×10^6 cells in 100 μl of serum-free medium. After the tumour diameter reached 0.5–1.0 cm, the tumours were excised and fixed for histological studies. Tissue sections were stained by periodic acid Schiff (PAS) and Alcian Blue.

Radiolabelling of cells

Mucins from both cell lines were metabolically labelled with [^{3}H]glucoasamine (27 Ci/mmol) (ICN Biochemicals, Irvine, California, U.S.A.) or [^{3}H]proline (99 Ci/mmol) (Amersham, Arlington Heights, Illinois, U.S.A.) and purified by gel filtration on a Sephrarose CL-2B column (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), as described in previous studies in this laboratory [22–24]. For glucosamine radiolabelling, subconfluent cells grown in six-well plates (Costar, Pleasanton, California, U.S.A.) were cultured in low-glucose DMEM containing 10% fetal calf serum for 24 h and labelled for 18 h with 10 μCi/dish [^{3}H]glucosamine in 2 ml of glucose-free DMEM containing 10% fetal calf serum [22, 23]. For mucin purification and characterisation, cells in 25 cm^2 flasks were labelled with 30 μCi/flask [^{3}H]glucosamine in 5 ml of glucose-free DMEM containing 10% fetal calf serum. For proline labelling, 100 μCi/dish [^{3}H]proline in 2 ml of DMEM with 10% fetal calf serum was used.

Harvesting of labelled cells

Cell culture media were collected, and protease inhibitor cocktail (0.5 μg/ml leupepsin, 0.7 μg/ml pepstatin, 0.2 mM PMSF and 1.0 mM EDTA) was added. The supernatant (medium fraction) was collected after centrifugation for 10 min at 1700*g*. The cell layers were washed twice with PBS containing protease inhibitor cocktail and scraped into 10 mM Tris–HCl, pH 8.0, then sonicated for 1 min. The supernatant, which contained the cytosol fraction, was collected after centrifugation for 1 h at $1 \times 10^5 g$. This was applied to the column after it was dialysed against deionised water at 4°C overnight and lyophilised [22–24].

Gel filtration of labelled glycoproteins

Samples were dissolved in 10 mM Tris–HCl, pH 8.0, and applied to a column of Sepharose CL-2B columns (1.5 × 50 cm) in 10 mM Tris–HCl buffer, pH 8.0 (18 ml/h). Sepharose column fractions (1 ml) were collected and radioactivity counted, and the void volume peaks were collected, dialysed and lyophilised in preparation for CsCl density gradient centrifugation and β-elimination.

Caesium chloride density gradient ultracentrifugation

The lyophilised samples were treated with 1 mg each of ribonuclease A and deoxyribonuclease I in 0.2 M sodium phosphate buffer, pH 6.4, 1 mM $MgSO_4$, 2 mM PMSF, and 0.02% sodium azide at 20°C overnight. After nuclease digestion, the samples were dialysed against PBS overnight

at 4°C. After centrifugation for 48 h at $1.6 \times 10^5 g$, aliquots were weighted and counted.

β-elimination and P4 column analysis of carbohydrate chains

After the aliquots of void volume fractions were dialysed and lyophilised, they were treated in 50 mM NaOH containing 1 M $NaBH_4$ at 50°C for 48 h. After neutralisation with acetic acid, the samples were lyophilised, and aliquots of samples were applied to a Sepharose CL-2B column to determine the extent of β-elimination and to a BioGel P4 column (1×115 cm) (Biorad, Richmond, California, U.S.A.) to analyse the length of oligosaccharide chains. P4 column chromatography was performed at room temperature and eluted at 5 ml/h with 0.1 M NH_4HCO_3. Blue dextran (void volume), ^{14}C-glucose (total inclusion volume), and variable-sized glucose units were used as internal standards [22–27].

Northern blot analysis

The cDNA probes were PUM for *MUC1* [28], SMUC41 for *MUC2* [29], SIB124 for *MUC3* [30], NT60 for *MUC5* AC *(MUC5)* [31], and NT44 for *MUC6* [32]. These probes are specific for unique mucin tandem repeat sequences. The relative signal obtained with these probes is indicative of the relative numbers of tandem repeats present in a sample [33, 34]. Total mRNA, extracted from each of the two cell lines, was purified using guanidium isothiocyanate and centrifugation through a CsCl cushion [35]. A total of 10 μg of mRNA was subjected to electrophoresis in a formaldehyde–agarose gel and transferred to a nylon membrane by capillary reaction. The filter was subsequently probed with the respective radioactive mucin gene cDNA probe. Loading equalities were checked by reprobing the filter with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe. The final wash was performed for 30 min at 65°C in $0.1 \times$ SSC, 0.1% SDS (except in the MUC3 cDNA probe, which was carried out at 55°C).

Immuno and lectin slot-blots

For the protein slot blots, a 10 μg cell homogenate was applied to a nitrocellular membrane using a Bio-Dot-Apparatus (BioRad Lab, Richmond, California, U.S.A.). To study the extent of exposure of the unique tandem repeat peptide of each mucin, the following lectin and rabbit polyclonal antibodies were used: anti-MRP (produced against MUC2 human intestinal mucin repeat peptide) [29], anti-M3P (produced against MUC3 human intestinal mucin repeat peptide) [30], and chicken polyclonal antibodies, anti-MUC5 MAb (against MUC5 peptide) [33], and anti-MUC6 MAb (against MUC6 peptide) [34]. Rabbit polyclonal antibody to hydrogen fluoride-treated human antral gastric mucin antibody (anti-Apo-A) was prepared as previously reported [32]. To study the nature of the mucin oligosaccharides, the following lectin and monoclonal antibodies were used: *Arachis hypogae*, specific for T antigen (Galβ1,3 GalNAc); 91S8, specific for Tn antigen (GalNAc) [26]; JT10e, specific for sialyl Tn antigen (Siaα2,6Ga1NAc) [36]; SNH3, specific for sialyl Le^x (Neu5Acα2-3Gal1-4[Fucα1-3]G1cNAc) (kindly provided by Dr S. Hakomori); 19-9, specific for sialyl Le^a (Neu5Acα2-3Galβ1-4 [Fucα1,4] G1cNAc); 91.9H, specific for sulfomucin (kindly provided by Dr T. Irimura); G4, specific for non-*O*-acetylated sialomucin; and G9, specific for *O*-acetylated sialomucin (kindly provided by Dr David Gold).

Immunohistochemistry

The streptavidin peroxidase method was performed as described previously [37] using the same antibodies used for immunoblot on formalin-fixed samples of 35 mucinous (colloid) cancers an 25 adenocarcinomas. The proportion of positive staining of cancer tissue was assessed in every low-power (10x) field and graded as follows: less than 20% (1), 21–40% (2), 41–60% (3), 61–80% (4), more than 80% (5). The score was then averaged for each specimen.

Cell adhesion assay

Matrigel 4 μg/well were used to coat the wells of 96-well plates (Costar, Pleasanton, California, U.S.A.) at 37°C for 2 h. The coated wells were washed with serum-free medium, and cells were added at a concentration of 1×10^5 cells/well in the serum-free medium. After incubation, the plates were gently washed, and attached cells were detected by 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyl-2,4 tetrazolium bromide [18, 27].

E-selectin 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) (kindly provided by Dr A. Aruffo) was measured on 96-well plates. ELAM-Rg (0.25 μg/well) or control chimeric protein CD7 and IgG1 was absorbed to the plate at 4°C overnight. The wells were blocked with 1% BSA, 1×10^5 cells were added, and the plates were incubated 1 h at room temperature with rotation. After washing with PBS, the attached cells were quantitated with the MTT colorimetric method [26, 37].

Liver colonisation assay

Tumour cells were grown in 75 cm^2 tissue flasks, harvested and suspended in serum-free DMEM (10^7 cells/ml). Athymic mice were anaesthetised and their spleen exteriorised through a flank incision. Cells (10^6) in 0.1 ml were injected into the splenic pulp with 30 gauge needle over 1 min followed by splenectomy 2 min later. The nude mice were sacrified 3 weeks later. The tumour burden of the nude mice livers were evaluated by liver weight and by photographing the liver-containing tumour nodules as described previously [17].

In vitro *invasion assay*

This was performed to evaluate the ability of tumour cells to penetrate a reconstituted basement membrane. Transwell cell culture chambers (Costar, Cambridge, Massachusetts, U.S.A.) were used. Reconstituted matrigel solution (6.6 μg) was applied to an 8 μm pore polycarbonate filter. Cells (2×10^5) were added and incubated for 48 h. The percentage of invasive cells was then determined by the MT colorimetric method [18, 37].

Assay of collagenase activity

Zymography was performed as described previously [38]. Briefly, cells were grown to confluence in 75 cm^2 flasks, and cultured in 6 ml serum-free DMEM. After incubation for 18 h, the conditioned medium was collected and centrifuged at 1000*g* for 10 min. The conditioned medium was

mixed at 4:1 (v/v) with sample buffer (10% SDS, 0.312 M Tris–HCl (pH 6.8); 0.1% bromophenol blue) and warmed to 37°C for 20 min. The substrate polyacrylamide gel electrophoresis was carried out in a 4% stacking gel and 10% dissolving gel containing type I Gelatin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) at a final concentration of 1 mg/ml. Following electrophoresis, the gels were washed to remove the SDS for 1 h in buffer composed of 50 mM Tris–HCl (pH 7.4) and 2% Triton X-100. Gels were incubated for 36 h in substrate buffer containing 5 mM CaC_2, 0.02% sodium azide at 37°C with or without 5 mM phenanthroline or 10 μg/ml aprotinin. Gels were stained with Coomassie Blue-250 and then destained.

Statistical analysis

Values are given as a mean standard deviation of at least five independent determinations. Student's *t*-test was used to determine statistical significance.

RESULTS

Xenografts of cell lines in nude mice

The human colorectal adenocarcinoma cell line, HM3, formed moderately-differentiated adenocarcinoma with numerous intraglandular collections of mucin. The colloid colon cancer cell line, C1a, exhibited extracellular collections of mucins in subcutaneous xenograft tumours. Alcian Blue/PAS staining of xenograft tissues is shown in Figure 1.

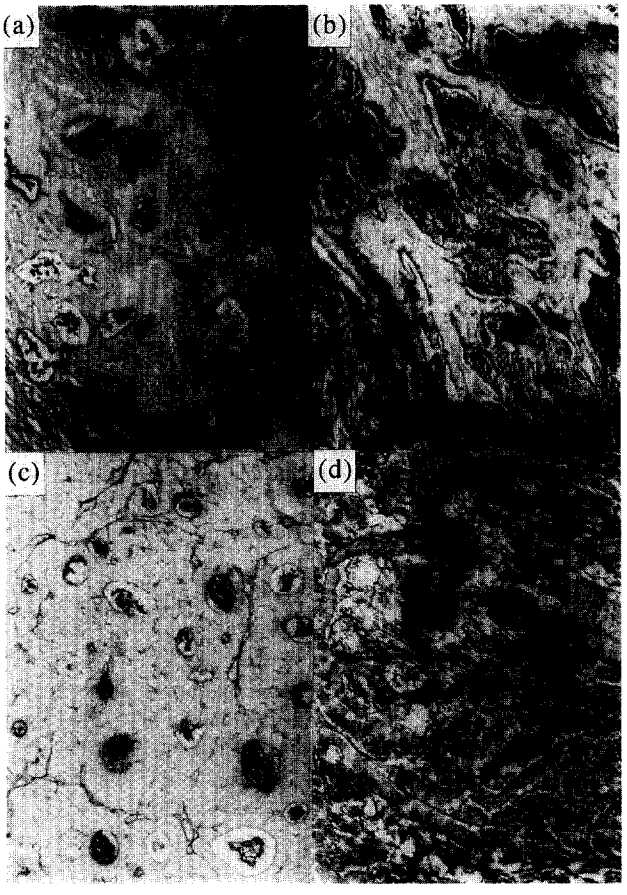

Figure 1. Alcian Blue/PAS stain of human cancer tissues and nude mouse xenografts of human colon cancer cell lines. (a) moderately well-differentiated colon adenocarcinoma tissue; (b) mucinous (colloid) colon cancer tissue. (c) In the nude mouse xenograft tumour, HM3 cells formed moderately-differentiated adenocarcinoma with numerous intraglandular collections of mucin, as indicated by dark stain mimicking moderately well-differentiated adenocarcinoma shown in (a). (d) C1a cells show extracellular collections of mucins as indicated by dark stain, thereby mimicking the mucin lakes of mucinous carcinoma shown in (b).

Purification and quantitation of mucins

The profile of [^{3}H]glucosamine-labelled glycoprotein in Sepharose CL-2B is shown in Figure 2. Table 1 shows the amount of radiolabelled glycoproteins in the void volume fractions collected from the Sepharose CL-2B column chromatography. When labelled with [^{3}H]glucosamine, C1a cells secreted approximately 1.5 times more mucin glycoproteins than that of HM3 cells, but when labelled with [^{3}H]proline, C1a cells secreted 5.5 times more mucin. The amount of mucin glycoproteins in the cytosol fraction of C1a cells was 1.4 times that of HM3 cells when labelled with [^{3}H]glucosamine and 2.5 times when labelled with [^{3}H]proline. In order to confirm that these high molecular weight glycoproteins were mucins, the void volume fractions were pooled, treated with nucleases and fractionated on a CsCl density gradient. Most of these high molecular weight glycoproteins had a density of 1.35–1.55 g/ml which indicates that these are mucin glycoproteins.

One of the important features of mucin is the *O*-linkage of glycoproteins to the mucin peptide core. When the void volume fractions on Sepharose CL-2B chromatography were β-eliminated by treating with $NaBH_4$ in mild alkali and applied to another Sepharose CL-2B column, almost all of the radiolabel disappeared in the void volume peak position and shifted to the position of very low molecular weight as expected in mucins (Figure 2c, d).

P4 column chromatography of the carbohydrate chain

To study the lengths of carbohydrate side-chain of mucin glycoproteins of both cell lines, the oligosaccharide side-chains were released from mucin peptides by β-elimination and separated by BioGel P4 column chromatography. C1a mucin was very different from that of HM3 in chain length. The shorter chain oligosaccharides (less than six glucose units) were 39.2% of the total carbohydrates of the mucins isolated from the medium fraction of C1a cells, while the longer chain oligosaccharides (more than 18 glucose units) were 25.9%. In contrast, the shorter chain oligosaccharides were 9.4% in the mucins isolated from the medium fraction of HM3 cells and the longer chain oligosaccharides were 65.4%. A similar pattern of oligosaccharide side-chain sizes was observed in the cytosol fraction of both cells (Figure 3).

Expression of mRNA

Northern blot analysis of total RNA extracted from HM3 cells and C1a cells showed that the steady-state level of *MUC2* mRNA was markedly increased in C1a cells compared to HM3 cells. *MUC1* mRNA was weakly expressed in both cell types. *MUC3*, *-5* and *-6* mRNA levels were decreased in C1a cells (Figure 4.)

Immunoblots

Antigenic expression of oligosaccharide side-chains and peptide moieties of mucins were examined in the cell homogenates by slot-blot analysis using lectin or antibodies. With antibodies and a lectin directed against mucin associ-

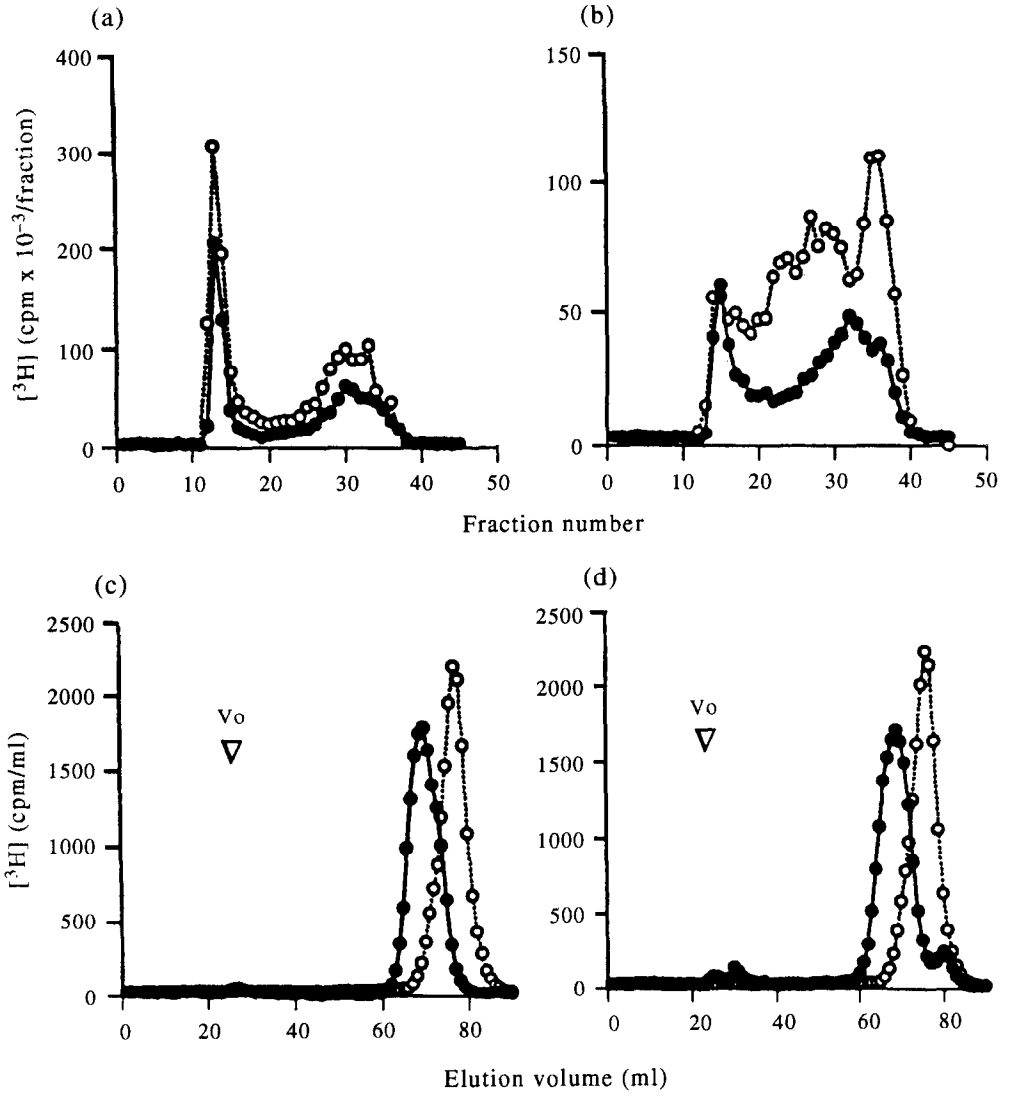

Figure 2. Sepharose CL-2B profile of [^{3}H]glucosamine-labelled glycoproteins of HM3 cells (closed circle) and C1a cells (open circle). (a) Medium fraction. (b) Cytosol fraction. Sepharose CL-2B profile of mucin oligosaccharide chains. The [^{3}H]glucosamine-labelled mucins from the HM3 and C1a cells were treated with alkaline borohydride, and released oligosaccharides were analysed on the Sepharose CL-2B columns. (c) Medium fraction, (d) cytosol fraction. The closed circle represents HM3 cells and the open circle represents C1a cells.

ated short-core region carbohydrate antigens (such as T, Tn and sialyl Tn), a marked increase in intensity was observed in the C1a cells compared with the HM3 cells (Figure 5). The ratio of the densitometric measurements of C1a to HM3 in the immunoblots were 1.73 (T), 3.79 (Tn), and 2.50 (sialyl Tn). The expression of a longer carbohydrate antigen, sialyl Le^x antigen, was markedly increased in HM3 cells, although the expression of sialyl Le^a, a positional isomer of Sle^x, showed only a slight increase (Figure 5). The ratios (C1a/HM3) were 0.16 in sialyl Le^x and 0.89 in sialyl Le^a. No difference in the level of expression of sulphomucin (91.9H) or *O*- or *N*-acetylneuraminase and epitopes (G4 and G9) was observed in both cell types. Anti-MUC2 antibody had a markedly increased expression in C1a cells

Table 1. Quantitation of mucin. [^{3}H]glucosamine (GlcN) or [^{3}H]proline (Pro) labelled high molecular weight glycoproteins were recovered in void volume after Sepharose CL-2B gel filtration

	Medium		Cytosol		Total	
	HM3	C1a	HM3	C1a	HM3	C1a
[^{3}H]GlcN	107 ± 7*	157 ± 8	24 ± 4	34 ± 1	131 ± 14	194 ± 14
[^{3}H]Pro	57 ± 2	312 ± 36	216 ± 34	533 ± 73	274 ± 59	845 ± 64
[^{3}H]GlcN/[^{3}H]Pro	1.88	0.50	0.11	0.06	0.48	0.23

*The values are expressed as cpm/mg x 10^{-3}.
Mean ± SE of three determinations

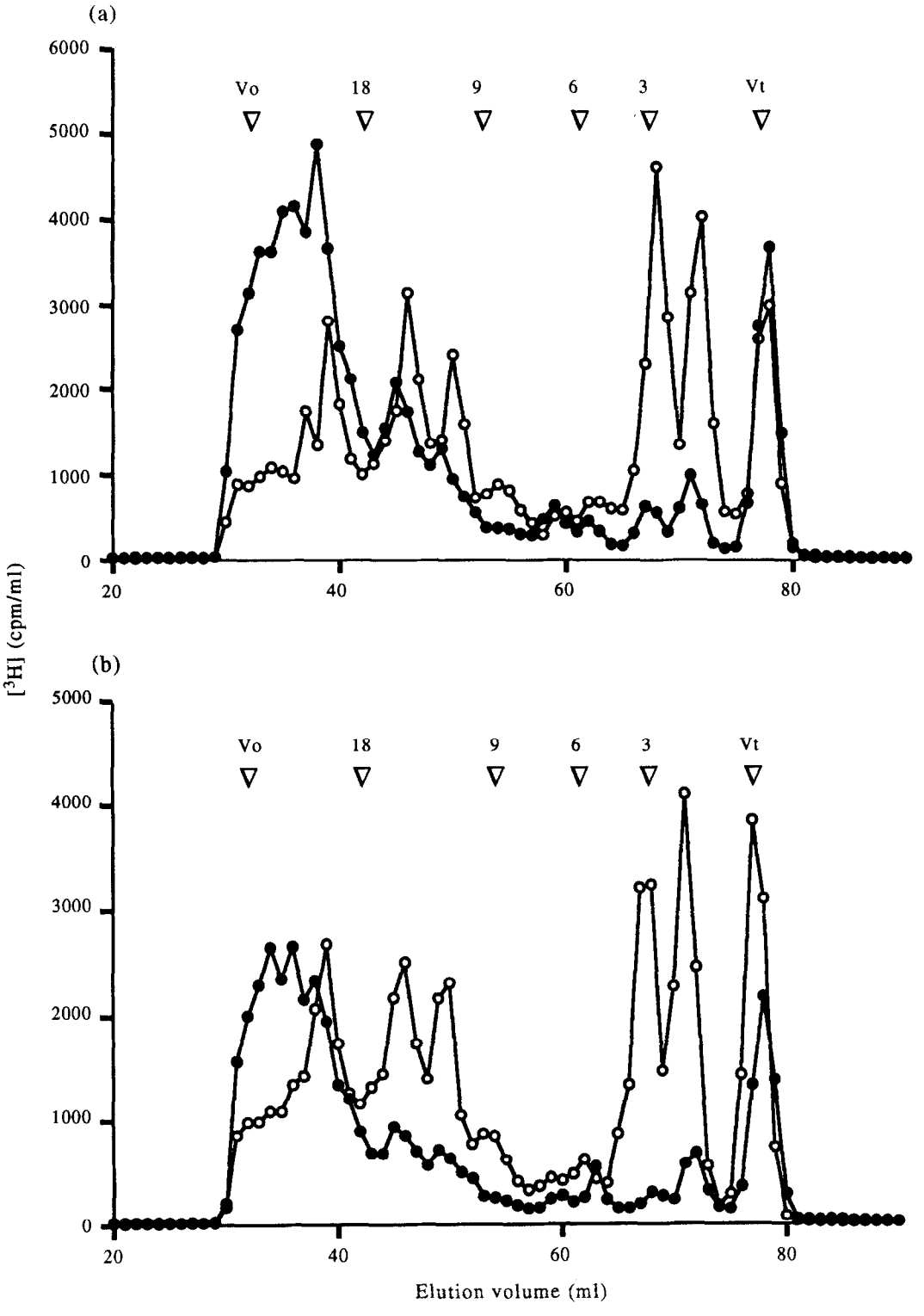

Figure 3. P4 column chromatography of oligosaccharide chain. The [^{3}H]glucosamine-labelled oligosaccharide chains of mucins from HM3 (closed circles) and C1a (open circles) cells were fractionated on BioGel P-4 after β-elimination. Arrows show the elution position of blue dextran (void volume) and glucose units. The last arrow on the right is [^{14}C]glucose (total inclusion volume, Vt). (a) Medium fraction, (b) cytosol fraction.

(2.76) compared with HM3 cells (Figure 6). There were no differences in the expression of MUC3, -5 and -6 apomucin peptides, but Apo-A expression was increased in C1a cells.

Immunohistochemical study

Immunohistochemical studies were carried out with the same antibodies described above on 35 colorectal adenocar-

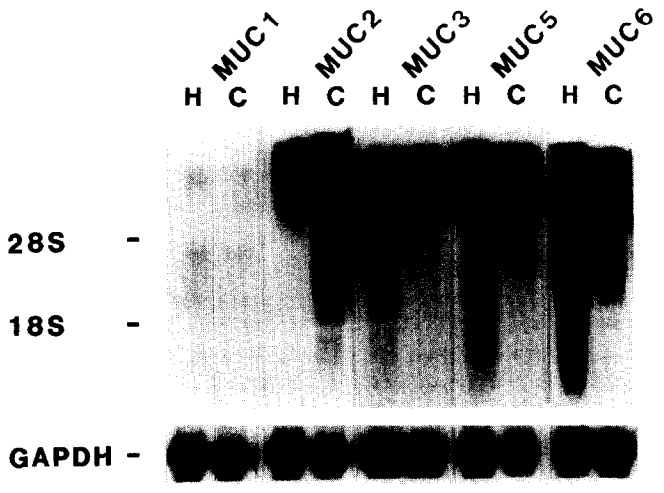

Figure 4. Northern blot analysis of mucin gene. Total cellular RNA was hybridised with MUC1, -2, -3, -5 and -6 cDNA probes. Loading equality was checked by reprobing the filters with GAPDH cDNA. Lane H: HM3 cells, Lane C: C1a cells.

cinomas and 25 colloid colorectal cancers in order to determine whether this cell line model can be applied to human cancer tissues. Antibodies against T, Tn, sialyl Tn and MUC2 apomucin antigens showed a significantly higher score in mucinous colorectal cancer tissues, while the antibody against sialyl Le^x showed a significantly higher score in colorectal adenocarcinoma tissues (Figure 7).

Adhesion, invasion and colonisation assays

When the adhesion of cells to matrigel was studied, HM3 cells showed a 2.4-fold higher degree of adhesion compared to C1a cells (Figure 8a). The binding of cancer cells to endothelial cells has been known to be an important step in distant metastasis. Sialyl Le^x on glycoproteins of the cell surface have been reported to be ligands for selectins on endothelial cells. Assays of adhesion of cells to a soluble chimeric protein, consisting of the extracellular region of E-selectin (ELAM-1) and a fragment of human IgG1, showed that HM3 cells had markedly increased binding compared to C1a cells (Figure 8b). Intrasplenic injection of nude mice with HM3 and C1a cells revealed that HM3 cells caused a 4-fold increase in the liver weight and tumour burden compared with C1a cells (Figure 9, Table 2).

The *in vitro* invasion assay closely resembles *in vitro* invasion in malignancy because it involves adhesion, disruption and penetration of the basement membrane by cancer cells.

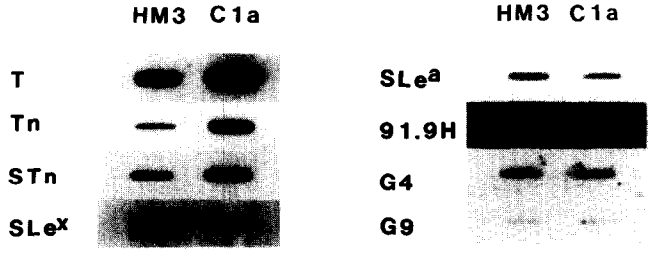

Figure 5. Immunoblot analysis of carbohydrate antigens. Antibodies or lectin used are lectin *Arachis hypogea* (T antigen), MAb 91S8 (Tn antigen), MAb JT10e (sialyl(s) Tn), MAb19-9 (SLe^a), MAb SNH3 (SLe^x), MAb 91.9H (sulphomucin), MAb G4 (non-*O*-acetylated sialomucin), MAb G9 (*O*-acetylated sialomucin).

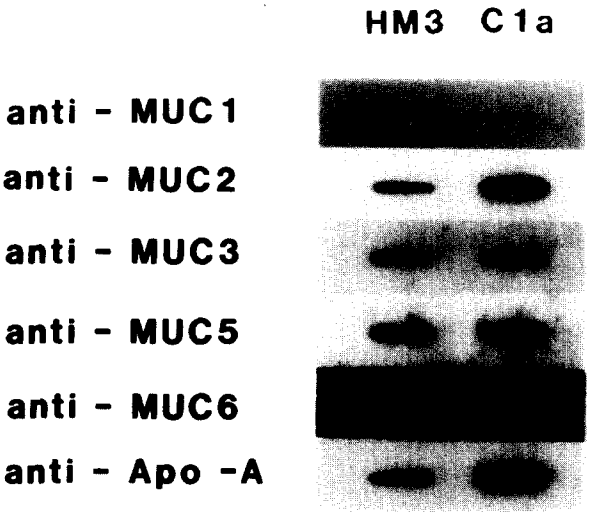

Figure 6. Immunoblot analysis of mucin peptides. MAb 139H (tandem repeat peptide of MUC1), anti-MRP (tandem repeat peptide of MUC2), anti-M3P (tandem repeat peptide of MUC3), MAb anti-MUC5 (tandem repeat peptide of MUC5), MAb anti-MUC6 (tandem repeat peptide of MUC6), anti-Apo-A (antral gastric apomucin).

In contrast to the results of the liver colonisation assay, C1a cells was more invasive than HM3 cells in the invasion assay (10.68 versus 7.09%) (Figure 10). C1a cells showed increased 72 KD (1.5x) and 92 KD (3.2x) type IV collagenase activity compared to HM3 cells (Figure 11).

DISCUSSION

The incidence of mucinous (colloid) carcinoma in patients with colorectal cancer is approximately 10–20% and it is characterised by abundant secretion of mucin and the formation of interstitial lakes of secreted mucin in the stromal tissues [39, 40]. Whether mucinous colorectal carcinoma carries a worse prognosis than well- to moderately-dif-

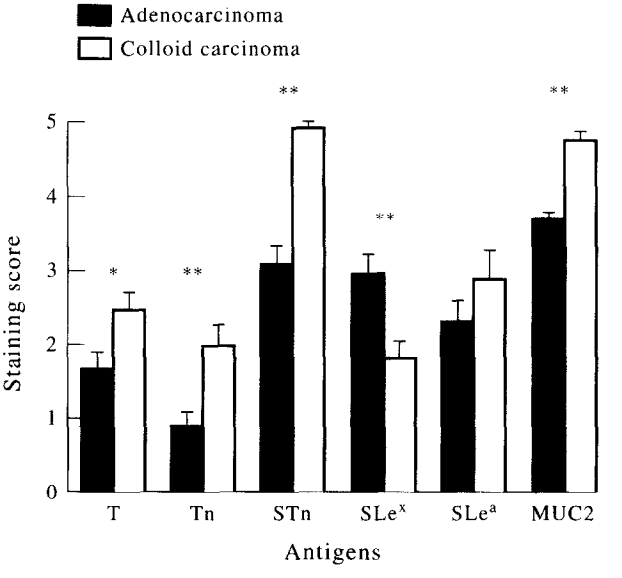

Figure 7. Immunohistochemical study of human colon cancer tissues. Fixed tissue specimens of adenocarcinoma ($n = 35$) and mucinous (colloid) carcinoma ($n = 25$) in Dukes' stage B or C were stained and scored as described in the Materials and Methods section. Mean values ± SE. *$P < 0.05$, **$P < 0.005$.

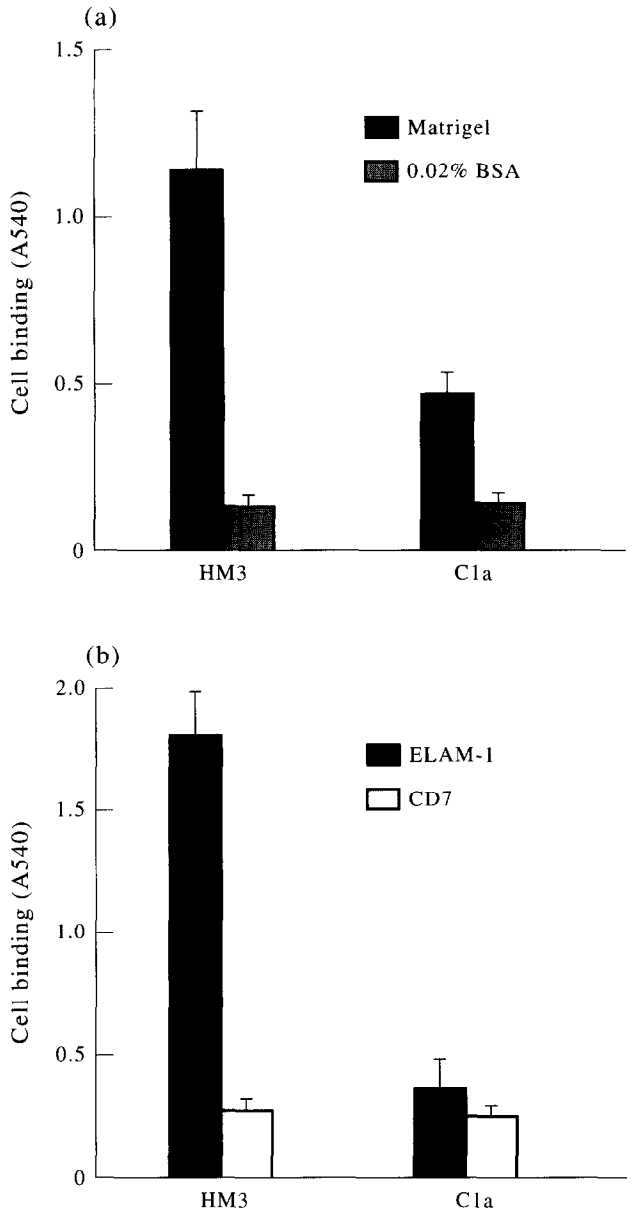

Figure 8. (a) Matrigel binding assay. Adhesion of HM3 and C1a cells to 4 μg/well of matrigel (closed column) are represented as mean ± SE of 18 (P <0.005) experiments. Open column shows control (0.02% BSA). (b) E-selectin binding assay. Binding of HM3 cells and C1a cells to chimeric protein of extracellular region of E-selectin and human IgG1 (closed columns). Open columns show binding to the control (chimeric protein of CD7 and IgG1). The mean values ± SE of 18 experiments are shown (P <0.0005).

ferentiated colorectal adenocarcinoma has been a subject of considerable debate [1, 6, 41, 42]. It is now, however, generally accepted that mucin production is not an independent prognostic factor, but mucinous cancers are more likely to present at an advanced stage and have been associated with a higher rate of recurrence after resection [6] as well as a higher incidence of incurability due to unresectable local extension and early and extensive invasion [1, 2, 7]. Mucinous colorectal carcinoma seems to occur in younger patients [43–45], tends to be proximal and generally presents at a more advanced stage than adenocarcinoma [7, 45]. However, at similar Dukes' stages, it is debatable

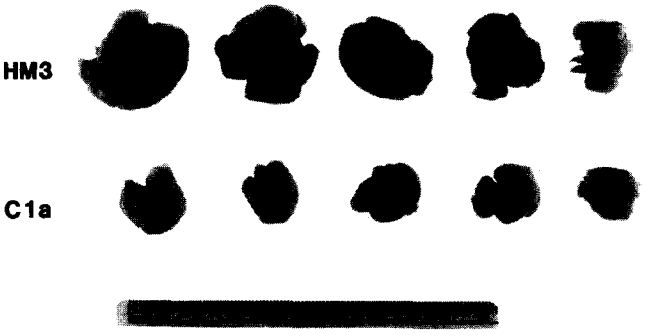

Figure 9. Livers from athymic nude mice 3 weeks after intrasplenic injection of colon cancer cells, HM3 and C1a.

whether mucinous colorectal carcinoma has a lower survival rate than colorectal adenocarcinoma [42].

Little has been known about the biological behaviour of mucinous colorectal carcinoma cells or the characteristics of the mucin it produces. We utilised a cell line model system in this study using two distinct cell lines: HM3, which is a mucin-secreting variant derived from moderately-differentiated human colorectal adenocarcinoma [21], and C1a, which is an adherent variant selected from a cell line derived from mucinous (colloid) colorectal carcinoma [20]. Previous studies in our laboratory, using colon adenocarcinoma cell lines, have demonstrated that there is a positive association between the amount of mucin secreted and a tumour's metastatic abilities [17, 18]. In liver colonisation assays, following intrasplenic injection or caecal wall injection in nude mice, the mucin-secreting colon adenocarcinoma cell line, HM7, has demonstrated higher metastatic activity than the low mucin-secreting adenocarcinoma cell line, LM3, or the parental cell line, LS174T. Similarly, colon adenocarcinoma cell line selected for high liver colonising activity, Lim6, synthesised and secreted greater amounts of mucin than the parental, LS174T cell line or LM3. Furthermore, inhibition of mucin glycosylation by benzyl-α-*N*-acetylgalactosamine in these mucin-secreting colon adenocarcinoma cell lines reduced liver colonisation after intrasplenic injection of these cells in nude mice [17].

In gastrointestinal cancers, many of the phenotypic markers for premalignant and malignant cells have been found to be present on the carbohydrate and peptide moieties of mucin glycoproteins. The expression of carbohydrate antigens may be due to extension and elongation of carbohydrate side-chain structures, modification of peripheral carbohydrate structures such as sialyl Le^x and sialyl Le^a including decreased *O*-acetylation of sialic acid [46], or the exposure of inner-core region carbohydrates such as T, Tn and sialyl Tn [9, 10, 47]. The expression of peptide-associated epitopes in cancer cells appears to be due either to

Table 2. Liver colonisation assay. Hepatic tumour burden 3 weeks after intrasplenic injection of 10^6 *tumour cells*

Cell line	Weight of mice (g)	Liver weight (g)	Number of metastasis bearing mice
HM3	22.02 ± 0.45*	4.31 ± 1.35	5/5
C1a	20.5 ± 0.67	1.10 ± 0.06†	4/5

*Mean ± SE. †P < 0.01.

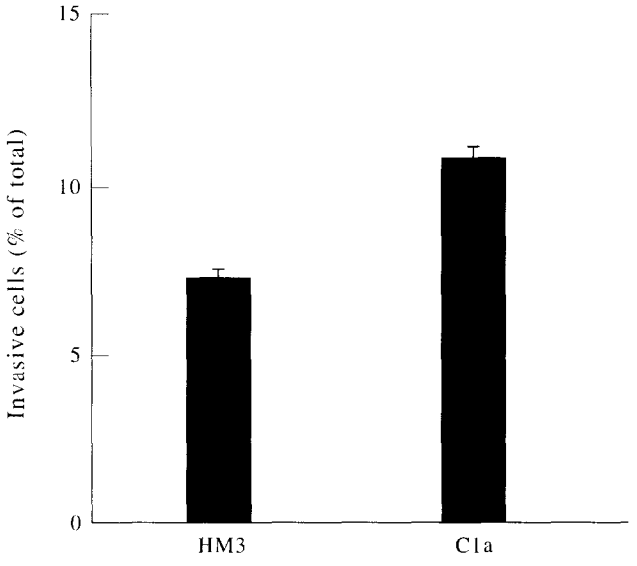

Figure 10. *In vitro* invasion assay. Percentage of cells penetrating through a reconstituted basement membrane to the lower compartment. The mean values ± SE of 12 experiments are shown ($P < 0.005$).

overexpression of mucin genes or to incomplete glycosylation or abnormal processing of apomucins [10, 48].

In the present study, using biochemical methods, we determined that there were significant differences in both the qualitative and the quantitative characteristics of mucins produced by the two cell lines. To date, at least eight different mucin genes have been identified, *MUC1–MUC8* [29–32, 49–51]. These mucin genes are expressed in a highly tissue- and cell-specific manner. For example, *MUC2* and *MUC3* mucin genes are highly expressed in the small and large intestine [52, 53], while *MUC5* and *MUC6* are highly expressed in the stomach [33, 53]. Furthermore, recent *in situ* hybridisation and immunohistochemical studies indicate that *MUC2* is expressed in goblet cells while *MUC3* is expressed in both goblet and absorptive cells of the intestine [52]. *MUC5* is expressed, predominantly in surface mucous cells in both the fundus and antrum of the stomach, while *MUC6* is expressed in the neck mucous cells of the fundus and pyloric gland cells [33, 53]. Using these much cDNA probes, Northern analysis reveals that both cell lines express all types of mucin mRNAs examined in a varying degree, indicating that dysregulation of tissue-specific mucin gene expression occurs in cancer cells. In addition, C1a cells express predominantly *MUC2* mRNA at higher levels than HM3 cells, suggesting that the *MUC2* mucin gene is upregulated in C1a cells.

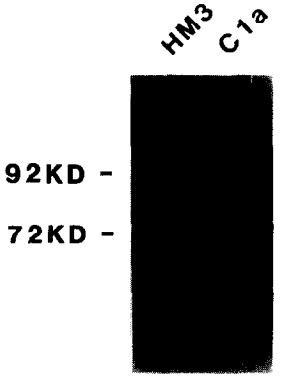

Figure 11. Type IV collagenase activity assay. Sample of conditioned medium from HM3 cells and C1a cells were analysed on the SDS–PAGE containing gelatin (1 mg/ml).

In the metabolic labelling study, the colloid cancer cell line C1a synthesised and secreted approximately five times more apomucin and 1.5 times more glycosylated apomucin than HM3 cells. The lower ratio of [^{3}H]glucosamine to [^{3}H]proline in the mucin fraction synthesised and secreted by C1a cells compared with HM3 cells suggests that C1a cells produced and secreted either more apomucins, more mucins with fewer carbohydrate side-chains or more mucins with shorter carbohydrate side-chains. The determination of the size of oligosaccharide side-chains of mucin by P4 column chromatography revealed that C1a cell mucins had much shorter oligosaccharide side-chains, which supports the third possibility. The markedly increased expression of mucin inner-core region shorter side-chain carbohydrate epitopes, such as T, Tn and sialyl Tn, in C1a cells is consistent with the results of the oligosaccharide side-chain analysis, and may be due to more incomplete glycosylation of apomucins in these cells. The increased expression of extended sialyl Le^x in HM3 cells is consistent with longer carbohydrate side-chains of mucins observed in HM3 cells. Western blot analysis using antibodies against different apomucins demonstrated increased MUC2 apomucin expression without alterations in the level of expression of MUC3, -5 or -6 apomucins in C1a cells. This result is consistent with the increased steady-state level of *MUC2* mRNA observed in C1a cells. The marked increase in the expression of MUC2 apomucin and *MUC2* mRNA suggests increased exposure of the MUC2 epitopes in C1a mucins due to incomplete glycosylation and/or abnormal processing of mucin in C1a cells.

Interestingly, the differences in patterns of expression of mucin-associated carbohydrate and apomucin antigens shown by Western blot studies in C1a and HM3 cells were very similar to those observed in immunohistochemical staining of mucinous (colloid) colorectal cancer tissues and well- to moderately-differentiated colorectal adenocarcinomas. These findings, together with the morphological and histochemical pattern of the xenografts derived from these cell lines, support our assertion that these cell lines serve as excellent models for the two histological types of colorectal carcinoma: mucinous (colloid) and moderately- to well-differentiated adenocarcinoma. However, the differences in the biochemical and molecular properties of mucins in the two cell lines could be due to clonal variation. Further studies on additional cell lines are needed to strengthen our conclusion.

Both sialyl Le^x and sialyl Le^a antigens, present on leucocytes, have been identified as ligands to E- and P-selectins present on activated vascular endothelium and on platelets, respectively [54]. These selectins have been thought to be involved in inflammatory processes. The sialyl Le^x and sialyl Le^a antigens are expressed on many colon cancer cells, which have also been demonstrated to bind to endothelial cells [55, 56]. Their binding is inhibited by pretreatment of the cells with antibodies to sialyl Le^x or sialyl Le^a, or by adding selectins in the binding assays. Recent studies suggest that L- and P-selectins interact primarily with mucin-type ligands on colon cancer cells, while E-selectins can recognise ligands on both mucin and non-mucin type

glycoproteins [14, 25]. Because of these findings, these antigens are thought to be involved in the adhesion of cancer cells to endothelial cells with subsequent extravasation resulting in metastatsis.

The higher expression of sialyl Le^x in HM3 cells is consistent with the higher binding of HM3 cells in the E-selectin binding assay and with the increased number of tumour nodules and increased total weight of livers in nude mice injected with HM3 cells in the liver colonisation assay. Although C1a cells secreted more mucin than HM3 cells, they appear to have less liver colonisation potential. This difference may be in part due to the truncation of carbohydrate side-chains resulting in the reduced expression of sialyl Le^x in mucinous cancer cells. In contrast to their decreased liver colonising propensity, C1a cells showed increased invasivenss on reconstituted basement membrane and increased collagenase type IV activity compared with HM3 cells. The increased collagenase activity may be responsible for the increased *in vitro* invasive properties of C1a cells and may contribute to enzymatic degradation of tissue stroma allowing extrusion of mucin into surrounding tissues in mucinous colorectal cancer. The viscous and lubricating physical properties of mucin may then overcome tissue resistance, dissect tissue planes, facilitating local spread.

These findings suggest that mucinous colorectal carcinomas and colorectal adenocarcinomas undergo different processes in tumour progression. Mucinous colorectal carcinomas spread by direct extensive local invasion, whereas colorectal adenocarcinomas spread by distant metastasis. This observation is consistent with clinical findings which show that mucinous colon cancer demonstrates increased local and abdominal invasion and more peritoneal seeding with less liver metastasis than adenocarcinoma [1, 41].

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