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Reduction of sialic acid O-acetylation in human colonic mucins in the adenoma-carcinoma sequence

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The oligo-O-acetylation of sialic acids found in normal colonic mucins is greatly reduced in colorectal cancer. Mucins prepared from cancer tissue in adenocarcinoma showed this reduction, while normal O-acetylation was detected in resection margin and control cases and total mucin sialic acid content was significantly decreased in cancer vs control samples. A reduction of the O-acetyl transferase activity catalysing the O-acetylation reaction was also found. A series of cultured human colorectal cell lines derived from the same premalignant adenomatous line, and representative of the adenoma-carcinoma sequence were examined and revealed a depletion of oligo-O-acetylation in the original diploid premalignant line, re-expression in a further premalignant line and reduction in malignant mucinous and adenocarcinoma cell lines. Reduction of sialic acid O-acetylation appears as an early event in the process of malignant transformation in human colorectal cancer.

Keywords: **sialic acids, O-acetylation, sialate O-acetyltransferase, colon cancer, mucin**

Abbreviations: **Bovine submandibular gland mucus glycoprotein (BSM), horse radish peroxidase (HRP), abbreviations for sialic acids Neu5Ac, Neu5Gc, Neu9Ac5Gc, Neu5,9Ac$_2$, Neu5,8,9Ac$_3$, Neu5,7,8,9Ac$_4$ as before [1]**

Introduction

The mucins synthesised and secreted by the human colonic mucosa are highly sialylated and sulphated [2–4]. Biochemical studies have shown that more than 50% of colonic mucin sialic acids are O-acetylated and 30% contain oligo-O-acetyl forms with either 2 or 3 acetyl esters per sialic acid residue [5–7]. Histochemical data suggest that total O-acetylation is at least 80% [8]. This high degree of O-acetylation is characteristic for the human colon and has not been detected in other human tissues.

The significance of the higher O-acetylation in the colonic mucins is related to the degradation of mucins by enzymes secreted by the normal enteric bacterial flora [5,9]. Stepwise removal of monosaccharides from the non-reducing terminal is limited by the relative activity of glycosidases required to degrade the entire oligosaccharide chain. As the sialic acids are largely terminal residues in these chains, their removal may be rate limiting. We have previously demonstrated that the presence of two or more acetyl esters on positions 7–9 of sialic acids in colonic mucins significantly reduces the action of enteric bacteria sialidase activity [5,9]. In addition, we detected the presence of a sialate O-acetyl esterase activity in individual bacterial strains and in faecal extracts from normal individuals [10]. The relative levels of esterase to sialidase activity suggests that a regulation exists at the level of sialic acid O-acetylation, which has a direct influence on the rate of sialic acid removal and hence mucin oligosaccharide degradation [5].

Although di-and tri-O-acetylated sialic acids were identified in colonic mucins, it is unknown whether there is a selective expression of these forms on specific oligosaccharide chains. Several antibodies have been raised which bind to saponification-sensitive epitopes in colonic goblet cells and membranes and correspond to the O-acetylated sialic acids identified by the mild periodic acid Schiff stain. The binding of the anti-sialyl-Tn antibody TKH2 was found to be blocked by O-acetylation [11,12], implicating these short oligosaccharides as carriers of acetyl esters. In addition, the increase in expression of sialyl Le(x) detected in colonic

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carcinoma metastases has been attributed to a reduction in sialic acid O-acetylation rather than an increased synthesis of this epitope [13]. This finding was also explained on the basis of reduced antibody binding due to O-acetylation. Further identification of oligosaccharide chains with O-acetylated sialic acids has not been made.

The mono-O-acetylated sialic acids are more widespread than the di- and tri-O-acetyl forms [1,14,15] The occurrence of Neu5,9Ac2 has been linked with functions in mouse erythrocyte membranes and the alternative complement pathway [16], human T-lymphocyte ganglioside antigens and T-cell differentiation [17,18]. They have also been implicated in blocking the binding of malaria parasites [19] and the sialic acid-dependent adhesion molecule CD22 [20,21] in lymphocyte interactions and haematopoietic differentiation. On the other hand, O-acetylated sialic acids serve as recognition sites for the binding of influenza C viruses [22,23]. Most Neu5,9Ac2 has been detected in N-linked oligosaccharides or gangliosides [1,14,20].

The existence of specific sialic acid 7(9)-O-acetyltransferases has been described [1,14]. Due to the migration of O-acetyl groups from the C7 to C9 OH position under physiological conditions it has been proposed that only one enzyme is necessary for the complete higher O-acetylation of sialic acids [24,25]. This has found support in the histochemical analysis of sialic acid O-acetylation in the human colon, where racial variation in O-acetylation phenotype has allowed identification of heterozygous individuals and prediction of a polymorphic autosomal gene for the O-acetyl transferase (*oat*) [26]. It has also been shown in bovine submandibular gland that the primary insertion site of the O-acetyl group is at C-7 [27]. This enzyme has not been purified from any tissue.

Histological analysis of sialic acid patterns in the normal human colon has shown that O-acetylated forms are associated with the sialomucin in the upper half of the crypts in the left colon. The reverse pattern is found in the right colon, with the lower crypts containing the O-acetylated sialomucins [28–30]. Conflicting results have been obtained with histochemical studies of the colonic mucosa from inflammatory bowel disease patients (Ulcerative colitis and Crohn's disease). Reduction [31] and lack of change [32] in goblet cell O-acetylation levels have been reported. However, loss of side chain O-acetylation has been detected in dysplasia and hyperplasia in ulcerative colitis [33,34], in colorectal adenomas and carcinomatous foci [35] and adenocarcinomas [30,36]. Recently we have reported a retention of sialic acid mono-O-acetylation, but with an abnormal pattern of Goblet cell expression [22].

No data are available for the early stages of colorectal cancer and the present study was designed to address this question using a series of cultured human colorectal cells representing stages in the adenoma-carcinoma sequence [37,38] and comparing these with individual cancer cases. The results show that O-acetylation is greatly reduced at a very early stage in malignant transformation and is a feature of both mucinous and adenocarcinoma cell lines and adenocarcinoma tissue. A preliminary report of this work has been presented [39].

Patients

Patients in this study included a group of five controls. Resected tissue was obtained from three patients with chronic constipation and two with rectal prolapse. All of these tissue samples showed normal histological mucosa. Tumour tissue was taken from seventeen patients with colorectal cancer. Tumours were located in the rectum (8), sigmoid colon (8) and descending colon (1). All were diagnosed as adenocarcinoma by routine clinical and histological assessment and only tissue samples corresponding to adenocarcinoma were taken for analysis. Resection margin was taken from eleven of these patients, at rectum (4) and sigmoid colon (7) locations.

Materials

D-[1-^{14}C]-Glucosamine (8.73GBq/mmol) and [^{3}H]-acetyl-CoA (120.25GBq/mmol) was obtained from Amersham International plc. Amersham, UK. Sepharose CL 2B, Sephadex G10 and Sephadex G25 superfine were obtained from Pharmacia, Milton Keynes, UK. Dowex ion-exchange resins were products of BioRad, Watford, UK. Immobilon P was purchased from Millipore, UK. Bovine submandibular gland glycoprotein was prepared as before [40]. The monoclonal antibody PR3A5, reacting with human colonic mucin [41] was a gift from Dr J. Burchell, Imperial Cancer Research Fund, London UK. Penta-O-acetyl-N-acetylneuraminic acid was synthesized as described before [42].

Methods

Colorectal cancer cell lines

The following cell lines were used at the passage numbers indicated PC/ AA, 15, 19 & 27 (premalignant, non-tumourigenic); PC/ AA, 80 & 90 (malignant, tumourigenic, mucinous carcinoma); PC/ AA/ C1, 72 (premalignant, non-tumourigenic); PC/ AA/ C1/SB10C, 109 (malignant, tumourigenic, adenocarcinoma); PC/JW, 15 (malignant, tumourigenic, adenocarcinoma). Details of the cell lines, their culture conditions and harvesting are as reported previously [37,38].

Colorectal tissue

Tissue from colorectal tumours was washed in PBS and samples taken for homogenization and histology. Colonic mucosal tissue was dissected free of the lamina propria and muscularis layers and washed in PBS as before [43].

Preparation of mucins

The preparation of mucin fractions from cell lines and tissue samples is based on previous work [43] and generates equivalent samples which can be used for comparison.

Metabolically labelled mucins

After labelling of individual cell lines with 370kBq D-[1-^{14}C]-glucosamine for 48 h, the medium was removed and the cells washed twice with 2.5 ml PBS containing inhibitors and 5 mM dithiothreitol for 20 min each. The medium and dithiothreitol washes were combined and taken for gel filtration on Sepharose CL 2B. The cells were scraped into 5 ml of PBS containing inhibitors and homogenized as described [43]. After centrifugation at 100,000 g for 60 min, the supernatant and membrane fractions were collected.

The medium and cellular soluble fractions were passed down columns (30 × 1 cm) of Sepharose CL 2B and the Vo fraction identified, pooled and lyophilized. Membrane fractions were resuspended in 1 ml of PBS and protein content measured as before [44].

Explants from colorectal tumours and control colonic mucosal tissue were incubated with 185 kBq D-[1-^{14}C]-glucosamine for 24 h as described before [43]. The medium, cellular soluble and total membranes were isolated and the Vo fractions from Sepharose CL 2B chromatography prepared as described.

Non-radioactive cells and tissue

Medium, cellular soluble and total membrane fractions were prepared from cell lines as described [45,46]. Mucins were prepared from the soluble fractions by caesium chloride density gradient centrifugation, gel filtration, desalting on Sephadex G10 and stored in solution at 4 °C before use [46].

Tissue mucin was prepared by scraping the colonic tissue mucosa, extraction in 6M-guanidine hydrochloride containing proteinase inhibitors and purified by density gradient centrifugation and gel filtration as before [43]. Purified mucins were stored at 4 °C in solution.

Tissue homogenates were prepared in 10 mM sodium formate pH 5,5 on ice with an Ultraturrax (Jahnke & Kunkel, Staufen, Germany) using three 10 sec bursts at maximum setting with 30 sec pauses between bursts. After centrifugation in sealed 13 ml tubes at 55,000 rpm (150,000 g) in a Beckman L70 ultracentrifuge with a Ti 17.1 rotor for 60 min, at 10 °C, the supernatant and membrane pellet were separated. The supernatant was applied to a 30 × 1 cm column of Sepharose CL 2B, eluted with 10 mM Tris/HCl pH 8.0 and the Vo fraction collected. The samples were dialysed and freeze dried. Membrane pellets were resuspended in 1 ml PBS buffer and protein content measured [44]

Analysis of mucins by slot blotting

Slot blotting of mucin fractions was performed as described before [46]. The amount of mucins was assessed using staining with wheat germ agglutinin-horse radish peroxidase (HRP) conjugate. The amounts in each fraction were quantified using bovine submandibular gland mucin (BSM) as standard after densitometric scanning of the individual slots and preparation of standard curves. Blots were scanned with a Hewlett Packard HP2C Scanner, their intensity was measured densitometrically using Optimas Bioscan Software.

The binding of the anti-colon mucin antibody PR3A5 was carried out on slot blots with equivalent amounts of mucin from each cell line or tissue sample standardized as above with wheat germ agglutinin and BSM. Samples were blotted corresponding to 10 μg of BSM when detected with wheat germ agglutinin-HRP.

The epitope recognised by PR3A5 contains a poly-O-acetylated sialic acid [41]. Thus, positive binding of this antibody to components containing this structure can be destroyed using mild alkaline treatment to saponify the O-acetyl esters.

The exact nature of this epitope has not been demonstrated but corresponds to the staining pattern detected histochemically with the mild PAS technique which is specific for O-acetylated sialic acids in the colon [8,47].

Preparation of total sialic acids

All samples were dissolved or suspended in 2M acetic acid as before [48,49]. To the radioactive samples 1 mg of BSM was added as carrier. Hydrolysis was carried out for 3 h at 80 °C, the hydrolysate was cooled and dialysed against 50 ml distilled water for 16 h at 4 °C. The dialysate was passed through a 5 ml column of Dowex 50W (50–100 mesh) H^+ form and washed with 10 ml water. The effluent was mixed with 0.5 ml 1M formic acid and freeze dried. Samples were dissolved in 5 ml 10 mM sodium formate pH 5.5 and passed over a column of 2 ml Dowex 3 × 4 (100–200 mesh) formate form, washed with 5 ml 10 mM sodium formate and the sialic acids eluted with 5 ml 1M formic acid. The samples were freeze dried, dissolved in water and the total sialic acid content determined by the periodic acid thiobarituric acid assay [49], radioactivity measured and suitable aliquots (at least 10,000 cpm) taken for thin-layer chromatography or HPLC.

Thin-layer chromatography

Thin-layer chromatography was carried out on 0.2 mm cellulose plates as before [49]. Samples were applied over 1 cm and the plates developed in butan-1-ol:propan-1-ol:0.1M-HCl (1:2:1 v/v/v). Standards of penta-O-acetyl-N-acetyl-D-neuraminic acid, synthesized as described before [42] and a mixture of sialic acids from mild acid hydrolysis of bovine

submandibular gland glycoprotein were run on both sides of each thin-layer plate. A minimum of 50 μg total sialic acid and > 10,000 cpm^{14}C were loaded per lane. Some samples were incubated with 100 mM NaOH at room temperature for 45 min to saponify the ester groups, passed through a 1 ml column of Dowex 50W H^+ form and freeze-dried before thin-layer chromatography. The lanes containing standards were cut off the plate and the position of the individual sialic acids determined by spraying with the orcinol/ferric chloride reagent [49]. Quantitation of bands was made by extracting the chromophore and measuring spectrophotometrically as before [50]. The nature of the bands was made by comparison of the R_f values [49]. Lanes with unknown samples were divided into four regions corresponding to the different sialic acid species (Fig. 3). The radioactive compounds in each lane were detected by scraping the cellulose layer from the plastic backing and eluting with 0.5 ml distilled water followed by liquid scintillation counting.

Identification of sialic acids by HPLC

Sialic acids were analysed fluorimetrically by HPLC using the method of Hara et al. [51]. Sialic acids (20 μg) were mixed with 20 μl of 1,2-diamino-4,5-methylene dioxybenzene solution and incubated for 1 h at 56 °C in the dark. The derivatised sialic acid mixture (10 μl) was injected onto an RP-18 cartridge in methanol-acetonitrile-water (7:9:84, v:v:v). The retention times of the sialic acids were compared with authentic sialic acids from bovine submandibular gland mucin [49].

Assay for O-acetyl transferase using a sialoglycopeptide substrate

Homogenates from fresh colorectal mucosal tissue or cultured cell pellets were made in phosphate-buffered saline (PBS) on ice as above. Samples of the homogenate were taken for protein determination using the Pierce dye binding assay [44]. The homogenate was centrifuged at 15,000 g for 5 min, the membrane pellet collected and resuspended in 200–500 μl of 100 mM sodium phosphate, 1 mM EDTA, 2% octyl glucoside buffer, pH 6.9, to give a concentration of approximately 300 μg protein/ml. Incubation for O-acetyltransferase activity contained 100 μl of this membrane suspension (approximately 30 μg protein), 1.26 kBq [^{3}H]-acetyl-CoA (45 nmole), 0.75 mg collocalia glycopeptide [52] (75 μg glycosidically bound sialic acid), in a final volume of 200 ml in 100 mM sodium phosphate, 1 mM EDTA, 2% octyl glucoside buffer, pH 6.9. Incubations were for 60 min at 37 °C and were stopped with 1 ml of ice cold PBS. The samples were centrifuged for 5 min at 15,000 g and the supernatant loaded onto a column (30 × 1 cm) of Sephadex G25 superfine and eluted with distilled water collecting 1ml fractions. The radioactivity in each fraction was determined and the glycopeptide fraction was identified by the orcinol/ferric chloride assay on 20 μl aliquots. Blank incubations were made with membranes which had been heated at 95 °C for 5 min. Activity was measured as the radioactivity in the total glycopeptide fraction per hour per mg protein. The value obtained for the blank incubation was subtracted from the test assay in each case to give the enzyme activity. The total glycopeptide from each positive sample was pooled, saponified in 0.1 M NaOH for 60 min at room temperature and rechromatographed on Sephadex G25 to confirm the O-acetyl ester link of radioactivity in this fraction. Two samples were subjected to mild acid hydrolysis without saponification, and analysed by thin-layer chromatography as described above. The minimum amount of activity detectable with this assay was 0.08 nmol sialic acid (125 dpm ^{14}C).

Statistical analysis

For statistical analysis, a comparison of continuous variables was made by means of the t-test. The number of experiments carried out in each case is shown in the tables and the legends to the figures.

Results

Detection of O-acetylated sialic acid epitopes with antibody PR3A5.

Secreted and cellular mucin prepared from the control colonic mucosa gave positive results with antibody PR3A5, while none of the cancer mucin samples was positive. The binding was completely abolished after saponification (Table 1). Mucins prepared from cultured cells gave a positive reaction only in the case of the premalignant, non tumorigenic PC/AA/C1 cell line and this was also susceptible to saponification (Table 1).

Total sialic acids in purified mucins from colorectal cells and tissue

The sialic acid content of purified mucin fractions from colorectal tissue samples and cultured cell lines is shown in Figures 1 and 2. Significantly lower levels were found in secreted mucin from cancer tissue ($p < 0.01$) compared with control and resection margin ($p < 0.005$), although this was only significant for cellular mucin ($p < 0.04$) in resection margin compared with cancer tissue (Fig. 1a). Membrane sialic acid was higher in cancer tissue ($p < 0.002$) but not in resection margin compared with control (Fig. 1a). Radioactive sialic acid prepared from metabolically labelled mucins from the same cases only showed a decrease from the secreted mucin in cancer patients compared with controls (Fig. 1b), although this did not reach statistical significance.

In the cultured cell lines lower levels were found in secreted mucins from the all cell lines except the intermediate premalignant PC/AA/C1 compared with the PC/AA

Table 1. Binding of monoclonal antibody PR3A5 to purified mucin from cell lines and resected colonic tissue. Secreted (S) and cellular soluble (CS) mucin purified from the cell lines described in methods, or tissue as indicated was blotted onto PVDF membrane and probed with PR3A5. Samples equivalent to 10 μg BSM detected by wheat germ agglutinin-HRP were used throughout. Identical samples were also tested after saponification in mild alkali. The positive blots were scanned densitometrically, as described in the Methods.

		PR3A5 Reactivity (optical units/10 μg mucin)	
Tissue/Cell line		*Direct*	*Saponified*
Colonic explants			
CONTROL	S	0.7 ± 0.02	0
(n=5)	CS	0.9 ± 0.03	0
RESECTION			
MARGIN	S	0.4 ± 0.04	0
(n=4)	CS	0.5 ± 0.04	0
CANCER	S	0	0
(n=10)	CS	0	0
Cell lines			
PC/AA 15	S	0	0
	CS	0	0
PC/AA 80	S	0	0
	CS	0	0
PC/AA/C1	S	0.2 ± 0.01	0
	CS	0.3 ± 0.03	0
PC/AA/C1/SB10	S	0	0
	CS	0	0
PC/JW	S	0	0
	CS	0	0

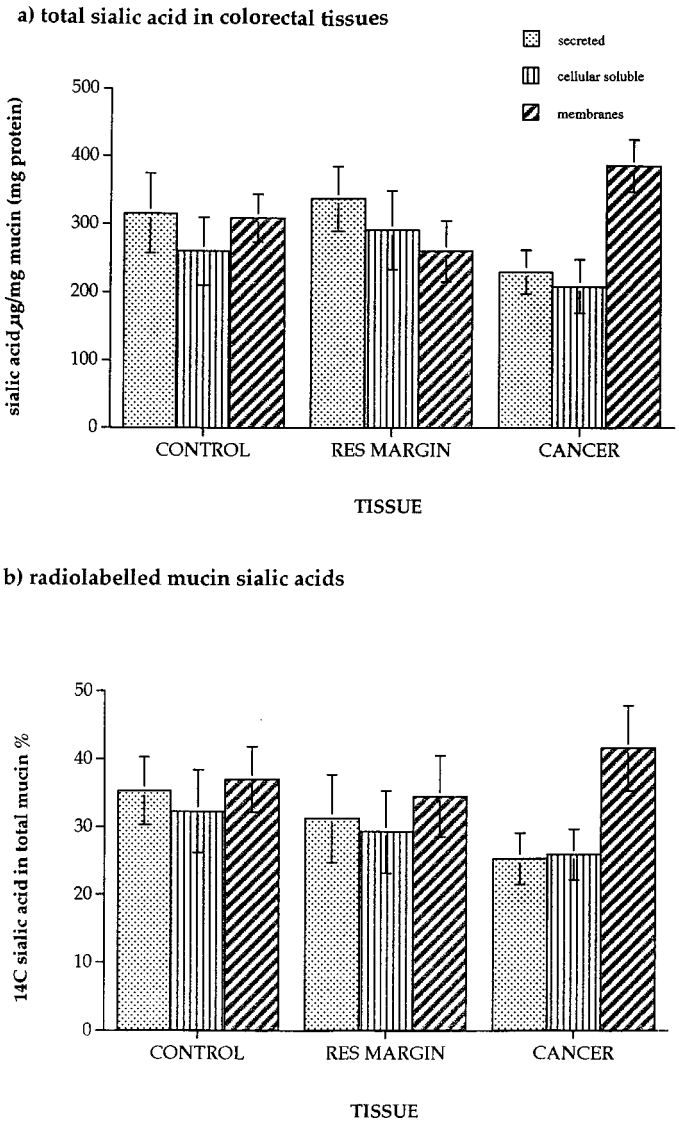

Figure 1. Sialic acid in mucins from tissue samples. Mucins and membranes were prepared from colorectal tissue in non-labelled a) and metabolic labelling b) experiments as described in the methods. The key for secreted, cellular soluble and membranes is indicated in a) and is the same for b). Control (n = 5), resection margin (n = 4) and cancer (n = 10) tissue was used in these experiments. Mucin was quantified by slot blotting with BSM as standard and sialic acids measured by the thiobarbituric acid assay. Results are expressed as μg sialic acid/mg mucin. Total membranes were isolated as single pellets and resuspended in PBS buffer. Sialic acid, measured in the same way, is expressed as μg sialic acid/mg protein (Fig. 1a). Radioactive sialic acids removed from total mucin and membranes in explant cultures is shown as ^{14}C % of the total radioactivity in the isolated mucin and membrane samples (Fig. 1b). Results are given as the mean ± SD for all experiments.

passage 15 line (Fig. 2a). All cell lines showed reduced sialic acids in the cellular mucin compared with the PC/AA passage 15, except for PC/JW. In contrast, membrane sialic acids were elevated in all lines relative to PC/AA passage 15. A similar pattern of reduced levels in mucins and increases in the membrane fractions was found for the radiolabelled sialic acids in the same cell lines (Fig. 2b) when compared with the PC/AA passage 15. The only exception was the PC/JW line which had elevated levels in the mucins (Fig. 2b).

O-Acetylated sialic acids of mucins in cultured colorectal cells and tissue

Metabolic labelling and thin-layer chromatography

Metabolic labelling of colorectal cells and tissue with D-[^{14}C]-glucosamine yielded a good incorporation into the mucin fraction to allow separation of the sialic acids (Fig. 1 & 2). Direct preparation of mucin and cells from tissue was in agreement with previous work [45,46,53].

The radioactive sialic acid samples were well separated into individual components by thin-layer chromatography (Fig. 3). In these samples only Neu5Ac, and not Neu5Gc, was detected as the parent sialic acid. This was confirmed after saponification in 0.1 M NaOH yielding a single band corresponding to Neu5Ac (data not shown).

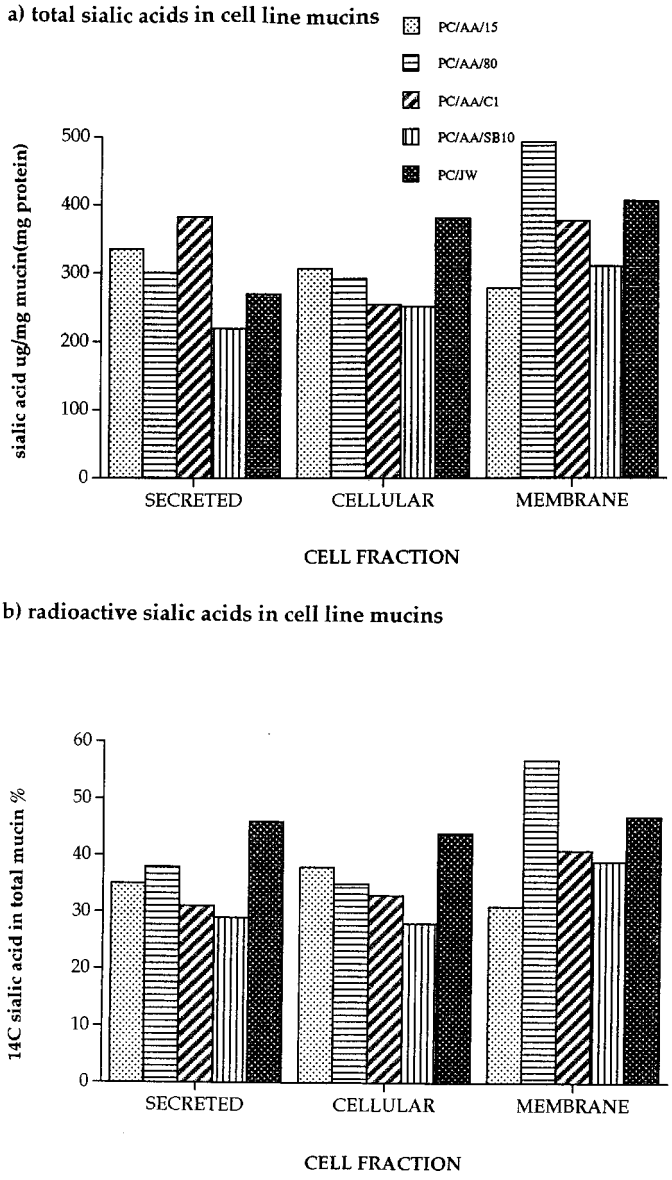

Figure 2. Sialic acid in mucins from cell lines. Mucins were prepared from cell lines in non-labelled a) and metabolic labelling b) experiments as described in the methods. Mucin and membrane sialic acids were quantified as described in the legend to Fig. 1. The key to the different cell lines is shown in a) and is the same for b). Radioactive sialic acids removed from total mucin and membrane fractions in cell culture experiments are shown as ^{14}C % of the total radioactivity in each sample. Results are given as the mean of two separate experiments for all cell lines.

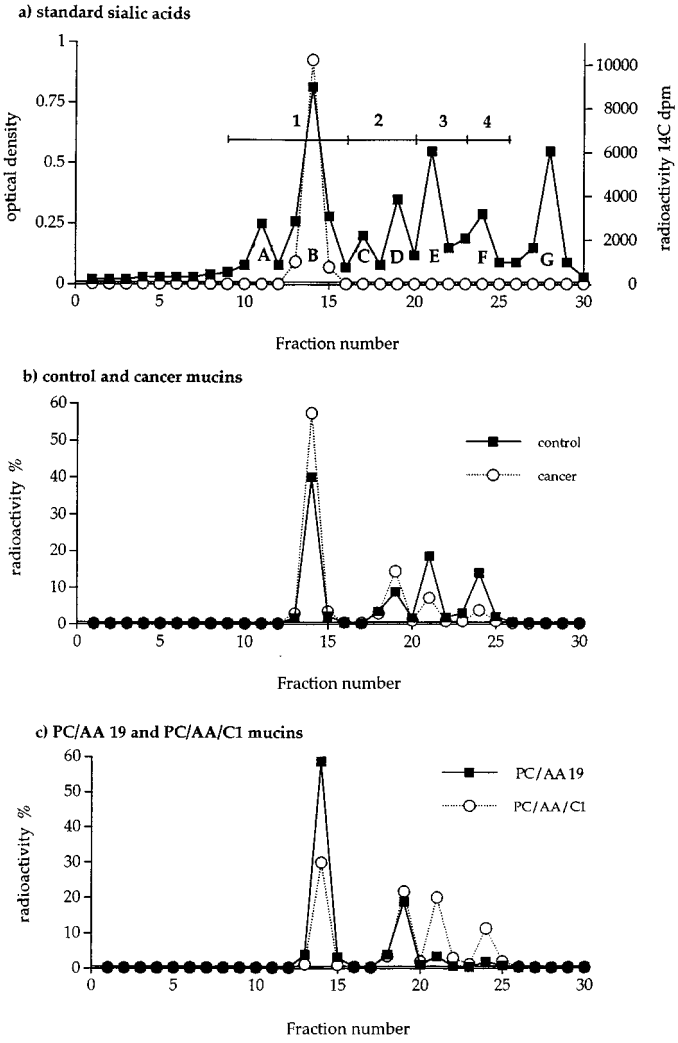

Figure 3. Profiles of [^{14}C]-sialic acids on thin-layer chromatography. Sialic acids were released by mild acid hydrolysis (see methods). a) sialic acid standards (■), A, Neu5Gc; B, Neu5Ac; C, Neu9Ac5Gc; D, Neu5,9Ac_2; E, Neu5,7(8),9Ac_3 and F, Neu5,7,8,9Ac_4 prepared from bovine submandibular gland mucin (abbreviations as described before [1]); G, synthetic penta-O-acetyl-N-acetylneuraminic acid. Radioactive standard N-acetylneuraminic acid (O). The bar shows the fractions 1–4, divided for scraping prior to radioactive measurement or colorimetric analysis. b) [^{14}C]-Sialic acids isolated from secreted control (■) and cancer tissue (O) mucins and c) [^{14}C]-sialic acids isolated from PC/AA 19 (■) and PC / AA /C1 (O) cell line mucins.

In the tissue from the control group O-acetylated sialic acids were found to comprise 42–53% of total sialic acids in secreted and cellular soluble mucins. Di- and tri-O-acetyl forms made up 34–38% of this fraction with 8–15% as mono-O-acetyl Neu5Ac (Table 2). In resection margin tissue total O-acetylated sialic acids were 45–48% of total sialic acids in secreted and cellular soluble mucins. Di- and tri-O-acetyl forms made up 23–25% of this fraction and 20–25% as mono-O-acetyl Neu5Ac. Comparison of control and resection margin showed a significantly higher mono-O-acetyl fraction in resection margin for secreted ($p < 0.01$) and cellular soluble ($p < 0.005$). The di- and tri-O-acetylated components were reduced in soluble (di- $p < 0.05$; tri- $p < 0.05$) and cellular soluble (di- $p < 0.02$; tri -NS).

In the cancer mucins only 22–31% of total sialic acids were O-acetylated ($p < 0.001$ and < 0.05 for soluble and cellular soluble respectively compared with the control

Table 2. Sialic acid O-acetylation patterns in control and colon cancer mucins. Cell lines as described in methods and tissue were labelled with [^{14}C]-D-glucosamine and the mucins isolated as above. The sialic acids released were separated on thin-layer chromatography and fractions 1–4 counted as indicated on the chromatogram in Figure 1. Subcellular fractions are S, secreted; CS, cellular soluble and M, membranes. Results are given as the mean ± SD for all experiments and are the range of two separate experiments for all cell lines.

		% Radioactivity in sialic acids Fraction				
Cell line/Tissue		*1*	*2*	*3*	*4*	*Total OAc*
Colonic explants						
CONTROL	S	47 ± 11	15 ± 5	18 ± 4	20 ± 6	53 ± 15
(n = 5)	CS	58 ± 14	8 ± 1	18 ± 5	16 ± 6	42 ± 12
	M	52 ± 11	27 ± 11	14 ± 4	7 ± 2	48 ± 17
RES MARGIN	S	52 ± 12	25 ± 5	10 ± 5	13 ± 3	48 ± 13
(n = 4)	CS	55 ± 10	20 ± 5	9 ± 2	16 ± 7	45 ± 14
	M	57 ± 9	31 ± 6	10 ± 4	9 ± 3	50 ± 13
CANCER	S	78 ± 12	15 ± 5	5 ± 1	2 ± 0.3	22 ± 6
(n = 10)	CS	69 ± 10	18 ± 5	7 ± 2	6 ± 1	31 ± 8
	M	57 ± 9	20 ± 4	15 ± 3	8 ± 1	43 ± 8
Cell lines (n = 2)						
PC/AA 15	S	71	23	4	2	29
	CS	73	14	6	7	27
	M	83	5	8	4	17
PC/AA 80	S	81	14	3	2	19
	CS	63	14	21	2	37
	M	77	9	9	5	23
PC/AA/C1	S	36	26	24	14	64
	CS	49	25	17	9	51
	M	56	16	18	10	44
PC/AA/	S	77	16	4	3	23
C1/SB10	CS	70	14	8	8	30
	M	67	11	4	18	33
PC/JW	S	72	17	6	5	28
	CS	63	15	17	5	37
	M	71	13	14	2	29

group; when compared with resection margin $p < 0.005$ and < 0.05 for soluble and cellular soluble respectively). The di- and tri-O-acetylated forms made up only 2–7% and were significantly reduced compared with control (di- $p < 0.002$; tri- $p < 0.001$ for soluble and di- $p < 0.004$; tri- $p < 0.002$ for cellular soluble), while this reduction was apparent in tri- ($p < 0.004$ and < 0.01 for soluble and cellular soluble respectively) but only for soluble ($p < 0.01$) compared with resection margin.

The mono-O-acetylated fraction remained in the same range as the controls (15–18%) in the soluble, but were lower in the cellular soluble fraction ($p < 0.005$). In resection margin there was no significant difference in either of these fractions. Examination of mucins from the cultured cell lines showed a reduction of di- and tri-O-acetylated sialic acids, with mono-O-acetylated forms making up the main part of the O-acetylated fraction (Fig. 3, Table 2). Significant proportions of higher O-acetylated forms were present only in the premalignant PC/AA/C1 cell line. The cellular fraction of PC/AA80 and PC/JW lines also showed similar levels as observed in control mucins (Table 2) but these were not present in the secreted mucins from the same cell lines. The AA/C1 cell line also showed an increase in the amount of mono-O-acetylated sialic acids. Otherwise, mono-O-acetylated sialic acids comprised 14–23% of the total, similar to the control and cancer mucins,

Membrane fractions from control, resection margin and cancer colonic tissues contained more mono-O-acetylated sialic acids (27, 31 and 20% respectively, Table 2) and none of the fractions showed any significant difference in the cancer samples compared with either control or resection margin groups. In the cultured cell lines there was a generally high (29–44%) content of membrane O-acetylated sialic acids in the AA/C1, AA/C1/SB10 and PC/JW lines.

The early passage and mucinous carcinoma lines contained only 17–23% with a lower proportion of mono-O-acetylated forms.

High-pressure liquid-chromatography

Sialic acids were derivatized and separated on HPLC as before allowing clear identification of non-substituted, mono- and higher O-acetylated sialic acids (Fig. 4). In these analyses the proportion of higher O-acetylated sialic acids was consistently lower than detected by metabolic labelling on thin-layer chromatography (Table 3). This result was also found for the BSM used as a standard in these experiments and indicates that the recovery of the higher O-acetylated sialic acids is poorer when analysed by HPLC.

The non-substituted sialic acid in human colonic samples was Neu5Ac only and in the fraction of mono-O-acetylated species Neu5,9Ac_2 predominated, with a small amount of Neu5,7Ac_2, the 7-O-acetyl isomer (Fig 4b). The fraction of higher O-acetylated sialic acids is assumed to comprise mainly the 7,9- and 8,9-di-O-acetyl derivatives of Neu5Ac, based on similar retention times compared with the corresponding fraction from BSM (Fig 4a). Significant amounts of higher O-acetylated sialic acids were detected only in control colonic and resection margin tissue mucins, and in the PC/AA/C1 cell mucin at approx. 10% (Table 3) compared with >35% in radiolabelling and thin-layer chromatographic methods. Similar amounts of mono-O-acetyl-sialic acid were found in the colonic tissue samples, but were not detected in the cultured cell mucins. However, the observation of a reduction in higher O-acetylation in tumour cells can also be detected using this technique (Fig. 4b and c). As found in normal colon, the relative amount of Neu5,9Ac_2 was larger than that of Neu5,7Ac_2 (Fig 4c)

Assay of O-acetyltransferase activity

O-Acetyltransferase activity was assayed using a collocalia mucin glycopeptide and radioactive acetyl-CoA. Significant levels of activity were detected only in the control colonic and resection margin tissue samples and the PC/AA/C1 cell line (Table 4). The radioactivity incorporated into the glycopeptide in positive assays (n = 42) was converted (87 ± 12%) into low molecular weight material ([^{14}C]-acetate) by mild alkali treatment. Two samples analysed for O-acetylated sialic acids after mild acid hydrolysis and TLC showed mono- and oligo-O-acetyl forms (data not shown). These experiments show the same pattern of O-acetylation detected with the metabolic labelling, HPLC and PR3A5 antibody methods.

Discussion

This study demonstrates that a reduction in higher O-acetylation of sialic acids in colonic mucins is an early event in malignant transformation. The use of human colonic cell

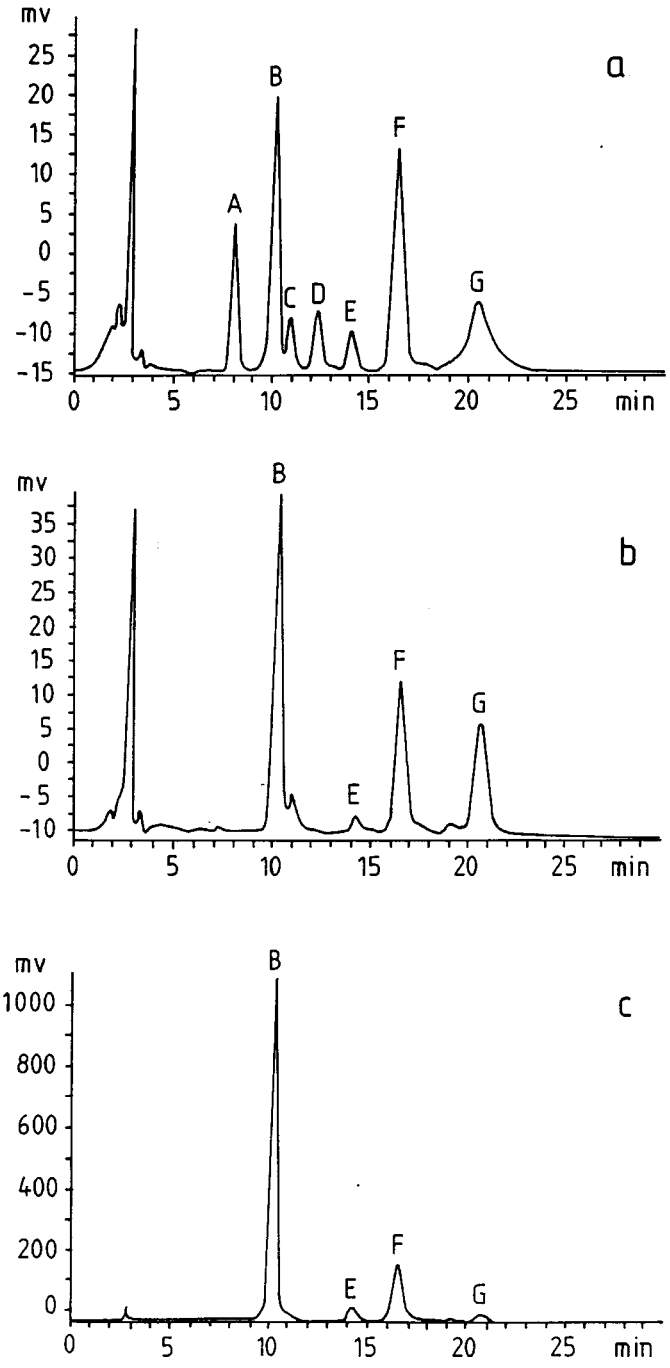

Figure 4. Profiles of sialic acids from normal and colon cancer mucins on HPLC. Sialic acids were isolated from different sources, derivatised with 1,2-diamino-4,5-methylene dioxybenzene and submitted to HPLC as described under Methods. a) sialic acid mixture from bovine submandibular gland glycoproteins; b) sialic acids from normal colonic mucin, and c) sialic acids from colon cancer mucin. A, Neu5Gc; B, Neu5Ac; C, Neu7Ac5Gc; D, Neu9Ac5Gc; E, Neu5,7Ac_2; F, Neu5,9Ac_2; G, higher O-acetylated sialic acids, mainly Neu5,7,9Ac_3 and Neu5,8,9Ac_3.

Table 3. HPLC analysis of sialic acids in secreted mucin from colonic tissue and cultured cells. Sialic acids were prepared as described in the methods and analysed by HPLC by the method of Hara et al. 1989 [51]. The proportion of each sialic acid derivative is expressed as a % of the total sialic acid in the sample.

		Sialic acid O-acetylation % of total			
Cell line/Tissue	*n*	*Non*	*Mono*	*Higher*	*Total OAc*
Colonic tissue					
NON-CANCER	3	76 ± 6	14 ± 3	10 ± 6	24 ± 6
RESECTION MARGIN	7	69 ± 4	23 ± 4	8 ± 2	31 ± 4
CANCER TISSUE	5	81 ± 4	19 ± 4	0	19 ± 4
Cell lines					
PC/AA 27	2	98 ± 2	0	2 ± 1	2 ± 1
PC/AA 90	2	100	0	0	0
PC/AA/C1	4	88 ± 8	2 ± 1	10 ± 7	12 ± 8
PC/AA/C1/SB10	2	98 ± 2	0	2 ± 1	2 ± 1
PC/JW	2	97 ± 2	0	3 ± 1	3 ± 1

lines representative of different stages in the adenoma-carcinoma sequence allows a clear demonstration of the very early stage at which this change takes place. The reduction is seen in both mucinous carcinoma and adenocarcinoma cell lines, and is confirmed in individual patients with colonic adenocarcinomas. These changes in sialic acid O-acetylation were established by the application of several independent methods including antibody binding, identification of individual sialic acids with and without metabolic labelling and the assay of the enzyme(s) responsible for O-acetyl transfer.

Table 4. O-Acetyltransferase assay in colorectal cell lines and mucosal tissue. Total mucosal tissue or cell homogenates were assayed for O-acetyltransferase activity as described in the methods. The results are the mean ± SD of duplicate assays for all samples and for two separate experiments for the cell lines. The lower level of detection was taken as 125 dpm (0.08 nmole)

Tissue/Cells		*O-acetyltransferase activity nmole/h/mg protein*
Colonic explants		
CONTROL	(n = 5)	18.1 ± 2.4
RESECTION MARGIN	(n = 4)	12.3 ± 2.3
CANCER	(n = 10)	1.5 ± 1.1
Cell lines (n = 2)		
PC/ AA 15		< 0.08
PC/ AA 80		1.9 ± 0.8
PC/ AA/ C1		10.1 ± 1.5
PC/ AA/ C1/ SB 10		0.9 ± 0.7
PC/ JW		2.7 ± 2.1

The analysis of the total sialic acid content of colorectal mucins with and without metabolic labelling shows a decrease in the cancer tissue (Fig. 1) in agreement with previous work [54,55]. The individual cell lines showed the same trend (Fig. 2), but this was less pronounced. There was an indication that the malignant PC/JW line shows a different regulation as increased sialylation of mucin was detected. In contrast to this reduction the membrane fractions showed an increase in sialic acid content, suggesting that the process of membrane glycoconjugate sialylation may be regulated separately, but is also modified as a result of malignant transformation. The possibility that increased sialic acid in the membrane fraction is due to insoluble mucin is unlikely as these fractions are exposed to dithiothreitol which will solubilise such secreted mucin. Calibration of the relative amounts of sialic acid using wheat germ agglutinin detection and BSM standard may be subject to errors due to tissue and molecular specific variation in glycosylation. However, individual variation at this level is impossible to calibrate and this method was used to provide a routine point for comparison of samples.

Several monoclonal antibodies have been described which show similar saponification-sensitive epitopes in the human colon and are thought to bind to structures containing O-acetylated sialic acids [41,56,57]. One of these antibodies, PR3A5 was used in the present study giving a direct demonstration of purified mucin O-acetylation in normal control colonic mucin. PR3A5 did not cross-react with cancer mucins purified from tissue or malignant cell lines (Table 1) in agreement with histological analysis [41] and biochemical data [46]. O-acetylated sialic acids were also detected unexpectedly in the premalignant cell line PC/AA/ C1 with immunoblotting (Table 1) and in cell pellets (data not shown). Metabolic labelling and enzyme assay confirmed the re-expression of O-acetylated sialic acids in this cell line.

The biochemical analysis supports the loss of O-acetylation in colorectal cancer tissue reported earlier using histochemical methods where no direct chemical analysis of sialic acids was made. Identification of O-acetylated sialic acids after metabolic labelling and mild acid hydrolysis on thin-layer chromatography gives recoveries of the higher (di- and tri-)-O-acetylated sialic acids of up to 53% (Table 2). Resection margin was included in this study to assess its value as a control relative to neoplastic tissue. Differences between resection margin and control groups found at the level of total sialic acid (Fig. 1) and for the O-acetylated forms (Table 2) confirmed that there is limited value of this tissue as a suitable control. It must be assumed that approximately 30% of the O-acetylation present in the start-

ing material is lost during this release and purification as has been noted before [48–50]. Thus, the in vivo level of O-acetylation is probably 80% or greater, and agrees with histochemical results using the mild periodic acid-Schiff (mPAS) method where quantitative assessment showed approximately 80% [8]. A loss of higher O-acetylated sialic acid was previously found by Hutchings et al. [6] in colonic cells removed from normal and cancer tissue, placed in primary culture and tested for sialic acid O-acetylation with metabolic labelling and paper chromatography. This study also reported a loss of O-acetylation in resection margin tissue from the cancer patients and is at variance with the results of histochemical studies [30,58,59].

The analysis of sialic acids prepared from tissue and cell culture mucins without metabolic labelling using HPLC (Table 3) confirms the loss of O-acetylation found with the other techniques, but reflects a poorer recovery of the O-acetylated forms presumably as a result of the derivatization before analysis.

Investigation of cultured human colorectal cell lines at different stages of the adenoma-carcinoma sequence by metabolic labelling, antibody PR3A5 binding and acetyltransferase assay gives an intriguing insight into the loss of O-acetylation during the progression to both mucinous and adenocarcinomas. Although statistical analysis of the metabolic labelling experiments was not possible the following observations can be made; O-acetylation of mucin sialic acids is greatly reduced in the earliest cell line studied, the diploid PC/ AA line at passages 15–27. Furthermore, this loss is maintained in both of the malignant cell lines derived from this initial line (PC/ AA passage 80–90 and PC/ AA/C1/SB10) and in an independent malignant colon cell line PC/JW. However, PC/ AA/C1, a premalignant cell line synthesises O-acetylated sialic acids, detectable by metabolic labelling, antibody PR3A5 binding and acetyltransferase assay. This may be interpreted to indicate re-expression of the O-acetyltransferase (*oat*) during the malignant process and provides a model for the further study of *oat* gene regulation.

Enzymatic assay of the O-acetyltransferase using a mucin glycopeptide substrate provides further indication for the deletion of higher O-acetylation (Table 4) in colonic cancer tissue and cells. Significant activity was only detected in the normal control colonic tissue homogenates and in the PC/AA/C1 cell line. About 10% of normal European individuals have been found to have a complete absence of colonic goblet cell sialic acid O-acetylation by histochemical analysis [26,58,59]. This is not believed to be a disease-related event but a genetic association with the expression of the O-acetyltransferase. It is seen in other populations with varying frequencies [26,36,60] and is consistent with the occurrence of the O-acetyltransferase as a polymorphic autosomal gene [26].

Although the di- and tri-O-acetylated sialic acids are diminished as a result of malignant transformation, a significant proportion of mono-O-acetyl sialic acid remains constant in the range of 10–15% (Tables 2 & 3; [6]). It is unclear whether this is a feature of the sialic acid preparation procedure reflecting greater stability of the mono-O-acetylated sialic acids, or whether it represents a separate phenomenon. The latter explanation raises the possibility that two separate enzymatic systems exist, yielding mono and higher O-acetylated sialic acids respectively, in the human colon. In the normal colon both systems would be present, but in malignant transformation only the higher O-acetylation is reduced. At present there is no evidence to support the existence of two separate systems. However, we have demonstrated that the mono-O-acetylation of sialic acids in the colon is maintained in control, cancer and inflammatory bowel disease tissue with an influenza C virus probe to demonstrate mono-O-acetylation [22].

In conclusion we have shown that sialic acid di- and tri-O-acetylation is greatly reduced as a result of colorectal cancer, and that this loss occurs at an early stage of malignant transformation. This study gives a more detailed assessment of previous histochemical data and raises new questions concerning the occurrence and enzymology of sialic acid O-acetylation in the colon. Transient re-expression of di- and tri-O-acetylation during the adenoma-carcinoma sequence has also been found in the cell culture model examined. Further study of the significance and relationship between 'normal' and cancer-related loss of sialate O-acetyltransferase is now required, as well as knowledge concerning genetic regulation of these events.

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