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O-acetylation and de-O-acetylation of sialic acids in human colorectal carcinoma

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A decrease in the level of O-acetylated sialic acids observed in colorectal carcinoma may lead to an increase in the expression of sialyl Lewis^X, a tumor-associated antigen, which is related to progression of colorectal cancer to metastasis. The underlying mechanism for this reduction is, however, not fully understood. Two enzymes are thought to be primarily responsible for the turnover of O-acetyl ester groups on sialic acids; sialate-*O*-acetyltransferase (OAT) and sialate-*O*-acetylesterase (OAE). We have previously reported the characterization of OAT activity from normal colon mucosa, which efficiently O-acetylates CMP-Neu5Ac exclusively in the Golgi apparatus prior to the action of sialyltransferase [Shen, Y., Tiralongo, J., Iwersen, M., Sipos, B., Kalthoff, H. & Schauer, R. (2002) *Biol. Chem.* **383**, 307–317]. In this report we describe the identification of a lysosomal and a cytosolic OAE activity in human colonic mucosa that specifically hydrolyses 9-*O*-acetyl groups on sialic acid. Utilizing matched resection margin and cancer tissue from colorectal carcinoma patients we provide strong evidence suggesting that the level of O-acetylated sialic acids present in normal and diseased human colon may be dependent on the relative activities of OAT to lysosomal OAE. Furthermore, we show that the level of free cytosolic Neu5,9Ac$_2$ in human colon is regulated by the relative activity of the cytosolic OAE.

Keywords: colon carcinoma, O-acetylation, sialate-*O*-acetylesterase, sialate-7(9)-*O*-acetyltransferase, sialic acids.

Sialic acids consist of a family of acidic nine-carbon sugars that are typically located at the terminal positions on a variety of glycoconjugates. The largest structural variations of naturally occurring sialic acids are at carbon 5, which can be substituted with either an acetamido, hydroxyacetamido or hydroxyl moiety to form 5-*N*-acetylneuraminic acid (Neu5Ac), 5-*N*-glycolylneuraminic acid (Neu5Gc) or deaminoneuraminic acid (Kdn), respectively [1,2]. Sialic acids can also undergo further modifications at any one of four hydroxyl groups, located at C-4, -7, -8 and -9. One such modification, the formation of O-acetyl esters, is found in nearly all higher animals and certain bacteria and has been found to play a pivotal role in modulating various biological processes [1,2].

The glycerol side chain of sialic acids present on human colonic mucins is highly O-acetylated. Chemical and histochemical analyses have shown that more than 50% of colonic mucin sialic acids are O-acetylated, with at least 30% containing di- and tri-O-acetylated sialic acid forms [3]. The significance of this high level of O-acetylation, which is characteristic for the human colon, is believed in part to regulate the degradation of mucins by bacterial enzymes [4]. For example, the presence of ester groups on sialic acids can hinder the action of enteric bacterial sialidase [5,6]. Interestingly, the gradual loss of sialic acid O-acetylation, particularly oligo-O-acetylated sialic acids, has been identified as an early alteration accompanying the adenoma-carcinoma sequence in cultured cells [7]. It has also been observed that the reduction of O-acetylation of sialyl Lewis^X, a tumor-associated antigen, is the primary alteration related to progression of colorectal cancer [8]. These observations, naturally, raise many questions concerning the occurrence and enzymatic processes involved in the O-acetylation and de-O-acetylation of sialic acids in human colonic tissues.

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Abbreviations: AcCoA, acetyl-CoA; DMB, 1,2-diamino-4,5-methylenedioxybenzene; Kdn, 2-keto-3-deoxynononic acid; MU, 4-methylumbelliferyl; 4-MUAc, 4-methylumbelliferyl acetate; Neu5Ac, 5-*N*-acetyl-neuraminic acid; Neu5,9Ac$_2$, 5-*N*-acetyl-9-*O*-acetylneuraminic acid; Neu5,7Ac$_2$, 5-*N*-acetyl-7-*O*-acetylneuraminic acid; Neu5,7,9Ac$_3$, 5-*N*-acetyl-7,9-di-*O*-acetylneuraminic acid; Neu5,8,9Ac$_3$, 5-*N*-acetyl-8,9-di-*O*-acetylneuraminic acid; Neu5,7(8)9Ac$_3$, 5-*N*-acetyl-7(8),9-di-*O*-acetylneuraminic acid; Neu5,7,8,9Ac$_4$, 5-*N*-acetyl-7,8,9-tri-*O*-acetylneuraminic acid; Neu2,7an5Ac, 5-*N*-acetyl-2,7-anhydro-neuraminic acid; Neu5Gc, 5-*N*-glycolyl-neuraminic acid; OAE, sialate-*O*-acetylesterase; OAE-C, cytosolic sialate-*O*-acetylesterase; OAE-L, lysosomal sialate-*O*-acetylesterase; OAT, sialate-7(9)-*O*-acetyltransferase.

Enzymes: sialate-*O*-acetylesterase (EC 3.1.1.53); sialate-7(9)-*O*-acetyltransferase (EC 2.3.1.45).

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Two enzymes are believed to be responsible for the turnover of O-acetyl ester groups on sialic acids. The introduction of acetyl groups into the glycerol side chain (i.e. at C-7, -8 and -9) of sialic acids is catalysed by acetyl-CoA:sialate-7(9)-*O*-acetyltransferase (OAT) [9,10]. This enzyme has, until now, proven inaccessible by purification or cloning [11–14]. However, a number of investigations have shown that this enzyme is a Golgi-localized membrane-bound protein that probably utilizes the OH group at position C-7 of sialic acids as the primary O-acetyl attachment site [10,11,15]. It is believed that migration of O-acetyl groups from C-7 along the glycerol side chain to C-9 [16] results in the di- and tri-O-acetylated forms observed in colonic mucins. We have recently shown that the OAT from human colonic mucosa preferentially utilizes CMP-Neu5Ac as the acceptor substrate [11]. This finding raises the possibility that sialate O-acetylation occurs in human colon via an alternative mechanism (Y. Shen, J. Tiralongo & R. Schauer, unpublished observation) to that previously postulated to occur in other systems [15].

The specific hydrolysis of 9-*O*-acetyl groups from sialic acids is catalysed by sialate-9-*O*-acetylesterase (OAE) [17,18]. In mammals two distinct forms of OAE, one in the cytoplasm and the other in the lysosomal compartment, have been described [17,18]. It is believed that lysosomal OAE is involved in the removal of 9-*O*-acetyl groups that are present on sialoglycoconjugates, while the postulated function of cytosolic OAE is to rescue any 9-*O*-acetylated sialic acids present in the cytosol [19], however, the latter has not been directly confirmed. To our knowledge, no OAE activities in human colonic mucosa have been described. However, an OAE activity detected in individual bacterial strains and in faecal extracts from normal individuals, which can facilitate the action of sialidase and thus the degradation of mucin oligosaccharides, has been reported [4,20].

Utilizing matched resection margin and cancer tissue at various stages of colorectal cancer development, a systematic survey of OAE and OAT activities along with the relative level of O-acetylated sialic acids, was undertaken. Here we show that the total level of sialic acid O-acetylation is significantly reduced in cancer mucosa, and that this reduction may be dependent on the relative activities of OAT to lysosomal OAE. Furthermore, we show that the level of free cytosolic Neu5,9Ac_2 in human colon, which has previously been found in porcine and bovine submandibular glands [9,21], is regulated by the relative activity of cytosolic OAE.

Materials and methods

Patient samples

Tissue was obtained from patients undergoing surgical resection of colorectal carcinomas. Fresh resection margin tissue, which showed normal histology, was obtained from the excised end of colon tissue resected for carcinoma. The colorectal carcinoma tissue that was obtained contained at least 80% cancer cells. The cancer stage assessment, based on the TNM classification system [22], was made by normal clinical and histological methods. The dissected tissue was washed in NaCl/P_i and frozen at −80 °C until required. Of the 13 tumour samples obtained; two were at Stage I, five at Stage II, four at Stage III and two at Stage IV. Each patient was informed about the study and gave written consent in accordance with the ethical guidelines of the Christian-Albrechts-University of Kiel, Germany.

Chemicals

All chemicals were of analytical grade except those for HPLC eluents that were gradient grade. Reversed phase columns (RP18, Lichrospher100, particle size 5 μm), HPTLC silica gel 60 (10 × 10 cm), HPLC solvents and all other chemicals unless otherwise stated were obtained from Merck (Darmstadt, Germany). Dowex 2 × 8 (200–400 mesh) was purchased from Fluka Chemie (Taufkirchen, Germany). CMP-Neu5Ac was obtained from Calbiochem-Novabiochem (Bad Soden, Germany). [^{3}H]AcCoA (specific activity: 7–28 Ci·mmol^{-1}) was obtained from Moravek Biochemicals (Bera, CA, USA). Mini Complete^{TM} protease inhibitor, Pefabloc SC and the acetic acid assay kit were from Roche Molecular Biochemical (Mannheim, Germany). 1,2-diamino-4,5-methylenedioxybenzene (DMB) was obtained from Dojindo Laboratories (Tokyo, Japan). 4-Methylumbelliferyl (MU), 4-methylumbelliferyl acetate (4-MUAc), 4-methylumbelliferyl-β-D-galactoside and acetyl-CoA (AcCoA) were purchased from Sigma-Aldrich Fine Chemicals (Deisenhofen, Germany). Centrex UF-0.5 (3 K MWCO) was from Schleicher & Schuell (Dassel, Germany). Mono- and oligo-O-acetylated sialic acids were purified from bovine submandibular gland mucins as described by Reuter and Schauer [23].

Preparation of lysosomal, microsomal and cytosolic fractions from human colonic mucosa

The lysosomes, microsomes and cytosol were prepared from the same homogenates using the differential centrifugation procedure described by Butor *et al.* [24]. Protein concentration was measured using the Micro-BCA protein assay reagent kit (Pierce, Rockford, IL, USA) as described by the manufacturer.

Sialyltransferase [25] and β-galactosidase [26] were used as the marker enzymes for the microsome and lysosome, respectively. In a typical preparation, β-galactosidase in the lysosomal fraction was enriched 2.5 times over the crude homogenate, while sialyltransferase was enriched twofold in the microsomal fraction. No β-galactosidase activity was observed in the cytosolic fractions isolated, indicating that lysosomes had not been disrupted. Sialyltransferase latency towards Triton X-100 indicated that approximately 75% of the microsomal membranes were intact and correctly orientated.

Fluorometric HPLC analysis of sialic acids

Sialic acids were prepared from the membrane and cytosolic fractions using the procedure described by Shen *et al.* [11]. For the purpose of GC-MS analysis, sialic acids were purified by sequential ion exchange chromatography as described by Reuter & Schauer [23]. For other purposes, sialic acids were purified on a column of Dowex 2 × 8.

Purified sialic acids were derivatised using DMB reagent and analysed by fluorometric HPLC utilizing the method described by Hara *et al.* [27]. The retention times of the

various sialic acids detected by HPLC were compared with authentic sialic acid standards. The identification of different sialic acids was additionally provided through mild periodate oxidation and ammonium treatment. Purified sialic acids were incubated prior to fluorometric HPLC with either 1 mM periodate for 20 min at 0 °C or 5% (v/v) ammonia solution for 1 h at 37 °C. Utilizing periodate oxidation, unsubstituted sialic acids such as Neu5Ac could be identified by monitoring, by HPLC, the disappearance of the corresponding peaks. Because O-acetylation of the glycerol side chain severely hinders periodate oxidation [1], O-acetylated sialic acids were identified following ammonium treatment by monitoring the decrease in the peak intensity by HPLC. This method was used to identify Neu5,7Ac_2, Neu5,9Ac_2 and oligo-O-acetylated sialic acids.

The amount of individual sialic acids separated by fluorometric HPLC was calculated via a standard curve constructed from known amounts of Neu5Ac (5–20 ng) against the corresponding area of the integrated peak.

GC-MS analysis of sialic acids from human colonic mucosa

Purified sialic acids were converted into their trimethylsilyl-methyl-ester derivatives and applied to a GC-system coupled with EI-MS (Fisons Instruments GC 8060/MD 800 system, Interscience, Breda, the Netherlands) and analysed according to a fragmentation scheme described by Kamerling & Vliegenthart [28].

Sialate-*O*-acetyltransferase (OAT) assay

OAT assays, either using endogenous sialic acid or CMP-Neu5Ac as acceptor substrates, were carried out as described in Shen *et al.* [11]. Briefly, OAT activity measured using endogenous substrates was performed by incubating 50 μg of protein in 30 μL of 50 mM potassium phosphate buffer, pH 7.0, containing 50 mM KCl, protease and esterase inhibitors, and [^{3}H]AcCoA (0.2 μCi, 8.3 μM) at 37 °C for 15 min. The reaction was stopped with 60 μL of 3 M propionic acid and membrane-bound sialic acids released by incubation at 80 °C for 2.5 h. Following the removal of membrane-bound proteins by centrifugation, the supernatant was lyophilized. The resulting residue was resuspended in ice-cold water and sialic acids purified as previously described [24]. The incorporation of [^{3}H]acetate into the glycerol side chain of sialic acids was subsequently quantified by radio-TLC. TLC was performed on silica gel 60 HPTLC plates and developed in methanol/chloroform/20 mM $CaCl_2$ (5 : 4 : 1, v/v/v).

OAT activity measured using CMP-Neu5Ac as the acceptor substrate was carried out by incubating 50 μg of protein in 30 μL of 50 mM potassium phosphate buffer, pH 7.0, containing 600 μM CMP-Neu5Ac, 50 mM KCl, protease and esterase inhibitors and [^{3}H]AcCoA (0.2 μCi, 8.3 μM) at 37 °C for 15 min. The reaction was stopped with 60 μL of 3 M propionic acid and heated at 80 °C for 15 min. After removal of membrane-bound proteins by centrifugation, the supernatant was lyophilized. The isolated sialic acids, following purification, were analysed and quantified by radio-TLC as described above.

Sialate-*O*-acetylesterase (OAE) assay

The OAE activity in different subcellular fractions prepared from carcinoma and resection margin mucosa was assayed using a number of different substrates. Nonspecific esterase assays were performed using the substrate 4-MUAc as described by Schauer *et al.* [18]. One unit of esterase activity equals 1 nmol of MU released per min under the conditions used.

OAE activity using Neu5,9Ac_2 and 5-*N*-acetyl-7(8),9-di-*O*-acetylneuraminic acid (Neu5,7(8)9Ac_3) as substrates were determined using the procedure outlined by Schauer *et al.* [18]. Acetic acid released from O-acetylated sialic acids was measured using a commercial test kit according to the manufacturers instructions. One unit of esterase activity equals 1 nmol of acetic acid released per min under the conditions used.

The hydrolysis of O-acetyl groups from Neu5,9Ac_2 and Neu5,7(8)9Ac_3 was also monitored by fluorometric HPLC. A sample of Neu5,9Ac_2-enriched (1 mM) or Neu5,7(8)9Ac_2-enriched (2.5 mM) sialic acid was incubated together with 50 μg of protein in NaCl/P_i at 37 °C for 1 h. The reaction products formed were subsequently identified and quantified by HPLC as described [27].

Results

The relative level of O-acetylated sialic acids is decreased in the mucosa from colorectal carcinoma patients

The content of glycoconjugate-bound sialic acid in the mucosa from matched resection margins and colorectal carcinoma tissue was determined by fluorometric HPLC and GC-MS analyses. As can be seen in Table 1, the predominant derivative of sialic acid, present as either glycoconjugate-bound or free in both resection margin and cancer mucosa, was Neu5Ac. Neu5Gc was not detected by HPLC or GC-MS. Apart from Neu5Ac, another molecule sensitive to mild periodate oxidation and eluting with a retention time relative to Neu5Ac (R_{Neu5Ac}) of 0.72, was observed. Despite this retention time indicating the presence of Kdn [1], confirmation by GC-MS could not be obtained. The exact nature of this molecule awaits elucidation, and is therefore referred to in Table 1 as unknown.

As has been reported previously [3,7], the resection margin obtained from colorectal cancer patients possesses significant levels of mono- and oligo-O-acetylated sialic acids (identified via their susceptibility to alkaline treatment). The principal mono-O-acetylated species detected was Neu5,9Ac_2 (18.0 ± 8.0%, 1.25 ± 0.55 μg·mg protein^{-1}), with a small amount of Neu5,7Ac_2 (1.2 ± 1.4%, 0.09 ± 0.15 μg·mg protein^{-1}) being observed. Neu5,7Ac_2 was not detected by GC-MS; this is probably due to the ability of O-acetyl groups at C-7 to migrate to C-9 during extended periods of storage [16].

GC-MS analysis revealed that the oligo-O-acetylated species observed by HPLC consisted of 5-*N*-acetyl-8,9-di-*O*-acetylneuraminic acid (Neu5,8,9Ac_3) and 5-*N*-acetyl-7,8,9-tri-*O*-acetylneuraminic acid (Neu5,7,8,9Ac_4), however, neither 5-*N*-acetyl-7,8-di-*O*-acetylneuraminic acid (Neu5,7,8Ac_3) nor 5-*N*-acetyl-7,9-di-*O*-acetylneuraminic acid (Neu5,7,9Ac_3) were observed (data not shown).

Table 1. Fluorometric-HPLC and GC-MS analysis of glycoconjugate-bound and free sialic acids isolated from the mucosa of matched resection margin and colorectal carcinoma tissue. Sialic acids were isolated and analysed by HPLC and GC-MS as described in Materials and methods. The proportion of individual sialic acids is expressed as a percentage of the total sialic acid in each sample. Values stated in parenthesis are μg sialic acid·mg protein^{-1}. HPLC analyses; n = 13 (values are stated as mean ± SD). GC-MS analyses; four matched colon sample pairs were pooled and the sialic acids isolated from the microsomal and cytosolic fractions were analysed following trimethylsilyl-methyl-ester derivatization by GC-MS. GC-MS bound; represents the average proportion of glycoconjugate-bound sialic acids isolated from the microsomal and cytosolic fractions. Neu2,7an5Ac is a by-product of sialoglycoconjugate hydrolysis by mild acid, and cannot be detected by fluorometric-HPLC. ND, not detected.

	% Sialic acids in resection margins				% Sialic acid in colorectal carcinoma			
	HPLC		GC-MS		HPLC		GC-MS	
Sialic acid derivative	Bound	Free	Bound	Free	Bound	Free	Bound	Free
Neu2,7an5Ac	ND	ND	3.5	ND	ND	ND	4.0	ND
Unknown[1,2]	2.4 ± 0.6	42.2 ± 20.2	ND	ND	2.5 ± 2.1	54.4 ± 22.5	ND	ND
Neu5Ac[2]	68.5 ± 11.4 (5.4 ± 0.9)	54.0 ± 22.3 (2.4 ± 1.0)	73.0	100	85.6 ± 10.5 (4.9 ± 0.6)	41.9 ± 24.8 (1.7 ± 1.0)	78.0	100
Neu5,7Ac$_2$[2,3]	1.2 ± 1.4 (0.09 ± 0.15)	ND	ND	ND	0.4 ± 0.9 (0.02 ± 0.04)	ND	ND	ND
Neu5,9Ac$_2$[3]	18.0 ± 8.0 (1.25 ± 0.55)	3.3 ± 4.2 (0.15 ± 0.19)	12.0	Trace	7.0 ± 4.0 (0.62 ± 0.35)	3.9 ± 5.0 (0.16 ± 0.20)	4.0	ND
Oligo-*O*-Ac-Neu5Ac[3]	3.9 ± 4.4 (0.3 ± 0.3)	ND	6.5	ND	ND	ND	ND	ND
Total *O*-Ac-Neu5Ac	23.1 ± 13.5 (1.55 ± 0.9)	3.3 ± 4.2 (0.15 ± 0.19)	18.5	Trace	7.5 ± 5.0 (0.64 ± 0.42)	3.9 ± 5.0 (0.16 ± 0.20)	4.0	Trace

[1] Unknown molecule with retention relative to Neu5Ac (R_{Neu5Ac}) of 0.72, as determined by fluorometric-HPLC, but not identified by GC-MS. [2] Susceptible to mild periodate oxidation. [3] Susceptible to ammonium treatment.

Interestingly, Neu5,7,8,9Ac$_4$ was only detected in the fine membrane fractions (microsomes) of resection margin mucosa, whereas Neu5,8,9Ac$_3$ was observed as glycoconjugate-bound sialic acid in both microsomal and cytoplasmic fractions (data not shown). The observation of tri-O-acetylated sialic acid in the fine membrane fraction but not in the cytoplasm provides some evidence for the presence of a migrase that may facilitate the formation of higher (tri-)O-acetylated sialic acid derivatives. Such a migrase, found in the microsomes from bovine submandibular glands, has been postulated to catalyse the rapid migration of O-acetyl groups along the glycerol side chain, subsequently followed by the transfer of another acetyl group [10].

It should be noted that the level of oligo-O-acetylated sialic acid reported here is probably an underestimation resulting from its coelution with a reagent peak (data not shown). Determination of oligo-O-acetylated sialic acid levels was therefore afforded by calculating the difference in peak intensity before and after alkaline treatment (reagent contamination is not sensitive to alkaline treatment).

The relative amounts of mono-, di- and tri-O-acetylated sialic acids in the mucosa from the corresponding matched colorectal carcinoma sample were also evaluated. As is shown in Table 1, the expression of oligo-O-acetylated sialic acid appeared to be completely eliminated. Similar findings have been observed in studies utilizing a series of human colorectal carcinoma cell lines [7] and tissue obtained from colorectal cancer patients [3,7]. The level of mono-O-acetylated Neu5Ac (Neu5,9Ac$_2$ and Neu5,7Ac$_2$) was also reduced with, in the case of Neu5,9Ac$_2$, only $\approx 7\%$ observed in cancer mucosa compared to $> 18\%$ in resection margin tissue. This is at odds with Corfield *et al.* [7], who observed that the level of Neu5,9Ac$_2$ remains constant as a result of malignant transformation.

The reduction in mono-O-acetylated sialic acid seen in cancer tissue compared to that in the corresponding resection margin was also observed at all stages of colorectal carcinoma (Table 2). Interestingly, the level of Neu5,9Ac$_2$ in the resection margin obtained from two Stage IV patients was dramatically reduced in comparison with that observed in Stage I, II and III patients. This suggests that not only is sialic acid O-acetylation decreased in the tumour itself but also in the resection margins obtained at a late stage in tumour development. It should be noted that in all cases the resection margins were assessed by routine clinical and histological methods as being normal.

A small but reproducible amount of Neu5,9Ac$_2$ was observed as cytoplasmic-free sialic acid by HPLC, with trace amounts also being detected by GC-MS. However, unlike glycoconjugate-bound Neu5,9Ac$_2$, no cancer related alterations in the level of Neu5,9Ac$_2$ were observed (Table 1).

OAE from human colonic mucosa specifically hydrolyses 9-*O*-acetyl groups on sialic acid

Sialate-*O*-acetylesterase (OAE) activity using the substrates Neu5,9Ac$_2$, Neu5,7(8),9Ac$_3$ and 4-MUAc was detected in the cytosolic and lysosomal fractions prepared from human colonic mucosa (Fig. 1). OAE activities, determined using bovine submandibular gland mucin as the source of glycosidically bound O-acetylated sialic acids, showed no

Table 2. Reduction in glycoconjugate bound Neu5,9Ac$_2$ and oligo-O-acetylated Neu5Ac in cancer mucosa at various stages of colorectal carcinoma. Sialic acids were isolated and analysed by fluorometric-HPLC. The proportion of individual sialic acids is expressed as a percentage of the total sialic acid in each sample. The values stated are the mean ± SD. The cancer stage was assessed using the TNM classification system [22]. ND, not detected.

	Resection margins (% sialic acid)		Colorectal carcinoma (% sialic acid)	
Stage	Neu5,9Ac$_2$	di- and tri-*O*-Ac-Neu5Ac	Neu5,9Ac$_2$	di- and tri-*O*-Ac-Neu5Ac
I ($n = 2$)	18.9	3.6	3.9	ND
II ($n = 5$)	18.5 ± 8.0	4.1 ± 4.5	7.5 ± 4.6	ND
III ($n = 4$)	20.4 ± 9.2	5.4 ± 4.9	6.4 ± 3.5	ND
IV ($n = 2$)	11.9	2.0	8.6	ND

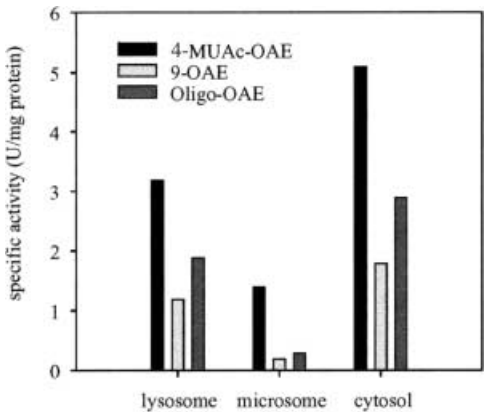

Fig. 1. The subcellular distribution of OAE activities in human colonic mucosa. Lysosomal, microsomal and cytosolic fractions isolated from four different resection margins were pooled and analysed. Nonspecific esterase activity (4-MUAc-OAE) was determined using 4-MUAc as substrate, one unit of activity equals 1 nmol of MU released per min. Sialic acid specific OAE activity was measured using Neu5,9Ac$_2$ (9-*O*AE) and Neu5,7(8),9Ac$_3$ (Oligo-OAE) as substrate, one unit of activity equals 1 nmol of acetic acid released per min.

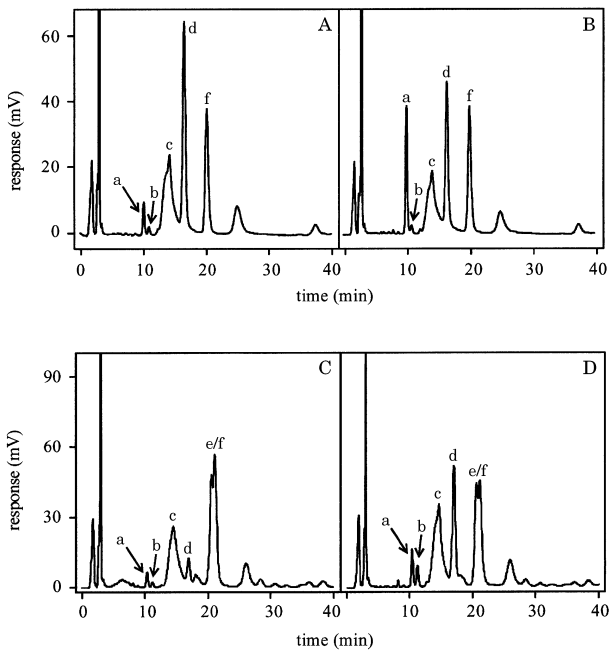

Fig. 2. The hydrolysis of O-acetyl groups from Neu5,9Ac$_2$ and Neu5,7(8),9Ac$_3$ by OAE-C. A heat-denatured cytosolic fraction (A) and a cytosolic fraction (B) were incubated with a 1 mM Neu5,9Ac$_2$ enriched sialic acid sample at 37 °C for 1 h. Similarly a heat-denatured cytosolic fraction (C) and a cytosolic fraction (D) were incubated with a 2.5 mM Neu5,7(8),9Ac$_3$ enriched sialic acid sample at 37 °C for 1 h. All resulting products were subsequently analysed by fluorometric-HPLC. a, Neu5Ac; b, Neu5,7Ac$_2$; c, reagent peak; d, Neu5,9Ac$_2$; e, oligo-O-acetylated Neu5Ac; f, reagent peak (not effected by incubation with cytosolic fraction).

observable differences in the cytosolic and lysosomal fractions compared with that obtained using free sialic acid substrates. Therefore, soluble free O-acetylated sialic acids and 4-MUAc were used throughout for the determination of OAE activity.

A small amount of activity was also observed in the microsomal fraction (Fig. 1). This activity is probably due to the presence of residual lysosome membranes. Therefore these results show that at least two OAE activities exist in human colonic mucosa, a soluble form localized in the cytosol (OAE-C), and a membrane-associated form that colocalized with β-galactosidase in the lysosomes (OAE-L). The presence of two OAE activities with altered localization has been observed previously in rat liver [24] and bovine brain [18].

To further examine the enzymatic hydrolysis of O-acetyl residues from mono-O-acetylated sialic acids, enzyme products were monitored by fluorometric-HPLC. As shown in Fig. 2, no degradation of O-acetyl groups from Neu5,9Ac$_2$ was observed when a heat-denatured cytosolic fraction was incubated with a sialic acid mixture enriched in Neu5,9Ac$_2$ (Fig. 2A, peak d). When the same mixture was incubated with a cytosolic fraction the observed amount of Neu5,9Ac$_2$ decreased, with a corresponding increase in the amount of Neu5Ac (Fig. 2B, peak a). Identical results were obtained when a lysosomal fraction was investigated using the same sialic acid mixture enriched in Neu5,9Ac$_2$ (data not shown).

Using a sialic acid mixture enriched in Neu5,7(8),9Ac$_3$, the process of de-O-acetylation catalysed by OAE-C was also monitored (Fig. 2C,D). Following the incubation of this mixture with a cytosolic fraction (Fig. 2D), a reduction in the amount of Neu5,7(8),9Ac$_3$ was observed. This reduction, in comparison with the control incubation performed with denatured cytosol (Fig. 2C), was accompanied by an increase in the level of not only Neu5Ac (peak

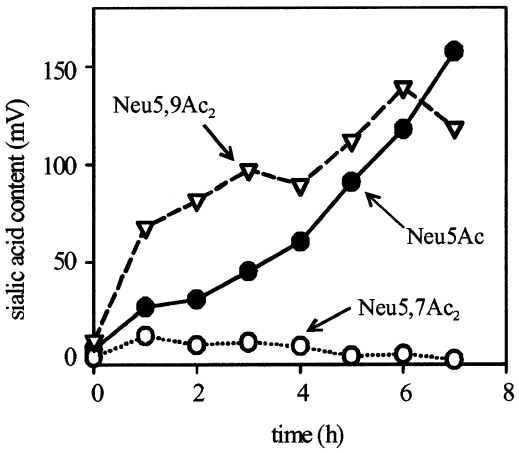

Fig. 3. The sequential removal of O-acetyl groups from Neu5,7(8),9Ac$_3$ by OAE-C. A sialic acid sample enriched in oligo-O-acetylated sialic acids was incubated with a pooled cytosolic fraction prepared from four resection margins at 37 °C and the resulting enzyme products quantified by fluorometric-HPLC at time points between 1 and 7 h.

a), but also Neu5,7Ac$_2$ (peak b) and Neu5,9Ac$_2$ (peak d). This suggests that a mixture of Neu5,7Ac$_2$ and Neu5,8Ac$_2$ is being released following the action of OAE-C on the primary ester at C-9. The 8-*O*-ester of Neu5,8Ac$_2$, considered to be extremely unstable [16], spontaneously migrates to position 9 which can subsequently be hydrolysed to give Neu5Ac. Neu5,7Ac$_2$, in comparison with Neu5,8Ac$_2$, is relatively stable with an isomerization half-life (of free Neu5,7Ac$_2$ to Neu5,9Ac$_2$) of approximately 6 h at physiological conditions (37 °C, pH 7.0) [16]. Therefore, the hydrolysis of side chain O-acetylated sialic acid is catalysed by an enzyme specific for 9-*O*-acetyl groups, with O-acetyl groups at position 7 and 8 being sequentially removed following migration to C-9. This proposed sequential de-O-acetylation of oligo-*O*-Ac-Neu5Ac is supported by the time course experiment shown in Fig. 3. As shown, the level of Neu5,9Ac$_2$ increases with time up to 6 h; this corresponds to the time point at which no further Neu5,7Ac$_2$ can be detected. Only following this point can a significant reduction in the level of Neu5,9Ac$_2$ be observed. The level of Neu5Ac, as expected, steadily increased during the entire incubation.

Altered OAT but not OAE activity in the mucosa from colorectal cancer

To explore the underlying mechanism responsible for the alteration of O-acetylated sialic acids in cancer mucosa, the activities of OAE-L, OAE-C and OAT in the resection margins and cancer mucosa from matched tissue samples were determined. As shown in Fig. 4, no significant difference (t-paired test, $p > 0.05$) in OAE activity when using Neu5,9Ac$_2$ (Fig. 4A) and oligo-*O*-Ac-Neu5Ac (Fig. 4B), was observed between resection margin and cancer mucosa in all subcellular fractions tested. OAE activity was also unchanged during cancer progression, with no alteration in OAE activities at different cancer stages (data not shown). No correlation could be observed between OAE activity and the expression of O-acetylated sialic acids in colorectal carcinoma.

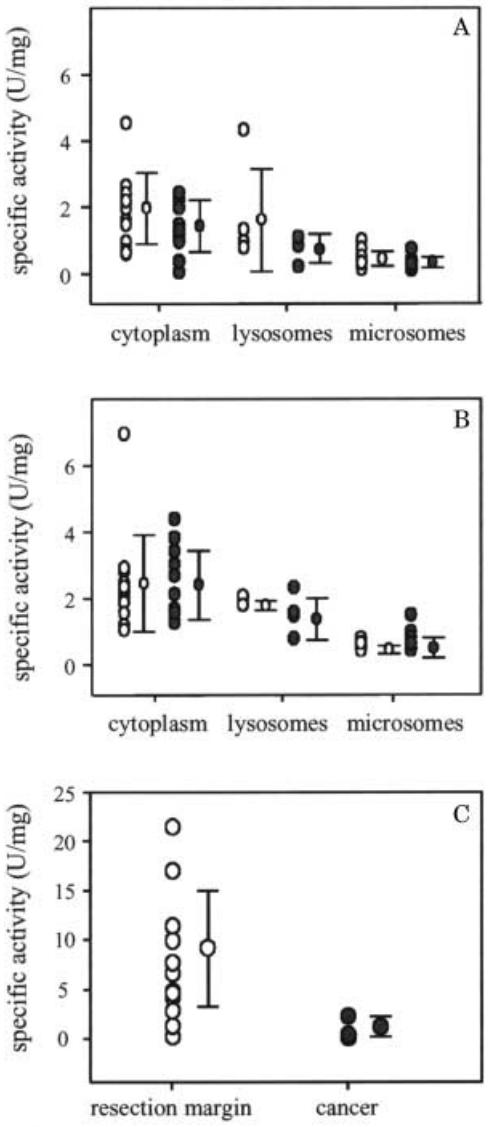

Fig. 4. The OAE specific activity in various subcellular fractions. OAE activity from resection margin (○) and colon cancer mucosa (●) was determined using Neu5,9Ac$_2$ (A) and Neu5,7(8),9Ac$_3$ (B) as substrate. The OAT specific activity in microsomal fractions (C) was determined as described previously [11]. The Bars show the mean ± SD ($n = 13$).

Unlike OAE activity, OAT activity using CMP-Neu5Ac as the acceptor substrate was significantly reduced ($P = 0.03$) in the microsomes isolated from cancer mucosa (Fig. 4C). We have shown previously that the OAT from normal colonic mucosa efficiently O-acetylates CMP-Neu5Ac exclusively in the Golgi apparatus, yet endogenous glycoconjugate substrates can also be O-acetylated [11]. However, no OAT activity was observed against endogenous substrates in the microsomes isolated from cancer mucosa. As was found for OAE, no correlation between the expression of O-acetylated sialic acids and OAT activity could be observed (Fig. 5A).

These results show clearly that the alteration in O-acetylated sialic acids in colorectal cancer cannot be attributed purely to the individual activities of OAE or OAT

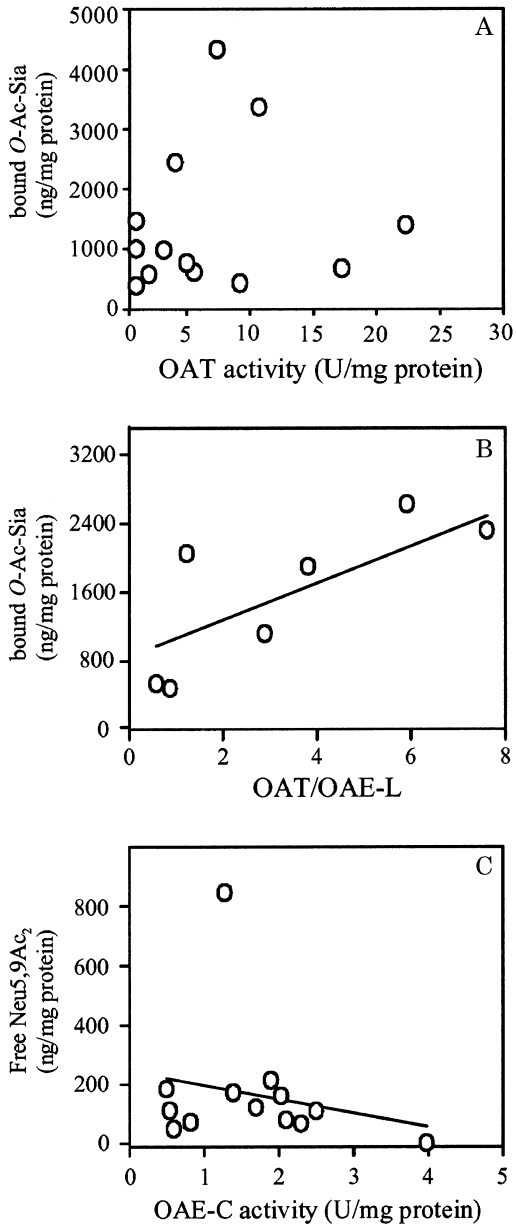

Fig. 5. The regulation of glycoconjugate-bound and free O-acetylated sialic acids. All correlations were assessed using Spearman rank coefficient (r_s). (A) The level of bound O-acetylated sialic acids is not regulated by OAT activity ($n = 13$, $r_s = 0.24$, $P = 0.005$); (B) positive correlation between glycoconjugate-bound O-acetylated sialic acid levels and the relative activity of OAT to OAE-L ($n = 7$, $r_s = 0.82$, $P = 0.003$); (C) positive correlation between free Neu5,9Ac_2 and OAE-C activity ($n = 13$, $r_s = 0.70$, $P = 0.005$). Enzyme activities and O-acetylated sialic acid levels were determined from individual resection margin mucosa as described in Materials and methods.

because no correlation between O-acetylated sialic acid levels and the individual activity measurements could be found. However, it has been reported that the removal of sialic acid, and therefore mucin oligosaccharide degradation, in human colon is regulated at the level of sialic acid O-acetylation by the relative levels of OAE and sialidase found in individual bacterial strains and faecal extracts from normal individuals [4,20]. Therefore we explored the possibility that the relative levels of OAT and OAE in both the resection margin and cancer mucosa regulate the level of O-acetylated sialic acids.

Figure 5B shows that in the resection margin from colorectal carcinoma patients, a significant positive correlation between the OAT:OAE-L activity ratio and the level of glycoconjugate-bound O-acetylated sialic acids was observed. This correlation, with a Spearman rank coefficient (r_s) of 0.82 ($P = 0.003$), was also found to occur in the corresponding matched cancer tissue (data not shown). This finding suggests that the relative levels of OAT to OAE-L activity might regulate the level of glycoconjugate-bound O-acetylated sialic acid in human colonic mucosa.

As shown in Table 1, a small but reproducible amount of free mono-*O*-acetylated-Neu5Ac (0.15 ± 0.19 ng·mg protein^{-1}) was detected in the cytoplasm from colonic mucosa. It has previously been proposed that a cytosolic OAE exists that is involved in the degradation of free O-acetylated sialic acids in the cytosol [19]. We have already shown here that an OAE-C activity is present in both the resection margin and cancer mucosa. Figure 5C shows that this activity regulates the level of free Neu5,9Ac_2 in the cytoplasm. A significant correlation ($r_s = 0.7$, $P = 0.005$) between OAE-C activity and free Neu5,9Ac_2 was observed not only in the resection margins (Fig. 5C) but also in cancer mucosa (data not shown).

Discussion

Previous studies have shown that the sialic acids present on mucins synthesized and secreted by the human colonic mucosa are highly O-acetylated [3,7], with histochemical studies suggesting that the level of O-acetylation is as high as 80% in normal colonic tissue [3]. On the other hand, a reduction in the level of sialate O-acetylation in colon cancer has been demonstrated [3,7,29], however, this reduction is presumably restricted to oligo-O-acetylation with mono-O-acetylation remaining constant [7,30]. In this study, utilizing matched colonic samples (resection margin and cancer tissue obtained from the same colorectal carcinoma patients) at all stages of cancer development, we revealed that a significant reduction in not only oligo-O-acetylated sialic acids, but also mono-O-acetylated species, occurs in cancer mucosa. This reduction in total O-acetylation was observed at all cancer stages, and mirrors observations made in cultured human colorectal cells representing stages in the adenoma-carcinoma sequence [7]. The exception in this case being that a reduction in total O-acetylation, rather than only oligo-O-acetylation, appeared as an early event in malignant transformation.

Differences in the relative level of sialic acid O-acetylation have previously been observed in the mucins isolated from resection margin and noncancer tissue [7]. These differences are probably the result of a premalignant field defect, rather than a local secondary effect of tumour growth [31,32]. However, in the resection margin from Stage IV patients we observed a significant decrease in the level of Neu5,9Ac_2 in comparison to earlier stages. This suggests that at a late stage in tumour development a local secondary effect occurs in colorectal carcinoma where the expression of O-acetylated sialic acids is decreased, even though the resection

margins obtained were all classified as normal by routine clinical and histological methods. Histochemical or immuno-histochemical analyses could provide conclusive proof for the alteration of sialic acid O-acetylation in resection margins from Stage IV patients, however, such data is currently unavailable.

A number of analytical techniques are currently available for the qualitative and quantitative determination of sialic acids [23]. In this report we utilized two very specific and powerful techniques, fluorometric-HPLC and GC-MS, for the detection and quantitation of sialic acids, in particular O-acetylated sialic acids, from human colon mucosa. The presence of all O-acetylated sialic acids that were detected by fluorometric-HPLC could be confirmed by GC-MS, however, the exact nature of the molecule present in the mucosa from human colon with an R_{Neu5Ac} similar to that of Kdn remains to be established.

OAE activities with different localizations have previously been reported to occur in a variety of mammalian tissue [18,24]. In accordance with this, two distinct OAE activities, one in the cytoplasm and another in the lysosomal compartment, were found to occur in human colonic mucosa. With regard to OAE-C we show here that this activity regulates the level of free 9-*O*-acetylated sialic acids. It is generally accepted that 9-*O*-acetylated sialic acids can occur freely in the cytosolic fractions isolated from mammalian cells [9]. Their presence in the cytosol we believe, is not an artefact of the method used to prepare subcellular fractions, but instead are free 9-*O*-acetylated sialic acids probably resulting from the action of a lysosomal sialidase followed by release into the cytolplasm [9,21]. Data presented here allows for the speculation that OAE-C probably rescues any 9-*O*-acetylated sialic acids that evade the action of the lysosomal esterase. Sialic acids rescued in this manner can then re-enter the sialic acid metabolic pathway.

The regulation of OAE-C and OAE-L at the molecular level is still open to debate. Findings provided by Takematsu *et al.* [19] suggest that, at least in mice, a single gene can encode two differently localized OAEs by differential usage of a signal peptide encoding exon at the N-terminus and that expression is regulated by independent promoters [19]. The results reported here support these findings, because apart from different localizations, no significant differences between the two activities, both in resection margin and cancer mucosa, were found. In particular, OAE-C and OAE-L were both shown to specifically hydrolyse 9-*O*-acetyl groups, with complete de-O-acetylation of oligo-O-acetylated sialic acids being achieved in a sequential manner. The stepwise removal of O-acetyl groups following migration of the remaining ester groups to position 9 is supported by the postulated pathway for the turnover of sialate O-acetylation reported by Butor *et al.* [24].

The expression of OAE-L mRNA in mouse tissue has been shown to be widespread, whereas OAE-C is restricted to liver, ovary and brain [19]. The expression of OAE-C and OAE-L mRNA, however, has not been studied in mouse colon. Nevertheless, based on our results one would expect message corresponding to both OAE forms to be present in colon. However, one cannot rule out the possibility that one or more other genes exist that can generate active OAE-C in colon or other tissue. Evidence for this is provided by Takematsu *et al.* [19], who report that in certain tissues OAE activity was detected that did not coincide with a protein cross-reacting with an antibody directed against a 69 amino-acid sequence shared by OAE-L and OAE-C. It is therefore apparent that further studies are required to clarify the regulation of OAE at the molecular level, including promoter analysis to prove the postulated differential promoter usage.

We have reported recently on the identification of a Golgi-localized human colon OAT activity that O-acetylates CMP-Neu5Ac [11] prior to the action of sialyltransferase (Y. Shen, J. Tiralongo, G. Kohla & R. Schauer, unpublished observation). Here we show that this activity is dramatically reduced in colon cancer in comparison with that observed in resection margins. Previously, using a mucin glycopeptide substrate, a reduction in OAT activity was observed in the homogenates of cancer tissues in comparison with that of normal colonic mucosa [7]. The expression of O-acetylated sialic acids in human colonic tissues shows racial variability [33,34], in which it is assumed that a single dominant gene encoding an OAT (*oat*) regulates sialate O-acetylation. For example, approximately 9% of apparently normal Europeans are believed to be homozygous (oat^-/oat^-) for sialate O-acetylation; the resulting loss of O-acetylated sialic acids in these cases, which is not believed to be a disease-associated event [35], is presumably regulated solely by the expression of OAT [33,34]. However, we show here that the level of OAT expression, assessed by direct activity measurements, does not correlate with the observed level of O-acetylated sialic acids in the corresponding mucosal sample. This was found to be the case in all matched-mucosal samples investigated. Instead, the level of O-acetylated sialic acids in human colon was found to correlate with the relative levels of OAT:OAE-L activity.

The tumor-associated over-expression of sialyl $Lewis^X$ and the sialyl-Tn antigen has been shown in colorectal carcinoma to correlate with cancer progression and metastases [8,36,37]. The finding that sialyl $Lewis^X$ is a ligand for E-selectin suggests that E-selectin-containing endothelial cells may interact with sialyl $Lewis^X$-bearing carcinoma cells, thus mediating extravasation of metastatic cells [38,39]. Immunohistochemical studies have shown that the expression of sialyl $Lewis^X$ and the sialyl-Tn antigen in normal and cancer mucosa is unaltered [35,40]. A subsequent study showed that the overexpression of sialyl $Lewis^X$ on MUC1 and MUC2 mucins during cancer progression is actually due to a reduction in O-acetylation and not, for example, the increased expression of mucin protein cores [8]. Taken together these studies indicate that sialate O-acetylation plays a pivotal role in regulating colorectal cancer progression, in particular its metastatic potential.

The finding reported here that the level of sialic acid O-acetylation may be dependent on the relative activities of OAT:OAE-L provides a significant insight into the regulation of this important modification in normal as well as in diseased tissue. Based on this information a reduction in or halting of colorectal cancer progression, and possibly metastasis, appears conceivable by regulating the relative levels of OAT:OAE-L.

To further elucidate the mechanism, regulation and significance of sialate O-acetylation in human colon, as well as in other biological systems, information regarding all the metabolizing and catabolizing steps is necessary. Currently,

sequence information for OAE-L and OAE-C is available [19,41], however, the complex genetic regulation and expression of these enzyme forms requires further elucidation. With regards to the sialate *O*-acetyltransferase, this enzyme has stubbornly avoided purification and cloning, remaining elusive despite the efforts of a number of groups. Nevertheless, the information reported here adds considerably to our understanding of sialate O-acetylation regulation in human colon, in particular the role of the sialic acid specific *O*-acetylesterase and -transferase in this process.

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