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Analysis of cancer-associated colonic mucin by ion-exchange chromatography: evidence for a mucin species of lower molecular charge and weight in cancer

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Cancer-associated mucins in the colon are antigenically distinct and glycosylated differently from their normal counterparts. Mucin-rich glycoconjugate preparations were made from nine non-neoplastic colons, seven colon cancers, and two different xenografts from mucin-producing human colon cancer cell lines, and radiolabeled with ^{3}H. The preparation was applied to a DEAE-cellulose ion-exchange column, and eluted with a discontinuous ascending NaCl gradient resulting in seven discrete fractions or 'species'. Over half of the ^{3}H-labeled glycoconjugates from specimens of non-neoplastic colonic epithelium eluted in fraction V (eluted with 0.25 NaCl). Significantly less of the ^{3}H-labeled glycoconjugates from specimens of colon cancer eluted in fraction V (34%, $P < 0.0005$), and there were significant increases in glycoconjugates eluted in fractions IV ($P < 0.008$), III ($P < 0.0005$), and II ($P < 0.028$). Additional samples were prepared without the radiolabeling procedures, chromatographed on a DEAE-cellulose ion-exchange column, and analyzed for monosaccharide content. Each of the fractions contained the monosaccharides expected in mucin-type glycoproteins, but only sialic acid was differentially expressed in the seven fractions or 'species', occurring principally in the more charged species. However, differences in sialic acid content were not sufficient to explain the differences in retention on the ion-exchange column, nor were differences in *O*-acetylation of the mucins. Mucin-type glycoconjugates from colon cancers are relatively less charged than those from the normal colon, and elute at lower ionic strengths. Of interest, cancer-associated mucins appear to be of lower molecular weight than their normal counterparts. Additional studies of oligosaccharide and apomucin structure will be required to explain the molecular basis of these differences in charge.

Introduction

Most epithelial surfaces secrete mucus, which is a complex mixture of mucin, lipid, and other solutes. After secretion, mucin forms a gel that lines and protects the epithelium. The principal glycoprotein constituents of mucus are mucins, which are a family of high molecular weight, carbohydrate-rich glycoproteins that express an extraordinary degree of microheterogeneity. Using histochemical and biochemical techniques, it has been shown that heterogeneity of mucin is seen as a function of its location within the gastrointestinal tract [1–3], in certain disease states [4–6], and with the processes of cellular differentiation and malignant transformation [7–11].

Using ion-exchange chromatography, Podolsky and Isselbacher fractionated colonic mucins into six discrete species and demonstrated that one of these (designated species IV) was selectively reduced in the colonic mucins of patients with ulcerative colitis [5]. Furthermore, it was demonstrated that this deficit persisted even in quiescent disease [12]. A similar alteration has been found by these investigators in an animal model of colitis [13]. It is not yet known how this biochemical abnormality is related to the histochemical abnormalities that have been described in inflammatory bowel disease [1,6,14–17].

Mucin secreted by colon cancer is immunologically and biochemically different from that in the normal colon [7,8,10], but details regarding the molecular structures of these different forms of mucin are not available. With the exception of the work done in ulcerative colitis [5,12], human colonic mucins in different disease states have not been analyzed using ion-exchange chro-

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matography. For the purposes of this study, mucins from specimens of normal and cancerous human colonic tissues were analyzed by a slight modification of the methods described by Podolsky and Isselbacher [5,12]. Samples of normal human colon, colon cancer, and xenografts generated from cultured human colon cancer cell lines were examined by extracting tissue glycoconjugates, radiolabeling them, and fractionating them using ion-exchange chromatography. A significant alteration in the relative proportions of the seven mucin species was seen, with a shift towards less charged mucins in glycoconjugate preparations from cancerous tissues. The shift was more pronounced in the xenograft specimens.

Materials and Methods

Reagents

Preparative and radiolabeling reagents. The following reagents were obtained from Sigma Chemical Company, (St. Louis, MO): sodium *m*-periodate ($NaIO_4$), galactose oxidase (EC 1.1.3.9), Tris buffer, ribonuclease A type IA (EC 3.1.27.5), deoxyribonuclease I (EC 3.1.21.1), β-mercaptoethanol, sodium dodecyl sulfate (SDS), and Sepharose 4B. NaB^3H_4 (specific activity 50–60 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Cesium chloride, optical grade, 99.99% purity, was purchased from Schwarz/Mann (Cleveland, OH). Acrylamide and *N,N'*-methylene bisacrylamide were purchased from National Diagnostics, Manville, NJ.

Monosaccharides for standards in the carbohydrate analysis were purchased from Sigma. Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). A 50% (w/w) NaOH solution was purchased from Fischer Scientific (Livonia, MI). Sodium acetate was purchased from Sigma. The chromatographic separations were performed on a Dionex BioLC gradient pump module with a model PAD 2 detector, and the monosaccharide separation performed on a (4.6 × 250 mm) HPIC-AS6 anion-exchange column equipped with an AG-6 guard column (Dionex, Sunnyvale, CA).

For ion-exchange chromatography, diethylaminoethyl (DEAE) cellulose (fine mesh) was purchased from Sigma.

Tissues

Fresh human tissues were obtained from the operating room, and immediately prepared for study or frozen at −70°C until use. Nine specimens of histologically normal colonic epithelium were obtained from patients as listed in Table I. A total of seven specimens of freshly excised human colon cancer, and xenografts derived from two different cell lines were utilized. The tumor cell line LS1714T was obtained from the American Type Culture Collection and was raised as a subcutaneous, mucin-producing xenograft in the nude mouse as previously described [18]. Xenografts from our different animals were utilized for replicate studies. Cell line RW-2982 was obtained from Lance Tibbetts, M.D. (Providence, RI). This cell line grows as a mucoid suspension in culture, and grows as bulky, noninvasive, mucin-producing masses after the injection of approximately 10^6 cells into the peritoneum of a nude mouse [19]. Subcutaneous tumors from these cells were obtained after 3–4 weeks of growth in the nude mouse.

TABLE I

Human tissues used as sources of glycoconjugates

Tissue designation	Source of tissue
NC ($n = 7$) ('normal-cancer', i.e. normal tissues from colons with cancer)	Histologically normal colonic epithelium from patients undergoing a resection for cancer; at least 6 cm from any neoplastic lesion; all specimens from the sigmoid colon
NN ($n = 2$) ('normal-normal', i.e. normal tissues from nonneoplastic colons)	Histologically normal colonic epithelium from patients undergoing resection for diverticulitis; at least 6 cm from inflammation; both specimens from the sigmoid colon
CA ($n = 7$) (cancer)	Colonic adenocarcinoma; CA-1, CA-5, CA-6, CA-12 from the sigmoid colon; CA-2, CA-11, CA-14 from the ascending colon or cecum
LS174T ($n = 4$) (cell line)	Subcutaneous xenograft in nude mice raised from human colonic cancer cell line LS174T
RW 2982 ($n = 1$) (cell line)	Subcutaneous xenograft from human colonic cancer cell line RW 2982

Extraction of glycoconjugates from tissues

Specimens of nonneoplastic colon were obtained by rinsing residual luminal debris from surgically excised colons and scraping the epithelial layer from the muscularis propria using the edge of a glass slide. Typically, 2–10 g of tissue were obtained from a 5–10 cm segment of sigmoid colon. This material was immediately suspended in 5 ml of phosphate-buffered saline (PBS), pH 7.4, for each gram of tissue obtained. This gelatinous suspension was readily dispersed by 30 s of sonication.

The specimens of cancer were immediately obtained from the operating room and sharply excised with a scalpel, trimming away adherent normal tissue (epithelium, muscle, or fat) or necrotic debris. The tissue was minced with a scalpel, after which 5 ml of PBS, pH 7.4, was added per g of tissue. The tissue was subjected to homogenization in a blender, followed by three 10-s

bursts of sonication using a Lab Line sonicator at a power setting of 90.

The xenografts derived from the cell line LS174T [18] and RW2982 [19] were sharply excised, minced, homogenized and sonicated in 5 vol. of PBS, in a manner similar to the other tissues. Each of these yielded a liquid suspension of lysed tumor cells in a liquid medium. The suspensions were centrifuged at 105000 × *g* for 60 min at 4°C. The supernatant was applied to a 5.0 × 100 cm column of Sepharose 4B equilibrated in PBS, pH 7.4, and developed at a rate of 1 ml/min, with continuous monitoring of the effluent for protein using A_{280}. Fractions of 10 ml were collected; the void volume peak was identified and pooled. This peak was dialyzed exhaustively against deionized water at 4°C, and lyophilized. This semi-purified mucin-containing preparation was utilized for radiolabeling and ion-exchange chromatographic analysis.

The semi-purified mucin-containing sample was purified further using equilibrium density ultracentrifugation according to the methods used by Podolsky and Isselbacher [5]. Briefly, a semi-purified sample derived from the cell line LS174T was subjected to nuclease digestion (DNase and RNase) for 17 h at 37°C in 10 ml PBS, pH 7.4, containing 1 mM $MgCl_2$ and 0.02% NaN_3, centrifuged at 15000 × *g* for 30 min, and the supernatant was dialyzed against PBS. 12.8 g of CsCl were added and the sample volume adjusted to 24 ml, with a starting density of 1.39 g/ml. The sample was centrifuged at 36000 rpm for 48 h in a Beckman L2-65B ultracentrifuge using an SW-41 rotor. Eight 1.5 ml fractions were pooled by aspiration and the specific gravity measured by weighing 1.00 ml. As previously described, the fractions with densities from 1.44 to 1.56 g/ml contain 'highly purified mucin' [5], and these were used for radiolabeling and ion-exchange chromatography to compare with the semi-purified or 'mucin-containing' samples described above.

Radiolabeling of glycoconjugates

The lyophilized high molecular weight peak was weighed and 10 mg was dissolved in 2.0 ml of PBS containing 1 mM $NaIO_4$. This was incubated in the dark for 45 min at 4°C, and dialyzed against PBS for 5 h at 4°C. The sample was further oxidized with galactose oxidase (5 units) for 45 min at 37°C, and finally radiolabeled by reduction with 4 mCi of NaB^3H_4 for 30 min at 37°C. The ^{3}H-labeled sample was then dialyzed exhaustively against 0.01 M Tris, pH 8.0 at 4°C.

Saponification of mucin using KOH

In order to remove *O*-acetyl groups from sialic acids or colonic mucins [20], the ^{3}H-labeled 'mucin-containing' preparations were subjected to mild base hydrolysis using 0.1 M KOH for 30 min at 25°C as previously described by Gold et al. [21]. To demonstrate that sialic acid or oligosaccharides had not been removed from the mucin by these conditions, the preparation was subjected to gel filtration through Sepharose 4B and the effluent monitored for radioactivity. The saponified preparation was then subjected to ion-exchange chromatography as described above.

Gel electrophoresis of ^{3}H-labeled mucins

^{3}H-labeled 'mucin-containing' samples were subjected to gel electrophoresis to determine the degree of contamination by radiolabeled, lower molecular weight, non-mucin components. A 10^5 cpm sample was added to the sample buffer in the presence of 1–5% β-mercaptoethanol final concentration, depending upon the volume of the sample, and 0.1% sodium dodecyl sulfate. The electrophoresis was run in a Tall Mighty Small electrophoretic apparatus (Hoefer, San Francisco, CA) at a constant current of 20 mA for 90–120 min. The gels were fixed, dried on paper, and autoradiograms were produced using standard x-ray film by development for 1–2 weeks.

Ion-exchange chromatography

The radiolabeled samples were diluted to $2.0 \cdot 10^6$ cpm/0.5 ml of sample using 0.01 M Tris, pH 8.0. The sample was applied to a 0.9 × 30 cm column of DEAE-cellulose equilibrated in 0.01 M Tris, pH 8.0. The column was washed with buffer free of NaCl, and then eluted with a discontinuous ascending NaCl gradient from 0.05 to 0.25 M, followed by a 5.0 M wash. The column was developed at 10 ml/h and 2 ml fractions were collected. Seven peaks were eluted over 4 h each (one at 0 mM NaCl, and one at each of the six different NaCl concentrations), consisting of 20 × 2 ml fractions, termed O–VI. Aliquots of 100 μl were added to 10 ml of liquid scintillant (Safety Solve) and monitored for cpm.

Measurement of carbohydrate

Separate samples were prepared for carbohydrate analysis in which the radiolabeling steps were omitted. For these experiments, a specimen of normal colon, one of colonic cancer, and a specimen of the xenograft from LS174T were selected on the basis of abundance of sample, from which glycoconjugates were extracted in PBS and subjected to gel filtration using Sepharose 4B. The void volume from each sample was pooled, dialyzed against distilled water, and concentrated by lyophilization for ion-exchange chromatography. 5 mg of each sample were dissolved in 0.01 M Tris buffer, applied to the DEAE-cellulose column, and the fractions collected as described above except that a 1.5 × 50 cm column was used and eluted at 20 ml/h, and 4 ml fractions were collected and monitored for neutral hexose [22]. Each pooled peak (or 'species') was dialyzed against distilled water and concentrated by

lyophilization. Each of the seven ion-exchange chromatography peaks was resuspended in 5 ml of water for carbohydrate analysis as follows.

Monosaccharides were measured by high-performance anion-exchange chromatography and pulsed amperometric detection according to the method of Hardy et al. [23]. Briefly, the samples were hydrolyzed in 2 M TFA at 100°C for 5 h. The TFA was removed using a nitrogen evaporator, and the samples were redissolved in water.

An aliquot of 50 μl from each sample (approximately 1 μg mucin) was injected to the column and the analysis of monosaccharides was carried out at an isocratic NaOH concentration of 23 mM for 15 min, using a flow rate of 1 ml/min at ambient temperature. The Dionex Eluant Degas Module was employed to sparge and pressurize the eluants with helium. The monosaccharides were detected by pulsed amperometric detection using a gold working electrode. A Dionex Basic Post Column Delivery System was used to obtain adequate sensitivity. Sodium hydroxide reagent (0.3 M) was mixed with the column effluent at a flow rate of 1 ml/min.

The following pulse potentials and durations were used for monosaccharide analysis: $E_1 = 0.10$ V ($t_1 = 300$ ms); $E_2 = 0.60$ V ($t_2 = 120$ ms); $E_3 = -0.80$ V ($t_3 = 300$ ms). The resulting chromatographic data were integrated using a Hewlett Packard 3392A integrator.

For sialic acid, the samples (20 μg mucin) were first heated at 80°C for 1 h in 0.1 M H_2SO_4, evaporated under nitrogen and redissolved in 1 ml water. 50 μl aliquots were injected into the same column and sialic acid was eluted with 100 mM NaOH containing 150 mM sodium acetate.

Results

Preparation of mucin-rich ^3H-labeled samples

The homogenization and sonication of tissues in PBS permitted the solubilization of mucosal glycoconjugates, and ultracentrifugation removed organelles and membrane contamination. The fractionation of the sample by column chromatography resulted in the isolation of a high molecular weight mucin-containing peak [18]; a typical chromatogram is illustrated in Fig. 1. The glycoconjugates in this peak were concentrated and oxidized with $NaIO_4$ (which oxidizes sialic acid residues), and galactose oxidase (which oxidizes galactose and galactosamine), and then radiolabeled by reduction with NaB^3H_4.

Equilibrium density centrifugation of radiolabeled mucins

The radiolabeled mucin-rich specimens were subjected to equilibrium density ultracentrifugation through a CsCl density gradient. The results demonstrated that 66.6% of the cpm were recovered in the high-density fractions (i.e., specific gravity ≥ 1.38 g/ml) as demonstrated in Table II.

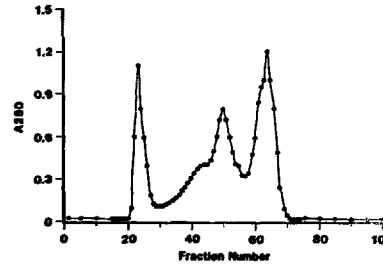

Fig. 1. Sepharose 4B chromatographic profile. The tissue extracts were subjected to gel filtration chromatography using Sepharose 4B, and the eluant monitored for protein (A_{280}). Three peaks were developed and fractions 21–28 were pooled as a 'mucin-containing' peak for the subsequent analyses.

Polyacrylamide gel electrophoresis and autoradiography

To further assess the purity of the radiolabeled 'mucin-containing' preparation, portions of samples derived from normal colonic mucin, colonic cancer, and LS174T were assessed by polyacrylamide gel electrophoresis and autoradiography. Samples containing approximately 10^5 cpm were applied to 10% and 4% polyacrylamide gels in the presence of both β-mercaptoethanol and 0.1% SDS. As indicated in Fig. 2, a broad band is seen at the top of the 10% gel for normal colonic mucin (lane A), whereas the bands from cancer-derived mucins (lanes B and C) enter the top of the gel. A small band is present in the dye front for both cancer-derived mucins. The radiolabeled bands from all three sources entered the 4% gel (lanes D–F),

TABLE II

Equilibrium density ultracentrifugation of ^3H-labeled glycoconjugates

A ^3H-labeled sample derived from the LS174T xenograft was subjected to equilibrium density centrifugation as described in the text. Eight 1.5 ml fractions were collected, the specific gravity determined by weight and volume, and the cpm determined in 50 μl aliquots.

Fraction	Specific gravity (g/ml)	cpm	% of total cpm
I	1.27	140	3
II	1.28	200	4
III	1.31	390	8
IV	1.33	610	12
V	1.36	806	16
VI	1.38	843	17
VII	1.47	1403	29
VIII	1.56	510	10

Fig. 2. Autoradiogram of radiolabeled mucins after polyacrylamide gel electrophoresis. ^{3}H-labeled samples derived from normal colon, colon cancer, and LS174T xenografts were used for polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol. All specimens were obtained from the excluded volume after Sepharose 4B column chromatography, but had not undergone equilibrium density centrifugation, and were therefore representative of the samples subjected to ion-exchange chromatography. Lanes A, B, and C represent normal colonic mucin, colon cancer-associated mucin and mucin from LS174T respectively, electrophoresed on a 10% polyacrylamide gel. Lanes D, E, and F represent the same three mucin-containing specimens electrophoresed on a 4% polyacrylamide gel.

although the cancer-derived bands (lanes E and F) migrated further than that from the normal colon (lane D).

TABLE III

Radiolabeling and column recovery efficiencies using $NaIO_4$ and galactose oxidase

Radiolabeling and column recovery efficiencies using $NaIO_4$ and galactose oxidase. Glycoconjugate-containing extract of the LS174T xenograft was prepared at 5 mg/ml and 1.250 ml used for radiolabeling with galactose oxidase, $NaIO_4$, or both, as described in Materials and Methods. After removal of unreacted NaB^3H_4 by dialysis, the efficiency of labeling was calculated in terms of mg of glycoconjugate present per $2 \cdot 10^6$ cpm. Column recovery represents the sum of all fractions recovered after application of $2 \cdot 10^6$ cpm to the DEAE-cellulose column.

Oxidation method	LS174T glycoconjugate (mg/$2 \cdot 10^6$ cpm)	Column recovery	
		cpm	%
Galactose oxidase	1.689	$1.158 \cdot 10^6$	58
$NaIO_4$	0.601	$1.416 \cdot 10^6$	71
$NaIO_4$ and galactose oxidase	0.387	$1.304 \cdot 10^6$	65

Ion-exchange chromatography

The ^{3}H-labeled 'mucin-containing' samples were fractionated by ion-exchange chromatography using DEAE-cellulose. The elution of seven mucin peaks (or 'species') [5]) produced by the xenograft derived from LS174T is demonstrated in Fig. 3, in which species 0 represents the labeled glycoconjugates that pass through the column, and fractions I–VI represent the fractions sequentially eluted with the discontinuous, ascending NaCl gradient. The continuous line represents cpm eluted; the dashed line represents the percent of the total eluted cpm in each species. As demonstrated, peaks 0 and I contain very little radioactivity, and majority of cpm were eluted in peaks IV and V.

To assess the individual roles of $NaIO_4$ and galactose oxidase in the generation of oxidized sites for reactive

TABLE IV

Ion-exchange chromatography of ^{3}H-labeled samples of nonneoplastic human colonic glycoconjugates

Percentage of cpm eluted in each DEAE ion-exchange chromatography species (0–VI) eluted using a discontinuous ascending NaCl gradient. NN refers to normal colonic epithelium derived from two patients with nonneoplastic disease (diverticulitis). NC refers to normal colonic epithelium derived from seven patients who also had a colon cancer in the resected specimen.

Sample	Glycoconjugate species (% of eluted cpm)						
	0	I	II	III	IV	V	VI
NN-3	3.71	1.37	1.42	1.60	24.54	55.11	12.25
NN-4	2.28	3.14	2.66	3.43	8.81	68.05	11.63
Mean	3.00	2.26	2.04	2.52	16.68	61.58	11.94
NC-1	2.57	4.19	1.60	2.64	40.25	43.05	5.70
NC-5	2.31	3.29	2.56	3.13	15.46	64.21	9.09
NC-6	2.68	8.85	3.43	3.94	10.09	62.66	8.35
NC-8	0.28	3.06	5.45	4.01	24.00	50.75	12.45
NC-9	1.16	5.60	3.03	2.46	25.75	54.37	7.63
NC-12	0.79	1.47	1.19	2.04	37.80	49.14	7.57
NC-13	0.31	1.15	1.72	3.57	32.72	49.65	10.88
Mean ± S.D.	1.44 ± 1.06	3.94 ± 2.65	2.71 ± 1.45	3.11 ± 0.76	26.58 ± 11.21	53.40 ± 7.64	8.80 ± 2.25

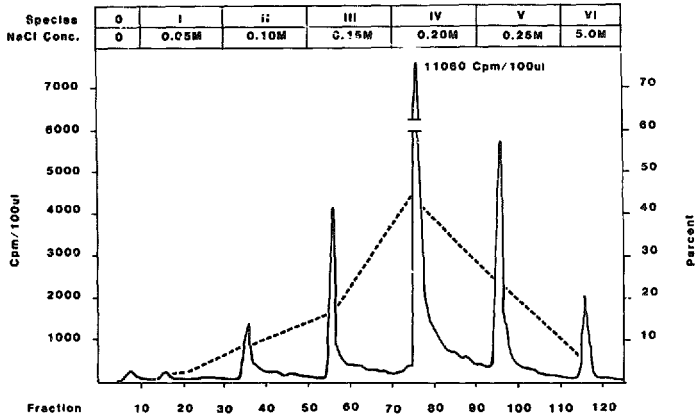

Fig. 3. Ion-exchange chromatography profile after labeling with galactose oxidase and $NaIO_4$. The mucin-rich peak obtained from a xenograft of the human colon cancer cell line LS174T was oxidized with both galactose oxidase and $NaIO_4$, and radiolabeled by reduction with NaB^3H_4. The ^3H-labeled glycoconjugates were applied to the DEAE-cellulose column, and eluted with a discontinuous ascending NaCl gradient (as indicated across the top). The cpm are indicated by the solid line; the percent of total cpm eluted is indicated by the dotted line.

radiolabeling, a sample of the LS174T xenograft preparation was separately oxidized by each of the two reagents. Table III demonstrates the relative labeling efficiency of the galactose oxidase and $NaIO_4$ methods separately, and the results using both together. Also depicted in Table III are the percent recoveries from the

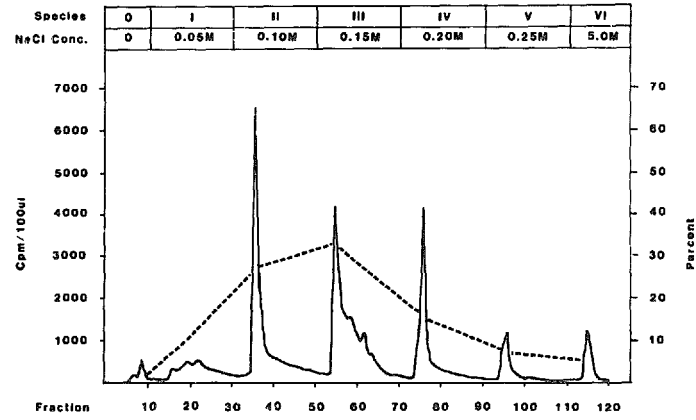

Fig. 4. Ion-exchange chromatography profile after oxidation with galactose oxidase only. This ion-exchange chromatography profile was obtained by extracting glycoconjugates from a xenograft derived from the human colon cancer cell line LS174T. In this instance, the glycoconjugates were oxidized with the galactose oxidase, and subsequently radiolabeled by reduction with NaB^3H_4. The cpm eluted are indicated by the solid line; the percent of total cpm eluted in each species is indicated by the dotted line. Using galactose oxidase only, species II and III were preferentially labeled.

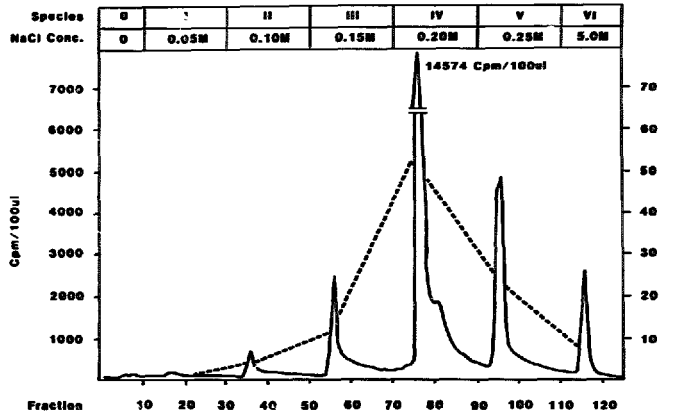

Fig. 5. Ion-exchange chromatogram obtained after oxidation with $NaIO_4$ only. This chromatogram was obtained from glycoconjugates obtained using a xenograft derived from the human colon cancer cell line LS174T that were oxidized using $NaIO_4$ followed by reduction with NaB^3H_4. Under these conditions, species IV was preferentially radiolabeled.

DEAE-cellulose columns, using each of the methods. Fig. 4 is a chromatogram demonstrating the cpm eluted in each ion-exchange species (continuous line), and the percent of the total cpm recovered in each of the glycoconjugate species (dashed line), after oxidizing with galactose oxidase alone. Fig. 5 is the corresponding chromatogram for a sample oxidized by using only $NaIO_4$. The galactose oxidase preferentially labeled fractions II and III, whereas $NaIO_4$ preferentially labeled fraction IV. As indicated in Table III, labeling

TABLE V

Ion-exchange chromatography of ^3H-labeled samples of glycoconjugates extracted from human colonic cancer tissues

Data are percentages of total cpm eluted in each glycoconjugate species. CA indicates a fresh surgically excised specimen of human colonic adenocarcinoma. LS174T and RW-2982 are subcutaneous xenografts, derived from cultured human colonic cancer cell lines.

Sample	Glycoconjugate species (% of eluted cpm)						
	0	I	II	III	IV	V	VI
Surgical specimens							
CA-1	2.90	3.76	2.85	15.21	47.32	23.02	4.94
CA-2	1.19	2.15	2.05	4.16	46.56	38.84	5.05
CA-5	5.39	5.55	5.73	5.63	19.41	46.33	11.96
CA-6	5.15	8.07	6.67	6.35	15.55	48.11	10.10
CA-11	0.64	1.60	2.74	7.43	52.66	26.97	7.96
CA-12	2.52	3.66	4.14	7.94	28.44	39.59	13.71
CA-14	1.00	2.67	7.26	15.80	54.95	12.69	5.63
Mean ± S.D.	2.68 ± 1.98	3.92 ± 2.24	4.49 ± 2.07	8.93 ± 4.66	37.84 ± 16.35	33.65 ± 13.10	8.48 ± 3.53
Xenografts							
LS174T-1	0.72	2.61	2.07	9.10	40.44	36.83	8.23
LS174T-2	3.91	2.87	3.27	7.51	43.53	34.27	4.64
LS174T-3	3.26	4.49	2.88	7.22	40.03	35.99	6.13
LS174T-4	0.73	1.99	8.23	15.31	43.78	23.52	6.44
Mean ± S.D.	1.90 ± 1.36	3.25 ± 1.15	4.11 ± 2.75	9.79 ± 3.77	41.95 ± 1.98	32.65 ± 6.18	6.36 ± 1.47
RW2982	0.36	2.10	8.04	18.58	57.47	8.87	4.58

TABLE VI

Ion-exchange chromatography elution profiles for all nonneoplastic colonic specimens and all colonic cancers

Values are mean ± S.D. of the percentages of ^{3}H-labeled glycoconjugates eluting at each salt concentration. Data were analyzed using Student's unpaired *t* test comparing all normals vs. all cancers; n.s. = not significant. NN, normal colonic epithelium from patients with diverticulitis; NC, nonneoplastic colonic epithelium from patients with cancer; CA, surgically excised human colon cancer specimens; LS, subcutaneous xenografts from LS174T cells; RW, subcutaneous xenografts from RW 2982 cells.

Glycoconjugate species (NaCl)		Percent of cpm eluting in each species		*P* value
		all normals (NN + NC)	all cancers (CA + LS + RW)	
0	(0 M)	1.79 ± 1.20	2.23 ± 1.75	n.s.
I	(0.05 M)	3.57 ± 2.45	3.55 ± 1.85	n.s.
II	(0.10 M)	2.56 ± 1.33	4.66 ± 2.37	$P = 0.028$
III	(0.25 M)	2.98 ± 0.85	10.02 ± 4.81	$P < 0.0005$
IV	(0.20 M)	24.38 ± 11.35	40.85 ± 13.35	$P = 0.008$
V	(0.25 M)	55.22 ± 8.20	31.25 ± 12.40	$P < 0.0005$
VI	(5.00 M)	9.51 ± 2.39	7.45 ± 3.04	n.s.

with $NaIO_4$ alone resulted in approximately 2.8-times the specific activity achieved with galactose oxidase alone.

Glycoconjugates were extracted from the nine samples of nonneoplastic human colon, radiolabeled, and subjected to ion-exchange chromatography as described above. The percents of cpm eluting in each of the seven species (O–VI) are depicted in Table IV. The first two specimens came from patients who had undergone resection of the sigmoid colon for diverticulitis (NN, see Table I). The last seven specimens were histologically normal colonic epithelium from patients who had undergone a resection for cancer (NC, see Table I). In both groups, nearly 80% of the cpm eluted in peaks IV and V, and more than half of the counts were found in peak V. The samples derived from patients who did not have cancer elsewhere in the colon had the largest percentage of cpm in peak V (mean 62%), however there were only two samples from this group.

The data derived from the samples of colon cancer are depicted in Table V. The first seven samples were derived from fresh surgical specimens of human colon cancer, and in four instances represent paired specimens that correspond to samples of nonneoplastic tissue (see NC-1, 5, 6, and 12 in Table IV). The cpm eluted in species V from the seven specimens of cancer are significantly less than the corresponding glycoconjugate species found in the nine specimens of nonneoplastic colon ($P < 0.005$). The loss of counts in species V from the seven specimens of colon cancer is reflected by a corresponding significant increase of cpm in species III ($P < 0.02$), and nonsignificant increases in species II and IV ($P = 0.06$ and $P = 0.12$, respectively). This observation, specifically a shift towards less-charged glycoconjugates species, was also seen and was more marked in the samples derived from the two xenografts, LS174T and RW2982 (Table V). When the combined data for all of the normal colonic specimens (NC and NN) were compared with the combined cancer specimens (CA, LS174T and RW2982), highly significant differences were seen for species II ($P < 0.028$), III ($P < 0.0005$), IV ($P < 0.008$) and V ($P < 0.0005$), with a shift away

TABLE VII

Carbohydrate analysis – normal colonic glycoconjugates (μmol)

Carbohydrates are expressed as μmol in 1 ml of the fraction eluted during ion-exchange chromatography using nonradiolabeled glycoconjugates. Molar percentages for each peak are given in parenthesis after each value in μmol. % total CHO refers to the percentage of carbohydrate in that species compared to the sum of carbohydrate for all seven species. % total cpm refers to that percentage of cpm present in that species when radiolabeled glycoconjugates were separated by ion-exchange chromatography (from Table IV). CHO, carbohydrate; Fuc, fucose; GalN, galactosamine; GlcN, glucosamine; Gal, galactose; n.d., none detected.

	Glycoconjugate species						
	0	I	II	III	IV	V	VI
CHO							
Fuc	0.670 (9.0%)	2.012 (16.0%)	3.537 (16.3%)	9.329 (17.3%)	12.561 (15.6%)	65.549 (10.8%)	28.537 (15.6%)
GalN	2.488 (33.5%)	1.855 (14.7%)	5.249 (24.2%)	8.869 (16.5%)	9.231 (11.5%)	115.566 (19.0%)	22.031 (12.0%)
GlcN	2.488 (33.5%)	4.525 (35.9%)	8.145 (37.6%)	18.145 (33.7%)	23.620 (29.3%)	136.335 (22.4%)	50.090 (27.3%)
Gal	1.778 (23.9%)	4.222 (33.5%)	4.722 (21.8%)	12.778 (23.7%)	19.611 (23.3%)	143.278 (23.5%)	56.500 (30.8%)
sialic acid	n.d.	n.d.	n.d.	4.693 (8.7%)	15.566 (19.3%)	148.738 (24.4%)	26.214 (14.3%)
Total μmol	7.424 (100%)	12.614 (100%)	21.653 (100%)	53.814 (100%)	80.589 (100%)	609.466 (100%)	183.377 (100%)
% total CHO	0.8	1.3	2.2	5.6	8.3	62.9	18.9
% total cpm	1.4	3.9	2.7	3.1	26.6	53.4	8.8

TABLE VIII

Carbohydrate analysis – colonic cancer-associated glycoconjugates

Carbohydrates are expressed as μmol in 1 ml of the fraction eluted during ion-exchange chromatography using nonradiolabeled glycoconjugates. Molar percentages for each peak are given in parenthesis after each value in μmol. % total CHO refers to the percentage of carbohydrate in that species compared to the sum of carbohydrate for all seven species. % total cpm refers to the percentage of cpm present in that species when radiolabeled glycoconjugates were separated by ion-exchange chromatography (from Table V). CHO, carbohydrate; Fuc, fucose; GalN, galactosamine; GlcN, glucosamine; Gal, galactose; n.d., none detected.

	Glycoconjugate species						
	0	I	II	III	IV	V	VI
CHO							
Fuc	9.329 (18.8%)	4.512 (14.6%)	13.415 (20.2%)	29.329 (15.0%)	237.622 (16.7%)	97.012 (17.2%)	32.134 (12.1%)
GalN	12.896 (27.4%)	9.774 (31.6%)	21.222 (32.0%)	31.403 (16.1%)	175.792 (12.4%)	112.127 (19.9%)	41.267 (15.5%)
GlcN	10.995 (23.4%)	11.176 (36.2%)	23.439 (35.4%)	59.457 (30.4%)	309.095 (21.8%)	119.910 (21.3%)	78.190 (29.4%)
Gal	13.833 (29.4%)	5.444 (17.6%)	8.222 (12.49)	59.611 (30.5%)	462.222 (32.6%)	160.778 (28.6%)	91.444 (34.3%)
sialic acid	n.d.	n.d.	n.d.	15.599 (8.0%)	235.146 (16.6%)	73.042 (13.0%)	23.333 (8.8%)
Total μmol	47.053 (100%)	30.906 (100%)	66.298 (100%)	195.399 (100%)	1419.877 (100%)	562.869 (100%)	266.368 (100%)
% total CHO	1.8	1.2	2.6	7.5	54.8	21.7	10.3
% total cpm	2.7	3.9	4.5	8.9	37.8	33.7	8.5

from peak V toward less charged glycoconjugates eluting in earlier fractions (II–IV) for the specimens derived from colon cancers (Table VI).

Ion-exchange chromatography was performed on 'highly purified mucin' obtained from the LS174T cell line as described in Materials and Methods (i.e., that subjected to additional nuclease digestion and ultracentrifugation through a CsCl gradient prior to labeling). The ion-exchange elution profiles were identical to those obtained using semi-purified mucin-containing samples (data not presented).

Monosaccharide analysis

The results of analysis of monosaccharides (specifically, fucose, galactose, glucosamine, galactosamine, and sialic acid) for each of the seven ion-exchange peaks are listed in Table VII (for normal colonic mucin), Table VIII (for colon cancer-associated mucin), and Table IX

TABLE IX

Carbohydrate analysis – glycoconjugates from cell line LS174T

Carbohydrates are expressed as μmol in 1 ml of the fraction eluted during ion-exchange chromatography using nonradiolabeled glycoconjugates. Molar percentages for each peak are given in parentheses after each value in μmol. % total CHO refers to the percentage of carbohydrate in that species compared to the sum of carbohydrate for all seven species. % total cpm refers to the percentage of cpm present in that species when radiolabeled glycoconjugates were separated by ion-exchange chromatography (from Table V). CHO, carbohydrate; Fuc, fucose; GalN, galactosamine; GlcN, glucosamine; Gal, galactose; n.d., none detected.

	Glycoconjugate species						
	0	I	II	III	IV	V	VI
CHO							
Fuc	1.037 (11.7%)	2.744 (16.4%)	6.159 (14.3%)	21.280 (13.8%)	78.415 (16.2%)	45.183 (8.9%)	35.122 (13.5%)
GalN	0.588 (6.6%)	2.443 (14.6%)	10.860 (25.2%)	26.335 (17.0%)	72.036 (14.9%)	72.579 (14.3%)	30.860 (11.9%)
GlcN	4.163 (47.0%)	9.412 (56.3%)	18.109 (42.2%)	54.072 (35.0%)	129.593 (26.7%)	95.430 (18.8%)	67.014 (25.8%)
Gal	3.056 (34.6%)	2.111 (12.6%)	6.389 (14.8%)	43.500 (28.1%)	135.056 (27.9%)	235.889 (26.5%)	118.056 (45.5%)
sialic acid	n.d.	n.d.	1.553 (3.6%)	9.515 (6.2%)	69.385 (14.3%)	58.414 (11.5%)	8.608 (3.3%)
Total μmol	8.844 (100%)	16.710 (100%)	43.151 (100%)	154.702 (100%)	484.485 (100%)	507.495 (100%)	259.660 (100%)
% total CHO	0.6	1.1	2.9	10.5	32.8	34.4	17.6
% total cpm	1.9	3.3	4.1	9.8	43.0	32.7	6.4

TABLE X

Sialic acid content (nmol) per μmol carbohydrate

The sialic acid content for each species in the samples analyzed in Tables VII–IX are expressed in nmol per μmol of the sum of the remaining carbohydrates detected.

Species	Colon sample		
	normal	cancer	LS174T
0	–	–	–
1	–	–	–
2	–	–	36
3	87	80	62
4	193	166	143
5	244	130	115
6	143	88	33

(for cancer-associated mucin derived from the tumor cell line LS174T). It is apparent that each ion-exchange species contains all five sugar moieties found in mucins. Table X lists the sialic acid content expressed as a fraction of the total carbohydrate content of each species for these samples. It appears from Table X that differences in sialic acid content do not entirely account for retention on the DEAE-cellulose.

Saponification of O-acetylated sialic acids

Radiolabeled mucin samples from the normal colon and xenograft of LS174T cells were saponified as described, and neutralized with 0.1 M HCl. A portion of each sample was subjected to gel filtration through Sepharose 4B, and all of the radioactivity eluted as a single high molecular weight peak, indicating that no degradation of radiolabeled groups had occurred. The saponified samples were chromatographed on DEAE-cellulose columns as described above. The saponified samples eluted identically to the untreated samples (data not shown), indicating that differences in *O*-acetylation did not account for the differential retention on the ion-exchange column see between the normal and cancer-associated specimens.

Discussion

In the experiments presented, glycoconjugates were tritium-labeled using a modification of the techniques developed by Podolsky and Isselbach [12], but the ion-exchange chromatography profiles we produced with extracts from the normal colons are different from those obtained by these investigators. Specifically, we subjected our tissue extracts to Sepharose 4B chromatography before the radiolabeling step, which could change both the labeling efficiencies of the mucins in the preparation and the relative recoveries of radiolabeled mucins. Moreover, Podolsky and Isselbacher initially equilibrated their DEAE-cellulose in 1.0 M Tris buffer prior to equilibration in 0.01 M Tris for the sample separations (personal communication). Although we observed no change in the elution profiles throughout the several dozen chromatographic runs necessary for this work that might suggest slow equilibration of the column with increasing amounts of Tris base, this difference cannot be excluded as an explanation of our results. However, a small number of samples has been chromatographed in our laboratory after equilibration of the ion-exchange resin in 1.0 M Tris, and the ^{3}H-labeled mucins were found to elute at lower ionic strength (data not presented). As a result, the altered representation of the charge in the seven mucin species seen in cancer-associated glycoconjugates may not be strictly comparable to those reported using extracts from samples of inflammatory bowel disease [5,12].

Because of the uniform reproducibility of the data with this method in our laboratory, we examined the individual contributions of $NaIO_4$ and galactose oxidase in the labeling of colonic glycoconjugates to explain the discrepancies between our results and those previously reported. These experiments demonstrated different oxidizing efficiencies with the two reagents; reduction with NaB^3H_4 resulted in almost 3-fold greater specific activity after oxidation with $NaIO_4$ alone compared with that produced after oxidation with galactose oxidase alone (Table III). Recovery of ^{3}H-labeled glycoconjugates from the DEAE-cellulose columns was similar with both methods. Also, galactose oxidase (which results in the labeling of galactose and *N*-acetylgalactosamine) tended to label glycoconjugate species II and III more prominently (Fig. 4), whereas $NaIO_4$ (which oxidizes sialic acid residues, and therefore would be expected to label the more acidic species) labeled species IV and V best. Since the efficiency of labeling with galactose oxidase was considerably less than that achieved with $NaIO_4$, it would appear that differences in the galactose oxidase activity could account for differences in the glycoconjugate species labeled from one laboratory to another. Alternatively, a difference in the source of the ion-exchange resin could produce different elution profiles. The principal difference between this work and that of Podolsky and Isselbacher is that the latter laboratory had a larger percentage of the tritium-labeled species in fractions I–III.

Although not strictly comparable, the ion-exchange chromatography profile reported by LaMont and Ventola [24] on nonradiolabeled rat colonic mucins demonstrated a predominant peak in mucin species IV when assayed for A_{280} and neutral hexose. Kim and Horowitz studied canine gastric mucins by ion-exchange chromatography in 1971 [25], again monitoring the eluant for A_{280} and neutral hexose. The principal eluting fractions were found at 0.2–0.3 M NaCl (corresponding to species IV and V using our gradient). Although similar to our results, comparisons of this sort are of limited value since they utilize different sources

of mucin (both with respect to species and organ), and the eluted fractions were monitored for protein (A_{280}) and neutral hexose, which detects different components of the mucosal glycoconjugates than that achieved by the use of the radiolabeling technique.

In spite of the above-stated considerations, there were highly significant differences in the ion-exchange chromatography elution profiles between glycoconjugates obtained from normal human colonic epithelium and colon cancers. The most important difference is that a charged species (V) accounted for the majority of the tritium-labeled glycoconjugates in the normal colon, whereas the profiles obtained from cancer specimens had a significant deficit of species V and corresponding increases in the less-charged species.

It was of interest to note that the specimens derived from human colons without cancer (NN) had the greatest percentage of tritium-labeled glycoconjugates in species V, although the number of these samples is small and it cannot be ascertained whether this is significantly different from that seen in the nonneoplastic mucosal specimens obtained from colons that were resected for cancer (NC). It has been reported by some investigators that glycoconjugate histochemistry is abnormal throughout the entire colon in the setting of colon cancer [26]. It is possible that 'normal-normal' colons are distinct from 'normal-cancer' colons, but the differences we have reported between NN and NC specimens are difficult to interpret at this time.

Very significant changes were seen in the cancer specimens with coordinant deficits in species V and increases in species II, III, and IV. These changes were even more pronounced in the subcutaneous xenografts derived from the mucin-producing colon cancer cell line LS174T, and the shift to more neutral species was exaggerated further in the xenografts derived from the human colon cancer cell line RW??82. This cell line demonstrates unusual growth characteristics in suspension culture and is a prodigious producer of mucin [19], but the biological significance of this shift towards less-charged glycoconjugates remains to be explored.

The biochemical basis of the production of less-charged glycoconjugates is unclear at this time. The method we employed for glycoconjugate preparation preferentially recovered mucin, however the 'mucin-containing' fraction obtained by Sepharose 4B column chromatography may also contain small amounts of contaminating proteins, lipids and other glycoproteins. The use of the radiolabeling techniques increases the ability to detect mucin glycoproteins by specifically labeling those residues abundantly represented in mucin oligosaccharides [27,28]. The analysis of the 'mucin-containing' preparation by CsCl density gradient ultracentrifugation (Table II) indicates that an insignificant proportion of the cpm is present in either lipids or proteins (which would band in low-density fractions). The autoradiograms of the polyacrylamide gels (Fig. 2) also indicate that the radiolabeled species are broad, high molecular weight bands characteristic of mucins. Of interest, the autoradiogram in Fig. 2 indicates that there is a difference between the molecular weights of reduced mucins from normal colons and cancers, which has never previously been appreciated. Moreover, the carbohydrate contents of the eluted species (Tables VII–X) are characteristic only of mucins [29]. Thus, the principal glycoconjugates that make up the eluted species are mucins.

The mechanisms that account for changes in the molecular charge of mucins are incompletely understood, however there are at least two possible explanations. First, the synthesis of a novel apoprotein less richly represented with basic residues in cancer-associated mucins could change the ionic strength with which a glycoconjugate species would be eluted from DEAE-cellulose. However, mucin glycoproteins from normal tissues tend to be densely glycosylated, and colonic mucins have relatively few nonglycosylated, 'exposed' portions of protein available for interaction with the ion-exchange resin [29]. The apparent differences in molecular weight between these mucins raises the possibility of the synthesis of a unique apomucin protein by cancerous tissues. However, since no mucin apoprotein has ever been purified or sequenced, and no apomucin gene has yet been cloned, it is premature to speculate further on this hypothesis.

Secondly, a change in oligosaccharide structures could account for differences in molecular charge and result in different ion-exchange chromatography profiles. The expression of the Thomsen-Friedenreich antigen on cancer-associated mucins [8–10] and the absence of this structure in the normal colon [30,31] has led to the hypothesis that cancer-associated mucins are aberrantly glycosylated, and perhaps incompletely glycosylated, compared to mucins from the normal colon [8]. This hypothesis has been supported by preliminary data from our laboratory using beta-eliminated colonic mucins, which suggest that cancer-associated mucins have shorter oligosaccharide chains compared to their normal counterparts [32].

Analysis of monosaccharide content in each fraction of the three nonradiolabeled samples revealed that each sample contained the five sugar residues found in mucin-type glycoconjugates, and the carbohydrate content of each fraction was proportional to the cpm eluted in the earlier experiments. Only sialic acid content was differentially expressed, tending to be below the limits of detection in species 0–III, and being highest in fractions IV and V. A trend towards lower relative sialic acid content in the cancer specimens is evident when expressed in terms of the total carbohydrate content. However, as demonstrated in Table X, sialic acid content alone probably does not account for the separation

of the seven peaks. Furthermore, the removal of *O*-acetyl groups from sialic acids produced no change in retention on DEAE-cellulose. A more complete analysis of this problem will require sequencing of oligosaccharide residues derived from cancer-associated mucins, characterization of the apomucins from normal and malignant tissues, and an understanding of the three-dimensional structure of mucin. From the data that are currently available, it is only possible to say that the mucins from colon cancer express at least some different carbohydrate sequences [8], are antigenically distinct from normal colonic mucins [7,33], contain fewer *O*-acetyl groups on sialic acids [21], and our work demonstrates that radiolabeled glycoconjugates derived from colon cancer have a different relative charge and molecular weight than do their normal counterparts.

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