Distribution of Mono-, Di-, and Tri-O-Acetylated Sialic Acids in Normal and Neoplastic Colon

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ABSTRACT

The purpose of this study was to compare the expression of O-acetylated sialic acids on normal colonie epithelial cells that to on primary and metastatic human adenocarcinoma of the colon and rectum. In 24 cases, the relative percentages of biosynthetically labeled mono-, mono-, di-, and tri-O-acetylated sialic acids were measured after hydrolytic release, separation, and identification by paper chromatography. In one case, the presence of di- and tri-O-acetylated sialic acids was confirmed by fast atom bombardment-mass spectral analysis. Differences were observed in the expression of sialic acids between normal colonie epithelium, “uninvolved” colon mucosa remote to a colonic adenocarcinoma, and colonie adenocarcinoma. The levels of mono- and tri-O-acetylated sialic acids accounted for the difference in the ratios of sialic acids expressed between normal and “uninvolved” colon mucosa, while the total amount of O-acetylation was unchanged. However, no difference was observed in the relative amounts of non- and O-acetylated sialic acids between either fresh and tissue culture-established colon carcinomas, or fresh and tissue culture-established liver metastasis derived from carcinoma of the colon. The relative expression of these O-acetylated sialic acids molecules appears to vary according to tissue type. This study suggests that individuals with adenocarcinoma of the colon express a field defect resulting in abnormal ratios of O-acetylated sialic acids.

INTRODUCTION

A number of N- and O-substituted derivatives of neuraminic acid (see Fig. 1) have been identified in biological samples (1). The presence of O-substituted sialic acid molecules in human colonie epithelial mucins was first suggested by the resistance of normal human colonie epithelial mucins to periodic acid-Schiff histochemical staining without prior saponification (2–4) as well as their resistance to bacterial neuraminidases (5–8).

The degree of O-substitution of sialic acids on human colonie epithelium appears to be a sensitive indicator of early malignant change (9–16). Using histochemical methods, these studies demonstrated a loss of O-substituted sialic acid molecules in colon-derived mucins at the onset of malignancy. In a few cases, sialomucins from normal and malignant tissue of human colon were fractionated and analyzed chemically (17–23). These investigations, however, were unable to prove unequivocally the O-acetylated nature of these molecules or to quantitate the relative amounts of each sialic acid species.

This study was designed to quantify the relative amounts of the sialic acids on normal, malignant, primary, and metastatic human colonie epithelial cells from both fresh and cultured specimens. Biosynthetically radiolabeled sialic acids from various human tissues were purified and analyzed by ascending paper chromatography. We demonstrate that mono-, di- and tri-O-acetylated sialic acid molecules can be

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3 The abbreviations used are: FAB-MS, fast-atom bombardment mass spectrometry; MEM, minimum essential medium; FBS, fetal bovine serum; Neu5Ac, N-acetylneuraminic acid; MDAH, M. D. Anderson Hospital; NK, natural killer; Neu4,5,7,8,9Ac5, A'-acetyl-4,7,8,9-tetra-O-acetylneuraminic acid.
Fig. 1. Nature and position of the N- and O-substituents of sialic acids. The parent sialic acid molecule is in the chair conformation with the individual carbons numbered 1-9. Sialic acids are glycosidically linked at the anomeric C-2 position to galactose, N-acetylgalactosamine, N-acetylglucosamine, or sialic acid. \( R_1 \), an acetyl substitution at the nitrogen group; \( R_2 \), R, \( R_4 \), \( R_6 \), possible O-acetyl substitutions.

by Dr. M. Pollack, Baylor College of Medicine, Houston, TX. Human hemangioblastoma P and its clone CI-6, human hepatocytes, Dor50 kidney cells, and splenic endothelial cells were provided by P. Belloni, MDAH. Human acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, and chronic myelogenous leukemia cells were obtained by pheresis and provided by Dr. J. Hester, MDAH. GRD, M40, and PHL cells were cultured in Leibovitz's L-15 medium (GIBCO) containing 10% FBS (Irvine Scientific, Santa Ana, CA), SKMel 40, 23, and 93DX6 cells in Dulbecco's modified Eagle medium (GIBCO) containing 10% FBS, and all other cell lines and fresh samples in RPMI 1640 medium (GIBCO) containing 10% FBS. Adherent cell lines were maintained by harvesting monolayer cultures with 0.25% trypsin and 0.02% EDTA (Irvine Scientific).

Clinical Characteristics of Patients. Surgical specimens were obtained from 11 patients with primary or metastatic disease. Median age of the patients (4 males, 7 females) was 56 years (range, 36–65). Eight of eleven patients had elevated levels of carcinoembryonic antigen (greater than 2.5 ng/ml) before surgery.

Preparation of Fresh Samples. Tumor specimens from primary human colon carcinoma or liver metastases including the respective uninvolved tissues were obtained immediately after surgery and were dissociated with collagenase and DNase, according to the technique of Giavazzi et al. (26). In brief, each tissue was dissected free of necrotic areas, connective tissue, and blood clots, then rinsed twice in cold (4°C) MEM containing antibiotics. With some specimens, the tissue was cut into 3- to 5-mm³ fragments using a sterile scalpel, pressed through a sterile 50-mesh stainless steel screen, and washed 5 times in MEM containing antibiotics. Each strip was placed in a 5-ml chloroform:methanol (2:1, v/v). Carrier NeuSAc (5 µg) containing 1.5 nCi of \([14C]\)Neu5Ac (specific activity, 244 mCi/mM; Amersham, Arlington Heights, IL) was added to each sample as an internal standard. The chromatogram was developed for 6 h (solvent migration; 20 cm), air-dried at room temperature, and cut sequentially into 0.2-cm strips (1 cm wide) beginning at the origin. Each strip was placed in a 5-ml liquid scintillation vial and soaked in 100 µl of water overnight. Four ml of liquid scintillation cocktail (Formula 963; New England Nuclear, Boston, MA) were added to each vial and assayed for levels of \(^3\)H and \(^14\)C. Using this Chromatographic system, all biosynthetically radiolabeled molecules (such as the biosynthetic precursors glucosamine, N-acetylgalactosamine, and N-acetylglucosamine) of the basis of the negative change of sialic acids (Fig. 1).

In some cases, another round of mild-acid hydrolysis was performed to measure the percentage of radiolabeled sialic acids resistant to removal. In all cases tested the second round of mild acid hydrolysis yielded less than 1% of the total radiolabeled sialic acids recovered in the first round of hydrolysis. The recovery of biosynthetically labeled sialic acids was assessed by repeating the purification procedure on purified material, yielding 87% of the starting material with no change in the relative percentages of non- and O-acetylated biosynthetically labeled sialic acids.

Paper Chromatographic Identification and Analysis. Biosynthetically labeled sialic acids from normal and malignant human cells were identified by ascending paper chromatographic analysis in a solvent system of n-butanol:propanol:0.1 N HCl (1:2:1, v/v) (28). Prior to analysis, Whatmann 3MM paper (Fisher Scientific, Houston, TX) was washed sequentially with 0.1 N hydrochloric acid, distilled water, and chloroform:methanol (2:1, v/v). Carrier Neu5Ac (5 µg) containing 1.5 nCi of \([14C]\)Neu5Ac (specific activity, 244 mCi/mM; Amersham, Arlington Heights, IL) was added to each sample as an internal standard. The chromatogram was developed for 6 h (solvent migration; 20 cm), air-dried at room temperature, and cut sequentially into 0.2-cm strips (1 cm wide) beginning at the origin. Each strip was placed in a 5-ml liquid scintillation vial and soaked in 100 µl of water overnight. Four ml of liquid scintillation cocktail (Formula 963; New England Nuclear, Boston, MA) were added to each vial and assayed for levels of \(^3\)H and \(^14\)C. Using this Chromatographic system, all biosynthetically radiolabeled carbohydrates other than sialic acids (N-acetylgalactosamine, N-acetylgalactosamine, and glucosamine) had slower rates of migration when compared to the migration of non- and O-acetylated species of sialic acids. Relative percentages of the separated sialic acids were determined by integrating the amount of \(^3\)H material in each peak. For each given cell type, zero values indicated the expression of less than 0.1% of the total sialic acids expressed. Nonparametric statistical tests were used to determine the significance of observed differences in the amounts of non-, mono-, di- and tri-O-acetylated sialic acids expressed on normal, malignant, and metastatic human colon cells. The Mann-Whitney test was used to compare data between groups, and the Wilcoxon paired-sample test was used to compare groups with paired data. Differences were considered statistically significant at P values of less than 0.05.

Synthetic O-Acetylation of Neu5Ac. Paper chromatographic standards of mono-, di-, and tri-O-acetylated sialic acids were prepared by per-O-acetylation of Neu5Ac to form N-acetyl-4,7,8,9-tetra-O-acetyl neuraminic acid (Neu5Ac,4,5,7,8,9Ac5), followed by partial de-O-acetylation with mild acid and base. In brief, 500 µg of Neu5Ac (Sigma) containing 0.5 µCi of \(^3\)H-radiolabeled Neu5Ac were resuspended in 200 µl of dry pyridine:acetic anhydride (1:1) and incubated overnight at room temperature. The acetylated material was dried under a stream of nitrogen, resuspended in water, and lyophilized. An aliquot of this material was...
treated with 2 N acetic acid at 80°C for 3 h, lyophilized, treated with 0.1 N ammonium hydroxide at 25°C for 1 h, and lyophilized. This partially de-O-acetylated material was resuspended in water and identified by paper chromatography.

FAB-MS Analysis. Cryopreserved nonradioactively labeled normal colon tissue (5 x 10^6 cells) was pressed through a 50-mesh stainless steel screen and washed three times in Hank's balanced salt solution prior to release and purification of the associated sialic acids as described above. The purified sialic acids were separated by paper chromatography, and material from the region corresponding to the migration of biosynthetically radiolabeled di- and tri-O-acetylated sialic acids (from the same tissue) was eluted in water and lyophilized. Material eluted from the paper directly adjacent to the region described above served as a control. Each sample was resuspended in glycerol:water (1:1, v/v) and 5 μl were placed on the probe plate. Positive-ion FAB-MS spectra were recorded on a Kratos M50 RF mass spectrometer (acceleration voltage, 8 kV) with a 3000 ppm sweep and 3.0-s scan time. The FAB gun used xenon (emission current, 0.15 mA; acceleration voltage, 8 kV). The glycerol served as a matrix for the sample material and an internal standard. Mass equivalents in daltons were determined by standardizing the spectrophotometer with triazine and counting signal peaks representing single mass units. Analysis was confined to the 185–500 region of the mass spectrum, since we were only interested in identifying the monosaccharides released from the human colon cells.

RESULTS

Separation of Synthetic O-Acetylated Sialic Acids by Paper Chromatography. The paper chromatographic migration profile of O-acetylated sialic acids was standardized using synthetic 14C-labeled O-acetylated sialic acids after partial de-O-acetylation of Neu4,5,7,8,9Ac5. In this chromatographic system, sialic acid molecules were separated according to their degree of oxygen substitution (Fig. 1). Fig. 2 represents the migration of these O-acetylated sialic acids expressed as a ratio of their migration divided by the migration of Neu5Ac. The Neu5Ac peak is referred to as 1.0, the mono-O-acetylated sialic acid peak as 1.24, the di-O-acetylated sialic acid peak as 1.40, the tri-O-acetylated sialic acid peak as 1.64, and the tetra-O-acetylated peak as 1.80. The migration of all radiolabeled sialic acid material (Fig. 4; Tables 1–3) was found to vary no more than ±0.02 units from the recorded peak values shown in Fig. 2.

To confirm that the synthetic O-acetylated sialic acids migrating at the 1.64 peak were isomers of tri-O-acetylated sialic acid, sialic acid material was eluted from the region for FAB-MS analysis. From this material, a positive ion molecular weight peak (436 mass units) was identified as the molecular weight peak of tri-O-acetylated sialic acid (data not shown).

FAB-MS Analysis of O-Acetylated Sialic Acids from Normal Colon. Sialic acid material released and purified from unlabeled, cryopreserved human colon cells (NC2) migrating on paper to the region corresponding to the migration of biosynthetically radiolabeled di- and tri-O-acetylated sialic acids from the same tissue (Fig. 3A) was analyzed using FAB-MS. Ion molecular weight peaks generated in the positive-ion mode that were not present in the paper control (paper directly adjacent to the migration of di- and tri-O-substituted sialic acids) are shown in Fig. 3B. By this subtractive analysis, molecular ions of 394 and 436 were the only masses detected, corresponding to the ion molecular weights of di-O-acetylated sialic acids and tri-O-acetylated sialic acids, respectively. The 416 and 458 peaks correspond to di- and tri-O-acetylated sialic acids, respectively, each complexed with a sodium ion, while the 185, 277, and 369 peaks correspond to the di-, tri-, and tetraglycerol peaks, respectively. Unfortunately, salt contamination causing signal suppression limited the quantitative aspect of this analysis.

Sialic Acids on Normal and Malignant Colon Cells. Biosynthetically radiolabeled sialic acids expressed on normal and malignant colon cells were separated and identified by paper chromatography. Table 1 represents the relative expression of non- (1.00), mono- (1.24), di- (1.40), and tri- (1.64) O-acetylated sialic acids on normal colon tissue, fresh “uninvolved” tumor mucosa (the excised end of colon tissue in resections for carcinoma), and fresh and cultured colon carcinomas. The uninvolved tumor mucosa and carcinoma tissue were taken from the same patient. In Fig. 4, paper chromatographic profiles of HC 10700 (A) and NC2 (B) demonstrate that the relative expression of tri-O-acetylated sialic acid (1.64) was high in the fresh normal colon tissue (45.2%) compared with carcinoma of the colon (1.1%). In the case of NC2, the spent medium was analyzed for secreted biosynthetically labeled sialic acids (non-, 22.9%; mono-, 13.1%; di-, 5.4%; and tri-, 58.6%) demonstrating similar expression compared with NC2 tissue (Table 1). The fresh uninvolved colon mucosa tissue expressed a greater proportion of mono-O-acetylated sialic acid compared to other tissues. In one case (HC 9434), the macroscopically normal mucosa resected adjacent to the frank colon carcinoma ("adjacent") was analyzed for the relative amounts of non- and O-acetylated sialic acids (non-, 82.8%; mono-, 13.1%; di-, 3.5%; and tri-, 0.6%). The relative amounts of sialic acids expressed on the adjacent tumor mucosa were similar to the sialic acids expressed on fresh colon adenocarcinoma (Table 1).

Statistical analysis comparing the tissue types in Table 1 demonstrated a significant difference in the expression of non-, di-, and tri-O-acetylated sialic acids on fresh normal colon...
**O-Acetylated Sialic Acids on Human Colon Cells**

**Table 1: Sialic acids released from normal and malignant colon cells**

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Species of sialic acid (%) of total sialic acids</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Non-OAc</td>
</tr>
<tr>
<td>Fresh normal colon mucosa</td>
<td></td>
</tr>
<tr>
<td>NC1</td>
<td>59.1</td>
</tr>
<tr>
<td>NC2</td>
<td>31.2</td>
</tr>
<tr>
<td>NC3</td>
<td>63.8</td>
</tr>
<tr>
<td></td>
<td>52.0 ± 10.6</td>
</tr>
<tr>
<td>Fresh uninvolved colon mucosa</td>
<td></td>
</tr>
<tr>
<td>HC 7091</td>
<td>68.8</td>
</tr>
<tr>
<td>HC 9445</td>
<td>64.0</td>
</tr>
<tr>
<td>HC 9434</td>
<td>66.4</td>
</tr>
<tr>
<td>HC 10139</td>
<td>63.8</td>
</tr>
<tr>
<td>HC 10700</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>66.6 ± 1.2</td>
</tr>
<tr>
<td>Fresh colon adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>HC 7091 (rectum)</td>
<td>87.5</td>
</tr>
<tr>
<td>HC 9445 (sigmoid)</td>
<td>86.2</td>
</tr>
<tr>
<td>HC 9434 (sigmoid)</td>
<td>91.7</td>
</tr>
<tr>
<td>HC 10139 (sigmoid)</td>
<td>86.7</td>
</tr>
<tr>
<td>HC 10700 (right colon)</td>
<td>84.0</td>
</tr>
<tr>
<td></td>
<td>87.2 ± 1.3</td>
</tr>
<tr>
<td>Colon adenocarcinoma cell lines</td>
<td></td>
</tr>
<tr>
<td>HT 29 (R)</td>
<td>88.8</td>
</tr>
<tr>
<td>HT 29 (F)</td>
<td>88.4</td>
</tr>
<tr>
<td>HC 2998</td>
<td>94.2</td>
</tr>
<tr>
<td>HC 8589</td>
<td>91.1</td>
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<tr>
<td>HC 84</td>
<td>90.8</td>
</tr>
<tr>
<td>CL 3.5</td>
<td>86.0</td>
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<tr>
<td></td>
<td>89.9 ± 1.1</td>
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</table>

*OAc, O-acetylated.

Migration ratio values relative to the migration of Neu5Ac on Whatman 3MM paper.

Mean ± SE.

Anatomical location of adenocarcinoma.


**DISCUSSION**

The chemical identification of enzymatically modified sialic acids has been limited in the past because of purification, analysis, and labeling techniques that either destroy or fail to measure O-acetylated sialic acids. To avoid these problems, we cultured human established cell lines and fresh tissue samples in the presence of radiolabeled [6-3H]glucosamine and measured biosynthetically radiolabeled sialic acids after separation by paper chromatography, on the basis of the number of O-substitutions on each sialic acid molecule (Fig. 1). By this technique, we have separated and identified varying proportions of non-, mono-, di-, and tri-O-acetylated sialic acids from the same patient. This survey demonstrates the presence of O-acetylated sialic acids on human cells with relative differences in the percentages of non- and O-acetylated species of sialic acid found on fresh tissues and established cell lines.

The levels of O-acetylation of sialic acids expressed on peripheral blood mononuclear cells (B- and T-cells and monocytes) and fibroblasts were different from those expressed on normal colon or uninvolved colon tissue (Table 1). However, the levels of O-acetylation of sialic acids expressed on fresh uninvolved (Table 2) and normal liver (Table 3) were similar.

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ically labeled O-acetylated sialic acids from human cells were converted to the non-O-acetylated form by saponification and crude mouse liver esterase treatments. Previous work by Reid et al. (20) provided indirect evidence for the expression of O-acetyl groups on sialic acids on rat colonic epithelial tissue by measuring the production of methyl acetate liberated from O-acetylated sialic acids in sodium methoxide by gas-liquid chromatography.

To estimate patterns and relative amounts of O-acetylated sialic acids, most chemical studies (18–23) were limited to measuring formaldehyde formation after periodate oxidation and colorimetric estimations of sialic acid concentration. Barker et al. (17), however, reported a change in the paper chromatographic mobility of sialic acids from a malignant gastric tumor, suggesting the presence of di-O-acetylated sialic acid. Another study (22) used thin-layer chromatography to separate five types of non- and O-substituted sialic acids from normal and malignant human colon epithelium. These and many other studies (2, 8, 9, 12–16, 29) have suggested that the loss of O-acetylation on sialic acid molecules expressed in human colon is a sensitive indicator of early malignant change, since side chain-substituted sialic acids of normal colonic mucosa (3, 4, 8, 11, 30) are predominantly di- and tri-O-substituted (31). Our data support these findings and demonstrate lower levels of O-acetylation in the colon carcinomas compared to normal colon mucosa (Fig. 4, Table 1) and differences between the ratios of O-acetylated sialic acids expressed on uninvolved tumor mucosa and normal colonic mucosa (Table 1). Furthermore, the relative amount of tri-O-acetylated sialic acid expressed on these tissues appears to be a sensitive indicator of premalignant change (Table 1). Fetal intestinal cells expressed levels of non- and O-acetylated sialic acids similar to colon carcinoma (32, 33) that adjacent and uninvolved tumor mucosa express abnormal patterns of O-acetylated sialic acids provide evidence of a premalignant field defect, rather than a local secondary effect of tumor growth. Reduced levels of O-acetylation in cases of ulcerative colitis and Crohn’s disease (34) support this suggestion.

When the relative amounts of each species of sialic acid were compared between colon carcinoma and uninvolved mucosa in the same patient, significant differences were observed (Table 1). However, this same analysis on hepatic metastasis derived from a primary colon adenocarcinoma and uninvolved liver tissue from the same patient demonstrated little difference in the expression of each species of sialic acid (Table 2). Furthermore, no significant difference was observed in the expression of non- and O-acetylated sialic acids between primary adenocarcinomas of the colon and liver metastasis derived from a primary colon adenocarcinoma (Tables 1 and 2). These findings
support earlier work by Gad (35) that showed that mucosubstance found in the secondary deposits in lymph nodes were similar to the main type of mucosubstance in the primary tumor as well as later work (36) histochemically demonstrating the expression of O-acetylated sialic acids in cases of gallbladder, pancreatic, and gastric cancer.

The relationship of total cellular sialic acid levels in normal, malignant, and metastatic tissues has been a long-standing observation detailed in histochemical analysis (10, 29, 32) and biochemical studies (37). In particular, cell surface sialic acid has been shown to be positively correlated with metastatic potential in animal models (38, 39). Alternatively, the increase in sialic acid levels, as detected by colorimetric assays, may be a function of the modulation of O-acetylated sialic acid species to the non-O-acetylated form (Neu5Ac). It is quite apparent that sialic acids may play an important role in antigen masking, glycoconjugate turnover, and cellular differentiation in many biological systems (1). The ability to identify abnormal ratios of O-acetylated sialic acids represents a possible diagnostic tool to evaluate patients who are genetically and culturally predisposed to the development of adenocarcinoma of the colon. However, the regulation and functional significance of O-acetylation on the sialic acid molecule remains to be determined.

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488


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