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Alterations of Membrane Glycopeptides in Human Colonic Adenocarcinoma

(lectins/glycosyltransferases/glycosidases)

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ABSTRACT **Membrane glycopeptides were examined in human colonic adenocarcinoma and normal colonic mucosa. The carbohydrates of membrane glycopeptides were found to be markedly reduced in tumor tissue and the relative proportions of the various sugars were altered. Although all of the sugars were lower in tumor tissue when compared to the adjacent normal mucosa, galactosamine, fucose, and sialic acid were more significantly reduced. Examination of the blood group activity and lectin-binding properties of membrane glycopeptides revealed that specific carbohydrate structures had changed in the tumor tissue. Most striking of these changes was the disappearance of glycoprotein-associated blood group A activity. Assay of the enzyme responsible for synthesis of the blood group A determinant showed that this glycosyltransferase activity was greatly diminished in tumor tissue. A galactosyltransferase and a fucosyltransferase were also significantly lower in the tumor tissue whereas the levels of another galactosyltransferase and a sialyltransferase were unaltered. Glycosidase activities in the normal and tumor tissues were similar. The results show that an alteration in glycoprotein biosynthesis occurred during tumorigenesis that resulted in a modified membrane glycoprotein composition and that these changes are probably a reflection of reduced levels of the enzymes responsible for glycoprotein synthesis.**

Glycoproteins and glycolipids are ubiquitous in mammalian cells and are major components of cellular membranes (1–6). Glycopeptides isolated from the plasma membranes of cells such as erythrocytes and fibroblasts have diverse immunological properties, among them blood group and virus agglutinating activities (3, 4). It has been shown that these properties are determined by the terminal sequence and linkage of sugars in glycoprotein and glycolipid structures. The surface membranes and the microsomal membranes of intestinal epithelial cells also contain various carbohydrate moieties (2, 7, 8).

Phenomena characteristic of tumor growth such as alteration of cell–cell interactions and disturbance of normal immunological processes strongly implicate cellular membranes in the process of tumorous transformation. Differences in composition of membrane glycoproteins resulting from viral transformation of cells and in the metabolic systems which elaborate and degrade them have been reported (9–17). Although a consensus has not been reached, the studies uniformly suggest that both quantitative and qualitative differences are induced in the membrane glycoproteins of cells after transformation. These changes apparently occur in both internally and externally located membranes (14, 15). The majority of studies were performed on cultured cell systems in which the effects of various tumorigenic agents can be measured easily. However, many of the cellular alterations which might be expressed in tumor cells only when present in the host cannot be examined in cell culture. A few such studies indicate that there are appreciable differences between the carbohydrate structures of normal and tumor tissues. ABH blood group activity, a property of normal gastrointestinal tissues and determined by carbohydrate structures, was found to be low or absent in tissues from primary gastrointestinal cancer (18–20).

In the present study, we examine membrane glycopeptides from normal and cancerous colonic mucosa obtained from patients with and without colon cancer for blood group activity and ability to bind lectins. Since considerable differences were observed in these activities between normal and cancerous tissues, we compared the carbohydrate compositions of these glycopeptides and determined the levels of some of the enzymes thought to be involved in the synthesis and degradation of the carbohydrate portion of the glycoproteins.

MATERIALS AND METHODS

Tissues. All specimens including cancer tissues were obtained at surgery. Normal appearing mucosal tissues were obtained from two sources; (*i*) from the adjacent normal appearing mucosa never closer than 5 cm from the periphery of cancer tissues in the same patient from whom the colonic cancer tissue was obtained and (*ii*) from normal appearing mucosa obtained from patients who underwent colectomy for diverticulitis and volvulus. The tissues were washed with cold 0.16 M NaCl–0.01 M cacodylate acetate buffer (pH 7.0) and stored at −60°.

Preparation of Membrane. Normal mucosal scrapings and minced tumors were weighed and homogenized in 4 volumes (w/v) of cold 0.16 M NaCl–0.01 M cacodylate acetate buffer (pH 7.0) using a Polytron homogenizer and passed through two layers of cheesecloth. The membrane fraction containing plasma, nuclear, mitochondrial, and microsomal membranes was then centrifuged at 120,000 × *g* for 60 min (21, 22). Examination of the pellet by phase microscopy revealed the presence of membranes without contamination by cell debris. The mean phospholipid to protein ratios of membrane pellets prepared from normal and tumor tissues were 1.25 and 1.24,

Abbreviations: con A, concanavalin A; α_1AGP-NANA, α_1-acid glycoprotein minus sialic acid; OSM-NANA, ovine submaxillary mucin minus sialic acid; α_1-AGP-NANA-GAL, α_1-acid glycoprotein minus sialic acid, minus galactose; NANA, *N*-acetylneuraminic acid.

TABLE 1. *Hemagglutination inhibition by membrane glycopeptides of normal and cancerous colonic mucosa*

	Dolichos biflorus (A_1)*		*Ulex europaeus* (H)		Human anti-Le^a serum (Le^a)		*Ricinus communis* (Gal)		Concanavalin A (Man, GlcN)	
Patients	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer
1	103†	—‡	—	—	—	—	—	222	77	55
2	10	—	—	—	2.0	—	—	139	123	17
3	10	—	—	—	0.3	—	—	136	10	34
4	24	—	—	—	—	—	—	—	6	15
5	8	—	—	—	1.0	16	—	62	8	31
6	64	—	—	—	0.6	1.6	—	202	32	101

* Human blood group activity (A_1, H, and Le^a) or sugar specificity (Gal, Man, and GlcN) exhibited by agglutinins.
† Numbers represent the amount of protein in μg that can cause hemagglutination inhibition.
‡ Indicates that no hemagglutination inhibition occurred with up to 300 μg of protein.

respectively, indicating that membrane pellets from normal and tumor tissues are comparable and that these pellets substantially contain membranes (23, 24).

Hemagglutination Inhibition by Membrane Glycopeptides. Membrane glycopeptides were prepared by treating membrane pellets with Pronase (1% of membrane protein) for 2 days, after inactivating the carbohydrate degrading enzymes by initial incubation of the pellet at 70° for 30 min. Inhibition of agglutination between various agglutinins and human erythrocytes was tested in 10,000 × *g* supernatant solutions containing membrane glycopeptides. Anti H lectin was prepared by saline extraction of the seeds of *Ulex europaeus;* blood group A specific lectin was prepared from the seeds of *Dolichos biflorus* (Anti A) (Pfizer Inc., New York, N.Y.) and *Ricinus communis* (RCA_{120}) (a gift of Dr. G. Nicolson); concanavalin A (con A) was prepared from jack bean meal (Sigma Chemical Co., St. Louis, Mo.) (25); human anti Le^a was a gift of the Irwin Memorial Blood Bank, San Francisco, Calif. The human erythrocytes, normally used for typing sera and obtained from Pfizer, Inc., were all type O, Rh negative except in reactions involving *Dolichos biflorus* lectins when type A, Rh negative human erythrocytes were used. Serial dilutions of lectins were tested against a constant amount of erythrocytes. The concentration of lectins selected for the assays was twice as high as the amount required for visible agglutination of cells. The assay mixture contained 25 μl of 1% erythrocyte suspension, 25 μl of 0.16 M NaCl, and 25 μl of the membrane glycopeptides.

Glycosyltransferase Assays. The activities of five glycosyltransferases were measured in the mucosal homogenates essentially as described previously (23, 26–28), i.e., a sialyltransferase, two galactosyltransferases, an *N*-acetylgalactosaminyltransferase, and a fucosyltransferase.

The acceptors used for the glycosyltransferase assays were for sialyltransferase, α_1AGP-NANA; for galactosyltransferase I, OSM-NANA; for galactosyltransferase II, α_1-AGP-NANA-GAL; for *N*-acetylgalactosaminyltransferase, 2′ fucosyllactose; and for α1-2 fucosyltransferase, lactose. Conditions for assay of these enzymes were modified slightly to obtain linearity of the reaction with respect to time and amount of enzyme.

Glycosidase Assays. The activity of five glycosidases were assayed using 4-methylumbelliferone derivatives of five sugars (Koch-Light Laboratories, Ltd., Colubrook-Bucks, England) and [^{14}C]GalNAc-fucosyllactose. The incubation mixture contained 20 nmol of the appropriate 4-methylumbelliferyl-glycoside, 50 μg of enzyme protein, and 100 μl of 0.1 M acetate buffer with 0.2% Triton X-100 in a final volume of 200 μl. Incubation was at 37° for 20 min, and the reaction was stopped by immersion of the reaction tubes in ice and immediate addition of 2 ml of 20 mM glycine–5 mM EDTA buffer (pH 10.4). Fluorescence of released free 4-methylumbelliferone was measured on a Perkin-Elmer fluorimeter at an excitation wave length of 365 nm and an analyzer wave length of 460 nm.

[^{14}C]GalNAc-fucosyllactose was prepared by the method previously described from this laboratory (29). The incubation mixture for assay of the *N*-acetylgalactosaminidase contained 20,000 cpm of [^{14}C]GalNAc-fucosyllactose, 1 mg of enzyme protein, and 50 μl of 0.1 M acetate buffer (pH 4.5) with 0.33%

TABLE 2. *Glycosyltransferase activities in normal and cancerous colonic mucosa*

Glycosyltransferase	Normal* (2)	Normal† (7) (cpm/mg of protein per hr × 10^{-3})	Cancer (7)
Sialyltransferase	57.9 ± 9.8	53.7 ± 11.6	42.6 ± 18.9
Galactosyltransferase I	7.8 ± 1.7	8.6 ± 4.8	7.7 ± 6.2
Galactosyltransferase II	70.8 ± 16.3	77.0 ± 19.0	41.2 ± 15.6‡
Fucosyltransferase	117.2 ± 7.7	117.1 ± 40.8	43.1 ± 33.6‡
N-Acetylgalactosaminyltransferase	155.2 ± 150.0	201.0 ± 115.0	36.7 ± 32.3‡

Numbers indicate mean values ± SD; numbers in parentheses indicate number of patients.
* Normal tissue obtained from patients without colonic carcinoma.
† Normal tissue obtained from patients with colonic carcinoma.
‡ Indicates $P < 0.005$ between normal and cancer tissues by Student's *t*-test.

Triton, X-100 in a final volume of 150 μl. The mixtures were incubated for 15 hr at 37° and the reaction was stopped by freezing the sample on dry ice. The entire mixture was then spotted on Whatman no. 1 paper and chromatographed for 4 hr in a solvent system of ethyl acetate–pyridine–H_2O (5:7:1, v/v/v). The standards used were [^{14}C]GalNAc-fucosyllactose, [^{14}C]GalNAc, and fucosyllactose. Radioactive areas on paper chromatograms were cut and counted in a Packard Tri-Carb liquid scintillation Spectrometer (30). Reducing sugars on paper chromatograms were detected by the silver nitrate reagent (31). All enzyme assays were performed under conditions in which the reaction was linear with respect to enzyme concentration and time.

Paper Chromatography. To determine if the breakdown of the substrate occurred, the reaction mixtures used for glycosyltransferase assays were applied to Whatman 3 MM paper with appropriate standard sugars and sugar nucleotides. Chromatography was for 18 hr at 25° and the solvent systems were as follows: for sialyltransferase, 1.0 M ammonium acetate (pH 7.5)–ethanol (3:7); for galactosyltransferases and for fucosyltransferase, ethyl acetate–pyridine–water (12:5:4), and for *N*-acetylgalactosaminyltransferase, 1.0 M ammonium acetate (pH 3.8)–ethanol (5:2). Product identification was carried out by paper chromatography after acid hydrolysis of the products as described previously (23–25).

Chemical Analysis. The membrane pellets were treated with 20 volumes (w/v) of chloroform–methanol (1:2, v/v) for 20 hr at 4°. Membrane proteins were then solubilized in 0.1 M NaOH, quickly neutralized with 0.1 M HCl, and hydrolyzed (8). Neutral sugar analysis was carried out on a Perkin-Elmer gas liquid chromotograph using inositol as an internal standard (29). The hydrolyzed samples were also analyzed for glucosamine and galactosamine using a model 120C Beckman amino acid analyzer (32). The membrane proteins were hydrolyzed and processed as described previously (8), and sialic acid was measured using the method of Aminoff (33).

RESULTS

Hemagglutination Inhibition by the Glycopeptides of the Normal and Cancerous Colonic Mucosa. The level of antigens in cancerous and adjacent normal colonic mucosa is shown in Table 1. Human blood group A activity could not be detected in the cancer tissue from any patient but high levels were present in adjacent normal tissues. No H activity was detected. Two patients who had high levels of Le^a activity in their normal mucosa had no detectable levels in the cancer tissue. The use of *Ricinus communis*, which recognizes the immediate precursor of blood group H structure, demonstrated higher levels of terminal galactose residues in cancerous tissues. Con A showed little difference between normal and cancerous tissues.

Glycosyltransferase Activity. Five glycosyltransferases were assayed using glycoprotein and oligosaccharide acceptors. Table 2 shows that there were no significant differences in the activities of these enzymes between the two normal groups. Cancer tissues had significantly lower activity of an *N*-acetylgalactosaminyltransferase and fucosyltransferase. Two galactosyltransferases were lower in cancer tissues but the difference was statistically significant only for one. The level of a sialyltransferase was not significantly different in normal and cancerous tissues. Mixing experiments showed that for all of the glycosyltransferases the differences between tissues were not due to activators or inhibitors. There was a wide variation in the activity of the *N*-acetylgalactosaminyltransferase among individuals, both in normal and in cancerous tissues. Regardless of this variation, all of the patients had a lower level of this enzyme in the tumor tissue. No such trend was apparent for the sialyltransferase.

Glycosidase Activity. Table 3 shows that of the six glycosidases examined, none was appreciably higher or lower in normal and cancerous tissues. Of interest is the observation that there was no statistical difference between normal and cancer tissue in the level of the enzyme responsible for degrading the A antigen.

Chemical Composition of the Membrane Fraction. The analysis of the carbohydrate content of the membrane fraction of normal and cancerous colonic mucosa as shown in Table 4 indicated that cancer tissues generally contained less of each of the sugars than did the membrane fraction from the normal tissues. Galactosamine, glucosamine, sialic acid, and fucose were reduced significantly in the cancerous tissues, whereas, galactose was reduced to a lesser extent. The mannose content was unchanged. The variable degree of reduction in the amount of each sugar in the membrane is evident when the molar ratios of each sugar are compared in normal and tumor tissues. Although not shown, the carbohydrate content of the supernatant fraction showed similar results. The protein content

TABLE 3. *Glycosidase activities in normal and cancerous colonic mucosa*

Glycosidases	Normal* (2)	Normal† (7)	Cancer (7)
		(nmol/mg of protein per min)	
β-Galactosidase	2.9 ± 0.08	2.3 ± 0.7	1.9 ± 0.5
α-Galactosidase	0.2 ± 0	0.2 ± 0.1	0.2 ± 0.1
β-*N*-Acetylgalactosaminidase	3.0 ± 0.1	1.7 ± 0.4	1.3 ± 0.4
β-*N*-Acetylglucosaminidase	5.9 ± 0	4.2 ± 1.0	3.0 ± 0.9‡
α-Mannosidase	0.7 ± 0	0.5 ± 0.1	0.4 ± 0.1
		(cpm/mg of protein per 15 hr)	
α-*N*-Acetylgalactosaminidase		917 ± 706	640 ± 657

Numbers indicate mean values ± SD; numbers in parentheses indicate number of patients.
* Normal tissue obtained from patients without colonic carcinoma.
† Normal tissue obtained from patients with colonic carcinoma.
‡ Indicates $P < 0.05$ between normal and cancer tissues by Student's *t*-test.

TABLE 4. *Carbohydrate composition of the membrane fraction of normal and cancerous colonic mucosa*

	Fucose	Mannose	Galactose	GlcNAc	GalNAc	NANA
			(nmol/mg of protein)			
Normal* pellet	32.7 ± 17.0†	27.6 ± 16.4	54.0 ± 28.1	63.9 ± 20.3	44.4 ± 18.3	41.5 ± 19.5
	(1.18)‡	(1)	(1.96)	(2.32)	(1.61)	(1.50)
Cancer§ pellet	9.1 ± 5.7	19.3 ± 8.1	24.0 ± 4.1	25.7 ± 10.6	8.3 ± 4.3	5.0 ± 3.8
	(0.47)	(1)	(1.24)	(1.33)	(0.43)	(0.26)
P¶	<0.02	$P > 0.05$	<0.05	<0.01	<0.005	<0.005

* Membrane pellet of normal tissue.
† Numbers indicate mean values from five patients ± SD.
‡ Numbers in parentheses are the molar ratios of each sugar when the value for mannose is taken as 1.
§ Membrane pellet of colonic tumor.
¶ Student's *t*-test.

of the whole homogenate was 27% higher and that of the membrane pellet was 38% higher in the tumor than in the normal tissues when expressed per g wet weight.

Identification of the Products of Enzymatic Reactions. After incubation, reaction products of the glycosyltransferases and glycosidases were chromatographed on paper to identify the appropriate reaction products and for the glycoslytransferases to determine if degradation of the substrates had occurred during the incubation. When the reaction mixtures for the glycosyltransferase assays were examined, only two radioactive spots were observed, one representing the product and another corresponding to nucleotide-linked sugar substrates. No radioactive sugar or sugar phosphate was detected. In all assays of the glycosyltransferases, acid hydrolysis of the product of each reaction revealed a single radioactive spot with a R_f corresponding to that of the sugar predicted to be transferred. Similarly, the reaction mixture for the assay of the *N*-acetylgalactosaminidase yielded a radioactive spot with an R_f identical to that of *N*-acetylgalactosamine. No other radioactive spot was observed except that of the substrate which remained at the origin.

DISCUSSION

The data presented in this study indicate that considerable alterations in composition and antigenic determinants of membrane glycoproteins occur in colonic tumors. Absence of blood group A activity of membrane glycopeptides prepared from colon cancer tissues is consistent with previously reported histological studies in which blood group A and B determinants in tumors of the cervix, urinary tract, and stomach were lost (18–20). Although we found a deletion in blood group A activity, no increase in H activity was detected. Le^a activity was reduced in some tumor tissues. The use of *R. communis* lectin, which recognizes the immediate precursor of H structure, demonstrated increased terminal galactose residues in cancerous tissues suggesting that more membrane glycoproteins with incomplete oligosaccharide side chains were produced. Thus, our data suggest that the absence of the blood group A determinant is not due to the lack of a terminal GalNAc alone but may be due to the failure of the tumor tissue to form the H determinant which is a prerequisite for the formation of the A structure.

The observed alterations may be due either to decreased synthesis or to increased degradation of glycoprotein antigens. The activity of the *N*-acetylgalactosaminyltransferase thought to be responsible for blood group A activity (34, 35) was markedly reduced in colon cancer tissues. The activities of a fucosyltransferase and a galactosyltransferase were also significantly lower in cancer tissues. Reduction in the activities of these glycosyltransferases in cancer tissues appears to be specific since there were no significant changes in a sialyltransferase and a galactosyltransferase level. It is of interest that the levels of activities of all five glycosyltransferases examined were similar in normal colonic mucosa obtained from patients with or without colon cancer. The decrease in glycosyltransferase activity was not due to inhibitors or activators as determined by mixing experiments, nor was it due to an elevation of nucleotide sugar degrading enzymes in tumor tissue, since paper chromatographic analysis of the glycosyltransferase reaction mixtures indicated that no degradation of the substrates had occurred. The protein content of the tumor tissues per wet weight was 27% higher than the normal mucosa but this factor alone cannot account for the marked reduction in the specific activities of these enzymes. Finally, glycosidase activities were not increased in tumor tissues, suggesting that the deletion of blood group activity in tumor tissues is mainly due to the reduction in several glycosyltransferase activities.

The glycoprotein alterations in cancerous tissue may be quantitative, qualitative, or both. Our data show that the total carbohydrate content of membrane proteins in cancer tissue is reduced to one-third of that in normal tissue. Furthermore, the ratios of the sugars in the normal differed from those of the cancerous tissue, suggesting that this decrease cannot be ascribed merely to fewer of the carbohydrate chains present in normal tissue. Although there may be a decrease in the normally occurring carbohydrate chains, this change must be accompanied by an alteration in the type of oligosaccharides present. In fact, the decrease in the glycosyltransferase reported here may permit incomplete oligosaccharides to accumulate and result in an altered carbohydrate composition. This suggestion is supported by the findings that the sugars commonly present on the nonreducing termini of the carbohydrate moieties of glycoproteins are those that are reduced in tumor tissue. It may be of interest to note that the sugars which are the major constituents of "serum type" glycoprotein, i.e., galactose, mannose, and glucosamine, remain relatively unchanged whereas the sugars associated with blood group structures, i.e., fucose and *N*-acetylgalactosamine, are reduced. This suggests that the synthesis of one type of oligosaccharide chain is unaffected by neoplasia but the synthesis of another family of oligosaccharides is greatly affected.

A marked decrease in most neutral and aminosugar contents of membrane carbohydrates was reported in simian virus 40-transformed 3T3 mouse fibroblasts (10, 13), whereas, others found an increase in sialic acid content (36). Contradictory results were also obtained for the sialyltransferase (12, 13, 17). In the present study using desialyzed fetuin or α_1-acid glycoprotein as acceptors, both normal and tumor tissues had similar sialyltransferase activities.

Several studies on the alterations in glycolipid composition and in levels of activity of glycolipid–glycosyltransferases in transformed cells have been reported (37–42). Although the results vary in many aspects, it appears that there is a buildup of incomplete glycolipids at the expense of those with completed oligosaccharide moieties which occurs concomitant with a reduction of some glycolipid–glycosyltransferases. Our finding that the enzyme responsible for the formation of the A determinant on glycoproteins is markedly reduced suggests either that one *N*-acetylgalactosaminyltransferase may utilize both glycoprotein and glycolipid acceptors or that the systems for the synthesis of blood group glycoproteins and glycolipids are closely linked and an alteration in one may be associated with a change in the other.

The present studies show that substantial alteration in glycoprotein biosynthesis occurs in tumor tissues. However, one of the shortcomings of studies where "tumor tissue" is compared to "normal tissue" is that the tumor may be due to the preferential growth of a cell type normally present in the tissue but not a major constituent or may represent a clone of cells derived from a different site. Although the adenocarcinoma tissue examined in the present study may reflect either of these possibilities, changes in glycolipid and glycoprotein biosynthesis have been reported using cultured cells (37–41) in which both normal and transformed cells are derived from the same cell line. Whether the alterations in the biosynthesis of glycoproteins and glycolipids are primary events or reflect changes in the tissues secondary to neoplastic transformation remains to be determined, but these changes seem to be a common manifestation of the malignant process.

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