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THE COMMON STRUCTURE IN FUCOSYLLACTOSAMINOLIPIDS ACCUMULATING IN HUMAN ADENOCARCINOMAS, AND ITS POSSIBLE ABSENCE IN NORMAL TISSUE*

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SUMMARY: Two major glycolipids accumulating in a human primary liver adenocarcinoma, but absent in normal liver, were characterized as lacto-N-fucopentaosyl(III)ceramide and difucosyllacto-N-nor-hexaosylceramide, (Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4Glcβ1→1Cer), a new type of glycolipid with Le^x-determinant. Comparison of glycolipids bearing Le^x-determinant in various cases of human colonic adenocarcinoma, in adjacent normal mucosa tissue, and in erythrocytes reveals a possibility that glycolipids accumulating in human adenocarcinoma, but not in normal tissue, have a common structural unit as identified below:

R→4GlcNAcβ1→3Galβ1→4Glcβ1→1Ceramide
3
↑
Fuc 1

Glycosphingolipids at the cell surface show dramatic changes associated with the process of development, differentiation, and oncogenesis (1). Two types of glycolipid change associated with oncogenesis have been noticed; a blocked synthesis with or without precursor accumulation, and a synthesis of a "neoglycolipid" that is absent/or present in very small quantity in normal cells or tissues (1,2). Typical examples of "neoglycolipid" are ganglio-triaosylceramide in mice lymphoma (L5178) and sarcoma (KIMSV) (2,3), Forssman or A-like antigen in human cancers of F^-(fsfs) or B/or O individuals (4,5), sialosyl-Le^a ganglioside in a large variety of human colorectocarcinoma and pancreas cancers (6,7), and GD_3 ganglioside in human melanoma (8).

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The search for the glycolipid antigen defined by the monoclonal antibody "SSEA-1", which is directed to mouse F9 embryonal carcinoma cells and to mouse preimplantation embryo (9), disclosed the presence of a series of glycolipids with Le^x determinant (Galβ1→4[Fucα1→3]GlcNAC) (10,11). The specificity of the antibody was also identified by inhibition of the reactivity by oligosaccharide lacto-N-fucopentaose III or its analogs (12). Some human adenocarcinoma accumulated glycolipids, lacto-N-fucopentaosyl-(III)-ceramide (13) and trifucosyl type 2 chain glycolipids which are reactive to SSEA-1 antibody (10); however, exact structural information on the glycolipids with Le^x determinants accumulating in human adenocarcinoma has been unknown. The present study is undertaken to compare the structure of glycolipids having Le^x determinant present in adenocarcinoma of human liver, colonic adenocarcinoma, normal liver, and normal colonic mucosa. The results indicate the presence of a common structural unit characteristic for the glycolipids accumulating in human adenocarcinoma.

MATERIALS AND METHODS

Tumors and adjacent normal tissues used in this study were obtained from the National Institutes of Health tumor procurement program and through the Sloan-Kettering Institute, New York, courtesy of Dr. Philip Levine. All cases were diagnosed as adenocarcinoma. The SSEA-1 antibody (9) was donated by Drs. Barbara Knowles and Davor Solter of the Wistar Institute, Philadelphia, Pennsylvania. Tissues were extracted by homogenization with isopropanol-hexane-water (55:25:20) (11), four-times partitioned according to Svennerholm's modification of Folch's procedure (14), separated by successive chromatography on DEAE Sephadex (15). Neutral glycolipids were purified by three stages of high performance liquid chromatography (11,16): i) Low pressure chromatography on an "Iatrobeads 6RS-8060" column (50 X 1 cm) with a gradient elution from isopropanol-hexane-water (55:40:5) to isopropanol-hexane-water (55:20:25). The total volume of solvent was 800 ml and collected over 200 tubes. Glycolipids distributed among fractions were as follows: lactosylceramide in fraction I(tube #23-29), ceramide trihexoside in fraction II(tube #30-37), fast migrating paragloboside in fraction III(tube #38-45), slow migrating paragloboside in fraction IV(tube #46-50), ceramide pentasaccharide with Le^a or Le^x structure in fraction V(tube #51-59), slow-migrating glycolipids with TLC mobility between H2 and H3 glycolipid (23) in fraction VI(tube #70-84), and in fraction VII(tube #85-99), and many other components with slower mobility than H3 in fractions VIII(tube #100-120), IX(tube #121-140), and X(tube #141-200); ii) The major fractions V, VII, and VIII were further purified on HPLC on an "Iatrobeads 6RS-8010" column with the same solvent system as above. The total solvent volume was 400 ml, collected over 100 tubes. Essentially pure ceramide pentasaccharide with Le^x structure was eluted between tubes #37-39. The major second component, in fraction VII, was eluted in tube #50-60; and the slower mnigrating major component was eluted in tube #70-80; iii) Each fraction was further purified on repeated HPLC. The purified glycolipid fractions were analyzed by using

Baker's HPTLC plates (see below) in chloroform-methanol-water (56:38:10) for polar glycolipids (sugar number > 6). Glycolipids reactive to SSEA-1 antibody were detected on Baker HPTLC plate, by a modified immunostaining procedure (11), as originally described by Magnani et al. (17). The sugar composition and the sugar linkages were determined by methylation analysis (18). The location of fucosyl residue was determined by methylation before and after preferential hydrolysis of fucosyl residue in 0.1N trichloroacetic acid at 100°C for 1 hour (19). The presence of N-acetyllactosaminyl structure was ascertained by its susceptibility to hydrolysis with endo-β-galactosidase of Escherichia freundii (20). The partially methylated sugars were separated on capillary column by gas chromatography and identified by chemical ionization mass spectrometry (21), and the permethylated glycolipid was analyzed by direct probe mass spectrometry (22).

RESULTS

Chemical Characterization of Glycolipids Accumulating in Human Adenocarcinoma

Two major glycolipids accumulating in human primary hepatoadenocarcinoma have been isolated through three steps of high performance liquid chromatography as described under Materials and Methods. 1) One major glycolipid (fraction V in HPLC; band 1 doublet, lane D, Fig. 2) was identified on methylation analysis as lacto-N-fucopentaosyl(III)ceramide (see Fig. 1, A-1). Defucosylated and permethylated glycolipid gave 3,6 di-O-methyl-GlcNAcMe, exclusively, (Fig. 1, A-2). Thus, the fraction did not contain Le^a structure. 2) The second major glycolipid (fraction VII in HPLC; band 3 doublet, lane D, Fig. 2) was characterized having a novel structure, identified as difucosylated lacto-N-norhexaosylceramide as shown in structure 3, Table I. The basis of structural identification is as follows: i) the glycolipid migrated on HPTLC slower than H_2 (23), but slightly faster than H_3 (23); ii) defucosylation of the glycolipid (19) gave a doublet migrating on HPTLC with a similar mobility as lacto-N-norhexaosylceramide derived from H2 glycolipid; iii) on methylation analysis, the glycolipid gave 2 mols of 2,3,4 tri-O-methyl-Fuc, 2,4,6 tri-O-methyl-Gal, and 1 mol of 2,3,4,6 tetra-O-methyl-Gal. 6-O-methyl-GlcNAcMe and 2,3,6 tri-O-methyl-Glc were clearly identified but the yield of these sugars was not stoichiometric (see Fig. 1, B-1); iv) The glycolipid gave an intense peak for 3,6 di-O-methyl-GlcNAcMe with the disappearance of 6-O-methyl-GlcNAcMe and 2,3,4 tri-O-methyl-Fuc on defucosylation (19). No trace amount of 4,6 di-O-methyl-GlcNAcMe was detected (Fig. 1, B-2); v) hydrolysis with endo-β-galactosidase resulted in a release of hepta-

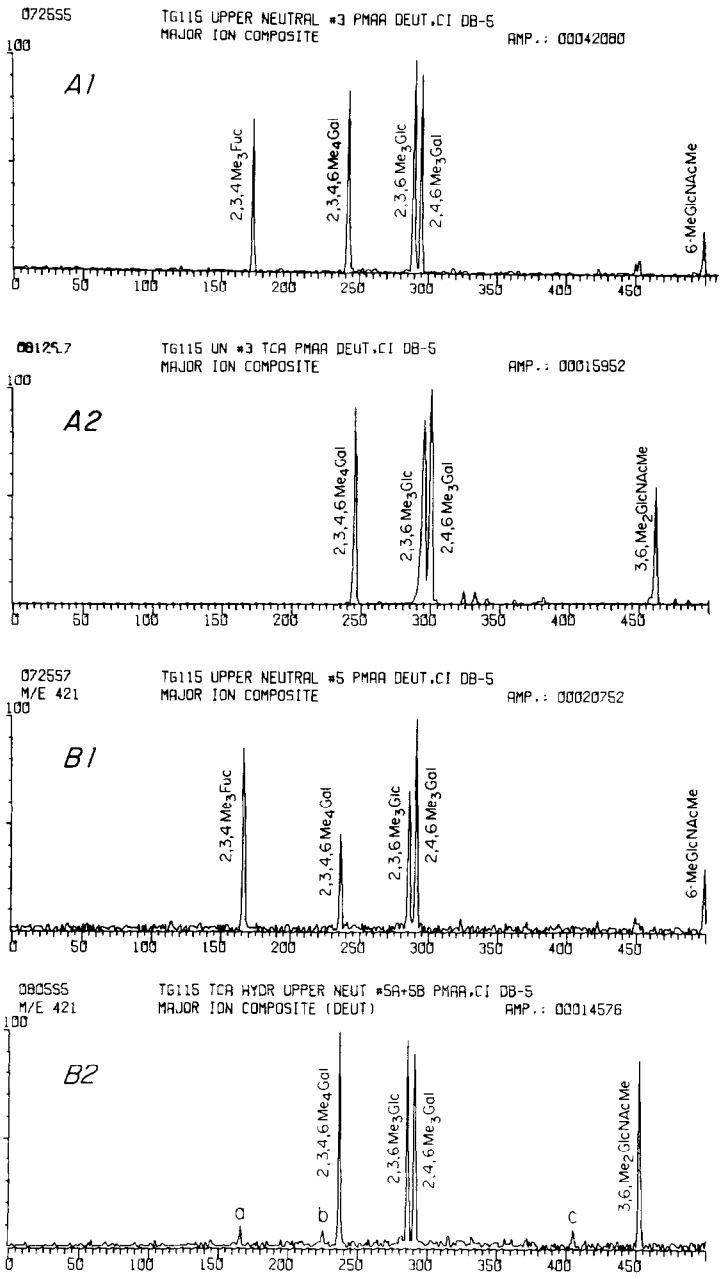

Figure 1: Chemical ionization mass chromatogram of partially O-methylated alditol acetates released from permethylated glycolipids before and after defucosylation. A-1, purified fraction V; A-2, fraction V after defucosylation and methylation; B-1, fraction VII; B-2, fraction VII after defucosylation and methylation. Each chromatogram is a summation of ions MH-32+, MH-60+, and MH+, separated on 30 m capillary column DB-5 bonded phase fused silica (J & W Scientific, Rancho, Cordova, CA 95670) with temperature programmed from 140 to 250°C. Determined in chemical ionization mode in methane in Finnigan 3300/6110 mass spectrometer.

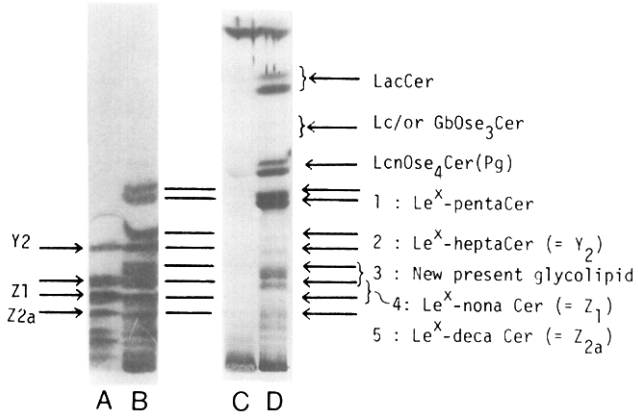

Figure 2: HPTLC of glycolipids isolated from human primary liver adenocarcinoma (lane D and B) and that from normal liver (lane C and A). Lanes C,D, orcinol reaction and lanes A,B, immunostaining with anti-SSEA-1 antibody. Bands are identified by arrow. The doublets of band 1 and band 3 stained by orcinol and by immunostaining are, respectively, lacto-N-fucopentaosyl(III)ceramide and difucosyl-lacto-N-norhexaosylceramide, the new glycolipid antigen. The lower band of doublet band 3 is overlapping with the upper band of doublet band 4, which is ceramide nonasaccharide with Le^x structure (= Z1-glycolipid, in reference 11). These doublets represent the differences in fatty acids (11, 27).

Table I.

Structure of Glycolipids Bearing Le^x Determinant.

From Tumor and Normal Tissue.

Glycolipid	Structure
1. Fr. IV-V	Galβ1→4 **GlcNAcβ1→3Galβ1→4Glcβ1→1Cer** * Fucα1 ↗3
2. Y_2 (ref. 11)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer ** Fucα1 ↗3
3. Fr. VII	Galβ1→4GlcNAcβ1→3Galβ1→4 **GlcNAcβ1→3Galβ1→4Glcβ1→1Cer** * Fucα1 ↗3 Fucα1 ↗3
4. Z_1 (ref. 11)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer ** Fucα1 ↗3
5. Z_{2a} (ref. 11)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer ** Fucα1 ↗3 Fucα1 ↗3
6. Fr. VIII-IX	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4 **GlcNAcβ1→3Galβ1→4Glcβ1→1Cer** *† Fucα1 ↗3 Fucα1 ↗3 Fucα1 ↗3

* Accumulate in adenocarcinoma of liver and colon and absent in normal liver, colon mucosa, and erythrocytes: Note the presence of the common structure as shown in bold print.

** Present in normal liver, colon and erythrocytes. † Structure is tentative.

saccharide and a doublet of ceramide mono-hexoside. The hydrolysis with the same enzyme after defucosylation gave a trisaccharide, a disaccharide, a ceramide trisaccharide, and a ceramide monohexoside. This degradation pattern was identical to that of lacto-N-norhexaosylceramide. 3) The slow migrating fractions VIII-IX eluted from HPLC column after "Z2a glycolipid" (see Table I, structure 5) contained heterogenous lactosaminolipids. Although complete characterization of these glycolipids remains for extensive future studies, the structure of one accumulated glycolipid present in the slow migrating fraction is assumed to have a trifucosylated lacto-N-nor-octaosylceramide structure as shown in Table I (structure 6) based on the following findings: i) the component migrated on HPTLC slower than Z2 glycolipid (11) of human erythrocytes which is difucosylated lacto-N-nor-octaosylceramide; ii) defucosylation (19) gave the same TLC mobility as lacto-N-nor-octaosylceramide.

The Pattern of Glycolipids with the Le^x Determinant

The two major glycolipids with Le^x determinant, accumulated in adenocarcinoma of liver and absent in normal liver, were band 1 and band 3 (see lane D, Fig. 2), which have been characterized as structure 1 and 3 in Table I. In normal liver, band 1 and band 3 were absent (Fig. 2, lane A,C). Glycolipids present in normal liver, marked as band 2 (doublet), 4 (doublet) and 5 were stained strongly by SSEA-1 antibody (lane A, Fig. 2). These bands showed the same TLC mobility as Y2 (a ceramide heptasaccharide with Le^x; structure 4, Table I), Z1 (a ceramide nonsaccharide with Le^x, structure 4, Table I), and Z2a (a ceramide octasaccharide with Le^x, structure 5, Table I), respectively (lane A, Fig. 2; lane 8, B, Fig. 3).

Neutral glycolipids of colonic adenocarcinoma and its adjacent normal mucosa were examined by HPTLC and by immunostaining of HPTLC with SSEA-1 antibody. The patterns of three cases are shown in Fig. 3. All three cases of tumor tissues contained a common band, stained by SSEA-1 antibody, showing the same mobility as difucosyllacto-N-norhexaosylceramide (lane 1,3,5). The glycolipid fraction prepared from normal mucosa tissue of the same wet

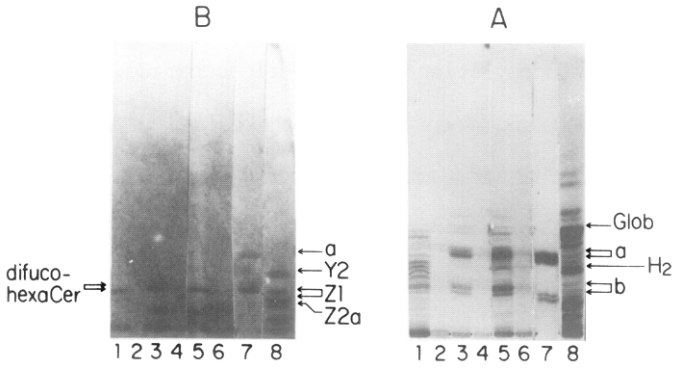

Figure 3: Glycolipids of colonic cancer (lanes 1,3,5) and from adjacent normal colonic mucosa (lanes 2,4,6). Lane 7, a reference for lacto-N-fucopentaosyl(III)ceramide (a), and difucosylated lacto-N-norhexaosylceramide (b) isolated from human liver adenocarcinoma. Lane 8, neutral glycolipids fraction of human erythrocytes isolated from the Folch's upper phase (11). Plate A is stained by orcinol reaction and plate B was revealed by immunostaining with SSEA-1 antibody.

weights as tumor tissue did not show any appreciable quantity of glycolipids, immunostained by SSEA-1 antibody (lane 2,4,6, Fig. 3).[1]

DISCUSSION

Normal human intestinal mucosa contain lacto-N-fucopentaosyl(II)ceramide, but not lacto-N-fucopentaosyl(III)ceramide (24), although lacto-N-fucopentaose III is the well-known component of human milk (25). The presence of exceptionally high concentration (30-100 μg/10 mg protein) of this glycolipid in some human adenocarcinoma may be characteristic of the malignant state. The second component is a new structure having two fucosyl residues linked to lacto-N-norhexaosylceramide. Although only four cases were demonstrated in Fig. 1-3, all these cases showed the accumulation of this glycolipid.

We noticed previously the presence of a lacto-series glycolipid having three fucosyl α 1-3GlcNAc substitutions, although the glycolipid was assumed to have a branched core (15). The core structure of tri-fucosyl glycolipid may also be a linear unbranched lacto-N-noroctaosyl (item 6, Table I).

[1] Lacto-N-fucopentaosyl(III)ceramide did not react with anti-SSEA-1 antibody on solid phase radioimmunoassay under the same conditions in which various Le^x glycolipids (Y2,Z1,Z2a) were strongly reactive (10). The reactivity of this glycolipid on TLC immunostaining required much higher concentration than other Le^x glycolipids. SSEA-1 antibody is, therefore, not suitable to detect lacto-N-fucopentaosyl(III)ceramide by these methods.

We have isolated and characterized a series of glycolipids with Le^x determinant from human erythrocyte membranes (16). The major glycolipids accumulated in human adenocarcinoma and those isolated and characterized from erythrocytes and presumably present in other normal tissues are listed in Table I. The structures 1, 3, and 6 which are accumulated in tumors have the common unit as shown below and as shown in bold print in Table 1.

R→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
↑
Fucα1

The structures 2,3, and 5 are present in normal tissue which are lacking the structure as identified above. Karlsson and Larson (26) suggested, on spectrometric basis, the presence of a similar structure, but with Le^b determinant in human meconium. Therefore, a synthesis of this structure could be an oncofetal expression. A crucial mechanism to cause the accumulation of these glycolipids having the common structure as above could be the activation of fucosyltransferase to GlcNAc residue of Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer as well as a possible induction of an aberrant GlcNAc transferase to the terminal Gal residue of Galβ1→4[Fucα1→3]GlcNAc structure.

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