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BIOSYNTHETIC PATHWAYS FOR THE Le^b AND Y GLYCOLIPIDS IN THE GASTRIC CARCINOMA CELL LINE KATO III AS ANALYZED BY A NOVEL ASSAY[1]

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ABSTRACT. The biosynthetic pathways for the difucosylated type 1 and 2 glycolipids, Le^b and Y, respectively, were investigated in the gastric carcinoma cell line KATO III, using a novel chromatogram binding assay. The type of fucosylation obtained was deduced from the binding pattern of monoclonal antibodies specific for the biosynthesized glycolipid products using microsomal fractions as the source of enzyme, pure glycolipids and non-radioactive GDP-fucose as acceptor and donor substrates, respectively.

The Le^b glycolipid (Fucα1→2Galβ1→3GlcNAc(4←1αFuc)β1→3LacCer) was synthesized mainly via the blood group H, type 1, precursor (Fucα1→2Galβ1→3GlcNAcβ1→3LacCer). However, the Le^a glycolipid (Galβ1→3GlcNAc(4←1αFuc)β1→3LacCer) also served as a precursor for the α1→2 fucosyltransferase, thus allowing conversion of Le^a to Le^b. This biosynthetic route represents either an "aberrant" specificity of the Fuc α1→2 transferase associated with these gastric carcinoma cells and/or a new member of the α1→2 fucosyltransferase family.

The Y glycolipid (Fucα1→2Galβ1→4GlcNAc(3←1αFuc)β1→3LacCer) was synthesized exclusively via the classical pathway using the blood group H type 2 glycolipid (Fucα1→2Galβ1→4GlcNAcβ1→3LacCer) as precursor. The X glycolipid (Galβ1→4GlcNAc(3←1αFuc)β1→3LacCer) did not serve as an acceptor substrate for the α1→2 fucosyltransferase(s) present.

The use of non-radioactive sugar-nucleotides as donor substrate, defined glycolipid precursors as acceptor substrates and of specific monoclonal anti-glycolipid antibodies for detection provides a rapid and highly specific assay for analyzing biosynthetic pathways of glycosyltransferases.

Le^b and Y antigens are difucosylated glycolipids which accumulate in gastrointestinal tumors (1-6). Biosynthesis of these glycolipids requires co-expression of several fucosyltransferases. At least four different fucosyltransferases have been implicated, namely the Lewis-transferase (or

Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; TLC, thin-layer chromatography.

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Fuc α1→4 transferase), the Fuc α1→3 transferase, and the blood group H- and secretor-transferases (or Fuc α1→2 transferases).

The Lewis-transferase in secretory organs is expressed in individuals who express the Lewis (Le) gene (7) and the secretor-transferase is present in individuals who express the secretor (Se) gene (7-9). Thus, expression of Le^b would not be expected in Lewis-negative, non-secretor individuals. However, not all regions of an organ in individuals who express the Le and Se genes necessarily express Le^b and Y antigens. For example, both Le^b and Y antigens are absent from the distal part of human colon in Le- and Se-positive individuals. Tumors that arise in the lower third of the colon in these patients, however, do express the antigens (3-6, 10). Those findings imply a tumor-associated expression of an Fuc α1→2 transferase(s) of either blood group H, secretor or a novel type.

The possible existence of two distinct Fucα1→2 transferases was first suggested based on the stereo-chemical differences that exist between the two precursor structures of type 1 (Galβ1→3GlcNAc) and type 2 (Galβ1→4GlcNAc) (11). Comparison of computer-generated hard-sphere molecular models (HSEA) of H type 1 and 2 chains demonstrated that the acetamido-group of the penultimate N-acetylglucosamine in the type 1 structure restricts access to the 2-hydroxyl group, whereas in the type 2 structure, this position is occupied by a smaller hydroxymethyl group. Thus a stereo-chemical basis exists that explains how a type 2-specific Fucα1→2 transferase, encoded by the H gene, is prevented from using the type 1 precursor.

Recently it was suggested that Fucα1→2 transferases are encoded by both blood group H and Se genes and that the two genes are differentially expressed in tissues of mesodermal and epithelial origin, respectively (9). In this model, the secretor-transferase is able to utilize both type 1 and 2 precursors, while the blood group H gene-encoded transferase might be restricted to type 2 substrates (12). Based on this model, we originally proposed that transformation of colon epithelial cells induces the Se gene-encoded transferase (6, 13), since colon epithelial cells express both Le^b and Y antigens. This report describes our efforts to identify the specificity of Fuc α1→2 transferase in the biosynthesis of difucosylated type 1 and 2 Le^b and Y glycolipids, respectively. We therefore investigated the biosynthetic pathways in a well-characterized gastric carcinoma cell line Kato III, which produces both Le^b- and Y-glycolipids (13).

METHODS

Fucosyltransferase assay. Kato III cells were harvested and homogenized in 0.25 M sucrose in a Potter-Elvehjelm homogenizer and centrifuged at 10,000 X g for 10 min at 4°C. The pellet was re-homogenized once and

centrifuged in the same manner. The two supernatants were pooled and centrifuged at 100,000 X g for 45 min at 4°C and the pellet thus obtained was dissolved in 50 mM cacodylate buffer at pH 6.0, containing 0.5% NP-40.

100 µg of microsomal fraction protein, as determined by the Lowry method (14), was incubated in a final volume of 100 µl of 50 mM cacodylate buffer, pH 6.5 containing 10 mM $MnCl_2$, 10 µg of glycolipid acceptor, 10 µg 5' guanosine-diphospho-fucose, and 0.5% NP-40. The reaction mixture was incubated for 24 hr at 37°C and terminated by the addition of an equal volume of methanol and then diluted to a total volume of 5 ml with methanol/water (1:1, v/v). The biosynthetic glycolipid products were adsorbed on reverse-phase Sep-Pak C_{18} cartridges (Waters/Millipore, Milford, MA 01757), pre-washed with 10 ml each of chloroform/methanol (2:1, v/v), methanol, methanol/water (1:1, v/v), and were subsequently eluted with 5 ml each of methanol and chloroform/methanol (2:1, v/v). The combined eluted fractions were evaporated to dryness under N_2, resuspended in 25 µl of chloroform/methanol (2:1, v/v), and 2 µl/lane analyzed in the thin-layer chromatogram binding assay.

GDP-fucose. Non-radioactive 5'-GDP fucose was synthesized as described (15).

Glycolipids. Table 1 gives the structures of purified glycolipids. Total glycolipid fractions of different origins were prepared by organic solvent extraction, mild alkaline degradation, dialysis, DEAE-Sepharose and silicic acid chromatography. Pure glycolipids were characterized by mass spectrometry and proton-NMR, all essentially as described (13, 16, 17). nLe4, X-5, H-5-2, and Y were prepared and characterized in our lab. Lc4, Le^a, H-5-1 and Le^b glycolipids were a kind gift by K-A. Karlsson, University of Göteborg, Göteborg, Sweden.

Monoclonal antibodies. The monoclonal antibodies used were CO 431 (anti-Le^b) (18), CO 514 (anti-Le^a) (18), $D_1$56-45 (anti-X) (19) and BR 55-2 (anti-Y + blood group B difucosyl type 2) (13). All monoclonal antibodies were used as serum-free hybridoma culture supernatant diluted 1:4 with 2% BSA in PBS at pH 7.3.

Chromatogram binding assay. Thin-layer chromatograms were immunostained according to the method modified as previously described (19). Briefly, glycolipids were separated on alumina-backed high-performance TLC plates (Merck-Bodman Chemicals, Gibbstown, NJ) with chloroform/methanol/water (60:35:8; v/v/v). The chromatograms were soaked for 1 min in

Table 1. Structures of Glycolipids Used in This Study

Type 1 chain	
Lc4	Galβ1→3GlcNAcβ1→3LacCer
H-5-1	Fucα1→2Galβ1→3GlcNAcβ1→3LacCer
Le^a	Galβ1→3GlcNAc(4←1αFuc)β1→3LacCer
Le^b	Fucα1→2Galβ1→3GlcNAc(4←1αFuc)β1→3LacCer
Type 2 chain	
nLc4	Galβ1→4GlcNAcβ1→3LacCer
H-5-2	Fucα1→2Galβ1→4GlcNAcβ1→3LacCer
X	Galβ1→4GlcNAc(3←1αFuc)β1→3LacCer
Y	Fucα1→2Galβ1→4GlcNAc(3←1αFuc)β1→3LacCer

diethyl-ether containing 0.5% polyisobutylmethacrylate (Plexigum P28, Röhm GmbH, Darmstadt, West Germany) and subsequently blocked for 2 hr in PBS solution, pH 7.3, containing 2% BSA and 0.1% NaN_3. The plates were then overlaid with monoclonal antibody solution and incubated for 2 hr at room temperature, followed by incubation with ^{125}I-iodinated goat anti-mouse $F(ab')_2$ specific antibody for 2 hr at room temperature. Binding of monoclonal antibody was detected by autoradiography.

RESULTS

To determine the glycolipid acceptor substrate specificity of fucosyltransferases in a gastrointestinal tumor cell line, we established a novel immunodetection system. Unlike all earlier described assays for glycosyltranferases, the present system employs non-radioactive 5'-GDP-fucose as a donor substrate together with monoclonal antibodies of defined specificity to detect reaction products. The assay is especially useful when assaying glycosyltransferase specificities in crude enzyme preparations, since several glycosyltransferases that utilize the same sugar nucleotide and endogenous precursors for the enzyme are present.

Fig. 1 illustrates the biosynthetic products obtained using Lc4, nLc4, H-5-1, H-5-2, Le^a and X glycolipids as acceptor substrates and the microsomal fraction from KATO III cells as enzyme source. Binding of the Le^a-specific monoclonal antibody CO 514, as indicated by the strong band in Fig. 1A, lane 1, demonstrate the expected transfer of α-L-fucose in an α1→4 linkage by the Le gene-encoded enzyme to the penultimate glucosamine of Lc4. Lanes 2 and 6, both panels of Fig. 1A, represent the control samples

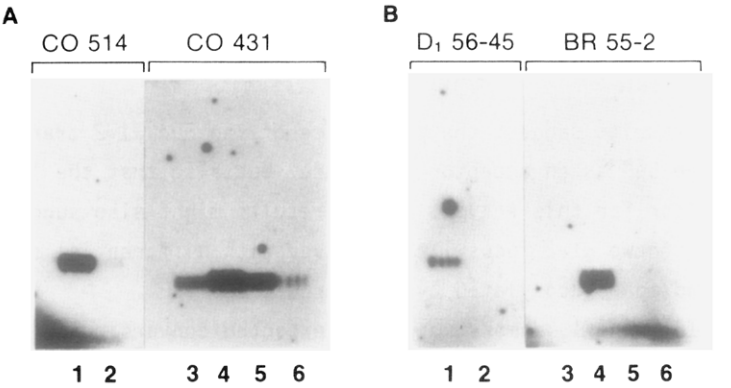

Figure 1. Autoradiograms obtained by the chromatogram binding assay performed on fractions from the fucosyltransferase assay. All assayed fractions contain microsomal protein and 5'-GDP-fucose with various acceptor substrate glycolipids or no acceptor substrate glycolipid as control. (A) Binding of type 1 chain-specific monoclonal antibodies CO 514 (anti-Le^a) and CO 431 (anti-Le^b). The acceptor substrate glycolipids are: lane 1, Lc4; lane 2, no acceptor substrate; lane 3, Lc4; lane 4, H-5-1; lane 5, Le^a; and lane 6, no acceptor substrate. (B) Binding of type 2 chain-specific monoclonal antibodies D_1 56-45 (anti-X) and BR 55-2 [anti-Y (B-7-2)]. The acceptor substrate glycolipids are: lane 1, nLc4; lane 2, no acceptor substrate; lane 3, nLc4; lane 4, H-5-2; lane 5, X; and lane 6, no acceptor substrate.

in which no external acceptors were added and the immunostained bands therefore represents the level of endogenous Le^a and Le^b antigens, respectively.

The biosynthetic pathway for Le^b glycolipid was tested with lactotetraosylceramide (Lc4), and α1→2 and α1→4 monofucosylated derivatives as acceptor substrates (H-5-1 and Le^a, respectively). When Lc4 was used as an acceptor (Fig. 1A, lane 3), Le^b antigen was formed as a result of the combined action of both α1→2 and α1→4 fucosyltransferases, as indicated by binding of the Le^b-specific monoclonal antibody CO 431. The results at this point do not reveal the order of attachment of the L-fucose residues in α1→2 and α1→4 linkages, since Lc4 is utilized as an acceptor by both enzymes.

The data presented in Fig. 1A, lane 4, demonstrating the strongest binding when H-5-1 is used as substrate, indicate that the Le^b glycolipid is formed mainly via H-5-1 glycolipid by the action of the Lewis-transferase (Fuc α1→4 transferase). Lane 5 in Fig. 1A shows the relative level of Le^b antigen formed when Le^a was added as an acceptor substrate and indicates that Le^a as well as Lc4 also serves as an acceptor for Fuc α1→2 fucosyltransferase in KATO III cells. Although the results obtained by the chromatogram binding assay only can be interpreted semi-quantitatively, the data suggest an increase in binding to the fraction in which Le^a was used as acceptor substrate, as compared with the control in lane 6 containing no exogenous acceptor substrate and in lane 4 where H-5-1 was used as acceptor substrate. The possibility that cross-reactivity of monoclonal antibody CO 431 (anti-Le^b) with Le^a glycolipid accounts for this finding was ruled out earlier (18).

These results suggest the preference of the Fuc α1→2 transferase for Lc4 over the Le^a as an acceptor structure, but also that the latter serves as an acceptor for this enzyme. These results might also suggest the expression of two α1→2 fucosyltransferases with differential specificity for non- and mono-fucosylated type 1 structures.

Fig. 1B, lane 1, demonstrates the expected conversion of nLc4 to X-glycolipid by the addition of an α1→3 fucose to the penultimate glucosamine as detected by monoclonal antibody D_1 56-45, specific for X-glycolipid. Lanes 2 and 6, Fig. 1B, respectively, represent the control samples where only GDP-fucose but no external acceptors were added.

The biosynthetic pathway for the Y-glycolipid was similarly tested. Lactoneotetraosylceramide (nLc4), α1→2 and α1→3 monofucosylated derivatives of nLc4, i.e., the H-5-2 and X glycolipids, respectively, were used as acceptor substrates. The difucosylated Y-glycolipid is only formed via H-5-2 (Fig. 1B, lane 4) and neither nLc4 or X-glycolipid are acceptor sub-

strates for any Fuc α1→2 transferase(s) present in the microsomal fraction obtained from the Kato III cell line (Fig. 1B, lanes 3 and 5, respectively).

DISCUSSION

The present results show that the Le^b determinant in KATO III cells can be biosynthesized from blood group H type 1 or Le^a determinants, since the Fuc α1→2 transferase(s) can use not only lactotetraosylceramide (Lc4) but also Le^a glycolipid as acceptor substrates (Fig. 2). However, the specificity of the Fuc α1→2 transferase(s) with respect to type 2 precursors is different. Formation of H-5-2 was not determined directly, since H-5-2 specific monoclonal antibody was not included in this assay. It is interesting that nLe4 does not produce the Y glycolipid (Fig. 1B, lane 4), which probably means that the nLe4 to X biosynthesis is much more efficient than the nLc4 to H-5-2 biosynthesis. H-5-2 glycolipid represents the only monofucosylated acceptor substrate because the X-glycolipids do not serve as an acceptor substrate for any fucosyltransferase present in the microsomal fraction of Kato III cells.

Earlier proposed pathways for biosynthesis of difucosylated carbohydrate structures of type 1 and 2, i.e., Le^b and Y, respectively, assumed the formation of the blood group H type 1 and 2 intermediates, respectively, prior to an α1→3/α1→4 fucosylation (20). The α1→4 and α1→3 monofucosylated glycolipids, i.e., Le^a and X, respectively, would therefore not be acceptors for α1→2 fucosyltransferase. However, it was reported that a gastric mucosa microsomal fraction from one individual was able to convert not only the precursor structure isolated from plasma of an Le^{a-b-} individual into Le^b antigen, but also that Le^b was formed from the Le^a glycolipid (21). Our demonstration that Le^a to Le^b conversion also exists in a human gastric carcinoma cell line is consistent with this finding.

Based on these results, it is difficult to evaluate whether the α1→2

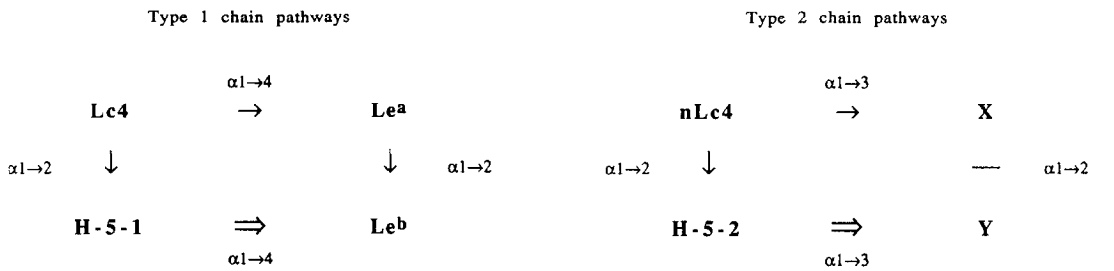

Figure 2. Schematic representation of biosynthetic pathways established for Type 1 and 2 chain glycolipids from Kato III cells. The fucosyltransferase activities are indicated by their respective linkage description.

fucosyltransferase activity in KATO III cells represents an "aberrant" tumor-associated activity or, instead, the normal Fuc α1→2 transferase associated with gastric mucosa but with new substrate specificity. Neither the normal gastric mucosa enzyme, nor the Lewis or secretor status of the patient, from which KATO III cells originated, are available. It was recently shown that the Le^b antigen in the colon adenocarcinoma cell line SW 1116 is formed exclusively via the traditional route and that Le^a does not serve as an acceptor for α1→2 fucosyltransferase (22). Thus, it seems reasonable to assume that the gastric Fuc α1→2 transferase is less specific than that normally present in colon epithelium or colon adenocarcinoma tissue. Likewise, the enzyme expressed in Kato III cells, which may also be present in normal gastric mucosa, is probably "aberrant", at least with respect to the general model for Fuc α1→2 transferases, and thus represents a new species in the Fuc α1→2 transferase family.

The existence of different forms of glycosyltransferases with restricted tissue localization raises the question of whether tumor-associated enzymes are normal transferases of either blood group H or secretor type, novel tumor-associated molecular species. It has been suggested that tumor-associated glycosyltransferases might express a less restricted specificity than transferases from normal tissues (23), analogous to glycosyltransferases with multiple specificity. For example, human milk α1→4 Lewis fucosyltransferase uses Galβ1→3GlcNac, Galα1→4GlcNAc and Galβ1→4Glc structures as acceptors (24), while lactose synthetase displays dual specificity in forming Galβ1→3GlcNAc and in the presence of α-lactalbumin Galβ1→4GlcNAc linkages (25). Also a sialyltransferase, which might express specificity analogous with that of α1→2 fucosyltransferase and which acts on a Le^a precursor saccharide was recently demonstrated in human seminal plasma (26).

Studies of α1→2 fucosyltransferases in non-cancerous tissues, i.e., in submaxillary glands (27-29), gastric mucosa and kidney (28), milk (30) and serum (31), suggest the involvement of more than one enzyme in determining non-cancerous Fuc α1→2 transferase activity. In a recent comparison of two variant forms of α1→2 fucosyltransferase from submaxillary glands and gastric mucosa in secretor and nonsecretor individuals (28, 29), the most striking differences were reflected in the substrate specificities of the transferases. The transferase from tissue in which the Se gene was expressed had a preference for type 1 chain acceptors, whereas the transferase in tissue from individuals homozygous for Se, i.e., Sese, preferred the type 2 chain acceptors. Comparison of substrate specificity of the α1→2 fucosyltransferase from human milk and plasma demonstrated that the

milk enzyme had much higher affinity for type 1 chain acceptors than type 2, whereas the enzyme from human plasma did not show this distinction (30). It was further shown that the type 2 antigens, independent of the secretor status and independent of H gene expression, are present in deep areas of the gastrointestinal mucosa (32), and vascular endothelium and digestive secretions (33), respectively. Consistent with these results is the detection of Le^b and Y expression in tumors of "nonsecretor" individuals (3), i.e., individuals who do not carry the Se gene. Thus the expressed enzyme might represent the product of another, perhaps tumor-associated, gene.

Together, these data suggest the existence of structural or regulatory genes other than H and Se for α1→2 fucosyltransferases and, as suggested for α1→3 fucosyltransferases (34, 35), the existence of a gene family encoding α1→2 fucosyltransferases.

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