Glycolipid Antigen Expression in Human Lung Cancer

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ABSTRACT

Several mouse monoclonal antibodies which recognize carbohydrate sequences distinguish between different types of human lung cancer immunohistologically. These antibodies bind to glycolipid antigens produced by the cancer cells. When these glycolipids are separated by thin-layer chromatography, immunostaining of the chromatograms yields complex patterns of antigen-positive bands. To determine whether glycolipid patterns are useful in the classification of lung cancer, 16 human lung cancer cell lines comprising the major histological types of primary lung cancer were studied. Neutral glycolipids and gangliosides were isolated and separated by thin-layer chromatography. Six anti-carbohydrate antibodies which recognize structurally related antigens were used for immunostaining. Neuraminidase treatment of the chromatograms was used to detect "cryptic" sialylated antigens. All the cell lines were unique with regard to the type, amount, and chromatography pattern of the glycolipid antigens produced. Small cell lung cancer cell lines synthesized the greatest variety of antigens, whereas cell lines with large cell cytology synthesized the least. Interestingly, there was an inverse relationship between expression of some glycolipid antigens and DNA amplification may influence the types of glycolipids expressed at the surface of lung tumor cells.

INTRODUCTION

Cell surface carbohydrates change during normal development and malignant transformation (2, 3), allowing some carbohydrate-specific antibodies to discriminate among various tumors and normal tissues (3-7). Many antigens recognized by these antibodies are in both glycoproteins and glycolipids (8). When glycolipids are separated by thin-layer chromatography these antibodies detect complex patterns of antigen-positive bands (4, 5). Several carbohydrate-specific monoclonal antibodies are able to recognize different human lung cancers (9-11). In the present study, six antibodies that detect structurally related carbohydrate sequences were used to examine the glycolipid expression of human lung carcinoma cell lines.

MATERIALS AND METHODS

Human Cell Lines. The cell lines (Table 1) were grown in RPMI 1640 medium or Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum (Gibco, Grand Island, NY). Classic SCLC* cultures and SCLC-v cell lines differ in histology, growth characteristics, radioisotopy, and levels of l-dopa decarboxylase (9, 12).

ABSTRACT

The antibodies and their carbohydrate specificities are described in Table 2. Antibodies 101 and 102 were gifts from I. Pastan, NIH. Antibody AK3-136 was a gift from D. Baker, Chembiomed, Ltd. Its specificity was determined by hemagglutination using RBC of the appropriate P phenotypes and by thin-layer chromatography immunostaining using glycolipids isolated from these RBC. The specificity of antibody 604A9 for the Y hapten was determined by radioimmunoassay and thin-layer chromatography immunostaining using purified standard glycolipids. The results obtained with both purified glycolipids and with those isolated from the lung tumor cell lines were identical to those found with antibody 101. Antibody 101 was previously shown to specifically bind the Y hapten (17).

Glycolipids. Glycolipid structures are presented in Table 3. Paragloboside, α-2-3sialylparagloboside, and blood group A active glycolipids were isolated from human RBC as previously described (18, 19). Lacto-N-fucopentaosyl III ceramide was a gift from D. Zopf, NIH, and α-2-3sialyllacto-N-fucopentaosyl III ceramide was a gift from H. Rauvala, Helsinki, Finland.

Glycolipids were isolated from the human cell lines as follows. Cell pellets (100 mg-1 g) were washed once with phosphate buffered saline, frozen on dry ice, and stored at -70°C. The lipids were extracted using chloroform, methanol, and water (20). The total lipid extract (1 ml extract from 200 mg of cells) was desalted by passage through 1-ml columns of Sephadex G-25 Fine (Pharmacia, Inc., Piscataway, NJ) previously equilibrated with chloroform:methanol:water, 60:30:4.5 (21). The neutral and acidic glycolipids were then separated by ion-exchange chromatography on 1-ml columns of DEAE-Sephadex (Sigma Chemical Co., St. Louis, MO) previously converted to acetate form (22). Phospholipids were removed by evaporating 1 volume of lipid extract and adding 1 volume of 0.2 M NaOH in methanol. Following incubation at 37°C for 90 min, 1 volume of 0.2 M HCl in methanol was added and the mixture was evaporated. The residue was redissolved in 1 volume of chloroform:methanol:water, 60:30:4.5 and desalted on G-25 Sephadex, as above.

Immunostaining. The method was described previously (5, 18). All incubations were done at room temperature. Glycolipids were chromatographed on aluminum-backed, Silica Gel 60, high-performance thin-layer chromatography plates (Merck, Darmstadt, West Germany) in chloroform:methanol:0.25% KCl 5:4:1. After drying, the plates were soaked in 0.1% poly(isobutylmethacrylate) (Polysciences, Inc., Warrington, PA) in hexane for 90 s. After drying, the plates were sprayed briefly with Buffer A (0.1 M NaH2PO4/Na2HPO4:0.15 M NaCl, pH 7.4) and then incubated in Buffer B (0.05 M Tris:0.1 M NaCl, pH 7.8) with 1% bovine serum albumin (Pentex Chemical Co., Naperville, IL) and 0.1% sodium azide for 45 min to decrease nonspecific binding of antibody. The plates were overlaid with a dilution of monoclonal antibody in Buffer B, 60 µl/cm2. The following concentrations or dilutions of antibodies were used: 534F8, 2 µg/ml; anti-MY28, a:1:100 dilution of ascites fluid; 102, a:1:30 dilution of culture supernatant; 102, a:1:100 dilution of ascites fluid. After drying, the plates were washed five times in Buffer A. They were then overlaid with 2 x 106 cpm/ml of affinity-purified goat anti-mouse or anti-human IgM (Kirkegaard and Perry, Gaithersburg, MD) labeled with 125I (ICN, Irvine, CA) to a specific activity of 50 µCi/µg using the Iodo-Gen (Pierce, Rockford, IL) method (23). Following a 1-h incubation, the plates were washed five times in Buffer A, dried under a heat lamp, and exposed in XAR-5 X-ray film (Eastman Kodak, Rochester, NY) for 16 h.

In some experiments the glycolipids were treated with Clostridium perfringens neuraminidase (Type VI; Sigma) by direct treatment of the
RESULTS

Neutral glycolipids isolated from the lung cancer cell lines were chromatographed and visualized with orcinol. The results with six cell lines are shown in Fig. 1. All the cell lines had similar patterns of the major glycolipids with minor differences.
Fig. 1. Major neutral glycolipids of lung cancer. Neutral glycolipids were isolated from the lung cancer cell lines as described in “Materials and Methods.” Neutral glycolipids from 2 mg wet weight of tissue culture cells were separated by high-performance thin-layer chromatography as described and visualized with 0.1% orcinol (11). Lane 1, NCI-H390; lane 2, NCI-H128; lane 3, NCI-H209; lane 4, NCI-H82; lane 5, NCI-N417; lane 6, NCI-H226. The glycolipid standards were ceramide monohexoside (CMH), Glc\(\alpha\)1-1ceramide; ceramide dihexoside (CDH), Gal\(\beta\)1-4Glc\(\alpha\)1-1ceramide; ceramide trihexoside (CTH), GaINAc\(\alpha\)1-3Gal\(\beta\)1-4Glc\(\alpha\)1-1ceramide; GM3, NeuNAc\(\alpha\)2-3Gal\(\beta\)1-4Glc\(\alpha\)1-1ceramide; GD3, NeuNAc\(\alpha\)2-8NeuNAc\(\alpha\)2-3Gal\(\beta\)1-4Glc\(\alpha\)1-1ceramide; GD1b, Gal\(\beta\)1-3GaINAc\(\alpha\)1-4[NeuNAc\(\alpha\)2-8NeuNAc\(\alpha\)2-3]Gal\(\beta\)1-1ceramide; ORI, origin.

Fig. 2. Type 2 chain-containing glycolipids of lung cancer detected by immunostaining. Glycolipid isolation, thin-layer chromatography, immunostaining, and autoradiography were performed as described in “Materials and Methods.” Neutral glycolipids were used in A–D; gangliosides were used in E. Monoclonal antibody 534F8 which detects Gal\(\beta\)1-4[Fuc\(\alpha\)1-3]GlCNAc\(\alpha\)R sequences was used for A; 102 which detects Fuc\(\alpha\)1-2Gal\(\beta\)1-4GlCNAc\(\alpha\)R was used in B; 604A9 which detects Fuc\(\alpha\)1-2Gal\(\beta\)1-4[Gal\(\beta\)1-3]GlCNAc\(\alpha\)R was used in C; anti-My-28 which detects Gal\(\beta\)1-4GlCNAc\(\alpha\)R was used in D; and the combination of neuraminidase and anti-My-28 which detects NeuNAcGal\(\beta\)1-4GlCNAc\(\alpha\)R was used in E. Glycolipids from 2 mg wet weight of tissue culture cells were used in lanes 1–6; glycolipids from 6 mg wet weight of cells were used in lanes 7–16. The cell lines are in the order described in Table 1. Lane 1, NCI-H390; lane 2, NCI-H128; lane 3, NCI-H209; lane 4, NCI-H82; lane 5, NCI-H146; lane 6, NCI-H187; lane 7, NCI-H69; lane 8, NCI-H82; lane 9, NCI-H60; lane 10, NCI-N417; lane 11, A549; lane 12, NCI-H128; lane 13, NCI-H12; lane 14, A427; lane 15, NCI-H226; and lane 16, NCI-H226. Structures for the ganglioside standards GM3, GD3, and GD1b are as in the legend to Fig. 1. The structures of paragloboside (PG) and \(a\)-2,3sialylparagloboside (SPG) are as in Table 3. ORI, origin.

There was no obvious distinction between SCLC and non-SCLC lines. In particular, NCI-H209, a SCLC line, and NCI-H82, a SCLC-v line had identical patterns (Fig. 1, lanes 1 and 3). In contrast, glycolipid immunostaining with carbohydrate-specific antibodies gave different results. The most striking differences were found with the SCLC and SCLC-v cell lines. For instance, using antibody 534F8 (Fig. 2A; Table 1), all six SCLC lines were strongly antigen positive, while NCI-H69 (Fig. 2A, lane 6) had a less complex pattern. NCI-H146 had a fast-migrating triplet band (Fig. 2A, lane 4), while NCI-H209 (Fig. 2A, lane 3) had a fast-migrating doublet band.

Similar results were obtained using antibodies 102 and 604A9 particularly with regard to the distinction between SCLC and non-SCLC cell lines (Fig. 2, B and C). Two mesothelioma cell lines were tested with contrasting results. NCI-H28 synthesized 3-fucosyllactosamine containing glycolipids (Fig. 2A, lane 15) but not H or Y containing glycolipids (Fig. 2A, lanes 2, 3, and 16). Interestingly, many of these precursor structures were in at least small amounts. Most cell lines produced only paragloboside (Table 3); however, several cell lines displayed fairly complex patterns of antigen-positive glycolipids (Fig. 2D, lanes 12, 13, and 16).
the small cell carcinoma lines which synthesize large, complex, fucosylated glycolipids only had paragloboside as an unsubstituted structure. Apparently, higher structures in these cells are almost completely substituted (Fig. 2A, B, C, and D, lanes 5). Only one cell line, an SCLC-v line, was antigen negative using anti-My-28 (Fig. 2D, lane 9).

Several oligosaccharide chains are substituted with sialic acid. Using neuraminidase to cleave covalently bound sialic acid, “cryptic” antigens can be detected with monoclonal antibodies (18). The combination of neuraminidase and anti-My-28 made it possible to detect glycolipids containing the sequence NeuNAcα2-3(6)Galβ1-4GlcNAc-R (Fig. 2E). Some cell lines were positive and displayed either complex (Fig. 2E, lanes 1–3 and 13) or simple (Fig. 2E, lanes 5 and 12) glycolipid patterns. Glycolipids containing sialylated 3-fucosyllactosamine sequences, NeuNAcα2-3(6)Galβ1-4[Fucα1-3]GlcNAc-R, were detected using neuraminidase followed by antibody 534F8 (Fig. 3B). Only two cell lines, both small cell carcinomas (NCI-N390 and NCI-H69), contained this antigen (Fig. 3, lanes 7 and 8). NCI-H69 also had both sets of bands that stained before and after neuraminidase treatment (Fig. 3, lanes 4 and 8). These bands may be branched glycolipids with one branch having a 3-fucosyllactosamine sequence and the other branch containing sialic acid. The combination of neuraminidase and antibodies 604A9 or 102 did not reveal any sialylated H type 2 or Y antigens.

The A and P1 structures are both genetically determined blood group antigens. Using antibody 33/25, none of the cell lines had A-active glycolipids (data not shown). Only one cell line, NCI-H226, had the P1 glycolipid (Table 3) when tested with antibody AK3-136 (data not shown).

**DISCUSSION**

Glycolipids are synthesized by the concerted action of various glycosyltransferases. These enzymes add single glycosyl residues to growing chains and the presence or absence of these enzymes determines which structures are produced. Many different disaccharide sequences occur in glycolipids (24), most of which are synthesized by separate glycosyltransferases. The expression of these enzymes varies during development and accounts for the characteristic carbohydrate structures of different tissues and for the developmental appearance of carbohydrate antigens (2, 3). The expression of glycosyltransferases also changes upon malignant transformation and leads to the appearance of glycolipid antigens recognized by monoclonal antibodies (2, 3).

Several previous studies have examined the major glycolipids found in human lung and lung carcinoma tissue (25–27). The glycolipids were detected by chemical staining. Although these studies demonstrated some differences between histological types of lung cancer, there was some question as to the relative glycolipid contribution of granulocytes containing the tissue (25). The present study uses human lung cancer cell lines which are free of granulocytes. Although individual cell lines had slightly different patterns of major glycolipids, no patterns characteristic of each histological type were found (Fig. 1); in fact, one small cell lung cancer cell line and one small cell lung cancer variant cell line had identical patterns (Fig. 1, lanes 3 and 4).

The immunostaining method using antiacarbohydrate antibodies detects nanogram amounts of particular glycolipids (6) and is, thus, both more sensitive and more specific than chemical staining. Six different antiacarbohydrate antibodies (Table 2) were used to detect the presence of eight structurally related glycolipid antigens (Table 3). A striking finding in our study was the marked heterogeneity of the different cell lines with regard to glycolipid antigen expression. This heterogeneity was demonstrated both in the types of antigens synthesized by individual cell lines and in the range of glycolipid patterns found for each antigen. The heterogeneity of glycolipid antigen synthesis confirms and extends previous findings (9). Intratumor heterogeneity of glycolipid expression is also probable but cannot be detected by this bulk method of glycolipid isolation. In general SCLC lines exhibited the greatest antigen diversity whereas cell lines with large cell cytology (small cell lung cancer variant and large cell lung cancer) demonstrated the least. For example, the NCI-H390 SCLC line synthesized six of the eight antigens tested (Fig. 2, lanes 9); however, even within the SCLC group there were differences in antigen expression, particularly with regard to the sialylated antigens. Only 4/6 and 2/6 SCLC lines had sialosylparagloboside (Figure 2A, lanes 1–6) and sialosylacto-N-fucosepentosyl III ceramide (Fig. 3, respectively).

The glycolipid patterns for individual antigens were also heterogeneous. The antigenic determinants recognized by the antiacarbohydrate antibodies in this study are at the nonreducing terminus and is, thus, both more sensitive and more specific than chemical staining. Six different antiacarbohydrate antibodies (Table 2) were used to detect the presence of eight structurally related glycolipid antigens (Table 3). A striking finding in our study was the marked heterogeneity of the different cell lines with regard to the sialylated antigens. Only 4/6 and 2/6 SCLC lines had sialosylparagloboside (Figure 2A, lanes 1–6) and sialosylacto-N-fucosepentosyl III ceramide (Fig. 3, respectively).

The A and P1 antigens are genetically determined blood group antigens on the RBC of 45 and 75% of individuals, respectively (28). Blood group A-active glycolipids have been isolated from normal human lung tissue (27). The antigen was also found on a lung carcinoma cell line using a monoclonal antibody (29). In our study, A glycolipids were not synthesized by any of the 16 lung carcinoma cell lines and P1 glycolipid was found in only one pleural mesothelioma cell line, NCI-H226.

With regard to the synthesis of these structurally related glycolipid antigens and the glycolipid patterns of individual antigens, each lung carcinoma cell line was unique; however, there was an interesting correlation between the synthesis of some of these glycolipid antigens and the amplification and expression of the c-myc oncogene. This was particularly evident
with regard to the fucosylated structures: 3-fucosyllactosamine, H Type 2, and Y (see Table 2). The degree of c-myc amplification had been determined previously for 14 of the cell lines described in Table 1 (13). In 13 cases, the present study found an inverse relationship between c-myc amplification and fucosylated glycolipid expression (see Table 1). The most striking contrast was between classic SCLC and SCLC-v (Figure 2, A–C, lanes 1–9). This relationship may be tissue specific since the human promyelocytic leukemia cell line HL-60 has amplified c-myc (13) and also produces significant amounts of these fucosylated glycolipid antigens (5, 8). In addition to amplification of c-myc, the SCLC-v cell lines differ from classic SCLC lines in histology, growth characteristics in vitro, radioresistance, amine precursor uptake and decarboxylation properties, clinical course, and response to therapy (10, 12, 30–33). This suggests that amplification of c-myc may influence both the expression of cell surface glycolipid antigens (9) and the behavior of human lung tumors in vivo and in vitro. Since changes in the glycolipids of cell membranes directly affects cell growth and response to growth factors (2, 34), it is possible that increased c-myc expression in human lung cancer regulates cell membrane glycolipids which then affect growth factor binding and cell growth. This possibility is being investigated.

ACKNOWLEDGMENTS

The authors would like to thank S. Stephenson, A. Gazdar, J. Fedorko, S. Rosen, and M. Nau for helpful contributions to this work, and J. Maltagliati and Val Remeter for expert secretarial assistance.

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