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Globotriaosyl Ceramide and Globoside as Major Glycolipid Components of Fibroblasts in Scirrhous Gastric Carcinoma Tissues

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Scirrhous gastric cancer is characteristic in that cancer cells proliferate and invade with prominent fibrosis. To search for the expression of specific carbohydrate chains in scirrhous gastric cancer, we have examined the glycosphingolipid composition of scirrhous cancer tissues (*n*=10) in comparison with that of non-scirrhous cancer tissues (*n*=10) by means of two-dimensional thin layer chromatography, followed by fast atom bombardment mass spectrometry of the individual glycolipids and immunostaining analysis. The major neutral glycosphingolipids from scirrhous gastric cancer tissues were identified as ceramide monohexoside, ceramide dihexoside, globotriaosyl ceramide (Gb_3) and globoside (Gb_4), while the major acidic glycosphingolipids were II^3 NeuAcα-LacCer, II^3 NeuAcα_2-LacCer and sulfatide. Relative concentrations of Gb_3 and Gb_4 in scirrhous gastric cancer tissues (Gb_3+ Gb_4 =58%) were two times higher than those in non-scirrhous gastric cancer tissues (29%). Orthotopic fibroblasts cloned from scirrhous gastric cancer tissues showed similar high concentrations of Gb_3 and Gb_4 to scirrhous gastric cancer tissues. Furthermore, immunohistochemical study revealed that Gb_3 and Gb_4 were expressed intensely on the fibroblasts. On the other hand, analysis of glycosphingolipids in four scirrhous gastric cancer cell lines yielded the following results. i) The contents of Gb_3 and Gb_4 were low (6%), compared with orthotopic fibroblasts (62%). ii) Significant amounts of Le^a (pentaglycosylceramide) and Le^b (hexa- and heptaglycosylceramides), which could not be detected in scirrhous cancer tissues, were observed. The results show that the major neutral glycosphingolipids such as Gb_3 and Gb_4 of scirrhous gastric cancer tissues were derived from orthotopic fibroblasts and not from the cancer cells.

Key words: Glycosphingolipid — Scirrhous gastric cancer — Fibroblast — Globotriaosyl ceramide — Globoside

Scirrhous gastric cancer has a characteristic histological progression with prominent proliferation of interstitial tissues, and the diagnosis is so difficult at early stages that the prognosis is poor, compared with that of non-scirrhous-type gastric cancer.[3)] One of reason for the difficulty of diagnosis at early stages is the small change of the mucosal lesion. When scirrhous gastric cancer cells invade the submucosa, the cancer cells proliferate diffusely with prominent fibrosis. The mechanism responsible for such characteristic biological behavior is not well understood. Recently, the existence of a specific interaction between scirrhous gastric cancer cells and orthotopic fibroblasts has been reported. i) Gastric orthotopic fibroblasts specifically stimulated the growth of scirrhous gastric cancer cells, but not that of well-differentiated gastric cancer cells.[4, 5)] ii) Scirrhous gastric cancer cells significantly stimulated the growth of peritoneal fibroblasts, but well differentiated adenocarcinoma cells did not.[6)] In an attempt to identify possible cell-to-cell interaction between scirrhous gastric cancer cells and orthotopic fibroblasts, we have searched for specific glycolipid antigen or adhesion molecules in scirrhous gastric cancer tissues by analyzing the glycolipid compositions of both cell types. Although chemical analysis of glycolipids in gastric cancer tissues has been already reported,[7–10)] no information has been available about the glycolipid composition of scirrhous gastric cancer. In this paper, we describe the glycolipid compositions of scirrhous and non-scirrhous gastric cancer tissues, scirrhous gastric cancer cell lines, and fibroblast cell lines from stomach bearing scirrhous cancer and from normal foreskin (as a reference).

MATERIALS AND METHODS

Glycolipids Glucosyl ceramide (GlcCer), lactosyl ceramide (LacCer), globotriaosyl ceramide (Gb_3), globoside (Gb_4), GM_3, GD_3 and sulfatide (SM_4) were purchased from Sigma Chemical (St. Louis, MO) as reference compounds for high-performance thin layer chromatography (HPTLC) and fast atom bombardment mass spectrometry (FAB/MS).

Tumor tissues Scirrhous (*n*=10) and non-scirrhous gastric cancer tissues (*n*=10) were obtained from patients accom-

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The designation of glycosphingolipids follows the IUPAC-IUB recommen-dations[1)] and the ganglioside nomenclature system of Svennerholm.[2)] modated in the First Department of Surgery at Osaka City University Hospital. Cancer tissues were trimmed from necrotic and adjacent materials, washed with saline to remove blood and mucous within 3 h after resection, and stored at −70°C until use. The remaining tissues were submitted for histologic diagnosis. Normal adjacent mucosa was analyzed as a control.

Cells and cell culture NF-8 cells are an orthotopic fibroblast cell line cloned from a human stomach with scirrhous cancer in our laboratory.[4] HS-27F cells are a fibroblast cell line derived from human normal foreskin. OCUM-1, OCUM-2M, OCUM-5 and Kato-III are cell lines established from human scirrhous gastric cancer tissues (including ascites or pleural effusion). The cells were cultured in Dulbecco's medium or RPMI 1640 medium with 10% fetal calf serum in a 5% CO_2 atmosphere at 37°C, and harvested at the semi-confluent stage. They were washed twice with phosphate-buffered saline (PBS) and stored at −70°C for analysis.

Isolation and purification of glycolipids Glycosphingolipids were extracted from tissues and cells according to the method of Siddiqui *et al*.[11] Briefly, the tissues were homogenized and lyophilized. After Folch's partition with 20 volumes of chloroform/methanol (2:1 and 1:2, by volume), 20 volumes of isopropanol/hexane/water (55:25:20, by volume) were added for further extraction. All extracts were combined and filtered. After mild alkaline hydrolysis with 0.3 *N* NaOH, dialysis, and lyophilization of the upper phase, we obtained major alkali-stable gangliosides and neutral glycosphingolipids containing more than five sugars. The lower phase was peracetylated and applied to a Florisil column to separate the phospholipid. After deacetylation, we obtained neutral glycosphingolipids containing less than five sugars.

Separation of individual glycosphingolipids Folch's lower and upper phase glycosphingolipids were developed on HPTLC plates coated with silica gel without fluorescent indicator (Merck, Darmstadt, Germany). The solvent systems were chloroform/methanol/0.02% aqueous $CaCl_2$ (60:35:8, by volume) as the first solvent system (two developments) and 1-propanol/water/28% ammonium hydroxide (75:25:5, by volume) as the second. Glycolipids were detected by spraying with 50% sulfuric acid (for all glycosphingolipids) or resorcinol-HCl (for gangliosides). Relative concentration of each glycolipid was estimated by color densitometry using a dual-wavelength flying-spot scanner CS-9000 (Shimadzu, Kyoto) connected to a computer system, PC-9801 RX (NEC, Tokyo). To obtain the purified glycosphingolipids, separated spots on the plate were visualized with iodine vapor and scraped off into a test tube, and then the glycosphingolipids were extracted with chloroform/methanol (2:1, 1:1, and 1:2, by volume) and isopropanol/hexane/water (50:25:20, by volume) successively.

FAB/MS analysis of the intact glycolipids Negative ion FAB/MS analysis was carried out with a JEOL SX102A mass spectrometer equipped with an MS-MD 7000 computer system (JEOL, Tokyo). Xenon gas was used as the primary beam. Each sample was dissolved in chloroform/methanol (1:1, by volume) to a concentration of approximately 5 μg/μl and 1 μl was applied to the target with a matrix solution of triethanolamine.

Gas chromatographic analysis of alditol acetates Dried samples were hydrolyzed with 2 *M* trifluoroacetic acid at 120°C for 2 h. After reduction with $NaBH_4$, acetylation was carried out overnight in a mixture of dry pyridine and acetic anhydride (1:1, by volume), at room temperature. Gas chromatographic analysis was carried out with a model 5890 series II (Hewlett-Packard, PA) equipped with a flame ionization detector and a 15 m×0.25 mm fused silica capillary column SP2380 (Supelco Inc., Bellefonte, PA). Column temperature was programmed at a rate of 5°C/min from 150°C to 230°C. The injection port and flame ionization detector were kept at 220°C.

Immunohistochemical staining Immunohistochemical staining was performed by the avidin-biotin peroxidase complex method. The tissues were embedded in OCT compound (Miles Laboratories, Inc., Elkhart, IN) and frozen in acetone at –20°C. The frozen sections were cut on a cryostat at a thickness of 6 μm, mounted on gelatin-coated slides, air-dried, and fixed with acetone for 10 min at 4°C. After blocking of endogenous peroxidase by treatment with 0.3% H_2O_2 and of non-specific binding with 10% normal horse serum, the sections were reacted overnight with mouse monoclonal antibody (mAb) against Gb_3 or Gb_4 (BGR 23, BGR 26)[12] or non-immune mouse IgG (Dako, Glostrup, Denmark) at 4°C. Next, the sections were incubated at room temperature for 30 min with biotinylated horse anti-mouse immunoglobulin as a secondary antibody and then for 30 min with avidin-biotinyl peroxidase complex (Dako). The peroxidase reaction was performed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride-hydrogen peroxidase (Dako) as a chromogen in Tris buffer (pH 7.2) for 5 min. Counter-staining was performed with Mayer's hematoxylin.

TLC-immunostaining Anti-Le^a and Le^b mAbs were purchased from Biotest (Dreieich, Germany). Anti-Le^x and Le^y mAbs were from Seikagaku Corporation (Tokyo). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Dako. The immunostaining procedure was carried out according to Tai *et al*.[13] An appropriate amount of glycosphingolipids was separated with aluminum-backed Polygram silica gel G plates (Machery-Nagel, Duren, Germany). The plates were blocked with 0.01 *M* PBS containing 1% polyvinylpyrrolidone, 1% bovine serum albumin (BSA) and 0.02% NaN_3, and then overlaid with anti-Le^a and Le^b mAbs diluted to 1000-fold in PBS supplemented with 1% BSA. After incubation with horseradish peroxidase-conjugated anti-mouse IgG, bound antibody was visualized with diaminobenzidine tetrachloride.

RESULTS

Neutral glycosphingolipid composition of scirrhous gastric cancer tissues Glycosphingolipid patterns in Folch's lower and upper phases from normal mucosa, non-scirrhous and scirrhous cancer tissues are shown in Figs. 1 and 2. In the lower phase of normal mucosa, four or five spots appeared and were tentatively identified as ceramide monohexoside (CMH), ceramide dihexoside (CDH), Gb_3, Gb_4, and ceramide pentahexoside by comparing the *Rf* values with those of reference compounds (Fig. 1). Each spot was further separated into three or four small spots due to the presence of multiple ceramide molecular species differing in long chain bases (sphingenine, sphinganine, or phytosphingosine), and fatty acids (hydroxy or non-hydroxy fatty acid).[14] Non-scirrhous gastric cancer tissues showed almost the same glycosphingolipid

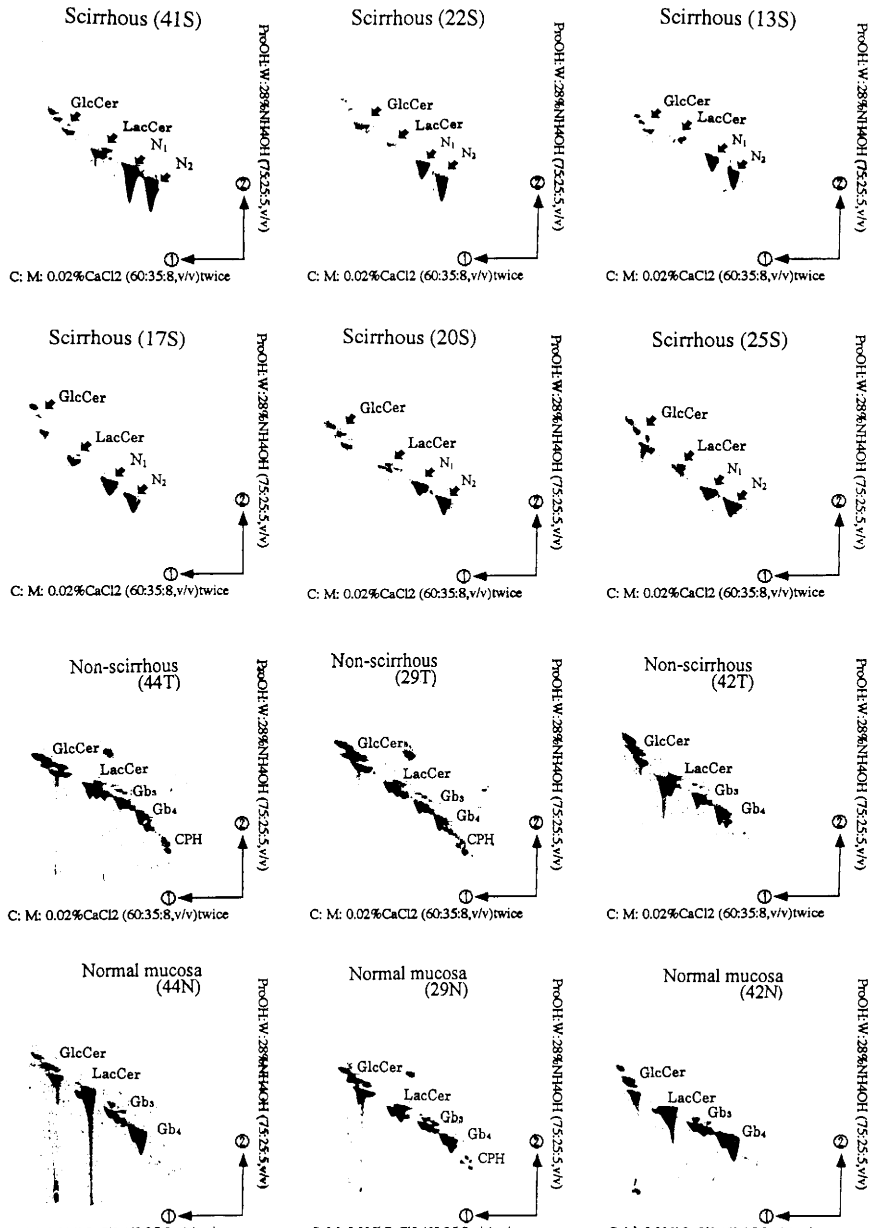

Fig. 1. Two-dimensional thin layer chromatograms of Folch's lower phase glycosphingolipids extracted from scirrhous gastric cancer tissue (S), non-scirrhous gastric cancer tissue (T) and normal gastric mucosa (N). Glycosphingolipids were developed with chloroform/methanol/0.02% aqueous $CaCl_2$ (60:35:8, by volume) in the first system and 1-propanol/water/28% ammonium hydroxide (75:25:5, by volume) in the second system. The spots were located with 50% sulfuric acid. Selected TLC plates with clearly demarcated bands are shown here. Sample numbers are indicated.

patterns as normal mucosa, but scirrhous gastric cancer tissues expressed different patterns. In scirrhous cancer tissues, N1 (corresponding to Gb_3) and N2 (corresponding to Gb_4) were highly expressed and these glycolipids each showed a single spot. The ceramide structures of N1 and N2 in scirrhous cancer tissues seemed to have no variation as compared with those of normal mucosa and non-scirrhous cancer tissues. Relative concentrations of N1 and N2 of scirrhous cancer tissues (N1+N2=58%) were twice those of the same spots of normal and non-scirrhous tissues (N1+N2=29%) as determined by TLC-densitometry (Table I). Structural analysis of N1 and N2 was performed. Based on alditol acetate analysis, N1 contained galactose and glucose in a ratio of 2:1, while N2 contained galactose, glucose and *N*-acetylgalactosamine in a ratio of 2:1:1. Negative ion FAB/MS of N1 (Fig. 3a) showed $(M-H)^-$ at *m/z* 1132 and *m/z* 1022 resulting from the ceramide moiety of nervonyl sphingenine and palmitoyl sphingenine, respectively. Fragment ions due to the loss of three hexoses were observed. N2 showed $(M-H)^-$

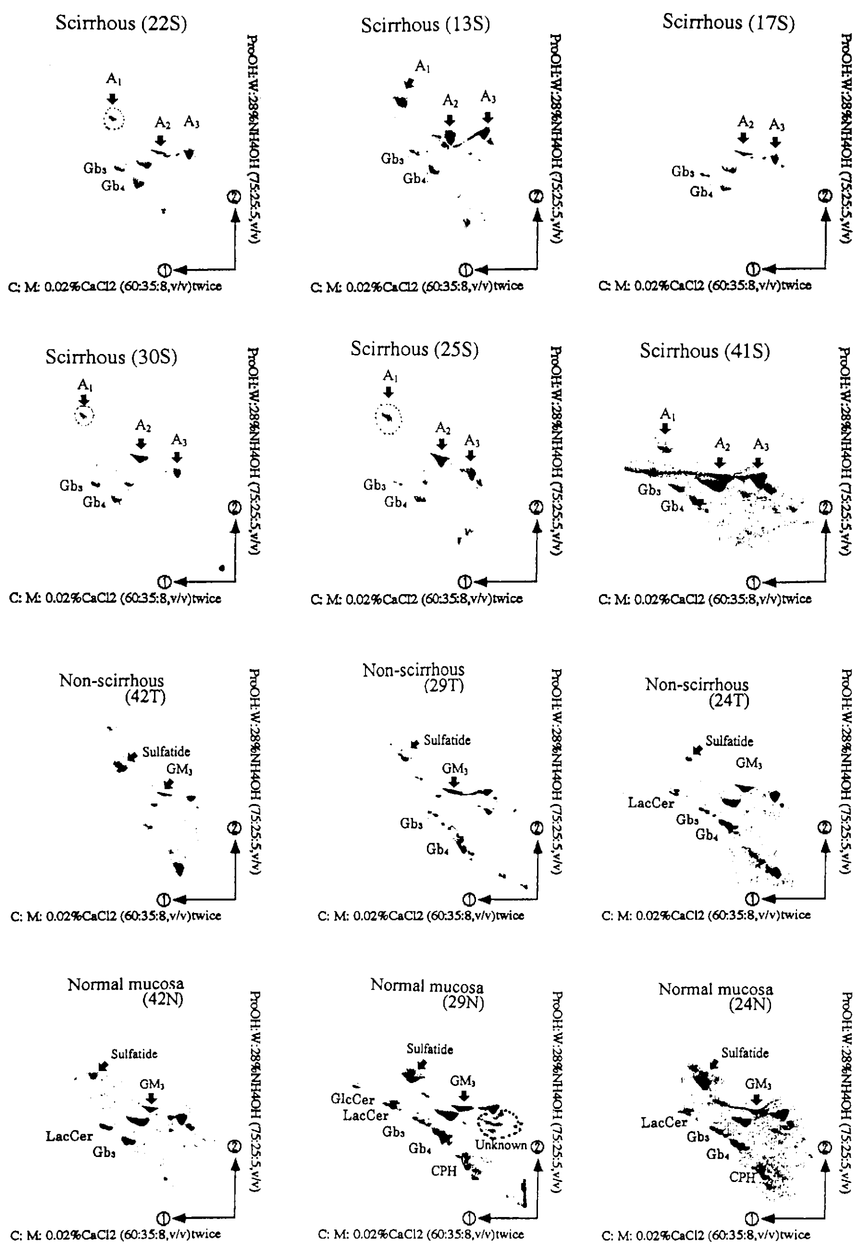

Fig. 2. Two-dimensional thin layer chromatograms of Folch's upper phase glycosphingolipids extracted from scirrhous gastric cancer tissue (S), non-scirrhous gastric cancer tissue (T) and normal gastric mucosa (N). The solvent system was the same as the lower phase described in the legend to Fig. 1.

Table I. Relative Concentrations of Glycosphingolipid Subclasses in Folch's Lower Phase, Prepared from Tissues (Scirrhous and Non-scirrhous) and Cell Lines (NF-8, HS-27F and OCUM-5) (%)

	CMH	CDH	Gb_3 [a)]	Gb_4 [b)]	Others
Scirrhous	24	16	29	29	2
Non-scirrhous	34	33	13	16	4
NF-8	23	15	32	30	—
HS-27F	48	20	22	10	—
OCUM-5	84	6	4	2	4

a, b) Gb_3 and Gb_4 corresponded to N1 and N2 in scirrhous gastric cancer tissues, respectively. Relative concentrations of Gb_3 and Gb_4 in scirrhous tissues were significantly increased (Mann-Whitney U test, $P<0.01$).

at *m/z* 1335 and *m/z* 1225 (Fig. 3b). The ceramide moiety was the same as that of N1. Fragment ions due to the loss of *N*-acetylhexosamine and three hexoses were observed. Based on the alditol acetate analysis and FAB/MS, we propose that the structure of N1 is Gal-Gal-Glc-Cer (Gb_3), and that of N2 is GalNAc-Gal-Gal-Glc-Cer (Gb_4).

Acidic glycosphingolipid composition of scirrhous gastric cancer tissues In Folch's upper phase from normal mucosa, scirrhous and non-scirrhous cancer tissues, three major acidic glycosphingolipids and several neutral glycosphingolipids were expressed (Fig. 2). The acidic glycosphingolipids were tentatively identified as SM_4, II^3NeuAcα-LacCer (GM_3) and II^3NeuAcα_2-LacCer (GD_3), based on a comparison of the chromatographic behavior with that of commercial standards. In more polar parts from normal mucosa, there were unknown ganglioside spots, but their *Rf* values did not coincide with those of any of the commercial standards. In non-scirrhous and scirrhous cancer tissues, those polar gangliosides were not detected and the TLC patterns were rather simple. Three spots in scirrhous cancer tissues were termed as A1 (corresponding to SM_4), A2 (corresponding to GM_3) and A3 (corresponding to GD_3), and their structure was analyzed. FAB/MS analyses of A1, A2 and A3 are shown in Fig. 4. A1 showed $(M\text{-}H)^-$ at *m/z* 906 and *m/z* 778 due to SM_4 with the ceramide moiety of 2-hydroxylignoceroyl sphingenine and palmitoyl sphingenine, respectively. The fragment ion at *m/z* 540 corresponded to lyso-sulfatide. A2 showed $(M\text{-}H)^-$ at *m/z* 1263 due to GM_3 with the ceramide moiety of lignoceroyl sphingenine. Fragment ions due to the loss of *N*-acetylneuraminic acid and two hexoses were observed. A3 showed $(M\text{-}H)^-$ at *m/z* 1554 due to GD_3 with the same ceramide moiety as A2. Fragment ions due to the loss of two *N*-acetylneuraminic acid and two hexoses were observed. These spectral patterns of A1, A2 and A3 corresponded to those of commercial SM_4, GM_3 and GD_3 (data not shown). From the above analytical results, A1, A2 and A3 were identified as SM_4, GM_3 and GD_3, respectively.

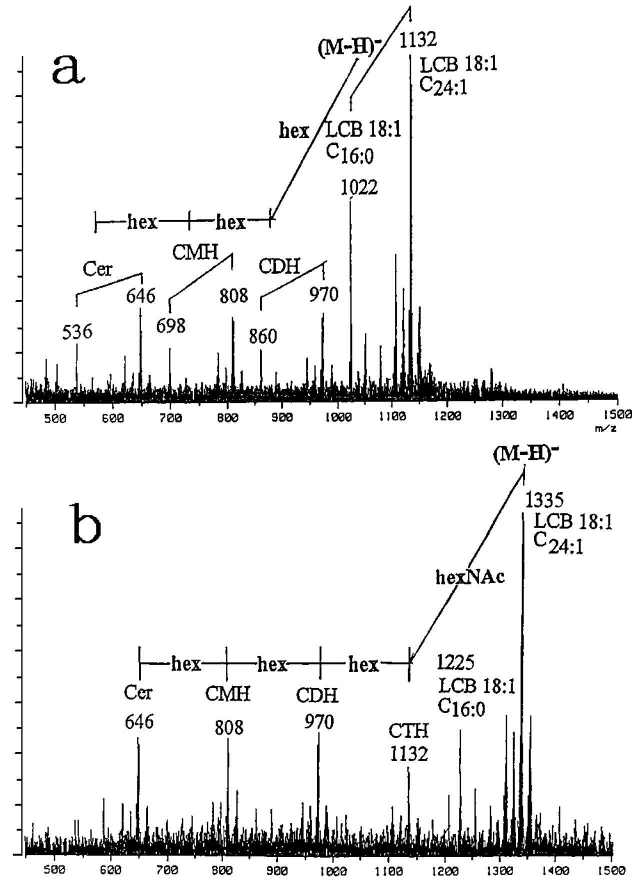

Fig. 3. Negative ion FAB/MS of N1 (a) and N2 (b). About 5 μg of each of N1 and N2 was analyzed by means of negative ion FAB/MS with triethanolamine as the matrix. Inset is the fragmentation mode of the glycolipid species.

Glycosphingolipid expression of fibroblast cell lines As scirrhous gastric cancer tissues show prominent proliferation of interstitial tissues, we have investigated the glycosphingolipid composition of two fibroblast cell lines, which were cloned from human stomach bearing scirrhous gastric cancer (NF-8) and normal foreskin (HS-27F) as references (Fig. 5). In the NF-8 and HS-27F cell lines, three gangliosides (GM_3, GM_2 and GM_1) and several neutral glycosphingolipids were detected in Folch's upper phase. NF-8 and HS-27F showed almost the same pattern. In the lower phases, both cell lines showed CMH, CDH, Gb_3 and Gb_4. FN1 and FN2, corresponding to Gb_3 and Gb_4, were highly expressed in NF-8 (Gb_3+Gb_4=62%) as compared with HS-27F (Gb_3+Gb_4=32%) (Table I). As

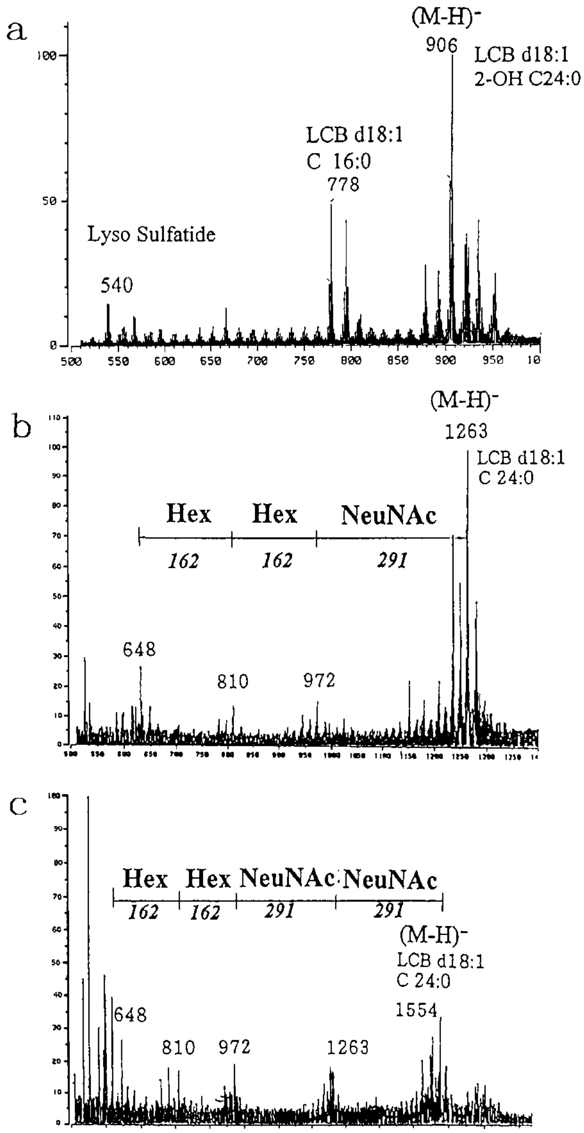

Fig. 4. Negative ion FAB/MS of A1 (a), A2 (b), and A3 (c).

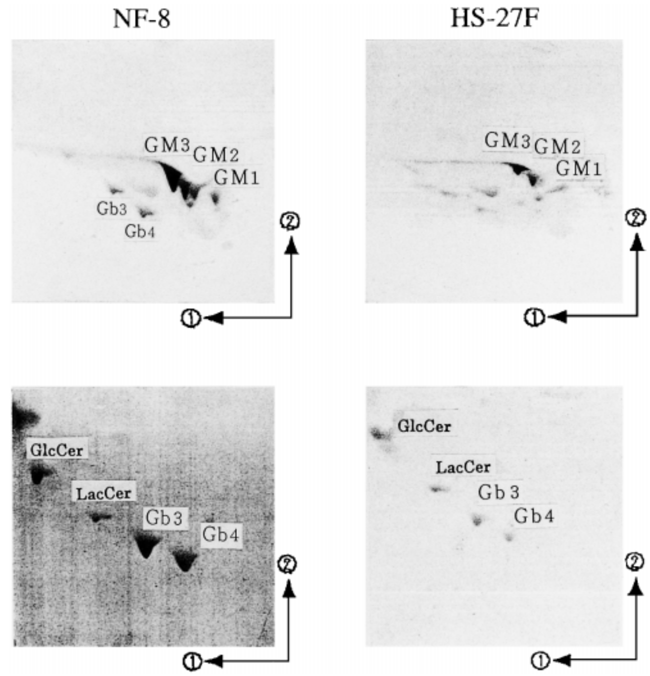

Fig. 5. Two-dimensional thin layer chromatograms of the glycolipids from fibroblast cell lines. The solvent system is that described in the legend to Fig. 1. TLC patterns of glycosphingolipids from NF-8 (derived from stomach) and HS-27F (derived from foreskin) are shown on the left and right, respectively. Upper and lower plates show glycosphingolipids in Folch's upper and lower phases from each cell line, respectively.

shown in Fig. 6, the negative ion FAB/MS spectra of FN1 and FN2 were essentially identical to those of N1 and N2, respectively, which were extracted from scirrhous gastric cancer tissues.

Immunohistochemical staining of scirrhous gastric cancer tissues Immunohistochemically, the interstitial cells in scirrhous gastric cancer tissues were stained strongly and cancer cells weakly with anti-Gb_3 and Gb_4 mAbs. By contrast, in normal tissues remote from the carcinoma, the staining was weak or almost negative even in the interstitial tissues (Fig. 7). We performed immunostaining of sequential sections with anti-human fibroblast mAb (Dako). In this case, the interstitial cells were strongly stained as well (data not shown). These results showed that Gb_3 and Gb_4 were expressed mainly on fibroblasts of interstitial tissues in scirrhous gastric cancer.

Glycosphingolipid expression of scirrhous gastric cancer cell lines Glycosphingolipid expression of scirrhous cancer cell lines is shown in Fig. 8. Scirrhous gastric cancer cell lines also contained Gb_3 and Gb_4, but as minor components. Instead, CMH and CDH were expressed as major components. Unknown neutral glycosphingolipids which may have long carbohydrate chains (N3, N4 and N5) were detected in Folch's upper phase. Based on analytical (FAB/MS and alditol acetate) and immunological (TLC-immunostaining) data, we propose that N3 is ceramide pentasaccharide bearing Le^a structure, while N4 and N5 are ceramide hexa- and heptasaccharides bearing Le^b structure (data not shown).

DISCUSSION

It has been well documented that chemical modifications of carbohydrate structure on the cell surface mem-

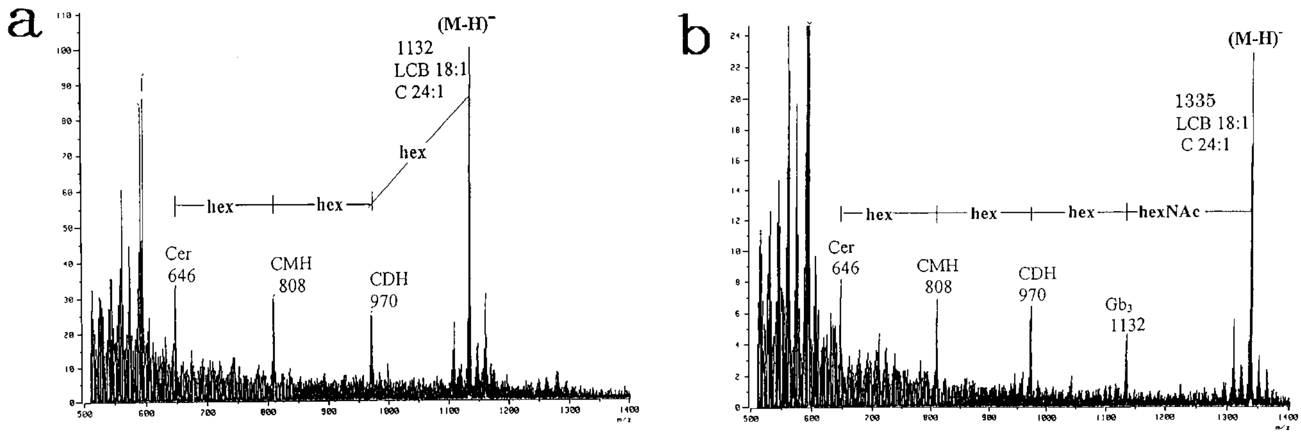

Fig. 6. Negative ion FAB/MS of FN1 (a) and FN2 (b). Quasi molecular ions at *m/z* 1132, *m/z* 1335 and all of the fragment ions were the same as those of N1 and N2 extracted from scirrhous gastric cancer tissues. FN1 and FN2 were essentially identical to N1 and N2, respectively.

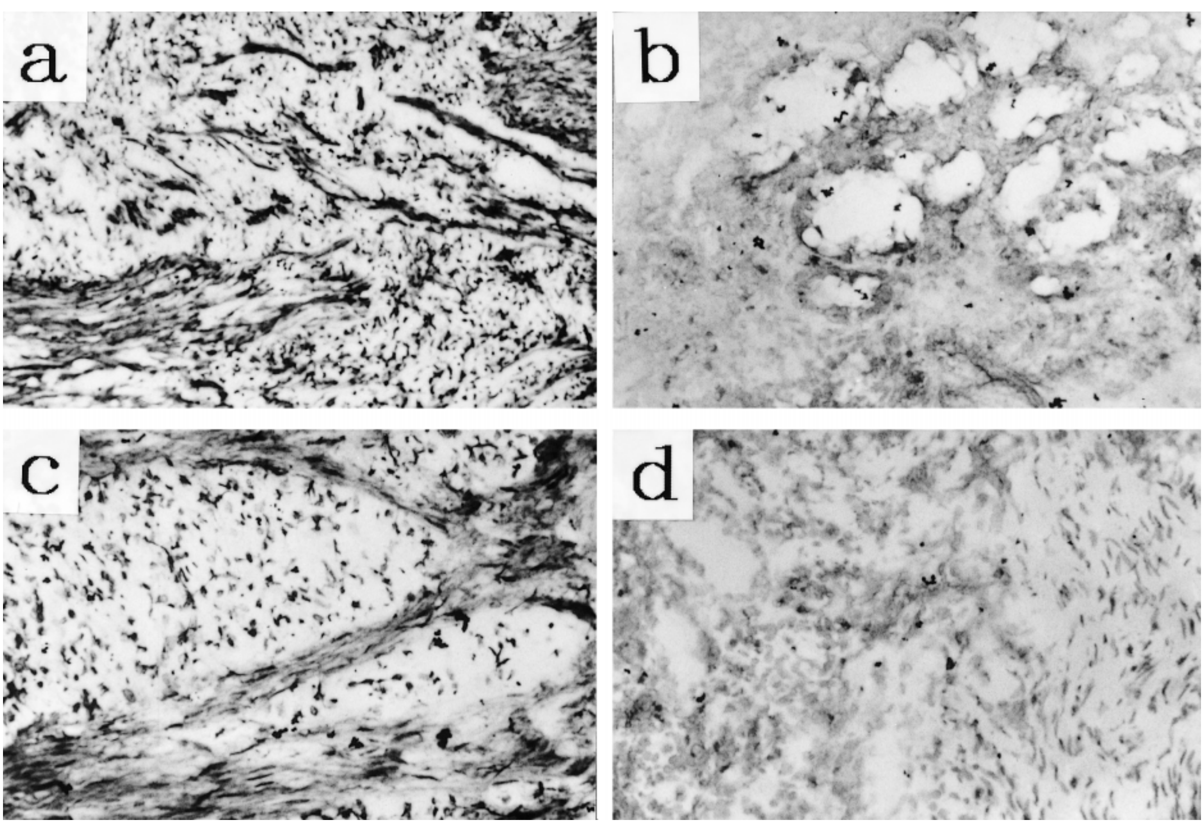

Fig. 7. Immunolocalization of Gb_3 (a and b) and Gb_4 (c and d) in scirrhous gastric cancer tissues (a and c) and normal gastric mucosa remote from carcinoma (b and d). Gb_3 and Gb_4 were expressed intensely in stromal cells (fibroblasts) and weakly in cancer cells in scirrhous gastric cancer tissues. In normal gastric mucosa, reactivity with anti-Gb_3 and Gb_4 monoclonal antibodies was very low even in the interstitial tissues.

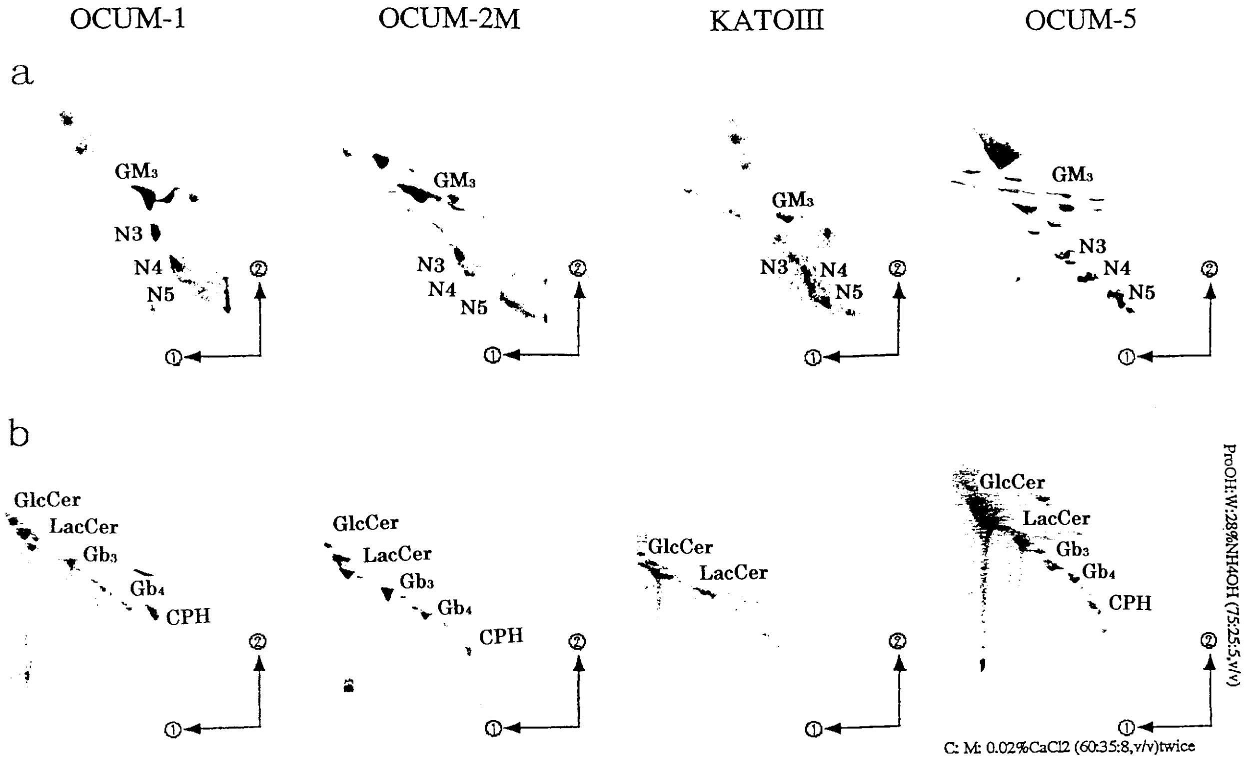

Fig. 8. Two-dimensional thin layer chromatograms of the glycolipids extracted from scirrhous gastric cancer cell lines. Plates a and b show glycosphingolipids in Folch's upper and lower phases, respectively.

Table II. Glycosphingolipid Component in Folch's Upper Phase, Prepared from Tissues (Scirrhous and Non-scirrhous) and Cell Lines (NF-8, HS-27F and OCUM-5)

Scirrhous cancer tissues	$GM_3 < GD_3$ (SM_4)
Non-scirrhous cancer tissues	$GM_3 > GD_3$ (SM_4)
Normal gastric mucosa	$GM_3 ≒ GD_3 ≒ SM_4$
NF-8	$GM_3 > GM_2 > GM_1$
HS-27F	$GM_3 > GM_2$ (GM_1)
OCUM-5	$GM_3 ≒ Le^a ≒ Le^b$

$GM_3 > GD_3$, the amount of GM_3 is larger than that of GD_3; ( ), trace amount; ≒, almost equal amounts.

brane occur in association with changes of cell density or cell-to-cell contact, cell morphology, cell adhesion, and cell differentiation, and are related to metastasis of cancer cells.[15–19]

Blood group glycolipids are expressed in human gastric cancer.[7, 8] However, no information about the glycolipid composition is available yet for scirrhous gastric cancer, probably because of its complex progression. To detect newly synthesized or shed glycolipid antigen, we analyzed the glycosphingolipid composition of human scirrhous gastric cancer tissues and compared the results with those of non-scirrhous gastric cancer tissues.

Recently, it has been revealed that the specific progression of scirrhous gastric cancer results from cellular interaction between cancer cells and interstitial cells, especially fibroblasts.[4, 5] Therefore, we performed the glycosphingolipid analysis of fibroblasts and scirrhous gastric cancer cells separately, and compared their compositions. The results are summarized in Tables I and II. In the present study, we did not find a new scirrhous gastric cancer-specific glycolipid antigen, but a marked increase of Gb_3 and Gb_4 in scirrhous gastric cancer tissues was observed. Also, we found that the main components of glycosphingolipids from fibroblasts in scirrhous gastric cancer were Gb_3 and Gb_4, which are the same as those of scirrhous gastric cancer tissues at the molecular level. However, it is not known whether or not these glycolipids correlate specifically with scirrhous-type cancer progression. In the previous reports, hamster and human lung fibroblasts did not show a specific increase of Gb_3 and Gb_4.[20, 21] Our data for the fibroblast cell line derived from foreskin also did not show an increase of Gb_3 and Gb_4. Although we have not yet determined the glycosphingolipid composition of fibroblasts derived from normal gastric mucosa, there is a possibility that orthotopic fibroblasts in scirrhous gastric cancer tissues have been affected by cancer cells and transformed specifically to synthesize Gb_3 and Gb_4 in high quantities.

Gb_3 was reported to be a tumor-associated glycolipid[22] because of its existence in Burkitt lymphoma cell line,[23] human teratocarcinoma cell line,[24] and murine myeloma cell line.[25] Furthermore, this glycolipid has also been reported to be related to the metastatic potential of murine fibrosarcoma cells; highly metastatic isolates were characterized by high levels of Gb_3, and a reduced level of Gb_3 was found in a weakly metastatic subclone.[26] As to Gb_4, it has been reported that NIL cell growth was inhibited by its exogenous addition.[27] However, our preliminary studies indicated that exogenous Gb_3 and Gb_4 had no effect on cell proliferation of scirrhous cancer cell lines (data not shown). Further investigations to clarify the role of Gb_3 and Gb_4 will be required.

Gangliosides have been proposed not only to be cell surface receptors for certain bacterial toxins and viruses, but also to regulate cellular functions including cell adhesion and differentiation.[28] GM_3 and GD_3 are major gangliosides in human gastric mucosa,[29] and in human stomach, they represent 42% and 26% of total glycosphingolipids, respectively.[30] In addition, more polar, unknown gangliosides were expressed in normal gastric mucosal tissues. These gangliosides may correspond to the more complex gangliosides reported by Keranen, having a monosialo- and disialotetraglycosyl structure containing *N*-acetylglucosamine instead of *N*-acetylgalactosamine.[30] In our study, the spots corresponding to polar gangliosides were absent and GD_3 was increased in scirrhous gastric cancer tissues as compared with normal and non-scirrhous cancer tissues. GD_3 is a possible precursor for GT_3, GD_2 and *N*-acetylglucosaminyl GD_2. We speculate that this biosynthetic pathway was blocked in scirrhous gastric cancer tissues, so that the more polar gangliosides were decreased and the precursor (GD_3) was increased. This is compatible with the idea of imperfect carbohydrate chain synthesis. On the other hand, neither the fibroblast cell lines nor the cancer cell lines expressed GD_3. As the structure of the ceramide moiety of GD_3 is essentially the same as that of GM_3, it is possible that GD_3 is newly synthesized from GM_3 in scirrhous gastric cancer tissues by the interaction of fibroblasts and cancer cells. In other words, sialyltransferase activity in scirrhous gastric cancer tissues may be activated.

Usually, sulfatides exist as normal components of mucosal epithelial cells exposed to acid, pepsin, and bile salts, such as gastric or duodenal mucosa. Therefore, it is suggested that sulfatide plays a role in mucosal protection.[31] In the case of scirrhous gastric cancer, the molar ratio of sulfatides to GM_3 was extremely low (data not shown). We speculate that sulfatides in scirrhous gastric cancer tissues originated from residual normal gastric mucosa under which scirrhous cancer cells invaded.

We characterized highly polar neutral glycolipids bearing Le^a and Le^b structure, which have already been reported to be expressed in various types of cancer tissues and cell lines.[32–34] In particular, Le^b has been proposed to be a cancer-associated glycolipid by these authors. Le^a and Le^b could not be detected in scirrhous gastric cancer tissues, probably due to the greater proliferation of the interstitial tissues than that of the cancer cells. However, we presume that these glycosphingolipids of cancer cells play some role in scirrhous-type cancer progression. Investigations of the biological functions of these glycolipids are in progress. In addition, the possible interaction between scirrhous gastric cancer cells and orthotopic fibroblasts which brings about glycolipids (Gb_3 and Gb_4) synthesis or interstitial proliferation should be clarified.

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REFERENCES

1) IUPAC-IUAB. Commission on biochemical nomenclature. The nomenclature of lipids. *Eur. J. Biochem.*, **79**, 11–21 (1977).
2) Svennerholm, L. Chromatographic separation of human brain gangliosides. *J. Neurochem.*, **10**, 613–623 (1963).
3) Sowa, M., Kato, Y., Nishimura, M., Yoshino, H., Kubo, T. and Umeyama, K. Clinicohistochemical studies on type 4 carcinoma of the stomach—with special reference to mucopolysaccharides and sialic acid in tumor tissues. *Jpn. J. Surg.*, **19**, 153–162 (1989).
4) Yashiro, M., Chung, Y. S. and Sowa, M. Role of orthotopic fibroblasts in the development of scirrhous gastric carcinoma. *Jpn. J. Cancer Res.*, **85**, 883–886 (1994).
5) Yashiro, M., Chung, Y. S., Kubo, T., Hato, F. and Sowa, M. Differential response of scirrhous and well-differentiated gastric cancer cells to orthotopic fibroblasts. *Br. J. Cancer*, **74**, 1096–1103 (1996).
6) Yashiro, M., Chung, Y. S., Nishimura, S., Inoue, T. and Sowa, M. Fibrosis in the peritoneum induced by scirrhous gastric cancer cells may act as "soil" for peritoneal dissemination. *Cancer*, **77**, 1668–1675 (1996).
7) Wang, S. M., Huang, T. W. and Hakomori, S. Immunohistochemistry of two glycolipid tissue antigens in human gastric carcinoma. *Cancer*, **52**, 2072–2076 (1983).
8) Uemura, K., Hattori, H., Ono, K., Ogata, H. and Taketomi, T. Expression of Forssman glycolipid and blood group-related antigens A, Le^x and Le^y in human gastric cancer and fetal tissues. *Jpn. J. Exp. Med.*, **59**, 239–249 (1989).

9) Dohi, T., Ohta, S., Hanai, N., Yamaguchi, K. and Oshima, M. Sialylpentaosylceramide detected with anti-GM_2 monoclonal antibody. *J. Biol. Chem.*, **265**, 7880–7885 (1990).
10) Breimer, M. E. Adaptation of mass spectrometry for the analysis of tumor antigens as applied to blood group glycolipids of human gastric carcinoma. *Cancer Res.*, **40**, 897–908 (1980).
11) Siddiqui, B., Whitehead, J. S. and Kim, Y. S. Glycosphingolipids in human colonic adenocarcinoma. *J. Biol. Chem.*, **253**, 2168–2175 (1978).
12) Kotani, M., Kawashima, I., Ozawa, H., Ogura, K., Ariga, T. and Tai, T. Generation of one set of murine monoclonal antibodies specific for globo-series glycolipids: evidence for differential distribution of the glycolipids in rat small intestine. *Arch. Biochem. Biophys.*, **310**, 89–96 (1994).
13) Tai, T., Sze, L., Kawashima, I., Saxton, E. R. and Irie, R. F. Monoclonal antibody detects monosialogangliosides having a sialic acid $\alpha 2 \rightarrow 3$-galactosyl residue. *J. Biol. Chem.*, **262**, 6803–6807 (1987).
14) Keranen, A. Fatty acids and long-chain bases of gangliosides of human gastrointestinal mucosa. *Chem. Phys. Lipids*, **17**, 14–21 (1976).
15) Hakomori, S. Structure and organization of cell surface glycolipids: dependency on cell growth and malignant transformation. *Biochim. Biophys. Acta*, **417**, 55–89 (1975).
16) Hakomori, S. Cell density-dependent changes of glycolipids concentrations in fibroblasts, and loss of this response in virus-transformed cells. *Proc. Natl. Acad. Sci. USA*, **67**, 1741–1747 (1970).
17) Kleiman, H. K., Martin, G. R. and Fishman, P. H. Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. USA*, **76**, 3367–3371 (1979).
18) Cheresh, D. A., Pierschbacher, M. D., Herzig, M. A. and Mujoo, K. Disialoganglioside GD_2 and GD_3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. *J. Cell Biol.*, **102**, 688–696 (1986).
19) Keranen, A., Lempinen, M. and Puro, K. Ganglioside pattern and neuraminic acid content of human gastric and colonic carcinoma. *Clin. Chim. Acta*, **70**, 103–112 (1976).
20) Gahmberg, C. G. and Hakomori, S. Surface carbohydrate of hamster fibroblasts. *J. Biol. Chem.*, **250**, 2438–2446 (1975).
21) Choi, B. O., Yamaki, T., Ibayashi, Y., Maeda, Y., Gasa, S. and Hashi, K. Interleukin 4 enhances ganglioside GD_3 expression on the human fibroblast cell line WI-38. *J. Biochem.*, **117**, 315–320 (1995).
22) Fredman, P. Glycolipid tumor antigen. *In* "Advances in Lipid Research," ed. R. M. Bell, Y. A. Hannun and A. H. Merril, Jr., Vol. 25, pp. 213–230 (1993). Academic Press, California.
23) Nudelman, E., Kannagi, R., Hakomori, S., Parsons, M., Lipinski, M., Wiels, J., Fellous, M. and Tursz, T. A glycolipid antigen associated with Burkitt lymphoma defined by a monoclonal antibody. *Science*, **220**, 509–511 (1983).
24) Kannagi, R., Levery, S. B., Ishigami, F., Hakomori, S., Shevinsky, L. H., Knowles, B. B. and Solter, D. New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3. *J. Biol. Chem.*, **258**, 8934–8942 (1983).
25) Kniep, B., Hunig, T. R., Fitch, F. W., Heuer, J., Kolsch, E. and Muhlradt, P. F. Neutral glycosphingolipids of murine myeloma cells and helper, cytolytic and suppressor T lymphocytes. *Biochemistry*, **22**, 251–255 (1983).
26) Mannori, G., Cecconi, O., Mungani, G. and Ruggieri, S. Role of glycolipids in the metastatic process: characteristics of neutral glycolipids in clones with different metastatic potentials isolated from a murine fibrosarcoma cell line. *Int. J. Cancer*, **45**, 984–988 (1990).
27) Laine, R. A. and Hakomori, S. Incorporation of exogenous glycosphingolipids in plasma membranes of cultured hamster cells and concurrent change of growth behavior. *Biochem. Biophys. Res. Commun.*, **54**, 1039–1045 (1973).
28) Bremer, E. G., Schlessinger, J. and Hakomori, S. Ganglioside-mediated modulation of cell growth. *J. Biol. Chem.*, **261**, 2434–2440 (1986).
29) Natomi, H., Sugano, K., Takaku, F. and Iwamori, M. Glycolipids composition of the gastric mucosa. A role of sulfatide in gastrointestinal mucosal defense? *J. Clin. Gastroenterol.*, **12**, 52–57 (1990).
30) Keranen, A. Gangliosides of the human gastro-intestinal mucosa. *Biochim. Biophys. Acta*, **409**, 320–328 (1975).
31) Slomiany, B. L., Liau, Y. H., Sarosiek, J., Tsukada, K., Mizuta, K., Rosenthal, K. W. and Slomiany, W. A. Sulfation of glycolipids by human gastric mucosa in disease. *Digestion*, **36**, 246–252 (1987).
32) Sogabe, T., Sowa, M., Okuno, M., Chung, Y.-K., Hotta, H. and Yano, I. Le^b, a novel glycosphingolipid antigen of human distal colorectal cancer. *Oncol. Rep.*, **3**, 857–862 (1996).
33) Blaszczyk, M., Yan Pak, K., Herlyn, M., Sears, H. and Steplewski, Z. Characterization of Lewis antigens in normal colon and gastrointestinal adenocarcinoma. *Proc. Natl. Acad. Sci. USA*, **82**, 3552–3556 (1985).
34) Iwamori, M., Sakayori, M., Nozawa, S., Yamamoto, T., Yago, M., Noguchi, M. and Nagai, Y. Monoclonal antibody-defined antigen of human uterine endometrial carcinomas is Le^b. *J. Biochem.*, **105**, 718–722 (1989).