A Novel Ganglioside Isolated from Renal Cell Carcinoma*

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In renal cell carcinoma (RCC), the level of higher gangliosides is correlated with degree of metastatic potential, and cell lines derived from metastatic deposits of RCC are characterized by high expression of disialogangliosides (Saito, S., Orikasa, S., Ohyama, C., Satoh, M., and Fukushima, Y. (1991) Int. J. Cancer 49, 329–334) and Saito, S., Orikasa, S., Satoh, M., Ohyama, C., Ito, A., and Takahashi, T. (1997) Jpn. J. Cancer Res. (Gann) 88, 652–659). We now report two disialogangliosides, G1 and G2, found in the RCC cell line TOS-1. G1 from TOS-1 cells was characterized as having a novel hybrid structure between ganglio-series (region I) and lacto-series type 1, characterized by high expression of disialogangliosides in addition to GM2 but do not express SLex or SLea (7). Therefore, an unknown mechanism mediated by glycoepitopes different from SLex, SLea, or their analogs should be considered for RCC metastasis (7). In immunohistological (8) and clinicopathological (9) studies using a series of mAbs directed to di- and monosialosylgangliosides of RCC, positive staining with these antibodies in original RCC tissue was correlated with later incidence of metastasis.

Disialosylgalactosylgloboside (DSGG) was identified previously as one of the major disialogangliosides from RCC tissues (10). However, further studies indicated that two other novel disialogangliosides are found in RCC tissue as well as TOS-1 cells, and that DSGG is the major disialoganglioside of ACHN but is absent from TOS-1 (see “Discussion”). We report here that the two major disialogangliosides (G1 and G2) present in TOS-1 cells and RCC are not DSGG but have entirely different properties. Their structures were characterized by 1H NMR spectroscopy and electrospray ionization mass spectrometry of the intact compound.

The expression pattern of glycosphingolipid (GSL)1 at the tumor cell surface may define the ability of the tumor cell to interact with specific target cell where the GSL antigen is recognized and may thus promote distant metastasis through the blood circulatory or lymphatic system. This general idea is supported particularly by tumor cell adhesion to E-selectin expressed on activated endothelial cells. This process is mediated by the E-selectin epitope expressed on tumor cells, which is identified as either sialosyl-Lea (SLea), sialosyl-Lea (SLea), or their analogs (for review, see Refs. 1–3).

An initial study of GSL patterns of renal cell carcinoma (RCC) and its metastatic deposits indicated that enhanced expression of higher gangliosides having TLC mobility similar to or slower than that of GM2 in original RCC tissue is correlated with metastatic potential (4). However, expression of SLea and dimeric Lea in RCC is higher in differentiated than in undifferentiated form and is not correlated with RCC metastasis (5, 6). This trend is opposite to that in other types of cancer (see above).

Two RCC cell lines derived from its metastatic deposits, TOS-1 and ACHN, are characterized by high expression of disialogangliosides in addition to GM2 but do not express SLea or SLea (7). Therefore, an unknown mechanism mediated by glycoepitopes different from SLea, SLea, or their analogs should be considered for RCC metastasis (7). In immunohistological (8) and clinicopathological (9) studies using a series of mAbs directed to di- and monosialosylgangliosides of RCC, positive staining with these antibodies in original RCC tissue was correlated with later incidence of metastasis.

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MK-1–8. G2 has the same lacto-series type 1 chain, with a vicinal disialosyl residue, and is identical to the structure described previously as a colonic cancer-associated antigen defined specifically by mAb FH9 (11). In this study, we describe isolation and detailed characterization of G1 and G2 and discuss the possible relationship of their expression to RCC malignancy.

MATERIALS AND METHODS

**Cells—**TOS-1 was derived from a renal metastatic lesion of an RCC patient (8). ACHN was purchased from American Tissue Culture Collection (ATCC) and was originally derived from a malignant pleural effusion of a patient with widely metastatic RCC. These cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 μg/ml streptomycin under a humidified atmosphere with 5% CO2 at 37 °C.

**Monoclonal Antibodies—**RM2 was established using TOS-1 cells (10), and FH9 was established in our laboratory (11). The anti-GM2 antibody MK-1–8 was kindly donated by Dr. Reiji Kannagi (Molecular Pathology, Aichi Cancer Center, Nagoya, Japan).

**Extraction of Glycolipids—**Glycolipids were extracted from packed cells as described previously (12). Briefly, ~130 ml of packed cells were extracted by homogenization and filtration with 15 volumes of isopropanol alcohol/methanol/water (55:25:20, v/v/v). The extraction/filtration procedure was repeated once more. Combined extracts were evaporated and divided into the upper and the lower phases by Folch’s partition (13). The upper phase was dialyzed against distilled water, dissolved in chloroform/methanol/water (30:60:8), and fractionated by DEAE-Sephadex A25 column chromatography into neutral glycolipids and gangliosides. Gangliosides were further separated into mono-, di-, and trisialosyl fractions by stepwise elution with 0.03, 0.13, and 0.45 M ammonium acetate in chloroform/methanol/water (30:60:8). The eluted gangliosides were dialyzed against distilled water and lyophilized. Each fraction was spotted onto an HPTLC plate and developed in a solvent system of chloroform-methanol-0.5% aqueous CaCl2 (50:40:10). The plates were air-dried and immersed in 0.5% orcinol and 2% sodium hydroxide acid.

**Purification of Gangliosides—**Gangliosides were separated on preparative HPTLC. 50 μl of sample were streaked across a HPTLC plate, developed in a solvent system of chloroform-methanol-0.2% aqueous CaCl2 (50:50:10), and visualized by spraying with 0.01% orcinol in 2 N sulfuric acid.

**Desialosylation of Ganglioside G2—**A 70-μg aliquot of ganglioside G2 was desialylated by heating in 1 ml of isopropanol alcohol/hexane/water (55:25:20, v/v/v; upper phase removed) containing 10% acetic acid in a sealed tube at 100 °C for 4 h. After cooling and drying under a N2 stream at 35–40 °C, the products were taken up in chloroform/methanol/water (30:60:5, v/v/v; Solvent A) and passed through a ~1-ml column of DEAE-Sephadex A-25 (acetate form, pre-equilibrated with solvent A), washing with 5–6 ml of the same solvent. The combined eluent, free of sialic acid and residual ganglioside, was dried under a N2 stream and prepared for NMR analysis as described below.

**Enzyme-linked Immunosorbent Assay—**Coating plates were coated with 50 μl of sample in 100% methanol for 1 h. Plates were blocked with FCS (5%) and incubated with peroxidase-linked secondary antibody for 1 h at room temperature. Each well was washed extensively in PBSt (0.05% Tween 20 (T-PBS), and incubated with peroxidase-linked secondary antibody for 30 min at room temperature. After washing in T-PBS, each well was

**RESULTS**

**Disialoganglioside Pattern of RCC Cell Lines TOS-1 and ACHN—**Four major disialoganglioside component bands (i, ii, iii, iv) were separated from TOS-1 cells, and two components (v, vi) were separated from ACHN cells (Fig. 1). mAb RM2 reacted strongly with iii and iv from TOS-1, which are termed G1. The ganglioside corresponding to components i and ii barely reacted with mAb RM2 but reacted strongly with FH9 and is termed G2. Similarly, components v and vi did not react with RM2, but reacted strongly with mAb 5F3, and are termed G3. Thus,
ACHN cells. Gliosides (G1, G2) from TOS-1 cells and disialoganglioside G3 from the middle of purified disialosyl fraction; lane 1, orcinol-sulfuric acid; lane 2, mAb FH9; lane 3, mAb RM2; B, monosialosyl (lane 1) and disialosyl (lane 2) ganglioside fractions of TOS-1 cells. Left, orcinol-sulfuric acid; right, anti-GM2 mAb MK1–8. Arrow indicates sulfatide in orcinol staining of disialosyl fraction. C, orcinol-sulfuric acid staining (left), mAb RM2 immunostaining (middle), and mAb 5F3 immunostaining (right) of purified disialogangliosides (G1, G2) from TOS-1 cells and disialoganglioside G3 from ACHN cells.

components i and ii showed the same reactivity with anti-carbohydrate mAbs and were assumed to have the same carbohydrate structure, as was also the case for iii and iv, and v and vi, respectively. These assumptions were confirmed by further studies of each component, which were purified by repeated preparative HPTLC, and subjected to (a) detailed immunochromatographic analysis using various mAbs, and (b) detailed chemical analysis by 1H NMR spectroscopy and mass spectrometry, as described under “Materials and Methods.” Immunostaining patterns with mAbs RM2, MK1–8, FH9, and 5F3 of purified G1, G2, and G3 are shown in Fig. 2.

Immunochromatographic Analysis of Purified G1, G2, and G3—G1 as above was strongly TLC immunostained with mAb RM2, but G2 was barely reactive. G1, but not G2, was stained with anti-GM2 mAb MK1–8. On the other hand, G2 was strongly stained with mAb FH9, which defines disialosyl type 1 chain (disialosyl LeC4Cer, i.e. IV2NeuAcIII2NeuAcLeC4Cer) (11), whereas G1 was not stained. Comparative reactivity of various gangliosides and GSLs with RM2 is shown in Fig. 3. These results are consistent with chemical analysis, indicating that G2 is identical to the FH9 antigen and G1 is GalNAc β1–4 linked to the terminal Gal of FH9 antigen.

Disialoganglioside bands v and vi from ACHN cells, defined by mAb 5F3, were identified as DSGG. These results will be described elsewhere, together with reactivity and tissue distribution pattern of the antigen defined by 5F3.

Glycosyl and Fatty N-Acyl Composition Analysis of TOS-1 Gangliosides—Monosaccharides were identified by GC-MS analysis of the per-O-trimethylsilyl derivatives of methyl glycosides produced from G1 and G2 following methanalysis and re-N-acetylation (data not shown). In the analysis of both G1 and G2, derivatives of Glc, Gal, GlcNAc, and NeuAc were identified in 1:2:1:2 ratio, but G1 was distinct from G2 in yielding an additional peak corresponding to a residue of GalNAc. Fatty acids in the ceramides of G1 and G2 were then identified as their methyl esters by GC-MS of hexane extracts of the methanolytes (data not shown). The predominant fatty acids from ganglioside G1 were 16:0, 24:1 (mostly nervonate), and 24:0, together with significant amounts of 18:0 and 22:0, and much smaller amounts of various other species, including 22:1, 18:1 (oleate), 16:1, 26:1, 14:0, 15:0, 23:0, and 26:0. The distribution from G2 was very similar, but with a much higher proportion of 16:0 fatty acid, and with some of the minor species near or below the limits of detection.

One and Two-dimensional 1H NMR Spectroscopy—For structural characterization, high resolution one-dimensional 1H and two-dimensional 1H–1H correlation NMR spectra were acquired at 800 MHz on both TOS-1 gangliosides in Me2SO-d6/2% D2O. One-dimensional 1H NMR spectra of G2 and G1 are reproduced in Fig. 4, panels A and B, respectively; two-dimensional TOCSY and NOESY spectra of G1 are represented in Fig. 5, panels A and B; chemical shift data (and 1H–1H coupling constants) are compiled in Table I. The one-dimensional NMR spectrum of the simpler compound, G2, clearly displays two characteristic α-NeuAc H-3eq resonances at 2.769 and 2.727 ppm, as well as four β-anomeric resonances (βJ1,2 ≈ 7–9 Hz) in the downfield region, of which at that 4.512 ppm could be tentatively assigned as the β-GlcNAc H-1, that at 4.160 ppm most likely as the β-Glc H-1, and those at 4.248 and 4.117 ppm as two β-Gal H-1, in agreement with the glycosyl composition analysis. Based on analogy to published NMR data, the rather upfield chemical shift of one of the β-Gal H-1 resonances suggested the possibility of a type I chain core, as only the terminal β-Gal H-1 of LeC4Cer has ever been observed upfield of 4.15 ppm (18). Three NAc methyl singlets (1.888, 1.875, and 1.759 ppm) are likewise consistent with the presence of two α-NeuAc and one β-GlcNAc residue in the ganglioside; moreover, the chemical shifts of the two more downfield singlets suggested the presence of NeuAc in both α2→3 and α2→6 linkages, respectively (10, 17, 20). The possibility that the two NeuAc residues are linked together (i.e. as a NeuAco2→8NeuAco2→X disaccharide structure) appears unlikely, as data previously shown.
published for disialosyl versus parent monosialosyl gangliosides (16, 33) shows that attachment of a second NeuAc α2→3 to the first results in substantial shift changes for key resonances of the newly internalized residue, upfield for H-3eq and downfield for H-3ax and H-4. For example, in comparing these resonances in NeuAc A of GD3 versus GM3, δ = 0.41, +0.33, and +0.28 ppm, respectively; these changes result in chemical shifts of 2.34, 1.69, and 3.83 ppm, respectively, for these signals in GD3 (33). There are no indications from the data for H-3eq, H-3ax, or H-4 of NeuAc in ganglioside G2 for such shielding/deshielding interactions affecting either of the NeuAc residues. The presence of two other resonances in the downfield region, a broad doublet at 4.091 ppm, and a narrow signal, resembling a β-Gal H-4 in its splitting pattern, but unusually deshielded at 4.293 ppm, are unique features compared with any previously published spectral data.

The spectrum of G1 clearly displays several similar features, in particular the latter two resonances essentially unaffected at 4.091 and 4.294 ppm, respectively. These similarities suggest a close structural relationship between G1 and G2, with the former most likely representing addition of a single monosaccharide residue to the latter. The additional residue is manifested by the appearance of a second downfield β-anomeric resonance tentatively assigned as β-GalNAc H-1. Significant glycosylation-induced shift changes are observable involving the β-Gal H-1 resonance at 4.117 ppm in G2 (apparently shifted to 4.207 ppm in G1), the β-GlcNAc H-1 resonance at 4.512 ppm (shifted to 4.590 ppm in G1), and the α-NeuAc H-3eq resonance at 2.769 ppm (shifted to 2.571 ppm in G1). Changes in chemical shifts of 2 of 3 NAc methyl singlets are also apparent, with those at 1.888 and 1.759 ppm, shifting to 1.871 and 1.793 ppm (coincident with the NAc signal for the β-GalNAc residue), respectively. These suggest the presence in G2 of a terminal NeuAcα2→3Galβ1→3GlcNAcβ1→trisaccharide to which, in G1, a β-GalNAc has been added in a position to affect resonances of these three residues most strongly. Comparison of the data on G2 and G1 available from two-dimensional NMR analysis shows that the 1H resonance undergoing the largest change is that for H-4 of the β-Gal correlating with the H-1 at 4.117 in G2 and 4.207 ppm in G1 (ΔδH,4 = 0.3 ppm), suggesting attachment of the GalNAcβ1→4 to this

![Diagram](http://www.jbc.org)
Gal in G1. Consistent with this tentative assessment, the chemical shifts of resonances associated with the terminal disaccharide, i.e., a-NeuAc H-3eq, H-3ax, H-4, and NAc methyl, b-Gal H-1, along with the newly added b-GalNAc H-1 and NAc methyl, are very similar in G1 to those found for the NeuAc a23(GalNAc b13Gal b13) trisaccharide of GM2 ganglioside (16, 21, 34). This supports by analogy that the structural relationship between G2 and G1 is the addition of GalNAc b134 to b-Gal of the terminal NeuAc a23Gal b13 disaccharide of the former, consistent with the reactivity of G1 but not G2 with anti-GM2 MAb MK-18.

Because neither reactivity with antibodies nor analysis of NMR spectra by analogy and chemical shift changes are completely unambiguous indicators of structure, and especially in view of the complexity and novelty of the one-dimensional NMR spectra, further glycosyl connectivity analysis was carried out by acquisition of 1H−1H NOESY data (shown for G1 only in Fig. 5, panel B), which is an indicator of spatial proximity between nuclei. With few published exceptions (see Ref. 35), the magnitudes of interresidue NOEs are greatest between those nuclei directly connected by glycosyl linkages. In the case of G2, strong interresidue NOEs can be clearly observed between b-Gal H-1 and b-GlcNAc H-3; between b-GlcNAc H-1 and H-3 of the other b-Gal; and between b-Glc H-1 and Cer H-1a/H-1b (an NOE between the internal b-Gal H-1 and the b-Gly residue is somewhat ambiguous, but this linkage is unlikely to be anything but Galβ1-4). These data are consistent with a type 1 chain, Lc4Cer core structure for G2. Unfortunately, no NOEs were observed between either of the NeuAc residues and those of the core glycan. The reactivity of G2 with MAb FH9, previously shown to react with a disialosyl-Lc4Cer having NeuAc residues linked α2→3 to Galβ1-3 IV and α2→6 to GlcNAcβ1-3 III, suggests the same structure for this ganglioside, and is certainly consistent with the NMR data. In the

Fig. 5. Downfield sections (3.00–4.90 ppm) of two-dimensional proton correlation NMR spectra of TOS-1 ganglioside fraction G1 in Me2SO-d6/D2O at 308 K. A, TOCSY (200 ms mixing time); B, NOESY (150 ms mixing time). Arabic numerals refer to ring protons of residues designated by roman numerals for the corresponding glycan sequence starting from b-Glc = I (see Table I). S refers to protons of the sphingosine backbone. Cross-peak labels marked by asterisks in B correspond to key interglycosidic NOE correlations, i.e., GalNAc V-1/Gal IV-4; Gal IV-1/GlcNAc III-3; GlcNAc III-1/Gal II-3; Gal II-1/Glc I-4; Glc I-1/S-1a, S-1b.
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Proton chemical shifts (ppm from tetramethylsilane) and $^{3}J_{1,2}$ coupling constants (Hz) for TOS-1 gangliosides in dimethylsulfoxide-d$_6$/2% D$_2$O at 308 K

| NeuAcO$_2$ | GalNAc$_1$ | GlcNAc$_1$ | 3Gal$_1$ | 4Gal$_1$ | 3Glc$_1$ | 4Glc$_1$ | 1Cer |
|-----------|-----------|-----------|--------|--------|--------|--------|------|
| G2        | A         | B         | V      | IV     | III    | II     | I    |
| H-1       | 4.117     | 4.512     | 4.248  | 4.160  | 3.421  | (a)    |      |
| ($^{3}J_{1,2}$) | (7.7) | (8.3) | (7.8) | (7.7) | 3.972 | (b)    |      |
| H-2       | 3.276     | 3.397     | 3.352  | 3.037  | 3.765  |        |      |
| H-3       | 2.769 (eq)| 2.727 (eq)| 3.962  | 3.659  | 3.275  | 3.307  | 3.884 |
| H-4       | 3.534     | 3.511     | 3.593  | 3.278  | 4.293  | 3.275  | 5.355 |
| H-5       | 3.070     |          | 3.070  | 3.275  | 4.293  | 3.275  | 5.355 |
| H-6       | 3.586 (a) | 1.933     |        |        |        |        |      |
| Nac       | 1.888     | 1.875     | 1.759  |        |        |        |      |
| G1        | A         | B         | V      | IV     | III    | II     | I    |
| H-1       | 4.809     | 4.207     | 4.590  | 4.249  | 4.160  | 3.414  | (a)  |
| ($^{3}J_{1,2}$) | (8.9) | (7.8) | (8.0) | (7.5) | (7.7) | 3.977 | (b)  |
| H-2       | 3.742     | 3.169     | 3.427  | 3.356  | 3.039  | 3.769  |        |
| H-3       | 2.571 (eq)| 2.719 (eq)| 3.360  | 3.759  | 3.605  | 3.277  | 3.878 |
| H-4       | 1.590 (ax)| 1.263 (ax)| 3.590  | 3.095  | 3.270  | 5.349  |        |
| H-5       | 3.690     | 3.506     | 3.526  | 3.989  | 3.323  | 4.294  | 3.275 |
| H-6       | 3.619     | 3.493     | 3.095  | 3.270  | 5.535  |        |      |
| Nac       | 1.874     | 1.871     | 1.793  | 1.793  | 1.933  |        |      |

* G2, G1: purified disialogangliosides from fractions G2 and G1, respectively.

case of G1, the same interresidue NOEs can be observed (including one between the internal $\beta$-Gal H-1 and $\beta$-Glc H-4); an additional strong NOE is observable between $\beta$-GalNAc H-1 and $\beta$-Gal IV H-4, again consistent with the Glycosyl composition and NMR analysis, and the relative abundances of the molecular species are essentially consistent with the fatty acid analyses, including the greater proportion of the 16:0 species in G2. As often is the case with ESI-MS, the higher molecular weight ganglioside G1 also produces a more abundant set of dicationized doubly charged molecular ions, $[M-2H+4Na]^{2+}$, observed at m/z 1050.5, 1105.5, and 1106.5 (panel B).

Both gangliosides readily lose 1 and 2 residues of NeuAc (as NeuAc–H2Na), giving abundant sets of Y fragments at m/z 1562/1672/1674 and 1249/1359/1361 for G2, and at m/z 1765/1875/1877 and 1452/1562/1564 for G1. The latter sets in each case represent double cleavage ions, which might be expected to be of low abundance, but in fact they are more abundant than the losses of single NeuAc residues. An additional loss of 1 HexNAc residue from G1 yields a set of Y fragments identical to that at 1249/1359/1361 observed for G2, and below this mass, the fragmentation patterns are very similar, except for the higher abundance of the 16:0 species in G2. Thus Y2 (HexCer) and Y1 (HexCer) fragments are observable at m/z 884/994/996 and 722/832/834 in both spectra, whereas a set of Y$_{34}$ fragments, which could be expected at m/z 1400/1510/1512, is not observed above noise level in either spectrum. Y$_{34}$ fragments at m/z 1087/1197/1199 are similarly not observed, although these represent a double cleavage not expected to be very abundant. An interesting double cleavage of one NeuAc residue and the sphingosine moiety at the C2–C3 bond ("G" fragmenta- tion) yields sets of fragments at m/z 1325/1435/1437 for G2 (panel A) and at m/z 1528/1638/1640 for G1 (panel B). Such cleavages of the sphingosine C2–C3 bond have been observed previously in positive ion ESI-MS of glycosphingolipids (37, 38).

The fragmentation data are consistent with the proposed structures for G1 and G2, but in the absence of certain ions as mentioned above, they are not unambiguous with respect to key parts of their putative sugar sequences. For example, in the absence of an observable set of Y$_{34}$ fragments, these data would strongly support the structures of G2 and G1 as IV$_{3}$NeuAc, III$^{\beta}$NeuAc-Lc$_{2}$Cer and IV$^{\beta}$-GlcNAc, IV$^{\beta}$NeuAc, III$^{\beta}$NeuAc-Lc$_{2}$Cer, respectively.

Electrospray Ionization Mass Spectrometry—Low resolution ESI mass spectra of both TOS-1 gangliosides were acquired by direct infusion as described previously (29). Single quadrupole mass spectra of G2 and G1 are reproduced in Fig. 6, panels A and B, respectively; assignments for fragments are summarized in Scheme 1, a and b (nominal, monoisotopic m/z are used throughout). The most abundant molecular adduct ions [M–2H+3Na]$^{+}$ are observed for G2 (panel A) at m/z 1975, 1855, and 1987, corresponding to a glycan formula Hex$_{2}$HexNAc$\cdot$NeuAc$_{2}$ in combination with ceramides consisting of d18:1 sphingosine predominantly N-acylated with 16:0, 24:1, and 24:0 fatty acids. In the spectrum of G1 (panel B), the most abundant molecular adduct ions are observed at m/z 2078, 2188, and 2190, corresponding to a glycan formula Hex$_{2}$HexNAc$\cdot$NeuAc$_{2}$ in combination with the same predominant ceramides. The additional HexNAc residue observed for G1 is consistent with the glycosyl composition and NMR analysis, and the relative abundances of the molecular species are essentially consistent with the fatty acid analyses, including the greater proportion of the 16:0 species in G2. As often is the case with ESI-MS, the higher molecular weight ganglioside G1 also produces a more abundant set of dicationized doubly charged molecular ions, [M–2H+4Na]$^{2+}$, observed at m/z 1050.5, 1105.5, and 1106.5 (panel B).
DISCUSSION

Many studies indicate that certain GSLs and gangliosides in tumor cells define their malignancy, in terms of metastatic potential (1). Our initial, comparative study on GSL and ganglioside patterns showed enhanced expression of GM2 or gangliosides higher than GM2 (including slow-migrating disialo-ganglioside species) in RCC having metastatic deposits and in the deposits themselves compared with RCC without metastatic deposits (4). Our subsequent studies were focused on the following: (a) establishment of RCC cell lines, and their tumor cell biological properties (8); (b) biochemical characterization of major gangliosides showing enhanced expression in RCC tissues, identified as monosialosyl- and disialosylgalactosylgloboside (MSGG and DSGG) (10); (c) production of mAb RM2 directed to TOS-1 cells, mAb RM1 directed to RCC (10), and mAb 5F3 directed to RCC gangliosides; and (d) application of these antibodies to compare expression of defined epitopes in RCC having metastatic deposits, the deposits themselves, and RCC without metastatic deposits. Expression of each of the antigens defined by RM2, RM1, and 5F3 was correlated with metastatic potential (9). This series of studies indicated that in addition to previously identified DSGG two other disialogangliosides are found as major components of RCC and TOS-1 cells: G1 (defined by mAb RM2) and G2 (defined by mAb FH9). Expression of SLe⁰ or dimeric Le⁰ in RCC was found to be correlated with degree of differentiation, but not with metastatic potential (5, 6). In contrast, expression of SLe⁰ and SLe⁰ in colorectal carcinoma was highly correlated with metastatic potential (1–3).

Our results clearly identify one major disialoganglioside (G1) expressed in RCC tissue and a cell line derived therefrom (TOS-1) as GalNAc β1–4 linked to terminal Gal of disialosyl Lac₃Cer, and the other disialoganglioside (G2) as disialosyl Lac₃Cer, i.e. FH9 antigen reported previously as colonic cancer-associated antigen defined by mAb FH9. Disialoganglioside fraction of RCC tissue in some cases was identified as DSGG, which shows nearly identical TLC mobility, and co-migration in various solvent systems, to that of a novel antigen now identified as GalNAcDSLc₄. This novel antigen is a hybrid-type composed of ganglio-series with the same structure as GM2 (region I), and lacto-series type 1 with a vicinal disialosyl residue (region II in Structure I in the Abstract, and Fig. 7). Ganglio-series structures are characterized by β1–4GalNAc linked to Gal, whereas lacto-series are characterized by

![FIG. 6. Positive ion mode electrospray ionization mass spectra of TOS-1 ganglioside fractions G2 (A) and G1 (B). Major sodiated molecular ion adducts [M–2H+3Na]+ (as well as [M–2H+4Na]+ for G1) and key fragments are labeled (nominal, monoisotopic m/z). For explanation of fragments, see text and Scheme 1.](http://www.jbc.org/)

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3 A ganglioside from RCC tissue showing the same TLC mobility as G1 (i.e. between that of GD1a and GD1b) but not showing reactivity with RM2, was found. mAb 5F3 was prepared against this ganglioside. Immunohistological studies of many cases of RCC, using mAbs RM1, RM2, and 5F3, showed clear correlation between metastatic potential and the epitopes defined by these mAbs (A. Ito, Dr. Med. Sci. (Ph.D. thesis) presented at Tohoku Univ. School of Medicine, Japan, 1997).
genes, or post-translational modification of the transferases. Three disialogangoligosides are present in RCC tissue. One, expressed in TOS-1 cells, is now identified as GalNAcDSLc4, defined by and reacting strongly with mAb RM2. Another, expressed in ACHN cells is now identified as DSGG, which was previously described as one of the major gangliosides of RCC tissue and is now defined by another mAb (5F3) but not by RM2. The third disialogangoligoside, expressed in TOS-1 cells, is now identified as disialosyl Le4, and is identical to the antigen of mAb FH9, described previously as a colon cancer-associated antigen (11).

In a previous publication (10), we mistakenly assigned the specificity of mAb RM2 as being directed to DSGG (G3), although RM2 was raised against TOS-1 cells. The mistake may have arisen because of the following: (a) disialogangoligoside fractions prepared from a few cases of RCC tissue contained DSGG and G1 (GalNAc disialosyl Le,Cer, abbreviated GalNAcDSLc4) in varying proportions; some RCC tissues contained globo-series structures as major component, whereas others contained ganglio/lacto-series structures as major component; (b) DSGG and G1 (GalNAcDSLc4) show identical TLC mobility, i.e. co-migrate together in various solvent systems; (c) the sample prepared from RCC tissue and used for structural analysis contained DSGG as major component but may have been contaminated with a small quantity of G1 showing reactivity with RM2. We have now clarified that RM2 is directed specifically to G1, whereas another mAb, 5F3, is directed specifically to DSGG. It is possible that RCC tissue expresses high level of G1 in some cases, and DSGG in other cases. Cell lines representing the former and latter situations are TOS-1 and ACHN respectively.

From a clinicopathological and histological point of view, RCC cases expressing either GalNAcDSLc4 or DSGG are highly malignant, and show initiation of lymph node and distant metastasis at an early stage (9). Preferential adherence of TOS-1 cells to lung tissue, determined by Stamper-Woodruff assay, is based on interaction of major disialogangoligoside present in TOS-1 with receptor present on peripheral endothelial cells. This process is strongly inhibited by RM2 (7) and is therefore presumed to be mediated by GalNAcDSLc4, rather than DSGG and its corresponding peripheral receptor. Interestingly, SLeα*, SLeα*, or their analogs, which bind to E-selectin, are not involved in this process. Studies on the identification of the receptors for both GalNAcDSLc4 and DSGG are essential to understand mechanisms for RCC metastasis and its possible inhibition.

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