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Monoclonal antibody (5F3) defining renal cell carcinoma-associated antigen disialosyl globopentaosylceramide (V^3NeuAcIV^{6}NeuAcGb5), and distribution pattern of the antigen in tumor and normal tissues

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Renal cell carcinoma (RCC) has been characterized by high expression of three types of disialogangliosides: two based on lacto-series type 1 structure (disialosyl Lc_4, GalNAc disialosyl Lc_4), the other based on globo-series structure (disialosyl globopentaosylceramide; disialosyl Gb5). The present study established a mAb, 5F3, directed to disialosyl Gb5. 5F3 was established after immunization with RCC cell line ACHN. The major disialoganglioside antigen isolated from ACHN cells, showing specific reactivity with 5F3, was characterized unequivocally as disialosyl Gb5 (V^3NeuAcIV^6-NeuAcGb5) by identification of the core structure as globopentaosylceramide (Gb5) after enzymatic and acid hydrolysis, and by 2-dimensional ^{1}H-NMR spectroscopy. 5F3 does not react with monosialosyl Gb5 (V^3NeuAcGb5), Gb5, or any lacto-series structures. 5F3 strongly stained 19 of 41 cases of primary RCC tissue. It reacted with proximal tubules (but not distal tubules) of kidney, microglial cells of cerebrum and cerebellum, goblet cells of stomach and intestine, smooth muscle of various organs. It did not react with parenchymatous cells of various organs, except for kidney epithelia and prostate stroma. Immunostaining of RCC tissue by mAb 5F3, in combination with staining by other antibodies directed to globo-series and lacto-series structures, has prognostic significance in defining metastatic potential of RCC.

Keywords: disialoganglioside, globo-series, monoclonal antibody 5F3, metastasis, renal cell carcinoma

Abbreviations: Glycosphingolipids are abbreviated as recommended by IUPAC-IUB (*J Biol Chem* 257: 3347–3351, 1982; 262: 13–18, 1987). Gangliosides are abbreviated according to Svennerholm (*J Lipid Res* 5: 145–55, 1964). BSA, bovine serum albumin; CM, chloroform-menthanol; DSGb5, disialosyl globopentaosylceramide (V^3NeuAcIV^{6}NeuAcGb5); $DSLc_4$, disialosyl Lc_4 (IV^3NeuAcIII^{6}NeuAcLc_4); ELISA, enzyme-linked immunosorbent assay; GalNAc$DSLc_4$, GalNAc-disialosyl Lc_4 (IV^4GalNAcIV^{3}NeuAcIII^{6}NeuAcLc_4); Gb5, globopentaosylceramide (IV^3GalGb4); GSL, glycosphingolipid; HPTLC, high-performance thin-layer chromatography; mAb, monoclonal antibody; MSGb5, monosialosyl globopentaosylceramide (V^3NeuAcGb5); RCC, renal cell carcinoma.

Introduction

Structure and function of glycosphingolipids (GSLs) are altered greatly in various types of cancer, and provide a basis for invasive and metastatic properties of tumor cells [1]. Renal cell carcinoma (RCC) has strong metastatic properties, *e.g.*, distant metastasis to lung or lymph nodes occurs before the

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initial diagnosis is made in many clinical cases. During examination of GSLs in extracts from a large number of surgically resected RCC tissues, an increase of slow-migrating gangliosides (having TLC mobility similar to or slower than that of GM2) was noted in primary and metastatic RCC tissues. Increase of gangliosides migrating on TLC to a position between ganglio-series gangliosides GD1a and GD1b was considered as a factor promoting RCC metastasis [2]. The mAb RM2 was established against RCC cell line TOS-1, and was found to react with slow-migrating RCC ganglioside [3]. The RM2 antigen, isolated from a large quantity of cultured TOS-1 cells, was identified recently as GalNAcDSLc$_4$ (IV^{4}GalNAcIV^{3}NeuAcIII^{6}NeuAcLc$_4$). Another disialoganglioside from TOS-1 cells was characterized as DSLc$_4$ (IV^{3}NeuAcIII^{6}NeuAcLc$_4$) [4], the same ganglioside previously identified as a colonic cancer-associated antigen defined by mAb FH9 [5]. RCC tissue, however, contains two major globo-series gangliosides, disialosyl globopentaosylceramide (DSGb5; V^{3}NeuAcIV^{6}NeuAcGb5) and monosialosyl globopentaosylceramide (MSGb5; V^{3}NeuAcGb5) [3]. A mAb directed to MSGb5 was established as RM1 [3], but we did not have a mAb directed to DSGb5. Slow-migrating gangliosides expressed in RCC appear to act as promoting factors for metastasis. These gangliosides are co-migrating and indistinguishable from each other by TLC with various solvent systems. It is therefore essential to have a mAb directed specifically to DSGb5. Here, we report establishment and epitope characterization of new mAb 5F3. Our results clarify: (i) the presence of three types of gangliosides expressed highly in RCC tissues: one based on lacto-series type 1 chain, the second a hybrid type between ganglio- and lacto- type 1, and the third having globo-series as core structure; (ii) expression pattern of the antigen defined by 5F3 in RCC tissues and in various normal tissues. Clinicopathological significance of expression patterns of various antigens defined by a series of mAbs directed to globo-series gangliosides (5F3, RM1), to lacto-series type 1 gangliosides (FH9), and to hybrid-type gangliosides (RM2) is to be discussed.

Materials and methods

Extraction of gangliosides from ACHN cells

ACHN cells were purchased from Dainihonseiyaku Co. (Tokyo, Japan); they were originally derived from malignant pleural effusion of a patient with widely metastatic RCC. Gangliosides were extracted from ACHN cells as described previously [4]. Briefly, ~30 ml of packed cells were extracted by homogenization and filtration with 15 volumes of isopropanol-hexane-water (55 : 25 : 20 v/v/v). The extraction/filtration procedure was repeated once more. Combined extracts were evaporated and divided into upper and lower phases by Folch's partition [6]. The upper phase was dialyzed against distilled water, dissolved in CM-water (30 : 60 : 8 v/v/v), and fractionated by DEAE-Sephadex A25 column chromatography into neutral GSLs and gangliosides. Gangliosides were further separated into mono-, di-, and trisialosyl fractions by step-wise elution with 0.03, 0.13, 0.45 M ammonium acetate in CM-water (30 : 60 : 8 v/v/v). The eluted gangliosides were dialyzed against distilled water and lyophilized. Each fraction was spotted onto Silica Gel 60 HPTLC plate (Merck, Darmstadt, Germany), developed in a solvent system of CM-0.2% aqueous $CaCl_2$ (50 : 40 : 10 v/v/v), and visualized by spraying with 0.5% orcinol in 2N sulfuric acid.

Purification of DSGb5

Gangliosides were separated on preparative HPTLC. 100 µl of sample was streaked across a HPTLC plate, developed in a solvent system of CM-0.2% aqueous $CaCl_2$ (50 : 50 : 10 v/v/v), and visualized by spraying with 0.001% primulin in acetone-water (4 : 1 v/v). Bands presumed to be DSGb5 were marked with a pencil under UV light. Marked bands were scraped from the plate by razor blade, and DSGb5 was extracted from the silica by sonicating for 20 min in isopropanol-hexane-water (55 : 25 : 20 v/v/v). The silica was removed by centrifuging at 1000 × g for 10 min and re-extracted as above, and the combined supernatants were dried under N_2 stream. Primulin was removed from a ganglioside sample by HPLC on a Lichrosorb-NH_2 column (0.4 × 25 cm, Merck) using a mobile phase consisting of acetonitrile: 5 mM phosphate buffer, pH 5.6, with a linear gradient from 80 : 20 to 71 : 29 over 35 min at a flow rate of 1.0 ml/min. The eluate was monitored at 215 nm.

Reference GSLs, and mAbs

MSGb5 was prepared from ACHN cells. GalNAcDSLc$_4$ and DSLc$_4$ were prepared from TOS-1 cells derived from back metastatic lesion of an RCC patient [7]. Gb5 was prepared by desialylation of MSGb5. $2 \rightarrow 3$ sialosylparagloboside ($2 \rightarrow 3$SPG; IV^{3}NeuAcnLc$_4$) was prepared from human placenta. $2 \rightarrow 6$ sialosylparagloboside ($2 \rightarrow 6$ SPG; IV^6-NeuAcnLc$_4$) was prepared from human colonic adenocarcinoma. Gb3, Gb4, Forssman antigen, and GM3 were purchased from Wako (Tokyo, Japan); GM2, GM1, asialo-GM1, GM1b, and GD3 from Seikagaku-kogyo (Tokyo); GD1a, GD1b, GT1b, GQ1b, and sialyl-Le^X from Diatron (Tokyo).

Anti-GalNAcDSLc$_4$ mAb RM2, raised originally TOS-1 cells, was established in our laboratory [3,4]. Anti-SSEA-3 mAb (directed mainly to Gb5 but also showing cross-reactivity with Gb4) was obtained from Developmental Studies Hybridoma Bank (Univ. of Iowa, Iowa City, IA).

Establishment of mAb 5F3 directed to DSGb5

ACHN cells (1×10^7) suspended in PBS were injected intraperitoneally into Balb/C mice on days 1, 6, and 11. On day 16, another 1×10^7 cells were injected both intraperitoneally and intravenously as the final boost. Three days later, host spleen cells were harvested and fused with mouse myeloma P3X63Ag8.653 cells. Hybridomas were screened by

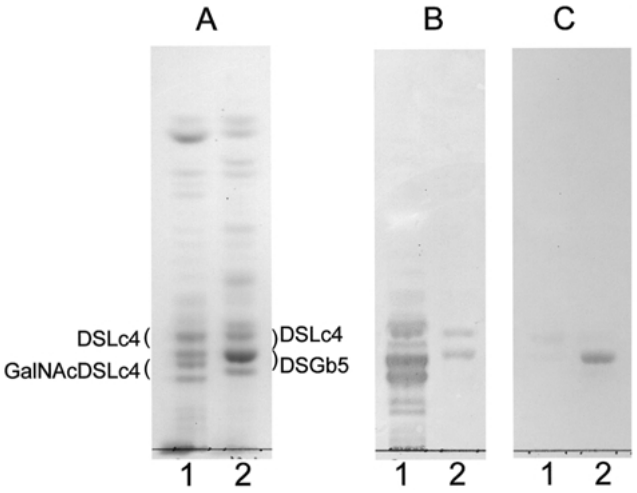

Figure 1. TLC immunostaining of TOS-1 and ACHN disialogangliosides. HPTLC was developed in a solvent system of CM-0.2% aqueous $CaCl_2$ (50 : 40 : 10). Bands were stained with mAbs as described in Materials and Methods and compared with patterns from orcinol-sulfuric acid staining. Lane 1, disialosyl fractions of TOS-1 gangliosides. Lane 2, disialosyl fractions of ACHN gangliosides. Panel A: Orcinol-sulfuric acid staining. Panel B: mAb RM2 immunostaining. Panel C: mAb 5F3 immunostaining.

ELISA as described below, and cloned by the limiting dilution method. Immunoglobulin isotype was determined using subclass specific antibodies.

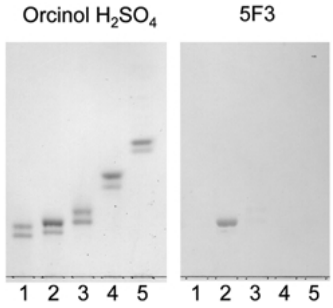

Figure 2. TLC immunostaining of purified GSLs with mAb 5F3. Purified GSLs were developed in a solvent system of CM-0.2% aqueous $CaCl_2$ (50 : 40 : 10), and bands were stained with mAb 5F3 as described in Materials and Methods. Lane 1, GalNAcDSLc4 purified from TOS-1. Lane 2, DSGb5 purified from ACHN. Lane 3, DSLc4 from TOS-1. Lane 4, MSGb5 from ACHN. Lane 5, Gb5 desialylated from MSGb5. Left, orcinol-sulfuric acid staining. Right, mAb 5F3 immunostaining.

Determination of specificity of mAb 5F3

The antibody specificity was determined by based on TLC immunostaining with various GSLs, by a modification of previously-described procedure [8]. GSLs were applied onto HPTLC plates for chromatography using a solvent system of CM-0.2% aqueous $CaCl_2$ (50 : 40 : 10 v/v/v). Plates were air-dried, and immersed in 0.5% poly(isobutyl metacrylate) (high molecular weight; Aldrich Chemical Co., Milwaukee, WI) in hexane-chloroform (9 : 1) for 40 sec, air-dried, blocked with 3%

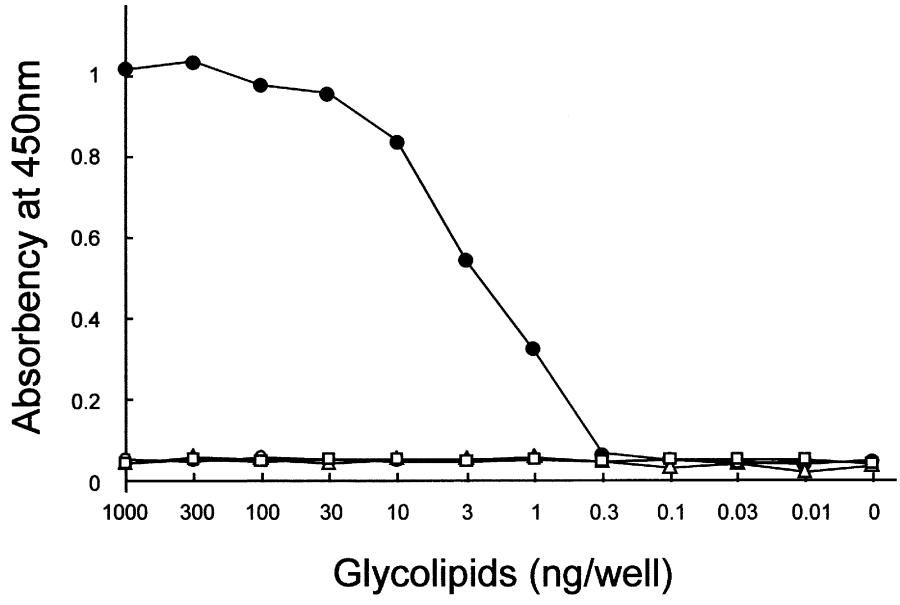

Figure 3. Reactivity of mAb 5F3 with various GSLs. Purified GSLs were dissolved in ethanol (1 ng/50 μl), and serially diluted solution was added to each well of 96-well flat-bottom polystyrene plate, air-dried, and blocked with 3% BSA in PBS. Each well was added with 5F3, and antibody-binding activity was determined by ELISA. ●, DSGb5 purified from ACHN. □, GalNAcDSLc$_4$ purified from TOS-1. ○, MSGb5 purified from ACHN. △, DSLc$_4$ purified from TOS-1, Gb5, Gb4, Gb3, Forssman glycolipid, asialo GM1, GM3, GM2, GM1a, GM1b, GD3, GD1a, GD1b, Le^x, SLe^x, 2 → 3 sialosylparagloboside, 2 → 6 sialosylparagloboside.

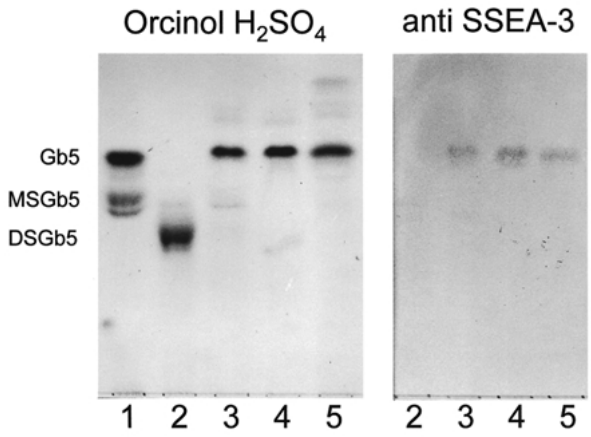

Figure 4. Analysis of desialylated product of DSGb5. Left panel, orcinol-sulfuric acid staining. Right panel, immunostaining by anti-SSEA-3 mAb. Lane 1, standard GSLs. Lane 2, no treatment. Lanes 3 and 4, treated with *Arthrobacter ureafaciens* sialidase for 1 h and 20 h, respectively, at 37°C. Lane 5, treated with 1% acetic acid for 1 h at 95°C.

BSA in PBS for 1 h, and reacted with culture supernatants of mAb 5F3 for 2 h at room temperature. Plates were washed, incubated with biotinylated goat anti-mouse IgM antibody for 1 h, incubated with Vector avidin-biotin solution (Burlingame, CA) for 30 min, and stained with 0.05% 3′, 3-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl (pH 7.6).

Reactivity was also confirmed by ELISA with various purified GSLs. GSLs were dissolved in ethanol (1 ng/50 µl), and serially diluted solution was added to each well of 96-well polyvinyl chloride plates (Sumitomo Bakelite, Tokyo), dried at 37°C, and washed in PBS. Each well was blocked with 3% BSA in PBS for 1 h, and reacted with mAb 5F3 for 2 h at room temperature. Each well was washed extensively in PBS containing 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 added with 0.1 M citric-acetate buffer (pH 6.0) containing 0.1 mg/ml of 3,3′, 5,5′-tetramethylbenzidine (TMBZ) and 0.01% H_2O_2. Reaction was stopped by addition of 0.5 M H_2SO_4, and absorbency of the solution at 450 nm was measured with a microplate reader.

Fluorescence-activated cell sorter analysis (FACS) of tumor cell lines and primary cultured cells

TOS-2 was derived from an axillary metastatic lesion of an RCC patient, TOS-3LN from a neck lymph node metastatic lesion of an RCC patient, and TOS-3 from a primary lesion of TOS-3LN [7]. SMKT-R-1, -R-2, -R-3, and -R-4, derived from primary lesions of RCC, were kindly donated by Dr. Noriomi

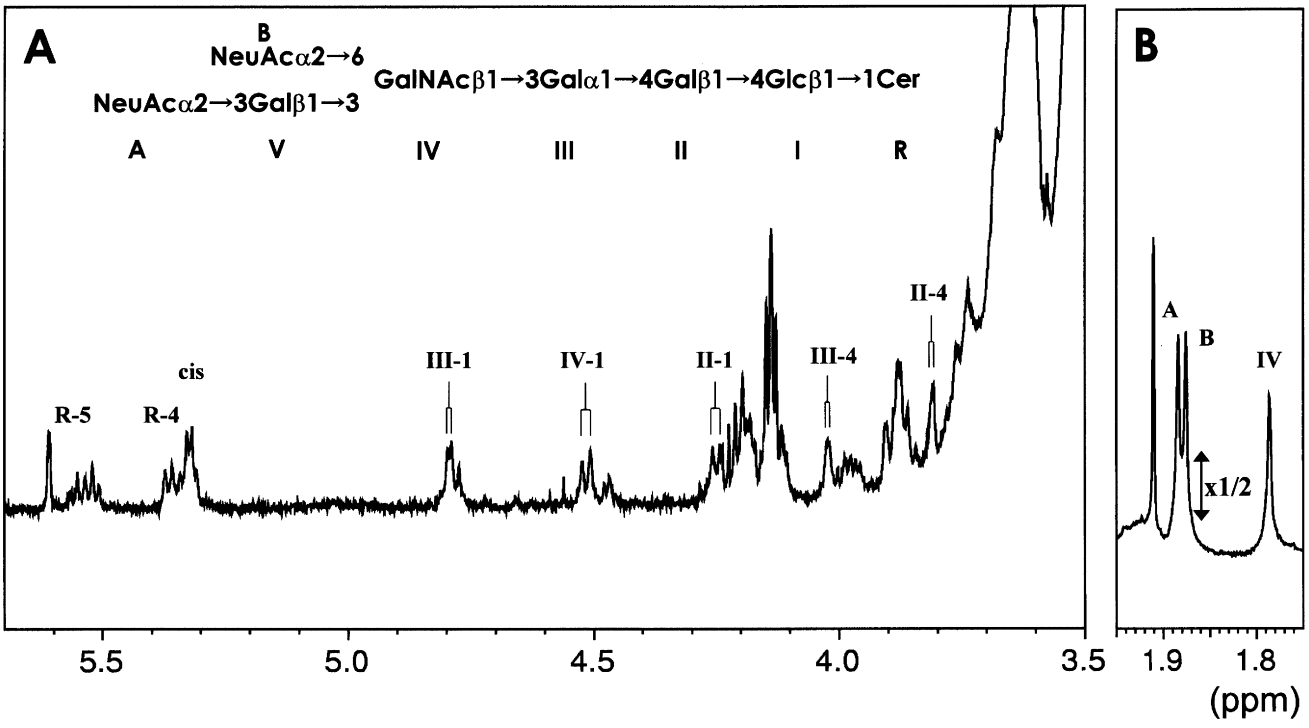

Figure 5. 500 MHz ^{1}H-NMR spectrum of downfield region (A) and methyl proton region of NAc group (B) of the ganglioside defined by mAb 5F3 dissolved in DMSO-d_6/2% D_2O. Spectrum was acquired at probe temperature of 308 K. Arabic numerals refer to ring protons of residues designated by *roman numerals or capital letters* in the corresponding structure drawn at the top of the figure. R refers to protons of the sphingosine backbone only and cis to vinyl protons of unsaturated fatty acids.

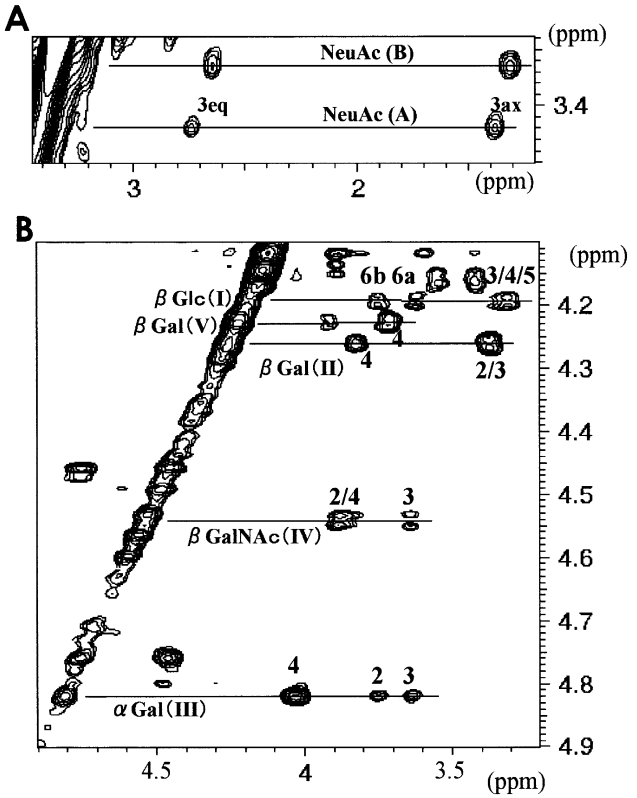

Figure 6. 2D-TOCSY of the ganglioside defined by mAb 5F3 in the sialic acid region (A) and anomeric region (B). Spectrum was acquired at probe temperature of 328 K and mixing time of 350 ms. Arabic numerals refer to ring proton of residues designated by roman numerals in the structure drawn in Figure 5.

Miyao (Dept. of Urology, Sapporo Medical College) [9,10]. 518-T and 518-N, 601-T and 601-N, 720-T and 720-N, 1023-T and 1023-N, and 1214-T and 1214-N, are primary cultured cells from tumor tissues and kidney tissues, respectively (each pair from the same RCC patient). Bladder cancer cell line T-24, prostate cancer PC-3, and embryonal carcinoma NEC-14 and NEC-8 were purchased from Dainihonseiyaku Co. (Tokyo, Japan). Seminoma cell line Tcam-2 was kindly donated by Dr. Yoshihito Mizuno (Dept. of Urology, Kobe University School of Medicine) [11]. Cells were detached with 0.02% EDTA, then 2×10^5–1×10^6 cells were distributed to Eppendorf tubes, incubated with 100 µl primary antibody for 90 min on ice, washed 2 times with 1% BSA in PBS, incubated with 50 µl of FITC-conjugated goat $F(ab')_2$ directed to mouse IgG and IgM (Tago, Burlingame, CA), and washed 2 times with 1% BSA in PBS. Cells were resuspended in PBS, and subjected to FACS analysis. Percent positivity was calculated in relation to negative control.

Immunohistochemical staining of fresh-frozen tissue sections with mAbs 5F3 and RM2

Human materials used in all experiments were obtained from surgery or autopsy, with informed consent of the patients. Avidin-biotin immunoperoxidase staining of fresh-frozen tissue section was performed using Vector staining kit (Burlingame, CA) as described previously [12]. Briefly, resected tissues were rapidly frozen to −80°C in OCT compound. Cryostat sections (5 µm) were mounted on microslides, air-dried, fixed in cold acetone for 2 min, blocked with 1% defatted milk in PBS for 1 h, treated with Vector Avidin/Biotin blocking kit (Burlingame, CA), and incubated with culture supernatant of hybridoma for 2 h at room temperature. Endogenous peroxidase was blocked with 0.03% H_2O_2 in PBS for 5 min. Sections were incubated with biotinylated secondary antibody for 1 h, incubated with Vector avidin-biotin solution for 1 h, and stained with 0.05% 3′,3-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl. Normal mouse IgM was used as a negative control.

Desialylation of DSGb5 and characterization of desialylated structure

DSGb5 (10 µg) was desialylated by treatment with sialidase derived from *Arthrobacter ureafaciens* for 1 h and 20 h at 37°C, or by heating with 1% acetic acid at 95°C for 1 h. The products were analyzed on HPTLC, and immunostained with anti-SSEA-3 mAb.

One- and two-dimensional ^{1}H-NMR spectroscopy

A ganglioside sample was dissolved in dimethyl sulfoxide-d_6/D_2O (98 : 2, v/v) after deuterium exchange with D_2O. One-dimensional ^{1}H-NMR spectra were acquired at 500 MHz on a Bruker (Karlsruhe, Germany) DMX-500 Fourier transform spectrometer. Spectra were recorded at 328 K and the residual HDO signal was suppressed using a presaturation pulse during the relaxation. Two-dimensional ^{1}H-^{1}H-TOCSY experiments were performed at 328 K with a mixing time of 350 ms.

Results

Establishment of mAb 5F3, and its immunostaining pattern with disialogangliosides of ACHN and TOS-1 cells

An IgM mAb 5F3 was established after immunization of mice with ACHN cells as described in Materials and Methods. A series of slow-migrating gangliosides corresponding to $DSLc_4$ and $GalNAcDSLc_4$ were detected in TOS-1 cell extract by orcinol-sulfuric acid reaction. Slow-migrating gangliosides indistinguishable from these were detected in ACHN extract by the same reaction (Figure 1A). However, antibody staining patterns of slow-migrating gangliosides from TOS-1 and ACHN were clearly distinguishable (Figure 1B, C). mAb 5F3, upon TLC immunostaining, showed a single ganglioside band in ACHN, but did not show any band in TOS-1 cells (Figure 1C). The IgM mAb RM2, originally established after immunization of mice with TOS-1, reacted strongly with

Table 1. Proton chemical shifts and $^3J_{1,2}$ coupling constants (Hz) for ganglioside reacted with mAb 5F3 in dimethylsulfoxide-d6/2% D_2O

			NeuAcα2 → 6				
	NeuAcα2 → 3Galβ1		*3GalNAcβ1*	*3Galα1*	*4Galβ1*	*4Glcβ1*	*1Cer*
	A	*V*	*B*	*IV*	*III*	*II*	*I*	*R*
				5F3-ganglioside				
H-1		[4.22][a]		4.521[b] [4.53]	4.790 [4.80]	4.250 [4.26]	[4.18]	
($^3J_{1,2}$)				(8.3)	(3.5)	(7.3)		
H-2				[3.85]	[3.76]	[3.36]	3.039 [3.05]	[3.79]
H-3eq	2.746 [2.75]		[2.64]	[3.64]	[3.64]	[3.38]		[3.93]
H-3ax	[1.39]		[1.32]					
H-4	[3.55]	[3.72]	[3.51]	[3.90]	4.023 [4.04]	3.810 [3.83]		[5.35]
H-5					[4.18]			[5.55]
H-6					[3.64]		[3.63]	[1.93]
					[3.43]		[3.76]	
Nac	1.884		1.876	1.786				
				V^3NeuAc-IV^6NeuAc-Gb5Cer[c]				
H-1		4.210		4.512	4.792	4.255	4.195	
($^3J_{1,2}$)		(7.9)		(8.5)	(3.7)	(7.3)	(7.9)	
H-2							3.045	
H-3eq	2.756		2.638					
H-3ax	1.378		1.330					
H-4		3.685			4.017	3.815		
H-5					4.185			
H-6								
Nac	1.889		1.881	1.789				

[a] data measured at 308 K.
[b] data measured by TOCSY at 328 K.
[c] data reported by Levery et al. (308 K).

disialoganglioside fraction of TOS-1, but also reacted, weakly, with disialoganglioside fraction of ACHN (Figure 1B).

Binding specificity of 5F3 to purified GSLs

Four purified gangliosides from RCC cells ($DSLc_4$, $GalNAcDSLc_4$, MSGb5, DSGb5), and neutral GSL Gb5 were analyzed by TLC with orcinol-sulfuric acid (Figure 2A) and by immunostaining with 5F3 (Figure 2B). Only DSGb5 showed a positive band with 5F3. Weak cross-reactivity was observed with $DSLc_4$ (Figure 2B, lane 3).

The binding specificity of 5F3 to purified GSLs was further confirmed by ELISA. 5F3 showed clear reactivity with DSGb5, but not with MSGb5, $DSLc_4$, Gb5, Gb4, Gb3, Forssman GSL, Gg4 (asialo-GM1), GM3, GM2, GM1a, GM1b, GD3, GD1a, GD1b, Le^x ceramide pentasaccharide, sialyl Le^x ceramide hexasaccharide, 2 → 3 sialosylparaglobo-side, or 2 → 6 sialosylparagloboside (Figure 3).

Characterization of desialylated DSGb5

Desialylated product of DSGb5 migrated to the same level as Gb5 on HPTLC plate, and showed positive reactivity to anti-SSEA-3 mAb (Figure 4).

One and two-dimensional ^{1}H-NMR spectroscopy

In order to identify the chemical structure of the ganglioside reactive with mAb 5F3, one and two dimensional ^{1}H-NMR spectra were acquired at 500 MHz on the ganglioside sample. One dimensional ^{1}H-NMR spectrum of the ganglioside is

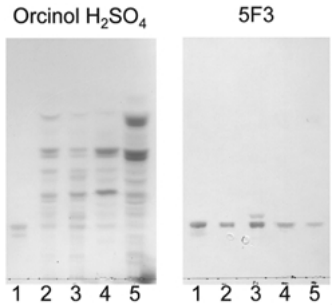

Figure 7. TLC immunostaining of ganglioside fractions extracted from RCC with mAb 5F3. HPTLC was developed in a solvent system of CM-0.2% aqueous $CaCl_2$ (50 : 40 : 10), and bands were stained with mAb 5F3 as described in Materials and Methods. Left panel, orcinol-sulfuric acid staining. Right panel, immunostaining with 5F3. Lane 1, DSGb5 standard. Lanes 2–5, total ganglioside fractions from RCC.

shown in Figure 5. Two-dimensional TOCSY for further assignment of overlapping signals is represented in Figure 6. Further analysis using TOCSY spectra permitted the distinction of signals in overlapping region. Chemical shifts of the structural reporter groups of the ganglioside are shown in Table 1. The one-dimensional ^{1}H-NMR spectrum was the same as that for DSGb5 reported by Levery et al. [13]. Two β-anomeric resonances ($^{3}J_{1,2} = 7–9$ Hz) at 4.250 and 4.521, and one α-anomeric resonance (J = 2–4 Hz) at 4.790 ppm were very close to those from β-Gal II, β-GalNAc IV, and α-Gal III in DSGb5 [13]. The spectra measured at 328 K showed better separation of each signals in the region 4.1–4.3 ppm. Two further β-anomeric resonances distinguished at 4.18 and 4.22 ppm were similar to those of β-Glc I and β-Gal V in DSGb5, respectively. A couple of the resonances of methyl protons at 1.884 and 1.876, and two sets of H-3ax and H-3eq at (1.39, 2.74 ppm) and (1.32, 2.64 ppm) from TOCSY analysis, show the presence of two NeuAc residues in α2-3 and α2-6 linkages, respectively. Collectively, these results suggest that the structure of the ganglioside reactive with mAb 5F3 is V^{3}NeuAcIV^{6}NeuAcGb5Cer as reported by Levery et al. [13].

Distribution pattern of DSGb5 defined by mAb 5F3 in RCC tissues and various cell lines

Expression of DSGb5 in RCC tissue from four cases of primary RCC were studied by TLC pattern with orcinol/sulfuric acid, and by immunostaining with mAb 5F3. The four cases were characterized by orcinol bands with the same mobility as DSGb5, which were all stained by mAb 5F3. No other bands were stained by this mAb (Figure 7). Of the four cases, distant metastases were observed in three (two to lung, one to pancreas). Cell surface reactivity of 5F3 with various cell lines derived from RCC or other types of tumor was analyzed by flow cytometry. Reactivity was expressed in terms of percent positivity relative to negative control (Figure 8). Among four RCC cell lines derived from metastatic deposits, three (ACHN, TOS2, TOS3LN) showed clear reactivity. Data from cell lines derived from primary RCC (TOS3), and from its lymph node metastatic deposit (TOS3LN), indicate that the latter have much higher reactivity. Reactivity was compared for three pairs of cell lines from normal vs. corresponding tumor tissue (518N vs. 518T; 601N vs. 601T; 720N vs. 720T); in each case, the tumor tissue had higher reactivity than normal tissue. Cell lines from primary lesion of RCC (SMKT-

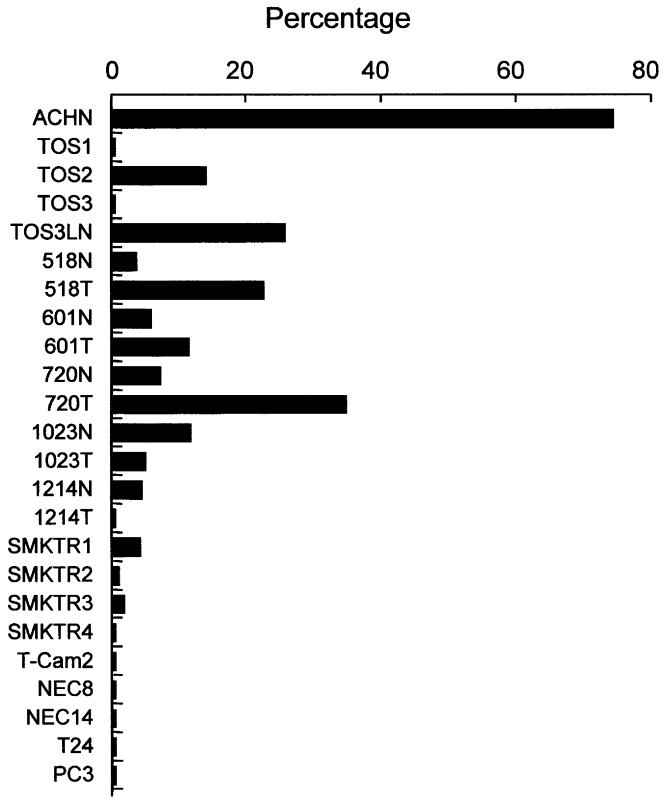

Figure 8. Reactivity of mAb 5F3 with various cell lines and primary cultured cells. Cells were detached, reacted with 5F3, treated with fluorescence-labeled secondary antibody, and subjected to FACS analysis as described in Materials and Methods. Percent positivity was expressed relative to negative control.

Table 2. Summary of immunohistochemical staining of human tissues and renal cell carcinomas with two mAbs

	Positive rate			*Positive rate*	
Tissue	*5F3*	*RM2*	*Tissue*	*5F3*	*RM2*
Normal kidney			Adrenal gland	0/4	0/4
(proximal tubules)	21/21	0/15	Ureteral mucosa	0/1	0/1
(distal tubules)	0/21	15/15	Bladder mucosa	0/3	0/3
Primary RCC	19/41	19/41	Prostate		
Metastatic RCC	2/7	4/7	(epithelium)	0/4	0/4
			(stroma)	4/4	4/4
Cerebrum					
(microglial cells)	1/1	0/1	Seminal vesicle	0/1	0/1
Cerebellum			Testis		
(microglial cells)	1/1	0/1	(seminiferous tubules)	0/3	0/3
			(stroma)	1/3	1/3
Lung	0/2	0/2			
Thyroid gland	0/1	0/1	Spleen	0/3	0/3
Aorta	0/1	0/1			
			Lymph node		
Stomach			(lymphoid nodule)	0/4	0/4
(epithelium)	0/2	0/2	(sinus)	1/4	4/4
(goblet cells)	2/2	2/2			
(stroma)	2/2	2/2	Cardiac muscle	0/2	0/2
Duodenum			Skeletal muscle	0/2	0/2
(epithelium)	0/1	0/1	Smooth muscle		
(goblet cells)	1/1	1/1	(bladder)	3/3	0/3
(stroma)	1/1	1/1	(ureter)	1/1	0/1
Jejunum			(GI tract)	4/4	0/4
(epithelium)	0/1	0/1			
(goblet cells)	1/1	1/1	Skin		
(stroma)	1/1	1/1	(epidermis)	0/2	0/2
Colon			(dermis)	0/2	0/2
(goblet cells)	3/3	3/3	(eccrine sweat gland)	0/2	2/2
(stroma)	3/3	3/3			
			Prostate cancer	0/3	0/3
Liver	0/3	0/3	Bladder cancer	0/3	0/3
Pancreas	0/3	0/3	Testicular cancer	0/3	0/3

R series) had very low reactivity. Cell lines from seminoma (T-Cam2), embryonal carcinoma (NEC8, NEC14), bladder cancer (T24), and prostate cancer (PC3) were negative (Table 2). No staining was observed for rat ascites hepatoma AH7974F expressing high level of GD1α (data not shown).

Immunohistology pattern of DSGb5 with mAb 5F3

In immunohistological staining, positive reactivity of primary RCC and metastatic RCC was 19 out of 41 cases and 2 out of 7 cases, respectively, for mAb 5F3, and 19 out of 41 and 4 out of 7, respectively, for mAb RM2. Of the 41 cases of primary RCC, 10 were positive for both 5F3 and RM2. Of these 10 cases, 8 were also positive for mAb RM1, *i.e.*, positive for all three mAbs. Of these 8 cases, metastasis was observed in 6—a remarkably high incidence of 75% (Ito A, Saito S, unpubl. data).

Interestingly, 5F3 stained luminal epithelia of proximal tubules (21 out of 21 cases), but not distal tubules (0 out of 21). RM2 showed the opposite pattern; it did not stain proximal tubules (0 out of 15 cases) but did stain distal tubules (15 out of 15 cases) (Table 2). RCC is regarded as being derived from proximal tubules, and 5F3 stained luminal epithelia of proximal tubules as noted above (Figure 9A). However, 5F3 staining patterns of RCC tissues correspond to the surface area surrounding the acinar tumor cell mass, or to the entire acinar mass (Figure 9B, C). This indicates that normal kidney epithelia have a clear polarity in distribution of DSGb5, and that such polarity is lost in tumor cells. DSGb5 is absent from human lung, thyroid, aorta, liver, pancreas, spleen, and many other tissues. It is expressed in goblet cells of gastrointestinal epithelia, microglial cells of cerebrum and cerebellum, stroma of prostate, and smooth muscle cells of bladder, ureters, and gastrointestinal tract (Table 2). Its high

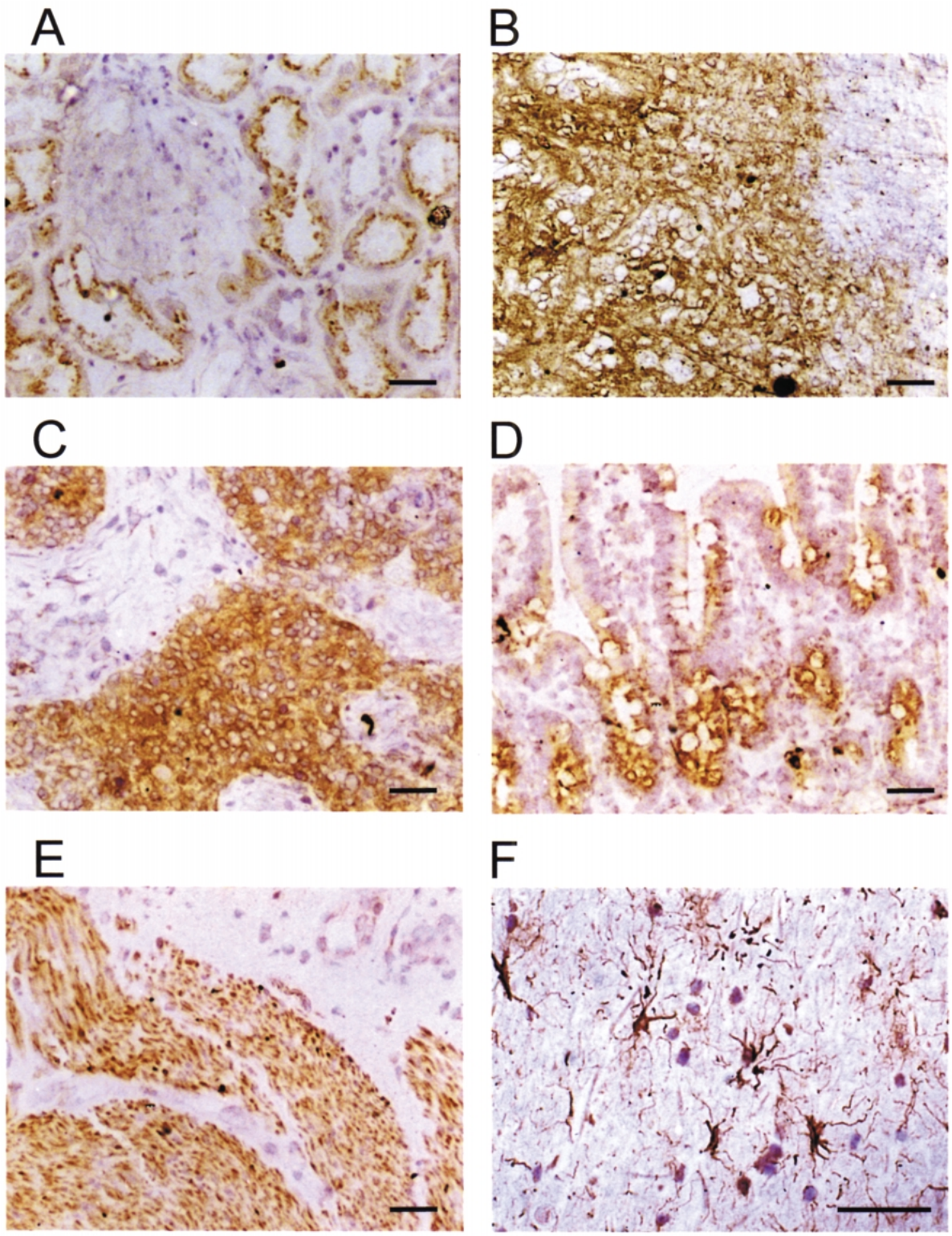

Figure 9. Immunohistochemical staining of fresh-frozen tissue sections of human normal kidney and RCC using mAb 5F3. A, normal kidney; B, primary RCC; C, RCC metastasis to adrenal gland; D, colon; E, urinary bladder (smooth muscle); F, cerebrum. Scale bar: 40 μm. Immunohistochemical staining with 5F3 and RM2 is summarized in Table 2.

concentrations in colon, bladder, and cerebrum are illustrated respectively in Figure 9D, E, and F.

Discussion

Extensive analysis with structural characterization of gangliosides in renal cell carcinoma (RCC) revealed accumulation of four types of gangliosides: ganglio-series (typically GM2) [4], globo-series (MSGb5 and DSGb5) [3], lacto-series type 1 ($DSLc_4$), and ganglio/lacto-type 1 hybrid ($GalNAcDSLc_4$) [4]. A preliminary study indicated that expression of slow-migrating gangliosides is correlated with metastatic potential [2]. Some of these gangliosides are indistinguishable on TLC, and identification must therefore be based on reactivity of bands with specific mAbs by TLC immunostaining. mAbs directed to GM2 (MK-1-8) [14], to $DSLc_4$ (FH9) [5], to $GalNAcDSLc_4$ (RM2) [4], and to MSGb5 (RM1) [3] have been well established. However, no mAb directed to one of the major disialogangliosides, DSGb5, has been available until now.

In our previous report, mAb RM2, established after immunization of mice with TOS-1 cells, was incorrectly assigned as being directed to "disialosyl galactosylgloboside" [3]. This mistake was based on reactivity of RM2 with the disialoganglioside isolated from RCC tissue. However, chemical analysis was not sufficiently sensitive to detect a small quantity of RM2-reactive ganglioside in the ganglioside fraction. The major ganglioside present in the specimen was correctly identified as "disialosyl galactosylgloboside", *i.e.*, DSGb5, by mass spectrometry and NMR spectroscopy. More recently, the real RM2 antigen was identified as $GalNAcDSLc_4$ [4]. It is clear from these observations that RCC tissues express three types of disialoganglioside, in addition to globo-series monosialoganglioside.

Expression patterns of all the above gangliosides should be studied systematically in order to establish exact correlations between ganglioside expression and metastatic potential/invasiveness of RCC. In this study, we successfully established mAb 5F3 directed to DSGb5. Using this new mAb, we characterized expression patterns of DSGb5 in primary RCC and metastatic lesions, as well as in normal kidney and many other human tissues. RM2 antigen (*i.e.*, $GalNAcDSLc_4$) is expressed in distal tubules, whereas 5F3 antigen (DSGb5) is expressed in proximal tubules. The two antigens are co-expressed, and the polarity of 5F3 antigen expression seen in normal proximal tubule epithelia is lost, in RCC.

Clinicopathological studies on expression patterns of the three types of slow-migrating gangliosides (globo-series, lacto-series type 1, and ganglio/lacto-type 1 hybrid) are still underway. However, it is expected that a combination of three antibodies including the newly-established 5F3 will allow systematic studies on ganglioside expression and metastatic potential/invasiveness of RCC. Interestingly, all three types of disialoganglioside as above bind strongly to siglec-7 [15]. The significance of this property is unclear at this time, but a few possibilities can be considered: (i) RCC cells aggregate with peripheral blood mononuclear cells, causing microembolisms that initiate metastasis. Such microembolisms may occur preferentially in lung microvessels, providing a basis for the high metastatic potential of RCC to lung [15]. (ii) Binding of RCC cells to siglec-7 expressed in NK cells inhibits NK activity [16].

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