Glycosphingolipid Antigens in Neural Tumor Cell Lines and Anti-Glycosphingolipid Antibodies in Sera of Patients with Neural Tumors

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Key Words

Sulfated glucuronosyl glycosphingolipids • GD3 • Neural tumors • Serum antibodies

Abstract

To characterize biomarkers in neural tumors, we analyzed the acidic lipid fractions of 13 neural tumor cell lines using enzyme-linked immunoabsorbent assay (ELISA) and high-performance thin-layer chromatography (HPTLC) immunostaining. Sulfated glucuronosyl glycosphingolipids (SGGLs) are cell surface molecules that are endowed with the Human Natural Killer-1 (HNK-1) carbohydrate epitope. These glycosphingolipids (GSLs) were expressed in all cell lines with concentrations ranging from 210 to 330 ng per 2×10^6 cells. Sulfoglucuronosyl paragloboside (SGPG) was the prominent species with lesser amounts of sulfoglucuronosyl lactosaminyl paragloboside (SGLPG) in these tumor cell lines as assessed by quantitative HPTLC immunostaining. Among the gangliosides surveyed, GD3 and 9-O-acetylated GD3 (OAc-GD3) were expressed in all tumor cell lines. In contrast, fucosyl-GM1 was not found to restrict to small cell lung carcinoma cells. In addition, we have analyzed serum antibody titers against SGPG, GD3, and OAc-GD3 in patients with neu-

ral tumors by ELISA and HPTLC immunostaining. All sera had high titers of antibodies of the IgM isotype against SGPG (titers over 1:3,200), especially in tumors such as meningiomas, germinomas, orbital tumors, glioblastomas, medulloblastomas, and subependymomas. Serum in a patient with subependymomas also had a high anti-SGGL antibody titer of the IgG and IgA types (titers over 12,800). The titer of anti-GD3 antibody was also elevated in patients with subependymomas and medulloblastomas; the latter cases also had a high titer of antibody against OAc-GD3. Our data indicate that certain GSL antigens, especially SGGLs, GD3, and OAc-GD3, are expressed in neural tumor cells and may be considered as tumor-associated antigens that represent important biomarkers for neural tumors. Furthermore, antibody titers in sera of patients with these tumors may be of diagnostic value for monitoring the presence of tumor cells and tumor progression.

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The nomenclature used for gangliosides is based on that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [1].

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Introduction

Previous studies have indicated that the human natural killer-1 (HNK-1) epitope is closely associated with the metastatic behavior of melanomas [2–4]. HNK-1 is a carbohydrate epitope closely associated with human natural killer cells. Its presence, however, on many neural cell adhesion molecules is well recognized. These molecules include glycoproteins, such as myelin-associated glycoprotein (MAG), neural cell adhesion molecule (NCAM), L1, L2, and P0, in neuroectodermal cells and neural tissues [4, 5]. In addition, this carbohydrate epitope has also been found to be in at least two sulfated glucuronosyl glycosphingolipids (SGGLs) in peripheral nerves, and their structures were determined to be sulfated glucuronosyl paragloboside (SGPG) and sulfated glucuronosyl lactosaminyl paragloboside (SGLPG) [6–8]. It is believed that the HNK-1 epitope is involved in the adhesive functions of these cells. Several reports have presented immunocytochemical evidence that the HNK-1 epitope is expressed in a variety of tumor cells, particularly those of neuroectodermal origin, including melanomas [2, 3, 9], brain tumors including neuroectodermal cells [5], gliomas [10–12], neuroblastomas [13–15], nerve sheath tumors [16], sarcomas [16], schwannomas [16], neurofibrinomas [16], and traumatic neuromas [16]. Its presence in other tumors such as lymphoma [17], small cell lung carcinoma [18], and thyroid carcinoma [19] has also been reported. In glioma cells, the expression of the MAG gene is regulated by the protein products [20, 22]. Interestingly, Noronha et al. [9] reported the cross-reactivity of anti-melanoma antibody with SGGLs, but it remains to be addressed whether SGGLs are expressed in tumor cells or whether their antibody titers are elevated in sera of patients with the various tumors. In this study, we investigated the presence of SGGLs in a variety of tumor cell lines and examined the anti-GSL antibodies in patients with various tumors. In addition, we demonstrated the expression of other GSLs in tumor cell lines and presented evidence that their expression might play an important role in tumor progression and metastasis.

Materials and Methods

SGGLs, including SGPG and SGLPG, were isolated and purified from bovine cauda equine based on a procedure developed in our laboratory [8, 23]. GD3 and 9-O-acetylated GD3 (OAc-GD3) were isolated from bovine buttermilk [24]. Fucosyl-GM1 (Fuc-GM1) was isolated from PC12 cells [25] (see fig. 1 for all the relevant glycosphingolipid antigens). Tumor cell lines were obtained from

American Type Culture Collection (ATCC, Manassas, Va., USA). Sera from patients with neural tumors and normal healthy controls were obtained at Teikyo University School of Medicine, Japan, with approval from the Ethics Committee of Teikyo University.

Cell Culture

Tumor cell lines were grown in Eagle's minimum essential medium (EMEM; Gibco, Gaithersburg, Md., USA), RPMI 1640 (Gibco), or Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, Md., USA) according to the recommendations of ATCC. Batches of cells were rinsed free of medium with phosphate-buffered saline (PBS, pH 7.4) and harvested by scraping with a rubber policeman. The cell pellets were set aside and stored at -20°C until used for analysis. Table 1 lists the 13 tumor cell lines used in the present study.

Lipid Isolation

Isolation procedures for the acidic GSLs from the tumor cell lines, using 2×10^7 cells each, were described previously [23, 26, 27]. Briefly, total lipids were extracted with chloroform-methanol (2:1 and 1:1, v/v) and chloroform-methanol-water (30:60:8, v/v; Solvent A) and filtered. The combined extracts were then applied to a DEAE Sephadex A-25 column (acetate form, 3-ml bed volume), which was further eluted with 30 ml of solvent A to remove neutral lipids. Acidic lipids were then eluted with 60 ml of chloroform:methanol:0.8 M sodium acetate in water (30:60:8, v/v; Solvent B). The acidic lipid fraction was evaporated to dryness and applied to a Sephadex LH-20 column [25] to remove salts. A portion of the acidic lipids was set aside for HPTLC, HPTLC immunostaining, and ELISA.

HPTLC and HPTLC Immunostaining

Acidic lipids from tumor cell lines were examined by HPTLC immunostaining as previously described [28]. Briefly, an aliquot of the acidic lipid fraction, equivalent to 100 μg protein, was applied onto an HPTLC plate and developed with chloroform:methanol:0.2% aqueous $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (55:45:10, v/v). The plate was immersed in 0.2% polyisobutyl methacrylate in n-hexane for 1 min. After drying, the plate was incubated for 2 h with a monoclonal anti-SGGL antibody derived from the serum of a patient (LT) who developed peripheral neuropathies and IgM paraproteinemia (1:1,000, diluted in 1% bovine serum albumin (BSA)-50 mM PBS, pH 7.4). After washing with PBS, the plate was incubated with peroxidase-conjugated anti-human IgM (1:2,000, diluted in BSA-PBS) for 2 h. After further washing with PBS, bands were visualized with the O-phenylenediamine peroxidase substrate [29]. In a separate experiment, an aliquot of the acidic lipid fraction from melanoma cells, SK-MEL28, was applied onto an HPTLC plate and developed as described above. The bands were visualized by spraying with the resorcinol-hydrochloric acid reagent followed by heating at 100°C .

For comparison, authentic samples of gangliosides and the acidic lipid fraction (containing SGGLs) from bovine cauda equina, and GD3 and OAc-GD3 from bovine buttermilk were applied onto the plates and analyzed as described above. After drying, the plate was incubated for 2 h with sera of patients with neural tumors (1:200, diluted in 0.3% BSA/1 M PBS [BSA/PBS]). After washing with PBS, the plates were incubated with peroxidase-conjugated anti-human IgM, IgG, or IgA (1:2,000, diluted in BSA

Fig. 2. HPTLC immunostaining profiles of the acidic lipid fraction from various tumor cell lines using a monoclonal antibody derived from the serum of a patient (L.T.) with IgM paraproteinemia. Lane 1, SGPG isolated from bovine cauda equine, 200 ng; lane 2, acidic lipid fraction from bovine cauda equine. All symbols for tumor cell lines used in figure 1 are listed in table 1. The immunostained bands are represented as SGGLs (SGPG and SGLPG) per 100 μ g protein.

in PBS) for 2 h. After further washing with PBS, bands were visualized with 4-chloro-naphthol (15 mg/25 ml PBS containing 0.02% hydrogen peroxide) [30].

ELISA

An aliquot of the acidic lipid fractions from tumor cell lines was dried under a stream of nitrogen and dissolved in 500 μ l of methanol. A 20- μ l aliquot was then placed in individual wells of a microtiter plate (Immulon 1B; Thermo, Milford, Mass., USA). Authentic standards, SGPG, GD3, OAc-GD3, and Fuc-GM1, in various amounts from 5 to 100 ng of pure GSL, were placed on the same plate. After drying of the GSL antigens, 100 μ l each of the LT serum (for detection of SGGLs, 1:1,000 diluted in BSA-PBS) or various mouse monoclonal antibodies (Mabs) (culture supernatant, 1:50 diluted in BSA-PBS), such as Mab R24 (anti-GD3), Mab D.1.1 (anti-OAc-GD3), or anti-Fuc-GM1 [31], was placed into the individual wells. After incubation overnight at 4°C, the plates were washed 5 times with PBS and then a 100 μ l-portion of peroxidase-conjugated anti-human IgM or anti-mouse IgG or IgM (1:3,000) was added to each well. The plates were then incubated at room temperature for 2 h. After washing, the plates were developed with the O-phenylenediamine peroxidase substrate [32]. Absorbance values at 490 nm were calculated by subtracting optical density (OD) for the wells without antigen.

In a separate experiment, 25 ng of the GSL antigen (SGPG, GD3, or OAc-GD3) was placed in individual wells of a 96-well plastic ELISA plate. The serum samples from patients with neural tumors were diluted serially starting at 1:400 (diluted in BSA-PBS), and a 100 μ l-portion of each dilution was added to each well. After incubation at 4°C overnight, the plates were washed

Fig. 3. HPTLC of the acidic lipid fraction of melanoma cells (SK-MEL28). Lane 1, authentic samples of GM3 and GM2; lane 2, authentic samples of GM3, OAc-GD3, and GD3; lane 3, ganglioside mixtures from bovine brain; lane 4, acidic lipid fraction of SK-MEL28 cells. Bands were visualized by spraying with the resorcinol-hydrochloric acid reagent followed by heating at 100°C.

and then a 100 μ l-portion of peroxidase-conjugated anti-human IgM, IgG, or IgA (1:3,000) was added to each well. The plates were then incubated at room temperature for 2 h. After washing, the plates were developed and read as described above. Anti-GSL antibody (1 $\times$ as specified) was the highest serum dilution at which the OD₄₉₀ was 0.2 or more.

Results

Expression of SGGLs and GD3 in Neural Tumor Cell Lines

Figure 2 shows comparative profiles of HPTLC immunostaining of SGGLs from neural tumor cell lines using the anti-SGGL antibodies from the serum of a patient (L.T.) with IgM paraproteinemia [8]. Each lane contained the equivalent of 100 μ g of protein. SGPG was abundantly expressed in all cell lines and was particularly abundant in astrocytoma (U87MG). SGLPG was also expressed in about half of the tumor cell lines and was particularly abundant in U87MG. Nontumor cells such as the immortalized motor neuron-like cell lines, NSC-34, CHO cells, and fibroblasts (data not shown) did not express SGGLs (fig. 2).

Figure 3 shows the HPTLC profile of the acidic lipid fraction of a melanoma cell line, SK-MEL28. This cell line

Fig. 4. HPTLC immunostaining profile of SGGLs using sera of patients with various neural tumors. Plate **a**, bands were visualized by spraying with the orcinol-sulfuric acid reagent followed by heating at 110°C. Plates **b**, **c**, and **d** were immunostained with patient or normal control sera, followed by treatment with appropriate secondary antibodies with human peroxidase-conjugated IgM (from **b** 1–9), IgG (from **c** 1–9), and IgA (from **d** 1–9), respectively. Plate 1, meningioma, 67M; plate 2, schwannoma, 57F; plate 3, hemangioblastoma, 70F; plate 4, anaplastic astrocytoma, 55M; plate 5, pituitary adenoma, 43M; plate 6, spinal ependymoma, 68M; plate 7, germinoma, 17M; plate 8, normal control; plate 9, LT serum from a patient with IgM paraproteinemia [Ariga et al., 1987]. Lane 1, ganglioside mixtures from bovine brain, 2 µg; lane 2, acidic lipid fraction from bovine cauda equina, 500 ng.

Table 2. Anti-glycosphingolipid antibody activities in sera of patients with various neural tumors

Age/sex	Tumor	SGPG			GD3 IgG	OAc-GD3 IgG
		IgM	IgG	IgA		
67/M	meningioma	6,400	3,200	1,600	800	<400
29/F	meningioma	>12,800	6,400	<400	3,200	<400
78/M	meningioma	6,400	3,200	<400	3,200	<400
63/M	meningioma	3,200	3,200	800	1,600	<400
69/M	meningioma	3,200	1,600	800	3,200	<400
50/F	meningioma	>12,800	3,200	1,600	3,200	<400
70/F	hemangioblastoma	3,200	1,600	<400	3,200	1,600
55/M	anaplastic astrocytoma	6,400	1,600	<400	800	800
23/M	astrocytoma grade II	6,400	<400	<400	1,600	<400
18/F	astrocytoma grade II	6,400	800	800	800	<400
43/M	pituitary adenoma	6,400	1,600	800	3,200	1,600
54/M	pituitary adenoma	3,200	3,200	<400	800	<400
68/F	cavernous sinus tumor	3,200	1,600	<400	<400	<400
68/M	spinal ependymoma	6,400	1,600	800	800	800
50/F	orbital tumor	>12,800	3,200	1,600	1,600	<400
14/F	subependymoma	>12,800	>12,800	>12,800	>12,800	>12,800
35/M	glioblastoma	>12,800	1,600	<400	800	<400
62/M	glioblastoma	6,400	3,200	1,600	800	<400
13/F	medulloblastoma	>12,800	800	<400	>12,800	3,200
17/M	germinoma	>12,000	1,600	<400	800	<400
57/F	schwannoma	6,400	6,400	<400	<400	<400

Patient sera with brain tumors and anti-GSL antibody titers were based on ELISA.

Fig. 5. HPTLC immunostaining of GSL standards using sera of patients with various neural tumors. Plate **a**, bands were visualized with the orcinol-sulfuric acid reagent followed by heating at 110°C. Plate **b** was immunostained with sera from human subjects. Plate 1, subependymoma, 14F; plate 2, medulloblastoma, 13F; plate 3, meningioma, 69M; plate 4, normal control. Lane 1, ganglioside mixtures of bovine brain, 2 µg; lane 2, SGPG isolated from bovine cauda equina, 500 ng; lane 3, GD3 and OAc-GD3 isolated from bovine buttermilk, 1 µg each.

expressed GD3 as the major ganglioside that co-migrated with an authentic sample of GD3.

Table 1 summarizes the expression of SGPG, GD3, OAc-GD3, and Fuc-GM1 in various tumor cell lines, as analyzed by ELISA using the patient serum or a specific mouse monoclonal antibody. ELISA was more sensitive than HPTLC immunostaining for detection of expression of SGPG. All tumor cell lines, including melanoma, expressed SGPG with concentrations ranging from 210 to 330 ng per 2×10^6 cells.

GD3 ganglioside was also predominantly expressed in all tumor cell lines, especially in small cell lung carcinoma (DMS79), astrocytoma (U87MG), neuroblastoma (SK-N-SH), and melanoma (SK-MEL28) (table 1). However, in neural tumor cell lines, PFSK-1, and medulloblastoma (DAOY), the level of GD3 expression was relatively low. The expression of GD3 varied widely, ranging from 250 to 2,600 ng per 2×10^6 cells. All cell lines also contained a small amount of OAc-GD3, and all small cell lung carcinomas expressed trace amounts of Fuc-GM1 (ranging from 12 to 32 ng per 2×10^6 cells).

Anti-GSL Antibodies in Patients with Neural Tumors

Table 2 shows antibody titers against SGPG, GD3, and OAc-GD3 in sera of patients with neural tumors. All sera had high IgM-type antibody titers against SGPG (titers over 1:3,200), especially in two meningiomas (29F and 50F), germinoma, orbital tumor, subependymoma, glioblastoma (35/M), and medulloblastoma, where the titers were over 1:12,800. Some sera had IgG-type antibody titers, e.g. meningioma 29F, schwannoma, and subependymoma; the latter also had a high antibody titer of the IgA type (titers over 1:12,800). No gender or age differences were observed with respect to the type and titer of the antibody. In subependymoma and medulloblastoma, the titer of anti-GD3 antibody was elevated; in medulloblas-

toma there was also a high-titer antibody against OAc-GD3.

Figure 4 shows HPTLC immunostaining profiles of SGGLs using sera of patients with various tumors. All sera from patients bearing tumors, but not from controls, had immunoreactivities against SGPG and SGLPG, in which the IgM-type antibodies were predominant. Figure 5 shows the HPTLC immunostaining profiles of GD3 and OAc-GD3 using two serum samples of subependymoma and medulloblastoma that showed high titer anti-GSL antibodies as evaluated by ELISA. Both serum samples had immunoreactivities for GD3 and OAc-GD3, but in medulloblastoma the activity for OAc-GD3 was relatively weak. Control serum and serum of a patient with meningioma 69M had no immunoreactivities for GD3 and OAc-GD3 (plate b-3).

Discussion

Metastasis is a multistep phenomenon involving several different cell-to-cell and cell-to-matrix interactions. Most of these interactions involve cell adhesion molecules such as MAG, NCAM, L1, and L2, and several of these are upregulated in metastatic tumors [3, 33, 34]. In particular, several reports have documented that the expression of MAG is associated with the metastatic process of melanomas, brain tumors, and neuroectoderm-derived cell lines (see 'Introduction'). Thus, adhesion molecules could be potential biomarkers useful for monitoring metastasis and tumor progression. All the above molecules express a common carbohydrate epitope, HNK-1. The minimal carbohydrate structure has been elucidated as the terminal sulfated disaccharide ($\text{HSO}_3\text{-3GlcA}\beta\text{1-3Gal}\beta\text{1-}$) using model compounds [35]. Molecules that bear the HNK-1 carbohydrate epitope also bind to li-

gands such as laminins, P-selectin, and L-selectin [36]. The HNK-1 epitope is also present on two GSLs, termed SGGLs, which are relatively abundant in the peripheral nervous system, as compared to the central nervous system [7, 8]. SGGLs are also expressed as a minor component in immortalized human cerebromicrovascular endothelial cells [37], human umbilical cord vein endothelial cells [38], and mouse neuroblastoma cells N18TG2 [39], but not in immortalized motor neuron-like cell lines, such as NSC-6, 8, 19, and 34 [39]. It is well known that aberrant GSL expression has often been observed in tumors, which provides an important diagnostic marker for tumor progression [40]. Because of its potential involvement in cell adhesion and metastasis, we hypothesized that the HNK-1 epitope could be expressed in certain tumors that express cell adhesion molecules such as MAG and SGGLs. Noronha et al. [9] demonstrated that an anti-melanoma antibody, 10C5, recognizes SGGLs obtained from human peripheral nerve by HPTLC immunostaining, but they did not show the expression of SGGLs in melanoma cells. In this study, we confirmed that NSC-34 did not express SGGLs (fig. 2). However, no systematic study on the expression of SGGLs in tumor cell lines has been reported.

In this study, we have provided evidence for the expression of SGGLs in a variety of neural tumor cell lines. Using a highly sensitive ELISA method and a specific monoclonal antibody (LT serum), we demonstrated that all tumor cell lines tested, including melanoma cells, expressed SGGLs with concentrations in the range of 210–330 ng per 2×10^6 cells (table 1). Analysis of the individual molecular species by HPTLC immunostaining revealed that SGPG was predominantly expressed in tumor cell lines, and approximately half of those cell lines also contained SGLPG. In tumors derived from neural origin, MAG was expressed in gliomas, neuroblastomas, and a small cell lung carcinoma as described above, in which expression of SGGLs was correlated with that of MAG.

GSLs are known to be immunogens. Interest in their immunological properties stems from recent recognition that SGGL antibodies are frequently encountered in patients with a variety of neurodegenerative diseases [41]. In this study, we demonstrated that anti-SGGL antibodies, mostly of the IgM type, were elevated in sera of patients with a variety of neural tumors. The titers of serum anti-SGGL antibodies of the IgM type were significantly elevated with titers of over 1:3,200. In fact, in 9 of 24 cases the titers were more than 1:12,800. Interestingly, in a case of subependymoma, anti-SGGL antibodies of both IgG and IgA isotypes were also present. In subependy-

omas, the number of cells in tumors was extremely low, and tumors contained GFAP-positive astrocytes and cellular fibrillated matrix associated with cell clusters. The IgA antibody is a normal part of the body's immune system, which protects against disease. Overexpression of the IgA type of anti-SGGL antibodies may be involved in tumor suppression in subependymomas, suggesting that this tumor type might be slow-growing and benign. These results suggest that aberrant SGGL expression, in particular, HNK-1 expression, may be caused by activation of glucuronosyltransferase or sulfotransferase. Our data further suggest that SGGLs could be a useful biomarker for tumor progression in certain cell lines and that elevated serum antibody titers against SGGLs should be of diagnostic value in patients with these tumors.

Many studies reported in the literature have documented that altered and elevated ganglioside expression may play a crucial role in tumor progression. In this study, we demonstrated that all tumor cell lines also expressed GD3 ganglioside, in addition to SGGLs. It is not surprising that GD3 could be a major surface marker of tumors, especially in neural tumors, such as melanoma [42, 43], brain tumors [44], astrocytoma [45, 46], glioma [47], neuroblastoma [48], meningioma [49, 50], and intracranial tumors [51]. Serum antibodies against GD3 or OAc-GD3 have been shown to be elevated in some patients with melanoma [52]. In addition, Fuc-GM1 has been shown to be present in trace amounts on all small cell lung carcinoma cells, as revealed by a specific anti-Fuc-GM1 monoclonal antibody [53, 54].

In conclusion, neural adhesion molecules are known to play a key functional role in cell-to-cell interactions [4]. Glycoconjugates expressing the HNK-1 epitope, including MAG and SGGLs, on the tumor cell surface may serve as important biomarkers for tumor cell differentiation and metastasis. Since aberrant GSL expression is a hallmark of tumorigenesis, GSL antigens should emerge as important markers for tumor progression. Expression of SGGLs bearing the HNK-1 epitope, which is known to be present on a variety of cell adhesion molecules on tumor cells, especially in those from neural origin, should provide a basis for future studies regarding the mechanisms underlying tumor progression.

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