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Occurrence of ganglioside GD3 in neoplastic astrocytes

An immunocytochemical study in humans

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Abstract GD3 immunocytochemical analysis was performed in 25 human specimens obtained by autopsy and biopsy from patients with astrocytomas, anaplastic astrocytomas, cerebellar astrocytomas and glioblastoma multiforme (GM), using the ABC method. Extraction of the ganglioside fraction from GM was used for thin-layer chromatography (TLC) analysis to confirm the specificity of anti-GD3 monoclonal antibody (DSG-1). Normal astrocytes were not immunoreactive for GD3. Neoplastic astrocytes of low- to high-grade tumours were GD3 immunoreactive. In GM, the multinucleated giant cells were also immunoreactive. All immunoreactivity present was within the cytoplasm. In TLC analysis, enzyme immunostaining of gangliosides from GM with DSG-1 showed only one positive band, which had the same TLC migration rate as GD3, indicating that GD3 of the ganglioside fraction from GM is the antigen detected by DSG-1. The presence of GD3 within the cytoplasm of neoplastic astrocytes showing invasive and proliferative properties, is of considerable interest. The implications and possible significance of the presence of GD3 in the cytoplasm in glioma cells are discussed.

Key words Ganglioside GD3 · Glioblastoma multiforme · Astrocytoma · Glioma · Immunocytochemistry · Thin-layer chromatography

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Introduction

Gangliosides, sialic acid-containing glycosphingolipids abundant in the central nervous system (CNS), have been assumed to be extensively involved in neural functions [2, 9, 33, 42, 46]. They have pivotal roles in the normal physiological functions of the CNS and may modulate the ability of the brain to modify its responses to signals from the internal milieu.

Gangliosides are involved in cell adhesion, migration and growth regulation of normal and neoplastic cells, and changes in ganglioside composition and metabolism occur in the neoplastic transformation of many types of cells [3, 17, 19, 20].

Malignant transformation is accompanied by an altered ganglioside pattern. Since gangliosides are suggested to be involved in cell adhesion and cell growth regulation, the aberrant ganglioside composition of tumour cells may play a part in the uncontrolled growth and metastatic and invasive properties of tumour cells.

In order to investigate ganglioside GD3 [$II^3\alpha$(NeuAcα2–8NeuAc)-LacCer, GD3] immunoreactivity in human neoplastic astrocytes, we carried out an immunocytochemical staining for GD3 using a mouse IgM anti-GD3 monoclonal antibody (DSG-1) [52] in astrocytomas, anaplastic astrocytomas, cerebellar astrocytomas and glioblastoma multiforme obtained from human autopsy and biopsy tissues.

Materials and methods

Formalin-fixed, 4-µm-thick paraffin-embedded sections from autopsy and biopsy specimens of patients with astrocytomas, anaplastic astrocytomas, cerebellar astrocytomas and glioblastoma multiforme (Table 1) were available. They were stained with haematoxylin-eosin and Klüver-Barrera stains and subjected to glial fibrillary acidic protein (GFAP, Dako) immunocytochemistry. Immunocytochemical analysis for the presence of GD3 was performed, using DSG-1 and the avidin–biotin-peroxidase complex (ABC) method [22], which was carried out using the Vectastain ABC Kit (Vector Laboratories; Burlingame, Calif.), with 3,3'-diaminobenzidine-tetrachloride (DAB) as the chromogen.

Table 1 Details of tissue speciness obtained for the study (*Cbll* Cerebellar astrocytoma, *AA* Anaplastic astrocytoma, *GM* glioblastoma multiforme, *M* GMale, *F* female, *NE* not examined, *LT.* left, *Rt.* right)

Case	Age/Sex	Localization	Brain weight (g)	Histological diagnosis
1	13/F	Midbrain ~lt.thalamus	Biopsy	Astrocytoma
2	31/F	Rt.frontal	Biopsy	Astrocytoma
3	33/F	Rt.frontal	Biopsy	Astrocytoma
4	52/F	Rt.parietal	Biopsy	Astrocytoma
5	42/F	Bil.frontal	1360	Astrocytoma
6	24/M	Lt.frontal	Biopsy	AA
7	48/F	Lt.frontal	Biopsy	AA
8	23/F	Cerebellum	Biopsy	Cbll astrocytoma
9	24/F	Cerebellum	Biopsy	Cbll astrocytoma
10	25/F	Lt.frontal	Biopsy	GM
11	45/M	Rt.frontal	Biopsy	GM
12	45/F	Rt.temporal	Biopsy	GM
13	48/F	Rt.parietal	Biopsy	GM
14	63/M	Lt.frontal	Biopsy	GM
15	65/M	Rt.temporal	Biopsy	GM
16	66/F	Lt.thalamus	Biopsy	GM
17	66/F	Lt.frontal	Biopsy	GM
18	39/M	Lt.fronto -temporal	1400	GM
19	48/F	Lt.frontal	1395	GM
20	49/M	Lt.frontal	1550	GM
21	62/F	Rt.frontal	1110	GM
22	72/F	Lt.parieto -occipital	1070	GM
23	77/M	Lt.temporal -thalamus	1360	GM
24	77/F	Rt.fronto -temporal	1080	GM
25[a]	64/M	Rt.parieto-temporo-occipital	1350	GM

[a] Immunostaining on TLC plate of gangliosides prepared from GM (Case 25) using DSG-1 (anti-GD3 monoclonal antibody [52]) was performed

To confirm the specificity of the reaction, DSG-1 was omitted or replaced with normal mouse serum, and the sections were pretreated with neuraminidase.

Extraction of ganglioside fraction from glioblastoma multiforme was performed by thin-layer chromatography (TLC). Lipids were extracted with 10 volumes each of chloroform–methanol (2:1, 1:1, and 1:2, v/v) and chloroform–methanol–water (30:60:8, v/v) at room temperature overnight. The lipids extracts were combined, evaporated and dried in vacuo. The total lipid extract was dissolved in a minimum amount of a mixture of chloroform–methanol (1:1, v/v), and then treated with 1 N sodium methylate (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 3 h. After neutralization with acetic acid, the hydrolysate was dialysed against distilled water and evaporated. Neutral glycosphingolipids and gangliosides were separated by passage through a DEAE-Sephadex A-25 column [33]. Gangliosides were eluded with chloroform–methanol–0.8 M sodium acetate (30:60:8, v/v). The ganglioside fraction was dialysed against distilled water and evaporated to dryness in vacuo.

The immunostaining of gangliosides from GM on TLC plate was performed according to the method of Magnami et al. [34] with slight modifications. Gangliosides from GM were spotted on a high-performance TLC plate (Silica Gel 60 F-254, Merck, Darmstadt, Germany) and developed with chloroform–methanol–water (60:40:9, v/v). The dried plate was soaked for 1 min in a 0.02% solution of polyisobutylmethacrylate (Tokyo Kasei Kogyo, Tokyo, Japan) dissolved in hexane, allowed to air-dry, and then blocked by incubation in PBS containing 1% bovine serum albumin (BSA; Wako), 1% polyvinylpyrrolidone (Wako), and 0.02% NaN3 (blocking buffer; Wako) at 37°C for 30 min. It was then rinsed five times with PBS containing 0.1% Tween 20 (washing buffer) and incubated with DSG-1 monoclonal antibody culture supernatant at 4°C overnight. After that, the plate was washed five times with washing buffer. After washing, the plate was reincubated with the 400-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgM antiserum (Cappel Laboratories, West Chester, Pa.) at 37°C for 1.5 h. As a final step, it was washed five times with washing buffer and incubated with peroxidase substrate solution consisting of 2 ml of 4-chloro-1-naphthol (Sigma) (0.3% in methanol) and 5 volumes of 100 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl and 0.01% H_2O_2 for 5 min at room temperature. The reaction was stopped by washing with water.

Results

Normal astrocytes were negative for GD3. In astrocytomas (Fig. 1) and anaplastic astrocytomas, neoplastic astrocytes were GD3 immunoreactive. In cerebellar astrocytomas (Fig. 2), the tumour was composed mainly of closely interwoven, elongated, and slender cells, showing GD3 immunoreactivity. In glioblastoma multiforme (Fig. 3A, B), fields showing a higher density of anaplastic glial tumour cells (undifferentiated glioma cells) demonstrated immunocytochemical variability from case to case. Fields with astrocytic features in high-grade tumours were GD3 immunoreactive, and multinucleated giant cells were also immunoreactive. The immunoreactivity was observed within the cytoplasm of the glioma cells. Oligodendrocytes were negative for GD3. Ependymal cells and choloid plexus were positive for GD3. Furthermore, GFAP-like immunoreactivity was observed in the neoplastic astrocyte.

No immunoreactivity was observed when the primary antibody was omitted or replaced with normal mouse serum. The immunoreactivity was reduced when the sections were pretreated with neuraminidase.

In order to confirm the nature of the molecule reactive with DSG-1 immunocytochemically, the total ganglioside fraction was prepared from glioblastoma multiforme and analysed by TLC immunostaining with DSG-1. The results are illustrated in Fig. 4. Enzyme immunostaining of gangliosides from GM with DSG-1 showed that only one positive band, with the same TLC migration rate as GD3, was stained. None of the other gangliosides in GM gave a positive reaction with DSG-1. This result suggests that only GD3 of the ganglioside fraction from GM is the antigen detected by DSG-1, and that the antigen immunocytochemically detected by DSG-1 is GD3.

Discussion

Gangliosides are cell surface molecules and are believed to be involved in cell adhesion, migration, and growth regulation of both normal and neoplastic cells [2, 9, 19, 20, 32, 33, 42, 46]. Some glycosphingolipids have been reported to be present intracellularly [14–16, 24, 45–48,

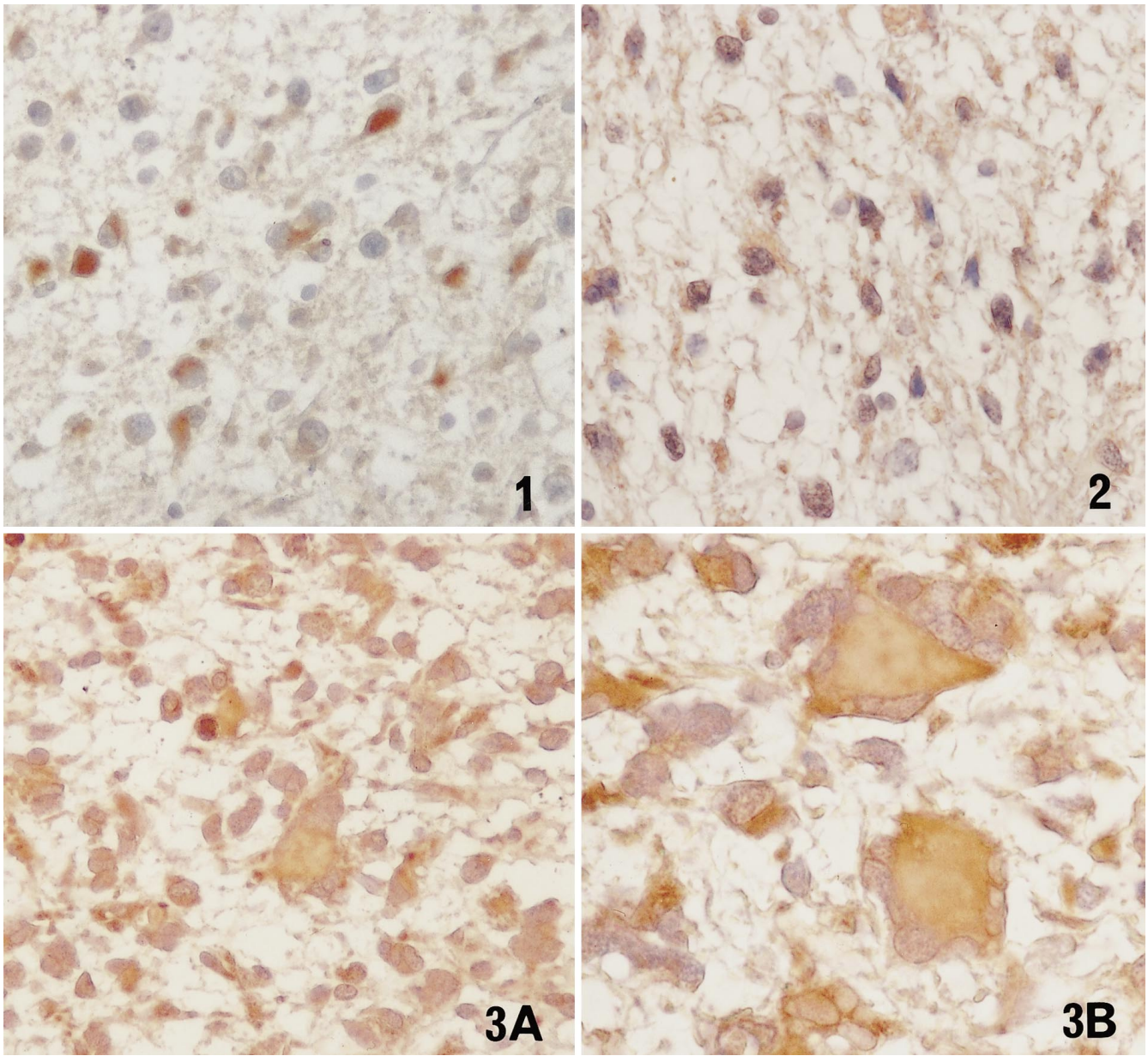

Fig. 1 Case 2. Astrocytoma. Diffusely distributed GD3-immunoreactive neoplastic astrocytes are observed. The peroxidase reaction product for ganglioside GD3 (RP) is present in the cytoplasm

Fig. 2 Case 9. Cerebellar astrocytoma. Pilocytic astrocytoma cells are diffusely distributed and the RP is also present in both cytoplasm and processes

Fig. 3 A, B Case 16. Glioblastoma multiforme. Cellular pleomorphism with multinucleated giant cells is evident. Neoplastic astrocytic cells including multinucleated giant cells are GD3 immunoreactive. GD3 immunoreactivity is observed in the cytoplasm

50]; we have demonstrated the presence of GD3 within the cytoplasm of neuronal cells [25] and reactive astrocytes [26] in the rat brain, and within the cytoplasm of neuronal cells in the human CNS [29], using DSG-1. In addition, our previous studies showed that the peroxidase reaction product for GD3 was present on the plasma membrane of choroidal cells [27] and neuroblasts (E13) during normal development [28], suggesting that GD3 is present not only in the plasma membrane but also within the cytoplasm.

Malignant transformation is accompanied by an altered ganglioside pattern [3, 4, 10–12, 18, 49). Since it has been suggested that gangliosides are involved in cell adhesion and cell growth regulation [19, 20], aberrant ganglioside composition of tumour cells might play an important role in the uncontrolled growth and the metastatic and invasive properties of tumour cells.

Gangliosides constitute a class of sialic acid containing glycosphingolipids that are ubiquitous in the body and present at high concentrations in the brain. They may be important in neoplastic cells in terms of their proliferation, adhesion, migration and invasion and in cell–cell recognition and differentiation [20, 31, 36, 38, 39].

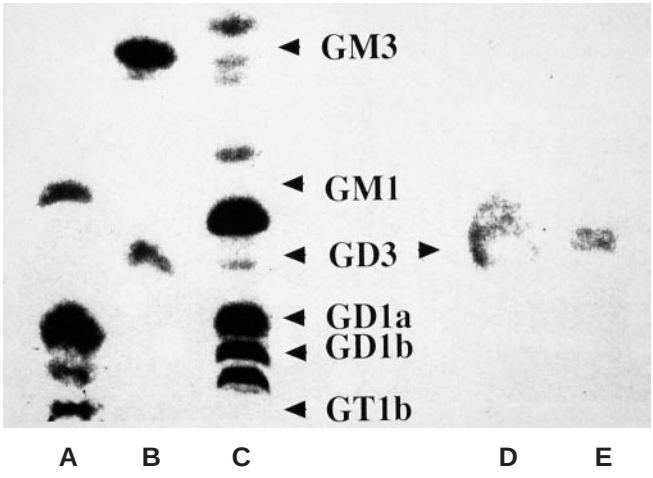

Fig. 4 Thin-layer chromatography (TLC) and TLC immunostaining. Immunostaining on TLC plate of gangliosides prepared from glioblastoma multiforme (case 25) using DSG-1. *Lane A* total bovine brain gangliosides, GM1, GD1a, GD1b and GT1b (*top* to *bottom*), *lane B* gangliosides GD3 and GM3, *lane C*, total gangliosides from glioblastoma multiforme, *lane D* ganglioside GD3, *lane E* total gangliosides from glioblastoma multiforme. *Lanes A–C* were visualized with resorcinol reagent. *Lanes D, E* were immunostained as described in "Materials and methods" using DSG-1 monoclonal antibody

GD3 is a structually simple ganglioside associated with malignant transformation, but its biological role has not been established. It has been shown that monoclonal antibodies to GD3 have a growth-inhibitory effect on melanoma cells that express GD3 [8, 53]. Studies on such melanoma cells have also shown that GD3 localised to focal adhesion plaques [5] and that attachment is inhibited with anti-GD3 antibodies [6].

In general, malignant gliomas contain a higher concentration of GD3. Its expression correlates with the degree of malignancy, and ganglioside composition in human gliomas correlates with the degree of biological aggressiveness [3, 7, 13, 40]. The increase in GD3 seems to be a characteristic of the neoplastic transformation of astrocytes, since it is only a minor ganglioside in astrocytes or glial cells [1, 21].

Koochekpour and Pilkington, using the LB1 monoclonal antibody [30], have reported observing localization of GD3 almost exclusively in vascular and perivascular areas with no GD3 immunoreactivity present in low-grade glioma or normal brain tissues. However, in the present study, GD3 immunoreactivity occurred in astrocytomas, anaplastic astrocytomas, cerebellar astrocytomas and glioblastoma multiforme regardless of the degree of malignancy. In the infiltrating fields showing the astrocytic feature of glioblastoma multiforme, GD3 immunoreactivity was also observed (cf. [35, 44, 51]). The GD3 immunocytochemical method used here is well established in our laboratory [28, 29], and the specificity of DSG-1 monoclonal antibody is documented in Fig. 4.

Cell motility is an important factor for invasion and various cytokines modulate the motility of glioma cells. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) and transforming growth factor-b (TGF-b) are examples [37, 41, 43].

We have recently reported that GD3 immunoreactivity is present within the cytoplasm of the reactive astrocytes [26] and neuroblasts (E16) during development [28]. Astrocytes are cells that participate actively in the process of lesion repair in the normally developed postnatal CNS [23], and we have therefore suggested that the GD3 in reactive astrocytes is involved in those astrocytic functions that are required for CNS repair processes [26]. Whether the present results indicate a role for GD3 in the cellular motility of human neoplastic astrocytes is open to speculation.

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