Human medulloblastoma gangliosides

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Introduction

Gangliosides are glycosphingolipid molecules predominantly expressed on the cell surface and widely found in tumors (Hakomori, 1984). Increasingly, these molecules have been implicated in the process of tumor formation and progression (Valentino et al., 1990; Hakomori, 1994). Abnormalities of glycosphingolipid metabolism which result in different patterns of ganglioside expression in tumors as compared to corresponding normal tissues are of interest for several reasons. One is that abnormal patterns of ganglioside expression may be diagnostic and/or prognostic markers for certain tumors. This has been well demonstrated in some tumors, such as human neuroblastoma (Schulz et al., 1984; Cheung et al., 1985; Ladisch and Wu, 1985; Wu et al., 1986; Ladisch et al., 1987) and melanoma (Watanabe et al., 1982; Natoli et al., 1986; Tsuchida et al., 1989). These ganglioside antigens may be useful as targets for the treatment of certain tumors with monoclonal antibodies (Houghton et al., 1985; Irie and Morton, 1986; Cheung et al., 1987; Vadhan-Raj et al., 1988; Hamanaka et al., 1989). Secondly, it is becoming recognized that alterations in tumor ganglioside structure may influence the biological functions of these molecules (Hakomori, 1990; Hakomori and Igarashi, 1993). For example, neuroblastoma ganglioside GD2 with a short fatty acyl chain (C16:0) has a much greater immunosuppressive activity than does GD2 with a longer fatty acyl chain (C24:0 or C24:1; Ladisch et al., 1994). For these reasons among others, full understanding of glycosphingolipid metabolism, particularly in defined and homogenous tumor cell systems, is of great interest.

The normal tissue which is richest in gangliosides is the brain (Svennerholm, 1963; Ledeen and Yu, 1982; Svennerholm et al., 1989). Therefore, the study of ganglioside expression in brain tumors is a logical endeavor, especially since valuable markers of brain tumors are generally lacking (Kurpad et al., 1995). Light and electron microscopic study sometimes has been used as the sole basis for their classification and diagnosis. One brain tumor that has been relatively little studied with respect to ganglioside composition is medulloblastoma. Medulloblastoma could be a particularly interesting tumor in which to investigate ganglioside metabolism because this is a very common and aggressive pediatric brain tumor.

We are therefore studying human medulloblastoma gangliosides. While data regarding the gangliosides of individual medulloblastoma tumors have been published (Gottfries et al., 1990; Shinoura et al., 1992; Fredman, 1994), to perform such studies on a cell line is also of value, because of the homogeneity of the cell population of a cell line in comparison with that of tumor biopsy specimens. Others have partially characterized medulloblastoma cell line gangliosides (Gottfries et al., 1991), but information on complete molecular structures is lacking. The one cell line which has had its ganglioside composition more fully characterized, TE-671 (Gottfries et al., 1989), unfortunately was recently identified as a sarcoma, not a medulloblastoma cell line (Chen et al., 1989; Stratton et al., 1989).

Here we have investigated the gangliosides of an established human medulloblastoma cell line, Daoy (Jacobsen et al., 1985). We report that Daoy cells have a high ganglioside content and marked heterogeneity of ganglioside ceramide structure. This latter finding is of particular importance since it provides support for the concept that heterogeneity of ceramide structure is a general characteristic of tumor ganglioside metabolism. Furthermore, now well characterized, the Daoy cell line should be a useful model for studies of the dynamic process of medulloblastoma tumor cell ganglioside shedding as well as for development of strategies to alter ganglioside metabolism.

Results

HPTLC analysis of total Daoy medulloblastoma gangliosides

The first important feature regarding Daoy human medulloblastoma cell gangliosides is that these cells have a high gan-
ganglioside content, 143 ± 13 (n = 3) nmol LBSA/10⁸ cells. HPTLC analysis reveals that Daoy cells contain G₃₂, G₅₃, and G₅₄ as major component gangliosides (Figure 1). Other gangliosides, including G₅₁, G₅₂, G₅₂, G₅₂, and G₅₂, are also present. By densitometric scanning of the HPTLC plate, the relative composition of the major gangliosides of Daoy cells has been determined—G₅₂ (63.9%), G₅₃ (13.0%), and G₅₄ (10.3%) (Table I). These results are consistent with the previous report (Gottfries et al., 1991). The other notable characteristic of Daoy human medulloblastoma cell gangliosides is that the major gangliosides (e.g., G₅₂) migrate as doublets as analyzed by HPTLC, due to the heterogeneity of ceramide structures (see below). Since culture conditions, such as cell density (Liepkalns et al., 1981; Schengrund and Repman, 1982; Kadowaki et al., 1990), fetal calf serum (Kemp and Stoolmiller, 1976), and pH of the medium (Iber et al., 1990), may affect ganglioside metabolism, we carefully controlled these conditions. Repeated evaluation, by HPTLC, of the gangliosides of Daoy cells which were harvested at different times over an 18 month period revealed the HPTLC pattern of these gangliosides to be constant.

HPLC isolation of Daoy cell gangliosides

The purified total Daoy medulloblastoma gangliosides were isolated by normal phase HPLC to yield individual gangliosides homogeneous in carbohydrate structure. Nine fractions containing ganglioside species were collected. These were identified by HPTLC analysis (Figure 2). The major gangliosides were also identified by mass spectrometry to be G₅₃ (lane 3), G₅₃ (lanes 4 and 5), G₅₁ (lanes 6 and 7), and G₅₄ (lanes 8 and 9).

By reversed-phase HPLC, the ceramide subtypes of one of the major component gangliosides, G₅₂, were isolated (Figure 3). For this purpose, total G₅₂ ganglioside was used. Among over 20 peaks obtained by the reversed-phase HPLC separation of G₅₂, 10 major ceramide subtypes, which constitute 85% of total G₅₂, were identified. These ganglioside subtypes are homogeneous in both carbohydrate and ceramide structure. G₅₂ ganglioside isolated from Daoy human medulloblastoma cells shows a much more complex profile when compared with G₅₂ isolated from human brain tissue (Figure 3). The former contains three major peaks as well as a large series of small peaks, suggesting significant heterogeneity in ceramide structure; the latter contains only two major peaks and several minor peaks.

Characterization of ceramide structures

Each of the gangliosides isolated by reversed-phase HPLC from Daoy medulloblastoma G₅₂ was structurally identified by negative-ion fast atom bombardment mass spectrometry (FAB MS) and collisionally activated dissociation tandem mass spectrometry (CAD MS/MS) to characterize ceramide structure. Figure 4 shows the mass spectrum of ganglioside G₅₂ (d18:1-C16:0), which is the fraction of ganglioside G₅₂ isolated by reversed-phase HPLC with retention time 27.0 min (Figure 3 and Table II). A single molecular ion [M-H]⁻ was clearly detected at m/z at 1354 (panels A and B). In addition to the molecular ion, there are four additional peaks in the mass spectrum (panel A) at m/z 1063, 860, 698, and 536. These resulted from the fragmentation along the oligosaccharide

![Figure 1](http://glycob.oxfordjournals.org/)

**Fig. 1.** HPTLC characterization of Daoy human medulloblastoma cell gangliosides. HBG, normal human brain gangliosides (4 nmol); Daoy, total gangliosides isolated from Daoy cells (10 nmol). Gangliosides were stained by resorcinol-HCl.

![Table I](http://glycob.oxfordjournals.org/)

<table>
<thead>
<tr>
<th>Gangliosides</th>
<th>% of total</th>
<th>nmol LBSA/10⁸ cells</th>
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<td>G₅₂</td>
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</tr>
<tr>
<td>G₂₂</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>G₂₂</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Others</td>
<td>1.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table I. Major component gangliosides in Daoy human medulloblastoma cells

Total Daoy medulloblastoma cell gangliosides were purified as described in Materials and methods and analyzed by HPTLC. The % of total for each ganglioside was obtained from densitometric analysis by scanning the TLC plate, and multiplied by the total ganglioside content (142.8 ± 13.4 nmol LBSA/10⁸ cells) to yield each component ganglioside content (nmol/10⁸ cells).
backbone, consistent with the sequence (from the nonreducing end) of N-acetyl neuraminic acid, N-acetylgalactosamine, galactose, and glucose. The peak at m/z 1152 also exists in the mass spectrum, corresponding to the loss of one N-acetylgalactosamine group. The ceramide ion [CerO]$^-$ was detected at m/z 646 (panels A and C). Together, these results are consistent with a carbohydrate structure of G$_{M2}$: GalNAc-(NeuAc-) Gal-Glc-Cer.

The ceramide structures were determined by the CAD-MS/MS (Domon and Costello, 1988). For this purpose, the [CerO]$^-$ ion at m/z 536 was used to produce daughter ions, which yielded structural information on the long chain base and the fatty acyl group. The presence of an S ion at m/z 280 and a T ion at m/z 296 (panel D) confirms that the long chain base is d18:1, the fatty acid is C16:0, and ceramide structure is d18:1-C16:0.

Similarly, ganglioside G$_{M2}$ (d18:1-C24:1), with a retention time 61.12 min in the reversed-phase HPLC profile (Figure 3 and Table II), was characterized (Figure 5). The molecular ion [M-H]$^-$ was detected at m/z 1464 (panels A and B). Four additional peaks were detected at m/z 1173, 970, 808, and 646, which resulted from the loss of N-acetyl neuraminic acid, N-acetylgalactosamine, galactose, and glucose. The peak at m/z 1262 corresponds to the loss of one N-acetylgalactosamine backbone, consistent with the sequence (from the nonreducing end) of N-acetyl neuraminic acid, N-acetylgalactosamine, galactose, and glucose. The peak at m/z 1152 also exists in the mass spectrum, corresponding to the loss of one N-acetylgalactosamine group. The ceramide ion [CerO]$^-$ was detected at m/z 646 (panels A and C). Together, these results are consistent with a carbohydrate structure of G$_{M2}$: GalNAc-(NeuAc-) Gal-Glc-Cer.

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Using this same approach, other ceramide structures of medulloblastoma ganglioside G$_{M2}$ were determined, as summarized in Table II. Several conclusions can be drawn. First, marked variation in the length of the fatty acyl chain, from C16 to C24, was found. The major ceramide structures of medulloblastoma G$_{M2}$ are d18:1-C16:0, d18:1-C24:1, and d18:1-C24:0, which are quite different from those of normal human brain ganglioside G$_{M2}$ (Figure 3, top panel), which contains predominantly the ceramide subspecies d18:1-C18:0 (peak 1) and d20:1-C18:0/d18:1-C20:0 (peak 2). Secondly, hydroxylation in the fatty acyl chain, which is not common in gangliosides from normal human brain tissue except in ganglioside G$_{M4}$ (Kadowaki et al., 1984), was also present in the form of d18:1-hC16:0. This indicates the occurrence of aberrant hydroxylation in medulloblastoma gangliosides, as previously found in human neuroblastoma tumor gangliosides (Ladisch et al., 1989). Thirdly, the formation of doublets by Daoy gangliosides on HPTLC was confirmed to be caused by the differences in ceramide structures. For example, the upper band of G$_{M2}$ contains mainly the gangliosides with ceramide structures d18:1-C24:1 and d18:1-C24:0; the lower band contains mainly the ganglioside with d18:1-C16:0.

Other Daoy medulloblastoma gangliosides

In addition to ganglioside G$_{M2}$, the predominant component ganglioside in Daoy cells, other gangliosides isolated by normal phase HPLC were identified by both HPTLC and negative-ion FAB-MS. Due to the limited amount of material, the reversed-phase HPLC isolation was not performed. Since ceramide structures of ganglioside G$_{M2}$ contain mainly one type of long chain base, d18:1, the major ceramide structures of other Daoy medulloblastoma gangliosides, including G$_{M3}$, G$_{M1}$, G$_{D18}$, and G$_{O2}$, were proposed based on the detection of [CerO]$^-$ ions as well as the molecular ions of each ganglioside (Table III). These results shows that Daoy gangliosides contain d18:1-C16:0, d18:1-C22:0, d18:1-C24:0, and d18:1-C24:1 as the major ceramide structures.

Discussion

Medulloblastoma is an aggressive human brain tumor. Full characterization of ganglioside structures in a medulloblastoma cell line, which we report here, will provide a model system for future study of medulloblastoma ganglioside metabolism. Qualitative analysis confirmed that the major gangliosides of the Daoy human medulloblastoma cell line are G$_{M3}$, G$_{M2}$, and G$_{D18}$. We also found a high ganglioside content (143 ± 13 nmol/10$^8$ cells), of the same order of magnitude as that of certain murine neural tumor cell lines (Dawson, 1979), and marked heterogeneity of ceramide structure. The most important findings of this study on the structure of the medulloblastoma cell gangliosides relate to that of the ceramide moiety. Using a combination of normal and reversed-phase HPLC separation, and negative-ion CAD MS/MS, we found enormous ceramide diversity. For example, over 20 peaks were seen and 10 major ganglioside ceramide species...
Fig. 4. Characterization of medulloblastoma ganglioside G₄₋₂ (d18:1-C16:0). This ceramide subspecies of Daoy ganglioside G₄₋₂ was identified by negative ion-FAB MS (A-C) and CAD-MS/MS (D). The molecular ion at m/z 1354 (A and B), ceramide ion [CerO]⁻ at m/z 536 (A and C), S ion at m/z 280 and T ion at m/z 296 (D) were clearly detected, indicating the structure of G₄₋₂ (d18:1-C16:0).
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Fig. 5. Characterization of medulloblastoma ganglioside G\textsubscript{M2} (d18:1-C24:1). This ceramide subspecies of ganglioside G\textsubscript{M2} was first identified by negative ion-FAB MS (A–C), and further characterized by negative-ion CAD-MS/MS (D). The presence of molecular ion at m/z 1464 (A and B), ceramide ion [CerO]\textsuperscript{−} at m/z 646 (A and C), S ion at m/z 390 and T ion at m/z 406 (405.88, D) confirms the structure of G\textsubscript{M2} (d18:1-C24:1).
were proposed based on the molecular ions and [CerO]⁻ ions. HPLC and analyzed by negative-ion FAB MS. The ceramide structures Individual Daoy medulloblastoma cells were isolated by normal phase dem-mass spectrometry (Domon and Costello, 1988). This is carbohydrate and ceramide structure. This allowed the subse-

diisopropyl ether/1-butanol partition (Ladisch and Gillard, 1985) followed by a reversed-phase HPLC (Gazzotti et al., 1993). HPTLC analysis of gangliosides was performed using a high rate of ganglioside shedding by the tumor. Such shedding (Chang et al., 1996) could affect tumor/host interactions (Hakomori and Igarashi, 1993). On the qualitative level, ceramide heterogeneity and the prominence of gangliosides with ceramide containing short fatty acyl chains may also be important, specifically with respect to possible biological functions of these molecules (Ladisch et al., 1994; Hakomori, 1996). The full characterization of the gangliosides of a human medulloblastoma cell line, Daoy, may now make this cell line a useful system for the study of the dynamics of ganglioside metabolism (including shedding) as well as providing a system in which to attempt to engineer cell glycosphingolipid metabolism in such a way as to inhibit or alter the synthesis of these biologically active molecules.

### Materials and methods

#### Cell culture

Daoy human medulloblastoma cells were cultured as adherent monolayers in 75 cm² flasks in Eagle's minimum essential medium (Bio-whittaker) and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) in a humidified 5% CO₂, 95% air atmosphere. The culture medium was changed every 3 days, and cell viability was assessed by trypan blue dye exclusion. Culture conditions, such as cell density (Liepkaunas et al., 1981; Schengrund and Repman, 1982; Kadowaki et al., 1990), fetal calf serum (Kemp and Stoolmiller, 1976), and pH of the medium (Iber et al., 1990), which may affect ganglioside metabolism, were carefully controlled.

#### Ganglioside isolation and quantification

The total lipids of the cells were obtained by chloroform/methanol extraction as described previously (Ladisch et al., 1983). Gangliosides were purified by diisopropyl ether/1-butanol partition (Ladisch and Gillard, 1985) followed by Sephadex G-50 gel filtration. The purified gangliosides were quantified as nmol lipid-bound sialic acid (LBSA) by the modified resorcinol method (Ledeen and Yu, 1982). HPTLC analysis of gangliosides was performed using 10 x 20 cm precoated silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol/0.25% aqueous CaCl₂/2H₂O (60:40:5, by volume) to separate gangliosides, which were stained with resorcinol/HCl (Ledeen and Yu, 1982).

#### Ganglioside purification by high performance liquid chromatography

Daoy human medulloblastoma gangliosides were first isolated by normal phase HPLC (Gazzotti et al., 1985). Briefly, total gangliosides (600 nmol LBSA) were dissolved in 50 μl water and chromatographed using the Perkin-Elmer HPLC system, on a LiChrosorb-NH₂ column (250 mm length, 10 mm i.d., Merck, Germany). The eluting solvent system was composed of acetonitrile/5% Sorensen's phosphate buffer (83:17), pH5.6 (solvent A), and acetonitrile/20% Sorensen's phosphate buffer (1:1), pH 5.6 (solvent B). The following elution program was used: 7 min of solvent A was followed by 53 min with a linear gradient from solvent A to solvent A–solvent B (66:34), and then to solvent A–solvent B (36:64) over 20 min (Gazzotti et al., 1985). The flow rate was 6.25 ml/min, and the elution profile was monitored by flow-through detection at 215 nm. Gangliosides isolated by normal phase HPLC were further separated by reversed-phase HPLC (Gazzotti et al., 1984) to obtain individual gangliosides which are homogenous in both carbohydrate and ceramide structures. G₄₄ (10

| Table II. Ceramide structures of Daoy human medulloblastoma G₄₄ |
|---|---|---|
| HPLC peak | % of total G₄₄ | Molecular weight | Ceramide structure |
| No. | Retention time (min) | | |
| 1 | 24.59 | 2.1 | 1371 | d18:1-hC16:0 |
| 2 | 27.00 | 14.3 | 1355 | d18:1-C16:0 |
| 3 | 29.43 | 2.0 | 1357 | d18:1-C16:0 |
| 4 | 37.32 | 2.9 | 1383 | d18:1-C18:0 |
| 5 | 48.26 | 3.2 | 1411 | d18:1-C20:0 |
| 6 | 49.77 | 3.6 | 1439 | d18:1-C22:0 |
| 7 | 53.07 | 6.0 | 1463 | d18:2-C24:1 |
| 8 | 55.17 | 3.9 | 1451 | d18:1-C23:1 |
| 9 | 61.12 | 35.7 | 1465 | d18:2-C24:1 |
| 10 | 75.78 | 11.9 | 1467 | d18:1-C24:0 |

Individual ceramide species of G₄₄ gangliosides from Daoy medulloblastoma cells were isolated by reversed-phase HPLC and analyzed by negative-ion FAB CAD-MS/MS. The percentage of total G₄₄ contributed by each ceramide subspecies was estimated by integration of the HPLC. Some additional ceramide ceramide subspecies were not conclusively identified, due to the limited amount available. They constitute 14.4% of total G₄₄. The major ceramide species of human brain G₄₄ (Figure 3, top panel) are d18:1-C18:0 (peak 1) and d18:1-C20:0/d20:1-C18:0 (peak 2).

specifically detected and characterized as having the carbohydrate structure of ganglioside G₄₄. This contrasts to only three prominent ceramide species in normal human brain ganglioside G₄₂. Curiously, C₂₄:0, which was not detected in human brain G₄₂, was quite elevated in the medulloblastoma cells. Whether this particular ceramide structure may be of some diagnostic value remains to be investigated. Overall, the findings of ceramide heterogeneity in this medulloblastoma cell line are in concordance with previous findings of marked ganglioside ceramide heterogeneity in tumor cell lines such as human neuroblastoma (Ladisch et al., 1994) and murine lymphoma (Li et al., 1993), as well as in tumor biopsies (Ladisch et al., 1989).

The present determination of complete molecular structures of medulloblastoma cell gangliosides represents a comprehensive study of ceramide structure. The strategy used here, applicable to the study of other tumors as well, was to obtain highly purified gangliosides which are homogeneous in both carbohydrate and ceramide structure. This allowed the subsequent detection of the ceramide ion and confirmation of ceramide structure (carbon chain length, degree of unsaturation, and hydroxylation) by collisionally activated dissociation tandem-mass spectrometry (Domon and Costello, 1988). This is valuable for example, in that d20:1-C18:0 G₄₂ and d18:1-C20:0 G₄₂ can easily be distinguished from each other by examining the daughter ions (S ion and T ion) produced from the ceramide ion. The m/z value of the S and T ions will indicate the structure of the fatty acyl group, and consequently the structure of the long chain base can be deduced. Another important advantage of this approach, the combined use of HPLC and mass spectrometry, is that a relatively small quantity (on the order of 1 nmol) of gangliosides is required, as compared with most standard methods.

The total lipids of the cells were obtained by chloroform/methanol extraction as described previously (Ladisch et al., 1983). Gangliosides were purified by diisopropyl ether/1-butanol partition (Ladisch and Gillard, 1985) followed by Sephadex G-50 gel filtration. The purified gangliosides were quantified as nmol lipid-bound sialic acid (LBSA) by the modified resorcinol method (Ledeen and Yu, 1982). HPTLC analysis of gangliosides was performed using 10 x 20 cm precoated silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol/0.25% aqueous CaCl₂/2H₂O (60:40:5, by volume) to separate gangliosides, which were stained with resorcinol-HCl (Ledeen and Yu, 1982).

#### Table III. Major ceramide species of other Daoy medulloblastoma cell gangliosides

<table>
<thead>
<tr>
<th>Gangliosides</th>
<th>[M-H]⁻</th>
<th>[CerO]⁻</th>
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Individual Daoy medulloblastoma cells were isolated by normal phase HPLC and analyzed by negative-ion FAB MS. The ceramide structures were proposed based on the molecular ions and [CerO]⁻ ions.
Structural characterization of gangliosides by mass spectrometry

The carbohydrate structure of gangliosides was characterized by negative-ion fast atom bombardment mass spectrometry (FAB-MS) without prior derivatization (Ladisch et al., 1989). Approximately 1 μl of ganglioside solution in methanol (0.1–2.0 nmol/μl) was mixed with 2 μl of tetraethylammonium (matrix) on the fast atom bombardment probe tip. Ions were formed by bombardment with a 6 keV beam of xenon atoms in a JEOL HX-110 double focusing mass spectrometer. The accelerating voltage was 10 keV, and the resolution was 3000.

To determine the ceramide structure of gangliosides, negative-ion fast atom bombardment collisionally activated dissociation tandem mass spectrometry (FAB CAD-MS/MS) was used (Donom and Costello, 1988; Ladisch et al., 1989). Helium was used as the collision gas in a cell located in the first field-free region. The [CerO]⁻ fragment ion was selected as the precursor ion for FAB CAD-MS/MS. The helium pressure in the collision cell in both cases was adjusted to reduce the abundance of the precursor by 75%. The JEDL data system was used to generate the linked scans at constant B/E.

Acknowledgments
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Abbreviations

1- CAD-MS/MS, collisionally activated dissociation tandem mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; LBSA, lipid-bound sialic acid; gangliosides are abbreviated according to the nomenclature of Svennerholm (1980). The abbreviations for ceramide structure follow the nomenclature of Breimer et al. (1974).

References


F. Chang et al.


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