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GANGLIOSIDES
IN
HUMAN PRIMARY BRAIN TUMORS

Dissertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University
by

Ching-Ching Sung, B. S.

The Ohio State University
1995

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Approved by
Advisor
Department of Pathology
DEDICATION

This work is dedicated to my father as his 60 year birthday gift.

To My Parents and Brother
With their love and support, I have finally reached my goals.

To My Lord
He makes everything possible.
ACKNOWLEDGMENTS

Especially thanks to my dear advisor and professional father, Dr. Allan Yates for his advice, support, and encouragement for so many years. Without him, I would not have completed all the studies.

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Sincerely thanks to my best friends, Jim Van Brocklyn, Hany Saqr, Rex Phung, Ginnie Yang, and Dianna Hynds for their support in many things. I would like to thank Teresa Franklin, Douglas Dangler, Scott Cross and Raymond Collins for their excellent technical assistance. Also thanks to Drs. Sandy Cottingham and Jiashun Li who helped me review histopathological slides. Thanks to Dr. Ming Zheng who helped me analyze survival data. I am also grateful to have Eleanor Borem and Cindy Fox's help in preparing manuscripts, clinical data management, and so on.

To all who accompany me during these years
Without you these things could not have happened. Thank You !!!
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LIST OF ABBREVIATIONS

ABC  avidin biotin complex
ANOVA  analysis of variance
ATCC  American type culture collection
BCIP  5-bromo-4-chloro-3-indolylphosphate
BSA  bovine serum albumin
CNS  central nervous system
Cer  ceramide
DEAE  diethylaminoethyl
FBS  fetal bovine serum
Fuc  fucose
Gal  galactose
GalNAc  N-acetylgalactosamine
Glc  glucose
GlcNAc  N-acetylglucosamine
GSL  glycosphingolipid
HPTLC  high performance thin layer chromatography
HRP  horse radish peroxidase
HRP-B  horse radish peroxidase conjugated cholera toxin B subunit
Lac  lactose
MAb  monoclonal antibody
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeoLac</td>
<td>neolactose</td>
</tr>
<tr>
<td>NeuAc</td>
<td>N-acetyleneuraminic acid</td>
</tr>
<tr>
<td>NeuGc</td>
<td>N-glycolyneuraminic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>SGGL</td>
<td>sulfoglucuronyl glycolipid</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
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CHAPTER I

INTRODUCTION

A. Historical Background of Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids found primarily in the external layer of the plasma membrane of all vertebrate cells and are particularly abundant in the central nervous system (CNS). The first historical description of such substances was of substances in lipid extracts of kidney and brain detected using the reactions with p-dimethylaminobenzaldehyde and orcinol (Landsteiner and Levene, 1927). Later, Klenk found such carbohydrate-rich glycolipids to be enriched in the gray matter of brain, and proposed the name "ganglioside", as they were known to be a component of neurons "Ganglienzellen" (Klenk, 1942). Fatty acids, sphingosine, galactose, glucose and sialic acid were identified upon hydrolysis of gangliosides (Klenk, 1942). In 1936, Blix found 9% hexosamine in a brain preparation; ten years later, the hexosamine of gangliosides was isolated and named D-galactosamine (Blix et al., 1948).
With chromatographic techniques, gangliosides were separated, and a system of nomenclature was devised (Svennerholm, 1956 and 1963). The four major mammalian gangliosides were characterized by Svennerholm (1962) as having the common backbone Gal-GalNAc-Gal-Glc-Cer, and the complete structures of these gangliosides were determined later by Kuhn and Wiegandt (1963). Gangliosides were one of the first substances postulated to be specific neuronal constituents. Although the concept of uniqueness to neurons proposed by Klenk was eventually found to be invalid, gangliosides were still found to be enriched in brain, particularly in gray matter. Yamakawa and Suzuki (1952) discovered a hexosamine-free ganglioside in erythrocyte membrane and named it hematoside. It was the first demonstration that gangliosides existed in extraneural tissues.

B. Ganglioside Structure

The structure of gangliosides consists of a hydrophilic portion (an oligosaccharide chain containing different numbers of sialic acid residues) and a hydrophobic portion, which is N-acylsphingosine (ceramide). The oligosaccharide portion of gangliosides faces the external environment of the membrane. Ceramide, formed by fatty acid linked to sphingosine via an amide bond, is embedded in the lipid bilayer of the membrane (Fig 1.1). Differences in the structure of gangliosides can occur in the sphingosine, fatty acid and/or the oligosaccharide backbone (Yu and Saito., 1989). Human brain gangliosides contain 80-90% 18:0 (stearic) fatty acids compared to 1% of gangliosides in red blood cells (Månsson et al., 1978). Lacto-series gangliosides in human red blood cells contain longer fatty acid chains (20-24 carbon).
Diversity of the oligosaccharide structure is a hallmark of gangliosides, as of glycosphingolipids generally. About 70 different oligosaccharide chain structures have been reported. In a recent review, 90 ganglioside structures were reported when variations in sialic acid structures were also taken into account (Yu and Saito, 1989).

Based on the oligosaccharide structures, gangliosides are classified into five families (Table 1.1): gala, hemato, ganglio, lacto and globo (Yu and Saito, 1989). The gala-family contains only one ganglioside which originates from galactosylceramide. All other families of gangliosides are derived from lactosylceramide and subdivided into four families according to types of the sugar linked to the galactose moiety of lactosylceramide. The hemato-family of gangliosides has a core structure of lactosylceramide with one or three sialic acid residues. Major mammalian adult brain gangliosides belong to the ganglio-family, characterized by the presence of N-acetyl galactosamine (GalNAc) as the third sugar attached to lactosylceramide. The lacto-family is different from the ganglio in that N-acetylgalcosamine (GlcNAc) is linked to lactosylceramide instead of GalNAc, and is further classified into lacto- and neolacto-series gangliosides based on the linkage between the third and fourth sugars, β1→3 and β1→4, respectively. The lacto and neolacto series are also referred to as type 1 and 2 lacto-series. The globo-family is characterized by having a galactose (Gal) as the third sugar linked to lactosylceramide. Recently gangliosides with complex branching oligosaccharide chain have been identified. The branching oligosaccharide structures consist of hybrids of various oligosaccharide backbone families (Yu and Saito, 1989). Whether these gangliosides should be classified into an
independent family or not has not been determined. With the advance of techniques in isolation and purification of glycosphingolipids, more and more gangliosides almost certainly will be identified.
### Table 1.1. Families of Gangliosides

<table>
<thead>
<tr>
<th>Family</th>
<th>Backbone Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gala</td>
<td>Galβ1→1Cer</td>
</tr>
<tr>
<td>Hemato</td>
<td>Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>Ganglio</td>
<td></td>
</tr>
<tr>
<td>gangliotriaose</td>
<td>GalNAcβ1→4Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>gangliotetraose</td>
<td>Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>Lacto</td>
<td></td>
</tr>
<tr>
<td>lactotriaose</td>
<td>GlcNAcβ1→3Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>lactotetraose</td>
<td>Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>neolactotetraose</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>Globo</td>
<td></td>
</tr>
<tr>
<td>globotriaose</td>
<td>Galα1→4Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>isoglobotriaose</td>
<td>Galα1→3Galβ1→4Glcβ1→1Cer</td>
</tr>
</tbody>
</table>

Glc: Glucose, Gal: Galactose, GalNAc: N-acetylgalactosamine, GlcNAc: N-acetylglucosamine, Cer: Ceramide
C. Ganglioside Nomenclature

There are several systems for ganglioside nomenclature. The most widely used and simplest is Svennerholm's (Svennerholm, 1980). Gangliosides are designated by the number of sialic acids as M (mono), D (di), T (tri), Q (quadra), P (penta), H (hexa) and S (sept). The length of the oligosaccharide is indicated as an arabic number according to the formula: 5-n, where n is equal to the number of sugars in the oligosaccharide. For example, GM1 represents the ganglioside which is made up of one sialic acid and four backbone sugars (Fig 1.2). Therefore, the longer the oligosaccharide chain, the smaller the designated number is.

D. Sialic Acid

Sialic acid is the defining characteristic of gangliosides which distinguishes them from neutral glycosphingolipids. It is a group name for all derivatives of N-acylated neuraminic acids (5-amino-3, 5-dideoxy-D-glycero-D-galacto-nonulosonic acid). Unsubstituted neuraminic acid is unstable due to a reaction between the C-2 keto and the C-5 nitrogen amino group. Thus, only N-acylated derivatives occur in nature. The amino group of neuraminic acid can be substituted by either an acetyl residue, yielding N-acetylneuraminic acid (NeuAc), or a glycolyl residue, to form N-glycolyneuraminic acid (NeuGc). The most common form of sialic acid in mammalian cells is NeuAc. NeuGc-containing gangliosides have been
found in bovine brains. However, they have not been reported to be present in normal human tissues including brains. The different species appear to have some biological significance. For example, the differentiation-dependent distribution of N-acetyl and N-glycolyl species of GM2 is found in human germ cell tumors (Miyaki et al., 1990).

Sialic acid is enzymatically synthesized by condensation of pyruvate and mannosamine (Schauer, 1982). Transfer of sialic acids onto oligosaccharide to form gangliosides is from cytidine monophosphate linked sialic acid. The highly electronegative charge of sialic acid (pK value around pH 2) contributes to the acidic properties of gangliosides. Sialic acids are linked to different positions of other sugars via α-ketosidic linkages, more frequently to galactose (by α2→3 or α2→6) and N-acetylgalactosamine than N-acetylglucosamine or sialic acid itself (Schauer, 1982). The positions and the types of sugars to which sialic acid is attached, along with the multiple forms of sialic acid substituents, contribute to the diversity of ganglioside structure and potential variable biological significance. Part of the results of this dissertation will demonstrate this phenomenon. In addition, sialic acids play important roles in binding and transport of cationic compounds, aggregation (via Ca^{2+} bridges), disaggregation (i.e., repulsive effect on red blood cells), functions for peptide hormones and viruses, maintenance of conformation of glycoproteins and prevention of degradation of proteins by proteases (Schauer, 1982).
Sialidases are the enzymes that release sialic acids from their α-ketosidic linkage on glycoproteins, glycolipids and oligosaccharides. They are widely distributed in a variety of bacteria, viruses and animal tissues. Sialidase activity was first found in *Clostridium perfringens* filtrates that had activities which destroyed erythrocyte receptors for influenza viruses and prevented agglutination (McCrea, 1947). A high level of sialidase activity was found in lactating mammary gland, followed by brain and liver, with trace amounts in the spleen and testes of rats (Rosenberg and Schengrund, 1976). The substrate specificity of sialidase is in part due to the difference in the steric position and linkage of sialic acids to the oligosaccharide chain. The internal sialic acid residue of GM1 is completely resistant to *Vibrio cholerae* sialidase, and slowly reacts with *Clostridium perfringens* sialidase only in the presence of bile salts. But sialidases from *Arthrobacter ureafaciens* can attack the internal sialic acid of GM1 easily. We used different substrate specificities of sialidases to identify the structures of some tumor gangliosides in combination with monoclonal antibodies.
E. Ganglioside Biosynthesis and Degradation

Biosynthesis of gangliosides involves a series of catalytic steps and intracellular transport from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane. The first steps in the synthesis of gangliosides involve the formation of ceramide. These reactions are catalyzed by membrane-bound enzymes on the cytosolic face of the endoplasmic reticulum (Sandhoff and Van Echten, 1993). Ceramides are produced from the condensation of the amino acid serine and palmitoyl-CoA to form the amino alcohol sphinganine, followed by acylation to form amide-linked fatty acid then addition of the 4-trans double bond (Sandhoff and Van Echten, 1993) (Fig. 1.3). Ceramides are then transported to the Golgi apparatus where the glycosylation takes place by sequential addition of monosaccharides (Trinchera et al., 1991). The glycosylation is catalyzed by multiglycosyltransferases utilizing sugar nucleotide donars (UDP-glycose + acceptor → UDP + glycose-acceptor; CMP for sialic acid; GDP for fucose).

The first two glycosylation steps which form lactosylceramide (glucosyltransferase and galactosyltransferase) occur on the cytosolic side of the Golgi (Trinchera et al., 1991). Further glycosylation, adding sialic acid residues or monosaccharides to the growing oligosaccharide, takes place on the luminal face of the Golgi membrane. How the lactosylceramides transfer from the cytosolic face to the luminal face of the Golgi has not been determined. Evidence indicates that the simple gangliosides GM3 and GD3 are formed in the early Golgi compartment, whereas more complex gangliosides such as GM1, GD1a, GT1b and GQ1b are synthesized.
Fig 1.3. The biosynthesis of sphingolipid from serine to lactosylceramide (from Sandhoff K and Van Echten G, Advances in Lipid Research (1993), 26: p. 123).
in a late Golgi or trans Golgi network (Van Echten et al., 1990; Young et al., 1990). The gangliosides are subsequently transferred to the plasma membrane via vesicular flow.

Ganglioside GM3, generated from the terminal sialylation of lactosylceramide, is the precursor of gangliosides of the "a", "b" and "c" series based on the number of sialic acid residues attached to the inner galactose (i.e., one, two, and three sialic acids, respectively) (Fig. 1.4). Glycosyltransferases and sialyltransferases involved in the biosynthesis of gangliosides are not specific with respect to the acceptor lipid. For example, Sialyltransferase IV generates GM1b from GA1, GD1a from GM1, GT1b from GD1b, and GQ1c from GT1c.

Degradation of gangliosides occurs by stepwise hydrolysis reactions. Gangliosides in the plasma membrane are internalized via endocytosis and transported to membrane-bound vesicles from which they are discharged into lysosomes for hydrolase digestion. During the vesicular flow, some gangliosides are recycled to the Golgi or even back to the plasma membrane. Degradation of gangliosides by hydrolases in the lysosome also occurs in stepwise reactions. Some of these products of degradation are recycled for biosynthesis of new glycosphingolipids. After sequential removal of monosaccharides, ceramide is then further broken down into sphingosine and fatty acids. A recent report showed that some of the hydrolases require cofactors called "sphingolipid activator proteins" (SAP) to assist the degradation of short oligosaccharide chains (Sandhoff and
Fig 1.4. The biosynthetic pathway of ganglioside a, b, and c-series (from Sandhoff K and Van Echten G, Advances in Lipid Research (1993), 26: p. 126).
Van Echten, 1993). It has been demonstrated that defects of these activator proteins cause some sphingolipid storage diseases (Fürst and Sandhoff, 1992). For example, a mutation affecting the initiation codon ATG of the SAP-precursor resulted in defects of several SAP proteins and in the accumulation of ceramide, glucosylceramide, lactosylceramide and GM3 in the patients' tissues (Schnabel et al., 1991).

F. Ganglioside Localization

Gross distribution

The patterns and concentrations of gangliosides vary in different tissues and in different species of animals. For example, "a" and "b" series gangliosides are widely distributed in mammalian brains; however, fish brains are rich in "c" series.

Regional differences in ganglioside compositions and patterns within the nervous system have also been recognized (Ando review, 1983). There is a higher proportion of GD3 and GD2 in disialoganglioside fractions of spinal cord than in brain and peripheral nerves; human peripheral nerves contain neolacto-series gangliosides which are not seen in normal adult brain. In general, cerebral and cerebellar gray matter contain a higher concentration of gangliosides (about 3-fold) compared to white matter (Ando review, 1983). Four major mammalian brain gangliosides are GM1, GD1a, GD1b and GT1b, which account for 80-90% of the total gangliosides in the brain. GM4 is abundant in the white matter, being located in the myelin and
Gangliosides are also present in non-neural tissues, such as intestine, mammary gland, liver, thyroid etc. (Ledeen, 1983). However, the concentration of gangliosides in lung, liver and spleen is lower than in brain gray matter by 15-fold (Ledeen, 1983). Gangliosides are present in the serum at a concentration of nmol/ml and are transported by lipoproteins similarly to cholesterol (Senn et al., 1989). They are transported mainly by low-density lipoproteins (LDL) (60%), followed by high-density lipoproteins (HDL) (25-30%) then very low density lipoproteins VLDL (12-14%) (Marcus, 1984). CSF also contains a very low concentration of gangliosides (pmol/ml) (Rodden, 1991).

**Cellular and Subcellular distribution**

Gangliosides constitute 5-10% of the total lipids of neuronal membranes (Ledeen, 1983). This high concentration of gangliosides in the neuronal membrane contributes to the carbohydrate-rich glycocalyx on the surface of neurons. Although the question of whether gangliosides are specifically localized at the nerve ending or are evenly distributed over the entire neuronal membrane has not been solved, some evidence shows that the growth cone membrane contains a higher ganglioside content than the
synaptic membrane (Sbaschnig-Agler et al., 1988). It has been demonstrated that gangliosides are present in clusters surrounding membrane-bound proteins as functional aggregates instead of being evenly distributed on the cell surface (Sharom and Grant, 1978; Lee and Grant, 1980). Gangliosides are not only found in the plasma membrane but also in the subcellular structures such as the Golgi apparatus, the endoplasmic reticulum and the lysosomes where biosynthesis and metabolism of gangliosides occur (see above). Intracellular gangliosides account for up to 24% of the total cellular gangliosides in rat liver cells; however, only 1% of total gangliosides are present in the Golgi where gangliosides are synthesized (Matyas and Morre, 1987). A recent study reported that a high concentration of gangliosides was isolated from the synaptic vesicles of electric organs of Torpedinidae (Ledeen et al., 1988).

G. Biological Functions of Gangliosides in Vivo and in Vitro

There is evidence that gangliosides play important roles in regulation of cell growth and differentiation (Yates et al., 1993; Van Brocklyn et al., 1993; Bremer et al., 1984 and 1986), cell-cell interaction (Hakomori, 1993), cell adhesion (Phillips et al., 1990, Zheng et al., 1993), and neuronal regeneration and repair (Yates, 1986; Schengrund, 1990). Recent studies showed that specific gangliosides inhibit human glioma cell growth by preventing platelet-derived growth factor (PDGF)-induced receptor dimerization, phosphorylation and increases in intracellular calcium concentration (Yates et al., 1993; Van Brocklyn et al., 1993 and Guan et al., 1992). Spiegel and Fishman (1987) showed that endogenous gangliosides can act as bimodal regulators of cell
growth causing both positive and negative effects, depending on the growth phase of the cells. They found that ganglioside GM1-cholera toxin B subunit can stimulate DNA synthesis of quiescent, nontransformed Swiss 3T3 cells but inhibit the growth of ras-transformed 3T3 cells as well as rapidly dividing normal 3T3 cells. Saqr et al. (1995) demonstrated that exogenously added gangliosides GM1, GD1a and GT1b can stimulate DNA synthesis in quiescent human glioma U-1242 MG cells in both confluent and sparse conditions.

In vivo and in vitro evidence suggests that gangliosides have neuritogenic and neurotrophic effects on nerve cells. Formation of aberrant neurites (meganeurites) is seen in patients with GM1-gangliosidosis (Purpura et al., 1978). Guzman-Harty et al. (1990) showed that there are changes in the patterns of gangliosides synthesized and transported by sensory neurons of the rat sciatic nerve following trauma. Several lines of investigation have shown that exogenous gangliosides induce neuritogenesis of neuroblastoma cells and improve the clinical status of some patients with neuropathies (Guzman-Harty et al., 1990; Schengrund, 1990). The cellular and molecular mechanisms responsible for the ganglioside neuritogenic and neurotrophic effects are not clear. They may be related to changes in membrane fluidity, modulation of membrane proteins and growth factor receptors, and cytoskeletal reorganization. Gangliosides are also involved in cell-cell interaction and cell adhesion to noncellular substrates. The function of the integrin receptor in fibronectin mediated cell adhesion has been shown to be modulated by different gangliosides (Kleinman et al., 1979 and Zheng et al., 1993). Sialylated fucosylated lacto- and neolacto series gangliosides (SLeα and SLeα) have been shown to be ligands for endothelial leucocyte adhesion
molecules (ELAM-1). Le<sup>x</sup>-Le<sup>x</sup> interaction in the presence of a bivalent cations was found to be involved in cell compaction in the morula stage of the mouse embryo (Hakomori, 1993). Glycosphingolipid-glycosphingolipid interaction is proposed as an early event in cell recognition, followed by the involvement of adhesion proteins and of integrin receptors in the cell-adhesion model (Hakomori, 1993).

Gangliosides can regulate permeability to ions and flux across neural membranes (Ledeen, 1983; Wu et al., 1990). Stokes' group (Guan et al., 1992) showed that gangliosides GM1 and GT1b inhibited PDGF-stimulated increase in intracellular Ca<sup>2+</sup> in Swiss 3T3 cells. In that study, the amount of the ganglioside taken up by the cells was not sufficient to buffer the increased Ca<sup>2+</sup> involved in PDGF-stimulated Ca<sup>2+</sup> influx. Experimental evidence also supports the hypothesis that Ca<sup>2+</sup> interacts weakly with gangliosides. The intrinsic association constant of Ca<sup>2+</sup> with gangliosides is very low (Langner et al., 1988). Thus, the inhibition of calcium influx by gangliosides is not simply due to the binding of gangliosides to Ca<sup>2+</sup> through their negatively charged sialic acid residues.

The activation of Na<sup>+</sup>, K<sup>+</sup> - ATPase by GM1 in vitro (Leon et al., 1981) and in diabetic animals has also been observed. Bianchi et al. (1988) proposed the hypothesis that the maintenance of normal levels of Na<sup>+</sup>/K<sup>+</sup> ATPase activity by gangliosides resulted in more efficient neuronal metabolism and enhanced axonal growth; the latter was seen in diabetic animals and clinical trials. Gangliosides as receptors for some bacterial toxins, viruses and glycoprotein hormones is well documented (Ledeen, 1983). For
example, ganglioside GM1 is a natural receptor for cholera toxin (Fishman, 1982); GD1b and GT1b are effective in both binding to and neutralizing tetanus toxin. The possible role of gangliosides as receptors for Sendai virus was first demonstrated by the observation that ganglioside-containing liposomes inhibited the agglutination of erythrocytes caused by Sendai virus (Ledeen, 1983).

The biological activity of gangliosides is assumed to depend mainly on the composition of the oligosaccharide; however, several studies suggest that the ceramide portion may also modify the binding of monoclonal antibodies to glycosphingolipids in vitro and may play an important role in biological functions in vivo (Karlsson et al., 1990; Kannagi et al., 1982).

H. Developmental Changes in Ganglioside Composition of Human Brains

During development, brain gangliosides change dramatically in concentration and pattern. The total ganglioside concentration in human frontal lobe increases 3-fold from gestational week 10 to 5 years of age, and maintains a constant level until 80 years of age. The developmental profile of ganglioside "a" and "b" series is dramatically different (Svennerholm et al., 1989). Ganglioside GM1 (GM1a) and GD1a increased 12 to 15-fold from gestational week 10 to age 5 years, the period of dendritic arborization, outgrowth of axons and synaptogenesis. However, the concentration of ganglioside "b" series (GD3, GD2, GD1b, GT1b, and GQ1b) was dominant at gestational week 10 and dropped until term; it then slowly increased through
to 50 years of age. Lacto- and neolacto-series gangliosides, characteristic fetal gangliosides (Molin et al., 1987), decreased near term to the point where they are undetectable using immune analysis (Svennerholm et al., 1989). A study of gangliosides and glycosyltransferases in human fetal brains during gestational weeks 10 to 22 was reported by Svennerholm (1991) and collaborators (Percy et al., 1991). The developmental profile of glycosyltransferase activities corresponds to the change in the ganglioside patterns observed in fetal brains. The decrease in the activity of GD3 synthase after gestational week 15 was accompanied by a decrease in b-series gangliosides, and was followed by a sharp increase in the level of activity of GM2 synthase and a-series gangliosides (Percy et al., 1991).

I. Gangliosides and Cancer

Gangliosides as Tumor Associated Antigens

Glycosphingolipids as tumor associated antigens have been well-documented in several biological systems. Glycosphingolipid alterations in hamster and human fibroblasts transformed by DNA viruses, RNA viruses, chemical carcinogens and radiation were shown in several studies (Hakomori's review, 1989; Yogeeswaran's review, 1983). Evidence from animal model systems (such as rat hepatoma, mammary carcinoma and mouse melanoma) transformed by chemical agents or spontaneous tumors also demonstrated glycosphingolipid changes in these tumors compared to their normal counterparts. Merritt et al. (1978) found that the ganglioside pattern showed a progressive simplication from hyperplasia to malignant
hepatoma in rat liver cells transformed by N-fluorenylacacetamide. With the introduction of hybridoma technique for production of monoclonal antibodies (Kohler and Milstein, 1975), several tumor associated gangliosides defined by monoclonal antibodies were reported (Dippold, 1980; Magnani, 1981). GD3 was found to be the most abundant ganglioside in human melanomas and therapy of melanomas with the anti-GD3 monoclonal antibodies R24 was found to be associated with partial regression of melanoma metastasis (Irie and Chandlen, 1989).

The changes in glycosphingolipid patterns in transformed cells and tumors vary among different types of cells and tumors. However, there are four basic changes in gangliosides associated with oncogenic transformation (Hakomori, 1985): a) Incomplete synthesis of normally existing gangliosides with the accumulation of precursor gangliosides, e.g. GD3 in human melanoma; b) Enhanced synthesis of neogangliosides, such as aberrant chain elongation, sialylation and fucosylation; c) Shifting of ganglioside synthesis from one series to another; d) Change of glycolipid organization in membranes. These changes can occur in various combinations.

**Immunological aspects of gangliosides**

Several lines of evidence suggest that gangliosides may have immunomodulatory properties on lymphocytes. Whisler and Yates (1980) and Ladisch et al. (1984) demonstrated the inhibitory effect of gangliosides on lymphocytes in response to different types of mitogens *in vitro*. A recent study from Chu et al. (1993) showed that gangliosides inhibit T-lymphocyte
proliferation by competing with interleukin-2 (IL-2) receptor for available IL-2. They also demonstrated the binding of IL-2 to gangliosides on TLC. Evidence showed that GM1-CD4 interaction induced CD4 down modulation through dissociation of protein tyrosine kinase p56lck from CD4 and triggered CD4 internalization (Saggioro et al., 1993). The increased level of serum or ascites fluid gangliosides in some human cancers (such as hepatoma, neuroblastoma, melanoma and mammary carcinomas) and tumor-bearing animals has been observed (Ladisch et al., 1987; Portoukalian et al., 1989). The mechanism and the biological significance of ganglioside "shedding" from tumor cells into plasma in the form of both membrane fragments and micelles are not clear. However, it may be at least part of the mechanism responsible for tumor cells escaping immunosurveillance (Ladisch et al., 1987). The rate of ganglioside shedding is associated with the metastatic potential of the tumor cells (Ladisch et al., 1987). Ladisch et al. (1983) suggested that the higher level of gangliosides in plasma, especially polysialogangliosides, may contribute to the immunosuppressed status of some tumor-bearing animals and human cancer patients.

**Gangliosides and human brain tumors**

Although the majority of the above studies have been done in non-neural tissues and cells, preliminary data from several groups on human brain tumors indicate that studies of gangliosides could be very useful in understanding the biology of gliomas. Human neural tumors have larger amounts of simple gangliosides and smaller amounts of complex gangliosides when compared to normal brain (Kostic and Buchheit, 1970; Yates et al.,
1979; Traylor and Hogan, 1980). A dramatic increase in the percentage of GD3 (20%) was observed in astrocytoma grade IV compared with that in normal brain (5%) (Taylor and Hogan, 1980). Eto et al. (1982) also found that primary gliomas have a high content of GD3, but metastatic tumors (multiple myeloma and adenoma) to the brain do not. In 1985, Berra's group demonstrated a correlation between the loss of polysialylated gangliosides and histological grade of malignancy in 38 human astrocytomas. Four major brain gangliosides (GM1, GD1a, GD1b and GT1b) were found to be decreased in gliomas, but the simple gangliosides GM3 and GD3 were predominant compared to normal brain (Fredman et al., 1986). However, they did not study the correlation between these gangliosides and the histological grades of tumors. The lacto-series ganglioside, 3'-isoLM1, was found to be a human glioma-associated antigen (Fredman et al., 1988). In a subsequent study of glioma tissues obtained at autopsy (Fredman et al., 1993), the presence and the amount of 3'-isoLM1 were highly variable from region to region. It was not present in any of the macroscopically homogenous tumor areas, but was elevated in the hemisphere contralateral to the tumor, making its relationship to glioma growth questionable. Therefore, it was necessary to conduct a study in more detail and in a larger sample size than any previously reported studies using specific methods to identify glioma-associated antigens.

J. Statement of the Problems

The incidence of human primary brain tumors has increased during the past two decades (Posner J.B., 1993), especially in the elderly. Brain tumors are the second cause of tumor mortality next to leukemia in young people and
the commonest solid tumor of childhood (Black, 1993). Due to the limitations of histological diagnosis of brain tumors by light microscopy, the NCI Glioma Marker Network was established to search for more objective markers to aid in the classification and prognosis of human brain tumors.

Gangliosides were chosen as one of the candidates because they have been found to change in many neoplastic tissues and cell lines compared to their normal counterparts, and they have significant biological functions relevant to tumor biology. Therefore, we have studied the ganglioside compositions and patterns of human primary brain tumors in a larger series than has been previously published in the literature and in more detail utilizing different methods including immunostaining with several monoclonal antibodies and cholera toxin to determine the structures and quantities of gangliosides. The results of these studies demonstrate that gangliosides may be of considerable value in the classification, diagnosis and prognosis of human gliomas. Based on the information presented above we formulated several questions for this study.

The questions we want to answer are as follows:

1) Are the compositions and patterns of gangliosides changed in human primary brain tumors compared to normal human brain? If they are changed, do they correlate with the degree of histological malignancy?

2) Does any specific pattern of gangliosides correlate with cell lineage of brain tumors? (e.g. astrocytic vs. oligodendrocytic)
3) Are any aberrant gangliosides, which are not expressed in normal adult brain, found in these tumors?

4) Is there any correlation between patient survival and the ganglioside pattern? If so, is this relationship restricted to certain types of tumors? Can gangliosides provide prognostic information in addition to that of histology?

5) What is the ganglioside composition of a cultured human malignant glioma cell line known to bear PDGF receptors?
CHAPTER II

METHODS

To study the ganglioside compositions of human brain tumors, we used the following methods to isolate and purify gangliosides from human tissues.

A. Tissue Specimens

Brain tumor specimens were obtained from surgical biopsies and autopsies of children and adults. Tissues were received from member institutions of the National Cancer Institute Glioma Marker Network (The Ohio State University, Barrow Neurological Institute and Mayo Clinic) and the National Cancer Institute Cooperative Human Tissue Network. Normal human brain tissues used as controls were from autopsies of patients with causes of death unrelated to neurological disease. The surgical samples were rinsed in cold saline and were snap frozen in isopentane cooled by liquid nitrogen, and transported to our laboratory on dry ice. The tissue specimens were kept in a -70° C freezer or the vapor phase of a liquid nitrogen freezer until processing.
Prior to lipid extraction, a frozen section slide of each tissue was prepared and stained with hematoxylin and eosin (H&E) for quality control to ascertain that the tissue used for extraction consisted of at least 80% of tumor. Most consisted entirely of tumor. The quality control slides were examined by Drs. Li and Cottingham.

B. Tissue Processing

For those tissues that qualified, we proceeded with chemical study. The frozen tissue was weighed and split into two parts, one for lipid analyses, the other for immunohistochemistry. The flow chart illustrating the processing of tumor tissue, ganglioside isolation and identification is shown in Fig 2.1. We require a minimum of 300 mg of tissue to conduct the complete study of glycolipids. To minimize the variability of histopathological diagnosis, a representative H&E stained paraffin embedded slide of a representative piece of each tumor tissue was reviewed by four neuropathologists-Drs. Allan J. Yates (The Ohio State University), Stephen W. Coons (Barrow Neurological Institute), Bernd W. Scheithauer (Mayo Clinic) and Peter C. Johnson (Barrow Neurological Institute). These were initially viewed independently and discrepancies resolved as much as possible by simultaneous viewing using a multiple headed microscope. For each tumor tissue, consensus diagnoses and grades were given using both the criteria of the World Health Organization and the St. Anne-Mayo system.
Fig 2.1. Flow chart illustrating the processing of tumor tissue, ganglioside isolation and identification.
C. Ganglioside Extraction, Isolation and Purification

Prior to chemical analysis, the tissues were thawed, blotted dry with a tissue paper for removal of excess water and weighed on an analytical balance (E. Mettler no. 1-911, Zurich, Switzerland). This weight was called "sample fresh weight". The tissue was placed in a glass homogenizing tube (Thomas B24) and lyophilized overnight using a freeze dryer (Virtis company, Gardiner, NY). The sample was weighed again after lyophilization to obtain the "sample dry weight". Water content was estimated as the difference between fresh and dry weights.

**Tissue homogenizing and total lipid extract**

Tissues were homogenized twice in organic solvents using a Thomas tissue grinder (Suzuki, 1965). For the first homogenization, 4 ml of water per gram sample fresh weight was added to the lyophilized tissue one hour before homogenization. Then 16 ml of chloroform/methanol (1:2) per gram fresh weight was added to make a final concentration of chloroform/methanol/water (1:2:20%) (Svennerholm and Fredman, 1980) and the tissue homogenized. The homogenate was centrifuged at 800 rpm for 15 minutes to separate a non-lipid residue pellet from a lipid extract supernatant. The non-lipid residue was homogenized a second time using 20 volumes (i.e., 20 ml solvent per gram sample fresh weight) of chloroform/methanol/water (1:1:5%). Both supernatants were collected into a round bottom flask and dried in a rotary evaporator. The dried total lipid extract was transferred to a tube using chloroform/methanol (2:1) and subjected to a Folch partition which separates
the acidic glycolipids into the upper phase from the neutral glycolipids into the lower phase. The non-lipid residue was saved in a homogenizing tube and placed into a desiccator for 2 days prior to weighing for the non-lipid residue weight.

**Separation of gangliosides from other lipids - Folch partition**

Folch et al. (1957) first described the solvent partition method for separation of gangliosides from other lipids by washing the total lipid extract [in chloroform/methanol (2:1)] with 0.2 volumes of water containing cation. This method results in most of the acidic lipids and other water-soluble materials partitioning into the aqueous upper phase (C/M/W, 3:48:47) and leaves the less polar substances in the lower phase (C/M/W, 86:14:1). We performed the modified Folch method using the initial partition with a total of 20 volumes of C/M 2:1 plus 0.1M KC1 in a ratio of 5:1. The lower phase was partitioned 3 more times using theoretical upper phase (C/M/W 3:48:47) without KC1 to recover less polar gangliosides such as GM3. All four upper phases were pooled and dried under nitrogen. This is the "crude ganglioside" portion. The lower phase was termed "washed lower phase". The washed lower phase was subjected to further purification for neutral glycolipid analyses.

After Folch partition, the upper phase containing gangliosides was passed through a reverse phase chromatography column to remove water-soluble contaminants such as salts.
**Bakerbond SPE C-18 Reverse Phase Chromatography (J. T. Baker, Phillipsburg, NJ)**

**Technique**

1. Dry sample under nitrogen
2. Add calculated amount of distilled water and 0.1M KCl into the sample to make a final concentration of 0.1M KCl. The amount of water and KCl calculated for each tumor varies with the original amount of KCl added in Folch partition.
3. Pre-equilibrate columns with 2 ml methanol then 2 ml 0.1M KCl. Take care that the column does not dry except where stated in this procedure.
4. Sonicate and vortex well, then load the sample onto the column.
5. Rinse tube with 1 ml 0.1 M KCl, sonicate and vortex, then add to column.
6. After passing first 2 ml through column, reload and pass same 2 ml through C-18 again. Allow columns to dry at this point.
7. Desalt column, two times or more, with one column volume (1 ml) of water each time. Collect each wash separately. Check last wash with 5 or 6 drops of 10% silver nitrate; precipitate indicates salt is still present.
8. When all salt has been removed, pull column dry at 80kpa for at least 20 minutes. Dry column reservoir with cotton swab.
9. Elute ganglioside sample with 5 ml C/M 1:1. Make sure that the column gets saturated with solvent.
10. Dry the ganglioside fraction under nitrogen, then reconstitute to a known volume.
E. Sialic Acid Quantitation of Ganglioside by Resorcinol

There are several methods to quantitate gangliosides. Most of these methods are based on the detection of sialic acids since they are the defining component of gangliosides. The resorcinol assay is a colorimetric method that reacts with both free and lipid-bound sialic acids. It was originally developed by Svennerholm (1957) and modified by Miettinen and Takki-Luukkainen (1959) using butanol:butyl acetate (15:85) to extract the chromophore. Since then, this method has been widely used for quantitation of gangliosides.

Resorcinol Reaction for Sialic Acid

Reagents needed:

- 5 N HCl
- 0.025 M CuSO₄ in 5 N HCl
- Resorcinol reagent: 200 mg resorcinol (Sigma, St Louis, MO) dissolved in 1 ml 0.025 M CuSO₄ reagent, and make to 100 ml with 5 N HCl.
- Butanol/Butyl Acetate (v/v): 15:85
Procedure:

1. Aliquot standards and samples in duplicate, into glass stoppered tubes. Use recrystallized quantitated N-acetylmuraminic acid standard (Sigma) at 0, 2, 3, 5, 7, and 10 μg. Samples after C-18 column are aliquotted using an estimated amount of ganglioside containing 3 μg sialic acids. This is based on the assumption that tumor tissue contains approximately 60 μg sialic acids per gram fresh weight.

2. Dry samples under nitrogen.

3. Add 1 ml 5 N HCl then 1 ml resorcinol reagent. Stopper and vortex immediately.

4. Put tubes in basket and place into a boiling water bath for 15 minutes. Place a weighted cover over the entire basket of tubes as stoppers tend to pop off.

5. Cool tubes in ice water (about 15 minutes).

6. Add 1 ml butanol/butyl acetate (15:85), shake tubes vigorously, then centrifuge at 1200 rpm to obtain 2 phases.

7. Transfer the purple upper phase to a silica class AA semi-micro microcell cuvette (Beckman, Fullerton, CA) and estimate absorbance using a DU series 60 spectrophotometer (Beckman, Fullerton, CA) against butanol/butyl acetate (15:85) as a blank.

8. The samples are read at three wavelengths: 470, 580 and 620 nm. (Multiple wavelength program is stored in program 0 and 1)

9. The linear standard curve is based on the corrected absorbance at 580 nm.
NOTE: The two-wavelength correction factor (Wherrett and Brown, 1969) is used for any potential monosaccharide contaminants in the sample.

1. Correction Factor = 1.073 - \[\text{absorbance at } 470 \text{ nm} \times 0.265\]
   \[\text{[absorbance at } 580 \text{ nm]}\]

For most ganglioside preparations of high purity, the correction factor is usually close to 1. If the correction factor is below 0.700, the 620 nm readings should be used instead of the 580 nm readings since the absorbance at 620 nm is more specific for sialic acid, although less sensitive than at 580 nm.

F. Ganglioside Separation Using Anionic Exchange Chromatography

Gangliosides are isolated and separated into mono- and polysialoganglioside fractions by elution from the anion exchange resin diethylaminoethyl (DEAE) Sephadex A-25 (Sigma, St. Louis, MO) with a discontinuous gradient of 0.02 M, 0.04 M and 0.25 M ammonia acetate in methanol.
Method of DEAE-Sephadex A-25 column

Preparation of DEAE-Sephadex

1. Suspend 100 grams of DEAE-Sephadex in 1 liter distilled water. This should be done by swirling in a 3-4 liter flask. Let it stand at room temperature for 1 hour.

2. Add 1 liter 1N NaOH, swirl several times, allow to sit at room temperature for 1 hour. Pour off supernatant.

3. Add water with swirling until flask is almost full. Let the slurry stand 15-20 minutes (to allow DEAE to settle). Pour off supernatant. Repeat step #3.

4. Transfer DEAE to a large Buchner funnel (which uses 18.5 cm filter paper) and wash with water (4-6 liters) until pH of filtrate equals that of the distilled water (pH = 6.5). Check with pH meter.

5. Transfer DEAE back to a flask and add 2 liters 0.5 M acetic acid (to make 2 liters, 0.5 M acetic acid, add 57.5 ml glacial acetate to 2 liters of solution). Swirl. Let the slurry stand 30 minutes. Pour off supernatant.
6. Repeat step #3, three times.

7. Transfer DEAE back to Buchner funnel and wash with water (at least 10 liters) until pH = 4.5-5.0.

8. The washed DEAE can be stored in the cold room at this point. It should be suspended in 3-4 liters water. Filter DEAE using Buchner funnel prior to further purification.

9. Repeat steps #1-7, except at step #1 there is no need to wait 1 hour (since it is not necessary for the DEAE swell again); and at step #5, prolong the time to 1.5 hours from 30 minutes.

10. Turn off the vacuum and fill the funnel with methanol. Gently stir DEAE using care not to dislodge the filter paper. Allow methanol to drain by gravity, taking care that the DEAE does not dry.

11. When methanol has drained, transfer the DEAE with a spatula to a reagent bottle, using a powder funnel. Add solvent A: C/M/W (30:60:8) until the bottle is 75% full, swirl several times and place in the cold room overnight.
12. Pour off the supernatant and add C/M/W (30:60:8). Allow to settle 20 minutes then decant the supernatant. Repeat step #12 several times. This step is very important for removal of sodium acetate. If not completely removed, it will severely interfere with the elution profiles of gangliosides.

13. Add a volume of C/M/W (30:60:8) equal to that of swollen DEAE. This is now ready to use and may be stored in the cold room for a minimum of one year. Make certain that the DEAE does not dry during storage by adding C/M/Water (30:60:8) occasionally.

**Packing the column**

1. Set up a vertically mounted 50 ml column (i.d. 0.5 cm X 32 cm)

2. The gel in solvent A (C:M:W, 30:60:8, v/v) is packed into the column to a bed volume of 3 ml. If air bubbles appear the bed must be repacked.

3. Before loading the column with gangliosides, the column is washed with 60 ml of Solvent A to remove sodium acetate, which is tested for in the first wash using 10% silver nitrate.

* The available capacity of this DEAE column (3 ml bed volume) is more than 50 µg sialic acid of a mixture of gangliosides.
Sample application

4. The desalted gangliosides (25 µg sialic acids) dissolved in 2 ml of solvent A are applied onto the bed surface of the column with a pipette and the outlet is opened.

5. When the entire sample has entered the bed, wash the column with 10 ml each of solvent A and methanol.

Elution

6. The column is sequentially eluted with 15 ml each of 0.02 M, 0.04 M (for monosialogangliosides) and 0.25 M ammonia acetate (for polysialogangliosides) in methanol, and collected in 3 tubes. Once beginning the elution, do not close or change the column outlet. This avoids changing the flow rate which will disturb the elution pattern. The flow rate should be controlled to be under 0.5 ml/min.

7. The fractions of 0.02 M and 0.04 M ammonia acetate in methanol are pooled for the monosialoganglioside fraction. The fraction of 0.25 M is the polysialoganglioside fraction.

8. Dry both fractions under nitrogen before desalting on a C-18 column using the following modified method.
*Modified C-18 Reverse Phase Column*

**Procedure:**

1. Dry mono- and polysialoganglioside fractions from DEAE-Sephadex A-25 column.

2. Add 2 ml of methanol to each fraction.

3. Pre-equilibrate columns with 2 ml of methanol. Take care that the column does not dry except where stated in this procedure.

4. Sonicate and vortex well, then load the sample onto the column.

5. After passing the first 2 ml of sample through the column, reload it and pass the sample through the C-18 column again. Allow columns to dry at this point.

6. Desalt columns: fifteen times for mono-fraction and thirty five times or more for poly-fraction with one column volume of water each time. Collect each wash separately. Check the last wash with 5 or 6 drops of 10% silver nitrate for precipitate indicating salt.

7. When all salt has been removed, pull the column dry at 80 KPa for at least 20 minutes. Dry the column reservoir with a cotton swab.
8. Elute the ganglioside sample with 5 ml of C/M 1:1. Make sure that the column becomes saturated with solvent.

9. Dry the ganglioside sample under nitrogen, then reconstitute it to a known volume.

G. Separation of Gangliosides on HPTLC

The desalted ganglioside samples from mono- and poly-sialoganglioside fractions or total gangliosides (estimated total 1.5 µg sialic acids) are spotted on a 0.5 cm lane of a glass backed silica gel HPTLC plate (Merck, Darmstadt, Germany) using a 10 µl Hamilton syringe. The pooled ganglioside standards of GM3, GM2, GM1, GD3, GD1a and GT1b are also spotted as references for TLC mobility. TLC plates are developed in an appropriate solvent system depending on the experimental purpose. The general solvent system is C/M/W (55:45:10, v/v/v) containing 0.2% CaCl₂. To separate NeuGc- from NeuAc-containing gangliosides, an alkaline solvent system is used as follows: C/M/5M NH₄OH/0.4% CaCl₂ (50:50:4:5). The developed plates are sprayed with resorcinol-hydrochloric acid and heated on a hot plate at 95°C for 20 minutes. Each ganglioside is visualized as a purple band and quantitated by densitometric scanning.
H. Densitometric Analysis of Gangliosides Separated on HPTLC Plates

The Shimadzu CS-9000 (Kyoto, Japan) Scanning Densitometer is an instrument that has automated quantitative and qualitative interpretation of densitometric scans of TLC, gels and autoradiographs and films. The CS-9000 has the zigzag technique, working curve linearizer, and background correction. The zigzag scanning provides more reliable quantitation of samples than linear mode scanning since it oscillates from side to side in the lane while reading the signal and background. In linear mode, the lane is read in a straight line through the middle of lane. Therefore, we set the scan mode in zigzag for each analysis of HPTLC plates. The parameters are set as follows:

Control parameters:

- photo mode: reflection
- scan mode: zigzag
- wavelength (λ): single, 580 nm
- Zero set mode: at start

Procedures:

1) Set the HPTLC plate onto the stage.
2) Select the lamp source to visible light.
3) Manually move the stage until the beam rests squarely before the first spot of the first lane to be scanned. Record the X and Y position.
4) Repeat step #3 until the beam is above the last spot of the first lane.
5) Record the new Y position.
6) Input the X and Y data into the "Lane Auto Scan Parameters".
7) Press "Measure".

For quantitation of unknown samples, we spotted a few lanes of standard compounds with known concentrations and scanned the plates to create a linear calibration curve. Based on the calibration curve, we quantitated the concentration of each immunopositive band in the HPTLC. The calibration curve is created by the external calibration method in a linear scale for the concentration axis and peak area axis. The data processing is stored in a 1D analysis program. The results of scanning are expressed either as percentage distribution of total ganglioside sialic acid or as concentration based on calibration curves.

I. Immunostaining HPTLC

When analyzing tumor gangliosides, especially those present in nanogram levels, a sensitive and specific method is necessary for detection and quantification. Because these gangliosides may be below the amounts detectable by resorcinol spray and/or not resolved from other major gangliosides with which they comigrate, immunostaining HPTLC was used. The method of immunostaining HPTLC for detection of glycosphingolipids was originally developed by Magnani (1981) and modified by Buehler and Macher (1986). The advantage of the modified method is the elimination of any hazardous radioactive materials by using an avidin-biotin enzyme system for detection. The immunostaining TLC method consists of two main steps: (i)
ganglioside separation on HPTLC in an appropriate solvent system; (ii) a specific species of gangliosides is detected by a specific binding compound. Recently, bacterial toxins (from cholera and botulinum), lectins and monoclonal antibodies have been employed for binding and identification of oligosaccharide structures of glycosphingolipids. We used cholera toxin and several antibodies (either generous gifts or hybridoma cultures in our laboratory) for identification of tumor ganglioside structures.

**Cholera Toxin B-Subunit**

Cholera toxin produced by *Vibrio cholerae* has two structural and functional components (Fishman, 1982). The A subunit of cholera toxin mediates its catalytic function by activating adenylate cyclase; the subsequent rise in cyclic AMP concentration results in chloride and water loss by the intestinal epithelial cells and causes the diarrhea. The B subunit mediates its binding function by binding to the membrane receptor which is GM1. The B subunit specifically binds to ganglioside GM1 at the low nanogram level on HPTLC plates (Wu and Ledeen, 1988). We used cholera toxin B-subunit with neuraminidase treatment to identify any ganglioside containing the GM1 oligosaccharide backbone. Since this assay is very sensitive and straightforward, it conserves ganglioside samples for other uses. The results of the cholera toxin assay lead us to believe that there are other families of gangliosides present in the tumor samples in addition to the ganglio family of gangliosides. Thus we used different antibodies against different types of oligosaccharide chains for further identification of gangliosides in these tumors.
Materials

0.4% polyisobutylmethacrylate in hexane

Neuraminidase from Clostridium perfringens: Sigma type V;

dissolve 1 unit in 5 ml acetate buffer to make
a concentration of 1 unit/5 ml.

Horseradish peroxidase conjugated B subunit (List Biological
laboratories Inc, Campbell, CA) code #105: dilute 4
µg B in 5 ml with 1 % BSA-PBS to make a
concentration of 800 ng B-subunit/ml.

4-chloro-1-naphthol: Sigma # C-8890

dissolve 10 mg in 3 ml methanol then add 15 ml
0.05 M Tris-HCl buffer (pH 7.5) and 5 µl 30% H₂O₂

Acetate buffer: pH 5.2, 0.05 M

Tris-HCl buffer: pH 7.5, 0.05 M

Technique

1. Spot gangliosides on aluminum backed silica gel-60 HPTLC plate.

0.1 ng sialic acid of GM1 can be detected on chromatograms.

2. Develop the plate in chloroform/methanol/0.2% CaCl₂ (55:45:10).

3. Air-dry.

4. Cut plates with razor into two parts. One is for resorcinol spray. The other is for immunostaining.
5. Immerse the plate for immunostaining in 0.4% polyisobutylmethacrylate for 2 minutes.

6. Air-dry.

7. Soak the plate with 0.05 M acetate buffer (pH = 5.2) for 1 hour.

8. Treat with neuraminidase for 2 hours at 37°C.

9. Remove neuraminidase by draining fluid from the plate.

10. Incubate with freshly made 4 μg B-subunit of HRP-B in 0.1% BSA-PBS for 1 hour on a rotary shaker.

11. Remove the solution. Wash with distilled water in a petri dish twice on a rotary shaker for 5 minutes.

12. Add 4-chloro-1-naphthol solution (freshly made).

13. Let stand for 20 minutes.

14. Wash the plate with distilled water.

15. Air-dry.

Antibodies

A list of the antibodies used in this study is provided in Table 2.1.
<table>
<thead>
<tr>
<th>Names of Abs</th>
<th>Epitopes Recognized by Abs</th>
<th>Species and Isotype</th>
<th>Sources</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1H11</td>
<td>Neolactotetraosyl (type 2 lacto-series)</td>
<td>Mouse IgM</td>
<td>Dainabot Inc., Japan.</td>
<td>Myoga et al., 1988</td>
</tr>
<tr>
<td>TE3</td>
<td>Lactotetraosyl (type 1 lacto-series)</td>
<td>Mouse IgM</td>
<td>Pacific Northwest Res. Institute, Seattle, WA.</td>
<td>Holmes and Greene, 1990</td>
</tr>
<tr>
<td>NS24</td>
<td>Sialylα2→3Galβ1→4GlcNAc</td>
<td>Mouse IgM</td>
<td>Univ. of Shizuoka, Japan.</td>
<td>Suzuki et al., 1991</td>
</tr>
<tr>
<td>CSLEX1</td>
<td>Sialyl Lewis X (SLe*)</td>
<td>Mouse IgM</td>
<td>ATCC hybridoma.</td>
<td>Chia et al., 1985</td>
</tr>
<tr>
<td>CF4-C4</td>
<td>Lewis A (Le*)</td>
<td>Mouse IgG1</td>
<td>ATCC hybridoma.</td>
<td>Young et al., 1983</td>
</tr>
<tr>
<td>HNK-1</td>
<td>SO3-3-GlcA</td>
<td>Mouse IgM</td>
<td>Kennedy Shiver Center for Mental Retardation.</td>
<td>Prasadarao et al., 1990</td>
</tr>
<tr>
<td>2-39M</td>
<td>N-glycolyl GM3</td>
<td>Human IgM</td>
<td>Memorial Sloan Kettering Cancer Center.</td>
<td>Furukawa et al., 1988</td>
</tr>
<tr>
<td>MK1-17</td>
<td>N-acetyl &amp; N-glycolyl GM2</td>
<td>Murine IgM</td>
<td>Aichi Cancer Center, Japan.</td>
<td>Miyaki et al., 1988</td>
</tr>
<tr>
<td>MK2-34</td>
<td>N-glycolyl GM2</td>
<td>Murine IgM</td>
<td>Aichi Cancer Center, Japan.</td>
<td>Miyaki et al., 1988</td>
</tr>
<tr>
<td>Anti-GA1</td>
<td>GA1 (asialo GM1)</td>
<td>Rabbit polyclonal</td>
<td>Wako Chemicals, TX.</td>
<td>Kasai et al., 1980</td>
</tr>
</tbody>
</table>
Technique

1. Aluminum backed silica gel-60 plates are cut to 10 cm × 10 cm using a razor. Prewash the plates in chloroform/methanol (2:1) and air dry.

2. Separate the ganglioside samples on aluminum backed silica gel-60 HPTLC in C/M/0.2%CaCl₂ (55:45:10).

3. Air dry the plate.

4. The plate is cut into two portions. One is for immunostaining, the other is for resorcinol spraying.

5. Coat the immuno-portion of the plate with 0.4% polyisobutylmethacrylate for 1 1/2 min by submerging the plate in the solution then air drying the plate.

6. Block non-specific binding with 1% BSA in PBS for 30 min in a humidified chamber.

7. Incubate with primary antibodies (dilution varies for each antibody) for 2 hours at room temperature.

8. Wash in PBS 3 times for 10 min each time in a rotary shaker.

9. Incubate in biotin conjugated secondary antibodies for 1 1/2 hour at room temperature.

10. Repeat step #8.

11. Incubate the plate in avidin-biotin-alkaline phosphatase complex solution (Vectastain ABC kit AK-5000) (Vector Laboratory, Burlingame, CA); (prepare the solution 30 minutes before use).

12. Wash the plate twice in PBS and once in 100 mM Tris/HCl pH = 9.5.
13. Apply alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) on the plate. Watch carefully for color development which occurs in about 10 minutes.

14. Stop the reaction by washing the plate in PBS and then in 5 mM EDTA/PBS.

15. Air dry.

J. Hybridoma Cultures

Hybridomas CSLEX1 and CF4-C4 were purchased from American Type Culture Collection (Rockville, Maryland). CSLEX1 secretes mouse IgM antibodies which react with sialyl Lewis X antigen: 

\[ \text{NeuAc} \alpha_2 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3) \text{GlcNAc}. \]

CF4-C4, a monoclonal antibody clone, produces mouse IgG1, which recognize Lewis A determinants containing \( \text{Le}^a(\beta_1 \rightarrow 3) \text{Gal}, \text{Le}^a(\beta_1 \rightarrow 6) \text{Gal} \) and \( \text{Le}^a (\text{Fuc} \alpha_1 \rightarrow 4) \text{GlcNAc} \rightarrow R. \)

Both hybridoma clones were cultured in RPMI medium supplemented with 15% fetal bovine serum and grown in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. The medium contained penicillin-streptomycin (100 units/ml-100 mg/ml), Fungizone (2.5 mg/ml) and Gentamycin (50 \( \mu \)g/ml). Medium was changed every 2-3 days by centrifugation at 750 rpm for 15 minutes at room temperature, and reinoculating cell pellets with new medium. Culture supernatants were collected after 3-4 days when cell density reached \( 10^7 \) cells/ml. After centrifugation at 3000 rpm for 15 minutes, the supernatant was frozen at
-70°C for long term storage. To adjust to neutral pH, tris buffer (0.05 M, pH = 7) was added in 1/20 volume of culture supernatant. Sodium azide 0.025% was added for preservation.

K. Monoclonal Antibody Isotyping And Quantification Of Proteins

To check further the isotypes and subclasses of mouse antibodies including the light chain for hybridoma culture supernatant of CSLEX1 and CF4-C4, we used a very simple and easy commercial kit, MabCheck (Sterogene, Arendia, CA). This kit contains several test strips onto which rat anti-mouse antibody isotypes have been coated in the form of separate bands. The diluted sample (400 ml with 800 ml diluent) and the strip were incubated together in a trough for 30 min at room temperature. The mouse antibodies of the test sample react with the corresponding isotype bands on the strip. The detection system consists of rat anti-mouse (kappa or lambda) conjugated alkaline phosphatases. Subsequent incubation with BCIP substrate produces a dark purple band in proportion to the amount of specific antibodies present in the sample. Total assay time is one hour. The sensitivity of the test is 0.15 mg/ml or lower. The results confirm that CSLEX1 contains mainly mouse IgMκ although there is a very weak band comigrating with IgG1. It indicates that the CSLEX1 secretes mouse IgM and a small amount of IgG1 as previously published. The lower panel showed that CF4-C4 is a pure mouse IgG1κ (Fig 2.2).
To determine the antibody concentration in culture supernatants of CSLEX1 and CF4-C4, we performed protein quantitation based on the bicinchoninic acid method using a QUICKMeasure kit (Sterogene, Arendia, CA). Mouse antibodies containing kappa light chains can be specifically captured onto the column and separated from the bovine antibodies present in the culture medium. The protein concentration of the eluted antibodies from the column are then measured by spectrophotometer at 562 nm based on external standard calibration curve. The antibody concentrations for both supernatants were obtained as follows: CSLEX1-0.93 mg/ml; CF4-C4-1.0 mg/ml.
Fig 2.2. Monoclonal antibody isotyping. Right panel: CSLEX1; left panel: CF4-C4. The first and second strips in both panels were kappa and lambda conjugated, respectively.
I. Immunohistochemical Staining

With the technique of immunohistochemistry, we were able to study the histological distribution of glycolipids in human brain tumor tissues. To prevent the elimination of lipid antigens due to fixation, we used frozen sections for the study. Staining results were determined as positive if more than 5% of tumor cells stained.

1. Frozen tumor tissues were cut on a cryostat at 6 μm in thickness, mounted on Superfrost/Plus slides, air dried and then fixed in 4% paraformaldehyde for 5 minutes. Positive and negative tissue controls were prepared in the same way (e.g. Colonic carcinoma and bovine kidney were used as positive tissue control for CSLEX1 and F1H11, respectively. Human normal adult brain was used for both antibodies as negative tissue control).

2. Rinse the slides once with PBS.

3. If primary antibody is CF4-C4 or F1H11, tissue sections were subjected to Arthrobacter urefaciens neuraminidase treatment (40 mU/ml in acetate buffer) for 2 hours at 37° C incubator prior to immunostaining.

4. Rinse twice with PBS.

5. All tissues were treated with 3% hydrogen peroxide in methanol for 15 minutes at room temperature to block endogenous peroxidase activity.
6. Rinse twice with PBS.

7. Tissues were incubated with 10% normal goat serum to block non-specific binding sites for 30 minutes at 37°C.

8. Dab dry the slides, except the treated tissue area.

9. Tissues were then exposed to the primary antibodies for 2 hours at 37°C. Mouse IgM and mouse IgG1 at the concentrations equal to the primary antibodies (Dako, Carpinteria, CA) were used as negative antibody controls for CSLEX1 and CF4-C4, respectively. The specificity of the antibodies used for negative control are directed towards *Aspergillus niger* glucose oxidase, which is neither present nor inducible in mammalian tissues.

10. Wash 3 times with PBS, each wash for 3 minutes on the shaker.

11. Secondary antibodies were added onto the tissues for 1 1/2 hours at 37°C. Peroxidase conjugated goat anti-mouse IgM (Sigma, St Louis, MO., cat # 102H8842) was used as the secondary antibody for CSLEX1. Peroxidase conjugated goat anti-mouse IgG1 (Boehringer Mannheim, Indianapolis, IN., cat # 100831) was used as the secondary antibody for CF4-C4.
12. Slides were thoroughly rinsed 3 times with PBS.

13. Color development was with the AEC chromogen system (Lipshaw Immuno, Pittsburg, PA., cat # 484700). Use 4 drops of each solution component 3-amino-4-ethyl-carbazole, 3% hydrogen peroxide, and sodium acetate buffer in 10 ml distilled water. Wait for the color change which occurs in approximately 10 min.

14. Slides were rinsed with distilled water.

15. Counter stained for 1 1/2 min in Gill's Hematoxylin #1 (Fisher Scientific, Fair lawn, NJ).

16. Rinse with running tap water.

17. Rinse with pH 9.5 Tris-Buffer (This turns the hematoxylin blue).

18. Rinse with distilled water.

19. Slides were air dried and coverslipped with glycerine jelly.

20. Positive staining was determined if more than 5% of the total tumor cell population stained, otherwise it was considered to be immunostaining negatively.
M. Data Management and Statistical Analyses.

The clinical data of patients involved in this study were obtained from the Department of Medical Records in The Ohio State University Hospital, Riverside Methodist Hospital, Columbus Childrens Hospital, Tumor Registry, Mayo Clinic, and Barrow Neurological Institute. Data including patient age, gender, birth date, date of death, date of the last follow-up and surgery, date of previous surgery if recurrent tumor, chemo-and radiotherapy information were entered into our database by Ms. Cindy Fox and Teresa Franklin. The data from neurochemical assays were stored in Microsoft Excel spreadsheets designed by Javier Agudelo and myself. The statistical analyses were performed by Dr. Dennis Pearl and Dr. Ming Zheng using the software package Data Desk (Data Description Inc., Ithaca, NY) and SAS (Statistical Analysis System Institute, Cary, NC). Survival analyses were done using Cox's proportional hazard regression model stratified by tumor grade and adjusted for the age of patients. The potential prognostic variables were selected by a stepwise regression method with a p-value no larger than 0.05 being maintained in the model. In order to check whether the proportional hazards model is appropriate for those potential prognostic variables, the survival curves were plotted on a log-log scale to see if they were parallel. Kaplan-Meier survival curves were generated for each significant prognostic factor, and the significance of the differences between curves were tested by log-rank test, log-likelihood test, and Wilcoxin test. New covariates were generated to determine any interaction between significant variates and the residual analyses which were run to check the fitness of the model.
CHAPTER III

GANGLIOSIDES AS DIAGNOSTIC MARKERS
OF
HUMAN ASTROCYTOMAS AND
PRIMITIVE NEUROECTODERMAL TUMORS.

Introduction

In several studies, changes in ganglioside compositions have been reported in human primary brain tumors compared to normal adult brains. However, most of the previous studies investigated gangliosides based on TLC mobility alone instead of using more specific methods to identify them. In addition, some aberrant tumor gangliosides present at very low concentrations could not be detected by the resorcinol spray. These minor gangliosides may contribute valuable information to the histological diagnoses and classification of brain tumors, but have been largely ignored in previous studies.
Rapid developments in chromatographic separations of gangliosides and production of monoclonal antibodies against oligosaccharides has greatly improved detection of minor gangliosides. We applied these techniques to thoroughly study the ganglioside compositions in a large series of astrocytomomas and primitive neuroectodermal tumors. There were two major goals in this study: 1) To identify and quantify glioma-associated gangliosides. 2) To determine the usefulness of gangliosides as diagnostic markers for astrocytomomas and primitive neuroectodermal tumors.
Gangliosides as Diagnostic Markers of Human Astrocytom as and Primitive Neuroectodermal Tumors

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Bernd W. Scheithauer, M.D.,§ Peter C. Johnson, M.D.,‡
and Allan J. Yates, M.D., Ph.D.*

Background. Limitations of classification schemes for brain tumors based solely on morphology have stimulated searches for molecular markers of nosologic and prognostic value. Gangliosides are logical candidates because there are high concentrations of them in the nervous system, there is evidence of their roles in regulation of growth and differentiation, and data from small series suggest correlations between ganglioside composition and glioma type.

Methods. Ganglioside compositions were determined for 70 primary human brain tumors: 16 low grade astrocytom as (LG), 12 anaplastic astrocytom as (AA), 34 glioblastoma multiformes (GBM), and 8 primitive neuroectodermal tumors (PNET). This method involved identification and quantitation of specific gangliosides using chemical analysis and immunoanalysis.

Results. Among all tumor types, histologic grade correlated with a progressive loss of 3b gangliosides (P < 0.0001). GQ1b was higher in LGs than in AAs (P < 0.001). Both GT1b and GD1b were higher in AAs than GBMs (P < 0.01 and 0.05, respectively) and lower in PNETs than in GBMs (P < 0.03). GM3 was higher in PNETs than in any astrocytoma group and higher in GBMs than in either AAs or LGs. There was a significant difference in the content of 3'-LMI among all groups (P < 0.005), between AAs and GBMs (P < 0.05), and between low grade ordinary and juvenile pilocytic astrocytom as (P < 0.01). The lacto-series ganglioside 3'-isolM1 was present in all groups except PNET.

Conclusions. These results indicate that patterns of gangliosides could be of considerable value in refining the classification and diagnosis of primary human brain tumors. Cancer 1994;74:3010-22.

Key words: glioma, brain tumor, glycolipid, ganglioside, astrocytoma, primitive neuroectodermal tumor, classification.

Limitations of light and electron microscopic study as the sole basis for classifying and diagnosing primary tumors of the central nervous system have stimulated a search for other markers to expand the diagnostic armamentarium. The number of such candidates is large, so the choice of which ones to study on rather limited amounts of material should be based on evidence that they may be involved in clinically relevant tumor biology. Gangliosides (Table I) are sialic acid containing glycosphingolipids that are present in all vertebrate cells, but they are especially enriched in the central nervous system. They also function as receptors for bacterial toxins and as tumor associated antigens.

Changes in ganglioside composition and metabolism occur during growth and differentiation and in neoplastic transformation of many types of cells. This is manifest in some types of tumors as the loss of expression of normally occurring gangliosides and in others as neosynthesis of specific gangliosides not normally detected in a mature cell or tissue type. The results of several studies provide convincing evidence that the
Gangliosides composition of primary tumors of the human central nervous system is different from that of normal brain tissue. However, there has been no comprehensive study of gangliosides in a large number of these tumors in which data generated using sensitive assays for minor gangliosides have been correlated with histologic diagnosis and grade. We report here the results of such a study on 70 tumors that demonstrate a strong correlation between ganglioside compositions and histologic types and grades of the major human brain tumors.

Materials and Methods

Human Glioma Specimens

Tumor tissue samples were received from members of the National Cancer Institute Cooperative Glioma Group (The Ohio State University, Barrow Neurological Institute and Mayo Clinic), and the National Cancer Institute Cooperative Human Tissue Network. Shortly after each specimen was surgically removed, it was frozen in liquid nitrogen until chemical analysis. Seventy specimens, each weighing 300–1000 mg, from 66 patients were studied. For one patient, we had samples from both the original and recurrent specimens; another sample was a recurrent specimen for which we did not have tissue from the first operation. Three specimens were duplicate analyses on two separate portions of the same tumor. The specimens were from 38 male and 28 female patients, giving a male-to-female ratio of 1.4. For all patients the mean age was 34 ± 23 years, with a median age of 37 years. Table 2 gives the gender and age distributions for each tumor type.

Table 3.1 Nomenclature and schematic structure of gangliosides

<table>
<thead>
<tr>
<th>Svennerholm</th>
<th>IUPAC-IUB</th>
<th>Schematic structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3</td>
<td>NeuAcα-LacCer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>NeuGc-GM3</td>
<td>NeuAcα-LacCer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GM2</td>
<td>NeuAcα-GgOse4Cer</td>
<td>GalNAcβ1 - 4(NeuAcα2 - 3)Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GM1, GM1a</td>
<td>NeuAcα-GgOse4Cer</td>
<td>Galβ1 - 3GalNAcβ1 - 4(NeuAcα2 - 3)Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GM1b</td>
<td>NeuAcα-GgOse4Cer</td>
<td>Galβ1 - 3GalNAcβ1 - 4(NeuAcα2 - 3)Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GD3</td>
<td>NeuAcα-LacCer</td>
<td>NeuAcα2 - 8 NeuAcα2 - 3 Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GD1a</td>
<td>NeuAcα- NeuAcα-GgOse4Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GD2</td>
<td>NeuAcα-GgOse4Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GD1b</td>
<td>NeuAcα-GgOse4Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GT1b</td>
<td>NeuAcα-NeuAcα-GgOse4Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>GQ1b</td>
<td>NeuAcα-NeuAcα-GgOse4Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>3'-LM1</td>
<td>NeuAcα-nLeOse3Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4GlcNAcβ1 - 3 Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>6'-LM1</td>
<td>NeuAcα-nLeOse3Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4GlcNAcβ1 - 3 Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>3'-isoLM1</td>
<td>NeuAcα-LeOse3Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4GlcNAcβ1 - 3 Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>3', 6'-isoLD1</td>
<td>NeuAcα-NeuAcα-LeOse3Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
<tr>
<td>3', 8'-isoLD1</td>
<td>NeuAcα-NeuAcα-LeOse3Cer</td>
<td>NeuAcα2 - 3Galβ1 - 4Glcβ1 - 1Cer</td>
</tr>
</tbody>
</table>

### Table 3.2 Tumors studied

<table>
<thead>
<tr>
<th>Consensus diagnosis</th>
<th>No.</th>
<th>Male</th>
<th>Female</th>
<th>Mean age*</th>
<th>Median age</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytic tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low grade</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>16 ± 19</td>
<td>7</td>
<td>0.01-46</td>
</tr>
<tr>
<td>anaplastic</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>38 ± 16</td>
<td>34</td>
<td>19-73</td>
</tr>
<tr>
<td>glioblastoma multiforme†</td>
<td>31</td>
<td>18</td>
<td>13</td>
<td>50 ± 17</td>
<td>50</td>
<td>5.5-81</td>
</tr>
<tr>
<td>Primitive neuroectodermal tumors</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>11 ± 9</td>
<td>9</td>
<td>0.6-38</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>38</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ages ± standard deviations and ranges are given in years.
† The group referred to as glioblastoma multiforme includes two cases of gliosarcoma.

Consensus diagnoses and grades were determined that were used in this study (Table 2). For statistical analyses, the 11 juvenile pilocytic and 5 Grade II fibrillary astrocytomas were combined into the category of low grade astrocytomas (LG). All anaplastic astrocytomas (AA) were judged to be Grade III, and glioblastoma multiformes (GBM) and primitive neuroectodermal tumors (PNET) were judged as Grade IV.

### Extraction, Isolation, and Purification

Before tissues were extracted, a frozen section slide of each was prepared and stained with hematoxylin and eosin and microscopically examined to ascertain that the tissue extracted consisted of tumor. Wet tumor tissue was weighed, lyophilized overnight, weighed again to determine dry weight, and rehydrated with 4 ml water per gram fresh weight for 1 hour before homogenizing. Tissue was homogenized with an additional 16 ml (final 20 ml) of chloroform-methanol (1:2) per gram fresh weight and centrifuged to pellet the nonlipid residues. The pellet was reextracted with chloroform-methanol-water (1:1:5%) and centrifuged again. The total lipid extract was dried and subjected to a Folch partition. The lower phase was washed an additional three times using theoretical upper phase without potassium chloride; all four upper phases were pooled. The pooled upper phase containing gangliosides was dried, dissolved in water, and adjusted to 0.1 M potassium chloride before passing through a Bakerbond SPE C-18 reverse phase column (J. T. Baker, Phillipsburg, NJ) to remove the salt. Results of neutral glycolipid analyses for these tumors have been reported elsewhere.

### Separation and Quantitation of Gangliosides

Total gangliosides were quantitated on the basis of sialic acid content using the colorimetric resorcinol-hydrochloric acid method. Aliquots of 43 specimens containing more than 40 μg sialic acid were separated on a DEAE-Sephadex A-25 (Sigma, St. Louis, MO) column eluting monosialogangliosides with 0.02 M and 0.04 M ammonium acetate in methanol, and polysialogangliosides with 0.25 M ammonium acetate. Both fractions were desalted by C-18 columns. Aliquots of 1.5 μg total ganglioside sialic acid were spotted on high performance thin layer chromatography (HPTLC) plates (Kieselgel 60, E. Merck, Darmstadt, Germany) and developed in chloroform-methanol-0.2% CaCl₂ in water (55:45:10). The solvent system chloroform-methanol-5 M ammonium hydroxide-0.4% CaCl₂ (50:50:4:5) was used to separate N-acetyl- from N-glycolyl-neuraminic acid containing GM3 (ganglioside nomenclature is according to Svennerholm). Separated gangliosides were detected by resorcinol spray and densitometrically scanned in the reflection mode (Shimadzu CS-9000, Shimadzu Corporation, Kyoto, Japan) at 580 nm. All specimens had these analyses performed on their total ganglioside extracts.

The same procedures were performed on the mono- and polysialoganglioside fractions isolated by DEAE-Sephadex A-25 chromatography of 43 tumors. There was no selection bias for the size of specimens with respect to diagnosis. Smaller specimens that were not subjected to DEAE-Sephadex chromatography were distributed as follows: 9 LG; 5 AA; 8 GBM; and 5 PNET. Chi-square analysis demonstrated that there was no statistically significant difference in the distribution of these tumors among the diagnostic groups.

### Identification of Specific Gangliosides by HPTLC Immunostaining

To confirm the identity of gangliotetraose series gangliosides (GM1, GD1a, GD1b, GT1b, GQ1b) on HPTLC, horseradish peroxidase conjugated Cholera toxin B subunit (List Biol Lab. Campbell, CA) was used with prior Clostridium perfringens type V (Sigma, St. Louis, MO) neuraminidase treatment on 11 representative specimens. N-glycolyneuraminic acid-containing GM3 was identified by immunostaining with antibody 2-39M from Kenneth Lloyd (Memorial Sloan-Kettering Cancer Center, New York, NY). Neolacto- and lactotetraosyl-containing gangliosides were visualized by HPTLC im-
Gangliosides in Brain Tumors/Sang et al.

### Table 3.3 Percentage distribution of sialic acid among gangliosides

<table>
<thead>
<tr>
<th>Tissue ganglioside</th>
<th>LG (n = 14)</th>
<th>AA (n = 12)</th>
<th>GBM (n = 34)</th>
<th>PNET (n = 8)</th>
<th>*GM (n = 4)</th>
<th>tGM</th>
<th>tWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3</td>
<td>6.7 ± 0.8†</td>
<td>7.0 ± 2.0</td>
<td>14.3 ± 1.9</td>
<td>33.3 ± 8.5</td>
<td>2.8 ± 0.3</td>
<td>2.7</td>
<td>4.8</td>
</tr>
<tr>
<td>GM2</td>
<td>1.5 ± 0.5</td>
<td>3.8 ± 0.7</td>
<td>3.9 ± 0.7</td>
<td>3.5 ± 2.0</td>
<td>2.4 ± 0.2</td>
<td>4.1</td>
<td>2.5</td>
</tr>
<tr>
<td>GM1</td>
<td>7.3 ± 1.5</td>
<td>13.6 ± 1.5</td>
<td>6.0 ± 1.0</td>
<td>1.0 ± 1.0</td>
<td>14.0 ± 2.8</td>
<td>14.9</td>
<td>21.6</td>
</tr>
<tr>
<td>GD3</td>
<td>30.1 ± 2.7</td>
<td>18.6 ± 2.1</td>
<td>29.6 ± 2.5</td>
<td>33.4 ± 6.7</td>
<td>5.8 ± 1.7</td>
<td>5.4</td>
<td>8.8</td>
</tr>
<tr>
<td>GD1*</td>
<td>8.0 ± 2.2</td>
<td>14.6 ± 1.8</td>
<td>8.1 ± 1.1</td>
<td>2.5 ± 2.3</td>
<td>26.4 ± 0.2</td>
<td>21.7</td>
<td>16.6</td>
</tr>
<tr>
<td>GD2§</td>
<td>8.1 ± 1.2</td>
<td>12.3 ± 1.3</td>
<td>12.8 ± 1.2</td>
<td>10.9 ± 6.0</td>
<td>5.9 ± 0.7</td>
<td>8.0</td>
<td>3.1</td>
</tr>
<tr>
<td>GD1b</td>
<td>14.1 ± 1.8</td>
<td>14.4 ± 1.5</td>
<td>8.6 ± 0.9</td>
<td>3.3 ± 1.6</td>
<td>22.4 ± 1.8</td>
<td>18.2</td>
<td>16.9</td>
</tr>
<tr>
<td>GT1b</td>
<td>12.1 ± 1.1</td>
<td>11.0 ± 0.9</td>
<td>7.5 ± 0.8</td>
<td>3.6 ± 2.4</td>
<td>17.6 ± 3.5</td>
<td>16.3</td>
<td>11.1</td>
</tr>
<tr>
<td>GQ1b</td>
<td>6.0 ± 0.8</td>
<td>2.1 ± 0.6</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 1.0</td>
<td>2.6 ± 0.3</td>
<td>5.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>


† Values from our laboratory using the procedures described in Materials and Methods.
§ Data shown as the mean ± SEM.

Data from all of the 70 specimens were used in the present analysis of summary statistics (Tables 3–6) and box-plots (Figs. 1–5). The data regarding ganglio series gangliosides and linear combinations of their measured values were analyzed using analysis of variance models with pairwise significance statements based on the least significant difference post hoc test. Thus, the P values reported for these pairwise comparisons are the corresponding experimentwise error rates. For each of these analyses, an examination of residual plots did not contradict the validity of the methods used. The Fisher-Irwin exact test was used in the analysis of the minor gangliosides to examine the differences between diagnostic groups in the presence or absence of the ganglioside being evaluated. All statistical studies of ratios involved an initial calculation of the ratios on a case-by-case basis; the calculated ratios were analyzed statistically.

### Results

#### Total Gangliosides

Total ganglioside sialic acid concentration per milligram dry weight was lower in all tumors than in normal brain tissue (Table 4). The group of PNET had the lowest concentration of all tumor groups, but there was no significant difference among grades of astrocytic tumors.

#### Ganglio Series Gangliosides

The amounts and proportions of ganglio series gangliosides in different human brain tumor diagnostic groups and normal brain are shown in Tables 3 and 4. These values were obtained by scanning densitometry of thin layer chromatographic plates sprayed with resorcinol (Fig. 6). Identification of gangliotetraose gangliosides was confirmed in 11 representative tumors using the neuraminidase/Cholera toxin method (Fig. 7). Oligosaccharide complexity was estimated by a sugar index calculated by the formula: sugar index = sum of [(proportion of ganglioside species) × (number of sugars per molecules)]. This index was higher for LG than for GBM (P < 0.01), with AA intermediate between these two. PNET was significantly lower than all of the astrocytoma groups (P < 0.01). The degree of sialylation was...
The table below shows the total and individual ganglioside contents of different tissue samples:

<table>
<thead>
<tr>
<th>Tissue ganglioside</th>
<th>LG (n = 19)</th>
<th>AA (n = 11)</th>
<th>GBM (n = 32)</th>
<th>PNET* (n = 7)</th>
<th>GM†</th>
<th>WM†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>860 ± 110</td>
<td>1080 ± 190</td>
<td>760 ± 60</td>
<td>500 ± 200</td>
<td>4800</td>
<td>1000</td>
</tr>
<tr>
<td>GM3</td>
<td>54 ± 10</td>
<td>64 ± 16</td>
<td>91 ± 9</td>
<td>100 ± 22</td>
<td>130</td>
<td>50</td>
</tr>
<tr>
<td>GM2</td>
<td>17 ± 6</td>
<td>43 ± 11</td>
<td>27 ± 6</td>
<td>23 ± 12</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>GM1</td>
<td>79 ± 29</td>
<td>156 ± 33</td>
<td>46 ± 9</td>
<td>20 ± 20</td>
<td>710</td>
<td>220</td>
</tr>
<tr>
<td>GD3</td>
<td>232 ± 32</td>
<td>196 ± 42</td>
<td>43</td>
<td>128 ± 22</td>
<td>260</td>
<td>90</td>
</tr>
<tr>
<td>GD1a</td>
<td>92 ± 36</td>
<td>184 ± 44</td>
<td>10</td>
<td>46 ± 45</td>
<td>1040</td>
<td>170</td>
</tr>
<tr>
<td>GD2a</td>
<td>76 ± 15</td>
<td>135 ± 31</td>
<td>10</td>
<td>48 ± 23</td>
<td>380</td>
<td>30</td>
</tr>
<tr>
<td>GD1b</td>
<td>122 ± 22</td>
<td>152 ± 32</td>
<td>68 ± 9</td>
<td>39 ± 32</td>
<td>870</td>
<td>170</td>
</tr>
<tr>
<td>GT1b</td>
<td>108 ± 16</td>
<td>124 ± 26</td>
<td>63 ± 0</td>
<td>52 ± 46</td>
<td>780</td>
<td>110</td>
</tr>
<tr>
<td>GQ1b</td>
<td>46 ± 7</td>
<td>25 ± 8</td>
<td>12 ± 3</td>
<td>15 ± 11</td>
<td>240</td>
<td>30</td>
</tr>
</tbody>
</table>


* There was no detectable GM1 in 6 of 7 PNET.


‡ Data are shown as the mean ± SEM. Some specimens from Table 3 were not large enough to perform total sialic acid estimations on them. Values for neolacto and lacto series gangliosides are in Tables 5 and 6, respectively.

The proportion of GT1b (Fig. 3) was greater in LG and AA than GBM, which was greater than for PNET (P < 0.05). The proportion of GD1b (Fig. 4) was significantly higher for LG than for GBM (P < 0.05), with the value for AA being intermediate. PNET had a lower proportion of GD1b than any of the astrocytomas (P < 0.05).

The combined proportions of GM1 and GD1a were higher in AA than in LG and GBM (P < 0.01), which were all higher than that in PNET (P < 0.001). The proportions of the other groups (P < 0.001) (Fig. 2).
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Table 3.6 Lacto series gangliosides

<table>
<thead>
<tr>
<th>Tissue ganglioside</th>
<th>Pilocytic (n = 11)</th>
<th>Ordinary (n = 3)</th>
<th>AA (n = 11)</th>
<th>GBM (n = 31)</th>
<th>PNET (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-isoLM1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number present (%)</td>
<td>3 (27.7)</td>
<td>4 (80)</td>
<td>2 (18)</td>
<td>17 (55)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Amount in specimens with isoLM1</td>
<td>65.1 ± 13.5*</td>
<td>315 ± 123</td>
<td>5.72†</td>
<td>49.3 ± 9.3</td>
<td>ND</td>
</tr>
<tr>
<td>3', 6'- or 3', 8'-isoLD1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number present (%)</td>
<td>3 (27.3)</td>
<td>4 (80)</td>
<td>0 (0)</td>
<td>11 (36)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Amount in specimens with isoLD1</td>
<td>57.1 ± 22.7</td>
<td>157 ± 75.5</td>
<td>ND</td>
<td>24.9 ± 8.3</td>
<td>ND</td>
</tr>
</tbody>
</table>


* Data (pmole/mg dry weight) are shown as the mean ± SEM only for those cases that contained lacto series gangliosides.
† Values are data for both specimens containing 3'-isoLM1.

0.01). Similar results were obtained for GM1 and GD1a individually. The ratio of GM2/GM1 (calculated for each tumor individually) is different in PNET than in astrocytic tumors, normal human brain gray matter, and white matter. However, the proportion of GM2 did not correlate with histologic grade of astrocytic tumors. It constituted only 1.5–3.9% of total gangliosides in all tumor groups, and the higher ratio of GM2/GM1 in PNET primarily was attributable to low levels of GM1.

GM1b. There were two GBM that contained GM1b, at 9.76 and 39.28 µg sialic acid/g fresh weight. These levels are 20- and 80-fold higher, respectively, than those found in normal brain.49

Neolacto Series Gangliosides

Neolacto series ganglioside contents of the different tumor groups as identified by F1H11 and NS24 are shown

![Graph](image)

**Consensus diagnosis**

Fig 3.1 The proportion of total 1b gangliosides inversely correlates with the degree of histologic grade (P < 0.001). Within the GBM group, the two recurrent tumors have low percentage values for 1b pathway gangliosides (6.8% and 8.1%). The low AA outlier (7.9%) is from a recurrent anaplastic astrocytoma for which we did not have tissue from the first surgical procedure. The low values from recurrent tumors may reflect effects of therapy or an aggressive subpopulation of cells. LG: low grade astrocytomas; AA: anaplastic astrocytomas; GBM: glioblastoma multiforme; PNET: primitive neuroectodermal tumor. Note that the data presented here and in the other graphs are in boxplots. The “box” in a boxplot extends from the 25th to the 75th percentiles. The horizontal line across the box marks the median. The shaded area on each box indicates the 95% confidence intervals around the median. The error bars extending from the bottom and top of the box depict the extent of the main body of the data. Outliers and extreme outliers are shown as circles and stars, respectively.

![Graph](image)

**Consensus diagnosis**

Fig 3.2 The proportion of GQ1b versus consensus diagnosis. GQ1b separates LG from all the other groups (P < 0.001).
The proportion of CTlb women in iiimmie CTlb separates LC end AA from GBM and CBM from PNET (P < 0.06). The AA and PNET outliers are the same tumors discussed in Figure 1.

in Table 4. Ganglioside 3'-LM1 was identified on the basis of its presence in the monosialoganglioside fraction, thin layer chromatography (TLC) mobility ahead of GM1, and immunopositive staining on TLC with F1H11 after neuraminidase treatment (Fig. 8). The presence or the absence of 3'-LM1 varied significantly across the diagnostic groups (P < 0.005). The ganglioside 3'-LM1 was positive in 9 of 16 (56%) LG, 4 of 12 (33%) AA, 26 of 34 (77%) GBM, and 8 of 8 (100%) PNET (Table 4). The concentration of 3'-LM1 (pmol/mg dry weight) ranked in the following order: PNET > GBM > AA and LG. The difference between GBM and AA is significant (P < 0.05). Among the LG, pilocytic astrocytomas have no or very low amounts of 3'-LM1, whereas the ordinary LG have higher amounts than do the pilocytic tumors (P < 0.01).

Ganglioside 6'-LM1 was identified in the same way as 3'-LM1, but its TLC mobility was slightly slower than that for GM1, and it was immunopositive with both F1H11 and MSG-15 (Fig. 9). This ganglioside was present in 1 of 16 (6%) LG, 2 of 12 (17%) AA, 15 of 34 (44%) GBM, and 2 of 8 (25%) PNET. This pattern for the pres-
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**Fig 3.7** Identification of gangliosides with gangliotetraosyl oligosaccharides using *Clostridium perfringens* neuraminidase and *Cholera* toxin B subunit after separation on HPTLC plate. Lane 1: GM1 standard (12 ng sialic acid). Lane 2: glioblastoma multiforme (30 ng total sialic acid). Lane 3: fibrillary astrocytoma (30 ng total sialic acid). Lane 4: anaplastic astrocytoma (30 ng total sialic acid).

Presence of 6'-LM1 across groups was marginally significant ($P < 0.05$).

Neolactotetraosyl gangliosides of uncertain identities were seen in some tumors. In the polysialoganglioside fractions of 25 tumors there were bands that stained positively with FIH11 in the region between GD1a and GD1b (21.9 ± 6.8 pmol/mg dry weight). This could account for 10–20% of the amount attributed to GD2 on the basis of resorcinol detection in these tumors. Such gangliosides comigrating with GD1b were present in five tumors (all smaller than 4 pmol/mg dry weight); another migrated between GD1b and GT1b in one tumor (5 pmol/mg dry weight). In three tumors there were FIH11 positive gangliosides that migrated with GT1b (all smaller than 4 pmol/mg dry weight).

FIH11 positive bands were seen between GD3 and GD1a in 17 tumors (11.4 ± 6.1 pmol/mg dry weight); some of these eluted in the mono- and some in the polysialoganglioside fractions. There was no significant correlation between any of these gangliosides and histologic diagnosis.

**Lacto Series Gangliosides**

The amounts of gangliosides of the lacto series identified and quantitated by TE3 are shown in Table 6. Murine monoclonal antibody TE3 reacts strongly with carbohydrates containing a terminal Gal$_{1-3}$GlcNAc structure, irrespective of chain length. Ganglioside 3'-isoLM1 was identified by its presence in the monosialoganglioside fraction, TLC mobility, and immunoreactivity with TE3 after neuraminidase treatment. It was present in 7 of 16 (43%) LG, 2 of 11 (18%) AA, 17 of 31 (55%) GBM, and 0 of 7 (0%) PNET. The presence/absence of 3'-isoLM1 was not statistically significantly different between any two diagnostic categories.

A ganglioside was seen in the polysialoganglioside fraction, which migrated between GD1a and GD2 standards on HPTLC and stained positively with TE3 after neuraminidase treatment, which could be either or both of 3',6'-isoLD1 or 3',8'-isoLD1. It was present in LG (43%) and GBM (36%) but not in AA or PNET. Only five tumors (three ordinary LG, one pilocytic, and 1 GBM) contained a ganglioside with similar characteristics, except that it migrated in the GT1b area. This is assumed to represent an isoLT1.

**Fig 3.8** Detection of gangliosides with sialylparagloboside oligosaccharides using neuraminidase and FIH11 immunostaining (lanes 1–4). Lane 1: 3'-LM1 standard (40 pmol). Lane 2: monosialoganglioside fraction eluted from DEAE-Sephadex column. Lane 3: polysialoganglioside fraction eluted from DEAE-Sephadex column. Lane 4: total unseparated gangliosides (2 mg sialic acid) Lane 5: ganglioside standards detected with resorcinol. Lanes 2, 3, and 4 were all from the same glioblastoma multiforme.

**Fig 3.9** Identification of 6'-LM1 by MSG-15 immunostaining. Lane 1: total gangliosides isolated from the same glioblastoma multiforme as in Figure 1. The arrow points to 6'-LM1, which migrates between GM1 and GD3. Lane 2: 3'-LM1 standard as a negative control. Lane 3: ganglioside standards detected with resorcinol.
GM3, GD3, and GD2

The proportions of GM3 and GD3 were elevated in all of the brain tumors relative to normal adult white or gray matter. The proportion of GM3 was higher in PNET than in any of the astrocytic tumors (P < 0.001) and was slightly higher in GBM than in AA or LG (Fig. 5). The proportion of GD3 did not correlate with the histologic grade or diagnosis. Thus, the ratio of GM3 to GD3 correlated with the histologic grade of malignancy overall. Correlating results of resorcinol stained with immunostained TLC plates indicates that GD2 overlaps with lacto and neolactopolysialogangliosides. More precise estimates of GD2 require additional study.

Discussion

Accurate classification of brain tumors is necessary to direct appropriate therapy and for proper interpretation of clinical investigations. However, classification based solely on histologic features often is inadequate for predicting tumor behavior and outcome. Several of the histologic criteria used to classify and grade brain tumors are somewhat subjective, resulting in significant inter- and intraobserver variability in diagnosis. In addition, subpopulations of tumor cells with similar histologic appearances but different biologic behaviors cannot be distinguished. Finally, there is heterogeneity in tumor response to treatment. Clinical responses to therapy and survival vary, even among patients with the same histologic type and grade of tumor. Improved classification of brain tumors requires identification of objective markers that correlate with histologic features: are predictive of biologic behavior, clinical progression, and response to therapy; and are relatively simple to assay. Current molecular probes fail to completely address these problems, in part because of a paucity of markers for specific cell types in the mature and developing nervous system.

A number of markers for different types of brain tumors have been reported. Cytogenetic abnormalities in chromosomes 7, 10, 17, as well as DNA aneuploidy as determined by flow cytometry have been reported to be significant in human gliomas. Several quantitative methods for measuring cellular proliferation also have been studied in gliomas. These include Ki-67 antibody labeling of proliferating pool cells, bromodeoxyuridine incorporation into S-phase cells, and silver staining of nucleolar organizing regions. Unfortunately, these markers have not been reliable in distinguishing between AA and GBM. Abnormal expression of growth factors or growth factor receptors has been found in human astrocytic tumors, such as amplification of the c-erb B gene (epidermal growth factor receptor gene) and the c-sis oncogene, which codes for PDGF-β. However, these have not been shown to be of sufficient value relative to tumor type to be applied diagnostically.

Aberrant glycosylation of lipid in tumors has been reported for several tumor types. More specifically, abnormal ganglioside expression in human gliomas has been found in several studies. Yates et al. reported a small series in which human neural tumors had larger proportions of simple gangliosides and smaller proportions of complex gangliosides when compared with normal brain tissue. Fredman et al. in a study of human gliomas, also found a general reduction in the ganglio series gangliosides normally present in adult human brain (GM1, GD1a, GD1b, and GT1b). In addition, Berra et al. demonstrated a correlation between the loss of polysialylated gangliosides and histologic grade of malignancy in 38 human astrocytomas.

Our study is based on a larger sample size than any previously reported series, and the statistical analyses of the results are more detailed. Although there was no correlation between total ganglioside and histologic type, we found that the degrees of oligosaccharide complexity and sialylation correlate significantly with the histologic grade of malignancy. These changes were attributable primarily to the loss of total lb gangliosides (GD1b, GT1b, GQ1b). However, the correlation between total lb ganglioside content and histologic grade of malignancy is almost certainly not attributable to contamination of tumor samples by residual brain tissue for three reasons.

First, GM1 and GD1a, also ganglio series gangliosides in normal human brain, did not demonstrate the same pattern of change. Second, the mean amount of neutral galactolipid in these tumors (mainly cerebroside, which is highly enriched in myelin) was only 9% of white matter values. In addition, the total lipid in tumors averaged only 3.6% of the fresh weight, which is only one-fourth its level in white matter and even less than that of gray matter (5.9%). The results of neutral glycolipid analyses of these tumors have been previously published. A third argument against contamination of the specimens with normal brain is the differential and progressive loss of lb gangliosides with increasing tumor grade. The decrease in GQ1b is seen first between LG and AA. The decrease in GT1b and GD1b occurs between AA and the Grade IV tumors (GBM and PNET). In contrast, all gangliosides from normal brain to believe that low grade tumors should have more contaminating normal brain than should high grade tumors.
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It is likely that the increases in GM3 and GD3 in these tumors are attributable to a block in the synthesis of 1b pathway gangliosides resulting in disproportionately large amounts of these precursors of gangliosides to the 1b series. As with previous studies, we found an increased amount of GD3 in all tumor types. However, in contrast to the study of Berra et al., there was no correlation between the amount of GD3 and tumor type or grade. GM3 and GD3 both separated as doublets on HPTLC, a finding that may be attributable to differences in fatty acids or sphingosine. A heavily staining band that migrated slightly behind known GM3 standards on HPTLC was seen in nine tumors (two AA, four GBM, two gliosarcoma, and one PNET), which accounted for 6.7-17% of total gangliosides in these tumors. The nature of this is not known with certainty, but it is not a NeuGc-containing GM3 because it was immunonegative on TLC with the 2-39M antibody. Because of its close migration to GM3 on HPTLC and its elution as a monosialoganglioside from the DEAE-Sephadex column, we suggest that it may be a subspecies of GM3.

The elevated GM3/GD3 ratio observed in PNET compared with the astrocytic group suggests that differences in the relative activity of sialyltransferases I and II probably exist in these tumors. A high ratio of GM2/GM1 also was observed in PNET because of lower amounts of GM1, indicating that PNET have significantly less capacity to synthesize gangliosides with a more complex oligosaccharide than do GM3, GD3, and GD2. It was reported that large amounts of GM3 and GM2 were found in a human medulloblastoma xenograft, but GD3 was not found. The differences between these results and ours could represent changes in ganglioside metabolism induced in the tumor cells by the environment in which they are growing.

The lacto series ganglioside 3'-isoLM1 is present in normal human fetal, but not adult, brain tissue. It also is present in brains of infants with polyunsaturated fatty acid lipidosis, which have considerable gliosis. This ganglioside has been found in small cell carcinoma of the lung, adenocarcinoma of the colon, and human embryonal carcinoma cells. Although gangliosides of the lacto series were not found in cultures or xenografts derived from a single malignant glioma, it was detected in a high proportion of human malignant glioma tissues, thus suggesting that it is a glioma associated antigen. The lacto series ganglioside 3'-isoLM1 subsequently was detected in all 14 Grade III and IV gliomas in which it was assayed. By immunohistochemical analysis using antibody SL-50, 3'-isoLM1 was present in 32% of 29 GBM, but in neither of the two AA studied. Although in the same study, only 2 of 16 malignant glioma cell lines and 6 of 8 xenografts of these cell lines contained 3'-isoLM1, in a study of tissues obtained at autopsy, it was found that its presence and concentration in pure glioma tissue is highly variable and demonstrates considerable regional heterogeneity. In addition, the amount of this ganglioside is elevated in uninvolved brain tissue on the side opposite to the tumor, making its relationship to tumor growth questionable. Collectively, these results suggest that the presence of 3'-isoLM1 in glioma and adjacent cells and tissues is highly dependent on their growth environments, but the exact cellular and molecular mechanisms involved are unknown. We found no correlation between the presence or amount of 3'-isoLM1 and histologic diagnosis.

Monoclonal antibody F1H11 is highly specific for the oligosaccharide of paragloboside, which is the parent compound of the neolacto series of gangliosides. It binds to i- and I-type glycolipid but does not recognize fucosyl-paragloboside or other glycolipids such as gangliosides. NS24 is a monoclonal antibody that recognizes the N-acetyleneuraminyl α2-3Galβ1-4GlcNAc residue in type II chains (i.e., neolacto series glycolipids). However, it does not bind to either of the neolacto series gangliosides 6'-LM1 or NGNA-3'-LM1, and it does not recognize i- or I-type glycolipid. MSG-15 binds to sialyl α2 → 6 paragloboside but not to sialyl α2 → 3 paragloboside. A ganglioside from the moniosialo-ganglioside column fraction migrating slightly behind GM1 stained positively with F1H11 after neuraminidase treatment and MSG-15 but not with NS24. It was eluted in the monosialo-ganglioside fraction on DEAE-sephadex column and had a relative mobility value (Rf:LM1) of 0.75. It is concluded from this information that it is the neolacto series ganglioside 6'-LM1. This ganglioside has been found in human meconium, colorectal carcinoma, carcinoma of the lung, pancreatic adenocarcinoma, and human hepatoma. However, to our knowledge this has never been reported previously in a human glioma. In our study, 6'-LM1 was present more frequently in GBM than in LG or AA.

With the exception of the five ordinary LG, the presence of the neolacto series ganglioside 3'-LM1 correlated with the degree of histologic malignancy. This ganglioside has been found in normal human fetal brain, cultures and xenografts derived from a malignant human glioma, a medulloblastoma xenograft, and medulloblastoma tissues. It was present in all eight PNET that we studied, but the lacto series ganglioside 3'-isoLM1 was not detected in any of these tumors. Gottfries et al. also found 3'-LM1 in all six medulloblastomas that they studied. However, they also identified 3'-isoLM1 with the SL-50 antibody in five of six medulloblastomas, albeit at extremely low levels. The reason for this difference between our results and those of Gottfries et al. is unclear.
We conclude that in astrocytic tumors, increasing degrees of histologic malignancy are correlated with a progressive decrease in lb gangliosides and an increase in the neolacto series gangliosides, mainly 3'-LM1 (Fig. 10). This indicates that, as tumors progress in degree of malignancy, there is a concurrent change in ganglioside metabolism, resulting in a switch from the lb pathway to the neolacto-ganglioside pathway. Another possibility is that there are changes in the proportions of different subpopulations of tumors cells that have different ganglioside compositions. However, these two possible bases for differences in ganglioside composition among different tumor types are not mutually exclusive. In either case, it is possible that patients with brain tumors containing larger amounts of gangliosides of the neolacto family may have poorer survival. Evidence for or against this requires a detailed survival analysis.

Neolacto and lacto series gangliosides both are derived from LA2, which contains GlcNAC, not GalNAC. Neolactotetraosylgangliosides, e.g., 3'-LM1, are different from lactotetraosylgangliosides, e.g., 3'-isoLM1, in that the former, unlike the latter, contains a terminal Galβ1—4GlcNAC, rather than Galβ1—3GlcNAC. As these tumors became progressively more malignant, the relative activities of glycosyltransferases responsible for synthesizing the neolacto family of gangliosides may be correlates, or even determinants, of biologic behavior.

Our results are of potential value in classification, diagnosis, and in determining the prognosis of human brain tumors. Several gangliosides in this study may be potential markers for classification and grading of these tumors. For example, the amount of GT1b is considerably less in LG than in AA. In addition, pilocytic astrocytomas clearly separated from ordinary LG on the basis of both 3'-LM1 and 3'-isoLM1. The amounts of GT1b and GD1b are significantly lower, and 3'-LM1 higher, in GBM than in AA. These same lb pathway gangliosides are even lower and 3'-LM1 higher in PNET than in GBM. The proportions of GM3 and 3'-LM1 were higher in PNET than in any of the astrocytic tumor groups studied, and PNET had extremely low levels of GM1. In contrast, very high levels of GM1 were present in AA.

Aniga and Yu45 identified GM1b as a minor ganglioside (2.6 μg/g fresh weight) in normal human brain. The two glioblastomas containing high levels of GM1b in our study had no or little neolactotangliosides, a finding that suggests that a portion of the ganglioside biosynthetic pathway in these glioblastomas is diverted toward GM1b synthesis, rather than toward the neolacto-ganglioside pathway (Fig. 10). The biologic significance of GM1b is unclear. However, the patient whose tumor had elevated GM1b is alive more than 701 days after surgery; the other patient died within 24 hours of surgery, with death not being attributable to rapid regrowth.

Gangliosides are associated with regulation of cell growth44 and cell adhesion.57,48 Specific gangliosides inhibit PDGF-stimulated DNA synthesis,13,49 changes in intracellular free calcium,70 receptor tyrosine phosphorylation,13,49 and receptor dimerization.14 The most inhibitory gangliosides of PDGF-stimulated DNA synthesis, receptor dimerization and tyrosine phosphorylation, are GT1b and GD1b, the same gangliosides that are deficient in the more malignant tumors in the current study. However, the least inhibitory ganglioside, GM3, is markedly elevated in high grade tumors, a finding that strongly suggests that a loss of structurally more complex gangliosides may be mechanistically related to the loss of growth control in these tumors. Supporting this notion are the findings of Zheng et al.,44 who found that GM3 plays an important role in supporting integrin receptor function in fibronectin-mediated cell adhesion, and of Kleinman et al.,71 who reported that polysialogangliosides inhibited cell adhesion to fibronectin. Both of these studies suggest that changes in ganglioside composition may be involved in tumor invasion. Thus, there is mounting evidence to indicate that gangliosides play a role in the biology of human gliomas. With monoclonal antibodies specific to glycolipids becoming more available, it may be possible to identify clinically significant subpopulations of tumor cells based on their glycolipid contents. Thus, the glycolipid profile might facilitate the classification and diagnosis of primary human brain tumors.

References

Gangliosides in Brain Tumors/Sung et al.


CHAPTER IV

GANGLIOSIDES IN HUMAN OLIGODENDROGLIOMAS

Introduction

Oligodendrogliomas are less common than astrocytomas, accounting for only 5% of intracranial gliomas. Oligodendrocytes, myelin-forming cells in the central nervous system (CNS), were postulated to be cell of origin of oligodendrogliomas by Bailey and Cushing (1926). Microscopically, they present a relatively uniform appearance. Tumor cells in oligodendrogliomas are small, round, closely packed with darkly staining nuclei surrounded by a clear halo. In addition to these features, it is not uncommon that many tumors contain neoplastic astrocytes in different proportions and types. These tumors are called mixed gliomas.

Due to their low incidence rate, there are very few previous reports of glycosphingolipids in human oligodendrogliomas. A study of neutral glycosphingolipid composition of oligodendrogliomas was reported by our group (Singh et al., 1994). In the current study, we investigated the ganglioside
compositions of 24 human oligodendrogliomas including different World Health Organization (WHO) histological grades. In the previous chapter, we demonstrated that specific ganglioside patterns exist in astrocytic tumors that correlate with histological malignancy. We also wanted to know whether this specific pattern found in astrocytic tumors can be applied to oligodendrogliomas. In addition, we compared the difference in ganglioside compositions between pure oligodendrogliomas and mixed gliomas.

**Methods**

**Tissue specimens**

Tumor specimens were obtained from The Ohio State University, Barrow Neurological Institute, Mayo Clinic and National Cancer Institute Cooperative Human Tissue Network. A total of 24 human oligodendrogliomas were studied, which consisted of 2 grade I, 13 grade II, 5 grade III and 4 grade IV tumors according to the WHO grading system. These included 16 pure oligodendrogliomas and 8 mixed gliomas. Representative hematoxylin and eosin stained slides from paraffin embedded tissues were examined by four neuropathologists from the three participating institutions. Disagreement was resolved by simultaneous viewing through a multiheaded microscope.
Ganglioside extraction, purification and identification

The methods for ganglioside isolation and identification were described in detail by Sung et al., 1994. In brief, tumor samples weighing 0.3-1.0 g were lyophilized overnight and extracted twice in 20 volumes of chloroform/methanol/water (1:2:20%, v/v/v) and (1:1:5%, v/v/v) with respect to their fresh weights. Total lipid extracts were then subjected to a Folch partition (1957). The lower phase was then washed an additional three times using theoretical upper phase (chloroform/methanol/water, 3:48:47, v/v/v) without KCl; and all four upper phases were pooled. The pooled upper phases containing gangliosides were dried, dissolved in water, and adjusted to 0.1 M KCl before passing through a Bakerbond SPE*C-18 reverse phase column (J. T. Baker, Phillipsburg, NJ) to remove the salt.

Total gangliosides were quantitated by the colorimetric resorcinol-HCl method based on the sialic acid content (Svennerholm;1957 and Miettinen and Takki-Luukkainen,1959). For each tumor sample, aliquots of 1.5 μg total ganglioside sialic acid were spotted on HPTLC plates (Kieselgel 60, E. Merck, Darmstadt, W. Germany) and developed in chloroform-methanol-0.2% CaCl₂ in water (55:45:10). The HPTLC plates were sprayed with resorcinol and heated to visualize separated gangliosides and densitometrically scanned in the reflection mode (Shimadzu CS-9000, Kyoto, Japan) at 580 nm.

Neolacto- and lactotetraosyl-containing gangliosides were identified by HPTLC immunostaining using the monoclonal antibodies F1H11 and TE3
after *Clostridium perfringens* neuraminidase (Type V, Sigma) treatment with the Avidin-Biotin alkaline phosphatase system modified from Buehler and Macher (1986). These gangliosides were quantitated by external calibration curves using known amounts of sialylparagloboside and lactotetraesylceramide (Eric Holms, Pacific Northwest Research Foundation, Seattle, WA and Bruce Macher, San Francisco State University, San Francisco, CA) as standards and scanned with densitometry scanner.

### Statistical Analysis

Data were analyzed by chi-square test, Mann-Whitney U test, and ANOVA for statistical significance.

### Results

Total ganglioside sialic acid content and percentage distribution of individual gangliosides for different grades and subtypes of tumors are shown in Tables 4.1 and 4.2. The differences in patterns of major gangliosides between pure oligodendrogliomas and mixed gliomas are not statistically significant. Neither does composition of major gangliosides correlate with WHO grade. However, tumors with a 1b ganglioside content (GD1b, GT1b and GQ1b) over 30% of total gangliosides occur more frequently in the WHO grade I & II (47%) and Grade III (40%) than in the grade IV (25%) group. This pattern follows that seen in our previous studies on astrocytomas (Sung et al., 1994).
Minor gangliosides with the neolacto- and lacto-series backbones were studied by immunostaining HPTLC using monoclonal antibody F1H11 and TE3 after neuraminidase treatment. Only 3 cases (two cases were grade II and one was grade I) of 24 have lactotetraosyl-containing gangliosides present, which was 3'-isoLM1. Major neolacto-series gangliosides in these tumors are 3'-LM1 and 6'-LM1. The presence of 6'-LM1 correlates significantly with higher grades (III and IV) of tumors (p=0.02 in chi-square test; p=0.01 in Mann-Whitney test) (Table 4.3). However, ganglioside 3'-LM1 and total neolacto gangliosides do not correlate with higher grade tumors. The presence of 3'-LM1 correlated with the low 1b ganglioside content (less than 30% of total gangliosides), chi square p=0.0078. Ganglioside 6'-LM1 does not have the same relationship.

Discussion

In a previous study (Sung et al., 1995) we found a similar pattern of ganglioside 1b decrease in astrocytic tumors with increase in the degree of histological malignancy, and those with 1b gangliosides less than 30% of total ganglioside content correlated with poorer survival. The percentages of gangliosides GT1b and GD1b among total ganglioside sialic acids were gradually decreased in grade III and IV oligodendroglomas, respectively. Ganglioside compositions of 24 oligodendroglomas were somewhat similar to that of anaplastic astrocytomas (AA) with higher 1b ganglioside content than either glioblastoma multiforme or primitive neuroectodermal tumors (Sung et al., 1994). Singh et al. (1994) reported that the neutral glycolipid composition
in oligodendroglialomas was also similar to AA in having high levels of ceramide monohezoside and infrequently having ceramide trihexoside or globoside.

We found that the presence of 6'-LM1 detected by immunostaining on HPTLC correlated with higher tumor grade; however, ganglioside 3'-LM1 or 3'-isoLM1 did not show this pattern. Here again we demonstrated that 6'-LM1 was an important glioma-associated ganglioside. Interestingly, ganglioside 6'-LM1 correlated with higher grades and poorer survival in astrocytic tumors (Sung et al., 1994 and 1995). Further studies for testing the prognostic value of 6'-LM1 and 1b gangliosides in oligodendroglialomas are required.

Recent evidence demonstrates that glial progenitor cells in vitro may develop into astrocytes or oligodendrocytes depending on culture medium (Raff, 1983). Choi et al. (1983-1986) postulated that oligodendroglia in the developing central nervous system may arise from astrogial precursors. This was based on the observations: (a) an increase in the mitotic activities of rat subpial astrocytes prior to the onset of myelination, followed by the appearance of oligodendrocytes in the same regions; and (b) the presence of transitional cells with both astrocytic and oligodendrocytic characteristics. Light and electron microscopic findings by Min and Scheithauer (1994) on 30 classic cases of oligodendrogliomas also suggest that oligodendrogliomas are related to or derived from a unique form of glial cell with considerable variation in cytological and ultrastructural features which are astrocyte associated. These examples point out the problems of traditional classification of gliomas based on morphological and cytological criteria, in which tumors are named according to the normal differentiated glial cells that they resemble and from
which they are assumed to be derived. Future information on this requires more studies on the multipotential capacity of glial progenitor cells. It would be particularly valuable to assign tumors to specific glial lineages. In order to do this, further understanding of the development of glial cell lineages is required, but this could provide additional methods for improved classification of oligodendrogliomas.
Table 4.1.

**Total Ganglioside Content And Percentage Distribution**  
Of Sialic Acid Among Gangliosides In 24 Oligodendrogliomas  
By WHO Histological Grade.

<table>
<thead>
<tr>
<th></th>
<th>Grade I&lt;sup&gt;a&lt;/sup&gt; &amp; II</th>
<th>Grade III</th>
<th>Grade IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n&lt;sup&gt;b&lt;/sup&gt;=15)</td>
<td>(n&lt;sup&gt;b&lt;/sup&gt;=5)</td>
<td>(n&lt;sup&gt;b&lt;/sup&gt;=4)</td>
</tr>
<tr>
<td><strong>Total NeuAc (μg)/mg Dry Weight</strong></td>
<td>0.99±0.14</td>
<td>1.02±0.12</td>
<td>0.90±0.29&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GM3</td>
<td>8.4±2.8</td>
<td>9.9±3.0</td>
<td>9.8±4.2</td>
</tr>
<tr>
<td>GM2</td>
<td>6.3±1.7</td>
<td>5.6±2.9</td>
<td>6.3±3.8</td>
</tr>
<tr>
<td>GM1</td>
<td>12.7±3.5</td>
<td>13.0±2.3</td>
<td>8.5±4.9</td>
</tr>
<tr>
<td>GD3</td>
<td>20.5±4.7</td>
<td>21.0±4.0</td>
<td>19.6±5.9</td>
</tr>
<tr>
<td>GD1a</td>
<td>12.3±3.8</td>
<td>16.6±2.6</td>
<td>12.2±7.6</td>
</tr>
<tr>
<td>GD2</td>
<td>9.1±1.9</td>
<td>7.1±0.4</td>
<td>9.7±1.2</td>
</tr>
<tr>
<td>GD1b</td>
<td>14.4±3.4</td>
<td>14.5±2.2</td>
<td>10.5±4.1</td>
</tr>
<tr>
<td>GT1b</td>
<td>12.5±3.4</td>
<td>10.1±1.2</td>
<td>10.4±3.6</td>
</tr>
<tr>
<td>GQ1b</td>
<td>2.7±0.7</td>
<td>2.5±0.7</td>
<td>2.3±0.6</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SEM.  
a: Only two cases were grade I.  
b: n is the number of cases studied.  
c: One specimen was not large enough to perform total sialic acid estimation.
### Table 4.2.

**Total And Individual Ganglioside Contents**

**In Pure Oligodendrogliomas And Mixed Gliomas.**

<table>
<thead>
<tr>
<th>Total NeuAc (μg)/mg Dry Weight</th>
<th>Pure (n=d,e=16)</th>
<th>Mixed (n=d=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>1.0±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>GM3</td>
<td>9.4±2.5</td>
<td>7.9±2.9</td>
</tr>
<tr>
<td>GM2</td>
<td>5.7±1.4</td>
<td>7.1±2.9</td>
</tr>
<tr>
<td>GM1</td>
<td>11.8±2.8</td>
<td>12.5±4.5</td>
</tr>
<tr>
<td>GD3</td>
<td>22.3±4.2</td>
<td>16.8±4.1</td>
</tr>
<tr>
<td>GD1a</td>
<td>14.3±3.5</td>
<td>10.9±4.2</td>
</tr>
<tr>
<td>GD2</td>
<td>9.3±1.7</td>
<td>7.7±1.2</td>
</tr>
<tr>
<td>GD1b</td>
<td>15.2±3.0</td>
<td>10.8±2.8</td>
</tr>
<tr>
<td>GT1b</td>
<td>13.6±3.0</td>
<td>7.7±1.9</td>
</tr>
<tr>
<td>GQ1b</td>
<td>2.8±0.7</td>
<td>2.1±0.5</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SEM.

- **d:** n is the number of cases studied.
- **e:** One specimen was not large enough to perform total sialic acid estimation.
Table 4.3.

Relationship Of Neolacto-Series Gangliosides And WHO Histological Grade.

<table>
<thead>
<tr>
<th>WHO Grade</th>
<th>3'-LM1</th>
<th>6'-LM1</th>
<th>Total Neolacto Gangliosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I and II</td>
<td>presence (5/15)</td>
<td>presence (1/13)</td>
<td>presence (6/15)²</td>
</tr>
<tr>
<td>(n=15)</td>
<td>33%</td>
<td>7.7%</td>
<td>62%</td>
</tr>
<tr>
<td>Grade III (n=5)</td>
<td>presence (2/5)</td>
<td>presence (3/5)</td>
<td>presence (3/5)</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>60%</td>
<td>60%</td>
</tr>
<tr>
<td>Grade IV (n=4)</td>
<td>presence (3/4)</td>
<td>presence (2/4)</td>
<td>presence (4/4)</td>
</tr>
<tr>
<td></td>
<td>75%</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>Chi-square test, p value</td>
<td>0.32</td>
<td>0.02</td>
<td>0.09</td>
</tr>
</tbody>
</table>

f: n is the number of cases studied.
g: Neolacto-series ganglioside other than 3'-LM1 and 6'-LM1 was detected in one case.
CHAPTER V

CORRELATION OF GANGLIOSIDE PATTERNS IN PRIMARY BRAIN TUMORS WITH SURVIVAL.

Introduction

Classification and grading of brain tumors based on histological criteria has major limitation. During the past decade, many researchers in neuro-oncological areas have searched for objective prognostic molecular markers. Unfortunately, none has been shown to be significantly better in predicting patient survival than the contribution of histological diagnosis, grade, and patient age.

In this study, we tested the value of ganglioside patterns in predicting patient survival. Our ultimate goal is to develop a diagnostic test using combined prognostic values of specific ganglioside patterns found in chapter III to define subtypes of human gliomas that can not be identified by existing histopathological techniques.
Correlation of Ganglioside Patterns of Primary Brain Tumors with Survival

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Background. Classification/grading schemes for brain tumors are based mainly on histologic examinations, but these have major limitations, which has led to a search for more objective prognostic markers. Gangliosides have several biologic effects relevant to tumors, and ganglioside compositions of primary brain tumors correlate with diagnosis. This led to the authors' hypothesis that ganglioside patterns of brain tumors might be useful as prognostic indicators.

Methods. Gangliosides in primary brain tumors of different histologic types from 84 patients were analyzed. Specific ganglioside patterns and several other relevant variables were examined for associations with survival using a Cox proportional hazards model. Kaplan-Meier survival curves were analyzed using the log-rank test.

Results. Patients in whom less than 30% of total tumor gangliosides consisted of 1b pathway gangliosides (GD1b, GT1b, and GQ1b) had significantly higher risk ratios than those with more than 30% 1b gangliosides (P = 0.03). The presence of 6'-LM1 (NeuAcα2→galβ1→GlicNAcβ1→galβ1→Glicα1→cer) was also associated with a higher risk ratio (P = 0.007). Combinations of 1b gangliosides and 6'-LM1 identified three groups of patients regardless of histologic diagnosis. Group A, with less than 30% 1b and the presence of 6'-LM1, had a median survival time of 331 days. Group B, with less than 30% 1b but no 6'-LM1, had a median survival time of more than 698 days. Group C, with more than 30% 1b had a median survival time of more than 778 days.

Conclusions. The correlation of ganglioside patterns with survival in this initial investigation suggests the potential of 1b gangliosides and 6'-LM1 to be used as prognostic indicators. Continuing research is being conducted to assess this possibility prospectively. Cancer 1995;75:891-9.

Key words: ganglioside, glycolipid, brain tumor, glioma, survival, prognosis, tumor marker, astrocytoma, oligodendroglioma, predictive indicator.

Introduction

Each year in the United States, there are 17,000 new cases of primary brain tumor diagnosed in adults, and the incidence is increasing, especially in the elderly.1-3 Brain tumors are the most common solid malignancy in childhood and the third most common cause of cancer death in young adults.4 During the last decade, there have been major advances in the clinical detection of brain tumors, mainly due to advances in diagnostic imaging techniques5; and a considerable amount has been learned about the biology of these neoplasms.6-14 Nevertheless, the neuropathologic diagnosis still rests mainly on histologic examination of tissues that have been stained with rather nonspecific dyes and with immunohistochemical stains, which in many cases are of marginal, questionable, or no prognostic value.15-16

During the last century, several classification/grading schemes based on histopathologic parameters have been developed.17-22 These have found wide utility because of their prognostic value, but they all have two major limitations: (1) there is a considerable range in the survival of patients within many of the diagnostic categories, and (2) they ultimately rely on subjective interpretations of histologic features. This has led to a search for more objective molecular markers of prognostic significance.23-28 The goal of these studies was to develop a panel of probes that could form the foundation for a classification system based on molecular abnormalities related to tumor behavior. These, in con-
junction with histologic findings, would presumably be prognostically more accurate. We have studied a series of primary human brain tumors to determine if their ganglioside compositions are of prognostic value. In the companion paper to this one, we report the results of ganglioside analyses for a series of primary brain tumors. In that study, we found a correlation between changes in specific gangliosides with increasing histologic grade of the tumors (i.e., decrease in 1b pathway gangliosides and the presence of 6'-LM1). In the present study, we report the results of survival analyses testing the ability of ganglioside patterns to predict survival for patients with brain tumors. Our results indicate that specific ganglioside patterns correlate with patient survival.

Methods

Patient Population and Ganglioside Analyses

The specimens analyzed were all surgical specimens obtained from member institutions of the National Cancer Institute Cooperative Glioma Group (The Ohio State University, Columbus OH; Barrow Neurological Institute, Phoenix AZ; and Mayo Clinic, Rochester MN), and the National Cancer Institute Cooperative Human Tissue Network. Diagnoses were classified according to the system of the World Health Organization and are based on independent reviews of histologic materials by four neuropathologists from the three participating institutions. In addition to the World Health Organization diagnosis and grade, four specific histologic parameters of prognostic significance (nuclear atypia, mitotic figures, endothelial proliferation, and necrosis) were recorded. Discrepancies in diagnoses and histologic findings were resolved by simultaneous review using a multiheaded microscope.

Values of ganglioside levels in the tumors under consideration are the ones derived from another recent study. Briefly, the analytic methods involved extraction of tissues with chloroform/methanol/water; purification of gangliosides using Folch partitioning, diethylaminoethyl-Sephadex, and reversed-phase column chromatography; separation of gangliosides using high performance thin layer chromatography, and visualization with the resorcinol spray. Separated gangliosides were identified on the basis of thin layer chromatography mobility in relation to known standards, visualization with horseradish peroxidase-conjugated Cholera toxin B-subunit after neuraminidase treatment, and immunostaining with the antibodies F1H11 and MSG-15 (Dainabot, Japan), TE3 (Eric Holms, Pacific Northwest Research Institute, Seattle, WA), and NS-24 (Y. Suzuki, University of Shizuoka, Japan). Visualized gangliosides were quantitated using scanning densitometry.

Statistical Analyses

Survival time was determined as the number of days between initial surgery for the tumor and either death or last follow-up. All deaths were due to the tumor or complications secondary to the tumor. Tumor grade, age, sex, radiotherapy, chemotherapy, the percentage composition of the gangliosides GM3, GM2, GM1, GD3, GD1a, GD2, the presence or absence of 3'-LM1, 6'-LM1, and 3'-isoLM1, and normal (> 30%) or abnormal (< 30%) levels of total 1b gangliosides (GD1b, GT1b, GQ1b) were examined for a possible association with survival. This analysis used a Cox proportional hazards model stratified by tumor grade and adjusted for the age of the subject. The association of the remaining covariates with survival was examined using a stepwise regression method with a P value no larger than 0.05 being required to maintain a risk factor in the model.

To determine interactions between significant variables, new covariates were created, and residual analyses were performed to monitor the appropriateness of the model developed. Kaplan-Meier survival curves were generated for each significant factor and the significance of overall differences in survival curves were tested by the log-rank tests. Along with the residual analysis, we further investigated whether the factors found to be significant in the overall analysis had a consistent relation with survival across tumor diagnoses and grades. In particular, the proportional hazards model was applied separately to all grade 3 and 4 tumors (as one analysis, stratifying for grade), to all grade 4 tumors, to all astrocytomas, to all fibrillary astrocytomas, and to grade 3 and 4 fibrillary astrocytomas (as one analysis stratifying for grade). For each of these diagnostic groups these analyses supported the robustness of the results.

Results

Patient Population

Eighty-four patients comprised the series (Table 1). This included 56 patients with astrocytic tumors: 16 patients with low grade astrocytomas (12 pilocytic and 4 fibrillary), 10 patients with anaplastic astrocytomas (AA) and 30 patients considered as having glioblastoma multiforme (GBM) that included 1 gliosarcoma. For purposes of discussion, low grade fibrillary astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme are all collectively referred to as fibrillary astrocytomas to distinguish them from pilocytic astro...
CNS Tumor Ganglioside Predicts Survival/Sung et al.

Table 5.1 Classification and grades of tumors used according to world Health Organization

<table>
<thead>
<tr>
<th>Consensus diagnosis</th>
<th>No. of patients</th>
<th>Mean ± standard deviation</th>
<th>Range</th>
<th>Male (%)</th>
<th>Female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytic tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade*</td>
<td>16</td>
<td>16 ± 19</td>
<td>0.02-46</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>10</td>
<td>35 ± 11</td>
<td>19-57</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>Glioblastoma multiforme†</td>
<td>30</td>
<td>49 ± 16</td>
<td>6-77</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>17</td>
<td>41 ± 17</td>
<td>7-68</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>Ependymoma</td>
<td>3</td>
<td>11 ± 3</td>
<td>9-15</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>PNET</td>
<td>8</td>
<td>11 ± 9</td>
<td>0.6-38</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>All 84 tumors by WHO grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>15</td>
<td>11 ± 12</td>
<td>0.02-43</td>
<td>46</td>
<td>53</td>
</tr>
<tr>
<td>Grade II</td>
<td>10</td>
<td>34 ± 12</td>
<td>15-46</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Grade III</td>
<td>14</td>
<td>38 ± 15</td>
<td>10-58</td>
<td>69</td>
<td>31</td>
</tr>
<tr>
<td>Grade IV</td>
<td>45</td>
<td>41 ± 21</td>
<td>0.6-77</td>
<td>59</td>
<td>40</td>
</tr>
</tbody>
</table>

WHO: World Health Organization; PNET: primitive neuroectodermal tumors
* Includes 12 pilocytic and four fibrillary astrocytomas.
† Includes one gliosarcoma.

Survival Analyses

Gangliosides of the 1b pathway. From our previous study, it was evident that gangliosides of the 1b pathway correlated with histologic grade. These included GD1b, GT1b, and GQ1b (nomenclature system is that of Svennerholm). Based on data in the literature and our own results, 30% was chosen as the normal lower limit for total 1b gangliosides (expressed as the percentage of total ganglioside sialic acid). Accordingly, patients were separated into two groups: those in whom 1b gangliosides accounted for more than 30% of the total tumor ganglioside sialic acid, and those with less than 30%. Of all 84 tumors, there were 28 in which 1b gangliosides were > 30% of the total ganglioside and 56 with < 30%. For those with > 30%, only 2 died as of the last follow-up; 50% had been followed at least 776 days and were still alive. For those living, follow-ups varied from 8 to 2709 days. In contrast, of the 56 patients with < 30% 1b gangliosides, 29 died and their median survival time was 638 days (Table 2). The estimated risk ratio for comparing these two groups was 5.0, with 90% confidence intervals ranging from 1.5 to 16.9 (P = 0.03).

Similar analyses were performed for all patients with grade 4 tumors regardless of cell lineage, all patients with grades 3 and 4 tumors, all astrocytomas, all fibrillary astrocytomas, and all grade 3 (anaplastic) and grade 4 (GBM) fibrillary astrocytomas. The grade 3 tumors of all types consisted of 10 AA, 3 oligodendrogliomas, and 1 ependymoma. The grade 4 group contained 29 GBM, 1 gliosarcoma, 6 oligodendrogliomas, 1 ependymoma, and 8 PNET. Each analysis for these different groups yielded similar results to those just discussed (Table 2). Kaplan-Meier survival curves for each of these tumor groups were generated and differences analyzed using the log-rank test. These analyses confirmed the statistical significance of the risk ratios and median survivals discussed above. Specifically, the overall differences in survival curves were significant with P = 0.001 for all tumors and all astrocytomas, P = 0.01 for fibrillary astrocytomas, P = 0.03 for grade 3 and 4 fibrillary astrocytomas, and P = 0.02 for all grade 4 tumors. This indicates that < 30% 1b gangliosides are associated with a much higher mortality rate than are > 30% 1b gangliosides, regardless of the primary brain tumor cell lineage or grade.

Ganglioside 6'-LM1. Similar analyses were performed examining the effects of 6'-LM1 on survival. For all patients those without measurable 6'-LM1 in their tumors had a median survival time of > 822 days, whereas for those whose tumors contained 6'-LM1 the median survival time was 393 days after surgery (Table 3). For those with grade 3 or 4 tumors of all types, the median survival times were > 698 days (without 6'-LM1) and 331 days (with 6'-LM1); for only those with grade 4 tumors these times were > 638 and 331 days (Table 3). For all diagnostic groups studied, the risk of...
### Table 5.2 Correlation of 1b gangliosides with survival

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>Total 1b gangliosides</th>
<th>50% survival (days)</th>
<th>Risk ratio</th>
<th>Value</th>
<th>90% confidence interval</th>
<th>Two-sided P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tumors (n = 84)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 30% (n = 28, 2 dead§)</td>
<td>29 ± 19</td>
<td>&gt; 776</td>
<td></td>
<td>5.0</td>
<td>1.5-16.9</td>
<td>0.03</td>
</tr>
<tr>
<td>&lt; 30% (n = 56, 29 dead¶)</td>
<td>36 ± 22</td>
<td>638</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL WHO grade 4 (n = 45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 30% (n = 7, 1 dead§)</td>
<td>40 ± 19</td>
<td>&gt; 491</td>
<td></td>
<td>6.4</td>
<td>1.2-34.8</td>
<td>0.07</td>
</tr>
<tr>
<td>&lt; 30% (n = 38, 25 dead¶)</td>
<td>41 ± 22</td>
<td>393</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All astrocytomas (n = 56)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 30% (n = 17, 1 dead§)</td>
<td>23 ± 20</td>
<td>&gt; 690</td>
<td></td>
<td>6.8</td>
<td>1.2-37.3</td>
<td>0.06</td>
</tr>
<tr>
<td>&lt; 30% (n = 39, 22 dead¶)</td>
<td>42 ± 20</td>
<td>444</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplastic astrocytomas and glioblastomas (n = 40)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 30% (n = 7, 1 dead§)</td>
<td>41 ± 14</td>
<td>&gt; 491</td>
<td></td>
<td>6.9</td>
<td>1.2-38.0</td>
<td>0.06</td>
</tr>
<tr>
<td>&lt; 30% (n = 33, 22 dead¶)</td>
<td>46 ± 16</td>
<td>435</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Age is shown as mean ± standard deviation.
† Determined as the number of days following surgery until last follow-up or death.
§ Number of patients

### Table 5.3 Correlation of 6'-LMI with survival

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>6'-LMI</th>
<th>50% survival (days)</th>
<th>Risk ratio</th>
<th>Value</th>
<th>90% confidence interval</th>
<th>Two-sided P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tumors (n = 84)</td>
<td>Absence (n = 60, 14 dead§)</td>
<td>31 ± 22</td>
<td>&gt; 822</td>
<td>2.7</td>
<td>1.5-5.0</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Presence (n = 24, 17 dead¶)</td>
<td>42 ± 17</td>
<td>398</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absence (n = 25, 10 dead§)</td>
<td>38 ± 24</td>
<td>&gt; 638</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Presence (n = 20, 16 dead¶)</td>
<td>45 ± 18</td>
<td>&gt; 331</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absence (n = 40, 12 dead§)</td>
<td>33 ± 25</td>
<td>&gt; 791</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Presence (n = 16, 11 dead¶)</td>
<td>44 ± 15</td>
<td>331</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absence (n = 26, 12 dead§)</td>
<td>44 ± 17</td>
<td>617</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Presence (n = 14, 11 dead¶)</td>
<td>46 ± 13</td>
<td>315</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Age is shown as mean ± standard deviation.
† Determined as the number of days following surgery until last follow-up or death.
‡ Number of patients
§ Includes one postoperative death at 14 days.
Ⅰ Includes one postoperative death at 1 day.
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Table 5.4 Correlation of 1b gangliosides and 6'-LM1 with survival

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>Patterns of expression of 1b gangliosides and 6'-LM1</th>
<th>Age* (yr)</th>
<th>50% survival (days)</th>
<th>Value</th>
<th>90% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tumors (n = 84)‡</td>
<td>Group A (1b &lt; 30% with 6'-LM1) (n = 20, 16 dead)</td>
<td>42 ± 17</td>
<td>331</td>
<td>9.1</td>
<td>2.6–32</td>
</tr>
<tr>
<td></td>
<td>Group B (1b &lt; 30% without 6'-LM1) (n = 36, 13 dead‡)</td>
<td>33 ± 24</td>
<td>&gt; 698</td>
<td>3.5</td>
<td>1.0–12</td>
</tr>
<tr>
<td></td>
<td>Group C (1b &gt; 30% with and without 6'-LM1) (n = 25, 2 dead‡)</td>
<td>29 ± 19</td>
<td>&gt; 776</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO Grade IV (n = 45)</td>
<td>Group A (n = 18, 14 dead)</td>
<td>43 ± 13</td>
<td>331</td>
<td>10.4</td>
<td>1.9–57</td>
</tr>
<tr>
<td></td>
<td>Group B (n = 20, 10 dead§)</td>
<td>39 ± 26</td>
<td>617</td>
<td>5.1</td>
<td>0.9–40</td>
</tr>
<tr>
<td></td>
<td>Group C (n = 7, 1 dead§)</td>
<td>40 ± 17</td>
<td>&gt; 928</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Age is shown as mean ± standard deviation.
† Determined as the number of days following surgery until last follow-up or death.
‡ For all tumors pairwise comparisons of groups A and C showed significant differences in survival (P = 0.003). Group B was significantly different from A (P = 0.008) and was marginally different from C in survival (P = 0.1). Pairwise comparisons for WHO grade IV showed that groups A and C had significant differences in survival (P = 0.03); group B was only marginally different from A (P = 0.07) and C (P = 0.1) in survival. All P values are for two-sided tests.
§ One postoperative death at 1 day.
1 One postoperative death at 14 days.

Combination of 1b gangliosides and 6'-LM1. The value of the combined use of 1b gangliosides and 6'-LM1 was tested by performing survival analyses using data for both sets of gangliosides (Table 4). Of 28 patients with over 30% 1b pathway gangliosides only 4 had 6'-LM1. Therefore, our patient population consists of three major subgroups: Those with less than 30% 1b and the presence of 6'-LM1 (Group A); those with less than 30% 1b but no 6'-LM1 (Group B); and those with greater than 30% 1b (Group C). Group A consisted of 1 patient with pilocytic astrocytomas, 1 patient with AA, 11 patients with GBM, 2 patients with PNET, 4 patients with oligodendrocytic tumors, and 1 patient with ependymoma. Group B consisted of 3 patients with pilocytic astrocytomas, 2 patients with low grade fibrillary astrocytomas, 6 patients with AA, 15 patients with GBM, 5 patients with PNET, 3 patients with oligodendrocytic tumors, and 2 patients with ependymomas. Group C consisted of 8 patients with pilocytic astrocytomas, 2 patients with low grade fibrillary astrocytomas, 3 patients with AA, 4 patients with GBM, 1 patient with PNET, and 10 patients with oligodendrocytic tumors. Combining all histologic diagnoses, the median survival time for Group A was 331 days, for Group B it was > 698 days, and for Group C it was > 776 days. The risk of death for patients in Group A was 9.1 times greater than patients in Group C. Kaplan–Meier survival curves clearly separated (P ≈ 0.0001) for Groups A, B, and C, with the patient population including tumors of several cell lineages and grades (Fig. 1). Of the 20 patients in Group A, 16 were dead, the longest survival among whom was 3.1 years past the initial surgical procedure. At the last follow-up, the four patients surviving had lived less than 2 years past surgery. This compares with only two deaths among the patients in Group C, one of whom died within 14 days of surgery for the tumor. Similar results were obtained for the more restrictive diagnostic groups of tumors, even among those of grade 4 alone (Fig. 2). Results of such analyses for astrocytomas grade 3 and 4 are shown in Table 5.

Other gangliosides. Results of similar analyses did not reveal any correlation with survival for 3'-LM1, 3'-isoLM1, gangliosides of the la series (GM1 or GD1a), GM3, GD3, or GD2.

Other Clinical and Histologic Features

Using data for all patients, after adjusting for tumor grade, there was no significant correlation of sex with...
The results showed that, across all diagnostic groups, the presence of 6'-LM1 significantly correlates with the presence of endothelial proliferation ($P = 0.03$) and tissue necrosis ($P = 0.004$), but not with the presence of mitotic figures or small cells. In contrast, < 30% 1b gangliosides significantly correlated with endothelial proliferation ($P = 0.003$), tissue necrosis ($P = 0.0008$) and mitotic figures ($P = 0.03$), but only marginally with small cells ($P = 0.08$).

Focusing on only those patients with the most malignant tumors (World Health Organization grade 3 or 4), we tested in our survival analysis the prognostic value of several histologic features considered to be of importance in determining histologic malignancy (endothelial proliferation, presence of mitotic figures, tissue necrosis, individual cell necrosis, giant cells, and small tumor cells). None of these histologic features was identified as significant ($P > 0.1$ in all cases) in our stepwise procedure or individually in a univariate analysis. Within this subgroup of grade 3 and 4 tumors, only the presence of small tumor cells approached significance among these histologic features ($P = 0.14$). In contrast, ganglioside patterns were able to separate the survival characteristics of these patients.

Two patients died postoperative deaths (1 and 14 days after surgery, respectively). These were included in all analyses as the deaths were tumor related. Excluding these two cases would have minimally strengthened the statistical significance of the findings for some studies. For example, it can be seen in Table 4 that both cases with postoperative deaths had ganglioside patterns that were found to correlate with longer survival.

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>Patterns of expression of 1b gangliosides and 6'-LM1</th>
<th>Age* (yr)</th>
<th>50% survival (days)</th>
<th>Risk ratio</th>
<th>90% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO grade III and IV fibrillary astrocytomas (n = 40)</td>
<td>Group A (1b &lt; 30% with 6'-LM1) (n = 12, 10 dead)</td>
<td>46 ± 12</td>
<td>316</td>
<td>10.6</td>
<td>1.9-60</td>
</tr>
<tr>
<td></td>
<td>Group B (1b &lt; 30% without 6'-LM1) (n = 21, 12 dead)</td>
<td>46 ± 19</td>
<td>444</td>
<td>5.1</td>
<td>0.9-28</td>
</tr>
<tr>
<td></td>
<td>Group C (1b &gt; 30% with and without 6'-LM1) (n = 7, 1 dead)</td>
<td>41 ± 14</td>
<td>&gt; 491</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Age is shown as mean ± standard deviation
† Determined as the number of days following surgery until last follow-up or death
‡ For WHO grade III and IV fibrillary astrocytomas pairwise comparisons of groups A and C showed significant differences in survival ($P = 0.02$) Group B was marginally different from A ($P = 0.10$) and C in survival ($P = 0.11$).
§ One postoperative death at 1 day
¶ One postoperative death at 14 days

Discussion

The main purpose of a classification scheme as well as of grading systems for tumors is to predict the clinical...
course and aid in therapeutic decision making. In an attempt to achieve these goals with respect to brain tumors, classifications have sometimes incorporated parameters of very different types. These include such factors as anatomic site (e.g., brainstem glioma and optic glioma), patient age (e.g., pilocytic astrocytoma and desmoplastic infantile ganglioglioma), and assumptions regarding "cell of origin" (e.g., glioblastoma and medulloblastoma). Although histologic parameters are of unquestionable value, they have obvious limitations, given their reliance on subjective interpretations of visual information. It is a well recognized fact that diagnostic discrepancies, such as interobserver variation with regard to histologic subtype and grade of gliomas, occur among even experienced neuropathologists. In the present study, this effect was minimized by having representative slides of each tumor independently reviewed by four neuropathologists from three different institutions. In this way a consensus diagnosis was obtained, one minimizing the bias encountered when only one or two neuropathologists from a single institution serve as reviewers.

Recognition of the limitations of histologic methods has led to a search for objective markers of predictive value. Given the ever increasing number of available molecular probes for different aspects of cell biology, it is difficult to choose which might be of the greatest prognostic value. Clearly, choices must be based on evidence linking candidate probes to some biologic feature critical to tumor progression. There is a large body of information indicating that gangliosides are involved in the regulation of cell division, differentiation, and migration of both normal and neoplastic cells. The mechanisms for this have not been elucidated entirely, but gangliosides inhibit ligand-stimulated autophosphorylation and dimerization of receptors for platelet-derived growth factor and epidermal growth factor. Furthermore, d- and tri-sialogangliosides are more inhibitory than are the monosialogangliosides tested, suggesting that the loss of lb gangliosides from the higher grade tumors may be involved in glioma invasion. We speculate that our two prognostic markers in combination may detect a population of patients who are at a greater risk for tumor recurrence.

Gangliosides added exogenously to culture medium inhibit the growth of normal and neoplastic cells. The mechanisms for this have not been elucidated entirely, but gangliosides inhibit ligand-stimulated autophosphorylation and dimerization of receptors for platelet-derived growth factor and epidermal growth factor. Furthermore, d- and tri-sialogangliosides are more inhibitory than are the monosialogangliosides tested, suggesting that the loss of lb gangliosides from the higher grade tumors may be involved in the biologic mechanisms underlying rapid growth.

Ganglioside 6'-LM1 is structurally related to the antigen sialylated Lewis-X (SLex) which for colon carcinoma correlates with both the metastatic potential and patient survival. Although primary tumors of the central nervous system rarely metastasize to distant organs, 6'-LM1 might be involved in glioma invasion.

A major problem inherent in histologic diagnosis and in primary brain tumor research, one that ganglioside analyses can not entirely overcome, is regional heterogeneity. It is well recognized that the smaller the diagnostic specimen, the greater is the chance of misdiagnosis. In our studies, even the smallest specimen analyzed (300 mg) was considerably larger than that usually made available for histologic study, thus...
providing information from a much larger number of tumor cells. In the eight tumor specimens that were large enough to allow ganglioside analyses on two separate pieces. 1b gangliosides were similar in all cases, suggesting that these ganglioside changes are regionally stable within the tumors. Ganglioside 6'-LM1 was present in both pieces in one case, absent from both pieces in one case, but present in one and absent from the other in two cases. In our statistical analyses we used the negative result for 6'-LM1 in the latter two patients, both of whom died. Thus our P values for these analyses are conservative, and it seems that if 6'-LM1 is present anywhere within the specimen it portends a shorter survival.

In summary, this study demonstrates a correlation between specific tumor ganglioside compositions and patient survival for the most common types of primary brain tumors. The predictive value of these ganglioside patterns is not simply due to a correlation with histologic diagnosis and grade, as it holds true regardless of diagnosis, and correlates with survival even in grade 4 tumors. Nevertheless, this study group of patients must be considered a "training set" in terms of the derived data. As a minimum, two additional studies will be required before ganglioside analyses on brain tumors can be justified as a routine clinical test of prognosis. First, this group of patients must be followed for a sufficient period of time to estimate 50% survival for all diagnostic categories analyzed. Second, this study must be repeated on another independent prospectively studied group of patients (i.e., a "verification set").

References

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CHAPTER VI

GLYCOLIPIDS IN A HUMAN GLIOMA CELL LINE.

Introduction

In our previous studies, we had demonstrated that ganglioside compositions in human primary brain tumors correlate with both histological malignancy and patient survival. Whether these tumors are astrocytic or oligodendroglial in origin, the accumulation of simple gangliosides (i.e. GM3 and GD3) and loss of the complex ones are the general pattern. Interestingly, GM3 when exogenously added to culture medium, has the least inhibitory effect on PDGF-stimulated cell growth compared to complex gangliosides (El Sayed, 1991; Van Brocklyn et al., 1993; Yates et al., 1993). One of the cell lines tested in these studies was human malignant glioma U-1242 MG, which is known to bear PDGF receptors.

PDGF is a potent growth factor for fibroblasts, smooth muscle cells and glial cells (Westermark and Heldin, 1993). It has been shown to be involved in regulation of cell growth, differentiation and in tumorigenesis.
Several cell lines derived from human tumors produce PDGF and express its receptor. Autocrine stimulation of cell growth by PDGF has been demonstrated in glioma cells (Betsholtz, 1989). Several lines of evidence indicate that modification of growth factors, their receptors, or their signal transduction pathways may lead to malignant transformation. Since gangliosides have been shown to have inhibitory effects on growth factor-stimulated cell growth, we conducted a study to determine the endogenous ganglioside composition of U-1242 MG.

In this study, neutral glycolipids and gangliosides extracted from U-1242 MG cells at a density of $10^5$ cells/ml were analyzed by different methods. We found that U-1242 MG cells have predominantly simple gangliosides and lack the complex ones, possibly contributing to the high proliferation rate of glioma cells.
Glycolipids of a Human Glioma Cell Line Bearing Receptors for Platelet-Derived Growth Factor (PDGF)

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Division of Neuropathology, Department of Pathology, The Ohio State University, Columbus, Ohio 43210

Glycolipids of U-1242 MG were characterised because results of previous studies showed that exogenous gangliosides, especially GM3, inhibit PDGF-stimulated growth of this human glioma cell line. GM3 and GM2 are the major gangliosides; both separate as doublets with thin-layer chromatography. The major neutral glycolipid is glucocerebroside with nonhydroxy fatty acids, but paragloboside, ceramide dibehexoside, globoside, and asialoGM3 (G4d) are also present. The coexistence in U-1242 MG of these gangliosides and the PDGF receptor, whose mitogenic signal is modulated by G4d in these cells, suggests a possible functional relationship among them with respect to growth regulation.


Growth inhibition was one of the first described biological responses of cultured cells to exogenously added gangliosides, and it has been suggested that this may be mediated through the ability of gangliosides and their metabolites to affect protein phosphorylation by regulating different protein kinases and phosphatases (1-3). Bremer et al. (4,5) have shown that gangliosides can inhibit i) growth of 3T3 fibroblasts stimulated by platelet-derived growth factor (PDGF), and ii) autophosphorylation of the receptors for PDGF and epidermal growth factor (EGF). The potency to cause these effects varied among different gangliosides, but GM3, a major ganglioside in fibroblasts, was one of the most effective. U-1242 MG is a cell line derived from a human malignant astrocytoma (8). If gangliosides are biological regulators of the PDGF receptor, then it would be expected that gangliosides which have been found to be more effective in inhibiting PDGF-stimulated growth and autophosphorylation of the PDGF receptor, such as GM3, might be present in U-1242 MG cells. In this paper we describe the results of glycosphingolipid analyses of this cell line which show that this is the case.

MATERIALS AND METHODS

For biochemical analyses, U-1242 MG cells were seeded into 150-cm² plastic cell culture flasks (Corning, Corning, NY) in Eagle's minimal essential medium containing 10% calf serum at 10,000 cells/cm². Cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C; culture media were changed every third day. When cultures were subconfluent they were harvested using 0.01% trypsin in 0.01% EDTA, and before the cells were pelleted, aliquots of cell suspension were removed for total protein determinations (using the method of Bradford (9)) and for quantitation of cell numbers. Total lipids were extracted from cell pellets by homogenizing them in chloroform/methanol/water (5:5:1, by vol) and by separating the non-lipid residue by centrifugation. Ganglioside and neutral glycolipid fractions were purified using methods previously described in detail (10,11). The procedure involves an initial Folch partition, followed by three sequential washes of the lower phase with theoretical upper phase containing water but no KCl. Triplicate aliquots of washed lower phase were weighed using a Cahn microbalance. Neutral glycolipids were purified from the lower phase by silicic acid column chromatography and alkaline methanalysis. Individual neutral glycolipids were separated and quantitated using high-performance liquid chromatography (HPLC) (10). Cerebrosides were qualitatively identified on the basis of their hexose content using thin-layer chromatography (TLC) on borate impregnated silica gel plates (12,13), and detected by spraying with diphenylamine followed by heating (14). Immunostaining for galactocerebrosides was performed after separating the neutral glycolipids on aluminum backed silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) using the monoclonal antibody Ol, provided by Dr. S.E. Pfeiffer (15), and the Avidin-Biotin alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) (16). Paragloboside was detected in a similar way using the antibody F1H11 from Dainabot (Chiba, Japan) (17). The pooled upper phases were desalted using a C18 reversed phase column, and total gangliosides were quantitated using the resorcinol reaction (18). Gangliosides were separated using HPTLC and quantitated using scanning densitometry. The usual solvent system was chloroform/methanol/0.2% CaCl₂ (5:5:10, v/v/v), but to separate N-acetylneuraminic acid (NeuAc) from N-glycolyl-neuraminic acid (NeuGc) containing gangliosides, the solvent system used was chloroform/methanol/0.5M ammonium hydroxide/0.4% CaCl₂/2H₂O (50:50:4:6, v/v/v/v), by vol. Immunostaining for NeuGc-containing GM3 was performed as described above for galactocerebrosides, but using the monoclonal antibody 2-39M (19). Antibody 2-39M, directed against NeuGc containing GM3, was provided by Dr. Kenneth Lloyd. The NeuGc-containing GM3 standard used as a positive control was from Matreya, Inc. (Pleasant Gap, PA).

RESULTS AND DISCUSSION

The amounts of total gangliosides and neutral glycolipids in U-1242 MG cells are shown in Table 1. The values for
both lipid classes expressed on the basis of total lipid and total protein are within the same range previously reported for the few other human glioma cell lines studied (10,11,20-22). G$_{M1}$ and G$_{M3}$ were the major gangliosides making up 57% and 43%, respectively, of the total gangliosides, with only trace amounts (not quantitated) of one which migrated just behind G$_{D1b}$ and another behind G$_{D1h}$. G$_{M1}$ was not detected. Both of the major gangliosides in U-1242 MG migrated as doublets (Fig. 1). In the basic solvent system, the faster migrating of the G$_{M3}$ doublet comigrated with G$_{M3}$ containing N-acetylgalactosaminic acid, while the other comigrated with N-acetylgalactosaminic acid-containing G$_{M3}$. However, on immuno-TLC, neither ganglioside reacted with monoclonal antibody 2-39M, which is specific for NeuGc-containing G$_{M3}$. Therefore, it seems most likely that these G$_{M3}$ gangliosides separate on the basis of either their sphingosine or fatty acid composition. The G$_{M3}$ doublet migrated as a single band in the basic system, indicating that both of the G$_{M3}$ bands in the neutral system contain only one type of sialic acid, probably NeuAc. The proportions of these gangliosides are shown in Table 2.

This relatively simple ganglioside pattern is remarkable, because other human glioma cell lines previously reported have had significant amounts of disialogangliosides (22), and some even had trisialogangliosides (23,24). The presence of G$_{M1}$ has been variable in previously reported human glioma cell lines, but gangliotetraose oligosaccharides have been present in all others studied (11,20,22-24).

The major neutral glycolipid in U-1242 MG is cerebroside which eluted on HPLC with a retention time equal to that of cerebroside containing nonhydroxy fatty acid (Table 3). It is approximately five times more prevalent than the cerebroside which coeluted with cerebroside containing hydroxy fatty acid (HFA). On immuno-TLC none of the cerebroside reacted with the G1 antibody (which reacted strongly with the galactocerebroside control). On borate impregnated TLC plates all of the cerebroside from U-1242 MG migrated with glucocerebroside. Therefore, the major neutral glycolipid in these cells is glucocerebroside, the majority of which contains nonhydroxy fatty acid. Glucocerebroside is both an anabolic precursor and a catabolic degradation product of all of the gangliosides and neutral glycolipids found in this cell line. Whether it has a biological role other than an intermediate metabolite of other glycolipids is presently unknown.

U-1242 MG cells also contain lesser amounts of ceramide dihexose (CDH), asialo-G$_{M3}$ (G$_{A3}$), and globoside (glob) (Table 3). CDH, G$_{A3}$, G$_{M3}$ and G$_{M1}$ are all closely related metabolically. Therefore it is conceivable that changes in the activities of sialytransferases and sialidases involved in their interconversions could modulate the functions of the PDGF receptor by altering the concentrations of these glycolipids in the plasmalemma. Paragloboside migrated as a triplet on both HPLC and HPTLC with approximately equimolar amounts in each band. Its identity was proven further by visualizing all three bands on immunostaining with the antibody F1H11. One major difference between the neutral glycolipids of this human glioma cell line and the other four described in the literature (10) is the presence of modest amounts of paragloboside in U-1242 MG which was not detected in any of the others. However, immunodetection was not used previously. The relationship of the neutral glycolipid composition to the function of the PDGF receptor, if any, involved in their interconversions could modulate the functions of the PDGF receptor by altering the concentrations of these glycolipids in the plasmalemma. Paragloboside migrated as a triplet on both HPLC and HPTLC with approximately equimolar amounts in each band. Its identity was proven further by visualizing all three bands on immunostaining with the antibody F1H11. One major difference between the neutral glycolipids of this human glioma cell line and the other four described in the literature (10) is the presence of modest amounts of paragloboside in U-1242 MG which was not detected in any of the others. However, immunodetection was not used previously. The relationship of the neutral glycolipid composition to the function of the PDGF receptor, if any.

**Table 6.1**

<table>
<thead>
<tr>
<th>Amounts of Total Gangliosides and Neutral Glycolipids in U-1242 MG Cells$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gangliosides</td>
</tr>
<tr>
<td>Per mg protein</td>
</tr>
<tr>
<td>$1.03 \pm 0.13$</td>
</tr>
</tbody>
</table>

$^a$Results for gangliosides are expressed as nmol sialic acid, for neutral glycolipids as nmol glycolipid. Values represent the means ± SEM for three separate experiments.

---

**Table 6.2**

<table>
<thead>
<tr>
<th>Percentage Distribution of Gangliosides in U-1242 MG Cells$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G$_{M3}$</td>
</tr>
<tr>
<td>Upper</td>
</tr>
<tr>
<td>28.0 ± 5.0</td>
</tr>
</tbody>
</table>

$^a$Values are based on the integration units of the areas under the peaks for at least five scans for each of three samples. Upper and lower refer to the relative migrations of the components of the doublets for each ganglioside separated by high-performance thin-layer chromatography.
is unknown. Although sphingosine is an inhibitor of protein kinase C (reviewed in ref. 25), there is little evidence that any neutral glycolipid has an effect on the activity of any protein kinase.

G\textsubscript{M1}, which comprises 57% of the total ganglioside in U-1242 MG cells, is a relatively potent inhibitor of both PDGF-stimulated growth of U-1242 MG and Swiss 3T3 fibroblasts (4). G\textsubscript{M1} also inhibits autophosphorylation of the PDGF receptor (4). Of relevance to the present study is the finding that sialosylparagloboside (SPG) was inactive in both respects (4). Although paragloboside is present in U-1242 MG, no SPG was found. The ganglioside composition reported here is compatible with the possibility that G\textsubscript{M1} could play a role in regulating the degree of autophosphorylation of the PDGF receptor, thus modulating signal transduction of the PDGF signal for mitogenesis in U-1242 MG cells.

The pattern of gangliosides reported here for U-1242 MG cells has not been seen in all human glioma lines studied so far, nor have PDGF receptors been found in all glioma cell lines examined (6,7). Thus, it is possible that glioma cells whose growth is influenced by PDGF may have ganglioside and neutral glycolipid contents similar to those described here for U-1242 MG. If this proves to be the case, then the gangliodip composition of glioma cells could play a role in their growth by modulating the function of the PDGF receptor and, thus, might be a marker for cells which interact with PDGF. This would have implications of both biological and clinical importance, and the U-1242MG cell line could be used as a model system to investigate the molecular mechanisms involved in the interactions of ganglioside with the PDGF receptor in human glioma cells.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Kenneth Lloyd and S.E. Pfeiffer for helpful discussions and the antibodies 2-39M and O1, respectively. We are also grateful to Dainabot (Chiba, Japan) for antibody F1H11. We also thank Ms. Julie Dolin for photographic assistance and Ms. Eleanor Boren for secretarial services. This work was supported by PHS Grants CA 16058 and CA 00486 awarded by the National Cancer Institute, DHHS.

REFERENCES


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During the past decade, many tumor associated antigens have been identified as being carbohydrate in nature. The aberrant glycosylation, sialylation, and/or fucosylation of glycolipids and glycoproteins in tumor cell membranes has also been reported (Hakomori, 1985). These tumor carbohydrate antigens are associated with cell adhesion, tumor invasion, and metastatic potential. However, most of these studies have been made on non-CNS tumors. To determine whether aberrant glycolipids are present in human primary brain tumors, we performed the following studies focusing on fucose-containing lacto and neolacto series gangliosides (SLe$^x$ and SLe$^a$), NeuGc-GM2 and sulfoglucuronyl glycolipids.
A. FUCOSE-CONTAINING LACTO AND NEOLACTO SERIES GANGLIOSIDES

Introduction

A number of fucosylated glycolipids and glycoproteins in which fucose is linked to internal and/or subterminal GlcNAc on lacto type I and type II chains via $\alpha 1\rightarrow 4$ or $\alpha 1\rightarrow 3$ linkages have been identified. Among these fucosylated antigens, sialyl Lewis X (SLe$^X$) and sialyl Lewis A (SLe$^A$) are known to be ligands of endothelial leukocyte adhesion molecule-1 (ELAM-1) (Phillips et al., 1990; Takada et al., 1993). SLe$^X$ and SLe$^A$ containing antigens were found to be highly expressed in stomach, colon, pancreas, bladder, and lung tumors and some cancer cell lines compared to their normal counterparts (Shigeyuki et al., 1991; Walz et al., 1990; Kasai et al., 1986). Recent studies showed that the expression of SLe$^X$ and SLe$^A$ correlated with tumor invasion in human colonic and gastric cancer (Dohi et al., 1993; Nakamori., 1993). No similar studies have been done on human brain tumors. Since SLe$^X$ and SLe$^A$ have the same oligosaccharide backbones as 3'-LM1 and 3'-isoLM1, respectively, and they have been found in human gliomas (Sung et al., 1994; Fredmen et al., 1988), we proposed the hypothesis that fucose-containing lacto and neolacto gangliosides are present in human brain tumors. Therefore, we conducted the following studies using immunochemical and immunohistochemical methods.
Methods

SLex and SLea ganglioside standards were obtained by chemical synthesis from Dr. Hasegawa (Hasegawa et al., 1993). Monoclonal antibodies CSLEX1 (Chia et al., 1985) and CF4-C4 (Young et al., 1983), recognizing SLex and Lea, were obtained from culture supernatants of two hybridoma clones purchased from ATCC. The positive staining of intra- and peri-vascular neutrophils provided internal positive controls. Human colorectal carcinoma tissues were used as immunohistochemical positive controls for both antigens (Dohi et al., 1994). Immuno HPTLC of ganglioside fractions were performed on seventy-five tumors (including 11 low grade astrocytomas, 11 anaplastic astrocytomas, 31 glioblastoma multiformes, 14 oligodendrogliomas, 5 primitive neuroectodermal tumors, and 3 ependymomas). Immuno HPTLC and immunohistochemical staining for SLea were done using neuraminidase Arthrobacter ureafaciens treatment prior to antibody incubations.

Results

Immuno HPTLC

None of the seventy five tumors had detectable level of SLex gangliosides (the lower limit of detection is 3 ng). For SLea only one case (primitive neuroectodermal tumor) was positive at a concentration of 9.7 ng/mg dry weight tissue.
One hundred and twelve cases were immunohistochemically studied including 16 pilocytic astrocytomas (LG.p), 8 low grade fibrillary astrocytomas (LG.f), 9 anaplastic astrocytomas (AA), 28 glioblastoma multiformae (GBM), 2 gliosarcomas, 11 PNET, 26 oligodendrogliomas (Oligo), 9 ependymomas, 1 astroblastoma, 1 pleomorphic sarcoma, and 1 pleomorphic xanthoastrocytoma. Among these tumors, SLeX immunopositivity of tumor cells was seen in 7 (44%) LG.p, 1 (12%) LG.f, 4 (50%) AA, 5 (16%) GBM, 3 (27%) PNET, 7 (27%) Oligo, 3 (33%) ependymoma, 1 astroblastoma, 1 pleomorphic sarcoma, and 1 pleomorphic xanthoastrocytoma. One pilocytic astrocytoma was notable for having immunopositivity limited to eosinophilic granular bodies. The staining intensity of the majority was weak; where staining of moderate intensity was seen, only the astroblastoma had a significant proportion of positive cells. The pattern of staining was localized predominantly to the surface membrane, but several cases showed a cytoplasmic reaction. There was no correlation between immunopositivity and histological grades using the world health organization system.

The immunopositivity of CF4-C4 both with and without neuraminidase treatment was only found in two cases (1 oligo, 1 pilocytic astrocytoma). Three cases (1 GBM, 1 AA, and 1 ependymoma) were positive only after neuraminidase treatment.
Discussion

The results showed SLe\(^x\) and SLe\(^a\)-containing gangliosides were not expressed in primary brain tumors. However, from the cases which were immunohistochemically positive but negative by immuno HPTLC suggest that SLe\(^x\) and SLe\(^a\) containing glycoproteins may be present in some primary brain tumors. In our previous study, gangliosides 3'-LM1 and 6'-LM1 correlated with the histological diagnosis and survival of 84 patients with primary brain tumors. Although SLe\(^x\) and 3', 6'-LM1 are all derived from the same ganglioside family with lacto-series type II chains as oligosaccharide backbones, human gliomas may lack the specific fucosyltransferases needed to synthesize the fucosylated neolacto gangliosides.

SLe\(^x\) was highly expressed in gastrointestinal, pulmonary, and pancreatic tumors. Recent studies showed that the immunohistochemical expression of SLe\(^x\) correlated with poor survival in patients with colorectal carcinoma (Nakamori et al., 1993). It may be possible that secondary brain tumors which metastasized from lung and colon carcinoma express these fucosylated gangliosides in contrast to primary brain tumors.

SLe\(^x\) gangliosides were not detected in normal human adult brain (Fukushima et al., 1984). However, considerable amounts of non-sialylated fucosyl-neolactotetraosylceramide (Le\(^x\)) (32-40 nmol/g whole brain) were found in human fetal brains from gestational week 22 to 23 (Ishikawa, 1987) compared to normal human brains of postnatal infants and adults. The latter contain a very low amount of
fucosyl-neolactotetrosylceramide (1.3-6.5 nmol/g of white matter, 0.6-1.0 nmol/g of gray matter) (Vanier et al., 1980). The subcellular localization of $\text{Le}^x$ in astrocytes (cell surface) and oligodendrocytes (cytoplasm) in normal human white matter was found to be different (Gocht, 1992). Interestingly, $\text{Le}^x$ immunostaining was seen in the cytoplasm of reactive astrocytes in central pontine myelinolysis and multiple sclerosis (Gocht, 1992).

Reifenberger et al. (1992) investigated $\text{Le}^x$ expression in neural tumors using immunohistochemistry on paraffin sections. They found that neither 21 glioblastomas nor 7 PNET studied expressed $\text{Le}^x$. Among four pilocytic astrocytomas, there were three positive cases with staining positivity less than 50% of tumor cells. Some immunopositive staining was seen in 6 out of 14 oligodendrogliomas (grade II), 1 out of 7 oligodendrogliomas (grade III), and 8 out of 15 ependymomas (grade I to grade IV). In contrast to Reifenberger's findings, a previous study by Budka and Majdic (1985) using immunofluorescence on frozen sections showed a relatively constant immunopositivity for $\text{Le}^x$ in tumor cells of gliomas including glioblastomas. Methodological differences may account for these contradictory results.

Although the expression of $\text{SL}^x$ and $\text{SL}^\gamma$ in human gliomas was absent, our preliminary data for $\text{Le}^\alpha$ indicate that $\text{Le}^\alpha$-containing neutral glycolipids are present in gliomas. We are continuing to study the carbohydrate epitopes $\text{Le}^x$ and $\text{Le}^\alpha$ in the neutral glycolipid fractions.
B. NeuGc-GM2

Introduction

The types of gangliosides vary considerably among different species of animals. Type of sialic acid is one example. Only NeuAc and its derivatives are found in normal human tissues, whereas, NeuGc-containing gangliosides are widely distributed in erythrocytes of cat, sheep, bovine and oriental species of dogs (Furukawa et al., 1988). There is no evidence that NeuGc-containing gangliosides exist in normal human tissues or human tumors such as colon, lung and melanoma (Furukawa et al., 1988; Miyake et al., 1988). Gangliosides isolated from human melanoma and astrocytoma cells grown in fetal bovine serum, but not in synthetic medium, expressed NeuGc species of GD3 and disialylparaglobosides (Furukawa et al., 1988). Although it is generally agreed that human tissues do not contain NeuGc-containing gangliosides because of lack of monooxygenase activity to convert N-acetyl to N-glycolyl sialic acid, NeuGc-GM2 was recently found in human non-seminomatous germ cell tumor tissues (Miyake et al., 1990).

In the previous study, we found some gangliosides that migrated between GM2 and GM1 on resorcinol-sprayed TLC plates. Most of these gangliosides were further identified as neolacto and/or lacto-series gangliosides using immunostaining TLC with monoclonal antibodies F1H11 and TE3. However, there were ten tumors (1 low grade astrocytoma, 3 anaplastic astrocytomatas, 3 glioblastomas, 1 PNET, and 2 oligodendrogiomas) having gangliosides migrating below GM2 standards but not showing...
immunoreactivity with either F1H11 or TE3. The percentage that these gangliosides made up of the total ganglioside sialic acid among the tumors varied from 0.6% to 6.5%. Also, among these ten tumors, seven cases did not have the band comigrating with GM2 standards on the HPTLC. Since NeuGc-GM2 migrates below NeuAc-GM2 on the TLC plate, we decided to determine whether this ganglioside was NeuGc-GM2 or a subspecies of NeuAc-GM2.

Methods

NeuGc-GM2 standard purchased from Matreya (Pleasant, PA) was spotted on the TLC plate as a positive control. Monoclonal antibodies MK1-17 and MK2-34 were generous gifts from Dr. Kannagi (Aichi Cancer Center, Nagoya, Japan). MK1-17 antibody recognizes both N-acetyl and N-glycolyl GM2. MK2-34 specifically detects N-glycolyl GM2 (Miyake et al., 1988). The solvent system used for separation of N-glycolyl GM2 from N-acetyl GM2 is chloroform/methanol/5M ammonium hydroxide/0.4% CaCl₂ (50:50:4:5). The immunostaining TLC method is described in detail above.

Results

NeuGc-GM2, defined by the antibody MK2-34, was not found in any of the ganglioside fractions of these tumors. On the other hand, all of these tumors showed immunopositivity to monoclonal antibody MK1-17. Five tumors contained GM2 upper bands, four cases had lower bands, and one had doublet bands on immunostaining TLC. The results suggest that the
gangliosides migrating below GM2 in these tumors were subspecies of NeuAc-GM2, not NeuGc-GM2. The difference in mobility may be due to the differences in the ceramide portion (sphingosine and/or fatty acid).

**Discussion**

The results indicate that identification of gangliosides based solely on TLC mobility is not sufficient, especially for tumor gangliosides where a variety of aberrant structures can be seen. In a previous study, we showed that there was no NeuGc-containing GM3 present in human gliomas detected by monoclonal antibody 2-39M which recognized NeuGc-GM3. Here again we demonstrate that NeuGc-containing GM2 was not expressed in the tumors. Our results do not support the presence of NeuGc-containing gangliosides in human tumors.
C. SULFATED GLUCURONYL GLYCOLIPIDS (HNK-1 ANTIGENS)

Introduction

During the development of the nervous system, there is a sequence of precisely regulated cellular events which include cell division, differentiation, neural crest cell migration, neuronal and glial differentiation, and axonal growth and synapse formation. Cell surface glycoconjugate molecules, glycolipids and extracellular matrix proteins play an important role in mediating cell-cell interactions, adhesion and migration. They include neural cell adhesion molecules (N-CAM), myelin-associated glycoproteins, sulfoglucuronyl glycolipids (SGGLs) and integrins (Jungalwala, 1994). The common characteristic feature of these molecules is that they all have a terminal 3-sulfoglucuronyl residue in the carbohydrate chain which is recognized by HNK-1 antibodies (Prasadara et al., 1990).

SGGLs were first identified in human peripheral nerves in patients with peripheral neuropathy and later found to be present in normal peripheral nerves of various animals (Chou et al., 1985; Chou et al., 1987). The developmental profile of SGGLs in cerebral cortex was different from cerebellum in rodent brains (Prasadara et al., 1990). The levels of SGGLs in the cerebellum increased dramatically after birth. In contrast to this, the expression of SGGLs in cerebral cortex decreased during postnatal development (Jungalwala, 1994). The structures of SGGLs contain neolacto-series oligosaccharide backbones which are the same as those of neolacto gangliosides. The only difference between neolacto gangliosides and SGGLs is
that SGGLs have a sulfated glucuronyl residue at the end of the oligosaccharide chain instead of sialic acid residues. Since the sulfoglucuronyl residues are acidic, they partition with gangliosides into the upper phase in Folch partition during lipid purification. We wanted to determine if SGGLs were present in the ganglioside fractions extracted from human gliomas, and primitive neuroectodermal tumors (PNET), which may derive from undifferentiated cells or bipotential cells with the capacity of both glial and neuroblastic differentiation.

**Methods**

HNK-1 antibodies and SGGL standards were obtained from Dr. F. B. Jungalwala. ImmunoTLC and immunohistochemistry techniques were described as before. Specimens from human fetal brains were obtained from autopsies performed on products of spontaneous or induced abortions at The Ohio State University Hospital. Mouse IgM (Dako, Carpinteria, CA) was used as a negative control.

**Results**

We have used HNK-1 antibody to study human primitive neuroectodermal tumors (PNETs) by immunoHPTLC and immunohistochemistry. The "ganglioside" fractions of 13 PNETs have non-detectable levels (the lower limit of detection: 2 ng) of SGGLs by immunostaining HPTLC. However, seven out of ten frozen sections of tumor tissues were immunohistochemically positive for HNK-1. Human fetal brains
at gestational ages 21 and 34 weeks were also strongly positive in the subependymal plate regions. The staining intensity was ranked as follows: fetal brain 21 weeks > 34 weeks > PNET.

**Discussion**

The immunohistochemical positive and immunoTLC negative results indicated that HNK-1 reactive antigens present in the seven PNETs were probably sulfoglucuronul glycoproteins, not SGGLs. The expression profile of HNK-1 antigens in the human CNS may be very important in cellular migration during early embryo development. In future studies of fetal brain antigens, HNK-1 should be included.
D. ANION EXCHANGE CHROMATOGRAPHY (DEAE-SEPHADEX)

Introduction

Gangliosides can be separated into mono-, di-, tri- and tetra-sialosyl gangliosides based on the number of negative sialic acid charges using anionic exchange chromatography. An anion exchanger is an insoluble material containing chemically bound positively charged groups and mobile counter ions. The principle of this separation is based on reversible adsorption. Mono-, di- and tri-sialogangliosides have different affinities for the ion exchangers due to their different number of negative charges. They can be released from ion exchangers separately by different concentrations of salt.

The reason for separation of gangliosides using DEAE-Sephadex column chromatography is that some of the monosialogangliosides have the same TLC mobility as GD3. With anion exchange chromatography and monoclonal antibodies techniques, we can determine the structure of these gangliosides based on their sialic acid number and antigenicity of the oligosaccharide backbone. Many procedures for isolation of large amounts of gangliosides employ anion exchange chromatography with a continuous gradient. For small amounts of tumor gangliosides, we needed to scale down the column size and set up our own elution parameters. This is summarized below.
Experimental technique

Choice of ion exchanger

There are several anion exchange resins such as DEAE-Sephadex, DEAE-Sepharose, QAE-Sephadex. Sephadex is a modified dextran which is made of (1→6) α-D-glucan. The dextran chains are cross-linked with ethylene oxide to give a three dimensional polysaccharide network and are coated onto silica beads. DEAE [-C₂H₄N⁺(C₂H₅)₂H] is the functional group and is attached to the glucose units of the dextran chains. DEAE-Sephadex A-25 type (Sigma, St. Louis, MO) was chosen by us because it has been widely used by other groups (Ledeen and Yu) and also gives us a better separation of gangliosides than DEAE-Spherodex M (IBF Biotechnics Inc., Savage, MD). In addition, DEAE-Sephadex is available in a dry powder form, and we can inexpensively prepare the acetate form in large amounts.

Choice of buffers / buffer volume and concentration

Ammonium acetate, sodium acetate and potassium acetate in methanol have been commonly used. Here we used ammonium acetate for buffers. The effects of different concentrations and volumes of ammonium acetate on ganglioside elution pattern is shown in Table 7.1.
Table 7.1.

The Effects of Different Concentrations and Volumes of Ammonium Acetate on Ganglioside Elution Pattern.

**Ganglioside elution profile**

(a mixture of 25 µg normal human brain ganglioside sialic acid and 5 µg GD3 standard from Dr. Yu)

<table>
<thead>
<tr>
<th>Eluting buffer</th>
<th>GM2</th>
<th>GM1</th>
<th>GD3</th>
<th>GD1a</th>
<th>GD2</th>
<th>GD1b</th>
<th>GT1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>concentration &amp; volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp#1. 0.02 M, 15 ml</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04 M, 15 ml</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10 M, 15 ml</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>trace</td>
</tr>
<tr>
<td>0.25 M, 15 ml</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp#2. 0.02 M, 30 ml</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10 M, 15 ml</td>
<td></td>
<td>trace</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>trace</td>
</tr>
<tr>
<td>0.25 M, 15 ml</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp#3. 0.04 M, 15 ml</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10 M, 15 ml</td>
<td></td>
<td>trace</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>trace</td>
</tr>
<tr>
<td>0.25 M, 15 ml</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

+: gangliosides mainly eluted in that fraction.

trace: trace amount of gangliosides were found in that fraction.
The data in Table 7.1 suggest that monosialogangliosides elute completely with 5 bed volumes of 0.02 M and 0.04 M ammonium acetate, while disialogangliosides elute only at higher concentrations followed by trisialogangliosides which elute at 0.25 M. In our experiments, we were interested in separation of mono- from polysialogangliosides. Thus, we did not study further the separation of di- and trisialogangliosides at specific concentrations and volumes.

Choice of sample size, and bed volume

The amount of gangliosides which can be applied to a column is dependent on the available capacity of the column. We chose an amount of gangliosides containing 25 µg sialic acid as our sample size and 3 ml of bed volume. Total capacity of DEAE-Sephadex is 3-4 mEq per gram of dry ion exchange. One gram of dry ion exchange is equal to 5-9 ml bed volume. Based on the above information, 3 ml bed volume is more than sufficient for 25 µg sialic acid exchange. We also tested the available capacity of the column (3 ml bed volume) by loading 50 µg ganglioside sialic acid. The void volume had no ganglioside detectable by resorcinol sprayed HPTLC. It indicates that the 3 ml bed volume is adequate for a 25 µg ganglioside sialic acid sample.
Choice of types of elution

There are two ways to elute gangliosides: a) continuous, b) stepwise ionic strength gradients. Stepwise ionic strength gradients are technicely more simple and easily prepared by using buffers of fixed ionic strength. Thus, we chose stepwise ionic strength gradients for elution.

Desalted sample

Before chromatography, the lipid extract must be desalted since the presence of salt interferes with the elution profile. We tested this by loading the sample (2 ml) containing 25 µg ganglioside sialic acid with 0.1 M K⁺ onto the DEAE column using the same eluting scheme. We found that the polysialoganglioside GT1b also eluted in the 0.02 M and 0.04 M ammonia acetate in methanol fractions with other monosialogangliosides. The results indicated that desalted samples are important for DEAE separation.

*These studies were done with Drs. Ariga, Yu, and Miyatani's advice.
E. MODIFIED C-18 REVERSE PHASE COLUMN

Introduction

The disadvantage of DEAE-Sephadex chromatography is that the sample after elution from the DEAE-Sephadex contains a considerable amount of salt. Salts make HPTLC spotting difficult and interfere with the separation of individual gangliosides. There are many ways for removal of salts from samples such as dialysis, gel filtration, and reverse phase chromatography. For large amounts of glycolipid, gangliosides above their critical micelle concentration can be safely dialyzed against water. However, the recovery of gangliosides is generally low (60-70%), especially for small amounts of ganglioside such as that extracted from tumors. In addition, dialysis takes 24 hours or longer. Gel filtration has been used for removal of water-soluble, low molecular weight contaminants from gangliosides. Gangliosides elute in the void volume, while lower molecular weight substances such as monosaccharides and salts are included in the gel. A widely used gel filtration column is Sephadex LH-20 (Byrne et al., 1985). There are two disadvantages to this method. First, the Sephadex LH-20 column requires calibration each time; and radiolabelled gangliosides are used for calibration. Second, the efficiency of separating gangliosides from salts with this column varies from batch to batch of Sephadex LH-20. An alternative procedure to dialysis and gel filtration is reverse phase chromatography. It was originally described by Williams and McCluer (1980) using Sep-Pak cartridges. Later, it has been found that the C-18 made by J. T. Baker gives more reproducible results. The principle of this method is that
nonpolar to moderately polar samples are extracted from polar solutions onto nonpolar sorbents. The sorbents are octadecylsilane (C18) bonded to silica gel packed in small columns. The hydrophobic portions of gangliosides will bind to C18 via hydrophobic-hydrophobic interactions and separate from the water soluble salts. Gangliosides are then eluted with chloroform/methanol. The methods were well developed in our lab and described in detail in Doreen Markowitz's Master Thesis (1984). Reverse phase chromatography has the following advantages. First, the overall recovery of the total gangliosides after C-18 reverse phase column is always high (90%). Second, this method does not require calibration and does not use radioactive materials. Also, the whole procedure takes only four to five hours.

**Experimental technique**

In order to remove large amounts of salts present in the DEAE-Sephadex fractions using C-18 column, we modified the method as following:

1) The samples are directly prepared in methanol without adding KCl.

2) There is no need to condition the column with 0.1M KCl. It is only necessary to condition the column with methanol before the sample is loaded.

3) To completely remove the salts, we wash the column with a large amount of water: 15 and 35 column volumes of water for monosialo- and polysialo-ganglioside fractions, respectively.
CHAPTER VIII

FINAL CONCLUSIONS

The results of this study provide considerable, valuable information about the relationship between gangliosides and gliomas. The conclusions from this study are as follows:

1. The degrees of sialylation and oligosaccharide complexity are inversely correlated with the degree of histological malignancy of brain tumors. These changes are largely due to the loss of total 1b gangliosides (GD1b, GT1b and GQ1b).

2. The amount and proportion of total gangliosides accounted for by gangliosides of the 1b series inversely correlate with the degree of histological grade of astrocytic tumors. This correlation is not due to contamination by brain tissue since GM1 and GD1a (also major gangliosides in normal human brain) did not demonstrate the same correlation. The proportion of GQ1b is significantly different between LG and AA (p<0.001). The proportions of GT1b and GD1b are
significantly different between AA and GBM ($p<0.01, 0.05$, respectively), and between GBM and PNET ($p<0.05$).

3. The proportions of GM3 and GD3 are elevated in all types of primary brain tumors studied. The proportion and absolute amount of GM3 correlate with histological grade of astrocytic tumors, but no such correlation is seen for GD3. However, GD3 is higher in PNET than any of the astrocytic tumors. The ratio of the amount of GM3 to GD3 correlates with histological degree of malignancy.

4. The pattern for the presence of 3'-LM1 and 6'-LM1 across the diagnostic groups of tumors is significant. The concentration of 3'-LM1 (pmol/mg dry weight) ranked in the following order: PNET > GBM > AA and LG. Among the LGs, pilocytic astrocytomas have no or very low amounts of 3'-LM1, while the fibrillary astrocytomas have higher amounts ($p<0.01$). Ganglioside 6'-LM1 was present more frequently in GBM than either AA or LG.

5. Gangliosides 3'-LM1 and 6'-LM1 are important glioma-associated antigens.

6. Another glioma-associated ganglioside, 3'-isoLM1, did not show any correlation with histological grades or survival.
7. With increasing degree of histological malignancy in astrocytic tumors there is a progressive loss of the 1b-series gangliosides and an increase in the neolacto series gangliosides, mainly 3'-LM and 6'-LM1.

8. In oligodendrogliomas, the presence of 6'-LM1 correlates with higher tumor grades. Tumors with a 1b ganglioside content (GD1b, GT1b and GQ1b) over 30% of total gangliosides occurred more frequently in the lower grade than in the higher grade tumors. This pattern follows that seen in our previous studies on astrocytom as.

9. Patients with primary brain tumors in which 1b gangliosides comprise more than 30% of total gangliosides have a better prognosis than those with <30%.

10. The presence of 6'-LM1 in a primary brain tumor is associated with a poor survival across all tumor grades.

11. The combination of 1b gangliosides and 6'-LM1 in a brain tumor provides significant prognostic information.

12. Gangliosides are important markers for classification, diagnosis and prognosis of human brain tumors.
13. GM3 and GM2 are the predominant gangliosides in the human glioma cell line U-1242 MG. This supports our findings in vivo that human gliomas lose the 1b complex gangliosides and express simple gangliosides. This cell line could be very useful as a model system to study the role of gangliosides in the regulation of cell growth in gliomas.

14. Neither fucosylated neolacto- and lacto-tetraosyl gangliosides (SLe¹ and SLe²) nor N-glycolyl containing gangliosides (NeuGc-GM3 and NeuGc-GM2), are expressed in human primary gliomas.

15. Sulfated glucuronyl glycolipids are not found in human primitive neuroectodermal tumors.
CHAPTER X

FUTURE STUDIES

The above findings suggest several directions for future studies. Some of these are:

1) Develop simple and rapid tests to detect 1b gangliosides and 6'-LM1 in specimens of tumors for clinical diagnoses and prognoses. Cholera toxin b-subunit is extremely sensitive and specific compared to resorcinol-spray for identification of 1b gangliosides after neuraminidase treatment. This assay would also allow us to perform the study on very small specimens (less than 0.3 mg fresh weight). In addition, we may be able to do blot immunostaining directly instead separating gangliosides on HPTLC before immunostaining. If 6'-LM1 antibody-coated 96 well plates are commercially available, we could add the ganglioside fraction and do very simple ELISA assay to determine the presence/absence of 6'-LM1 in the tumors for a large series of clinical screening. The determination of cutoff values and quality controls would be a very important part of the experimental design.
2) Create a panel of all promising markers including gangliosides, neutral glycolipids, and other markers from the NCI Glioma Marker Network to improve the classification and prognosis of human gliomas which hopefully would be beneficial for patient therapy.

3) Immunohistochemical data indicate that 6'-LM1 was present in the small tumor cells of human gliomas. A large series of immunohistochemical studies will be required to confirm this result.

4) The importance of glycosyltransferases and glycosidases in the regulation of ganglioside biosynthesis has been recognized. N-acetyl glucosyltransferase and sialyltransferase $\alpha 2\rightarrow 6$ probably would be the key enzymes that control the regulation of aberrant neolacto series ganglioside synthesis in human gliomas.
REFERENCES


Markowitz, DL (1985) Master's Thesis, The Ohio State University, Columbus, Ohio.


