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In vivo growth conditions suppress the expression of ganglioside GM2 and favour that of lacto series gangliosides in the human glioma D-54MG cell line

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The human glioma D-54MG cell line grown *in vitro* primarily expresses ganglio series gangliosides, particularly GM2. Subcutaneous injection of these cells into nude mice produced xenografts with an increased content of the human glioma-associated lacto series gangliosides, primarily 3′-isoLM1, an alteration that was dose dependent, with the highest dose (1×10^8) resulting in a phenotype that was most like that of the inoculum. After one passage *in vivo*, the lacto series dominated and reached a proportional level that was kept throughout the 10 passages. The mRNA levels of the GM2-synthase clearly coincided with GM2 expression and was 20 times higher in cells grown *in vitro* than in those grown *in vivo*. These results support the view that ganglioside expression in human gliomas is strongly influenced by environmental factors.

Keywords: Ganglioside antigens, gliomas, ganglioside metabolism, glioma cell line

Abbreviations: The gangliosides have been designated according to Svennerholm (*Eur J Biochem* (1977) **79**: 11–21) GM3, II^3NeuAc-LacCer; GM2, II^3NeuAc-GgOse$_3$Cer; GM1, II^3NeuAc-GgOse$_4$Cer; GD3, II^3(NeuAc)$_2$-LacCer; GD2, II^3(NeuAc)$_2$-GgOse$_3$Cer; GD1a, IV^3NeuAc, II^3NeuAc-GgOse$_4$Cer; GD1b, II^3(NeuAc)$_2$-GgOse$_4$Cer; GT1b, IV^3NeuAc, II^3(NeuAc)$_2$-GgOse$_4$Cer; 3′-LM1, IV^3NeuAc-nLcOse$_4$Cer; 3′-isoLM1, IV^3NeuAc-LcOse$_4$Cer; 3′,6′-isoLD1, IV^3NeuAc, III^6NeuAc-LcOse$_4$Cer; 3′8′-LM1, IV^3(NeuAc)$_2$-nLcOse$_4$Cer. MAb(s), monoclonal antibody (ies); the designation LM1 is used when both 3′-isoLM1 and 3′-LM1 and LD1, when both 3′6′-isoLD1 and 3′8′-LD1 are included.

Introduction

When serially transplanted as subcutaneous xenografts in nude mice the D-54MG cell line predominantly expresses the lacto series gangliosides 3′-isoLM1 and 3′,6′-isoLD1 [1, 2]. These ganglioside antigens have been shown to be associated with the primary brain tumours, human malignant glioma and medulloblastoma, but not with human meningiomas [2–4]. Analyses of autopsy brains from individuals with malignant gliomas showed that 3′-isoLM1 appeared in highest concentrations in areas surrounding macroscopic tumour tissue and in anatomical structures along which tumour cells may migrate [2, 5]. The expression of 3′-isoLM1 and 3′6′-isoLD1 is not exclusively found in primary brain tumours, but in human normal brain they are restricted to the developmental periods with astroglial proliferation [6]. The fetal expression of lacto series gangliosides has also been demonstrated in primary cultures of rat fetal astrocytes [7]. Recent studies have shown that human glioma cells and fetal rat astrocytes invade normal rat brain in a similar manner [8, 9]. These results support the hypothesis that 3′-isoLM1 and 3′6′-isoLD1 are associated with proliferative astrocytes, either neoplastic or non-neoplastic, and may

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play a role in cell-cell attachment during development and migration. Their restricted appearance in normal adult brain makes them promising as targets for immunotherapy of malignant primary brain tumours.

The large proportions of 3′-isoLM1 and 3′6′-isoLD1 gangliosides in the human glioma D-54MG cell line-induced xenografts make this cell line a potential experimental model for studies concerning these gangliosides as targets for immunotherapy and their biological function in human gliomas. However, when grown *in vitro*, the ganglioside phenotype of D-54MG is dominated by ganglio series gangliosides (e.g. GM2) with virtually undetectable lacto series gangliosides [3, 4, 10]. This study was undertaken to further investigate the influence of environmental factors present in *in vivo* cultures of the glioma cell line D-54 MG on the synthesis of the lacto series gangliosides, in particular the effect of serial transplantation *in vivo*. Cells grown in monolayer culture and serially transplanted as subcutaneous xenografts in nude mice were analysed for ganglioside expression and for *in vitro* activity of the glycosyltransferases involved in ganglioside biosynthesis; for GM2 synthase, mRNA expression was also determined.

Materials and methods

Chemicals

Silica Gel, 60, mesh and high performance thin-layer chromatography (HPTLC) plates and alumina backed HPTLC plates were purchased from Merck AG, (Germany). Sephadex G-25 was from Pharmacia, (Sweden). The ion-exchange resin Spherosil-DEAE-Dextran, prepared as described previously [11], and cholera toxin B-subunit (CT-B) were kindly provided by Institute Merieux, (France), and 5′-bromo-4′-chloro-3′-indolylphosphate was from Sigma (USA). Gangliosides and neutral glycolipids used as standards were isolated and characterized by fast atom bombardment-mass spectrometry (FAB-MS) as previously described [1, 12].

Antibodies

The following monoclonal antibodies(MAbs) were used in the study: DMAb-1, specific for the terminal sugar sequence GalNAcβ1-4(NeuAcα2-3)Gal found in GM2 [13]; C-50 binding to NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAc- found in Fuc-3′-isoLM1 [14]; SL-50 specific for NeuAcα2-3Galβ1-3GlcNAc- found in 3′-isoLM1 [3]; DMAb22 binding to NeuAcα2-3Galβ1-3(NeuAcα2-6)GlcNAc- found in 3′6′-isoLD1 [4]; DMAb20 binding to GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Gal- in GD2 [15]; DMAb8 binding to NeuAcα2-8NeuAcα2-3Galβ1- as in GD3 and 3′8′-LD1 [16]; GD1b:1b binding to Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Gal- as in GD1b [17]; SL-1 binding to NeuAcα2-3Galβ1-4GlcNAc- found in 3′-LM1 and Cto5:2 binding to the CT-B with the same specificity as described by Lindholm *et al.* [18]. Affinity-purified alkaline-phosphatase conjugated goat anti-mouse IgM + IgG (heavy + light chain) used in TLC-ELISA was purchased from Jackson Immunoresearch Laboratories Inc. (USA).

Restriction enzymes and probes

Taq 1 was from BRL, Life Technologies, Inc. (USA). All probes used in the RFLP (restriction fragment length polymorphism) analyses, pYNZ (1p), pJCZ67 (7q), pTBQ7 (10p), pYNZ22 (117p) and pTHH59 (117q) were obtained from the American Type Culture Collection (USA).

As probe for GM2 synthase we amplified a 413 bp fragment (from nt 637 to nt 1110 of the GM2 synthase cDNA sequence, EMBL Accession No.M83651) by reverse transcriptase PCR from U563MG cDNA. PCR primer sequences were 5′-CTGGACCAACTCAACAGG (PC271) and 5′-CTTGGTGGTTACTTGAGACACG (PC272). The PCR product was subsequently cloned into the pCRII^{TM} vector (Invitrogen, USA). The identity of the generated probe was confirmed by sequencing the insert from both ends. Sequencing was performed with a Pharmacia automated DNA sequencer using oligonucleotides complementary to the SP6 and T7 promotors of the pCRII^{TM} vector.

Characteristics and growth conditions of the D-54 MG cells

D-54 MG is the Duke University maintained subline of A172, the permanent glioblastoma multiforme-derived cell line established by Giard *et al.* [19]. The karyotype, morphology, growth characteristics, and antigenic phenotype of this line and derived clones have been extensively described [20, 21]. This cell line was used to induce nude mouse xenografts; details of the establishment and propagation of this xenograft system have been published [22].

The propagation, storage, and testing of the cell line to insure the absence of HeLa cell, inter- or intra-cell line contamination, or *Mycoplasma* infection have been published elsewhere [20, 23, 24]. The cell line was grown in 10% fetal calf serum-Richter's Zinc Option medium (10%) and passed at confluence with 0.125% trypsin or 0.125% trypsin-0.02% EDTA. Cultured cells, passage 58, were expanded directly from an aliquot of cells used to inject mice (see below), harvested from roller culture bottles (1.9×10^8 cells) and used for the isolation and structural characterization of gangliosides. The cell pellet was washed three times in serum-free medium, the supernatant aspirated, and the pellet frozen at −20 °C until extraction. D-54 MG subcutaneous xenografts established from cultured cells, passage 56, were used for xenograft establishment. Three groups of five

recipients were established; each group received either 1×10^8 ($P_{0:a}$), 5×10^7 ($P_{0:b}$) or 1×10^7 ($P_{0:c}$) cells to initiate xenografts. The two higher doses ($P_{0:a}$ and $P_{0:b}$) resulted in rapidly growing but relatively low-weight tumours, which arose within a week after implant. The mean weights of four tumours at harvest from two animals per group were 0.6 g in both the higher dose groups as compared to 1.3 g when 1×10^7 ($P_{0:c}$) cells were injected. A single tumour obtained after injection of 1×10^7 ($P_{0:c}$) was used to initiate ten serial passages *in vivo* (P_{1-10}) [25]. The tumours were immediately frozen by immersion in liquid nitrogen and stored at −20 °C until the extraction was performed.

Extraction and separation of gangliosides

Extraction and separation of gangliosides from cells grown *in vitro* and as xenografts was performed as described previously [26]. In brief, cells and xenografts were homogenized in water, and the lipids were extracted by adding methanol and chloroform to a final ratio of chloroform:methanol:water (4:8:3 by vol). Most of the phospholipids and neutral glycolipids were separated from the gangliosides by silica gel column chromatography. The column was first eluted with eight bed volumes of chloroform:methanol:water (65:25:4 by vol) whereafter the gangliosides were eluted with 10 bed volumes of chloroform:methanol:water (30:60:20 by vol). The ganglioside fraction was dialysed against running tap water for 48 h at +4 °C and then applied to a Spherosil-DEAE-Dextran column. Monosialogangliosides were eluted with 10 bed volumes of 0.02 M potassium acetate and oligosialogangliosides were eluted with 10 bed volumes of the same solution, but in a 0.5 M concentration.

Isolation of individual gangliosides for structural characterization was performed by repeated preparative thin-layer chromatography of the ganglioside fractions using chloroform:methanol:0.25% aqueous potassium chloride (50:40:10 by vol) and/or chloroform:methanol:2.5 M ammonia (50:40:10 by vol) as developing solvents.

Analytical methods

The total ganglioside sialic acid was determined with the resorcinol method and individual ganglioside quantified by densitometric scanning at 620 nm of HPTLC-plates after chromatography in chloroform:methanol:0.25%KCl (50:40:10 by vol) and the gangliosides were visualized using the resorcinol reagent [26].

Sialidase hydrolysis was performed with *Vibrio cholera*-sialidase (EC 3.2.1.18, Behringwerke, Marburg-Lahn, Germany) as previously described [1]. The formed products were characterized by their TLC migration as compared to structurally characterized standards of gangliosides and neutral glycolipids. Quantitative determination of the neutral glycolipids formed was performed by densitometric scanning at 515 nm after visualization with orcinol. Ganglioside products were quantified as described above. The structures of gangliosides were determined by analyses of the partially methylated alditol acetates after permethylation [12] and fast atom bombardment-mass spectrometry, FAB-MS on a VG 7070 mass spectrometer equipped with a fast atom gun [1].

Protein was measured using bicinchoninic acid [27].

TLC-ELISA

The procedure recently described by Fredman *et al.* [26] was used. Ganglioside fractions from the tumour cells and ganglioside standards were applied to a HPTLC-plate and the chromatogram developed in chloroform:methanol:0.25% aqueous potassium chloride (50:40:10 by vol) or chloroform:methanol:2.5 M ammonia (50:40:10 by vol). The plate was then incubated first with the MAb reacting with the ganglioside antigen to be determined, then with alkaline phosphatase conjugated goat anti-mouse antibody and substrate (5-bromo-4-chloro-3-indolylphosphate).

The gangliotetraose series of gangliosides was determined with a modification of the method described above [28]. Briefly, after chromatogram development, the plate was incubated with *Clostridium perfringens* sialidase to hydrolyse all the gangliosides in this series to GM1. Thereafter, the plate was incubated with CT-B, which specifically binds to GM1 ganglioside, then with the murine MAb to CT-B, and finally, with the anti-mouse antibody conjugated to alkaline phosphatase. Binding was visualized by incubation with 5-bromo-4-chloro-3-indolylphosphate as described above.

The detection limit for ganglioside antigens with MAbs using the procedure described above was 3 pmol for 3′,8′-LD1, 2 pmol for 3′-LM1, 3′-isoLM1, Fuc-3′-isoLM1, GM2, GD2 and GD1b and 1 pmol for 3′,6′-isoLD1. The affinity between GM1 and CT-B allowed detection of 0.2 pmol of the gangliotetraose series gangliosides.

Glycosyltransferase activities

A crude membrane-enriched fraction prepared from *in vitro* cultured D-54MG cells as well as those grown *in vivo*, provided the transferases, the activities of which were measured. For each *in vivo* passage 500 mg tissue from five tumours was pooled and mixed with a knife homogenizer and the crude membrane fractions were prepared from 100 mg of this mixture. The fraction was prepared according to modification [29] of the procedure described by Momoi *et al.* [30]. Briefly, the cells were sonicated in ice-cold 0.32 M sucrose, and the disrupted cell suspension immediately centrifuged at $1000 \times \mathbf{g}$ for 10 min. The supernatant was used in the transferase assays, and its protein content determined with the bicinchoninic acid method [27].

The assay conditions (Table 1) are the same as those

Table 1. Assay conditions for determination of glycosyltransferases.

Parameters	*Unit*	*Glycosyltransferase*				
		GM3 Synthase	*GD3 Synthase*	*GM2 Synthase*	*GM1 Synthase*	*LA2 Synthase*
Lipid acceptor		LacCer	GM3	GM3	GM2	LacCer
	mM[a]	0.3	0.9	0.9	0.4	0.3
Nucleotide sugar		CMP-NeuAc	CMP-NeuAc	UDP-GalNAc	UDP-Gal	UDP-GlcNAc
	mM[a]	0.8	0.6	0.4	0.4	1.0
		(8000 dpm nmol^{-1})	(10 000 dpm nmol^{-1})	(10 000 dpm nmol^{-1})	(11 000 dpm nmol^{-1})	(11 000 dpm nmol^{-1})
Enzyme	μg protein	20–60	20–60	20–60	20–60	20–60
Detergent		Triton CF-54 Tween 80 (2:1)	Triton X-100	Octylglucoside	Triton CF-54	Triton X-100
	mg l^{-1} [a]	2.0	1.0	8.0	1.0	1.0
Buffer		HEPES	HEPES	HEPES	HEPES	HEPES
	M[a]	0.2	0.2	0.2	0.2	0.2
pH		6.25	6.25	8.00	7.25	7.25
Mn^{2+}	mM[a]	–	–	6.0	6.0	5.0
CDP-Choline	mM[a]	–	–	5.0	5.0	5.0

[a]Final concentration in the incubation mixture. The assays were performed in a total volume of 50 μl at 37 °C for 60 min.

described in a previous report [29]. The reactions were stopped by adding ice-cold 0.05 M NaAc buffer (pH 4.4). Thereafter the radioactive precursor was removed by chromatography on LiChroprep RP-18. The precursor was eluted with NaAc buffer, water and water/methanol (3:1 by vol) and the glycolipids with methanol, chloroform: methanol (2:1 by vol). The radioactivity in the glycolipid fraction was counted with a scintillation analyser. All assays were performed in triplicate with and without glycolipid acceptor.

RFLP-analyses

Southern blots of xenograft (*in vivo* passage no. 10) and cell line DNA were digested with *Taq*I and probed with five variable number tandem repeat probes [31]. The probes were pYNZ2 (1p), pJCZ67 (7q), pTBQ7 (10p), pYNZ22 (117p), and THH59 (117q). This set of probes was selected because we have found that, in more than 50 cell lines, the collective pattern of bands obtained by hybridization of these probes was unique to each DNA sample.

Northern blot analyses

Total RNA was extracted by homogenization of cells in 4 M guanidiniumisothiocyanate followed by ultracentrifugation over a cesium chloride cushion as decribed [32]. For Northern blotting 20 μg of total RNA were electrophoresed over a denaturing 1% agarose gel and blotted to Hybond-N^+ membranes (Amersham). The membranes were hybridized with the β1,4*N*-acetylgalactosaminyltransferase (GM2 synthase) [33] probe labelled with [^{32}P]dCTP by random priming. Hybridized membranes were exposed to phosphor storage screens (Molecular Dynamics, USA) which were then analysed using a Molecular Dynamics PhosphorImager. Densitometric analysis of mRNA expression was performed with a PhosphorImager using ImageQuant. Control probings of Northern blots for β-actin were done in order to assess variations in RNA loading. As a probe for β-actin we used the plasmid HHCPD 08, which was obtained from American Type Culture Collection (USA).

Results

RFLP-analyses

RFLP analyses of cells grown *in vitro* and after 10 passages *in vivo* are shown in Fig. 1. With all five probes the bands in the xenograft lanes completely matched those in the cell line lanes, indicating a high probability of these two DNA samples being derived from a common source.

Ganglioside analyses

The concentration of gangliosides (Table 2) was highest in the *in vitro* grown cells, and decreased during the first two to three passages *in vivo* to a plateau around 150 nmol g^{-1}. The first generation of solid tumours (P_0) all had similar total ganglioside content. Monosialylated gangliosides dominated in cells grown *in vitro* as well as *in vivo*. The tumours resulting from injection with the highest dose of D-54MG cells ($P_{0:a}$) showed only a slight alteration in the ganglioside pattern (Table 2), while the first generation tumour obtained with lower numbers of injected cells ($P_{0:b}$ and $P_{0:c}$) showed a marked increase of lacto series gangliosides, the largest increase with the lowest injected dose. After one passage *in vivo* (P_1), the lacto series

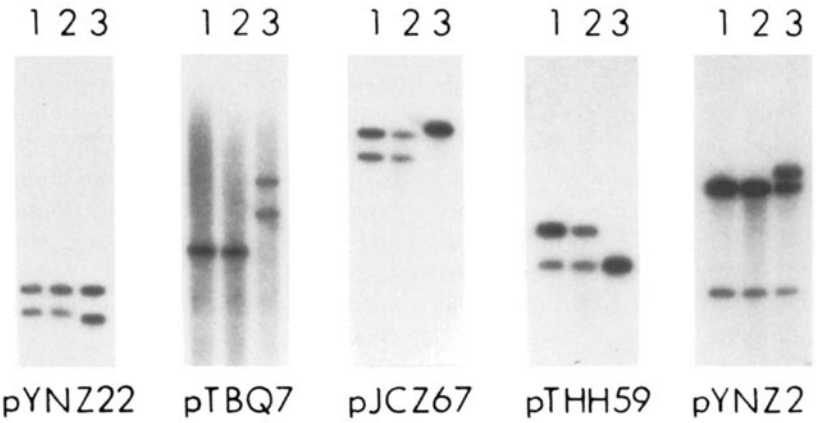

Figure 1. Genomic DNA from D-54MG cultured cells, passage 58 (lane 1); from D-54MG murine xenograft cells, passage 9 (lane 2); and from R-21 unrelated control cells (lane 3) was digested with *Taq* I, electrophoresed, Southern blotted, and hybridized to the indicated probes. Auto-radiographs of washed filters are shown. Each probe hybridized to similar sized bands in cultured and xenograft DNA.

Table 2. Ganglioside composition in D-54 MG cells cultured *in vitro* or *in vivo* as subcutaneous xenografts in nude mice. The mice were injected with a 1×10^8 ($P_{0:a}$), 5×10^7 ($P_{0:b}$) or 1×10^7 ($P_{0:c}$) cells as described in Materials and methods. The initial tumours (passage 0 *in vivo*) obtained by injection with 1×10^7 cells, were serially transplanted *in vivo*, passages 1–10. Each experiment included five tumours and 100 mg wet weight of each tumour were pooled for analyses. The cells grown *in vitro* were expanded from an aliquot of cells, which was used to inject the mice and were harvested at passage 58 *in vitro*. Ganglioside determination and structural chararcterization was performed as described in Materials and methods.

Cell culture	*Passage no.*	*Total (nmol sialic acid per g)*	*Gangliosides*						
			Lacto series		*Ganglio series*				
			3′-isoLM1	*3′6′-isoLD1*	*GM3*	*GM2*	*GM1*	*GD1a*	*GD2*
			(% of total ganglioside sialic acid)						
In vitro	58	307	3	ND	24	45	6	14	6
In vivo	$P_{0:a}$	202	5	2	28	39	9	9	7
	$P_{0:b}$	232	13	5	34	28	6	5	6
	$P_{0:c}$	217	24	3	34	20	4	7	6
	P_1	187	58	8	12	11	2	2	6
	P_2	173	47	5	23	11	3	1	8
	P_3	104	40	3	27	14	ND	3	5
	P_4	147	45	4	24	15	3	1	5
	P_5	148	52	4	23	11	1	1	5
	P_{10}	148	45	3	20	14	1	1	3

ND, not determined.

reached a proportional level that was kept throughout the 10 passages.

The major ganglio series ganglioside in *in vitro* cultured cells, both in quantity and proportion (Table 2), was GM2 followed by GD1a. Other gangliosides of the ganglio series identified were GM1 and GD2. Ganglioside 3′-isoLM1 of the lacto series comprised only 3% of the total ganglioside sialic acid. The lacto series gangliosides in the D-54 MG cells increased markedly in content upon transfer to *in vivo* cultures (Table 2), mainly due to an increase of the monosialylated 3′-isoLM1. The neolacto series 3′-LM1 was detected in all solid tumours as well as in the *in vitro* cultured cells, but constituted <3% of the ganglioside sialic acid. The disialylated forms of these gangliosides, 3′6′-isoLD1 and 3′8′-LD1, have a very close migration rate on TLC, regardless of which organic solvent is used. As immunodetection of 3′6′-isoLD1 with the DMAb22 antibody is inhibited by the presence of 3′8′-LD1, no accurate value for the relative proportions of these gangliosides could be obtained. However, the data indicated that the predominant fraction was 3′6′-isoLD1,

and the quantitative value was based on the amount of the neutral glycolipid LA1 formed after sialidase hydrolyses. Ganglioside 3′6′-isoLD1 was not even detected in cells grown *in vitro*, but *in vivo*, it constituted 3–8% of the total ganglioside sialic acid without any correlation to passage number. Ganglioside GM3, the first ganglioside product in the biosynthesis of the ganglio series (Fig. 2) showed no significant proportional alteration. The disialylated form of GD3 was only detected in two preparations, where they constituted 1% of the total ganglioside sialic acid.

Glycosyltransferase activities

The glycosyltransferase activities measured by radiolabelling in the glycolipid fraction are presented in Fig. 3 (A–D). The GM2- and GM1-synthase activities were approximately one magnitude higher than those of GM3-synthase and, in particular, of LA2-synthase. A very low activity consequently resulted in a low number of cpm, and below 100 cpm the built-in uncertainty in the method compromises quantification. Therefore, values below 0.1 nmol per mg protein h^{-1} are unreliable. In the case of GD3-synthase, the activity in all enzyme preparations was too low to be evaluated and thus no data are presented.

Except for GM2-synthase, enzyme activity was detectable without addition of glycolipid acceptor (Fig. 3A–D). An extreme example was found in the GM3-synthase assay of cells grown *in vivo*, where no additional product formation was obtained by adding the glycolipid substrate LacCer. In monolayer cultured cells the expected increase in measured activity by addition of exogenous substrate was found. The glycolipid products formed in the GM2- and LA2- synthase assays (Fig. 3 A,C) migrated as authentic GM2 and LA2, respectively; also, the GM2-synthase product was identified by the GM2 MAb, DMAb-1.

GM2- and GM1-synthase activities were lower in the cells grown *in vitro* than those grown *in vivo*, but no correlation to passage number or number of injected cells was observed. GM3-synthase levels were as high in the cells grown *in vitro* as those grown *in vivo*, and there was a large variation between passages. It was noted that among the initial tumours (P_0) those obtained with the lower doses ($P_{0:a}$ and $_{0:b}$) of cells had the lowest GM3-synthase activity. The activity of the LA2-synthase was low, and in the cells grown *in vitro* the signal was below the background, but showed a tendency to correlate with the proportion of the lacto series gangliosides in the glioma cells. Two solid tumours of D-54 MG cells (P_5) were used to prepare both a Golgi-enriched fraction [29]

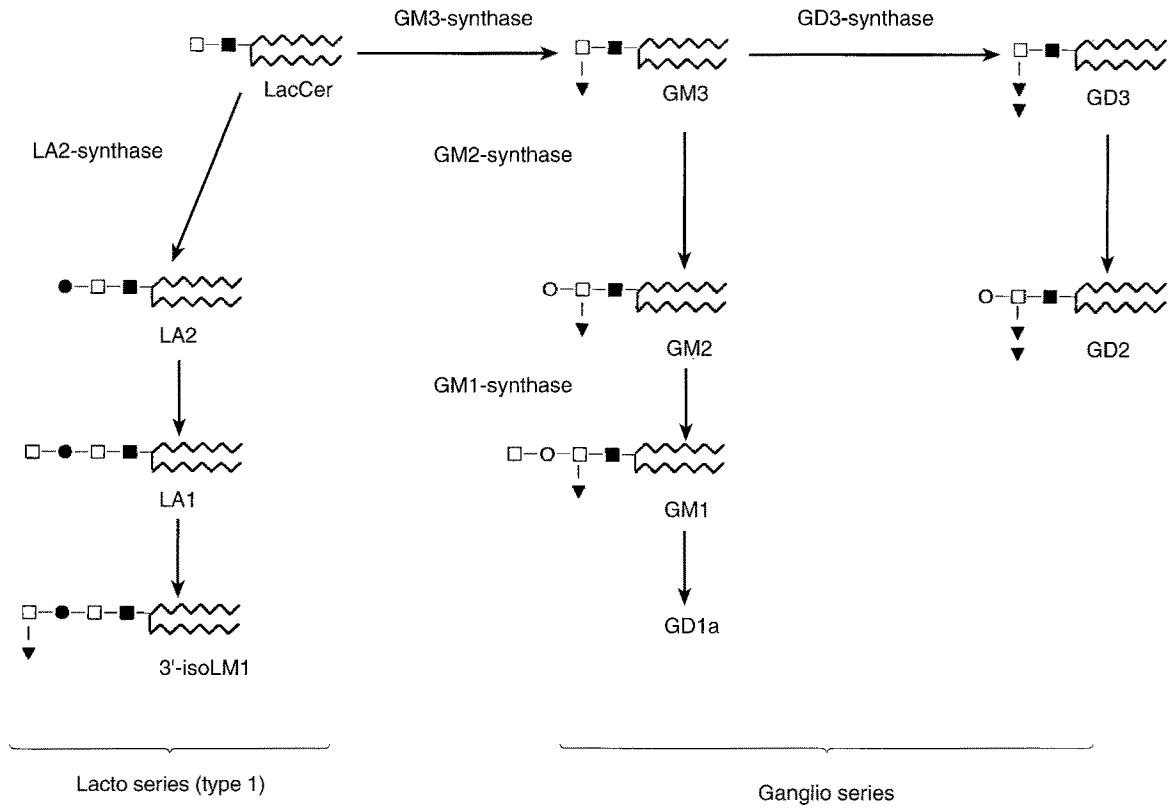

Figure 2. Schematic drawing of the biosynthetic pathway of lacto and ganglio series gangliosides. Symbols are as follows: ■, glucose; □, galactose; ○, galactosamine; ●, glucosamine and ▽, sialic acid.

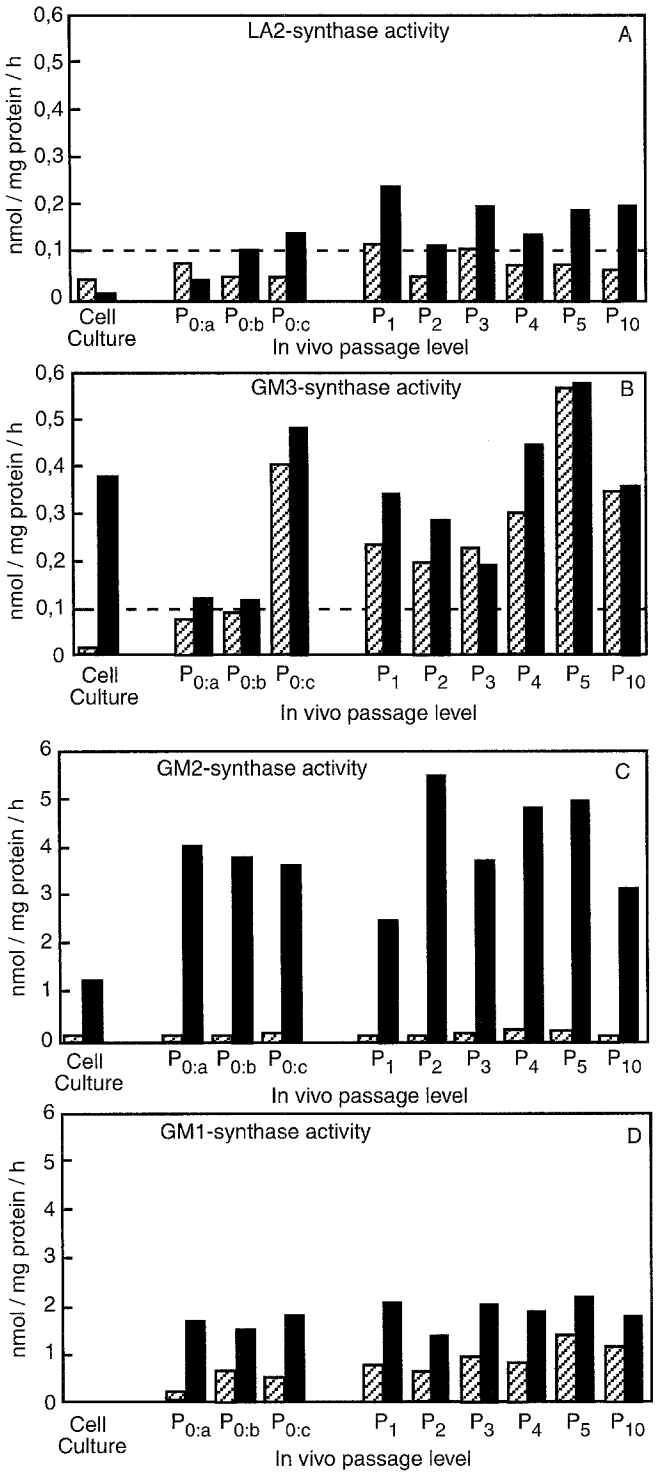

Figure 3. Glycosyltransferase activities in D-54MG cells grown *in vitro* or *in vivo* as xenografts serially transplanted in nude mice. The preparation of a membrane enriched fraction that was used as enzyme source and the assay of the glycosyltransferase activities are described in Materials and methods. The chequered bars represent the activities without exogenous lipid acceptor and filled bars those with exogenous acceptor. The CV for the assay was <10% for a specific activity >0.5 nmol per mg protein h^{-1} and <15% when the specific activity was <0.5 nmol per mg protein h^{-1}.

and a crude membrane-enriched fraction as used throughout this study. There was no significant difference between the glycosyltransferase activities measured as described in those two preparations.

Northern blot analyses

Northern blot analysis of D-54MG grown *in vitro* versus D-54MG grown as xenografts in nude mice revealed a dramatic difference in the abundance of GM2 synthase mRNA (Fig. 4). While D-54MG cells grown *in vitro* showed strong signals for the 5.2 kb and the 3.0 kb transcripts of GM2 synthase, the same cells grown *in vivo* revealed a dramatic decrease in the expression of GM2 synthase. Quantitative densitometric analysis of the transcript signal intensities demonstrated approximately 20-fold higher expression of the 5.2 kb transcript and approximately five-fold higher expression of the 3.0 kb transcript *in vitro*.

Discussion

The aim of this study was to investigate the expression of the glioma-associated lacto series gangliosides 3′-isoLM1

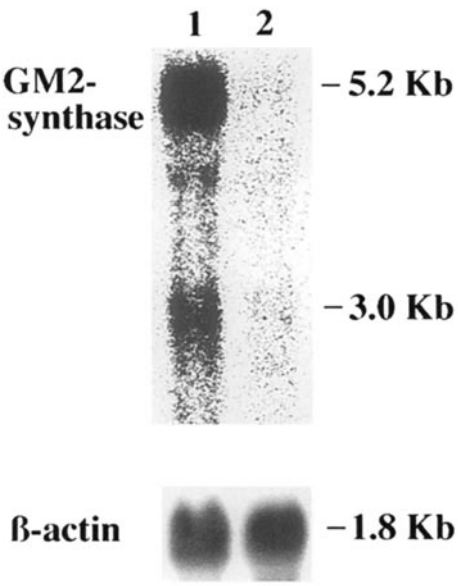

Figure 4. Northern blot analysis of GM2 synthase (β1,4*N*-acetylgalactosaminyltransferase) [33] mRNA abundance in D-54MG cells grown *in vitro* [1] or as nude mice xenografts [2]. Probing of the blot for GM2 synthase revealed a strong expression of the 5.2 kb and the 3.0 kb transcripts in cells grown *in vitro*. In contrast the signal intensity was much lower under *in vivo* growth conditions. Comparative densitometric analysis revealed about a 20-fold higher expression of the 5.2 kb transcript and about a five-fold higher expression of the 3.0 kb transcript *in vitro*. The same blot was reprobed for β-actin as control for RNA loading. The respective transcript sizes in kilo-bases (kb) are indicated on the right side.

and 3′6′-isoLD1 [3–5, 34] in the human glioma cell line D-54MG upon xenograft establishment and, for the first time, during serial transplantation *in vivo*. The transfer of the cells to an *in vivo* milieu immediately led to an alteration in ganglioside phenotype; the most apparent alteration was an increase in the lacto and a decrease in the ganglio series gangliosides, dominated by 3-isoLM1 and GM2, respectively. This alteration was dependent on the dose of injected cells in P_0, and in the tumours obtained by injecting the highest dose of cells (1×10^8), the alteration was only marginal. This could not be explained by the lower tumour mass and/or more rapid growth rate, as these two parameters were similar in the solid tumours obtained after injection of 5×10^7 cells. One possible explanation might be that the tumours derived from the larger inoculum contained a proportionately larger percentage of cultured cells (i.e. GM2-rich cells) as opposed to cells which had divided *in vivo*, presumably enriched in the lacto series. One can also speculate that environmental factors would initially have a lesser effect on the entire population of injected tumour cells than on growing and dividing populations in subsequent passages.

The proportion as well as the total amount of the lacto series gangliosides increased at the first passage *in vivo* (from P_0 to P_1), and remained relatively constant throughout passages 1–10. The ganglioside values found in these tumours grown *in vivo* are in agreement with our previous report on gangliosides in D-54MG cells that were serially transplanted up to 20 times. Altered expression of gangliosides in tumour cell lines transferred to growth *in vivo* has been noted in the human glioma cell line U-118 MG [26] and in the medulloblastoma DAOY cell line [34]. As in this study on the D-54MG cell line, a common finding was a reduction in ganglioside GM2. These cell lines, grown *in vitro* as well as *in vivo*, express only minor amounts, if any, of the glioma-associated gangliosides 3′-isoLM1 and 3′6′-isoLD1. They express instead the neolacto series 3′-LM1 and 3′8′-LD1, which also are increased upon *in vivo* growth, but these gangliosides are not associated with human gliomas. A reduction of GM2 has also been noted in melanoma cells [35].

Altered ganglioside expression is expected to be correlated with changes in the relative activity of the involved glycosyltransferases. Sandhoff and co-workers [36] have shown that glycosyltransferase activity in non-dividing cells, primary nerve cells, correlate with ganglioside composition. However in the D54MG glioma cells GM2-synthase activity was lower in the cells grown *in vitro* than in those grown *in vivo* despite the fact that GM2 was the major ganglioside in the *in vitro* cultured cells. GM1-synthase was also lower in cells grown *in vitro*, although this ganglioside was expressed as frequently in the solid tumours; and GD1a, its disialylated form, was even more abundant *in vitro*. GM3 is the first product in the ganglio series (Fig. 4) and the decrease of ganglio series gangliosides in xenografts could be expected to be reflected in a lower GM3 synthase activity, but no such correlation was seen. There was one marked difference, however, between cells grown *in vitro* and those grown *in vivo*; the former did show an expected increase in GM3-synthase activity with the addition of the exogenous substrate, LacCer, while the latter did not. This may reflect differences in location and/or accessibility of endogenous and exogenous LacCer. The absence of measurable activity of GD3-synthase was in accordance with the low content of GD3, and in low level of GD2, both belonging to the b-series (Fig. 3). The activity of the LA2-synthase appeared to correspond to the relative amount of lacto series gangliosides, although the very low activities detected do not allow a firm conclusion. Thus, there was no consequent correlation between the cells content of an individual ganglioside and the *in vitro* activity of the glycosyltranferase involved in the synthesis. Similar results were obtained in our previous study of glycosyltransferase activity and gangliosides in medulloblastoma cell lines [29] There are reports [36–38] that show that the LA2-synthase activity, in the same order of magnitude as in this study, in non-neuroectodermal tumour cell lines correlates with ganglioside expression.

However, measurement of β1,4*N*-acetylgalactosaminyltransferase expression by Northern blotting revealed significantly higher levels in D-54MG cells grown *in vitro* than *in vivo*. The mRNA levels of this transferase, cloned by Nagata *et al.* [33], thus correlated with the abundance of GM2 in contrast to the *in vitro* measured activity of the enzyme. This result indicates that higher expression of GM2 in the cells grown *in vitro* is dependent upon regulatory mechanisms at the gene level. A possible explanation for the lack of correlation between transferase activity and ganglioside expression observed here is that factors influencing the transferase activities and/or intracellular transport of formed products [36, 39] *in vivo* are potentiated or inhibited in the *in vitro* assays. We conclude that measurements of glycosyltransferase activities by using optimized *in vitro* assays, which also contain detergents to solubilize the enzymes, do not reflect the activity *in situ*.

It was not within the scope of this study to rule out a clonal selection of cells upon transfer. However, RFLP analyses showed no difference between cells grown *in vitro* and those grown *in vivo*. Moreover, if clonal selection occurred, this study has shown that D-54 MG cells with a high expression of the glioma-associated lacto series gangliosides are favoured in *in vivo*, both in xenograft and in *in situ* tumours [4], a finding that also speaks against spontaneous mutation. Most likely, the environment is involved in such a selection. The possible

influence of matrices, serum factors and cell density have been reported [40–42].

This study has shown that ganglioside composition in the human glioma cell line D-54MG was altered by transfer from *in vitro* to *in vivo* cultures favouring the glioma-associated lacto series gangliosides that are glioma-associated. It was also shown, for the first time that, in the case of this cell line, at least one passage *in vivo* was necessary to obtain a stable ganglioside pattern. *In vivo* modulation of ganglioside expression in human glioma cells, either by clonal selection or altered ganglioside metabolism, may also influence the growth and invasive properties of these cells in the brain.

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