Neuro-Oncology 13(9):950–960, 2011.
doi:10.1093/neuonc/nor108
Advance Access publication July 31, 2011

Targeting the GD3 acetylation pathway selectively induces apoptosis in glioblastoma

Suzanne M. Birks, John Owusu Danquah, Linda King, Reinhardt Vlasak, Dariusz C. Gorecki, and Geoffrey J. Pilkington

Cellular and Molecular Neuro-oncology Research Group, Institute Biomedical and Biomolecular Sciences, University of Portsmouth, Portsmouth, UK (S.M.B., G.J.P.); Insect Virus Research Group, School of Life Sciences, Oxford Brookes University, Oxford, UK (J.O.D., L.K.); Department of Molecular Biology, University Salzburg, Salzburg, Austria (R.V.); Molecular Medicine Research Group, Institute Biomedical and Biomolecular Sciences, University of Portsmouth, Portsmouth, UK (D.C.G.)

The expression of ganglioside GD3, which plays crucial roles in normal brain development, decreases in adults but is upregulated in neoplastic cells, where it regulates tumor invasion and survival. Normally a buildup of GD3 induces apoptosis, but this does not occur in gliomas due to formation of 9-O-acetyl GD3 by the addition of an acetyl group to the terminal sialic acid of GD3; this renders GD3 unable to induce apoptosis. Using human biopsy-derived glioblastoma cell cultures, we have carried out a series of molecular manipulations targeting GD3 acetylation pathways. Using immunocytochemistry, flow cytometry, western blotting, and transwell assays, we have shown the existence of a critical ratio between GD3 and 9-O-acetyl GD3, which promotes tumor survival. Thus, we have demonstrated for the first time in primary glioblastoma that cleaving the acetyl group restores GD3, resulting in a reduction in tumor cell viability while normal astrocytes remain unaffected. Additionally, we have shown that glioblastoma viability is reduced due to the induction of mitochondrially mediated apoptosis and that this occurs after mitochondrial membrane depolarization. Three methods of cleaving the acetyl group using hemagglutinin esterase were investigated, and we have shown that the baculovirus vector transduces glioma cells as well as normal astroctyes with a relatively high efficacy. A recombinant baculovirus containing hemagglutinin esterase could be developed for the clinic as an adjuvant therapy for glioma.

Received February 11, 2011; accepted June 8, 2011.

Corresponding Author: Suzanne M. Birks, Department of Cellular and Moleculare Neuro-oncology, St Michaels Building, White Swan Road, University of Portsmouth, Portsmouth, Hampshire, PO1 2DT, UK (suzanne.birks@port.ac.uk).

Keywords: 9-O-acetyl GD3, baculovirus, brain tumor, hemagglutinin esterase.

Central nervous system (CNS) tumors account for approximately 2% of all cancers diagnosed in the United Kingdom but represent a disproportionately high mortality rate; in fact, brain tumors are the leading cause of neoplasm-related death in patients younger than age 35 years. Gliomas are the most frequent CNS primary tumor, and glioblastomas are the most common and of the highest malignancy grade—grade IV—as classified by the World Health Organization. Glioblastomas are highly refractory to current therapeutic strategies due to their characteristic local diffuse infiltration of normal tissue and heterogeneous nature—for example, they show differences in epigenetic regulation and differentiation status.[1] GD3 is a ganglioside—a member of a family of glycosphingolipids containing sialic acid—that consists of a ceramide backbone, 2 sugar residues, and 2 sialic acid residues. It is found ubiquitously as a constituent of the plasma membrane in all mammalian cells, where it is clustered in the lipid rafts, with its hydrophobic ceramide portion anchored in the membrane and the hydrophilic tail protruding into the extracellular environment.[2,3] GD3 has been shown to play crucial regulatory roles in both normal physiological processes (such as embryogenesis) and pathological conditions (such as tumor onset and progression).[4] In particular, GD3 is a major ganglioside constituent of the embryonic brain, where it has been reported to mediate migration as well as induce apoptosis in the supernumerary progenitor cells.[5] Following maturation of the CNS, GD3 expression diminishes considerably; in the adult brain, GD3 is considered a minor ganglioside constituent and is replaced by more complex gangliosides.[6] There is accumulating evidence suggesting that GD3 is being

upregulated as a result of initial oncogenic transformation and that it may play key roles in controlling the induction of invasion, angiogenesis, apoptosis, and immunosuppression.[3,7–11] The buildup of GD3 has been shown in recent years to induce apoptosis in a variety of cell lines as well as in isolated mitochondria, and several mechanisms have been proposed for this, including direct interaction with the mitochondrial permeability transition pore (MPTP), enhanced production of reactive oxygen species, suppression of the NFκ-B survival pathway, and promotion of CD95/Fas-mediated apoptosis.[12–17] However, while high levels of GD3 are reported in glioblastomas[18] and have also been shown in our laboratories (unpublished data), these cells do not display any signs of apoptosis. This phenomenon has been linked to acetylation, a postsynthetic modification that results in the addition of an acetyl group to the 9-carbon position of the terminal sialic acid to form 9-O-acetyl GD3 (GD3^A). Unlike GD3, GD3^A is found mainly intracellularly and appears to inhibit the pro-apoptotic potential of GD3,[19–23] thus promoting tumor cell survival while still allowing cell surface-associated GD3 to assist cell migration.

It has been shown in other cell lines (eg, HEK-293, Jurkat, U87) that cleaving the acetyl group from GD3^A appears to restore pro-apoptotic GD3[20,22,23] and that this may represent a potential therapeutic strategy. Hemagglutinin esterase (HE) from influenza C virus plays a significant role in cellular attachment and infection by the virus but has also been shown to bind specifically to 9-O-acetylated sialic acids and to induce efficient degradation of GD3^A to GD3.[24–27] In this study we used HE to cleave the acetyl group from GD3^A in biopsy-derived glioblastoma cell lines to selectively induce apoptosis. As part of this study, we investigated whether the baculovirus Fas-mediated *Autographa californica* nucleopolyhedrosisvirus (AcNPV) may be a suitable expression vector system for delivering *HE* to glioblastoma cells. The baculovirus is an insect-specific virus that is nonpathogenic in mammalian cells and is rapidly gaining credibility as a gene therapy vector due to its large cloning capacity, good biosafety record, and ability to efficiently transduce a large number of mammalian cells as a non integrating vector.[28–31] One of the major incentives for developing a recombinant baculovirus for use as a brain tumor cell gene delivery system is its ability to transduce quiescent cells (eg, the cancer stem cell population, the invading population) as well as mitotic cells. Moreover, unlike most mammalian viruses, baculovirus does not trigger immune/inflammatory responses against infected cells—a great advantage in our approach where treatment safety and specificity result from the expected lack of effect in normal cells, even if targeted by the vector.[31]

We show here that the acetylation status of GD3 critically affects glioblastoma cell survival and that cleaving the acetyl group from GD3^A results in tumor cell death via mitochondrially mediated apoptosis.

Materials and Methods

Cell Culture

Three glioblastoma cell lines were used throughout: SNB-19 (passages 38–42 since receipt in laboratory) (Deutsche Sammlung von Mikroorganismen und Zellkulturen brain bank); University of Portsmouth (UP) AB (passages 12–16), an in-house–derived adult biopsy cell line; and IN699 (passages 15–18), a pediatric biopsy-derived cell line (Institute of Neurology). Additionally, CC-2565 (passages 4–6), a normal human cerebral astrocyte line (Lonza Wokingham), and SC-9 amplified using specific primers (Invitrogen), which incorporated flanking restriction endonuclease sites (Table 1) and Phusion proofreading polymerase (New England Biosciences) before resulting amplicons were cloned, under the control of the cytomegalovirus (CMV) promoter, into either pcDNA3.1 (Invitrogen) to form pcDNA3.1-HE-CMV or a modified form of pBacPAK8 (Clontech), in which the polyhedron promoter had been replaced with the CMV promoter, to produce pBacPAK-HE-CMV. Insect *Sf9* cells were transfected with pBacPAK-HE-CMV and *flash*BACGOLD virus DNA (Oxford Expression Technologies) to produce the recombinant baculovirus AcHE-CMV. The virus was then amplified to high titer ($1–2 \times 10^8$ plaque-forming units (PFU)/mL) in *Sf9* cells, titrated by plaque assay and characterized. HE was expressed with the C-terminal transmembrane anchor.

Treatment of Cells with HE

Cells were treated with HE in 3 different regimens: (1) exogenous administration of 10-mU recombinant HE to the cell culture monolayer for 60 min before assays; (2) monolayers transfected with 1.6 μg/100-μL pcDNA3.1-HE-CMV using Lipofectamin 2000 (Invitrogen) and HE expressed for 24 h; (3) cells transduced with AcHE-CMV by adding virus to monolayers at a multiplicity of infectionof 50 PFU/cell and incubating for 1 h before removing and replenishing with SFM and incubating for a further 16 h. Cells were plated at a density of $\sim 1 \times 10^5$ cells/well.

Flow Cytometry

The expression of intracellular GD3 and GD3^A was quantitatively determined by flow cytometry. Briefly, cells were harvested (and for intracellular analysis of

Table 1. Specific primer sequences incorporating flanking restriction endonuclease sites for amplification of HE from pCHE4

5′ for pcDNA3.1-	GGTCTCGAGATGTTTTTCTCATTACTC
3′ for pcDNA3.1-	GATATCTTTAGTTCTGCAGATGG
5′ for pBacPAK8	TCTAGAATGTTTTTCTCATTACTCTTG
3′ for pBacPAK8	GAGCTCTTTAGTTCTGCAGATGG

GD3 and GD3^A cells were permeabilized using Cytofix Cytoperm [BD Biosciences]) before incubating with primary antibody (mouse anti-human GD3 clone MB3.6 [Chemicon, Millipore] or mouse anti-human 9-O-acetyl GD3 clone D1.1 [Zymed, Invitrogen]), followed by incubation with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Expression data were collected using a 4-color multiparameter FACS [fluorescence-activated cell sorting] Calibur flow cytometer (BD Biosciences), and acquisition and analysis of data were carried out using CellQuest Pro Software. Using this technique enabled the analysis of both the mean percentage of the cell population expressing either GD3 or GD3^A (% gated expression) and the mean amount of antigen expressed by the positive cells (increase in fluorescence fold). Thus we were able to track the increase or decrease in GD3^A and GD3, respectively, after treatments. Monitoring differences in fluorescence fold was crucial for this study, since even subtle changes in the critical ratio between the 2 ganglioside forms may be sufficient to induce apoptosis.

Cell Viability Assay

Cell viability was assessed using the CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega). The solution was added posttreatment, absorbance (at 490 nm) was recorded using a Labtech Polar Star Optima plate reader, and viability was calculated from the ratio between the sample and control readings.

Annexin V/Propidium Iodide

Apoptosis was detected using annexin V/propidium iodide (PI) staining. Briefly, cells were harvested and incubated with annexin V antibody conjugated to an Alexa Fluor 488 (Invitrogen) and PI (BD Biosciences). Analysis was carried out by flow cytometry, as previously described in the section 'Flow Cytometry' above. To provide evidence of a direct link between GD3 amounts and the induction of apoptosis, double staining was also carried out using the annexin V and GD3 antibodies.

Depolarization of the Mitochondrial Membrane

The JC-1 probe (Sigma-Aldrich) was used to determine the degree of membrane depolarization in response to HE treatment. The dye was added after treatment to cell monolayers, where it could enter the mitochondrial matrix in cells with intact mitochondrial membranes and exist as red aggregates (emitting at λ590 nm). However, in cells with depolarized membranes, the dye is excluded and remains in the cytoplasm as a green monomer (emitting at λ530 nm). Fluorescence at each wavelength was recorded using a plate reader as previously described, and the ratio was used to calculate the extent of depolarization.

Western Blotting

Cells were lysed with radioimmunoprecipitation assay buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Pierce Biotechnology) and scraped to obtain lysates; 20 μg of protein was loaded and resolved in 15% (for cytochrome [cyt] c detection) or 10% (for HE detection) sodium dodecyl sulfate gels and then transferred to nitrocellulose membranes before probing with primary antibody (1:1000 mouse anti-human cyt c clone 7H8.2C12 [Zymed, Invitrogen] or mouse anti-HE antibody 8B3A5 [Applied Biotech]), followed by goat anti-mouse horseradish peroxidase for the secondary antibody (Dako). Signals were detected with enhanced chemiluminescence reagent (GE Healthcare), and images were acquired and analyzed using the Syngene imaging system and GeneSnap Image Acquisition software.

Triacetin Assay

The lysates obtained for western blotting were also used in the triacetin assay to confirm presence of the active enzyme. The triacetin assay uses HE to degrade triacetin into glycerol and acetic acid—1 unit of enzyme is defined as the amount that will produce 1 μM of acetic acid from triacetin per min at pH 6.5 at 30°C. Briefly, cell lysate was added to a 5% triacetin solution (Sigma-Aldrich), and a 5-mM NaOH solution (Sigma-Aldrich) was gradually added until it was no longer possible to maintain pH at 6.5, and the units of enzyme were calculated from the volumes used and the time taken.

Modified Transwell Boyden Chamber Assay

The invasive potential of cells in vitro was assessed using a modification of the method described by Albini.[32] Briefly, treated cells were harvested and seeded onto transwell units with an 8-μm pore polycarbonate filter (Corning) coated with 0.5 mg/mL growth factor reduced matrigel (BD Biosciences). Added to the lower chamber as a chemoattractant was 10 ng/mL platelet-derived growth factor-AB (Sigma-Aldrich). After 8 h incubation, the upper side of the filter was wiped and the bottom side was fixed with methanol and stained with Giemsa (Sigma-Aldrich) and mounted onto slides; 9 fields per slide were counted.

Statistics

Statistical significance was assessed by 2-way analysis of variance (ANOVA) and the Tukey–Kramer post-hoc test ($P \leq .05$). Unless otherwise specified, data presented here are representative of 3 independent experiments carried out in triplicate ($n = 3$), and values shown are the arithmetic means ± standard errors of the means (SEMs).

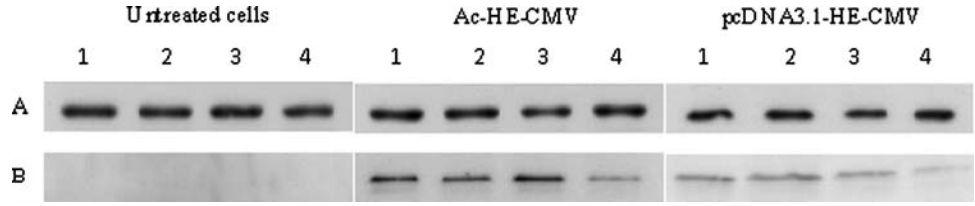

Fig. 1. Western blots after transduction with AcHE-CMV or transfection with pcDNA3.1-HE-CMV. (1) SNB-19, (2) IN699, (3) UPAB and (4) CC-2565 cells. Expression of 44-kDa actin as protein loading control (A) and 120-kDa HE (B) are shown. Results are representative of 3 independent experiments ($n = 3$).

Table 2. Units/mL HE expressed in SNB-19, IN699, UPAB, and CC-2565 cells after transduction with AcHE-CMV or transfection with pcDNA3.1-HE-CMV as determined by the hydrolysis of triacetin

		Units/mL Enzyme
	Blank	0
	Control	860
UPAB	Mock	0
	Transfected with pcDNA3.1-HE-CMV	60
	Transduced with AcHE-CMV	400
SNB-19	Mock	0
	Transfected with pcDNA3.1-HE-CMV	120
	Transduced with AcHE-CMV	400
IN699	Mock	0
	Transfected with pcDNA3.1-HE-CMV	120
	Transduced with AcHE-CMV	360
CC-2565	Mock	0
	Transfected with pcDNA3.1-HE-CMV	20
	Transduced with AcHE-CMV	80

Note: Control is recombinant HE produced in and purified from *Sf9* insect cells.

Results

Cell Targeting and Expression of HE

We have conducted initial studies into the deacetylation of GD3^A using recombinant HE enzyme produced in *Sf9* cells (data not shown). This enzyme is reported to target only 9-O-acetylated sialic acids associated with the cell surface, thus we created pcDNA3.1-HE-CMV to obtain a proof of principle that intracellular delivery of HE would result in a greater effect on the GD3:GD3^A ratio and the induction of apoptosis. HE expression after transfection with pcDNA3.1-HE-CMV or transduction with AcHE-CMV in the 3 glioblastoma cell lines and in CC-2565 was analyzed by western blotting. We confirmed that HE complementary DNA was successfully introduced into cells, resulting in protein synthesis. Figure 1 shows that control cells expressed no HE, while all treated cells showed some degree of expression. Additionally, the triacetin assay used for quantification of the amount/activity of HE produced in each cell line confirmed that the enzyme was correctly processed and active (Table 2). We also tested for the presence of the enzyme in the cell medium by western blot and found that it had not been secreted from the cells in any significant amount due to the c-terminal transmembrane anchor that ensures membrane binding of the enzyme.[25,27] The amount of enzyme produced by different cells varied greatly depending on the cell line, and this was particularly noticeable in the heterogeneous UPAB line.

In an attempt to eliminate variable transfection efficacies, AcHE-CMV was developed once we had shown the improved rates of deacetylation and apoptosis in response to intracellular expression of HE. Using AcEGFP (enhanced green fluorescent protein)-CMV, we have previously shown that the baculovirus is a suitable vehicle for gene delivery to glioblastoma cells, and the preliminary assessments described above indicated that more HE was produced after transduction with AcHE-CMV than as a result of pcDNA3.1-HE-CMV transfection.

Characterization of Glioma and Glial Cells for GD3 and GD3^A Expression

The intracellular expression of GD3 and GD3^A before and after the various treatment regimens was assessed by flow cytometry (Fig. 2). This was a crucial determinant of the existence of a critical ratio between GD3 and GD3^A and whether this ratio could be manipulated in order to induce mitochondrially mediated apoptosis. The percentage of the cell population expressing intracellular GD3 and GD3^A as well as the average amount in each cell line (fluorescence fold) after the 3 treatment regimens were compared with ganglioside expression in untreated cells and are shown in Figure 2. The phenotype of the cell affects the expression; for example, nonneoplastic CC-2565 cells demonstrate the lowest expression of the gangliosides, while the highest levels were seen in the pediatric glioblastoma IN699.

All 3 treatment regimens caused a reduction in levels of GD3^A in the glioblastoma cell lines, and this effect was most significant after transduction with AcCMV-HE. However, the subsequent increase in GD3 expression was greater than the reduction in GD3^A levels. For example, in UPAB after exogenous administration of HE, GD3^A expression decreased 3-fold (expressed as fluorescence fold change), while the GD3 expression increased 10-fold. Additionally, CC-2565 expresses very low levels of GD3^A, and none of the 3 treatment

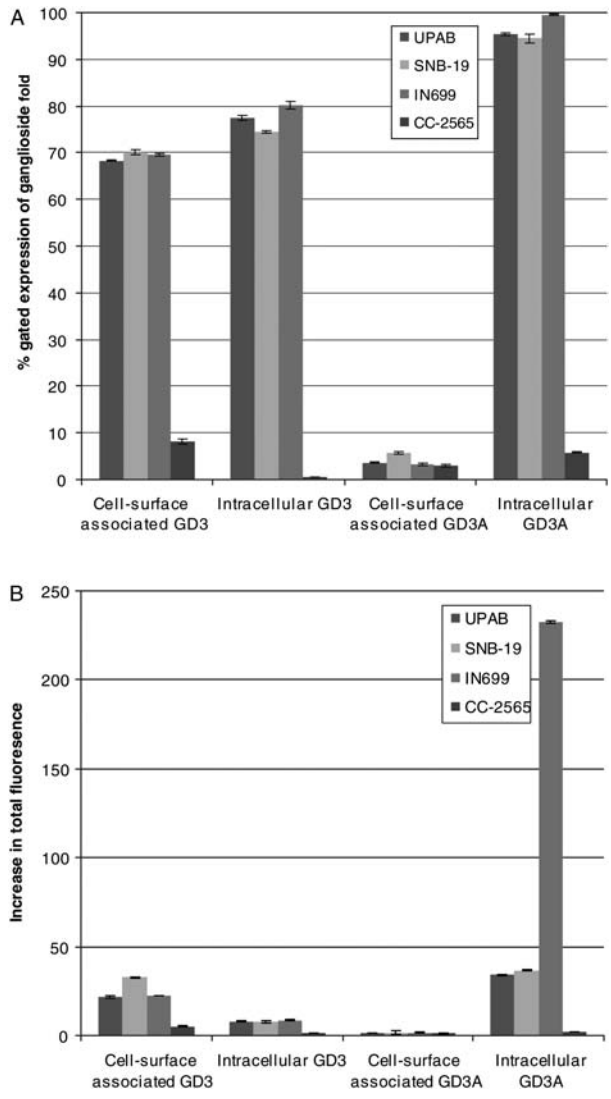

Fig. 2. (A) Percentage of the cell populations of UPAB, SNB-19, IN699, and CC-2565 expressing GD3 and GD3^A after treatment by exogenous administration of HE, transfection with pcDNA3.1-HE-CMV, or transduction with AcHE-CMV and compared with untreated cells. (B) Amount of intracellular GD3 and GD3^A as shown by fluorescence intensity in UPAB, SNB-19, IN699, and CC-2565 cells after exogenous administration of HE, transfection with pcDNA3.1-HE-CMV, or transduction with AcHE-CMV and compared with untreated cells. Results are representative of 3 independent experiments carried out in triplicate ($n = 3$), and values shown are the arithmetic means $\pm$ standard errors of the means (SEMs). Statistical significance was assessed by 2-way ANOVA and the Tukey-Kramer post-hoc test ($P \leq .05$).

regimens appeared to affect it or to increase GD3 expression, which was found in less than 1% of the cell population.

Effects of GD3 Deacetylation on Cell Viability

Cell viability was assessed in the 4 cell lines in response to the exogenous addition of HE and transduction with AcHE-CMV (Fig. 3). CC-2565 astrocytes appeared unaffected by HE treatment, with viability remaining close to 100% in all experiments. In the glioblastoma cell lines, however, cell viability was statistically significantly reduced in response to the treatment regimen.

Effects of GD3 Deacetylation on the Induction of Apoptosis

Both treatment regimens resulted in the induction of apoptosis in the glioblastoma cell lines. Importantly, no significant effect was seen in CC-2565 after any of the treatment regimens, with only ~5% of the population apoptotic in any of the 4 conditions assessed (Fig. 4A). There was, however, some variability in treatment efficacy with transduction, with AcHE-CMV causing the greatest apoptosis in SNB-19 and exogenous administration triggering the greatest apoptosis in UPAB cells. AcHE-CMV induced apoptosis in ~55% of the SNB-19 population and over 40% of the IN699, which is very similar to the transduction efficacies we have previously determined using this vector expressing green-fluorescent protein as a reporter gene (unpublished data). Linear regression analysis shows that there appears to be a very strong cause-and-effect relationship between the percentage of the population that is apoptotic and the percentage expressing GD3 after treatment (supplementary data). Here we demonstrate that increasing levels of GD3 show a positive correlation with the levels of apoptosis detected. A direct link was shown by carrying out double staining experiments; cells expressing high levels of GD3 were also positive for annexin V (Fig. 4D), whereas cells with little or no GD3 showed no signs of early apoptosis. For example, we show that after treating SNB-19 cells with AcHE-CMV, ~48% of the population is positive for both GD3 and annexin V. Around 21% of the population stained positively for GD3 but not for annexin V; however, this occurrence can be explained by the fact that the critical ratio may not have yet been achieved in those cells. Depolarization of the mitochondrial membrane is believed to be a critical step in the induction of GD3-evoked mitochondrially mediated apoptosis. JC-1 was used to determine the extent of depolarization, and it was found that the phenotype of cells (cell line) affects the extent of depolarization (Fig. 4B). For example, CC-2565 controls remained unaffected regardless of the treatment, while the glioblastoma cell lines displayed depolarization in response to all 3 treatment regimens. The differences between the 2 regimens were more marked in the homogeneous cell lines SNB-19 and IN699, with exogenous administration having the least effect and transduction with AcHE-CMV resulting in the most depolarization of the membrane.

Western blotting for cyt c was used to analyze mitochondrially mediated apoptosis. Figure 4C shows that cyt c was not detected in any of the untreated glioblastoma cell lines and was not detectable in CC-2565 cells, regardless of whether these were treated or untreated. However, cyt c was present in varying degrees in the glioblastoma cell lines in response to both treatment regimens. The greatest amount of cyt c

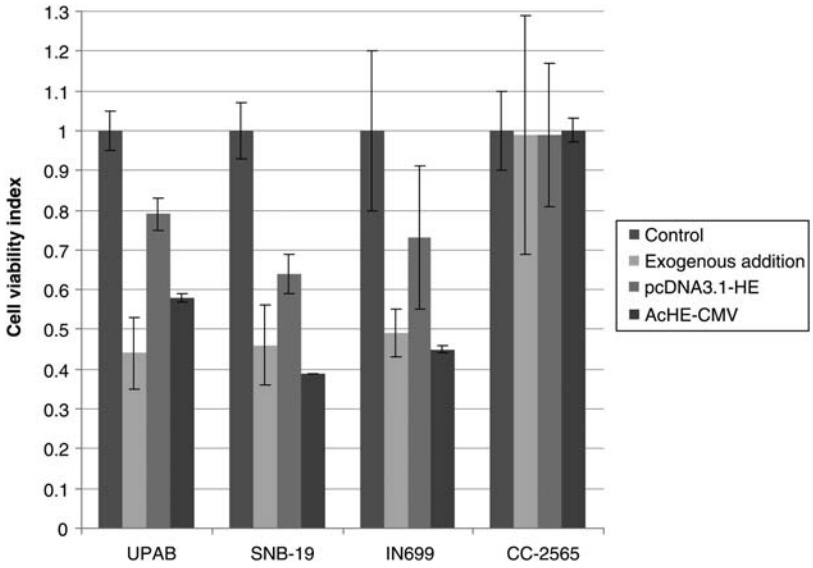

Fig. 3. Cell viability shown for UPAB, SNB-19, IN699, and CC-2565 after exogenous HE administration or transduction with AcHE-CMV and compared with untreated cells. Results are representative of 3 independent experiments carried out in triplicate ($n = 3$), and values shown are the arithmetic means ± standard errors of the means (SEMs). Statistical significance was assessed by 2-way ANOVA and the Tukey-Kramer post-hoc test ($P \leq .05$).

in UPAB was detected in response to exogenous administration, with HE levels being significantly reduced after transduction. Transduction with AcHE-CMV and exogenous administration of HE resulted in the similar amounts of cyt c being released in IN699; however, interestingly, the treatment method did not appear to have an effect on the amount of cyt c released in SNB-19.

Effect of GD3 Deacetylation on the Invasive Potential

Since elevated levels of GD3 are known to be associated with the diffuse local infiltration of glioblastoma cells into the normal brain, it was investigated whether cleaving the acetyl group from GD3^A (which results in an increase in intracellular GD3) would lead to the promotion of diffuse cellular invasion (Fig. 5). As expected, CC-2565 cells displayed minimal invasion whether treated or untreated, while in the 3 glioblastoma cell lines, invasion was reduced after treatment. Treatment method significantly affected the invasiveness of the glioblastoma cell lines, with transduction with AcHE-CMV resulting in the greatest decrease in invasive potential in all 3 cell lines. Additionally, linear regression analysis indicated that invasive potential is inversely dependent on the amount of GD3 expressed (supplementary data). For example, we show that with increasing amounts of deacetylated GD3 in the cell there also appears to be a reduction in invasive potential. Despite these data conflicting with investigations previously carried out in our laboratory,[8] we suggest that this is due to cells expressing high levels of GD3 undergoing apoptosis before they can invade through the transwell membrane, as opposed to GD3 suppressing invasion.

Discussion

The main aim of this work was to identify the existence of a critical ratio between GD3 and GD3^A in glioblastoma cell survival. Additionally, we have shown that the baculovirus is a suitable vector for delivering *HE* to glioblastoma cells in order to maximize cleaving of the acetyl group from GD3^A, with resultant apoptosis and depolarization of the mitochondrial membrane potential.

GD3:GD3^A Critical Ratio Controls Glioblastoma Cell Survival

We have shown in our preliminary studies that high levels of GD3 are expressed both intracellularly and on the surface of glioblastoma cells. In contrast, very little GD3 was detected in the normal human astrocytes CC-2565. Similarly, CC-2565 showed very little expression of GD3^A, while in the 3 glioblastoma cell lines it was present but detected only intracellularly, although at extremely high frequency (ie, over 90% of the cell population were positive). This finding was consistent with work by Testi and colleagues[20,33,34] and Yu and colleagues[35,36] showing that a critical ratio between GD3 and GD3^A exists and dictates cell survival and apoptosis.[20,22,33–36] The expression of GD3 and GD3^A in the glioblastoma cells but not in CC-2565 appears to support the cancer stem cell hypothesis, as it is the undifferentiated progenitor cells that enable tumor self-renewal, and it has been widely acknowledged that ganglioside expression is upregulated in undifferentiated cells but disappears from those cells with a stellate appearance—that is, in the normal astrocytic population

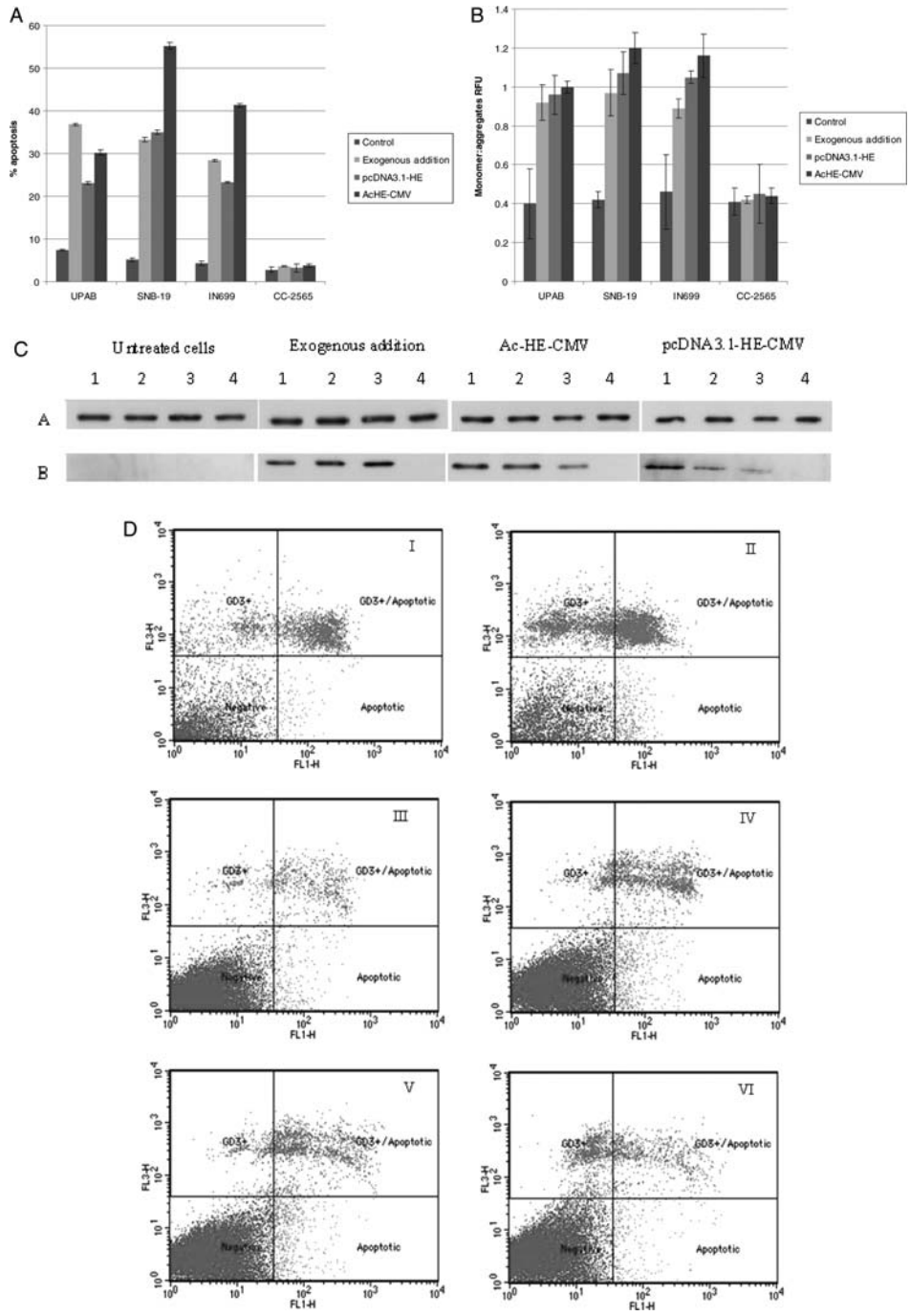

Fig. 4. (A) Percentage of apoptotic cells shown for UPAB, SNB-19, IN699, and CC-2565 after exogenous HE administration or transduction with AcHE-CMV and compared with untreated cells. Results are representative of 3 independent experiments carried out in triplicate ($n = 3$), and values shown are the arithmetic means $\pm$ standard errors of the means (SEMs). Statistical significance was assessed by 2-way ANOVA and the Tukey–Kramer post-hoc test ($P \leq .05$). (B) Effect of deacetylation on the mitochondrial membrane potential in SNB-19, IN699, UPAB, and CC-2565 cells either untreated, after exogenous administration of HE, or after transduction with AcHE-CMV. Relative fluorescence units (RFU) are calculated from the ratio of JC-1 monomers to aggregates, and higher RFU indicates greater depolarization. Results are representative of 3 independent experiments carried out in triplicate ($n = 3$), and values shown are the arithmetic means $\pm$ standard errors of the means (SEMs). Statistical significance was assessed by 2-way ANOVA and the Tukey–Kramer post-hoc test ($P \leq .05$). (C) Western blots on SNB-19, IN699, UPAB, and CC-2565 cells either untreated, after exogenous administration of HE, or after transduction with AcHE-CMV. Presence of 44-kDa actin as protein loading control (A) and 13-kDa cyt c (B) are shown. Results are representative of 3 independent experiments ($n = 3$). (D) Cells positive for GD3 after deacetylation also stain positively for annexin V, indicating a direct link between GD3 and the induction of apoptosis. Results are representative of 3 independent experiments carried out in triplicate ($n = 3$). SNB-19 after treatment with exogenous HE (I), transduction with AcHE-CMV (II), IN699 after treatment with exogenous HE (III), transduction with AcHE-CMV (IV), UPAB after treatment with exogenous HE (V), transduction with AcHE-CMV (VI).

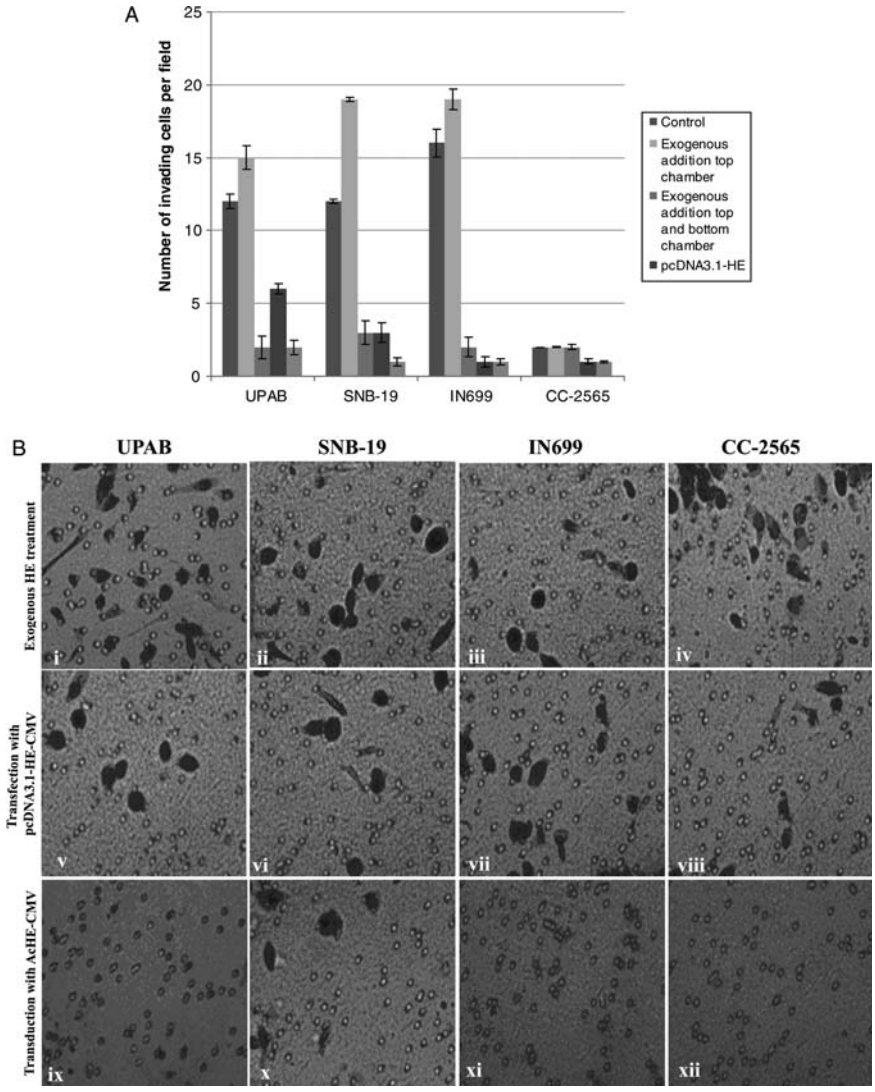

Fig. 5. (A) Effect of deacetylation by exogenous HE addition or transduction with AcHE-CMV on the invasive potential of UPAB, SNB-19, IN699, and CC-2565 cells as determined by the modified Transwell Boyden chamber assay. (A) The average number of cells per captured field is shown. Results are representative of 3 independent experiments carried out in triplicate ($n = 3$), and values shown are the arithmetic means $\pm$ standard errors of the means (SEMs). Statistical significance was assessed by 2-way ANOVA and the Tukey–Kramer post-hoc test ($P \leq .05$). (B) UPAB (i, v, ix), SNB-19 (ii, vi, x), IN699 (iii, vii, xi), and CC-2565 (iv, viii, xii). No scale bar is shown but the 8-μm pres serve as a reference point.

in the adult brain.[37,38] Furthermore, the highest levels of GD3 and GD3^A were seen in the pediatric glioblastomas IN699; this cell line has been shown in our laboratories to express high levels of CD133 (Donovan PhD thesis 2010, University of Portsmouth). CD133, a transmembrane glycoprotein, has been well documented as a putative marker of brain tumor stem cells, and this finding suggests that GD3 expression may indeed be upregulated in response to the glycosylation status of CD133. At present, no literature has been published on the topic, and thus it is impossible to predict the significance of this finding on the clinical application or discuss in detail the biological reason for this phenomenon. Despite this, Yu et al and Varki et al have both recently reviewed the role of glycosphingolipids in neural stem cells and reported the presence of GD3 in mouse neural progenitor cells, which they hypothesized may function to restrict cell proliferation, potentially by regulating Ras-MAPK signaling.[39,40] Additionally, the coexpression of GD3 and CD133 has since been further linked in a study on Caco-2 and HT-29-D4 colon cancer cells after the development of a polyclonal anti-CD133 antibody that recognizes the N-terminal extracellular region as opposed to the extracellular loops at the C-terminus region, which can become unglycosylated and thus do not truly reflect CD133 expression.[41] The authors of this study suggest that the binding of GD3 and other gangliosides may modulate the accessibility of the N-terminus of CD133 and thus serve to regulate cell-cell contact.

For the first time we have shown cleavage of the acetyl group from GD3^A in biopsy-derived human

glioblastoma cells in vitro and observed a concomitant increase in the amount of GD3 expressed by the cells. Furthermore, we showed that the process of deacetylation resulted in a reduction in cell viability, which was inversely dependent on the amount of GD3 expressed. The issue of whether the observed increase in GD3 is due simply to the cleaving of the acetyl group remains to be resolved. For example, in UPAB, the increase in GD3 is greater than the decrease in GD3^A, which suggests another phenomenon acting in concert with the act of deacetylation, as this should simply decrease GD3^A and increase GD3 to the same degree. Previous studies have reported that upon activation of death receptors such as tumor necrosis factor–α receptor or CD95/Fas, many cells begin the *de novo* synthesis of GD3, which then may contribute to mitochondrially mediated apoptosis.[42–44] In light of these studies and the findings reported here, further investigation would be required to rule out this phenomenon. We confirmed that this reduction in cell viability was linked to the induction of mitochondrially mediated apoptosis: as shown by the annexin V/PI assay as well as by changes in cyt c levels. Additionally, the JC-1 probe, which detects mitochondrial membrane depolarization, indicated that apoptosis was induced by membrane depolarization, which corroborates the current literature data that GD3 induces mitochondrially mediated apoptosis through direct interaction with the MPTP.[14–16] However, one of the most important questions arising from this study is how GD3 actually interacts with the mitochondrial membrane and the molecular mechanism behind the propagation of apoptosis. Garofalo and colleagues have already shown the existence of a complex consisting of GD3, voltage-dependent anion channel 1 (VDAC-1), and the fission protein hFis1 on the outer mitochondrial membrane;[44] additionally, modulation of VDAC has been reported to alter channel conductance in the mitochondrial membrane and is thought to be involved with the opening of the MPTP.[45,46] Thus we hypothesize that GD3 may interact with VDAC to cause a conformational change or recruit other pro-apoptotic molecules, which either results in the formation of a channel or alters ion exchange/oxidative phosphorylation to induce rupturing of the membrane. We hypothesize that the acetyl group blocks the ability of GD3^A to interact with the MPTP and that the role of GD3 acetylation in glioblastoma appears to suppress GD3 interaction with the mitochondria. Additionally, it may support the activation of the NFκ-B survival pathway; however, any biological function or supplementary role in tumor progression for GD3^A has yet to be elucidated.[12,13] Furthermore, we investigated whether cleaving the acetyl group from GD3^A and increasing the intracellular concentration of GD3 would cause an increase in invasive potential; however, it was found that cell death is achieved before GD3 can be trafficked to the plasma membrane and impact on invasion.

The Baculovirus as a Potential Therapeutic Vector

Through the exogenous addition of recombinant HE from influenza C virus, we achieved a proof of principle: this treatment caused a decrease in GD3^A expression with an increase in GD3 and induced apoptosis with varying degrees of success within the 3 glioblastoma cell lines. In the next step, 2 expression vectors were developed: pcDNA3.1-HE-CMV and AcHE-CMV for transfection/transduction studies and intracellular expression of HE. We found that the baculovirus (with no specific pseudotyping) was relatively efficient at transducing the 4 cell lines and producing active HE—shown through western blotting and the triacetin assay—and we believe that modifications such as the use of tumor-homing peptides or specific ligands/binding receptors may improve this virus specificity and efficacy even further. Additionally, the use of agents such as cyclohexamide has been shown to increase the size of the nuclear pores and may enhance nuclear import.[47] Unfortunately, as is the case with other therapeutic approaches, the more heterogeneous cell lines are more difficult to transduce, and thus the rate of deacetylation and subsequent mitochondrially mediated apoptosis were reduced in these cell lines. At present it is difficult to establish whether the reduced effects in these more heterogeneous cells are due to poor transduction efficacy alone or variability of expression/processing of HE. A major biological feature of glioblastomas is their heterogeneous nature, and these differences in epigenetic regulation and differentiation status are a barrier to transduction with the baculovirus. However, the most significant benefit of using this virus is that it is able to transduce quiescent cells as well as the mitotic ones, and thus the invasive population that have been arrested from the cell cycle can also be targeted. Another significant benefit of developing the baculovirus further for use in our future studies is its large (130 kb) double-stranded genome, which is capable of containing more than one insert. In this respect we envisage combining deacetylation with other established and/or novel glioblastoma markers, such as cathepsins, matrix metalloproteinases, neural/glial antigen 2, etc.[48]

Conclusions and Clinical Implications

A treatment that selectively targets neoplastic cells without affecting normal cell populations represents the Holy Grail for brain tumor research. The critical ratio between GD3 and GD3^A is clearly one of the factors that contribute to the survival of glioblastoma and to its ability to evade the immune response; however, it may also be exploited in order to overcome the therapeutic resistance demonstrated by glioblastoma. Modifying GD3 postsynthesis caused no damage to normal astrocytes (unlike many currently used therapeutic modalities). Clearly, targeting the biochemical pathways used exclusively or predominantly by brain tumor cells minimizes harm to "innocent bystanders."

Additionally, although the effect on normal neurons has not been specifically investigated in this study, current literature indicates that we can expect a similar result to that obtained with normal astrocytes. Previous studies have shown that the process of deacetylation should have little effect on neurons in the developed brain, as both antigens have been shown to be significantly downregulated following maturation.[5,6]

The viral origins of the enzyme used in this proof-of-principle study do not make it an optimal candidate for therapeutic applications in vivo. Influenza C virus HE is a strong antigen: ~70–90% of adults have preexisting antibodies toward it, and treatment could trigger an immune response that would disable the enzymatic effect and/or lead to cell death. Therefore, future developments should exploit human sialic acid 9-O-acetylesterase or inhibition of the sialic acid–specific O-acetyltransferases.[49] Importantly, a gene therapy approach allows for more selective targeting of tumor cells: for example, use of vectors containing the target gene under the control of cell-specific regulatory elements will prevent unwanted expression in other brain cell populations. In this respect we envisage that baculoviruses containing *HE* or GD3^A-specific 9-O-acetylesterase enzymes could be developed for the clinic and used as a safe and specific adjuvant therapy or twin treatment to target the infiltrative population left behind by surgical resection or combat the side effects of many anti-angiogenics. Crucially, not only has increased GD3 in the cell been shown to enhance chemosensitivity, but the hypoxic environment may reduce the threshold for the critical ratio.[13,38] Two of the most important aspects that must be considered in the development of the baculovirus containing *HE* as a potential therapy are which population of cells we are targeting and whether the therapy can be improved in order to reach the cells we want to kill.

These findings may bear a significant impact on our future studies and indeed on our therapeutic targeting of the tumor stem cell population. We acknowledge that the levels of apoptosis that we are currently reporting (30–55%), while significant in vitro, are not sufficient in a therapeutic setting, and clearly further investigations are required to elucidate the in vivo situation—for example, to elucidate whether cells at the hypoxic center of the brain tumor will be accessible to this approach. We intend to use ex vivo rat brain slices to investigate the cell tropism shown by the baculovirus and to allow us to specifically target populations of cells by pseudotyping the virus and identifying biological barriers to transduction. Through these investigations, we hope that we can improve on these proof-of-principle data to achieve a clinically relevant outcome.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

Acknowledgments

This work was funded with many thanks to Charlie's Challenge and Ali's Dream children's brain tumor research charities (Ph.D grant) (SB) and through a Ph.D scholarship funded by Oxford Brookes University (JD).

Conflict of interest statement. None declared.

References

1. Buckner JC, Brown PD, O'Neill P, Meyer FB, Wetmore CJ, Uhm JH. Central nervous system tumours. *Mayo Clin Proc*. 2007;82:1271–1286.
2. Zeller CB, Marchase RB. Gangliosides as modulators of cell function. *Am J Physiol*. 1992;(6 Pt 1):C1341–C1355.
3. Birkle S, Zeng G, Gao L, Yu RK, Aubry J. Role of tumor-associated gangliosides in cancer progression. *Biochimie*. 2003;85:455–463.
4. Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyo)lipid metabolism. *Cancer Res*. 1996;56:5309–5318.
5. Ogiso M, Ohta M, Harada Y, Kubo H, Hirano S. Developmental change in ganglioside expression in primary culture of rat neurons. *Neuroscience*. 1991;41:167–176.
6. Hilbig R, Rosner H, Merz G, Segler-Stahl K, Rahmann H. Developmental profiles of gangliosides in mouse and rat cerebral cortex. *Dev Genes Evol*. 1982;191:281–284.
7. Ziche M, Alessandri G, Gullino PM. Gangliosides promote the angiogenic response. *Lab Invest*. 1989;61:629–634.
8. Merzak A, Koochekpour S, McCrea S, Roxanis Y, Pilkington GJ. Gangliosides modulate proliferation, migration, and invasiveness of human brain tumour cells *in vitro*. *Mol Chem Neuropath*. 1995;24:121–135.
9. McKallip R, Li R, Ladisch S. Tumor gangliosides inhibit the tumor-specific immune response. *J Immunol*. 1999;163:3718–3726.
10. Kudo D, Rayman P, Horton C, et al. Gangliosides expressed by the renal cell carcinoma cell line SK-RC-45 are involved in tumor-induced apoptosis of T cells. *Cancer Res*. 2003;63:1676–1683.
11. Sa G, Das T, Moon C, et al. GD3, an overexpressed tumor-derived ganglioside, mediates the apoptosis of activated but not resting T cells. *Cancer Res*. 2009;69:3095–3104.
12. Colell A, Garcia-Ruiz C, Roman J, Ballesta A, Fernandez-Checa J. Ganglioside GD3 enhances apoptosis by suppressing the nuclear factor-κB –dependent survival pathway. *The FASEB J*. 2001;15:1068–1070.
13. Lluis JM, Llacuna L, von Montfort C, et al. GD3 synthase overexpression sensitizes hepatocarcinoma cells to hypoxia and reduces tumor growth by suppressing the cSrc/NF-κB survival pathway. *PLOS One*. 2009;4:1–11.
14. Scorrano L, Petronilli V, Di Lisa F, Bernardi P. Commitment to apoptosis by GD3 ganglioside depends on opening of the mitochondrial permeability transition pore. *J Biol Chem*. 1999;274:22581–22585.

15. Garcia-Ruiz C, Colell A, Paris R, Fernandez-Checa JC. Direct interaction of GD3 ganglioside with mitochondria generates reactive oxygen species followed by mitochondrial permeability transition, cytochrome c release, and caspase activation. *The FASEB J*. 2000;14:847–858.
16. Omran OM, Saqr HE, Yates AJ. Molecular mechanisms of GD3-induced apoptosis in U-2142 MG glioma cells. *Neurochem Res*. 2006;31: 1171–1180.
17. Chakrabandhu K, Huault S, Garmy N, et al. The extracellular glycosphingolipid-binding motif of Fas defines its internalisation route, mode, and outcome of signals upon activation by ligand. *Cell Death and Differentiation*. 2008;15:1824–1837.
18. Pilkington GJ. Glioma heterogeneity *in vitro*: the significance of growth factors and gangliosides. *Neuropath and Applied Neurobiol*. 1992;18:434–442.
19. Varki A. "Unusual" modifications and variations of vertebrate oligosaccharides: are we missing the flowers for the trees? *Glycobiology*. 1997;6:707–710.
20. Malisan F, Franchi L, Tomassini B, et al. Acetylation suppresses the proapoptotic activity of GD3 ganglioside. *J Exp Med*. 2002;196:1535–1541.
21. Chen HY, Varki A. O-acetylation of GD3: An enigmatic modification regulating apoptosis? *J Exp Med*. 2002;196:1529–1533.
22. Kniep B, Kniep E, Ozkucur N, et al. 9-O-acetyl GD3 protects tumor cells from apoptosis. *Int J Cancer*. 2006;119:67–73.
23. Mukherjee K, Chava AK, Mandal C, et al. O-acetylation of GD3 prevents its apoptotic effect and promotes survival of lymphoblasts in childhood acute lymphoblastic leukaemia. *J Cell Biochem*. 2008;105: 724–734.
24. Ariga T, Blaine GM, Yoshino H, et al. Glycosphingolipid composition of murine neuroblastoma cells: O-acetylesterase gene downregulates the expression of O-acetylated GD3. *Biochemistry*. 1995;34:11500–11507.
25. Vlasak R, Krystal M, Nacht M, Palese P. The influenza C virus glycoprotein (HE) exhibits receptor-binding (hemagglutinin) and receptor-destroying (esterase) activities. *Virology*. 1987;160:419–425.
26. Garcia-Sastre A, Villar E, Manuguerra JC, Hannoun C, Cabezas JA. Activity of influenza c virus O-acetylesterase with O-acetyl–containing compounds. *Biochem J*. 1991;273:435–441.
27. Rosenthal PB, Zhang X, Formanowski F, et al. Structure of the haemagglutinin-esterase-fusion glycoprotein of influenza C virus. *Nature*. 1998;396:92–96.
28. Boulaire J, Zhao Y, Wang S. Gene expression profiling to define host response to baculoviral transduction in the brain. *J Neurochem*. 2009;109:1203–1214.
29. Matilainen H, Rinne J, Gilbert L, Marjomaki V, Reunanen H, Oker-Blom C. Baculovirus entry into human hepatoma cells. *J Virol*. 2005;79: 15452–15459.
30. Paul A, Jardin BA, Kulamarva A, Malhotra M, Elias CB, Prakash S. Recombinant baculovirus as a highly potent vector for gene therapy of human colorectal carcinoma: molecular cloning, expression and *in vitro* characterisation. *Mol Biotechnol*. 2010;45:129–139.
31. Lehtolainen P, Tyynela K, Kannasto J, Airenne KJ, Yla-Herttula S. Baculoviruses exhibit restricted cell type specificity in rat brain: a comparison of baculovirus- and adenovirus-mediated intracerebral gene transfer *in vivo*. *Gene Ther*. 2002;9:1693–1699.
32. Albini A. Tumor and endothelial cell invasion of basement membranes. The Matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. *Pathol Oncol Res*. 1998;4:230–241.
33. Rippo MR, Malisan F, Ravagnan L, et al. GD3 ganglioside directly targets mitochondria in a bcl-2–controlled fashion. *The FASEB J*. 2000;14:2047–2054.
34. Malisan F, Testi R. GD3 ganglioside and apoptosis. *Biochemica et Biophysica Acta*. 2002;1585:179–187.
35. Birkle S, Ren S, Slominski A, Zeng G, Gao L, Yu RK. Down-regulation of the expression of O-acetyl-GD3 by the O-acetylesterase cDNA in hamster melanoma cells: Effects on cellular proliferation, differentiation, and melanogenesis. *J Neurochem*. 1999;72:954–961.
36. Birkle S, Gao L, Zeng G, Yu RK. Down-regulation of GD3 ganglioside and its O-acetylated derivative by stable transfection with antisense vector against GD3-synthase gene expression in hamster melanoma cells: Effects on cellular growth, melanogenesis, and dendricity. *J Neurochem*. 2000;74:547–554.
37. Mendez-Otero R, Constantine-Paton M. Granule cell induction of 9-O-acetyl gangliosides on cerebellar glia in microcultures. *Dev Biol*. 1990;138:400–409.
38. Paris R, Morales A, Coll O, Sanchez-Reyes A, Garcia-Ruiz C, Fernandez-Checa J. Ganglioside GD3 sensitizes human hepatoma cells to cancer therapy. *J Biol Chem*. 2002;277:49870–49876.
39. Yanagisawa M, Yu RK. The expression and functions of glycoconjugates in neural stem cells. *Glycobiology*. 2007;17:57R–74R.
40. Lanctot PM, Gage FH, Varki AP. The glycans of stem cells. *Curr Opin Chem Biol*. 2007;11:373–380.
41. Taieb N, Maresca M, Guo X-J, Garmy N, Fantini J, Yahi N. The first extracellular domain of the tumour stem cell marker CD133 contains an antigenic ganglioside-binding motif. *Cancer Letters*. 2009;278: 164–173.
42. Garcia-Ruiz C, Colell A, Morales A, Calvo M, Enrich C, Fernandez-Checa JC. Trafficking of ganglioside GD3 to mitochondria by tumor necrosis factor–α. *J Biol Chem*. 2002;277: 36443–36448.
43. Kang Y, Kang S-K, Lee Y-C, et al. Transcriptional regulation of the human GD3 synthase gene expression in Fas-induced Jurkat T cells: a critical role of transcription factor NF-κB in regulated expression. *Glycobiol*. 2006;16:375–389.
44. Garofalo T, Tinari A, Matarrese P, et al. Do mitochondria act as "cargo boats" in the journey of GD3 to the nucleus during apoptosis? *FEBS Letters*. 2007;581:3899–3903.
45. Hengartner MO. The biochemistry of apoptosis. *Nature*. 2000;407: 770–776.
46. Pastorino JG, Hoek JB. Integration of energy metabolism and control of apoptosis in tumor cells. In: Singh, KK, Costello, LC, eds. Mitochondria and Cancer.. Springer Science; 2009.
47. Feldherr CM, Akin D, Cohen RJ. Regulation of functional nuclear pore size in fibroblasts. *J Cell Sci*. 2001;114:4621–4627.
48. Chekenya M, Pilkington GJ. NG2 precursor cells in neoplasia: Functional, histogenesis and therapeutic implications for malignant brain tumours. *J Neurocyt*. 2002;31:507–521.
49. Arming S, Wipfler D, Mayr J, et al. The human Cas1 protein: A sialic-acid–specific O-acetyltransferase? *Glycobiology*. 2011;21:553–564