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Induction of GM1a/GD1b synthase triggers complex ganglioside expression and alters neuroblastoma cell behavior; a new tumor cell model of ganglioside function

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Abstract

Neuroblastoma is the most common extracranial solid tumor in children and tumor ganglioside composition has been linked to its biological and clinical behavior. We recently found that high expression of complex gangliosides that are products of the enzyme GM1a/GD1b synthase predicts a more favorable outcome in human neuroblastoma, and others have shown that complex gangliosides such as GD1a inhibit metastasis of murine tumors. To determine how a switch from structurally simple to structurally complex ganglioside expression affects neuroblastoma cell behavior, we engineered IMR32 human neuroblastoma cells, which contain almost exclusively (89%) the simple gangliosides (SG) GM2, GD2, GM3, and GD3, to overexpress the complex gangliosides (CG) GM1, GD1a, GD1b and GT1b, by stable retroviral-mediated transduction of the cDNA encoding GM1a/GD1b synthase. This strikingly altered cellular ganglioside composition without affecting total ganglioside content: There was a 23-fold increase in the ratio of complex to simple gangliosides in GM1a/GD1b synthase-transduced cells (IMR32-CG) vs. wild type (IMR32) or vector-transfected (IMR32-V) cells with essentially no expression of the clinical neuroblastoma marker, GD2, confirming effectiveness of this molecular switch from simple to complex ganglioside synthesis. Probing for consequences of the switch, we found that among functional properties of IMR32-CG cells, cell migration was inhibited and Rho/Rac1 activities were altered, while proliferation kinetics and cell differentiation were unaffected. These findings further implicate cellular ganglioside composition in determining cell migration characteristics of tumor cells. This IMR32 model system should be useful in delineating the impact of ganglioside composition on tumor cell function.

Keywords

complex gangliosides; GD2; neuroblastoma; cell migration; GM1a/GD1b synthase; molecular switch

Introduction

Neuroblastoma (NB), originating from neural crest and presenting in the adrenal gland and in sympathetic ganglia, is the most common extracranial solid tumor in childhood and the most common cancer of infancy [1]. The outcome of neuroblastoma varies widely according to patient age and clinical stage: children older than 1 year with metastasis have a poor

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prognosis whereas infant NB has a better prognosis and often undergoes spontaneous regression. Although a number of biochemical markers (e.g., ferritin, lactate dehydrogenase, GD2 ganglioside) have been identified and correlated with the clinical behavior and prognosis[2, 3], specific factors causing these differences in outcome remain to be elucidated. Here we have developed a model enabling direct assessment of the role of tumor cell ganglioside metabolism in this context.

Gangliosides are membrane-bound glycolipid molecules that are highly expressed in many tumor cells and actively shed into their microenvironment [4]. They consist of a ceramide backbone and a carbohydrate portion containing sialic acid. In humans the monoganglioside GM3, derived from lactosylceramide, is the common ganglioside precursor of the other gangliosides, the biosynthesis of which occurs via a series of glycosylations in two main pathways, the “a” pathway (GM2, GM1a, GD1a, GT1a) and the “b” pathway (GD3, GD2, GD1b, GT1b, GQ1b) [5], resulting in increasingly structurally complex molecules (Fig. 1). Gangliosides can also be grouped into structurally simple (SG) and complex (CG) molecules, with the enzyme GM1a/GD1b synthase (UDP-Gal:betaGlcNAc-beta-1,3-galactosyltransferase) converting its substrates, the simple gangliosides, GM2 and GD2, to the corresponding initial complex ganglioside products, GM1a and GD1b (Fig. 1).

Prominent expression of simple gangliosides (GM3, GM2, GD3, GD2) characterizes many aggressive tumors, such as melanoma and neuroblastoma[6–9], and in some cases a correlation between their expression and outcome of these patients has been demonstrated. For example, ganglioside analysis of biopsy samples of different types of human brain tumors, such as astrocytomas and oligodendrogliomas [10, 11] and of neuroblastoma [7] showed that low expression of structurally more complex “b” pathway gangliosides (CbG) was associated with lower survival of patients. Consistent with these findings, in our studies of human neuroblastoma, high CbG expression strongly predicted a favorable outcome, whereas low or absent CbG content correlated with unfavorable clinical behavior and aggressive biological phenotype in primary NB [12]. The correlation between higher tumor CbG content and lower neuroblastoma malignancy in vivo suggested by this and other [13] studies raises the question of whether this change in ganglioside expression has specific biological effects on the tumor cell.

Supporting this possibility are a number of ganglioside effects on cell behavior in vitro. High expression of complex gangliosides (CG), both CbG and complex “a” pathway gangliosides (CaG) such as GM1a and GD1a has been suggested to inhibit in vitro correlates of aggressive tumor cell behavior, such as cellular proliferation and migration, and to enhance differentiation: Exposing tumor cells to complex gangliosides such as GM1, GD1a, GD1b, or GT1b in vitro [14–16], or increasing structurally complex ganglioside expression by exposure of cells to retinoic acid [17] inhibits the proliferation and stimulates the differentiation of the cells. And in vivo, in certain murine tumors, a low propensity to metastasize was correlated with high expression of the complex gangliosides GM1a and GD1a, while inhibition of their expression caused more rapid cell migration [18–20].

To establish whether increasing the level of complex gangliosides alters key biological properties of neuroblastoma cells here we have constructed a cell model of stable, constitutive and selective high expression of CG by overexpression of the enzyme GM1a/GD1b synthase.

Materials and Methods

Reagents and cell culture

Human NB IMR32 cells (ATCC, Manassas, VA), were cultured in Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS), and 1% each of L-glutamine, penicillin/streptomycin, and non-essential amino acids (NEAA) (all from Lonza) at 37°C in 5% CO_2. Y27632 [(R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexane-carboxamide, 2HCl] was purchased from Calbiochem (Gibbstown, NJ), G418 sulfate from Clontech (Mountain View, CA) and the Rho/Rac1 activation assay kit from Cell Biolabs (San Diego, CA).

Stable transduction

The human GM1a/GD1b synthase (UDP-Gal:betaGlcNAc-beta-1,3-galactosyltransferase) cDNA (GeneCopoeia, Germantown, MD) was cloned into a retroviral vector, pLXIN (Clontech, Mountain View, CA), and stably transduced into IMR32 cells as recommended by Clontech. The human GM1a/GD1b synthase cDNA clone was generated by PCR using 5′-CGGACTCTAGCCTAGATCTGTTAACCATGCAGCTCAGGC-3′ as the forward primer and 5′-GAAAGCTGGGTTGCGGATCCACTCGAGCTAGCTCTG-3′ as the reverse primer. The PCR product was digested with HpaI and BamHI followed by ligation into the HpaI/BamHI restriction sites of the retroviral expression vector, pLXIN. The GM1a/GD1b synthase open reading frame in the recombinant plasmid GM1a/GD1b synthase/pLXIN was verified by sequencing and then transfected into the packaging cell line, AmphoPack™-293, using CalPhos™ transfection kit (Clontech). 48 hours after transfection, the culture medium containing retrovirus-packaged recombinant DNAs was collected and used to infect IMR32 cells for 24 hours. Stable transductants were selected with G418 (600ug/ml) and maintained in culture medium supplemented with 300ug G418/ml. The primers for identifying the newly integrated GM1a/GD1b synthase gene were the forward primer 5′-CCTACATGTGACCTGGGAAG and the reverse primer 5′-CCAAAAGACGGCAATATGGTG. The IMR32-CG allele yields a 1.5kb product, the IMR32-V allele a 300bp product, and the control untransformed IMR32 allele yields no product.

Western blot

Cells were grown to 70% confluence, washed twice with cold PBS, and incubated in lysis buffer (20mM Tris-HCl, pH=7.5, 1% Triton, 1mM Na_2EDTA, 1mM EGTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 15 minutes. Protein concentrations in the lysates were measured by the Pierce BCA Protein Assay Kit (Pierce Biotechnology, IL) and equal amounts of total protein from each sample were separated by SDS-PAGE (Bio-Rad, CA) and transferred to PVDF membranes that were then incubated in blocking buffer (Tris buffered saline, pH 7.2, containing 0.05% Tween-20 and 3% BSA) for 1h, followed by the primary antibodies (diluted in the blocking buffer) for an additional hour at room temperature. Primary antibodies were used to detect GM1a/GD1b synthase (B-1,3-Gal-T4, Santa Cruz Biotechnology, CA, 1:200), neuronal cell adhesion molecule (NCAM) (Santa Cruz, 1:250), neuron specific enolase (NSE, Abcam, MA, 1:2000), α-tubulin (Sigma, MO, 1:1000), Rho and Rac1 (Cell Biolabs, CA, 1:1000). The membranes were washed 3 times and incubated for 1 hour in blocking buffer containing horseradish peroxidase (HRP)-linked secondary antibodies (1:2000). Detection was performed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, IL). Rho and Rac1 activity were detected in the cell lysates using the Rho/Rac1 activation assay combination kit (Cell Biolabs, CA) in which Rho-GTP and Rac1-GTP were concentrated separately by Rhotekin

RBD and PAK PBD agarose beads and detected by western blot, using anti-Rho and anti-Rac1 mouse monoclonal antibodies.

Ganglioside purification and quantification

To assess the effect of the enzyme transduction on the composition of the cellular gangliosides, these were extracted and purified as previously described [21]. Briefly, lyophilized cell pellets were extracted twice with chloroform/methanol (1:1 v/v) at 4°C with stirring. The total lipid extract was partitioned in diisopropyl ether/butanol/0.1% aqueous NaCl (6:4:5 v/v) to separate the gangliosides, which were further purified by Sephadex G-50 gel (Sigma, MO) chromatography and examined by HPTLC using 10×10cm precoated silica gel HPTLC plates (MERCK, Nutley, NJ). The plates were developed in chloroform/methanol/0.25% $CaCl_2 \cdot H_2O$ (60:40:9 v/v) and stained with resorcinol. Individual gangliosides were identified by comparison to migration of human brain ganglioside standards and quantified by HPTLC densitometry.

In a separate experiment, the transformed IMR32-CG and control IMR32 cells were metabolically radiolabeled and gangliosides analyzed by HPTLC autoradiography as in our previous work [22]. Briefly, the cells were incubated with 1.0 μCi/ml each of D-[1-^{14}C]-glucosamine hydrochloride and D-[1-^{14}C]-galactose for 24 h, harvested and the gangliosides purified. The gangliosides were separated by HPTLC as above, and the plate exposed to Kodak X-Omat RP film to reveal the radiolabeled gangliosides.

Cell proliferation and migration assays

Cell proliferation was assessed by cell count of sextuplicate cultures at various time points. Briefly, 3×10^4 IMR32 and IMR32-CG cells were seeded/well in 12-well plates. Cells were counted daily starting at two days after seeding and doubling times were calculated according to the equation: t = T total/N; $N=3.32(\log N_t - \log N_0)$. As a second method to quantify the cell growth, the tetrazolium/formazan assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, WI) was used and here the cells were seeded at 3×10^3cells/well in 96-well plates and assayed in sextuplicate.

To evaluate cell migration, the Boyden Chamber Assay (QCM™ Haptotaxis cell migration assay, Millipore, MA) with a porous (8 μm) membrane precoated with fibronectin to facilitate optimal cell migration was used. IMR32 cells were plated in the upper chambers at 3×10^5 cells/300μl medium/chamber. The lower wells contained 500 μl culture medium. The chambers were incubated for 18 hours at 37°C in 5% CO_2 to allow cells to migrate from the upper chamber toward the lower well. Cells migrating through the pores of chamber membrane and adherent on the undersurface of the membrane were stained with crystal violet and quantified by spectrophotometry (O.D. = 570nm). A second method used to assess cell migration was the wound-scratch assay. Cells were seeded near confluence in 6-well plates and scratched 24 hours later with a 1000uL pipet tip. The wound scratches were photographed by phase-contrast microscopy every 24 hours and the wound areas measured using AxioVision software.

Statistical analysis

Data are expressed as the mean and standard deviation (SD). Statistical analyses were performed using the t-test. $P<0.05$ was considered significant. The densities of protein (Western blots) and ganglioside bands (HPTLC) were quantified using Image J software.

Results

Overexpression of GM1a/GD1b synthase cDNA in human IMR32 NB cell line

To be able to study the consequence of increased expression of complex gangliosides (CG) on the biological properties of human neuroblastoma cells, we constructed a CG overexpression model by stable transduction of human GM1a/GD1b synthase cDNA (1.2 kb), subcloned into retroviral vector pLXIN, into IMR32 human neuroblastoma cells. The resulting GM1a/GD1b synthase/pLXIN transfected cells were termed IMR32-CG. To assure that the retroviral vector alone was not responsible for any changes in phenotype observed, IMR32 cells were transfected with the empty pLXIN vector (IMR32-V). Stable transduction of the GM1a/GD1b synthase cDNA and overexpression of GM1a/GD1b synthase were successful (Fig. 2), and IMR32-V was used as a control.

The GM1a/GD1b synthase cDNA transductants in IMR32-CG were examined by PCR amplification (Fig. 2A). Genomic DNA was extracted from untransduced IMR32 cells, from IMR32 cells transduced with vector pLXIN alone (IMR32-V), and from IMR32 cells transduced with GM1a/GD1b synthase/pLXIN plasmid (IMR32-CG). The primers designed for PCR amplification to detect the integrated genes are complementary to the nucleotide sequence of the retroviral vector pLXIN, which is not present in human genomic DNA. The distance between the forward and reverse primers is ~300 base pairs. PCR amplification showed that no product was detected in IMR32; a 300 bp product was amplified from IMR32-V cells and the expected 1.5 kbp PCR product was detected in IMR32-CG cells. These results confirmed successful stable transduction of the GM1a/GD1b synthase gene into two separate batches of IMR32 cells.

The expression of GM1a/GD1b synthase was detected by western blot using a polyclonal goat anti-GM1a/GD1b synthase antibody (Fig. 2B). Expression of GM1a/GD1b synthase in the IMR32-CG cells was significantly (about 5 times) higher than that in IMR32 cells. There was no significant difference in synthase levels between IMR32-V and IMR32 cells. Thus GM1a/GD1b synthase was specifically increased following stable transduction of the gene into IMR32 cells.

Ganglioside expression following GM1a/GD1b synthase overexpression in IMR32 cells

We purified total gangliosides of IMR32, IMR32-V and IMR32-CG cells to examine the effect of overexpression of GM1a/GD1b synthase on ganglioside synthesis and expression. We analyzed their ganglioside patterns by HPTLC and scanning densitometry (Fig. 2C and Table 1) to determine both the relative and absolute content of the individual gangliosides. The striking change in ganglioside expression was that gangliosides in IMR32-CG cells had been substantially converted from the structurally simple SG species, GM3, GM2, GD2, and GD3, that constituted 89% of the total gangliosides of control (IMR32 and IMR32-V) cells and that characterize many neuroectodermal tumors and tumor cell lines [23], to complex gangliosides; there was almost complete disappearance of GM2 and GD2 (the substrates of GM1a/GD1b synthase) and total SG dropped to only 26% of the total gangliosides of IMR32-CG cells. Conversely, complex gangliosides (including GM1a, GD1a, GD1b and GT1b), which comprised only 11% of the gangliosides in control IMR32 cells, increased to 74% of total gangliosides in IMR-CG cells with very high expression of GD1a, the appearance of GD1b, and an increase in GT1b. Together with an essentially unchanged total ganglioside concentration (27–31 nmol/10^8 cells), the shift from 89% to 26% in gangliosides upstream of GM1a/GD1b synthase and from 11% to 74% in gangliosides downstream of the enzyme demonstrate successful GM1a/GD1b synthase gene transduction and CG overexpression. These findings were confirmed by sensitive HPTLC-autoradiography (Fig 2D) which also revealed that the clinical NB ganglioside marker, GD2, had essentially

disappeared in IMR32-CG cells, compared to its prominent presence (as expected) in the parent IMR32 cells. IMR32-CG cells therefore constitute a useful system to study effects of CG expression on the phenotype of these cells.

Comparison of biological behavior of IMR32-CG, IMR32, and IMR32-V cells

Cell morphology—Cell morphologies of the three IMR32 cell populations cultured in growth medium were similar (Fig. 3A). Under the condition of subconfluence, all three spread well on the plate surface and there were no differences observed in either cellular size, outgrowth of cellular extensions, or the number of neurites.

Cell proliferation—The proliferation of IMR32 and IMR32-CG cells was compared by cell counting and cell growth curves were generated (Fig. 3B). There was no significant difference in proliferation between the IMR32 and IMR32-CG cells. Both had calculated cell doubling times of about 30 hours. The tetrazolium/formazan assay, used as an alternative method to evaluate cell growth (Fig. 3C), gave a result consistent with that obtained by cell counting.

Cellular differentiation—The role of CG in cellular differentiation of NB can be evaluated by observing cellular morphology and by comparing the expression levels of differentiation markers. Morphological signs of neuronal differentiation in NB cells include outgrowth, prolongation and arborization of cellular extensions. As seen in Figure 4A, there was no difference in the degree of cellular extensions, comparing IMR32-CG to IMR32 and IMR32-V. Expression levels of NSE and NCAM, two widely used neuroblastoma differentiation markers, [24–26], were also examined and found to be comparable in IMR32 and in IMR32-CG cells (Fig. 3D). This suggests that there was no difference in the stage of differentiation caused by overexpression of GM1a/GD1b synthase or complex gangliosides.

Cell migration—The migration of tumor cells is an important property that contributes to tumor malignancy and prognosis. To investigate the effect of the induction of GM1a/GD1b synthase and overexpression of CG on the migration of IMR32 cells, two experimental approaches were taken. The first was the wound scratch assay, in which, within two days after scratching, the IMR32 or IMR32-V-seeded plates was more completely healed than those of IMR32-CG cells (Fig. 4A, 4B). Likewise, in the Transwell assay system, migration of CG-overexpressing IMR32 cells was impeded compared to that of wild type or pLXIN-transduced IMR32 cells (fig. 4C). Taken together, the data suggest that the increased expression of CG and/or the increased ratio of complex to simple gangliosides in the cell, while not altering cellular morphology, proliferation or differentiation of IMR32 cells, markedly affects cell migration.

Impeded migration of IMR32-CG is associated with changes in Rho/Rac1 signaling

The Rho family of GTPases is important in regulating cell migration. Members of this family include Rho, Rac and cdc42, all of which cycle between an inactive GDP-bound conformation and active GTP-bound conformation and are known to affect cell migration as well as to be influenced by gangliosides [27–29]. Given the findings of impeded cellular migration after CG overexpression, we therefore evaluated the Rho/Rac1 signaling pathway by quantifying the active forms of Rho and Rac1 (Rho-GTP and Rac1-GTP) in IMR32, IMR32-V and IMR32-CG cells by western blot. Rho GTP was increased in IMR32-CG cells to approximately three times that of IMR32 or IMR32-V cells (Fig. 5A) while Rac1 GTP in IMR32-CG cells was reduced to only half that of IMR32 or IMR32-V cells (Fig. 5B). These findings suggest that the inhibitory effect of the shift to high expression of CG, on IMR32 cell migration, might depend at least in part on the Rho/Rac1 signaling pathway, and that this pathway plays a major role in the inhibition of migration observed in IMR32-CG cells.

Discussion

Gangliosides, as a class of molecules, are known to have multiple effects on cell function, and to influence tumor progression. Whether structural characteristics of gangliosides expressed by tumor cells impart specific biological properties on these cells is therefore a question of substantial interest. One approach to probe this possibility has been to transfect specific glycosyltransferase genes into tumor cells to alter cellular ganglioside composition, and then to study the consequences on the cell phenotype. Examples of successful transfection into tumor cells using non-retroviral vectors are stable transfection of the genes encoding GD3 synthase [30], GM3 synthase [31] and GM1 synthase [32]. Here we have optimized stable expression of human GM1a/GD1b synthase in IMR32 human neuroblastoma tumor cells by taking advantage of a retroviral vector to carry the cDNA, integrating the cDNA into the IMR32 cell genome stably and efficiently. By highly expressing GM1a/GD1b synthase, we induced complex ganglioside expression in a cell line that normally contains predominantly simple gangliosides, creating a cell population constitutively expressing markedly increased levels of complex, and reduced levels of simple, gangliosides.

We chose the IMR32 cell line because of its very high expression of simple gangliosides (89% of total gangliosides) and low expression of their downstream complex ganglioside products, representative of many human neuroblastoma cell lines [23], and because its phenotype (highly tumorigenic in immunosuppressed mice[33]) mirrors that of aggressive human neuroblastoma in vivo. We therefore felt it would lend itself well as a model to detect any increased abundance of complex gangliosides and any effects on key NB cell phenotypic characteristics. In fact, after stable transfection of IMR32 cells with GM1a/GD1b synthase cDNA, CbG (GD1b+GT1b) were increased >3-fold, CaG (GM1a+GD1a) increased >7 fold, and total CG increased (from 3.3 nmol/10^8 cells to 19.7 nmol/10^8 cells. As total SG simultaneously decreased from 27.5 to 7.0 nmol/10^8 in IMR32-CG cells, the overall total ganglioside content was essentially unchanged. The resulting dramatic change in the ratio of complex to simple gangliosides—a 23 fold increase compared to that of IMR32 and IMR32-V cells—demonstrates that activating this single enzyme provided the key to almost complete conversion of simple to complex ganglioside expression. Of additional significance, the universal human neuroblastoma ganglioside tumor marker, GD2 [9], very prominent in the parent IMR32 cells (9nmol/10^8 cells, 29% of total gangliosides), is seen to be essentially absent in IMR32-CG cells.

What are the consequences of the induction of complex ganglioside synthesis? The main biological effect we observed in IMR32-CG cells was decreased cell migration. This finding is consistent with earlier studies in which the degree of cell migration was positively correlated with the intrinsic expression of structurally simple gangliosides such as GM3 and GD3 [30, 34, 35], and inhibition of migration was associated with high expression of complex gangliosides such as GM1, GD1a, and GT1b [18–20, 36]. Whether this biological effect is due to the higher concentrations of complex gangliosides, to the associated lower concentrations of simple gangliosides, and/or to a change in the simple/complex ganglioside ratio, remains to be determined, and our findings linking decreased cell migration to altered constitutive complex ganglioside synthesis should now also be confirmed in additional neuroblastoma cell lines.

In contrast to our findings here, in some cells when the cellular ganglioside composition was altered by *exogenous addition* of specific gangliosides, complex ganglioside enrichment was linked to increased cell migration and simple ganglioside enrichment to decreased migration. For example, GD1a enhanced HUVEC migration induced by VEGF [37], whereas GM3 enrichment suppressed their migration [38]. Also, GM3- and GD3-treated

human epidermal Langerhans cells also had less ability to migrate towards a chemokine [39], and GM3 enrichment inhibited epithelial cell migration on fibronectin and collagen matrices [40] and GM3 but not GM1 inhibited CD9-facilitated cell migration [41]. Clearly, the impact of intrinsic cellular vs. exogenously added gangliosides in different cell types is likely a complex issue that will require further study.

Regarding the regulation of cell migration, Rho/Rac1 signaling is considered to be one pathway having a pivotal role. Increased Rho and decreased Rac1 activity act to regulate lamellipodial and filopodial protrusions through regulating the polymerization of actin, focal adhesion, and cell body contraction [42–47]. In IMR32-CG cells Rac1 activity was decreased while Rho activity was increased, compared to the IMR32 parent cells, suggesting this pathway as one possible explanation for how the greater ganglioside complexity in IMR32-CG may contribute to the inhibition of IMR32-CG cell migration. To further delineate signaling mechanisms linking intrinsic ganglioside expression and cell migration, the IMR32-CG cells developed here should be a useful model system that may identify other biological effects and related signaling pathways or effects as well. For example, while this manuscript was in preparation, transfection of GMl/GD1b synthase into the melanoma cell line SK-MEL-37 was reported. The induced gangliosides were predominantly found in the glycolipid enriched microdomains/lipid raft membrane fractions (where they may affect cell signaling), and cell proliferation and invasion were suppressed, further supporting the concept that complex gangliosides impact cell biology [48].

A number of interesting clinical associations underscore the potential clinical impact of complex gangliosides, or the shift from simple to complex ganglioside expression in NB, and the consequent change in cell properties: *(i)* The higher CbG level in IMR32-CG cells [3 fold higher than in IMR32 cells] mirrors the higher CbG levels in nonprogressive neuroblastoma tumors [2.3 fold higher than in progressive tumors]. This result came from evaluating the ganglioside composition of 74 non-progressive or progressive neuroblastoma tumors, and finding that CbG comprised 41% of total gangliosides in non-progressive tumors vs. 18% of total gangliosides in tumors that progressed, suggesting that high content of CbG strongly predicts a favorable outcome in NB patients [12]. *(ii)* The ganglioside content of nine neuroblastoma cell lines established from tumors of patients who had a poor prognosis provided evidence consistent with the findings above, in that these all had a high expression of simple gangliosides (with the structurally simple ganglioside GD2 comprising up to 60% of total gangliosides) and a low content (1–20% of total gangliosides) of the complex gangliosides, CbG (the products of GM1a/GD1b synthase) [23]. (iii) The >7-fold increase of CaG expression caused by GM1a/GD1b gene transduction of IMR32 cells here was very similar to the >6-fold increase in complex gangliosides induced by treatment of neuroblastoma cells in vitro with retinoic acid, which induced GM1a/GD1b synthase activity [49]; retinoic acid has been shown clinically to be an effective agent in the treatment of neuroblastoma in the maintenance setting [50, 51].

In conclusion, by successfully selectively overexpressing complex gangliosides in human neuroblastoma cells, the present work underscores an important functional consequence of a shift to high cellular expression of CG—decreased cell migration, a possible mechanism at the cellular level contributing to clinical observations that high complex ganglioside content predicts a good prognosis in NB. In this regard, CG might be a biomarker useful to predict clinical response, to stratify patients with NB for purposes of tailoring anti-cancer treatment, or to monitor effectiveness of treatment. Even more intriguingly, we speculate that pharmacologically increasing the content of CG in NB might impede tumor progression, and in this regard suggest a novel therapeutic target in human NB.

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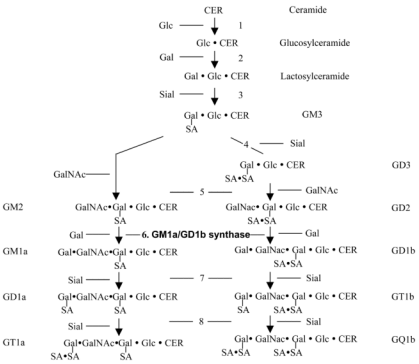

Figure 1.

Major pathways of human ganglioside biosynthesis. GM3, derived from lactosylceramide, is the common precursor of both “a” and “b” pathway gangliosides, each consisting of a ceramide backbone (CER), a carbohydrate chain (glc=glucose, gal=galactose, GalNac=N-acetylgalactosamine), and one or more sialic acid (SA) residues. Parallel steps in both pathways are catalyzed by the same glycosyltransferase. (1) Glucosylceramide synthase, (2) Lactosylceramide synthase, (3) GM3 synthase, (4) GD3 synthase, (5) GM2/GD2 synthase, (6) GM1a/GD1b synthase, (7) GT1b/GD1a synthase, and (8) GQ1b/GT1a synthase.

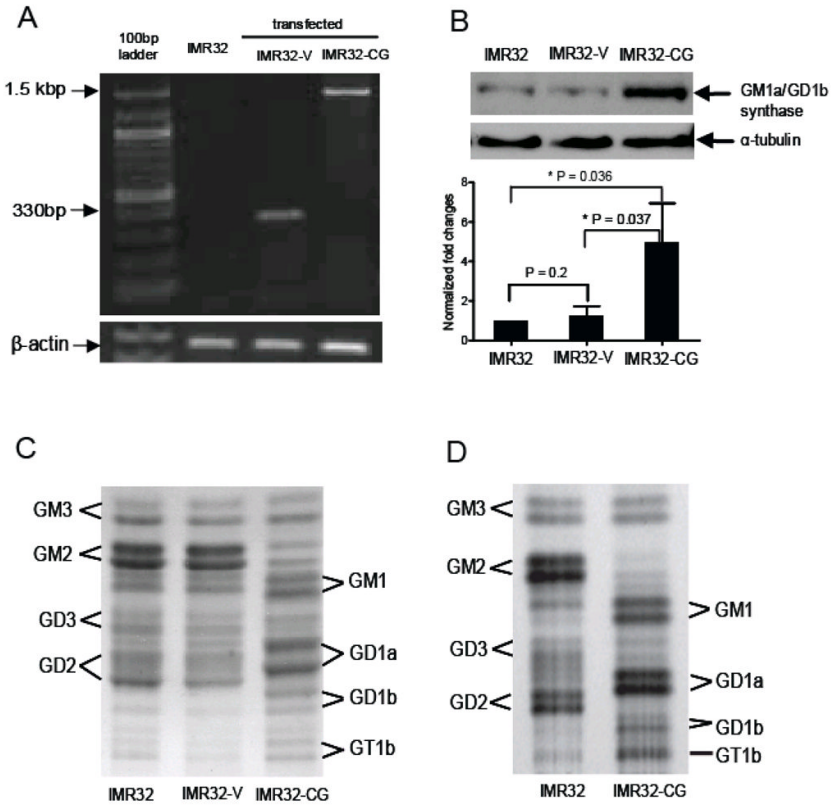

Figure 2.

Confirmation of stable transduction of IMR32 NB cells with GM1a/GD1b synthase. (A) cDNA by PCR. The length of PCR products obtained from GM1a/GD1b synthase/pLXIN transduced cells (IMR32-CG) and pLXIN transfected cells (IMR32-V) are ~1.5 kbp and 300 bp respectively. As control non-transduced cells, no band was seen in wild type IMR32 cells. (B) Expression of GM1a/GD1b synthase, analyzed by western blot. The bars are the mean ± SD fold changes in three separate experiments. Key: *$p<0.05$. (C) IMR32 cell ganglioside composition. Gangliosides were extracted, purified, and analyzed by resorcinol staining of the HPTLC. Note that each ganglioside is visualized as a doublet, reflecting the known heterogeneity of ganglioside ceramide structures. (D) Autoradiogram of wild type and IMR32-CG cell gangliosides. Note the disappearance of GD2 in the IMR32-CG cells.

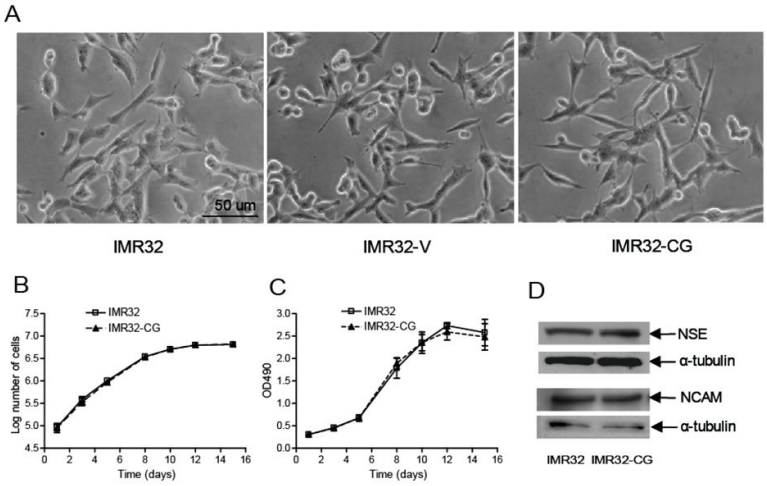

Figure 3.

Biological behavior of IMR32-CG cells. **(A)** Cell morphology. Wild type IMR32 cells, vector-transduced IMR32-V cells, and CG overexpressing IMR32-CG cells were analyzed by microscopy at subconfluence. Representative images (400x) are shown. **(B, C)** Cell proliferation assays. Cell growth of IMR32 and IMR32-CG cells was compared by cell counting (panel B) and tetrazolium/formazan assay (panel C). The mean ±SD of six separate experiments are shown; no significant difference was observed. (D) Cellular differentiation markers. Expression of NSE and NCAM by IMR32 and IMR32-CG cells was analyzed and compared by western blot.

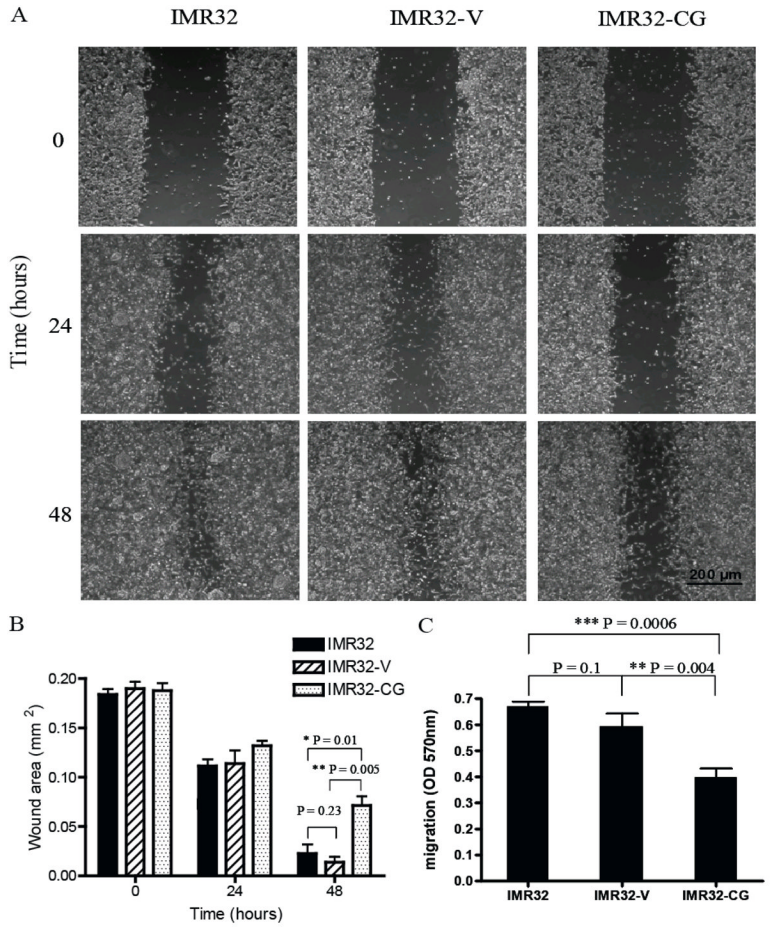

Figure 4.

Cell migration. Migration of IMR32, IMR32-V and IMR32-CG cells was assessed by the wound scratch (A, B) and Transwell (C) assays. Healing of the wound was assessed every 24 hours and photographed (panel A) and quantified (panel B). (C) Migration in the Transwell assay. 3×10^5 cells were seeded in each well, incubated for 18 hours and then stained with crystal violet. The number of migratory cells was quantified by spectrophotometry. Bars represent the mean ±SD of 3–4 experiments.

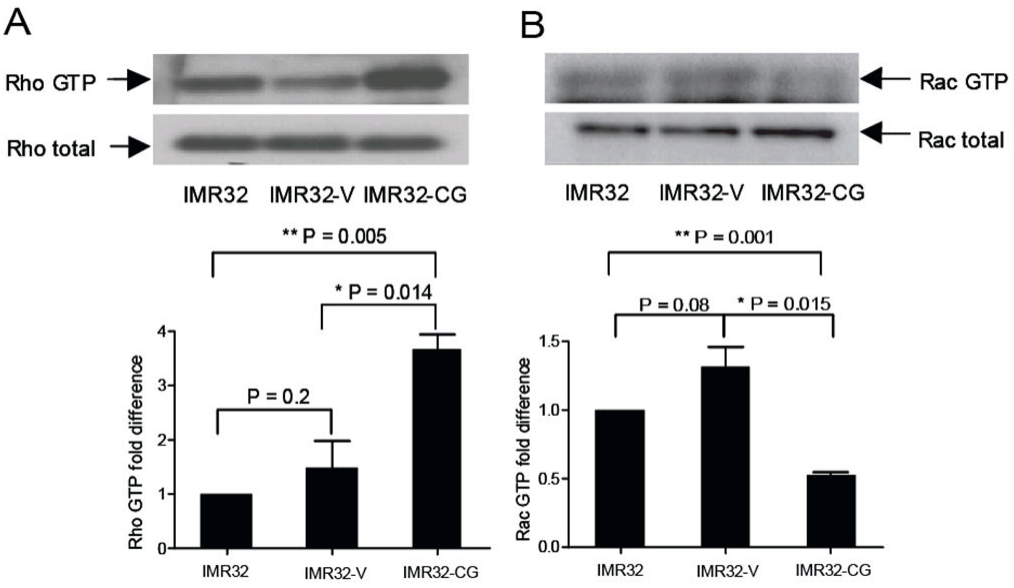

Figure 5.

Rho/Rac1 signaling in IMR32, IMR32-V and IMR32-CG. Rho GTP/total Rho (A) and Rac1 GTP/total Rac1 (B) were quantified in the three cell populations by western blot. Bars represent the mean ± SD of normalized fold differences of three separate experiments.

Table 1

Effect of GD1a/GM1a synthase overexpression on IMR32 cell ganglioside content

Cell	Total	Simple Gangliosides (SG)					Complex Gangliosides (CG)					CG/SG
	SG+CG	GM3	GM2	GD3	GD2	Total SG	GM1	GD1a	GD1b	GT1b	Total CG	
IMR32	31[1]	4	12	4	9	**28(89)**[2]	2	≪1	<1	<1	**3(11)**	0.12
IMR32-V	28	4	12	3	6	**25(89)**	2	≪1	<1	<1	**3(11)**	0.12
IMR32-CG	27	4	2	1	≪1	**7(26)**	7	9	2	2	**20(74)**	2.8

[1] nmol/108cells; mean of three separate experiments; SEM routinely <10% of the mean.

[2] % of total gangliosides