Alterations in neuroblastoma ganglioside synthesis by induction of GD1b synthase by retinoic acid

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Recent findings link increased expression of the structurally complex ‘b’ pathway gangliosides GD1b, GT1b, GQ1b (CbG) to a favourable clinical and biological behaviour in human neuroblastoma (NB). Seeking a model to probe these observations, we evaluated four human NB cell lines. Very low CbG content (4–10%) in three of the four cell lines (LAN-5, LAN-1, SMS-KCNR) reflected the ganglioside pattern observed in the most aggressive NB tumours. Pharmacological alterations of complex ganglioside synthesis in vitro by a 5–7 day exposure to 5–10 μM retinoic acid, which is employed in maintenance therapy of disseminated NB, included markedly increased (i) relative expression of CbG (6.6 ± 2.0-fold increase, P = 0.037), (ii) relative expression of the analogous ‘a’ pathway gangliosides, termed CaG (6.4 ± 1.4-fold increase in GM1a and GD1a; P = 0.010), and (iii) total cellular ganglioside content (2.0–6.3-fold), which in turn amplified the accumulation of structurally complex gangliosides. Substantial increases (2.7–2.9-fold) in the activity of GD1b/GM1a synthase (β-1,3-galactosyltransferase), which initiates the synthesis of CbG and CaG, accompanied the all-trans retinoic acid (ATRA)-induced ganglioside changes. Thus, increased CbG synthesis in NB cell lines is attributable to a specific effect of ATRA, namely induction of GD1b/GM1a synthase activity. Since the shift towards higher expression of CbG and CaG during retinoic acid-induced cellular differentiation reflects a ganglioside pattern found in clinically less-aggressive tumours, our studies suggest that complex gangliosides may play a role in the biological and clinical behaviour of NB.

Keywords: neuroblastoma; gangliosides; retinoic acid; GD1b synthase

Increasing evidence has implicated gangliosides, a specific class of cell surface glycosphingolipids, in the biological and clinical behaviour of many types of tumours including human neuroblastoma (NB). Gangliosides are overexpressed and actively shed by tumour cells (Wu et al., 1986; Valentino et al., 1990; Li and Ladisch, 1991) and have a number of biological properties that could conceivably alter tumour–host interactions to influence the survival of the malignant cells that carry these molecules (Hakomori, 1996). Our previous studies have linked specific ganglioside changes in human NB tumours to differences in the clinical and biological behaviour of this tumour (Kaucic et al., 2003). We have linked alterations in the expression of complex ‘b’ pathway gangliosides (CbG) downstream of GD1b/GM1a synthase (GD1b, GT1b and GQ1b) to differences in the biological phenotype and clinical behaviour that can be used to predict patient outcome in NB (Hettmer et al., 2003). Further, recent experimental evidence suggests that GD1b, GT1b and GQ1b can modulate a number of biological processes that are believed to counteract malignant transformation and progression, including tumour cell proliferation, host immune function and signal transduction mechanisms (Hynds et al., 1995; Fukumoto et al., 2000; Kanda et al., 2001; Kanda and Watanabe, 2001). We hypothesise that high CbG content may contribute to reduced tumour aggressiveness, and that alteration of CbG expression might be a potential therapeutic target in NB.

One pharmacological agent that is well known to alter cellular ganglioside metabolism and successfully used in the oral maintenance therapy of disseminated NB is retinoic acid (Matthy et al., 1999). Previous in vitro studies have shown that morphological signs of neuronal differentiation in response to retinoic acid are accompanied by an increase in total ganglioside content and a
relative increase in the expression of certain complex gangliosides of both the 'a' and 'b' pathway in NB cells (Li and Ladisch, 1992; Rebhan et al, 1994), as well as in normal and transformed embryonal cells (Levine and Flynn, 1986; Rizzo et al, 1995; Liour et al, 2000). On the basis of these findings, we have undertaken a comprehensive analysis of retinoic acid-induced changes in ganglioside expression and metabolism in human NB cell lines.

Here, we demonstrate that low CbG levels in some NB cell lines are analogous to the ganglioside pattern observed in clinically and biologically unfavourable NB tumours, providing an in vitro model to study pharmacological agents that might alter the metabolism of complex ganglioside subspecies. We further demonstrate that treatment with retinoic acid markedly enhances the activity of GD1b/GM1a synthase, resulting in increased expression of the complex gangliosides downstream of this enzyme, namely GD1b and GT1b (CbG), and GM1a and GD1a (CaG). Our results thus identify a specific effect of retinoic acid on the expression and biosynthesis of complex gangliosides in NB cells.
MATERIALS AND METHODS

Cell culture

Human NB cell lines LAN-1, LAN-5, SMS-KCN and SMS-KCNR were derived from three children with disseminated NB and have been previously described. SMS-KCN and SMS-KCNR were established from the same patient, from the primary tumour and bone marrow at relapse, respectively (Kohl et al., 1983; Reynolds et al., 1986; Rettig et al., 1987).

LAN-1 and LAN-5 were maintained in Waymouth's MB 752/1 medium (GIBCO, Grand Island, NY, USA), and SMS-KCN and SMS-KCNR in RPMI 1640 medium (GIBCO, Grand Island, NY, USA). The medium was supplemented with 2 ml/l-glutamine and 10% heat-inactivated foetal calf serum (GIBCO, Grand Island, NY, USA), respectively. Cells were grown in adherent monolayer cultures using 75-cm² and 175-cm² cell culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C in a 5% CO₂ atmosphere in a humidified incubator.

Retinoic acid (all-trans or 13-cis; Sigma Chemical Co., St Louis, MO, USA) was dissolved in ethanol to a concentration of 10⁻² M and kept as stock solution for up to 4 weeks at ~70°C, protected from light. For each experiment, stock solutions were diluted with growth medium to a final concentration of 5 or 10 μM retinoic acid. These concentrations are equivalent to the serum levels effective in the oral maintenance therapy of disseminated NB with 13-cis retinoic acid (13-cis RA) (7.2 ± 5.3 μM) (Reynolds and Lemons, 2001). The final concentration of ethanol in the culture medium was ≤ 0.2% (v/v).

Cells were seeded at 7 × 10⁴ cells cm⁻². After 24 h, the medium was replaced with a medium containing retinoic acid. Culture medium was replaced with fresh medium containing retinoic acid every other day. Untreated control cells were grown with and without ethanol. Cells were harvested after 5–7 days of exposure to retinoic acid, followed by ganglioside purification or extraction of crude membrane extracts, as described below. Viable cells were counted using trypan blue dye exclusion and the cellular protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). A bovine serum albumin standard was used.

Ganglioside purification and quantification

Methods for extraction and purification of gangliosides were previously described (Ladisch and Gillard, 1985). Briefly, total lipid extracts of cell pellets were obtained by extracting the lyophilised cell pellets twice with chloroform/methanol (1 : 1 v/v) at 4°C with stirring. Gangliosides were isolated by partitioning the dried total lipid extract in diisopropyl ether/1-butanol/17 mM aqueous NaCl solvent (60 : 40 : 9 by volume), and the gangliosides were stained with resorcinol. Absolute cellular ganglioside concentrations of human brain gangliosides (HBG) as the standard were measured by incorporation of radiolabelled sugars into ganglioside precursors (Kemp and Stoolmiller, 1976; Pohlitzen et al., 2000).

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Processing of glycosyltransferase assays

For each glycosyltransferase assay, buffer mixtures containing 30 nmol GM3 (Sigma Chemical Co., St Louis, MO, USA), 0.01 Ci ¹⁴C-labelled CMP sialic acid ([sialic-4,5,6,7,8,9-¹⁴C], 150 mCi mmol⁻¹), 10 nmol ‘cold’ CMP sialic acid, 2.5 μmol of MES buffer (pH 5.9), 2.5 μmol of KCl, 0.1 μmol of MnCl₂ and 0.3 μmol of MgCl₂.

To assess β,1-4-N-acetylgalactosaminyltransferase (GD2 synthase) activity, 30 nmol GD3 (Matreya Inc., Pleasant Gap, PA, USA), 0.01 μCi ¹³C-labelled CMP sialic acid ([sialic-4,5,6,7,8,9-¹³C], 150 mCi mmol⁻¹), 10 nmol ‘cold’ CMP sialic acid, 2.5 μmol of MES buffer (pH 5.9), 2.5 μmol of KCl, 0.1 μmol of MnCl₂ and 0.3 μmol of MgCl₂.

For evaluation of β,1-3-galactosyltransferase (GD1b synthase) activity, the reaction mixture contained 10 nmol GD2 (Sigma Chemical Co., St Louis, MO, USA), 0.01 μCi ¹³C-labelled UDP galactose ([galactose-¹³C(U)], 250 mCi mmol⁻¹), 20 nmol ‘cold’ galactose, 2.5 μmol MES (pH 6.5) and 2.1 μmol MnCl₂.

For each glycosyltransferase assay, buffer mixtures containing MES (Calbiochem, San Diego, CA, USA), KCl, MnCl₂ and MgCl₂ (Sigma Chemical Co., St Louis, MO, USA) were freshly prepared. Radiolabelled and ‘cold’ sugar donors were obtained from Perkin–Elmer Life Sciences Inc. (Boston, MA, USA) and Calbiochem (San Diego, CA, USA), respectively. All enzyme assays were performed in duplicates with the appropriate blank.

Separation of lipid-soluble reaction products

Lipid-soluble compounds in the reaction mixture were isolated by passing them through columns of Sephadex G25 Superfine (1 × 3–5 cm in
To measure the rate of incorporation of radiolabelled sugars, the column effluents were transferred in part (40%) to glass scintillation vials, evaporated to dryness under a stream of nitrogen, and dissolved in 10 ml Betafluor (National Diagnostics, Superfine (Sigma Chemical Co., St Louis, MO, USA) was suspended in water (5 g per 60 ml), stored overnight at 4°C and shaken vigorously. Before pouring the columns, the gel was brought to room temperature and washed twice with the original volume of methanol (≥ 30 min each time). One-third of the supernatant was decanted, the mixture was shaken vigorously to obtain a homogenous solution and the Pasteur pipettes were filled to the top. After the solvent had drained, the gel was overlayed with 1.5 ml methanol and equilibrated with chloroform/methanol/water (120:60:9 v/v). The stopped samples were applied to the columns, the sample tubes washed with 1 ml chloroform/methanol/water (120:60:9 v/v) and elution of the products was completed by adding 2 ml of the latter (Pohlentz et al, 2000).

**Determination of glycosyltransferase activities and product identification** To measure the rate of incorporation of radiolabelled sugars, the column effluents were transferred in part (40%) to glass scintillation vials, evaporated to dryness under a stream of nitrogen, and dissolved in 10 ml Betafluor (National Diagnostics, Mannville, NJ, USA). Radioactivity (measured as d.p.m.) was determined in a Beckman LS6500 liquid scintillation counter. Quench correction was accomplished using the AutoDPM program. Enzyme activity (pmol h⁻¹ mg protein⁻¹) was calculated from the ratio of the products and the radioactivity of the total sample. The remainder of the sample was used to confirm the identity and the purity of the product by TLC autoradiography.

**Statistical analysis**

The relative expression of individual gangliosides and ganglioside subsets (CbG and CaG) in each of the four cell lines studied were expressed as the mean ± s.e.m. for three to five separate experiments. For the three cell lines that were responsive to retinoic acid, the relative ganglioside content was similarly reported as the mean ± s.e.m. and, alternatively, the per cent of the expression of parallel control cells for two separate experiments per cell line. Data from six separate experiments involving three different cell lines were normalised by relating results in each experimental group to their respective controls by expressing the experimental (exp) levels as a per cent of the control (ctrl) levels (exp/ctrl × 100). Control values of zero were set to 1 before the computation. To test the hypothesis that experimental values differed from controls, we employed a t-test to examine whether the experimental values differed from 100%. The same method was used to compare glycosyltransferase activities among retinoic acid-treated and control cells in retinoic acid-responsive cell lines (LAN-1, LAN-5, SMS-KCNR).

To examine whether experimental values differed from controls in studies of 13-cis RA, mean treatment values were compared to control values (no treatment with 13-cis RA) using a t-test.

**RESULTS**

**Ganglioside expression in human NB cell lines**

The impetus for this study were our findings (Hettmer et al, 2003) and those of others (Schengrund et al, 1985; Sung et al, 1995; Yates et al, 1999), suggesting that differences in tumour tissue CbG (GD1b, GT1b and GQ1b) expression are linked to the clinical and biological behaviour of NB and other tumours. In order to obtain an in vitro model that permits a more dynamic view of NB ganglioside biosynthesis, we analysed ganglioside expression in four well-described NB cell lines, LAN-1, LAN-5, SMS-KCNR, and SMS-KCN.

To analyse ganglioside expression, we grew NB cells to 80–90% confluence, then extracted total cellular gangliosides and separated them by HPTLC (Figure 2). Gangliosides were measured (i) as absolute cellular ganglioside content (nmol lipid-bound sialic acid (LBSA) 10⁶ cells⁻¹) and (ii) as relative percentages of individual gangliosides out of total cellular gangliosides. Absolute cellular ganglioside content differed widely among the NB cell lines studied (69 nmol LBSA 10⁶ cells⁻¹ for SMS-KCN, 57 nmol LBSA 10⁶ cells⁻¹ for LAN-5, 30 nmol LBSA 10⁶ cells⁻¹ for LAN-1 and 15 nmol LBSA 10⁶ cells⁻¹ for SMS-KCNR). Nevertheless, there were clear similarities in the relative ganglioside content of the four cell lines (Table 1). The most prominent components were the ‘b’ pathway dialysialoganglioside GD2 (26–60%) and the ‘a’ pathway monosialoganglioside GM2 (22–35%). Complex ‘b’ pathway gangliosides represented only minor components (≤10%) in three of the four NB cell lines studied (10% in LAN-5, 4% in LAN-1 and 8% in SMS-KCN), and were composed of the disialylated molecule GD1b (2%) and the trisialylated ganglioside GT1b (2–8%). The fourth cell line, SMS-KCN, contained 21% CbG (2% GD1b and 19% GT1b). There were no detectable amounts of the most complex molecule, GQ1b, in any of the cell lines studied.

The prominent position of GD2 as a major ganglioside component in the NB cell lines studied (44% in LAN-5, 36% in LAN-1, 60% in SMS-KCNR and 26% in SMS-KCN) mirrors the

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**Table 1** Ganglioside expression in neuroblastoma cell lines

| Ganglioside expression (% of total cellular gangliosides)* | LAN-5 | LAN-1 | SMS-KCNR | SMS-KCN |
|----------------------------------------------------------|-------|-------|----------|---------|
| GM3                                                      | 0 ± 0 | 2 ± 1 | 0 ± 0    | 1 ± 0   |
| GM1a                                                    | 35 ± 8| 35 ± 12| 22 ± 4   | 34 ± 5  |
| GD1a                                                    | 2 ± 1 | 1 ± 0 | 1 ± 0    | 2 ± 1   |
| GD2                                                      | 2 ± 0 | 0 ± 0 | 3 ± 0    | 17 ± 5  |
| CaG                                                      | 4 ± 1 | 1 ± 0 | 4 ± 0    | 18 ± 5  |
| GD3                                                      | 8 ± 3 | 22 ± 4| 7 ± 2    | 1 ± 0   |
| GD2                                                      | 44 ± 5| 36 ± 8| 60 ± 6   | 26 ± 4  |
| GD1b                                                    | 2 ± 1 | 2 ± 1 | 2 ± 1    | 2 ± 1   |
| GT1b                                                    | 8 ± 2 | 2 ± 1 | 7 ± 3    | 19 ± 3  |
| GQ1b                                                    | 0 ± 0 | 0 ± 0 | 0 ± 0    | 0 ± 0   |
| CbG                                                      | 10 ± 3| 4 ± 2 | 8 ± 3    | 21 ± 4  |

*Values represent means ± s.e.m. of three independent experiments, except for LAN-5 which consisted of five independent experiments.
ubiquitously high expression of GD2 that characterizes NB tumours (Wu et al., 1986). Importantly, the low CbG expression observed in each of the cell lines is also analogous to the CbG levels found in clinically and biologically unfavourable NB tumours (Hettmer et al., 2003). We therefore used these four NB cell lines as an in vitro model to determine whether retinoic acid, as a pharmacological agent, might alter the expression and biosynthesis of CbG.

Retinoic-acid induced changes in CbG expression in NB cell lines

Retinoids are a class of vitamin A derivatives that differ in their isomeric conformations, and include all-trans retinoic acid (ATRA), 9-cis retinoic acid and 13-cis RA. Owing to its superior pharmacokinetics (higher and more sustained plasma levels), 13-cis RA is the isomer that is primarily used clinically. However, 13-cis RA isomersise in vivo to both ATRA and 9-cis retinoic acid, which are believed to be the active compounds in vivo in nonocular tissue (Lovat et al, 1994). Since retinoic acid treatment has been associated with changes in the ganglioside complement of various cell types (Li and Ladisch, 1992; Rebhan et al., 1994; Liour et al., 2000), and the oral maintenance therapy of disseminated NB with 13-cis RA results in improved event-free survival (Matthey et al., 1999), we sought to specifically explore the effect of retinoic acid on CbG expression. We, therefore, first treated NB cells in vitro with 10 μM ATRA, then evaluated ATRA-induced changes in the composition of individual gangliosides and in total cellular ganglioside content in LAN-1, LAN-5, SMS-KCN and SMS-KCN cells (Table 2). Strikingly, ATRA induced a shift from synthesis of simpler gangliosides towards more complex species within both the 'a' and 'b' pathways in three of the four cell lines (LAN-1, LAN-5 and SMS-KCN). In these three cell lines, the redistribution towards complex gangliosides included increased relative expression of GD1b and GT1b ('b' pathway) and GM1a and GD1a ('a' pathway), compounded by a substantial increase in total cellular ganglioside content, making the increase in the absolute content of CbG and CaG even more striking (Table 2). In LAN-5 cells for example, the ATRA-induced changes in cellular ganglioside content included increased relative expression of GD1b+GT1b (CbG) and GD2 (from 6 to 22%), a 2.2-fold increase in absolute ganglioside content (from 6 to 22%), a 1.5-fold increase in CaG content (from 6 to 22%) and a 2.2-fold increase in total ganglioside content (from 6 to 22%). In SMS-KCN cells, the ATRA-induced changes in cellular ganglioside content included increased relative expression of GD1b+GT1b (CbG) and GD2 (from 6 to 22%), a 2.2-fold increase in absolute ganglioside content (from 6 to 22%) and a 2.2-fold increase in total ganglioside content (from 6 to 22%).

Table 2  Effect of ATRA on neuroblastoma cell ganglioside expression

|                | GpG1b+GpT1b (CbG) | GD2     | GD3     | GD1a+GpM1a (CaG) | GM2 | GM3 | Total | CbGb | CaGb |
|----------------|-------------------|---------|---------|------------------|-----|-----|-------|------|------|
| LAN-1          |                   |         |         |                  |     |     |       |      |      |
| Ctrl           | 3 ± 2             | 29 ± 1  | 18 ± 1  | 1 ± 0            | 48 ± 2| 3 ± 1| 30    | 0.9  | 0.3  |
| ATRA           | 13 ± 3            | 29 ± 1  | 6 ± 5   | 8 ± 2            | 44 ± 5| 1 ± 0| 70    | 9.1  | 5.6  |
| LAN-5          |                   |         |         |                  |     |     |       |      |      |
| Ctrl           | 6 ± 4             | 48 ± 3  | 5 ± 2   | 4 ± 2            | 38 ± 6| 0 ± 0| 57    | 3.4  | 2.3  |
| ATRA           | 22 ± 5            | 32 ± 3  | 3 ± 1   | 9 ± 3            | 35 ± 4| 0 ± 0| 112   | 24.6 | 10.1 |
| SMS-KCN        |                   |         |         |                  |     |     |       |      |      |
| Ctrl           | 10 ± 5            | 64 ± 9  | 8 ± 4   | 4 ± 1            | 16 ± 1| 0 ± 0| 15    | 1.5  | 0.6  |
| ATRA           | 40 ± 3            | 27 ± 2  | 1 ± 0   | 30 ± 1           | 5 ± 4 | 0 ± 0| 94    | 37.6 | 28.2 |
| SMS-KCN        |                   |         |         |                  |     |     |       |      |      |
| Ctrl           | 23 ± 8            | 21 ± 2  | 1 ± 0   | 24 ± 1           | 31 ± 7| 1 ± 0| 69    | 15.9 | 16.6 |
| ATRA           | 23 ± 4            | 11 ± 1  | 6 ± 5   | 18 ± 6           | 43 ± 6| 6 ± 4| 50    | 11.5 | 9.0  |

*Values represent means ± standard errors of two independent experiments. **Calculated from the absolute total ganglioside content and the mean relative content of CaG or CbG. ATRA = all-trans retinoic acid; Ctrl = control; CbG = complex b' pathway gangliosides; CaG = complex a' pathway gangliosides.

Table 3  Relative changes in neuroblastoma cell ganglioside expression in ATRA-responsive cell lines

|                | GpG1b+GpT1b (CbG) | GD2     | GD3     | GD1a+GpM1a (CaG) | GM2 | GM3 | Total | CbGb | CaGb |
|----------------|-------------------|---------|---------|------------------|-----|-----|-------|------|------|
| LAN-1          |                   |         |         |                  |     |     |       |      |      |
| Exp            |                   |         |         |                  |     |     |       |      |      |
| a              | 250               | 96      | 6       | 1000             | 104 | 33  | 22    | 60   | 80   |
| b              | 1500              | 104     | 53      | 600              | 80  | 50  | 100   | 60   | 80   |
| LAN-5          |                   |         |         |                  |     |     |       |      |      |
| Exp            |                   |         |         |                  |     |     |       |      |      |
| a              | 300               | 57      | 67      | 220              | 97  | 100 | 22    | 97   | 100  |
| b              | 850               | 76      | 57      | 300              | 91  | 100 | 22    | 97   | 100  |
| SMS-KCN        |                   |         |         |                  |     |     |       |      |      |
| Exp            |                   |         |         |                  |     |     |       |      |      |
| a              | 740               | 38      | 25      | 967              | 50  | 100 | 22    | 97   | 100  |
| b              | 307               | 45      | 0       | 750              | 7   | 100 | 22    | 97   | 100  |
| Mean ± s.e.m.  | 659 ± 202         | 69 ± 11 | 35 ± 12 | 640 ± 137      | 72 ± 15 | 80 ± 13 |
| P-value        | 0.037             | 0.040   | 0.002   | 0.010           | 0.117 | 0.180 |

*For each cell line, two separate experiments (a and b) were analysed. **Mean ± s.e.m. of normalised values for all three cell lines (two experiments per cell line; six experiments total). Bold values are statistically significant (P < 0.050). t-tests. ATRA = all-trans retinoic acid; CbG = complex b' pathway gangliosides; CaG = complex a' pathway gangliosides.
Experimental Therapeutics

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Table 4 Effect of ATRA on neuroblastoma (NB) cell glycosyltransferase activities

| Enzyme activity (pmol h⁻¹ × mg protein)ᵃ | GD1b synthase | GD2 synthase | GD3 synthase |
|------------------------------------------|---------------|--------------|--------------|
| LAN-5                                    | 215           | 1416         | 572          |
| ATRA                                     | 572           | 1384         | 669          |
| SMS-KCNR                                  | 172           | 518          | 364          |
| ATRA                                     | 497           | 927          | 511          |
| SMS-KCN                                  | 186           | 1143         | 333          |
| ATRA                                     | 283           | 769          | 405          |

ᵃActivities of GD1b synthase, GD2 synthase and GD3 synthase in NB cells treated with 10 μM all-trans retinoic acid (ATRA) are compared to untreated cells (Ctrl). Enzyme activities were determined by incorporation of radiolabelled sugars into ganglioside precursors. Values represent means from duplicate samples of one to two experiments. ATRA = all-trans retinoic acid; Ctrl = control; CbG = complex 'b' pathway gangliosides; CaG = complex 'a' pathway gangliosides.

Figure 3 Effect of all-trans retinoic acid (ATRA) on cell morphology (A) and cellular ganglioside expression (B) in the human NB cell line SMS-KCNR. Cells were plated at 7 × 10⁴ cells and treated with ATRA 10 μM on days 1–7. Photomicrograph (× 100) was obtained on day 6. Cells were harvested on day 7, and gangliosides were purified and separated by HPTLC (Ctrl, medium alone; Ctrl*, medium containing 0.1% ethanol; ATRA, medium containing 10 μM ATRA). GD2, GD1a and GT1b are doublets in tumour samples. The lower GD1a band and the upper GD2 band overlap.

Figure 4 Effect of 13-cis retinoic acid (13-cis RA) on CbG (GD1b, GT1b; black bars) and CaG (GM1a, GD1a; grey bars) expression in LAN-1 human NB cells. Bars represent means ± s.e.m. Ganglioside subsets are expressed as a percentage of total cellular gangliosides. Experimental and controls values were compared using a t-test (*P < 0.050).

Expression (69% vs 50 nmol 10⁸ cells⁻¹). When normalised data from the three responsive cell lines were analysed together (Table 3), ATRA treatment clearly resulted in significant increases in the cellular content of CbG (658 ± 202% of control, P = 0.037) and CaG (640 ± 137% of control, P = 0.010), and, concomitantly, significant decreases in the cellular content of GD2 (69 ± 11% of control; P = 0.040) and GD3 (35 ± 12% of control; P = 0.002).

To illustrate these changes, Figure 3 summarises the ATRA-induced changes in cell morphology and ganglioside expression of the NB cell line SMS-KCNR. The morphological changes in response to ATRA, including rounding up of the cell body, extension of long processes with the appearance of neurites and cell aggregation into tight clusters (Figure 3A), are consistent with previous studies evaluating the differentiating effects of ATRA on NB cells (Li and Ladisch, 1992; Melino et al, 1997). Qualitative changes in the composition of cell-surface gangliosides accompanied morphological signs of differentiation in response to ATRA. The most prominent ganglioside components in untreated SMS-KCNR cells were the 'b' pathway ganglioside GD2 and the 'a' pathway ganglioside GM2. Treatment with ATRA increased the proportions of the CbG (GD1b and GT1b), as well as the CaG (GD1a and GM1a), such that these two subgroups predominated relative to other ganglioside species (Figure 3B). In view of the increase in total cellular ganglioside content (Table 2), this relative change resulted in significant absolute changes in the membrane expression of complex ganglioside molecules. Thus, the mean four-fold increase in relative CbG expression translated into a 20-fold increase in absolute cellular CbG expression in SMS-KCNR cells, indicating a striking accumulation of these biologically active molecules.

Since 13-cis RA is currently the only retinoid compound in clinical use to treat NB (Reynolds and Lemons, 2001), we also exposed one NB cell line, LAN-1, to 13-cis RA at two different concentrations (5 μM, 10 μM). We observed that 13-cis RA caused a concentration-related increase in the expression of structurally complex gangliosides in LAN-1 cells (Figure 4). As with ATRA-treated cells, the increase in CbG expression was paralleled by a concurrent increase in CaG, suggesting a specific effect of retinoids on the activity of GD1b/GM1a synthase (β-1,3-galactosyltransferase), which initiates the synthesis of both CbG and CaG, from GD2 and GM2, respectively.
Effect of retinoic acid on the activity of ganglioside glycosyltransferases in human NB cells in vitro

To delineate the metabolic basis for the ATRA-induced shift towards expression of complex gangliosides, we determined the effect of ATRA treatment on the activity of three key enzymes in the synthesis of these molecules. Precursor molecules enter ‘b’ pathway ganglioside biosynthesis through conversion of GM3 into GD3 by GD3 synthase (β-2,8-sialyltransferase), GD2/GM2 synthase (β-1,4-N-acetylglactosaminyltransferase) and GD1b/GM1a synthase (β-1,3-glactosyltransferase) catalyse the two subsequent enzymatic reactions resulting in the synthesis of the most complex gangliosides in each pathway (van Echten and Sandhoff, 1993) (Figure 1). We measured the activities of GD3 synthase, GD2 synthase and GD1b synthase in crude membrane extracts from LAN-5, SMS-KCNR, SMS-KCN, using excess GM3, GD3 and GD2 as exogenously added ganglioside precursors.

When the retinoic acid-responsive LAN-5 or SMS-KCN cells were exposed to 10 μM ATRA for 6–7 days (Table 4), we observed a marked increase (2.7–2.9-fold, \( P < 0.050 \)) in GD1b synthase activity. This was accompanied by a lesser (0–1.8-fold, \( P = 0.198 \)) increase in GD2 synthase activity, and only small changes in GD3 synthase activity (1.2–1.4, \( P = 0.161 \)). Thus, in both LAN-5 and SMS-KCN cells, increased expression of complex gangliosides downstream of GD1b synthase was associated with a significant (over two-fold) increase in GD1b synthase activity. In contrast, in SMS-KCN cells, the cell line that was unresponsive to ATRA treatment, GD1b synthase activity was only slightly increased (1.5-fold) in ATRA-treated vs control cells.

Figure 5 illustrates the autoradiographic visualisation of enzyme products resulting from ATRA-induced changes in ganglioside metabolism in SMS-KCNR cells, confirming the major increase in GD1b synthase activity (Figure 5A), a comparatively minor increase in GD2 synthase activity (Figure 5B) and almost no change in GD3 synthase activity (Figure 5C). It is interesting to note that in the case of the ATRA-treated cell samples, the parallel blank (which does not contain exogenously added ganglioside precursors) also shows the synthesis of GD1b and GM1a from the trace amounts of endogenous gangliosides present in the cell pellet (Figure 5A), further confirming the substantial increase in the activity of GD1b/GM1a synthase following treatment with ATRA. Taken together, our data demonstrate that the increase in GD1b synthase activity is specifically associated with increased expression of complex ganglioside species, induced by ATRA in NB cells in vitro, and provides strong evidence that elevated CbG and CaG expression can be accounted for by a specific effect of ATRA on this enzyme.

**DISCUSSION**

Treatment with retinoic acid is shown here to induce a dramatic shift from synthesis of simpler gangliosides towards predominant expression of structurally complex ‘a’ and ‘b’ pathway ganglioside molecules downstream of GD1b/GM1a synthase in some NB cell lines. The increase in total cellular ganglioside content combined with the relative changes in the proportion of complex gangliosides results in a significant increase in the absolute cellular content of these molecules. The findings are consistent with the previous observations of a dramatic increase in membrane expression and shedding of total cellular gangliosides and enhancement in GD1a and GT1b content in LAN-5 NB cells treated with retinoic acid (Li and Ladisch, 1992).

Among the four cell lines employed in this study, the NB cell line SMS-KCN appears to be biologically distinct. Three differences are evident. First, SMS-KCN cells consist of two morphologically distinct cell populations, one neuroblast-like (N-type) and the second substrate-adherent (S-type) with epithelial or fibroblast-like morphology (Rettig et al, 1987), while LAN-1, LAN-5 and SMS-KCNR exhibit a uniform phenotype with small, round, neuroblastic cells that have short neuritic processes. Second, constitutive CbG content in SMS-KCN was higher (1.8- to 5.2-fold) than in the other cell lines studied. Third, treatment with retinoic acid did not induce the alterations in cellular ganglioside biosynthesis observed in the other cell lines. These differences in ganglioside metabolism

**Figure 5** Effect of all-trans retinoic acid (ATRA) on the activities of GD1b synthase, GD2 synthase and GD3 synthase in the human NB cell line SMS-KCNR. HPTLC autoradiograms demonstrate reaction products from assays measuring activities of GD1b synthase (A), GD2 synthase (B) and GD3 synthase (C). Enzyme assays were performed in duplicate (1,2) with an appropriate ‘blank’ (Ctrl, medium alone; ATRA, 10 μM).
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do not have an obvious explanation, but it is possible that they are accounted for by constitutive differences between N-type and S-type cells.

One possible explanation for the retinoic acid-induced changes in ganglioside composition in LAN-1, LAN-5 and SMS-KCNN cells is an increased rate of synthesis of certain gangliosides. A previous study in embryonal carcinoma cells demonstrated enhanced activities of various ganglioside glycosyltransferases at different stages of retinoic acid-induced neuronal differentiation, and a parallel increase in mRNA expression, suggesting that retinoic acid activated the transcription of these glycosyltransferases or stabilised their expression (Osanai et al, 1997; Liour et al, 2000).

Our data support that the retinoic acid-induced increase in the expression of complex gangliosides in NB cells transfected with GD3 synthase occurs in parallel with trk-A dimerisation (Fukumoto et al, 2000), which in turn is associated with improved prognosis in NB (Nagawara et al, 1993). Taking these findings together, we hypothesise that CbG may impede tumour progression, either by acting directly on the tumour cell or by increasing the host resistance.

Similarly, increased tumour CbG expression might contribute to the success of retinoic acid in the maintenance therapy of disseminated NB. The beneficial role of retinoic acid is generally attributed to a reversal of the malignant state through differentiation of the tumour cells into more mature ganglion-like cells (Matthay et al, 1999; Reynolds and Lemons, 2001). Substantial experimental evidence suggests that complex gangliosides are biologically relevant molecules during cellular differentiation and development. First, outgrowth of cellular extensions, one aspect of neuronal differentiation, can be induced by treating NB cells in vitro with exogenous gangliosides, and the CbG species GT1b and GQ1b have been repeatedly described as the two most potent ganglioside molecules with differentiating properties (Tsujii et al, 1983; Leskawa and Hogan, 1985). Secondly, increased expression of GD1b and GT1b in murine NB cells transfected with GD3 synthase occurs in parallel with morphological signs of increased cellular differentiation (Kojima et al, 1994). Thirdly, absence of all gangliosides downstream of GM2/GD2 synthase (Cbg + GD2 and CaG + GM2) in genetically altered mice with a disrupted gene for that enzyme is associated with decreased myelination and axon degeneration in the central and peripheral nervous system (Sheikh et al, 1999), although brain development and gross behaviour is normal (Takamiya et al, 1996). Finally, although retinoic acid-induced neurite outgrowth in NB cells is inhibited by abrogation of cellular ganglioside synthesis in LAN-5 cells grown in a serum-supplemented environment (Li and Ladisch, 1997), reduction of cellular ganglioside synthesis is associated with decreased NGF-induced outgrowth of neuritic processes in a NB cell line grown in serum-free medium, and is reversible by exogenous addition of gangliosides (Rosner, 1998).

Previous findings and our demonstration of induction of CbG (and CaG) synthesis by retinoic acid underscore the potential role of complex gangliosides in cellular differentiation and other cellular functions important to reversion of the malignant phenotype. Consequently, the specific role of complex gangliosides compared to structurally simpler molecules in influencing cellular differentiation and other cellular functions in NB and other neuroectodermal tumours will be important to delineate.

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(Srinivas and Colburn, 1982). Proliferation of and IL-8 production by human metastatic melanoma cells is inhibited by GD1b, GT1b and GQ1b (Kanda et al, 2001). Platelet-derived growth factor (PDGF)-mediated cell growth and PDGF receptor activation in NB and gloma cells are inhibited by complex ganglioside species (Hynds et al, 1995). These gangliosides also modulate immunoglobulin synthesis in peripheral blood mononuclear cells (Kanda and Tamaki, 1998, 1999a,b) and cause a shift from Th-2 to Th-1 cytokine production in PHA-stimulated T cells (Kanda et al, 2001). Finally, increased expression of GD1b and GT1b in rat pheochromocytoma cells transfected with GD3 synthase occurs in parallel with trk-A dimerisation (Fukumoto et al, 2000), which in turn is associated with improved prognosis in NB (Nagawara et al, 1993). Taking these findings together, we hypothesise that CbG may impede tumour progression, either by acting directly on the tumour cell or by increasing the host resistance.

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