Deficiency of ganglioside biosynthesis in metastatic human melanoma cells: relevance of CMP-NeuAc:LacCer α2–3 sialyltransferase (GM3 synthase)

Noureddine Zebda, Sandrine Pedron, Abdelhadi Rebbaa, Jacques Portoukalian*, Odile Berthier-Vergnes**

INSERM U218, Centre Léon Bérard, 28 Rue Laënnec, 69373 Lyon Cx 08, France

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Abstract The glycosphingolipid patterns were analyzed on two clones derived from a human melanoma cell line and selected for their respectively high and low metastatic ability in immunosuppressed newborn rats. Conversely to the weakly metastatic cells which exhibited a pattern similar to that of the parental cell line, highly metastatic human melanoma cells appeared to be deficient in ganglioside biosynthesis. An accumulation of lactosylceramide was found in the latter cells, with low amounts of GM3 as the only ganglioside detected and a fourfold decreased activity of GM3 synthase (EC 2.4.99.9). After subcutaneous injection of metastatic cells in newborn rats, the cells proliferating in the tumor induced at the injection site re-expressed the four common gangliosides of melanoma: GM3, GM2, GD3 and GD2, whereas the cells growing in the lungs as metastatic nodules were deficient in ganglioside synthesis and showed an accumulation of lactosylceramide. Taken together, our results suggest that the human melanoma cells which are able to escape from the primary tumor and invade the lungs have an impaired ganglioside biosynthesis with a deficient GM3 synthase.

Key words: Human melanoma; Metastasis; Glycosphingolipids; Gangliosides; GM3 synthase; Sialic acid

1. Introduction

Gangliosides are ubiquitous membrane-associated sialic acid-containing glycosphingolipids implicated in a broad range of biological functions. Their location on the outer leaflet of the lipid bilayer of the plasma membrane make them specific mediators for cellular adhesion and intercellular recognition phenomena [1,2] involved in the multi-step process of the metastatic diffusion of cancer cells [3]. Although ganglioside patterns exhibit cellular lineage-dependant specificity [4], changes in the structure, synthesis and cell surface exposure of glycosphingolipids are associated with metastatic phenotype in various experimental tumor systems [5–7].

Human melanoma tissues and cell lines express GM3, GM2, GD3 and GD2 as major gangliosides, whereas normal melanocytes essentially synthesize GM3 ganglioside [8–10]. Mouse and human monoclonal antibodies (MAbs) to disialogangliosides GD2 and GD3 have thus been used in clinical trials to treat melanoma patients [11–13]. In the same way, active immunotherapeutical trials using ganglioside vaccines have been conducted [14–16]. However, little is known about the gangliosides exposed on human malignant melanocytes that are able to escape from the primary tumor to establish distant metastases.

We recently reported evidence suggesting that deficient sialylation of glycoproteins is involved in the metastatic capacity of human malignant melanocytes [17]. In a spontaneous metastasis model of human melanoma recently established in our laboratory [18,19], PNA-binding glycoproteins are expressed in human melanoma cells able to give lung metastases whereas these cell surface glycoproteins in non-metastatic cells are sialylated and do not bind PNA lectin [20]. Sialyltransferases being also essential enzymes in the biosynthesis of gangliosides, we determined the patterns of gangliosides of metastatic (T1C3) and non-metastatic (IC8) clones of our melanoma model, as well as those of the corresponding tumors grown in immunosuppressed newborn rats. The present data indicate that human melanoma cells able to give lung metastases are defective in ganglioside biosynthesis and accumulate their common precursor, lactosylceramide (LacCer) because of a lowered activity of CMP-NeuAc:LacCer a2–3 sialyltransferase (EC 2.4.99.9).

2. Materials and methods

2.1. Reference compounds and reagents

Gangliosides GM3, GM2, GD3 and GD2 were purified from human melanoma tumors [16]. Glycosphingolipids: CMH and CTH were purified from human thyroid tumors [21]. Lactosylceramide (LacCer), used for CMP-NeuAc:LacCer a2–3 sialyltransferase (EC 2.4.99.9) assay was isolated from human melanoma tissue and shown to be pure according to the procedure previously described [22]. CMP-[^{14}C]NeuAc was purchased from Amersham (Les Ulis, France, 10.5 GBq/mmol). Anti-thymocyte serum (ATS) was prepared according to Bailly et al. [19].

2.2. Cells and cell culture

Two clones (IC8 and T1C3) derived from M4Be human melanoma cell line were used in this study. The procedures for selection and cloning and the culture conditions have been described elsewhere [18, 20]. Cell cultures were regularly tested for absence of mycoplasma and preserved metastatic capability. The metastasis incidence and the number of lung nodules in immunosuppressed newborn rats were assessed using a standardized protocol as described [18,19].

*Corresponding author. Fax: (33) 78 74 83 90.

***Present address:* INSERM U346, Clinique Dermatologique (Pavillon R), Hôpital Edouard Herriot, 69437 Lyon Cx 03, France.

Abbreviations: Cer, ceramide; CDH, ceramide dihexoside; CMH, ceramide monohexoside; CMP-NeuAc, cytidine 5′-monophosphoryl *N*-acetylneuraminic acid; CTH, ceramide trihexoside; GlcCer, glucosylceramide (Glcβ1–1Cer); HPTLC, high-performance thin-layer chromatography; LacCer, lactosylceramide (Galβ1–4Glcβ1–1Cer); MAb, monoclonal antibody; NeuAc, *N*-acetylneuraminic acid; TLC, thin-layer chromatography. Gangliosides are referred to according to Svennerholm [37].

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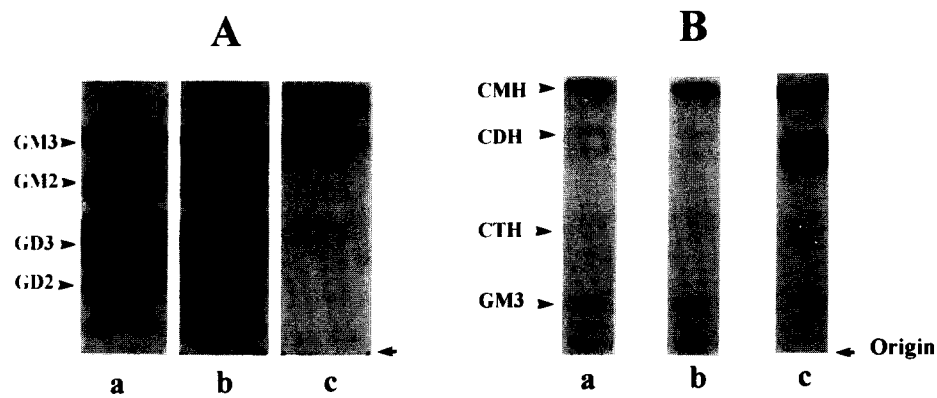

Fig. 1. HPTLC patterns of gangliosides (A) and neutral glycosphingolipids (B) extracted from the low metastatic parental human melanoma cell line M4Be (a) and IC8 clone (b) and from the high metastatic T1C3 clone (c). Glycosphingolipids corresponding to 2 mg cellular proteins were chromatographed on HPTLC plates and visualized by spraying with orcinol-sulfuric acid. Migrations of reference glycosphingolipids are indicated on the left. 9-O-AcGD3 migrated just below GM2. In panel A, bands above GM3 are residual neutral glycosphingolipids. In panel B, GM3 and bands below are resorcinol-positive (not shown).

2.3. Tumors and metastases

In vivo experiments were performed according to Bailly et al. [18,19]. Briefly, newborn rats less than 24 hours old received simultaneously an s.c. injection of 0.1 ml melanoma cell suspension (10^6 cells) in the ventral area and an optimal dose of anti-thymocyte serum (ATS) in the dorsal area. ATS injection was repeated on days 2, 7 and 14 and the animals were killed on day 21. Surgical excision was performed on s.c. tumors that were growing at the injection site with all clones regardless of their metastatic potential, and the lungs were collected for anatomo-pathological examination.

2.4. Glycosphingolipid extraction

Gangliosides and neutral glycosphingolipids from cultured melanoma cells, rat s.c. tumors, and rat lungs were isolated according to a procedure already described [23]. The glycosphingolipids remaining in the lower phase of partition were purified by the acetylation procedure of Saito and Hakomori [24].

2.5. Thin-layer chromatography

The gangliosides and neutral glycosphingolipids were separated by thin-layer chromatography on aluminium-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), using as solvent systems chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 (by vol.) or chloroform/methanol/water, 65:25:4. The glycosphingolipids were visualized by spraying with orcinol-sulfuric acid and heating at 160°C. They were identified by comigration with reference compounds and by immunodetection on thin-layer plates as described [25] using specific mouse monoclonal antibodies (MAbs). MAbs GMR6 (IgM) directed to GM3 ganglioside and GMB28 (IgM) to GM2 [26] were kindly provided by Dr. Tadashi Tai (Tokyo Metropolitan Institute of Medical Science). MAbs 4G2 (IgG3) to GD2 [27], 4F6 (IgG3) reactive with GD3 and 7H2 (IgG2a) specific for O-acetylGD3 were produced in our laboratory. Their relative amount was estimated by scanning the HPTLC plates on a CS-930 Chromatoscan densitometer (Shimadzu, Kyoto, Japan).

2.6. CMP-NeuAc: LacCer α2–3 sialyltransferase (GM3 synthase, EC 2.4.99.9) assay and characterization of the reaction products

Subconfluent monolayer cells were mechanically disrupted, rinsed twice with PBS and centrifuged at 1800 rpm for 10 min. Cell pellets were resuspended in a cold 10 mM sodium cacodylate buffer, pH 6.5 containing 1% Triton CF-54, 20% glycerol and 0.5 mM dithiothreitol. After centrifugation at 4°C for 15 min at $10,000 \times g$, the supernatant was assayed for protein concentration and GM3 synthase activity. GM3 synthase activity was determined in duplicates using a procedure modified from Ruan and Lloyd [28]. The reaction mixtures were made of 50 nmol CDH, various concentrations of CMP-[^{14}C]NeuAc (36900 cpm/nmol), 100 mM sodium cacodylate buffer, pH 6.5, 1% Triton CF-54, 8.33 mM $MnCl_2$ and 400 to 800 μg cellular proteins in a total volume of 120 μl. After various incubation periods at 37°C under gentle shaking, the reaction was stopped by addition of 120 μl ethanol. Unreacted CMP-NeuAc and degradation products were removed by reversed-phase chromatography on a C18-bonded silica gel column. The eluted fraction was divided into two equivalent portions: the first one was dried and counted in a scintillation spectrometer after addition of Ultima-Gold (Packard, Meriden, CT, USA); the remaining portion was subjected to TLC as described above, followed by fluorography after spraying TLC plates with Enlightning (New England Nuclear, Paris), then exposure to X-ray films (Hyperfilm-MP, Amersham).

2.7. Ganglioside assay

Gangliosides were assayed according to their sialic acid content by using Svennerholm's resorcinol method [29] as modified by Jourdian et al. [30].

2.8. Determination of glycosphingolipid content

Glycosphingolipids were assayed by their sphingosine content using the fluorimetric method of Naoi et al. [31].

2.9. Protein assay

Proteins were quantified using a colorimetric protein assay (Biorad, Ivry-sur-Seine, France) according to Bradford [32].

3. Results and discussion

The parental M4Be human melanoma cell line and its derived IC8 clone, both unable to give lung metastases, greatly differed from the metastatic T1C3 clone by their glycosphingolipid pro-

Table 1
Distribution of gangliosides of high and low metastatic human melanoma cells

Ganglioside	M4Be	IC8	T1C3
GM3	19.2%[a]	19.9%	79.2%
GM2	5.8%	7.3%	6.2%
9-OAcGD3	7.4%	10.0%	2.1%
GD3	50.0%	44.5%	12.5%
GD2	12.8%	9.4%	traces
GT1b	4.8%	8.9%	traces
Total lipid-bound sialic acid (μg/g proteins)	1.88 ± 0.02	1.91 ± 0.02	0.48 ± 0.01
Total sphingoglycolipids (nmol/mg proteins)	11.07 ± 0.03	11.69 ± 0.05	10.64 ± 0.05

[a]Values are expressed in nmoles per cent nmoles of total gangliosides. Values of total sphingoglycolipids and total lipid-bound sialic acid are the mean ± S.E. of three different assays.

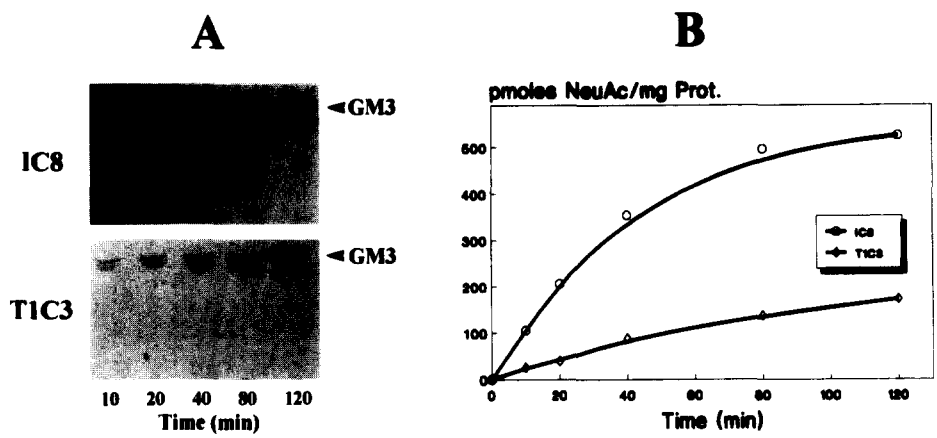

Fig. 2. Time course of CMP-NeuAc: LacCer a2–3 sialyltransferase (GM3 synthase) activity in crude extracts of low (IC8) and high (T1C3) metastatic human melanoma cells. Sialyltransferase was assayed in duplicates at different incubation periods in the presence of 50 nmol LacCer and 32 nmol CMP-[^{14}C]NeuAc. (A) Autoradiograms of the reaction products. GM3 indicates the migration of standard unlabeled GM3 (B) Kinetics of [^{14}C]NeuAc incorporation into lipid-soluble components (see section 2).

files. As shown in Fig. 1A (lanes a,b), the major gangliosides in M4Be and IC8 cells were identified as GM3, GM2, 9-O-AcGD3, GD3 and GD2. In contrast, the T1C3 cells almost exclusively contained GM3, with low amounts of GM2, 9-O-AcGD3 and GD3, and no GD2 (Fig. 1A, lane c; Table 1). Although GM3 represented the major ganglioside in these cells, its amount was about two to threefold lower than in M4Be and IC8 cells. In T1C3 cells only, a strong band in the zone of ceramide dihexoside: CDH was observed, with trace amounts of ceramide trihexoside: CTH (Fig. 1B, lane c), which is synthesized from CDH by the transfer of an additional galactose. The accumulating CDH was identified as lactosylceramide (LacCer), with no traces of galabiosylceramide, by the TLC mobility of the peracetylated derivative as previously described [33]. Thus, the defect in the ganglioside synthesis in the metastatic melanoma cells was accompanied by a concomitant accumulation of LacCer, which is the common precursor of the gangliosides in the biosynthetic pathway known to exist in human melanoma cells [28]. Although T1C3 cells yielded fourfold less

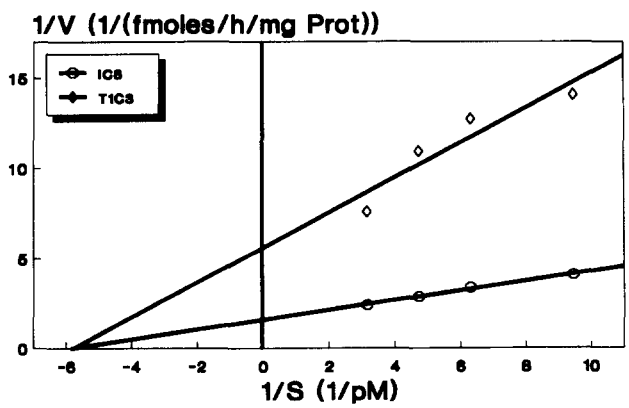

Fig. 3. Determination of the apparent enzymatic parameters of GM3 synthase from low (IC8) and high (T1C3) metastatic human melanoma clones. Complete incubation mixtures containing various concentrations of CMP-[^{14}C]NeuAc (S, substrate) as indicated, were incubated 40 min at 37°C. Kinetic parameters V_{max} and K_m were calculated by linear regression from double reciprocal Lineweaver-Burke plots ($1/V$ versus $1/S$). K_m IC8 and T1C3 = 173 μM; V_{max} IC8 = 178 pmol/h/mg protein; V_{max} T1C3 = 643 pmol/h/mg protein.

gangliosides than M4Be and IC8 cells (Table 1), the amount of total glycosphingolipid was not different (11.07±0.03 nmol/mg protein). IC8 and T1C3 crude cell homogenates were assayed for CMP-NeuAc:LacCer a2,3 sialyltransferase (GM3 synthase, EC 2.4.99.9) activity in the presence of donor CMP-[^{14}C]NeuAc and exogenous acceptor LacCer purified from human melanoma tumors [22]. The reaction velocity of the GM3 synthase activity increased linearly with incubation time (Fig. 2). In both cell clones, [^{14}C]GM3 was synthetized (Fig. 2A). However, a weak activity was seen in T1C3 cells, regardless of the incubation time in the assay, in contrast to the high activity in IC8 cells (Fig. 2B). The apparent activity found in T1C3 cells was probably engendered by the high endogeneous LacCer content since, when LacCer was omitted in the assay, T1C3 cells synthesized GM3 in a similar rate (data not shown). GM3 synthase is weakly operative in metastatic melanoma cells, due to a low ability to transfer sialic acid to LacCer, rather than to a decrease in the affinity towards CMP-NeuAc substrate. In fact, when the apparent kinetic constants towards CMP-NeuAc substrate were estimated from the double reciprocal Lineweaver-Burke plots (Fig. 3), we found V_{max} to be fourfold lower in T1C3 cells (178 pmol NeuAc/h/mg protein) than in IC8 cells (643 pmol NeuAc/hr/mg protein), whereas the apparent K_m was similar (173 μM).

Our results strongly suggest that the human melanoma cells which have potential to give lung metastases exhibit a deficient ganglioside biosynthesis. To have further information about such alterations of biosynthesis in the metastases, the glycosphingolipids were compared between IC8 and T1C3 melanoma cells grown either as s.c. tumors or as lung metastases (Fig. 4). Although IC8 and T1C3 cells drastically differed in their glycosphingolipid profiles in culture, the same pattern as cultured IC8 was found in both IC8 and T1C3 when grown in subcutaneous tumors in rats. By contrast, the T1C3-induced lung metastases exhibited the same glycosphingolipid pattern as cultured T1C3 cells with GM3 as the most prominent ganglioside accompanied by a strong accumulation of LacCer, whereas the tumor-free lungs from rats injected with IC8 cells contain exclusively GM3 without accumulation of neutral glycosphingolipid (Fig. 4), as in normal rat lungs (not shown). The rather simple ganglioside composition of the lung metastases

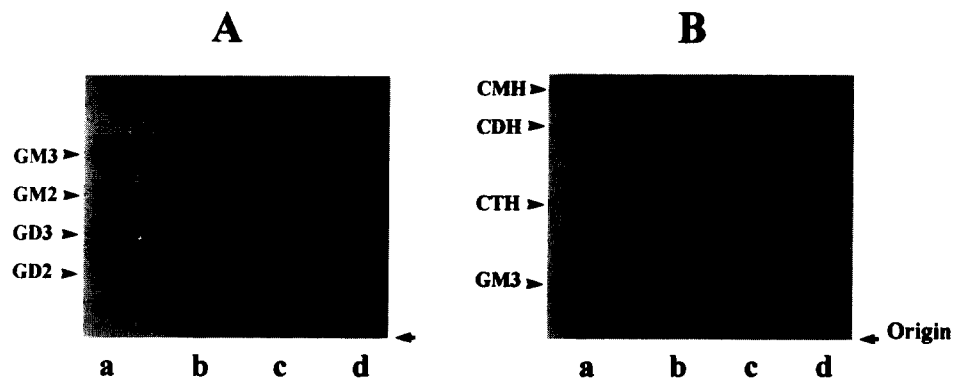

Fig. 4. Thin-layer chromatogram of gangliosides (A) and neutral glycosphingolipids (B) extracted from subcutaneous rat tumors (a,c) and related rat lungs (b,d) harvested three weeks after s.c. inoculation of low metastatic IC8 cells (a,b) or high metastatic T1C3 cells (c,d). In A, bands above GM3 are residual neutral glycosphingolipids. In B, GM3 and bands below are acidic glycolipid components.

in contrast to the more complex patterns found in the s.c. tumors may be interpreted in terms of the well-known phenomenon of heterogeneization along with the tumor growth [34]. Therefore, our data strongly suggest that human melanoma cells which escape from the s.c. tumors to proliferate in the lungs are those which have a low activity of GM3 synthase and only traces amont of GD2 and GD3. Our findings are quite consistent with the reported clinical outcome of melanoma patients treated by immunotherapy using MAbs against GD2 and GD3. Indeed, such trials were shown to induce complete or partial regressions of primary tumors, whereas the effect was only weak on metastases [11–16].

Glycolipids may play a critical role in the interactions between tumoral and host cells. In this respect, GM3 was recently described to interact with LacCer [35]. Thus, LacCer which is highly expressed on metastasizing cells could interact with GM3 in the lung parenchyma. The nearly total absence of disialogangliosides in highly metastatic cells is also a striking feature and we are currently investigating the relevance of GD3 synthase in the onset of the metastatic spread of T1C3 cells. The recent cloning of GD3 synthase from melanoma cells by Nara et al. [36] shall offer valuable tools for such studies.

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