GD2 ganglioside biosynthesis is a distinct biochemical event in human melanoma tumor progression

Jan Thurin, Magdalena Thurin, Meenhard Herlyn, David E. Elder*, Zenon Steplewski, Wallace H. Clark, jr* and Hilary Koprowski

The Wistar Institute of Anatomy and Biology, 3601 Spruce Street and **Pigmented Lesion Study Group, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

Received 9 September 1986

Gangliosides from cell cultures established from melanocytic lesions, representing different stages of melanoma tumor progression, were analyzed by chemical and immunological means on thin-layer chromatograms. The GD2 ganglioside and *N*-acetylgalactosaminyl transferase, which catalyzes the biosynthesis of GD2 from its precursor GD3, were detected in cultures established from advanced primary and metastatic melanomas, but not in cultures of normal melanocytes. Immunohistochemical studies on tissue sections from all progression stages confirmed GD2 expression only in these advanced lesions. A distinct biochemical event thus coincides with the onset of faster growth and acquisition of metastatic competence in human melanoma tumor progression.

Glycolipid *Melanoma* *Glycosyltransferase* *Tumor progression* *Melanocyte*

1. INTRODUCTION

Human melanoma provides one of the best tumor progression models available, because the early developing lesions can be studied with relative ease as they develop in the skin. The lesional steps that may culminate in metastatic melanoma are: (i) the common acquired nevus; (ii) melanocytic nevus; (iii) dysplastic nevus; (iv) primary cancer without competence for metastasis (radial growth phase); (v) primary cancer with metastatic competence and accelerated proliferation (vertical growth phase); and (vi) metastatic melanoma [1]. Cells from the melanocytic system are all rich in gangliosides, which are synthesized by stepwise addition of activated sugars from the common precursor lactosylceramide (Galβ1→4GlcCer). Fig.1 demonstrates the synthesis of the five major melanoma-associated gangliosides (GM3, GM2, GD3, 9-*O*-acetyl GD3, and GD2) [2–15]. Using monoclonal antibodies [16] against melanoma gangliosides [7,8] to detect differences in ganglioside expression during tumor progression [1,17,18], we provide evidence that biosynthesis of ganglioside GD2 (NeuNAcα2→8NeuNAcα2→3Gal(4←1βGalNAc)β1→4Glcβ1→1Cer) from its precursor ganglioside GD3 (NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→4Cer) coincides with an accelerated growth rate and the acquisition of metastatic competence in the progression of melanoma.

Abbreviations: Abbreviations for gangliosides follow the Svennerholm nomenclature, L. Svennerholm, 1963; chromatographic separation of human brain gangliosides (J. Neurochem. 10, 613) and otherwise the IUPAC-IUB Commission on Biochemical Nomenclature 1977 (Eur. J. Biochem. 79, 3512) were followed; mAb, monoclonal antibody; TLC, thin-layer chromatography

2. MATERIALS AND METHODS

2.1. *Cells and monoclonal antibodies (mAbs)*

Melanocytic, nevic and fibroblastic cell cultures

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

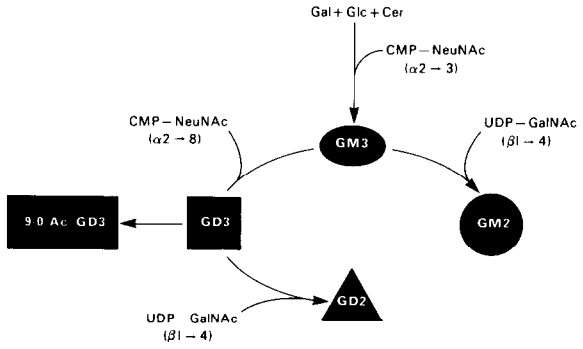

Fig.1. Schematic presentation for biosynthesis of major ganglioside species in the melanocytic system.

and primary and metastatic cell lines as well as hybridomas were established and maintained as described [17,18].

2.2. *Glycolipid fractions and thin-layer chromatography*

Total acid glycolipid fractions were obtained as described [7]. Approx. 30 μg of each fraction were applied per lane to glass-backed HPTLC plates (Merck/Bodman, Gibbstown, NJ) relative to the amount of protein. The resorcinol reagent was used for detection [19].

2.3. *Chromatogram binding assay*

Identical amounts of the same fractions as above were applied onto alumina-backed HPTLC plates (Merck/Bodman, Gibbstown, NJ) to perform the assay as described [7,20,21]. For detection ^{125}I-labeled goat anti-mouse F(ab')$_2$ (Capell, Worthington, PA) was used at 1500 cpm/μl. Primary antibodies were used as undiluted hybridoma culture supernatants, and autoradiograms developed using XAR-5 film (Eastman/Kodak, Rochester, NY) for 20 h at −70°C.

2.4. *Glycosyltransferase assay*

The assay was performed essentially as described [22] and repeated three times with each cell line, with identical results. 10 nmol UDP-[1-^{14}C]GalNAc (Amersham, Arlington, IL) and 40 μg GD3, purified and characterized as described [7], were added to microsomal preparations corresponding to 1 mg protein. After incubation at 37°C for 3 h, the lipids were extracted and half the extract used for TLC. For autoradiography, film was exposed to the thin-layer chromatogram for 20 h at −70°C.

2.5. *Immunohistochemistry*

Immunohistochemistry was performed as described [8] on frozen sections by the peroxidase anti-peroxidase technique (Ortho Pharmaceuticals, Raritan, NJ). Previous studies [8], using the more sensitive avidin-biotin immunoperoxidase methods, revealed reactivity in dysplastic nevi and radial growth phase melanomas. Here these lesions and normal melanocytes were uniformly negative. Reactivity was observed in the same lesions with other mAbs, including some that bind GD3 ganglioside.

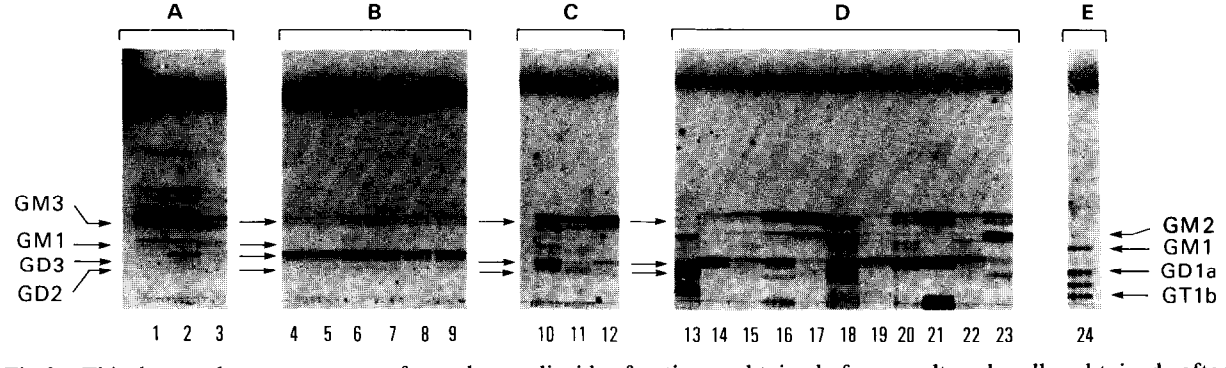

Fig.2. Thin-layer chromatograms of total ganglioside fractions obtained from cultured cells obtained after chromatography in chloroform/methanol/0.2% $CaCl_2$ in H_2O (60:40:9, v/v). Gangliosides were detected with resorcinol [19]. Fractions were obtained from cultures of (lanes): fibroblasts (1–3); melanocytes (4–9); dysplastic nevus (10); congenital nevus (11); radial growth phase melanoma (12); vertical growth phase and metastatic melanoma (13–23); total human brain ganglioside fraction (24). Arrows indicate R_f values for gangliosides.

3. RESULTS AND DISCUSSION

Fig.2 shows thin-layer chromatograms of total ganglioside extracts from cell cultures established from tissue at different stages in the progression of melanoma. This assay reveals not only the presence of particular gangliosides, and therefore their biosynthesis, but also gives information regarding their expression, because the amount expressed on the cell surface of gangliosides GD2 and GD3 correlates with the extractable amount of these gangliosides in the cell [8]. Lanes 1–3 show gangliosides from cultured fibroblasts (negative controls) established from the same normal foreskins that were the source of the melanocytes for the cultures shown in lanes 4–6. A stable pattern of ganglioside expression was observed in normal fibroblasts (lanes 1–3) and normal melanocytes (lanes 4–9 and six additional normal melanocyte cultures, not shown). GM3 was the major ganglioside in fibroblasts, and GM3 and GD3 were the major species in melanocytes, with GD3 predominating. The latter finding contrasts somewhat with that of Carubia et al. [13], who reported that GM3 was the predominant species in cultured melanocytes. Thus, ganglioside biosynthesis in melanocyte cultures is halted at the GM3 and GD3 stages (see fig.1), in contrast to human brain and fibroblasts, where extension of the neutral carbohydrate chain in GM3 and GD3 with Galβ1→3GalNAcβ1→4- results in brain-type ganglioside biosynthesis [14,15].

Chromatograms of gangliosides from cultures of vertical growth phase and metastatic melanoma lesions (fig.2, lanes 13–23) illustrate the aberrant biosynthesis of gangliosides at the later stages of melanoma progression. All of the melanoma-associated ganglioside antigens were present, but the relative amounts of each differed in different cultures (lanes 13–23), with GD3 being the most abundant species.

The same ganglioside extracts were also tested in a chromatogram binding assay with mAbs that detect melanoma-associated gangliosides (fig.3). Using antibody ME361 (row 1), which detects GD2 as the major antigen and cross-reacts to a lesser extent with GD3 [7], GD2 was detected in abundance in the extracts of cell cultures established from vertical growth phase and metastatic melanoma lesions but was not detected in cultures from lesions prior to this stage in progression. Antibody ME311 (row 2), which detects 9-*O*-acetyl GD3 [7], revealed gradually increasing amounts of that ganglioside in the stages of progression from normal melanocytes to metastatic melanoma. Antibody ME24 (row 3), which detects GD3 as the major antigen and reacts slightly with GD2 (unpublished), detected GD3 in all cell extracts and GD2 in extracts of some cultures from advanced lesions (lanes 13–16, 18–20, 23). Among earlier lesions, only that established from a congenital nevus (fig.3, row 1, lane 11), a lesion that does not fit into the tumor progression scheme (no.1), had detectable amounts of the GD2 ganglioside. The onset of GD2 expression can therefore be localized between the steps defined as radial growth phase and vertical growth phase melanoma, thus coinciding in time with a higher proliferation rate and the acquisition for metastasis.

Results of immunoperoxidase analyses, using antibody ME361 on frozen sections, are consistent with this conclusion and also indicate that the finding is relevant in vivo by showing intense staining in 6/7 vertical growth phase and 19/23 metastatic melanoma sections, as compared with staining of melanocytes in 0/10 sections of normal skin, 0/18 compound and/or dysplastic nevi sections, and 1/10 radial growth phase melanoma sections. The weak occasional binding observed in early lesions probably reflected the presence of GD3, the ganglioside expressed throughout melanoma progression and in fibroblasts, as determined by antibodies ME361 and ME24 (fig.3, rows 1 and 3, respectively).

The biosynthesis of carbohydrate chains is catalyzed by glycosyltransferases [14,15]. Synthesis requires availability of an activated sugar, which in the case of GD2 biosynthesis is UDP-GalNAc (fig.1), as well as precursor glycolipid. The turnover rate of these components is important in the final balance of ganglioside biosynthesis. Our observations (fig.2, lanes 13–23) indicate that abnormalities in biosynthesis occur randomly in the advanced stages of melanoma. To determine the basis for elevated GD2 biosynthesis, we studied the glycosyltransferase activity in microsomal preparations of cell cultures from vertical growth phase and metastatic lesions relative to that of cultured melanocytes. In the assay used, both UDP-[1-^{14}C]GalNAc and pure precursor

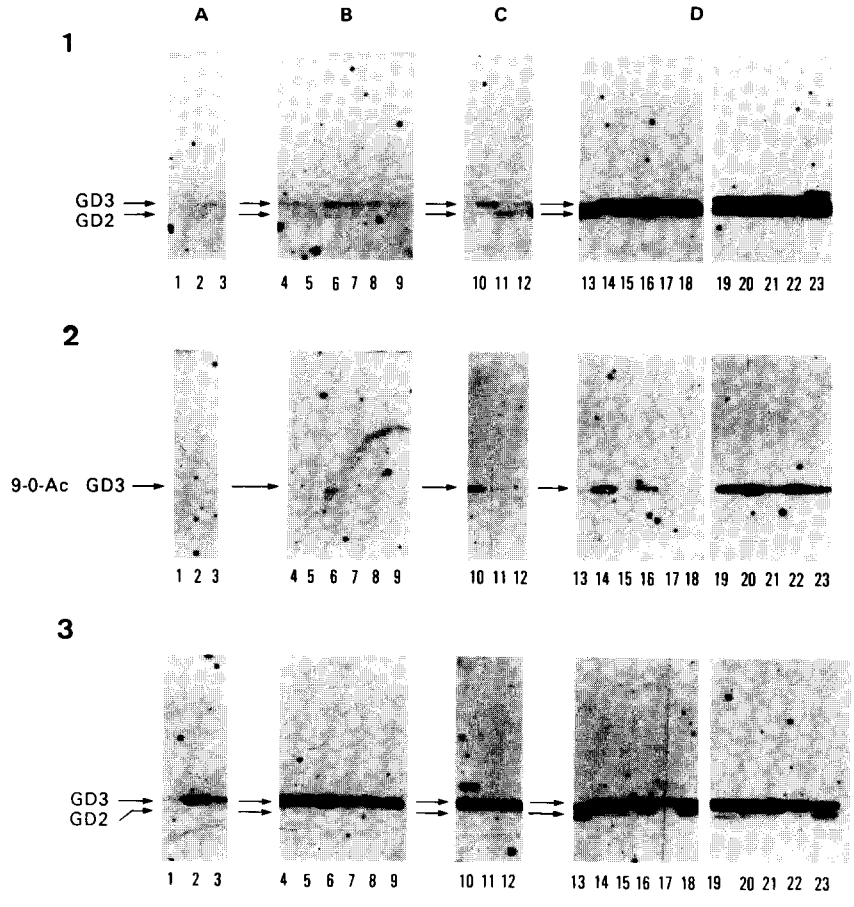

Fig.3. Autoradiograms of chromatograms identical to those in fig.2 but reacted with mAbs. Rows: 1, ME361, detecting GD2 and, to a lesser extent, GD3 [7]; 2, ME311, detecting 9-*O*-acetyl GD3 [6]; 3, ME24, detecting GD3 and, to a lesser extent, GD2 (unpublished).

GD3 ganglioside were present in excess, so that GD2 biosynthesis, i.e., addition of GalNAcβ1→4- to GD3 (see fig.1), over that generated from possible endogenous substrates could be followed. Following TLC of the ^{14}C-labeled products extracted (fig.4), bands were observed at the R_f value of GD2 in extracts of microsomal preparations obtained from vertical growth phase and metastatic melanoma cells (lower bar, lanes 1 and 2, respectively), but not in extracts of cultured melanocytes (lane 3). A control in which a microsomal preparation from a vertical growth phase-derived cell culture was assayed without GD3 precursor was also negative (lane 4). The band with R_f value of GM2 (lane 1, upper bar) was not a biosynthetic product from endogenous substrate, because ^{14}C-labeled GM2 was not recovered when GM3 ganglioside was used as substrate in the assay (not shown). This band probably reflects sialidase activity which produced GM2 from ^{14}C-labeled GD2. Because the induced enzymatic activity only catalyzes the formation of GD2 from GD3, and not that of GM2 from GM3 (fig.1), it appears that the GD3 ganglioside is highly preferred as substrate.

The relationship, if any, between the onset of GD2 biosynthesis and the concomitant increase in proliferation that characterizes vertical growth phase melanoma lesions is unclear, although gangliosides are known to influence the affinity and phosphorylation of growth factor receptors [23,24]. In particular, Bremer et al. [24] have

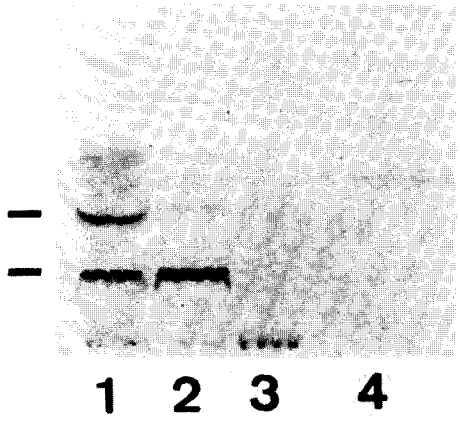

Fig.4. Autoradiogram of the thin-layer chromatograms of the reaction products of a glycosyltransferase assay. Products of extracts of vertical growth phase melanoma cell line, metastatic melanoma cell line, and cultured melanocytes, all incubated with GD3 ganglioside, are seen in lanes 1, 2, and 3, respectively. Lane 4 contains products from a vertical growth phase melanoma cell line extract incubated as in lane 1 but without GD3 substrate. The bars on the left indicate the R_f values for GD2 (lower) and GM2 (upper).

demonstrated that gangliosides added exogenously to EGF-receptor rich A431 cells are incorporated into the cell membrane. They have suggested a 'lateral' mode of interaction between ganglioside and growth factor receptor that results in increased affinity and decreased phosphorylation of the receptor. Enhanced EGF-receptor expression has been correlated with trisomy of chromosome 7 in advanced melanoma [25]. In view of our present results, analysis of GD2 as it interacts with the EGF receptor in melanoma seems warranted. Similarly, analysis of the human immune response to GD2 ganglioside, which has been described as immunogenic in humans [9–12], could now take into consideration the abrupt expression of this antigen at the vertical growth phase stage, which has been correlated with disappearance of the T cell infiltrate known to be present at the earlier stages of melanoma progression [1].

Finally, the GD2 ganglioside has been implicated in the attachment of melanoma cells to solid substrata [26], which makes out observation that the induction of GD2 ganglioside expression in vertical growth phase melanoma, coinciding with the acquisition for metastasis, more interesting, since taken together they implicate a possible role of this ganglioside in the in vivo metastatic process.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (Bethesda, MD) grants CA-25874, CA-21124, CA-25298 and CA-10815. We thank Roxanne Fisher, Ro Stewart, Maureen Coleman, and Angela Lang for expert technical assistance. The editorial assistance of Marina Hoffman was greatly appreciated, as were valuable discussions with Dr Leila Diamond.

REFERENCES

[1] Clark, W.H., jr, Elder, D.E., Guerry, D., iv, Epsten, M.N., Greene, M.H. and Van Horn, M. (1984) Human Pathol. 15, 1147–1165.
[2] Nudelman, E., Hakomori, S.-I., Kannagi, R., Levery, S., Yeh, M.-Y., Hellström, K.-E. and Hellström, I. (1982) J. Biol. Chem. 257, 12752–12756.
[3] Pukel, C.S., Lloyd, K.O., Travassos, L.R., Dippold, W.G., Oettgen, H.F. and Old, L.J. (1982) J. Exp. Med. 155, 1133–1147.
[4] Cheresh, D.A., Varki, A.P., Varki, N.M., Stallcup, W.B., Levine, J. and Reisfeld, R.A. (1984) J. Biol. Chem. 259, 7453–7459.
[5] Brodin, T., Hellström, I., Hellström, K.-E., Karlsson, K.-A., Sjögren, H.-O., Strömberg, N. and Thurin, J. (1985) Biochim. Biophys. Acta 837, 349–353.
[6] Dubois, C., Magnani, J.L., Grünwald, G.B., Spitalnik, S.L., Trisler, G.D., Nirenberg, M. and Ginsburg, V. (1986) J. Biol. Chem. 261, 3826–3830.
[7] Thurin, J., Herlyn, M., Hindsgaul, O., Strömberg, N., Karlsson, K.-A., Elder, D.E., Steplewski, Z. and Koprowski, H. (1985) J. Biol. Chem. 260, 14556–14563.
[8] Thurin, J., Thurin, M., Kimoto, K., Lubeck, M.D., Herlyn, M., Elder, D.E., Smereczynska, M., Karlsson, K.-A., Clark, W.M., jr, Steplewski, Z. and Koprowski, H. (1986) Cancer Res., submitted.
[9] Watanabe, T., Pukel, C.S., Takeyama, H., Lloyd, V.O., Shiku, H., Li, L.T., Travassos, L.R., Oettgen, H.F. and Old, L.J. (1982) J. Exp. Med. 156, 1884–1889.

[10] Tai, T., Cahan, L.D., Tsuchida, T., Saxton, R.E., Irie, R.F. and Morton, D.L. (1985) Int. J. Cancer 35, 607–612.
[11] Tai, T., Paulson, J.C., Cahan, L.D. and Irie, R.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5392–5396.
[12] Cahan, L.D., Irie, R.F., Singh, R., Cassidenti, A. and Paulson, J.C. (1982) Proc. Natl. Acad. Sci. USA 79, 7629–7633.
[13] Carubia, J.M., Yu, R.K., Macala, R.J., Kirkwood, J.M. and Varga, J.M. (1984) Biochem. Biophys. Res. Commun. 120, 500–504.
[14] Fishman, P.H. and Brady, R.O. (1976) Science 194, 906–915.
[15] Roseman, S. (1970) Chem. Phys. Lipids 5, 270–297.
[16] Koprowski, H., Steplewski, Z., Herlyn, D. and Herlyn, M. (1978) Proc. Natl. Acad. Sci. USA 75, 3405–3409.
[17] Herlyn, M., Balaban, G., Bennicelli, J., Guerry, D., iv, Halaban, R., Herlyn, D., Elder, D.E., Maul, G.G., Steplewski, Z., Nowell, P.C., Clark, W.H., jr and Koprowski, H. (1985) J. Natl. Cancer Inst. 74, 283–289.
[18] Herlyn, M., Thurin, J., Balaban, G., Bennicelli, J.L., Herlyn, D., Elder, D.E., Bondi, E., Guerry, D., iv, Nowell, P., Clark, W.H., jr and Koprowski, H. (1985) Cancer Res. 45, 5670–5676.
[19] Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611.
[20] Magnani, J.L., Brockhaus, M., Smith, D.F. and Ginsburg, V. (1983) Methods Enzymol. 83, 235–241.
[21] Hansson, G.C., Karlsson, K.-A., Larson, G., McKibbin, J.M., Blaszczyk, M., Herlyn, M., Steplewski, Z. and Koprowski, H. (1983) J. Biol. Chem. 258, 4091–4097.
[22] Taniguchi, N., Yokosawa, N., Gasa, S. and Makita, A. (1982) J. Biol. Chem. 257, 10631–10637.
[23] Bremer, E.G., Hakomori, S.-I., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) J. Biol. Chem. 259, 6818–6825.
[24] Bremer, E.G., Schlessinger, J. and Hakomori, S.-I. (1986) J. Biol. Chem. 261, 2434–2440.
[25] Koprowski, H., Herlyn, M., Galaban, G., Palmiter, G., Ross, A.H. and Nowell, P. (1985) Somatic Cell Mol. Genet. 11, 297–302.
[26] Cheresh, D.A., Harper, J.R., Schultz, G. and Reisfeld, R. (1984) Proc. Natl. Acad. Sci. USA 81, 5767–5771.