GANGLIOSIDES AND 'GALACTOPROTEIN A' ('LETS'-PROTEIN) OF TEMPERATURE-SENSITIVE MUTANT OF TRANSFORMED 3T3 CELLS*

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1. Introduction

A transformed 3T3 cell line with SV40 virus isolated by Renger and Basilico [1], showing temperature-sensitive expression of transformed phenotypes, is of particular interest in that the growth behavior of these cells seems to depend on their cellular alteration rather than viral mutation. Three parameters typical of malignant growth in vitro have been described at 32°C: (a) high saturation density in culture, (b) ability to form colonies on monolayer of normal 3T3 cells, (c) lack of contact inhibition of DNA synthesis, and (d) high agglutinability by lectins [2]. These phenotypes disappear at 39°C, and growth behavior mimics that of non-transformed 3T3 cells. Mora [3] studied ganglioside synthetase activities of Renger and Basilico's temperature-sensitive cell line and described that the synthetase activity for GM_2-ganglioside (i.e. *N*-acetylgalactosaminyltransferase) was essentially the same at 32 and 39°C, whereas the same enzyme activity of Swiss mouse 3T3 cells transformed by SV-40 virus was significantly low. This paper describes, however, a significant change in chemical quantity, synthetase activity and organizational status of GM_2-ganglioside in the same cell line. The surface glycoprotein profile of this cell line did not show significant difference between 32 and 39°C.

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Ganglioside designation is made according to Svennerholm [18]. 3T3SV, Swiss 3T3 fibroblasts transformed with Simian Virus 40; PBS, phosphate-buffered saline.

2. Materials and methods

2.1. *Cells and cell cultures*

The temperature-sensitive mutant of mouse 3T3 cells transformed with SV40 virus (3T3SVTS) and their parent cell line 3T3ME were donated by Dr Claudio Basilico of the Department of Pathology, New York University School of Medicine, New York, N.Y., and have been cultured in Dulbecco's modified minimum essential medium, supplemented with 10% calf serum. Growth characteristics and temperature-sensitivity of the phenotypes of these cells were published [1,2]. The temperature-sensitive mutant expresses its transformed phenotype at 32°C, which is typical of malignant growth in vitro, namely high saturation density in culture, lack of contact inhibition of DNA synthesis, and some morphology changes. We have observed these parameters, but the change of the agglutinability by Con A and wheat germ lectin was not tested because of technical difficulty.

2.2. *Ganglioside labeling pattern with [^{14}C]galactose*

[^{14}C]1-Gal (52.9 mCi/mmole) in 70% ethanol solution was purchased from New England Nuclear. 10 μl of the above solution, containing 1 μCi, was mixed with 2 ml of Dulbecco-modified Eagle's medium immediately before use. The culture medium containing radioactive galactose is added at a nearly

Table 1
The radioactivity incorporation of [^{14}C]galactose into individual ganglioside of 3T3SVTS and 3T3ME cells at two different temperatures. (Mean value of 2 separate experiments)

	Activity incorporated into gangliosides (cpm/mg of residue)			
	3T3SVTS		3T3ME	
	32°C	39°C	32°C	39°C
GM_3	2095	6055	1280	3200
GM_2	407	2170	358	960
GM_1	98	210	51	102
GD_{1a}	611	1590	103	261
GD_{1b}	293	843	268	622
GT	42	122	64	150

Cells were labeled with [^{14}C]gal (see text) and harvested. The cell pellets were extracted with 30 times volume of chloroform–methanol, 2:1, homogenized for 3 min, and the extract was filtered with glass-fiber paper (Whatman #GF/C) fitted on Millipore assemblage. The cell residues on glass fiber paper were weighed after drying. The filtrate was partitioned with 0.1 M KCl and the lower layer was partitioned twice with the 'theoretical upper layer' [10]. The non-radioactive human brain ganglioside mixture was added to the upper phases and dialyzed against ice-cold distilled water for 2 days. The dialyzate was then evaporated to a dryness in a rotary evaporator, and the residue was dissolved in chloroform–methanol, 2:1. The extracts were evaporated under nitrogen and the residue was analyzed by thin-layer chromatography on Silica gel G, developed with chloroform–methanol–concentrated ammonia–water, 60:35:1:7. The autoradiogram was taken with X-ray film (XO mat film, Kodak), for 5 days at room temperature. The Silica gel thinlayer spot, corresponding to the spots on X-ray film was scraped, and counted in a scintillation counter after solubilized with NCI-solubilizer.

confluent stage of cell culture, and incubated for 3 h at 32°C or 39°C, respectively. Cell sheets were washed with PBS and harvested by scraping with a rubber policeman. Then cell suspensions were centrifuged and washed 3 times with PBS. Further procedure see the footnote of table 1.

2.3. *Chemical quantitation of gangliosides*

Cell pellets, after harvest of culture, were extracted with chloroform–methanol, 2:1, fractionated, and analyzed, according to the method as described by MacMillan and Wherret [4] by resorcinol reagent.

2.4. *Determination of UDP-*N*-acetylgalactosamine: hematoside* N*-acetylgalactosaminyl-transferase activity*

UDP-[^{14}C]*N*-acetylgalactosamine was purchased from New England Nuclear. *N*-Acetylhematoside, used as substrate, was prepared from dog erythrocytes. The assay conditions were the same as was described by Nigam et al. [5].

2.4. *Determination of* N*-acetylgalactosaminidase activity for GM_2-ganglioside*

GM_2-ganglioside, labeled at the *N*-acetylgalactosaminyl residue of the molecule, was prepared enzymatically from hematoside and UDP-[^{14}C]*N*-acetylgalactosamine by the procedure described in the above paragraph. The homogenate of freshly harvested cells was prepared and incubated with the radioactive substrate according to the description of Cumar et al. [6], and the radioactive *N*-acetylgalactosamine, separated through a column of Sephadex G-25, was counted.

2.5. *Cell surface labeling*

Surface labeling was processed according to the method previously described, which was performed on cell sheet [7,8]. 10 μl of galactose oxidase (Kabi, Stockholm: containing 10 units) per plate (8 cm diameter) and 10 μl of tritiated sodium borohydride (1 mCi) per 0.5 ml cell suspension were used. Cell surface-labeling with galactose oxidase and [^{35}S] methionine sulfone hydrazide was carried out according to the method as previously described [7]. The extent of glycolipid exposure was determined on glycolipid analysis of the surface-labeled cells according to the procedure as described [8]. The glycoprotein patterns of surface-labeled cells were analyzed on slab gel electrophoresis followed by autoradiogram [7,9].

3. Results

The radioactivity incorporation of [^{14}C]galactose into individual ganglioside of 3T3SVTS and 3T3ME cells at 32 and 39°C is shown in table 1.

The difference in GM_2-ganglioside level between

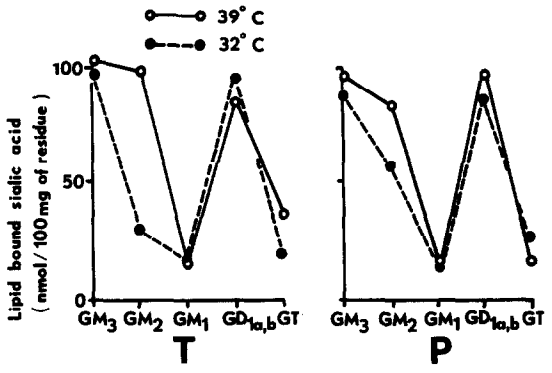

Fig.1. The relative chemical quantities of each ganglioside at 32°C and 39°C. The relative chemical quantity is expressed taking GM_3-ganglioside as 100%. (o——o) The relative chemical quantity at 39°C. (•- - -•) The relative chemical quantity at 32°C. T, TS mutant; P, parent 3T3ME cells. Abscissa: kind of ganglioside, Ordinate: % of quantity of each ganglioside to GM_3-ganglioside. The quantity of gangliosides were expressed as nmoles of lipid bound sialic acid per 100 mg of cell residue. The value for GM_3-ganglioside of 3T3 TS (T) at 32°C was 97 nmole, at 39°C was 101 nmole; that of 3T3 ME (P) at 32°C was 88 nmole and that at 39°C was 95 nmole. Mean value of two separate experiments.

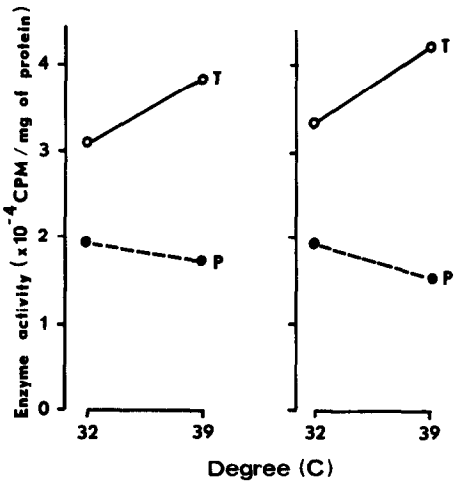

Fig.2. The enzymatic activity of UDP-GalNAc:hematoside-acetylgalactosaminyl transferase at 32°C and at 39°C of TS-mutant (o——o) and of parent cells (•- - -•). The results of two separate experiments are shown.

32 and 39°C was more remarkable when the chemical quantity was compared. As shown in fig.1, the chemical quantity of GM_2-ganglioside was much higher at 39°C as compared to 32°C, where transformed phenotypes were suppressed. In the parent cell the difference was not remarkable. The chemical level of GD of TS mutant was higher at 32°C than at 39°C, whereas that of parent cells was higher at 39°C than at 32°C.

The enzyme activity for GM_2-ganglioside synthesis (UDP-*N*-acetylgalactosamine: hematoside-*N*-acetylgalactosaminyl transferase) was higher at 39°C as compared to 32°C in TS mutant. However, the activity of the parent ME cell did show a slight decrease at 39°C (see fig.2). *N*-Acetylgalactosaminidase activity for GM_2-ganglioside of TS mutant slightly decreased at 39°C was about 20% less than that at 32°C and can be regarded as significant (data not shown).

The extent of surface exposure of GM_2-ganglioside was determined by specific activity of GM_2-ganglioside labeled by the galactose oxidase procedure. As shown in table 2, significant increase of the specific activity in GM_2-ganglioside of TS-mutant was observed at 32°C, whereas no difference was observed in GM_1-ganglioside.

The surface-labeled pattern of glycoproteins was studied and the results are shown in fig.3. The pattern is essentially identical for permissive and non-permissive temperatures.

4. Discussion

The correlation between the transformed phenotype, such as increased cell saturation density and lack of 'contact inhibition' as observed at 32°C, and the ganglioside changes as observed at the same

Table 2
Extent of glycolipid exposure in 3T3SVTS and 3T3ME cells

	Specific activity (cpm/n mole)			
	3T3SVTS		3T3ME	
	32°C	39°C	32°C	39°C
GM_2	4527	1507	2419	1974
GM_1	2161	2453	3607	3331

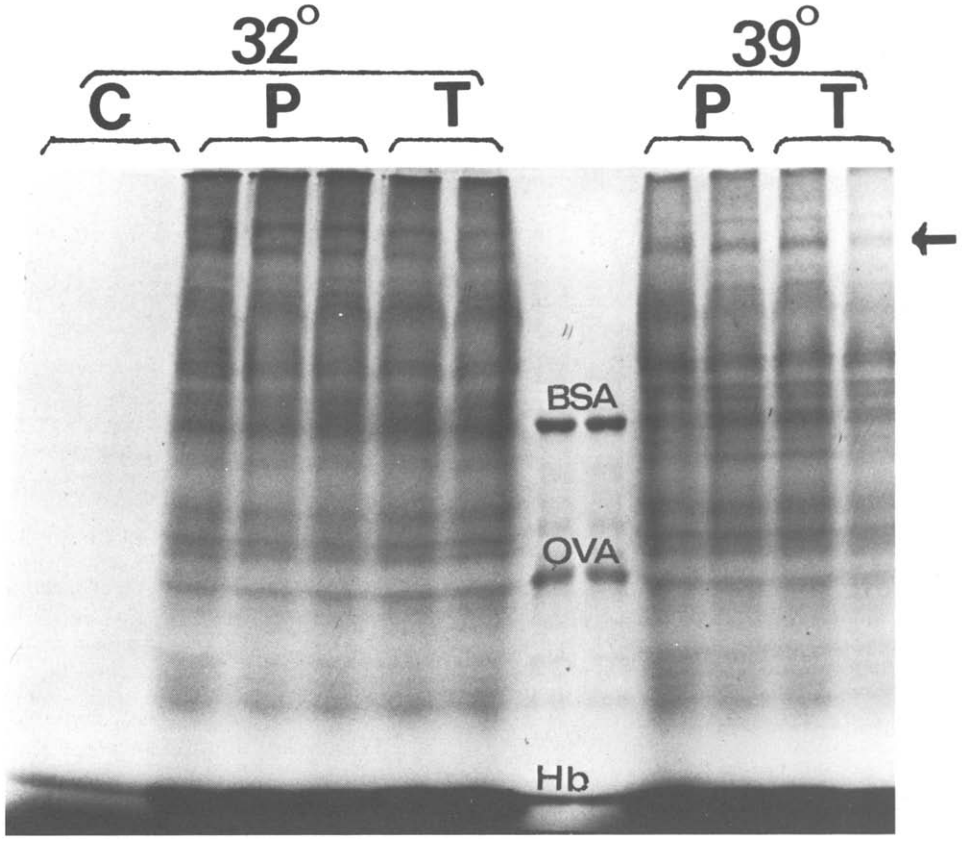

Fig.3. Profile of cell surface glycoprotein of 3T3SVTS and 3T3ME as revealed by neuraminidase, galactose oxidase and [^{35}S] methionine sulfone hydrazide method [7]. C, Control cells, namely cells were treated with [^{35}S] methionine hydrazide alone without galactose oxidase; P, parent 3T3ME cells; T, temperature-sensitive mutant 3T3SVTS. These cells were treated with neuraminidase and galactose oxidase and then treated with [^{35}S]methionine sulfone hydrazide. Two or three slots for each sample were run for testing reproducibility. Arrow indicates the location of 'galactoprotein a'. BSA, bovine serum albumin; OVA, ovalbumin; Hb, hemoglobin.

temperature have been described. The results clearly indicate that GM_2-ganglioside change must be an important molecular basis for membrane phenotype expressed at 32°C. The change of GM_2-ganglioside was indicated by decrease of chemical quantity and by loss of cryptic status of GM_2-ganglioside. A similar change of glycolipids associated with transformation of various cells has been described [11,12]. The results of this study contradict with that of Mora [3] who claimed the absence of ganglioside changes in this mutant cell line. In his studies, cellular ganglioside change was attributed to the expression of virogenom, and the absence of ganglioside change of this mutant was ascribed to the host cellular mutation rather than viral mutation. Present study indicate, however, that ganglioside change may not be associated with virogenom expression, but is due to the change of growth regulation.

The deletion of the high molecular weight glycoprotein, termed 'galactoprotein a' or 'LETS protein', has been regarded as a common phenotype observed in many transformed cells [12–17]. Unexpectedly, however, the change of this protein was not observed to be associated with the change of phenotype. The absence of a correlation between LETS protein and the transformed phenotype in some transformed 3T3

cell was briefly mentioned under the discussion of a paper by Hogg [17].

The change of carbohydrate structure in glycoprotein, however, is difficult to detect by gel electrophoresis, unless changes occurred to an extraordinarily large extent. It is thus possible to have overlooked structural change in carbohydrates of glycoprotein, whereas ganglioside changes have been clearly detected as associated with the change of cell growth control in this mutant.

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References

[1] Renger, H. C. and Basilico, C. (1972) Proc. Natl. Acad. Sci. USA 69, 109–114.
[2] Noonan, K. D., Renger, H. C., Basilico, C. and Burger, M. M. (1973) Proc. Natl. Acad. Sci. USA 70, 347–349.
[3] Mora, P. T. (1973) in: Membrane mediated information (Kent, P. W., ed.) Vol. I, pp. 64–84. American Elsevier Publishing Company.
[4] Macmillan, V. H. and Wherrett, J. R. (1969) J. Neurochem. 16, 1621–1624.
[5] Nigam, V. N., Lallier, R. and Brailovsky, C. (1973) J. Cell Biol. 58, 307–316.
[6] Cumar, L. A., Brady, R. O., Kolodny, E. H., MacFarland, V. W. and Mora, P. T. (1970) Proc. Natl. Acad. Sci. USA 67, 757–764.
[7] Itaya, K., Gahmberg, C. G. and Hakomori, S. (1975) Biochem. Biophys. Res. Commun. 64, 1028–1035.
[8] Gahmberg, C. G. and Hakomori, S. (1973) Proc. Natl. Acad. Sci. USA 70, 3329–3333.
[9] Laemmli, U. K. (1970) Nature 227, 680–685.
[10] Suzuki, K. (1965) J. Neurochem. 12, 629–638.
[11] Hakomori, S. (1973) in: Advances in Cancer Research (Weinhouse, S., ed.) Vol. 18, pp. 265–315. Academic Press, Inc., New York.
[12] Gahmberg, C. G. and Hakomori, S. (1975) J. Biol. Chem. 250, 2438–2446.
[13] Ruoslahti, E., Vaheri, A., Kuusela, P. and Linder, E. (1973) Biochim. Biophys. Acta 322, 352–358.
[14] Hynes, R. O. (1973) Proc. Natl. Acad. Sci. USA 70, 3170–3174.
[15] Wickus, G. G., Branton, P. E. and Robbins, P. W. (1974) in: Control of Proliferation. (Clarkson, B. and Baserga, R. eds.) pp. 541–546. Cold Spring Harbor Laboratory Press.
[16] Teng, N. N. H. and Chen, L. B. (1975) Proc. Natl. Acad. Sci. USA 72, 413–417.
[17] Hogg, N. M. (1974) Proc. Natl. Acad. Sci. USA 71, 489–492.
[18] Svennerholm, L. (1964) J. Lipid Res. 5, 145–155.