ENHANCED GLYCOLIPID:α-GALACTOSYLTRANSFERASE ACTIVITY IN CONTACT-INHIBITED HAMSTER CELLS, AND LOSS OF THIS RESPONSE IN POLYOMA TRANSFORMANTS*

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Summary - The activity of UDP-gal:lactosylceramide α-galactosyltransferase enhanced 2-3 fold in hamster BHK and NIL cells when the growth of these cells was contact-inhibited as compared to the same enzyme activity of the same cells at lower cell population density. The activity of this enzyme in polyoma transformed NIL-2E and BHK was 10-50% of the activity of the growing normal cells, and was not influenced by cell population density. The activity of UDP-gal:glucosylceramide β-galactosyltransferase did not increase as cell population density increased and was not effected by transformation. The activity of α-galactosidase was higher in polyoma-transformed cells.

It is widely believed that the partial escape of malignant cells from growth controls *in vivo* relates to their lack of contact inhibition *in vitro* (1) Recent studies carried out in several laboratories have focused on the chemical changes of surface membrane of animal cells which accompany the malignant transformation. The changes in composition of membrane glycolipids and glycoproteins have been correlated with a loss of contact inhibition (2-8). In our previous work, it was found that the concentration of CTH, hematoside, and disialosyl-hematoside increase on cell-to-cell contact of contact-inhibitable cells (BHK and 8166 cells), and those glycolipids that increase on cell-to-cell contact decrease or disappear after malignant transformation of the cells (9). A similar cell-density dependent change of glycolipid pattern in hamster NIL cells has been observed by ^{14}C palmitic acid labelling experiment (10). Among various glycolipid species, a most sensitive response

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1 Contractions: CMH(ceramide monohexoside glc→cer), CDH(ceramide dihexoside: βGal 1→4 Glc→cer), CTH(ceramide trihexoside:αGal 1→4 βGal 1→4 βGlc→cer)

depending on cell-density and on malignant transformation was demonstrated by CTH (9) whose structure was recently identified as α-D-Galp(1→4)β-D-Galp(1→4)β-D-Glcp(1→1)ceramide (11). The present paper reports cell density-dependent enhancement of biosynthesis of CTH in hamster BHK and NIL cells in comparison with their viral transformants. The enhanced α-galactosidase activity in the transformed cells is also reported.

EXPERIMENTAL PROCEDURE

Glycolipids and sugar nucleotides: CMH^1, CDH^1, and CTH^1 were prepared from human erythrocytes according to the method previously described (12). CTH labelled with ^{14}C at the terminal α-galactosyl residue was prepared by biosynthesis (method of preparation, and the radioactivity, see Tables). UDP-^{14}C-gal was purchased from New England Nuclear, Boston, and UDP-^{12}C-gal was purchased from Sigma Chemical Co., St. Louis, Mo. The purity of UDP-gal used in this work was found to be more than 90% by examination on paper chromatography. The biosynthesized glycolipid was purified on thin-layer chromatography and has been characterized by degradation with α- and β-galactosidases as previously described (11,20).

Cells and cell culture: Hamster fibroblasts, BHK C13/21 cells (13) with low passage having a relatively large quantity of CTH was donated by Dr. Ian MacPherson, Imperial Cancer Research Fund, London. The other kind of hamster fibroblasts, NIL-2E cells (14) were donated by Dr. Leila Diamond, Wistar Institute, Philadelphia, Pa. These cell lines grew as monolayer and were characterized by the presence of CTH (9,10). Saturation densities of BHK, and NIL-2E cells grown on "Falcon" plastic dish were approximately $1.5 \times 10^5/cm^2$ and $1.2 \times 10^5/cm^2$ respectively. BHK and NIL-2E cells were transformed by polyoma virus and isolated according to the method of MacPherson and Montagnier (15). Enzyme activities and glycolipid patterns were determined using the freshly transformed cells within 10 passages in order to avoid any subsidiary effect of successive cultivation. Cells were cultured and harvested in the same condition as described previously (9) and cells

grown at different population densities were obtained as either described previously (9) or by plastic dividers.

Preparation of particulate fraction carrying enzyme activities: Fractions prepared by the following conditions gave the highest enzymatic activity: one part volume of packed cells was mixed with 10-15 parts volume of a "medium A" of Basu, Kaufman and Roseman (16) containing 0.3% Triton X 100, and disrupted by low energy sonication; the cell suspension in a conical centrifuge tube, kept in ice water, sonicated with "Biosonik III," (Brownwill Scientific, Rochester, New York) using a "Needle" probe (12.5 x 0.25 cm). An output intensity of the ultrasonic vibration was set at 60 watts (dial at "20") and run for 90 seconds in an ice-cold bath. The homogenate was centrifuged at 800 xg for 15 minutes (the precipitate was designated as P-1). The supernatant fluid was then centrifuged in a Sorvall centrifuge at 12,000 xg for 25 minutes. The precipitate was designated as P-2. The supernatant was centrifuged in Spinco Model L at 105,000 xg for one hour. The precipitate was designated as P-3. The supernatant was used directly for enzyme assay; fractions P-1, P-2 and P-3 were suspended in the "medium A" of Basu et al (16) by the aid of a small homogenizer, specially made in this laboratory. The protein content of the suspensions were approximately 0.5 - 1 mg/0.1 ml. Aliquots of 0.1 ml were used for various enzyme assays.

Enzyme assay: The activities of UDP-gal:lactosylceramide α-galactosyltransferase and of UDP-gal:glucosylceramide β-galactosyltransferase were determined according to the published basic methods (16,17). The condition as described in Table I has been found to be optimal and used for comparison of the activities of enzymes derived from various cells. The activities of α-galactosidase was determined by ^{14}C-labelled CTH as the substrate (see Table II). The condition of analysis was similar to that of published data (11,18).

RESULTS AND DISCUSSION

Properties of enzymes and biosynthesized products: The enzyme activity of UDP-gal:glycolipid galactosyltransferases from BHK and NIL cells were

found to be most effectively released by a short period of low intensity sonication, and was present in the 12,000 xg supernatant, and quantitatively sedimented by 105,000 xg. Regardless of the method of disruption of cells, the maximal synthesis of CTH and CDH was demonstrated under the conditions

TABLE I

Activities of UDP-gal:glycolipid α- and β-galactosyltransferase of "Fraction P-3" prepared from Normal and Transformed BHK and NIL cells.

The complete assay system was prepared as follows: 20 ul of chloroform-methanol solution of acceptor lipid (0.05 μmole of either CMH or CDH), 20 μl of chloroform-methanol solution containing 800 μg of "Cutscum" (see, Ref. 17) were delivered in a small conical tube and solvent was evaporated under nitrogen stream. The residue was added with 10 μl of 1 M cacodylate buffer pH 6.1, 20 μl of 0.15 M $MnCl_2$ and heated to dissolve. To this solution was added 20 μl of ^{14}C-UDP-galactose which contained 1.4 x 10^5 cpm/80 mμ moles, and agitated on a "Vortex mixer", then added 100 μl of the enzyme suspension ("P-3") in "Medium A" of Basu, et al (16). The mixture was incubated while shaking at 37°C for 1 hour. The reaction was stopped by the addition of 10 μl of 0.25 M EDTA and 10 ul of 0.5 M KCl and extracted with 500 μl of chloroform-methanol 2:1. The mixture was vigorously shaken on a "Vortex" mixer followed by centrifugation. The lower layer was analyzed by thin-layer chromatography or by silica gel coated paper chromatography (16) The synthesized CTH and CDH was calculated from cpm per mg of P-3 protein per hour. (Essentially, the same changes were obtained when the activity was expressed by cpm per DNA content of total cells). The value shown below were arithmetic mean of separate experiments of complete system and the number of experiments were indicated in parenthesis. The values of experiment of incomplete system were: 20-30 μμmoles/mg/hour for the minus CDH or CMH, and 10-20 μμmoles/mg/hour for the minus Mn^{++}.

	Cell population densities	Glycolipid synthesized in μμmoles/mg P-3 protein/hour (complete system) CTH synthesis	CDH synthesis
BHK	sparse (≤5 x 10^4/cm^2)	109 (4)	122
	confluent (>10^5/cm^2)	385 (5)	167
*PY-BHK	Low (≤10^5/cm^2)	55	
	High (>10^5/cm^2)	48 (4)	121
NIL-2E	sparse (≤5 x 10^4/cm^2)	385 (2)	247 (2)
	confluent (>10^5/cm^2)	1052 (2)	257 (2)
*PY-NIL	Low (≤10^5/cm^2)	26	400 (2)
	High (>10^5/cm^2)	38 (2)	206

*PY: polyoma transformed

specified in Table I. A similar condition with pH 6.1 dimethylglutarate buffer was described by Hildebrand and Hauser (17). The α-galactosidase for CTH ("ceramide trihexosidase") (18) was released by either brief sonication or by the Potter homogenization, but the activity was localized either in the 12,000 xg sediment or in the 105,000 xg supernatant fraction. The P-3 fraction (105,000 xg sediment) was, however, essentially freed from α-galactosidase activity. An identical enzyme distribution pattern was obtained from either BHK or NIL-2 cells. No appreciable UDP-gal:lactosylceramide α-galactosyltransferase activity could be demonstrated in any fraction derived from PY-NIL-2E cells (see Table I).

The biosynthesized CTH or CDH whose terminal galactose was labelled with ^{14}C, was indistinguishable from the reference sample of CTH or CDH both with regard to behavior on thin-layer chromatogram under three different test conditions and with regard to hydrolyzability of the material by means of α- and β-galactosidases (11,19,20). The radioactive galactose was quantitatively released from the biosynthesized CTH by fig α-galactosidase, and from the biosynthesized CDH by jack bean β-galactosidase in the presence of sodium taurocholate, and the released activity located exclusively at the galactose position on paper chromatogram. No galactose was released from CTH by a similar treatment with jack bean β-galactosidase, and from CDH by fig α-galactosidase (11,20). Thus, the biosynthesized CTH and CDH were labelled with C^{14}-galactose exclusively at the terminal α- or β-galactosyl residue, respectively. The observed galactosyltransferase activities were, therefore, UDP-gal:lactosylceramide α-galactosyltransferase and UDP-gal:glucosylceramide β-galactosyltransferase, respectively.

Change of enzyme activities: The activity of UDP-gal:lactosylceramide α-galactosyltransferase of either BHK, or NIL-2E cells increased remarkedly as the cell population density reached at 1-3 x $10^5/cm^2$ as the cell growth became contact-inhibited (Table I), and the activity of the polyoma-transformed cells was only 10-50% of that of normal growing cells, and did

not exhibit any variation depending on cell population density.

In contrast, the activity of UDP-gal:glucosylceramide β-galactosyltransferase was not overly dependent on cell population density and was unchanged or became higher after polyoma-transformation (Table I).

The activity of α-galactosidase that hydrolyzes the α-galactoside of CTH was found either in the supernatant fraction or in the 12,000 xg sediment after sonication, but very little activity was present in P-3 fraction. The activity was consistent regardless of whether the cells were growing at sparse cell density or at high cell population density.

TABLE II

Activity of α-galactosidase which catalyzes hydrolysis of terminal α-galactoside of ceramide trihexoside of various cells.

The substrate CTH labelled with ^{14}C at the terminal α-galactoside (specific activity: 36,000-67,000 cpm/μmole) was delivered in a microtube, evaporated under nitrogen and dissolved in 100 μl of 0.05 M citrate buffer pH 5.0 containing 100 μg sodium taurocholate. Dissolution was achieved by heating followed by agitation on Vortex mixer and standing for a few minutes in an ultrasonic bath. To the solution was added 100 μl of cell homogenate or a suspension of P-2 fraction in 0.32 M sucrose containing 1 mM EDTA and 0.1% mercaptoethanol. After the indicated incubation period, the reaction mixture was shaken with 1 ml of chloroform-methanol (2:1), the upper layer which contained liberated ^{14}C-galactose was counted in scintillation counter after addition of "Aquasol" counting fluid (New England Nuclear).

		α-galactosidase activity for CTH (mμmole/mg/16 hours)	
	Cell population densities*	Whole homogenate obtained by 50 strokes of the Potter homogenizer	P-2 fraction obtained from sonicate
NIL-2E	sparse	13.4	13.5
	confluent	12.6	12.3
+PY-NIL	Low	17.6	
	High	13.7	44.5
BHK	sparse	8.5	
	confluent	5.5	12.5
+PY-BHK	High	10.5	23

* Designations for cell population densities are the same as in Table I.
\+ PY: polyoma transformed

The malignant transformed cells showed an increased level of the enzyme activity over that of normal cells (Table II).

An α-galactosyl extension, possibly occurred on contact inhibition, and lack of such response in virally transformed cells have been verified on enzyme basis. Also, the "incomplete" synthesis of the carbohydrate chain (2,3) apparently observed in the transformed cells may now be explained on an enzyme basis. The lack of α-glycosyltransferase activation on cell-to-cell contact, and an enhanced activity of α-galactosidase resulted in a very low quantity of CTH in the transformed cells. It is unknown, however, whether these enzyme changes are the result of a loss of contact inhibition or these changes cause loss of contact inhibition. A search for these two possibilities are in progress.

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