INCORPORATION OF EXOGENOUS GLYCOSPHINGOLIPIDS IN PLASMA MEMBRANES* OF CULTURED HAMSTER CELLS AND CONCURRENT CHANGE OF GROWTH BEHAVIOR

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Received August 17,1973

Summary - Globoside added to culture medium was taken up by NIL cells and accumulated as a component of plasma membrane. This was evidenced by the recovery of ^{3}H-labelled globoside from plasma membrane fractions and by the higher chemical quantity of globoside found in NIL cells cultured in medium containing globoside. Concomitantly the following changes in growth behavior were manifested: a reduction in growth rate due to an extended prereplicative phase and a reduced saturation density which may result from changed adhesive properties of cells.

Glycosphingolipids of animal cells are localized in plasma membrane at much higher concentrations than in intracellular membranes (3-7). The synthesis of a particular glycolipid of cells in culture is greatly enhanced when cells become confluent (8-10), especially at the early stage of cell-to-cell contact (11). Those glycolipids present in cells that show contact-sensitive behavior have been lost in various transformed cells thus far examined, which have also lost "contact inhibition" (8-12). These data point to the possibility that glycosphingolipids in plasma membrane could be a regulator of cell growth, although no direct evidence for this hypothesis has been provided.

Marcus and Cass observed that glycosphingolipid Lewis antigens are readily taken up by erythrocytes to alter Lewis specificities of erythrocytes. Glycoprotein Lewis antigens under the same conditions were not

* This investigation was supported by National Cancer Institute research grants CA10909 and CA12710 and by American Cancer Society grant BC-9C.

Abbreviations: globoside, a trivial name for a ceramide tetrasaccharide whose structure was established as GalNAcβ1→3Galα1→4Galβ1→4Glc→Ceramide (1,2); TLC, thin-layer chromatography.

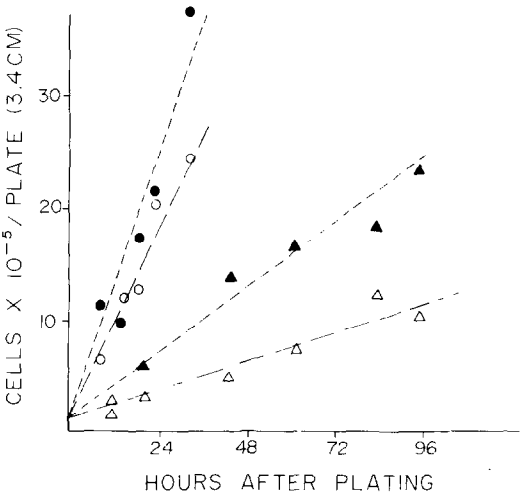

FIGURE 1. Growth Curves of NIL and NILpy Cells in Media with and Without Globoside: Points with solid triangle, NIL in normal Eagle's medium; open triangle, NIL in globoside medium (8 x 10^{-4} M); solid circle, NILpy cells in normal medium; open circle, NILpy cells in globoside medium (8 x 10^{-4} M). A chloroform-methanol solution of globoside was heated at 100°C and evaporated to dryness; the sterile residue was dissolved in a suitable volume of sterile distilled water by alternative heating in a boiling water bath and mixing on a "Vortex" mixer, and it was placed in sonicating bath. The resulting transparent aqueous solution was then mixed with an equal volume of two times concentrated Eagle's medium, supplemented with 20% fetal calf serum.

taken up (13). It has been well known that cultured cells derive many of the lipid components from serum (14).

If cell growth is indeed regulated by the quantity and the organization of sphingoglycolipids in membrane, cell growth behavior could be altered by increasing the level of glycolipid in membrane. The present study is aimed at demonstration of incorporating exogenous glycolipids into plasma membrane and examination of concurrent alteration of growth behavior of cells in culture.

MATERIALS AND METHODS

NIL cells originated from and were donated by Dr. Leila Diamond, Wistar Institute, Philadelphia (15) and were transformed and cultured in this laboratory as described previously (11,12).

Globoside was prepared from human erythrocyte membrane by the proc-

edure previously described (16,17) and was characterized as described previously (18). For uptake study, globoside was labelled with galactose oxidase and tritiated sodium borohydride (19). Globoside was added in culture medium as described in the footnote to Figure 1.

Cells were synchronized by allowing a confluent cell sheet to remain for at least three days without feeding, and then were trypsinized and plated at densities of 1-2 x $10^4/cm^2$.

Plasma membranes were prepared by Stone's method (private communication from Dr. K. Stone, Department of Microbiology, Duke University, N. Carolina), which is essentially a combination of Atkinson's method (rupture by Dounce homogenization in hypotonic solution) (20) and of biphasic partition with polyethyleneglycol and dextran (21). Recovery of plasma membrane was checked by labelling of surface proteins with ^{125}I and lactoperoxidase (22). Each step of the procedure was monitored under a phase-contrast microscope.

Chemical quantity of glycolipids was determined as previously described (8,11). For *^3H-thymidine uptake* and *mitotic index*, see the footnote to Figure 2.

RESULTS

Uptake of Globoside: The chemical quantity of globoside in NIL cells that were cultured for 24 hours in globoside-enriched medium was found to have increased 60-100%, as compared with the same cells at the same population density in medium without globoside (see Table I).

^3H-labelled globoside was taken up rapidly by trypsinized NIL cells. When cells were immediately placed in globoside-enriched medium after trypsinization, they absorbed 55% more ^3H globoside in 24 hours than cells which were first allowed to settle and assume a fibroblastic appearance in 12 hours.

The majority of ^3H globoside taken up was recovered from plasma membrane. Because of the delicacy of cell rupture, the recovery of plasma

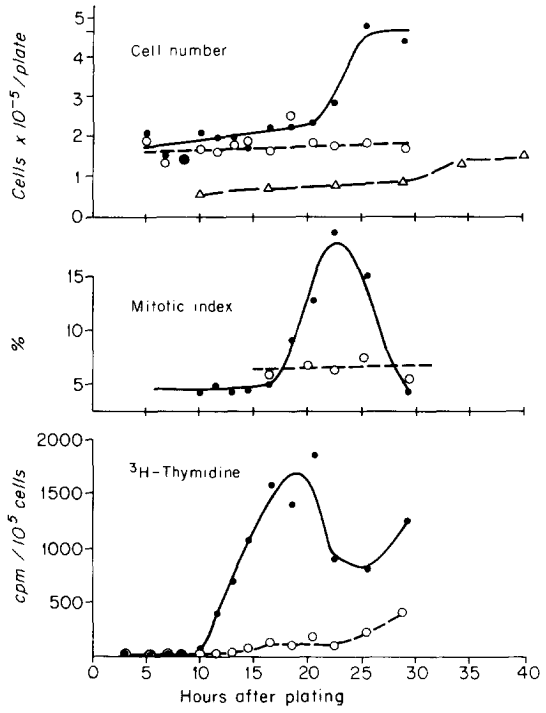

FIGURE 2. Effect of Globoside Medium on Synchronized Cell Cycle of NIL Cells: Points with solid circle, NIL in normal Eagle's medium; open circle or triangle, NIL in globoside-enriched Eagle's medium. Cell number counted in hemocytometer; mitotic index determined by counting cell number in mitotic phase against the total number of cells in random microscopic sight fields. ^{3}H-thymidine uptake was determined after the culture (in 3.5 cm dish) was incubated with 2 ml medium containing 4×10^{5} cpm ^{3}H-thymidine for 30 minutes, followed by washing the monolayer culture 3 times with saline; precipitate occurring on treatment with 7.5% trichloroacetic acid was counted after collection on a "Millipore" filter. Note that S-phase, indicated by the peak of thymidine uptake, was followed by a mitotic peak, after which the cell number doubled. All these changes were not observed in NIL cells grown in globoside medium within 10-22 hours, but they occurred 25-30 hours later.

membrane in the "plasma membrane fraction", as specified at the interphase of biphasic partition, is variable. The value of ^{3}H-globoside in the "plasma membrane fraction" was therefore corrected by the proportion of the surface label activity of ^{125}I (22) distributed among various subcellular fractions. In a typical experiment, 83% of the ^{3}H counts taken up by NIL cells was associated with plasma membrane. Of the counts associated with the "plasma membrane fraction", 2% were retained on a fine filter after

TABLE I

Glycolipid Levels in NIL Cells Grown in Globoside-Enriched Eagle's Medium As Compared to That Grown in Normal Eagle's Medium

	Normal Medium	Globoside-enriched Medium (8×10^{-4} M globoside)
	*Glycolipid levels in n moles**	
Globoside	12	27
CTH (Gal→Gal→Glc→Cer)	10	15
CDH (Gal→Glc→Cer)	10	11
Forssman glycolipid	26	21

* Per 10 mg of delipided cell residue; cells were extracted with chloroform-methanol, glycolipid fraction was prepared by acetylation (18). Values are for confluent cells.

chloroform-methanol extraction, and the remainder was recoverable from a TLC two-dimensional system as ^{3}H globoside.

Changes in Growth Behavior and Cell Cycle: Growth curves in medium with and without globoside are shown in Figure 1. Obviously, growth rate was slowed in medium with added globoside. Saturation density of NIL cells grown in control medium with daily feeding was 4.5-5 x $10^5/cm^2$, while those grown with 0.4 μmole/ml of added globoside attained 3.5-4 x $10^5/cm^2$, and with 0.8 μmole/ml of globoside, 2.7-3 x $10^5/cm^2$. This reduction in saturation density was characterized by circular spaces in the cell sheet, which were surrounded by well-oriented spindle-shaped cells. Such an arrangement persisted even after renewal of medium. This property led us to imagine that the cells may have greater adhesion for each other than for the substrate under conditions of growth in globoside medium. In fact, the confluent cell sheet formed in globoside-medium, though containing the circular spaces, was difficult to separate into individual cells by EDTA or trypsin, and instead resulted in clumps of cells.

In the presence of 0.8 μmole of globoside, the cells were slow to assume a fibroblastic appearance and showed an extended G_1 phase. After

30 hours the cells in globoside medium had not yet doubled, while the control cells doubled after 20 to 25 hours. An extended experiment (lower tracing, Figure 2, top) demonstrated doubling for the experimental conditions at 30-35 hours. Thymidine uptake gave the location of S-phase for control cells at 10-20 hours immediately followed by mitotic phase, but those changes for cells grown in globoside medium were only beginning to occur at 25 hours or later (see Figure 2).

DISCUSSION

Of a number of plasma membrane components, only particular glycolipids increase their concentration significantly when cells are "contact inhibited". It is deemed that such an increase of glycolipid in plasma membrane occurring on cell-to-cell contact is a necessary signal to inhibit S-phase initiation (23) and that tumor cells lack such a mechanism (12,23). Present studies support this possibility more realistically, i.e. artificial enforcement of glycolipid concentration in plasma membrane occurred when cells were cultured in medium with enriched concentration of glycolipid, which results in a greatly delayed growth rate, due to the extended lapse of prereplicative phases, especially G_1 phase.

The mechanism of incorporation of globoside into cells is unknown; it may not result from simple pinocytosis, but could be an incorporation of exogenous glycolipids in the lipid bilayer of plasma membrane, i.e. exogenous glycolipids are integrated into membrane and function in the same fashion as preexisting counterparts in membranes. The fact that exogenous globoside incorporated into plasma membrane and that such an incorporation proceeded at a greater rate when the "fluid dynamic" state of the membrane bilayer is greater, such as in membrane of cells after trypsinization, would support the idea that exogenous glycolipids are integrated into plasma membrane.

Significantly, Nigam et al (24) recently found that core lipid moieties of Salmonella lipopolysaccharides greatly reduce the growth

rate of tumor cells in vitro. As these lipid moieties can affect cells at very low concentrations, and as they have a structure foreign to animal cells, they may not be integrated into the membrane, but rather attach or interact on the surface of plasma membrane and may thus affect growth behavior. The mechanism of the effect of "core lipid" may be different from that of natural glycosphingolipid.

The present study is only a preliminary model experiment as to the relation of cell growth and membrane glycolipids, but could open a wide variety of studies regarding the functions of membrane glycolipids in general.

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