# MECHANISM OF CELL CONTACT-DEPENDENT GLYCOLIPID SYNTHESIS: FURTHER STUDIES WITH GLYCOLIPID-GLASS COMPLEX*

G. Yogeeswaran, R. A. Laine, and S. Hakomori

Department of Pathobiology
School of Public Health and Community Medicine

University of Washington, Seattle, Washington 98195

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Summary - Glycosphingolipids were linked covalently to fine glass particles (diameter 2-10 μ) or glass plates bearing aminopropylsilane. When NIL and BHK cells contacted such a glass complex, the glycolipids on glass were glycosylated. The glycosylation occurred under physiologic conditions with or without exogenously added sugar nucleotide and required no detergents. It took place to a lesser degree when the glycolipids-glass complex made contact with cells transformed by polyoma virus (BHKpy, NILpy). Thus, cell contact-dependent glycolipid synthesis (reference no.: 1-5 of the text) could be ascribable to the activation of cell-surface localized glycosyltransferases on cell contact. Such a surface mechanism is not normally operating or is defective on the surface of transformed cells.

## INTRODUCTION

Enhanced synthesis of a particular glycolipid was observed when non-transformed cells contacted each other. The phenomenon was not observed on cell contact of virally transformed cells (1-7). A related study using a galactose-binding protein demonstrated cell contact-dependent surface structure (8). The enzyme activity for synthesis of CTH was greatly enhanced when hamster NIL or BHK cells contacted each other, while the enzyme responsible for synthesis of CDH was unchanged in the same cells (5). In some cells, however, the activation of the synthesis occurred at the very early stage of cell contact, and the phenomenon could be easily overlooked when glycolipid

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Abbreviations: BHK: baby hamster kidney fibroblasts; BHKpy: BHK cells transformed with polyoma virus; NIL 2E: hamster embryo cells isolated by Diamond, cloned by MacPherson; NIL 2K: a clone from NIL 2E isolated by Kijimoto (see reference 6): NILpy: polyoma virus-transformed NIL 2E or NIL 2K cells: UDP: uidine diphosphate; CMP: cytidine monophosphate: PBS: phosphate buffered isotomic saline; CDH: ceramide dihexoside βGal1→4βGlc→ceramide; CTH: ceramide trihexoside αGal1→4βGal1→4βGlc→ceramide; Globoside: βGalNAcl→3αGal1→4βGal1→4 βGlc→ceramide.

synthesis of sparse cells was simply compared with that of confluent cells (6, also Yogeeswaran and Hakomori, unpublished observation). Roth et al. (9) and Roseman (10) postulated the presence of galactosyltransferase in retinal cell surfaces based on the observation that UDP-galactose does not penetrate into cells; while galactosylation occurred when cells were incubated with UDP-galactose. 3T3 SV cells adhered to a galactose-Sephadex bead complex, but did not adhere to glucose or N-acetylglucosamine derivatives (11). Bosmann (12) and Jamieson et al. (13) showed a change in platelet galactosyltransferase when the platelets aggregated, suggesting the presence of this enzyme on the surface of platelets. Enhanced synthesis of hematoside on platelet aggregation was reported by Chatterjee and Sweeley (14). However, clear evidence as to the surface-localization of glycosyltransferase has not been presented so far. This is a preliminary note on the demonstration of cell contact-dependent glycolipid synthesis using glycolipids affixed covalently on glass beads or glass plates.

## MATERIALS AND METHODS

*Preparation of glycolipid-glass complexes:* "Pyrex" glass tubes were crushed in a mortar by pounding with a pestle for 20 minutes. The glass powder was suspended in 500 ml water in a measuring cylinder. Larger glass particles sedimented quickly, and the supernatant containing smaller particles was centrifuged at 500 rpm for 5 minutes; the larger particles were eliminated, and the supernatant was centrifuged at 1,000 rpm for 10 minutes, giving relatively uniform glass particles with diameters of 2-10 μ. Both such glass powder and Bellco microscope cover glasses (1x2 cm) were washed and boiled with 5% nitric acid, washed with water, and treated with α-aminopropyltriethoxysilane (A-1100 solution, Union Carbide Corporation) according to the method of Venter et al. (15; and personal communication from Dr. J. C. Venter, University of California, San Diego), then washed exhaustively with water. The quantity of alkylamine groups attached to glass was determined by $^{14}C$-formaldehyde in 0.1 M borate buffer pH 9.0 and sodium borohydride.

Glycolipids were prepared from human erythocytes and tritium-labelled by galactose oxidase and tritiated sodium borohydride (16). Glycolipids were acetylated, and the olefinic double bond of sphingosine was converted to a carboxyl group by oxidative ozonolysis according to the method previously described ("acetylglycosylceramide acid") (17,18). The "acetylglycosylceramide acid" was first treated with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide, and the resulting activated ester was coupled to alkylamine glass in dioxane, with shaking for 18 hours. The glass was twice washed with dioxane followed by six washings with chloroform-methanol 2:1. Then the complex was deacetylated in chloroform-methanol 2:1 containing 0.1% sodium methoxide (19). After 30 minutes the reaction was stopped with ethyl-

acetate and the complex was exhaustively washed with chloroform-methanol 2:1 and stored in this solvent until used.

*Cell toxicity test of the alkylamine glass or glycolipid-glass:* Cells were seeded on aminoalkyl glass, or on glycolipid-glass, or these glass materials were added to cell sheets. The cell growth and viability of cells were examined. Cell growth appeared normal when compared with controls. A Trypan blue exclusion test was performed on cells on which glass beads were loaded. No mechanical hazard to the cell was detected. Cells with glass bead materials added were fixed with glutaraldehyde, dried with increasing concentrations of alcohol and subjected to critical point drying. They were then coated and observed under the scanning electron microscope.

*Detection of cell contact-dependent glycosylation of glycolipids on glass surfaces with endogenous glycosyldonor:* BHK and BHKpy cells were grown in Eagle's medium with 2x amino acids and vitamins containing [$^{14}$C]-galactose (0.6 μCi/ml) for 24 hours. The medium was taken out, cells were washed with phosphate-buffered saline pH 7.0, and then incubated in the same PBS with glycolipid-glass beads or glycolipid glass plates with which cells were brought into contact for 90 minutes. In separate experiments 2 x $10^5$ cells were seeded onto glycolipid-glass material and cultured in Eagle's medium containing [$^{14}$C]-galactose (0.4 μCi/ml). Glass beads or plates were separated from cells and washed exhaustively with 10% sodium dodecylsulfate to eliminate any cellular components or cellular debris, followed by washing with chloroform-methanol, and the $^{14}$C radioactivity of the glass materials was counted in a scintillation spectrometer. Control experiments with alkylamine glass without glycolipid were carried out.

*Detection of cell contact-dependent glycosylation of glycolipids in glass surfaces with exogenous glycosyldonor:* Subconfluent cell cultures of NIL 2E, NIL 2K, and BHK 21/C13 were prepared in 3 cm Petri dishes. Twenty hours after seeding the medium was removed and the cell sheet was washed three times with PBS pH 7. To twelve dishes of each cell line were added the following substances for determination of different glycosyltransferases: 1) galactosyltransferase: to each Petri dish were added 10 μl of [$^3$H] UDP-gal (10 μl = 700,000 cpm/0.6 μ moles); 50 μl $MnCl_2$ (6 μ moles), and 10 mg CDH-glass powder suspended in 0.1 M phosphate buffer pH 6.5, or two glass plates per dish; 2) sialyltransferase: to each Petri dish were added 5 μl of [$^{14}$C] CMP-sialic acid (5 μl = 700,000 cpm/2.5 μ moles); 50 μl $MgCl_2$ (6 μ moles), and 10 mg CDH-glass powder suspended in 0.1 M phosphate buffer pH 6.5, or two glass plates per dish; 3) for N-acetylgalactosaminyltransferase: add 10 μl UDP-galNAc ( = 56,000 cpm/4 μ mole); 50 μl $MnCl_2$ (6 μ moles), 10 mg glass powder-globoside compound or two plates per dish with 450 μl of 0.1 M phosphate buffered saline pH 7.4. To collect the glass powder, the plates were washed gently with PBS. The glass powder was centrifuged, and washed twice with water, then 6% sodium dodecylsulfate was added. The mixture was heated at 90°C for 2 minutes and again centrifuged. This was repeated twice, then it was washed twice with chloroform-methanol 2:1 followed by centrifugation. The residue was counted in a scintillation spectrometer.

*Analysis of glycosylated glycolipid residues of glass surfaces on cell contact:* Approximately 2 x $10^6$ cells were seeded in 10 cm Petri dishes. Two dishes of each cell line were used for each analysis. Cells were grown for 36 hours and showed subconfluent appearance. Cell layers were washed four times with PBS, 2 ml of CDH-glass bead suspension in PBS containing 50 mg of glass powder, 100 μl of $MnCl_2$ (12 μ moles) and 2.5 μCi (1 μ mole) of UDP-[$^{14}$C]-galactose were added, and the mixture was incubated for 90 minutes at 37°C. During the incubation, cell morphology seemed normal and the cells were viable as evidenced by a Trypan blue exclusion test. Glass powder was washed off the cell layer with PBS and centrifuged. The glass powder from two plates of cells was combined and washed twice with 5 ml of PBS, and twice with 5 ml of water. Then 2 ml of 2% sodium dodecylsulfate was added, heated at 100°C for 2 minutes and centrifuged. Particles were further washed twice with water and chloroform-

Table I. Glycosylation of glycolipids on glass by contact with cell surfaces and exogenously added labelled sugar donor (sugar nucleotides). Numbers expressed by radioactivity (cpm) found on glass with approximately 4 x $10^5$ cells.

| Cells | Glycolipid affixed on glass | Sugar nucleotide added | Suspected reaction on glass | Experiments with glass plates A* | B* | glass beads A* | B* | Control experiments 1† | 2† |
|---|---|---|---|---|---|---|---|---|---|
| NIL 2E | CDH | CMP-Sialic acid | CDH→Hematoside | | | 158 | 162 | 10 | 28 |
| | CDH | UDP-gal | CDH→CTH | 112 | 115 | 182 | 140 | 4 | 12 |
| | Globoside | UDP-galNAc | Globoside→ Forssman glycolipid | 45 | 50 | 144 | 140 | 8 | 20 |
| NIL 2K | CDH | UDP-gal | CDH→CTH | | | 199 | 188 | 7 | 25 |
| | Globoside | UDP-galNAc | Globoside→ Forssman glycolipid | 43 | 49 | 198 | 200 | 6 | 15 |
| BHK C13/21 | CDH | CMP-Sialic acid | CDH→Hematoside | 455 | 460 | 172 | 152 | | 10 |
| | CDH | UDP-gal | CDH→CTH | 95 | 105 | 124 | 110 | | 8 |
| BHK C13/21py | CDH | CMP-Sialic acid | CDH→Hematoside | 204 | 271 | 82 | 72 | | |
| | CDH | UDP-gal | CDH→CTH | | | 25 | 26 | 20 | 12 |

*A,B two sets of experiments with the identical conditions: cells (2 x $10^5$) seeded in small Falcon Petri dishes (3 cm diameter) in Eagle's medium. After 20 hours, the medium was removed and the plates were washed 3 times with PBS pH 7.0, and incubated with glycolipid-glass and labelled sugar nucleotides as described in the text.

†Control 1: The same cells with the same cell number incubated with alkylamine-glass without glycolipid and with labelled sugar nucleotide. Control 2 cells added with Eagle's medium, washed with PBS and incubated with alkylamine-glass and labelled sugar nucleotide.

Table II. Analysis of Biosynthetic Product on Glass-Beads

Approximately 2 x $10^6$ cells of NIL 2E or BHK C13/21 cells grown on Falcon Petri-dishes. After 36 hours, the cells were washed with PBS four times, and incubated with 2 ml phosphate buffer pH 6.5 containing a suspended glass beads-ceramide lactoside complex [100 µl $MnCl_2$ (12 µ moles) and 2.5 µCi (1 µ mole) of UDP-[$^{14}$C]-galactose]. The weight of glass beads in the added suspension was approximately 50 mg, containing 5.2 µ moles of ceramide lactoside. After incubation for 90 minutes at 37°C, glass beads were collected and extensively washed with water, sodium dodecylsulfate and organic solvents and analyzed by gas-chromatography after methanolysis (see the text).

| Cells | Total cpm $^{14}$C-galactose incorporated on glass | Radioactivity (cpm) found in galactose peak on gas chromatography after methanolysis |
|---|---|---|
| BHK-21 | 15,600 | 9,280* |
| NIL 2E | 74,000 | 36,000* |

*Some radioactivity (less than 1,000 cpm) was found also in the glucose area, this could be a tailing of galactose.

methanol 2:1. The particles were dried under nitrogen and subjected to methanolysis with 1 N methanolic HCl overnight. The methanolysate was treated according to Laine et al. (20) and analyzed by gas chromatography on SE 30. Each sugar peak was collected and its radioactivity was determined in a scintillation counter.

## RESULTS

*Glycosylation of glycolipids on glass by contact with cell surfaces and with exogenous sugar donors (UDP-gal, UDP-galNAc, or CMP-sialic acid):* When cells grown on Petri dishes were contacted with glycolipids affixed to glass and in the presence of UDP-[$^{14}$C]-gal, UDP-[$^{14}$C]-galNAc, or CMP-[$^{14}$C]-sialic acids, glycosylation of glycolipids on glass could be observed by radioactive incorporation. No radioactivity was incorporated on glass without glycolipid residues. Interestingly, glycosylation on glass by contacts with BHKpy cells was found to be only 20-50% of the value observed by contacts with BHK cells (see Table I).

*Analysis of biosynthesized product on glass:* Preliminary analysis of radioactive sugars incorporated onto glycolipids bound to glass material is reported in Table II.

Table III. Glycosylation of glycolipids-glass by contacts with cells, determined with endogenous sugar donor.

| Cells | Glycolipids on glass | Cover slips (cpm taken up on glass-glycolipid) | Powdered glass (cpm taken up on glass-glycolipid) |
|---|---|---|---|
| Prelabeled with $^{14}$C-gal* | | | |
| BHK | CDH | 220 | 70 |
| | Globoside | 105 | 65 |
| Labelled during growth and contact† | | | |
| BHK | CDH | 500 | 350 |
| | Globoside | 250 | 240 |
| NIL 2E | CDH | 940 | 175 |
| | Globoside | 680 | 150 |
| BHKpy | CDH | 260; 220 | 180; 190 |
| NILpy | CDH | 320; 260 | 65; 68 |

*Cells were cultured in Eagle's medium with 3 μ Ci $^{14}$C-galactose (uniformly labelled) per 5 ml medium for 48 hours. Radioactive medium removed, washed with PBS twice and then cells were incubated with 20 mg of glass beads or 20 cover slips (1 x 0.5 cm) coupled CDH or globoside, $MnCl_2$, $MgCl_2$ (6 μ moles each in 100 μl) and 1 ml PBS pH 7.0 for 90 minutes. Cell number seeded: 2 x $10^5$ per plate.

†2 x $10^5$ cells were seeded in the same amount of glass material as above and grown for 48 hours in the Eagle's medium containing $^{14}$C-galactose (2 μCi/5 ml of the medium).

*Glycosylation of glycolipids on glass by contact with cell surfaces as determined with endogenous sugar donor:* Glycosylation on glass-glycolipid was observed at a high level when cells were grown on cover slip-glycolipid complexes in medium containing $^{14}$C-galactose. Glycosylation was also observed when prelabelled cells with $^{14}$C-galactose were grown on a cover slip-glycolipid complex (see Table III).

*Distribution of glycolipid-glass beads on cells:* Scanning electron micrography showed that glass beads added on cultured cells clustered and adhered to cells, but the cells retain their normal morphology (Fig. 1 and Legend).

## DISCUSSION

Enhanced glycolipid synthesis at cell contact is a general cell biological phenomenon observable in all non-transformed cells but not in transformed cells

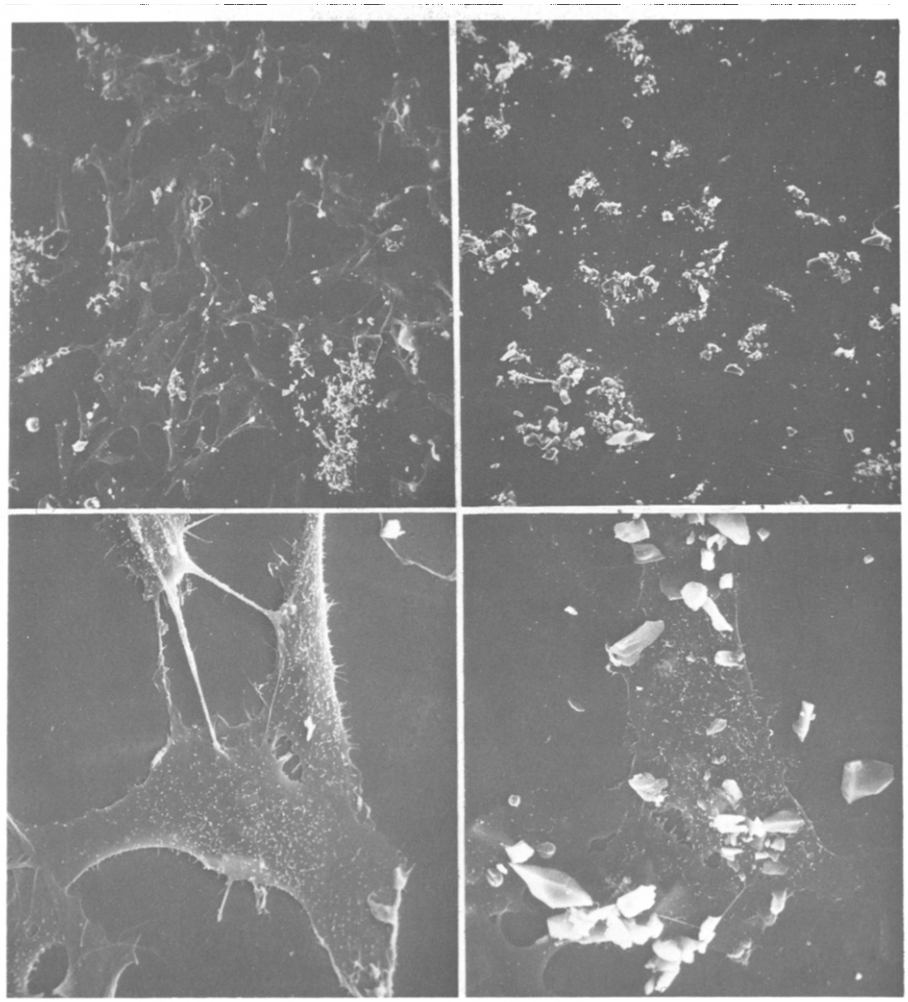

Fig. 1. Scanning electron micrograph of NIL 2K cells associated with globoside-glass beads. A: 90 minutes, B and D: 24 hours after incubation with glass beads. C: control cells. Magnification A, B 100x; C, D 1000x. Note that glass beads selectively adhere on certain cells at 90 minutes ("A"), possibly due to phase of cell cycle. They are distributed and adhered on every cell after 24 hours ("B"). Cell morphology of cells in contact with glass beads is normal ("D") as compared with control cell ("C").

so far tested in this laboratory (8, and Yogeeswaran and Hakomori, unpublished results). The phenomenon can be plausibly explained by a possible intercellular glycosylation on cell-to-cell contact through activation of cell surface glycosyltransferases. An analogous view is being postulated for chick retina (10) and mouse 3T3 cell adhesion (8,21). Evidence is presented here which demon-

strates that glycosylation of glycolipids covalently attached to a glass surface occurred on contact with the surfaces of cultured cells. The glycosylated glycolipidyl groups on glass were unequivocally characterized by observation of radioactive sugar peaks on gas-chromatography. The glycosylation of glycolipidyl groups on glass was observed with or without the presence of exogenous sugar nucleotide, i.e., glycosylation on glycolipids was observed when glass materials contacted a) cells pre-labelled with [$^{14}$C]-galactose, b) cells grown in the medium containing [$^{14}$C]-galactose, or c) cells added with exogenous [$^{14}$C]-sugar nucleotides. One possibility of contact-dependent glycolipid synthesis may proceed via an unknown intermediate bound to the surface membrane; e.g., UDP-Gal + MX → MX-Gal, MX-Gal + Gal-Glc-Cer-G → Gal-Gal-Glc-Cer-G + MX, where MX is an unknown membrane mediator for surface glycosylation; G is glass. Preliminary experiments indicate that "MX" could be a retinol derivative bound in surface membrane since the glycosylation of glycolipids on glass was greatly enhanced when cells contact glass material in the retinol enriched medium. This may be an important clue for further studying the mechanism of "functional cell contact" (22), the absence of which may be a common denominator of malignant cell surface.

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