CONTACT-DEPENDENT ENHANCEMENT OF NET SYNTHESIS OF FORSSMAN GLYCOLIPID ANTIGEN AND HEMATOSIDE IN NIL CELLS AT THE EARLY STAGE OF CELL-TO-CELL CONTACT

SHIGEKO KIJIMOTO and SEN-ITIROH HAKOMORI

Biochemical Laboratory, Departments of Pathobiology and Microbiology
University of Washington, R.D. 98, Seattle, Washington 98195, USA

Received 17 May 1972
Original figure received 26 June 1972

1. Introduction

The chemical quantity of some glycolipids increases when the growth rate of cells decreases on cell-to-cell contact [2,3]. In a previous paper, the cell density-dependent increase of the concentration of ceramide trihexoside and hematoside in BHK cells, disialohematoside and hematoside of human 8166 diploid cells was reported [2], and the enhanced activity of UDP-galactose: lactosylceramide α-galactosyltransferase was observed in contact-inhibited BHK and NIL cells as compared to growing cells [4]. The enhanced uptake of isotope in glycolipids from [^{14}C] palmitic acid was reported in contact-inhibited NIL cells, as compared to growing cells [3]. More recently, Forssman glycolipid was found in NIL cells [5], and the structure of this glycolipid antigen has been established [6]. In this article we are reporting the change of chemical quantity and net synthesis of Forssman glycolipid and hematoside on cell-to-cell contact at various cell population densities.

2. Materials and methods

2.1. *Cells and cell culture*

NIL 2E cells, which is the contact-inhibitory subclone of NIL cells [7], were donated by Dr. Leila Diamond of the Wistar Institute in Philadelphia. NIL 2K cells were isolated from these NIL 2E cells and showed a different morphology (cobblestone appearance) and a lower saturation density. The saturation densities of 2E and 2K cells were $2-2.5 \times 10^5$ and $1-1.8 \times 10^5/cm^2$, respectively. These cells were cultured in Eagle's medium reinforced with two times amino acids and vitamins and supplemented with 10% fetal calf serum. The NIL 2E cells were transformed with polyoma virus, and a transformed colony was isolated by soft agar procedure [8]. The transformed cells were analyzed within several passages.

Cultures with different cell population densities were prepared as follows: 2×10^6 cells seeded in the area of 145 cm^2 and 50 cm^2; 4×10^6 cells in the area of 50 cm^2.

2.2. *Method of glycolipid analysis*

Approx. 0.5–2 ml of packed cells were extracted with chloroform–methanol (2:1), and the glycolipid fraction was prepared by acetylation procedure as described previously [9].

Quantitation of glycolipids in some experiments was performed by gas chromatography of an extract from a thin-layer plate, according to the method of Vance and Sweeley [10]. Visual comparison of spot intensities on thin-layer chromatography with the known quantities of standard glycolipids was also used in other experiments, as previously described [2].

The following standard samples were prepared in our laboratory: NANA(2 → 3)Galβ(1 → 4)Glc → Cer (*N*-acetyl-hematoside) [12,13]; NGNA(1 → 3)Galβ (1 → 4)Glc → Cer(*N*-glycolylhematoside) [12]; GalNACβ(1 → 3)Galα(1 → 4)Galβ(1 → 4)Glc → Cer (Globoside) [12,15]; Galα(1 → 4)Galβ(1 → 4)Glc → → Cer(CTH) [14,15]; Galβ(1 → 4)Glc → Cer(CDH)

* Supported by the National Cancer Institute Research Grants CA 12710 and CA 10909 and by the American Cancer Society Grant BC9-B. A preliminary note of this paper was published [1,5].

Table 1
Identification and variation of the quantity of glycolipids in NIL 2E cells at different growth phases and in polyoma-transformed cells.

Structures and abbreviation of glycolipids found in NIL 2E cells	Chemical quantity of glycolipids in μg per 100 mg of protein		
	Growing phase	Confluent phase	Polyoma-virus transformed
Galβ→Glcβ→Cer(CDH)	10–15	10–15	30–35
Galα→Galβ→Glcβ→ →Cer(CTH)	15–20	36–45	6–15
GalNACβ→Galα→Galβ→ →Glcβ→Cer(Globoside)	24–35	35–40	10–20
GalNACα→GalNACβ→ →Galα→Galβ→Glcβ→ →Cer(Forssman)	35–45	75–85	< 5
NANA→Galβ→Glcβ→ →Cer(Hematoside)	70–90	120–160	80–95

[10,12]; Glc → Cer(CMH) [10,12]; GalNACα(1 → 3) GalNACβ(1 → 3)Galα(1 → 4)Galβ(1 → 4)Glc → Cer (Forssman GL) [6].

2.3. *Determination of net synthesis of glycolipids*

In order to compare net synthesis of each glycolipid in cultured cells under different cell population densities, cells on Petri-dishes with varying cell population densities were incubated for 1 hr at 37° with a medium having the following concentration of galactose: one ml of glucose-free medium contained 2 μCi of [^{14}C]-galactose and 1 mg of ^{12}C-galactose. Other conditions are described in table 2.

2.4. *Radioautograph of immunoprecipitin by anti-Forssman glycolipid serum and by anti-globoside serum*

Anti-sera were obtained according to the procedure of Koscielak et al [16]. The glycolipid fraction of NIL

Table 2
Net synthesis of glycolipids as determined by the amount of galactose incorporated into various glycolipids of NIL cells during 1 hr at 37° by intact cells at different population densities.

Cell population densities (growth phase)		μμmoles of galactose incorporated into each glycolipid of 10^7 cells during 60 min		
		NIL 2E	NIL 2E-py	NIL 2K
0.2–0.4 × 10^5/cm^2 (growing)	CDH	68	293	107
	CTH	122	46	122
	Globoside	122	28	452
	Forssman	550	20	1152
	Hematoside	500	450	822
1–1.4 × 10^5/cm^2 for 2E and 2E-py	CDH	74	367	102
	CTH	166	15	417
0.4–0.6 × 10^5/cm^2 for 2K	Globoside	160	24	638
(early confluency)	Forssman	1470	46	1377
	Hematoside	2120	850	2553
2 × 10^5/cm^2 for 2E and 2E-py	CDH	47	139	97
	CTH	174	19	391
0.7–0.9 × 10^5/cm^2 for 2K	Globoside	178	24	378
(confluency)	Forssman	800	95	933
	Hematoside	1770	750	1290

The cells were washed with phosphate-buffered saline (pH 7.2) and were then collected by centrifugation. The cell pellet (0.2–0.3 ml) was extracted with 5–10 ml of chloroform–methanol (2:1); it was then heated at 50° for 30 min, followed by two more extractions with chloroform–methanol (1:1) under the same conditions. The extracts were combined and evaporated under nitrogen to dryness. The residue was re-extracted with 0.5 ml of chloroform–methanol (2:1) and centrifuged in a small conical tube. The supernatant was analyzed by thin-layer chromatography after being subjected to acetylation procedure [9], or it was analyzed directly by two-dimensional chromatography. The modified method of Gray [11] was used. The chromatogram was developed with chloroform–methanol–water (65:25:4) for one direction and with tetrahydrofuran–2-butanon–methanol–water (10:6:4:1) for the other direction. The chromatogram was developed with reference glycolipids. The glycolipid spots, as revealed by iodine vapor, were cut out and the radioactivity was counted.

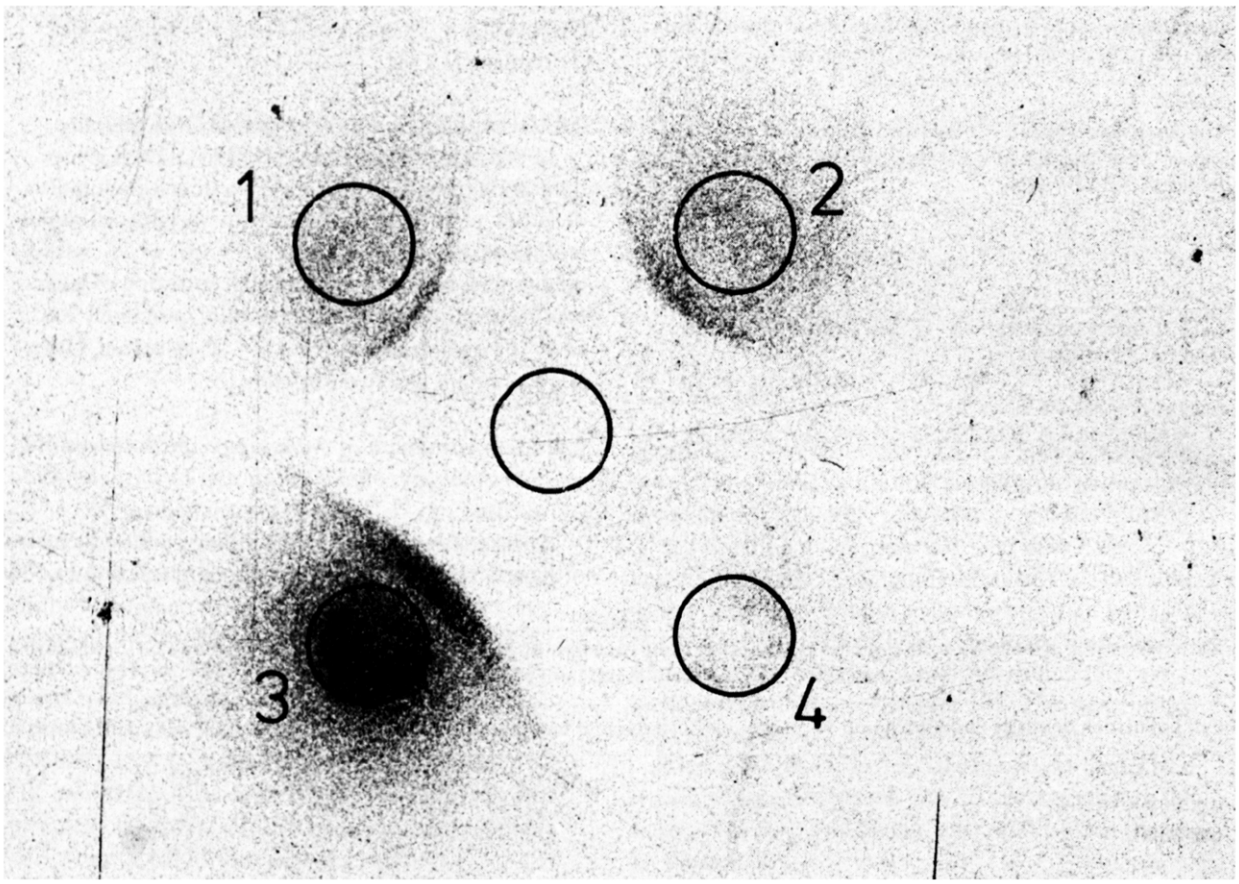

Fig. 1. Radioautogram of precipitin lines formed between anti-Forssman antiserum and total glycolipid fraction [9] of NIL cells grown under different conditions. Center: anti-Forssman glycolipid rabbit antiserum prepared by the method of Koscielak et al. [16]. 1: total glycolipid fraction of actively growing NIL cells; 2: total of early stage of confluent NIL cells; 3: total of highly confluent NIL cells; 4: total of polyoma-transformed NIL cells. The condition of labelling is the same as in table 2. Precipitin lines were radioautographed by exposing the film for one week. Note that an increase of the intensity of precipitin line at the early stage of contact inhibition.

cells grown under different cell population densities and of polyoma-transformed NIL cells was then prepared by "method 2" acetylation procedure [9], and the fraction was dissolved in 100 μl saline per 10 mg cellular protein; 20 μl was placed on an immunodiffusion well. Immunodiffusion analysis was performed according to the method of Sharples and LoGrippo [17] using anti-globoside and anti-Forssman glycolipid. After two days, the gel was washed and dried, and a radioautogram on Kodak "Royal X-Omat" film was taken.

3. Results and discussion

Table 1 shows the chemical quantity of glycolipids in NIL 2E cells and in polyoma virus-transformed cells at different phases of cell growth. Forssman hapten glycolipids and hematoside increased significantly when cells showed a decreased rate of growth at high cell population densities.

A typical example of net synthesis of glycolipids as determined by the amount of galactose incorporated into various glycolipids of NIL 2E cells and NIL 2K cells at various cell population densities is shown in

table 2. It is particularly noteworthy that cell-density dependent enhancement of the synthesis of Forssman glycolipid and hematoside was more remarkable at a relatively early stage of cell confluency as compared to after complete confluency. In polyoma-transformed cells, an increase of CDH synthesis was observed, in striking contrast to the synthesis of all other glycolipids, which decreased to a great extent.

The synthesis of hematoside in sparsely growing cells was about the same as that of transformed cells. The enhanced net synthesis of hematoside was extremely remarkable at an early stage of cell confluency. Enhanced synthesis of hematoside was also observed in transformed cells at higher cell densities. The degree of enhancement by an increased cell population was, however, much less than that of non-transformed cells.

Increased synthesis of Forssman hapten glycolipid at the early stage of cell contact was further substantiated by a larger radioactivity incorporation into the precipitin line formed between total glycolipid of NIL cells and anti-Forssman glycolipid antisera (see fig. 1).

It is known that the activity of Forssman antigen increases when BHK cells are transformed [18–20], and this phenomenon was recently interpreted as "cryptic Forssman" becoming "exposed" by malignant transformation, rather than a real increase of synthesis [21,22]. In these studies, however, the presence of Forssman glycolipid was not determined, and in fact, we were unable to demonstrate any significant amount of Forssman glycolipid in BHK cells or their transformants (unpublished data). The Forssman activity in BHK cells must be due to the presence of a specific glycoprotein rather than a glycolipid. NIL cells were the first cells for which we were able to demonstrate the presence of Forssman glycolipid [5]; therefore, the change of this antigen on cell-to-cell contact and on malignant transformation has been well-studied using these cells. Increase in the net synthesis of this antigen at the early stage of cell-to-cell contact and its complete deletion by malignant transformation are remarkable behavioral features of this antigen, which are demonstrated in these cells.

Cell-contact dependent increase in the synthesis of Forssman glycolipid was not remarkable in NIL K cells, whereas that of hematoside was extremely remarkable at the early stage of cell-to-cell contact (see table 2, last column).

The findings described in this paper further suppor the hypothesis of "contact extension" of glycosyl resi due on cell-to-cell contact and further point out that the mechanism appears at the early stage of contact in hibition. A similar phenomenon is present in malignant cells as to the change of hematoside, although it appears to a lesser degree (see table 2, second column)

The increase of chemical quantity of glycolipids on cell-to-cell contact was much smaller than the enhanced isotope incorporation, as previously described [2]. This suggests that both synthesis and degradation of glycolipids were enhanced on cell contact. The enzyme systems for the synthesis and degradation of hematoside, ceramide trihexoside and Forssman antigen must be contact-sensitive, and those glycolipids function as recognition sites on cell membranes for intercellular association and contact inhibition. Presence of such contact-sensitive glycolipids at lower concentrations in malignant cells should be closely related to the loss of contact inhibition and the loss of intercellular association of transformed cells.

References

[1] S. Kijimoto and S.Hakomori, Federation Proc. 31 (1972 874 Abs.
[2] S. Hakomori, Proc. Natl. Acad. Sci. U.S. 67 (1970) 1741
[3] P.W. Robbins and I. MacPherson, Proc. Roy. Soc. Londo 177 (1971) 49.
[4] S. Kijimoto and S. Hakomori, Biochem. Biophys. Res. Commun. 44 (1971) 557.
[5] S. Hakomori, S. Kijimoto, and B. Siddiqui, Federation Proc. 30 (1971) 1043 Abs.
[6] B. Siddiqui and S. Hakomori, J. Biol. Chem. 246 (1971) 5766.
[7] L. Diamond, Int. J. Cancer 2 (1967) 143.
[8] I. MacPherson and L. Montagnier, Virology 23 (1964) 291.
[9] T. Saito and S. Hakomori, J. Lipid Res. 12 (1971) 257.
[10] D.E. Vance and C.C. Sweeley, J. Lipid Res. 8 (1967) 62
[11] G.M. Gray, Biochim. Biophys. Acta 144 (1967) 511.
[12] T. Yamakawa, R. Irie, and M. Iwanaga, J. Biochem. 48 (1961) 490.
[13] L. Svennerholm, Acta Chem. Scand. 17 (1963) 860.
[14] S. Hakomori and G.D. Strycharz, Biochemistry 7 (1968) 1279.
[15] S. Hakomori, B. Siddiqui, Y-T. Li, S-C. Li, and C.G. Hellerqvist, J. Biol. Chem 246 (1971) 2271.
[16] J. Koscielak, S. Hakomori, and R. Jeanloz, Immunochemistry 5 (1968) 441.

[17] N.S. Sharples and G.A. LoGrippo, Henry Ford Hospital Bulletin 13 (1965) 55.
[18] M. Fogel and L. Sachs, Exp. Cell Res. 34 (1964) 448.
[19] C.H. O'Neill, J. Cell. Sci. 3 (1968) 405.
[20] H.T. Robertson and P.H. Black, Proc. Soc. Exp. Biol. Med. 130 (1969) 363.
[21] A. Makita and Y. Seyama, Biochim. Biophys. Acta 241 (1971) 403.
[22] M.M. Burger, Nature New Biol. 231 (1971) 125.