Tumor Res. **31**, 31—42 (1996)

Cell Density Regulates Antibody Accessibility and Metabolic Turnover of Gangliosides in Human Glioma Cells

Junpei YOSHIKAWA*, Toshiaki YAMAKI, Haozhe PIAO, Hongwei YU, Kohshi TATEWAKI, Yukihiro IBAYASHI, and Kazuo HASHI

Department of Neurosurgery, Sapporo Medical University School of Medicine, S-1, W-16, Sapporo, Hokkaido 060, Japan

ABSTRACT

The effects of cell density on the gangliosides of 4 human glioma cell lines were studied. The cell lines used were KG-1C (GM3-dominant), A172 and H4 (GM2-dominant), and Hs683 (GM3, GM2-co-dominant) cells. All these cell lines showed higher immunofluorescence with anti-ganglioside antibodies in FACS analysis at sparse density than at confluent density. Steric hindrance from cell surface proteins had been removed by the pretreatment of the cells with trypsin. The chemical content of gangliosides was consistent throughout the time of cell growth. The mechanisms of crypticity of gangliosides at confluent culture were under investigation. We first evaluated the metabolic turnover rate of gangliosides at different cell densities. The results clearly showed a more rapid turnover of gangliosides at sparse density from approximately 2 to 4 fold in terms of radioactivities of incorporated tritium into gangliosides. The profiles of labeled gangliosides were also different between the sparse and confluent cultures. We speculate that better accessibilities of antibodies toward gangliosides should be facilitated by the same mechanism which should, in turn, provide easier access of carbohydrate-hydrolysis enzymes to gangliosides at sparse cell density in order to keep an enhanced turnover rate.

Key words: Glycolipid, Glioma, Cell density, Immunoreactivity, Turn over

INTRODUCTION

Glycolipids are located at the cell surface of most mammalian cells, and it has been suggested that they may play a role in normal growth regulation. Since glycolipids are considered to function in various cell surface phenomena such as cell-to-cell or cell-to-matrix contact (1), it is possible that analysis of glycolipid

* To whom requests for reprints should be addressed

profiles in an individual case may provide information concerning the biological behavior of gliomas in terms of infiltrative or proliferative potentials, or response to treatment. Furthermore, monoclonal antibodies against those glycolipid antigens in gliomas can be utilized in various immunodiagnosis or therapy based on the laboratory analysis of an individual tumor. However, the presence of a given glycolipid alone does not always indicate the active involvement in binding with external ligands such as antibodies, lectins, or carbohydrate-oriented enzymes. Receptors that are present in the membrane and yet to fail to bind with external ligands are said to be cryptic (2), (3). In our previous study of glycolipids of human glioma cell lines, we quantified the chemical content of the major ganglioside components and analyzed the data with regard to the accessibilities of anti-ganglioside antibodies toward those residing in the cell membrane and those extracted and purified from the cells (4). The study supported the concept of crypticity of glycolipid carbohydrates in the sense that it revealed more a consistent antibody binding in parallel with the chemical quantity with isolated gangliosides than with those indigenous in the cell membrane. In fact, the capabilities of antibodies to bind with cell-surface gangliosides were often unpredictable. In another study, we have found that anti-GM3 ganglioside antibody reactivity toward KG-1C glioma cells, of which glycolipid component was almost exclusively GM3, showed an inverse correlation with cell density although the chemical content was consistent regardless of the density (5). The present study was designed to collect more evidence to support the correlation of the reactivity of anti-ganglioside antibodies with the cell density using cell lines with different ganglioside profiles, to analyze the turnover rate of those gangliosides and to see if it could account for certain aspects of the background mechanism of glycolipid crypticity.

MATERIAL AND METHODS

CELLS: Human glioma cell lines used were H4 and Hs683 glioma cells (American Type Culture Collection), and A172 and KG-1C glioma cells (Japanese Cancer Research Resources Bank). The cells were cultured in Dulbecco's MEM with 10% FCS supplemented with 0.1mM of the non-essential amino acids, 2mM L-glutamine, and 1mM sodium pyruvate without the addition of antibiotics at 37°C with 5% CO_2 atmosphere; the cells were maintained in 15cm-diameter culture dishes (CORNING, USA) with 50ml of the culture media.

FLOWCYTOMETRIC STUDY: The cells, cultured either in sparse or confluent densities, were detached with trypsin and EDTA, and were reacted with the primary antibodies against ganglioside antigens (anti-GM3, M2590; anti-

GM2, GMB28; anti-sialylparagloboside (LM1), DU-PAN-2) for 1hr at room temperature. After washing, the cells were reacted with FITC-conjugated goat anti-mouse immunoglobulin for 1hr at room temperature. The cells were then washed again with PBS twice, and were fixed with 0.5% formaldehyde in PBS, and 1×10^4 cells were counted with FACScan (Beckton-Dickinson, CA). The cell number is plotted on the ordinate, and the fluorescence intensity is plotted on the abscissa.

METABOLIC LABELING AND EXTRACTION OF GLYCOLIPIDS: The cells, cultured either in sparse or confluent densities, were harvested from the dishes with Trypsin-EDTA (GIBCO BRL, USA) followed by washing with PBS. The cells in culture were labeled with [^{3}H]-glucosamine (6), which was incorporated into the neuraminic acid of the gangliosides. The total lipid was extracted with chloroform/methanol/water (CMW) = 4 : 8 : 3 (v/v/v) by 2 consecutive 24hr extractions followed by a final 2hr extraction at 40°C. The whole extract was applied to a gel filtration column (Sephadex LH-20, Pharmacia, Sweden) for desalting. Total glycolipid collected was further applied to an ion-exchange column (Sephadex A-25, acetate form, Pharmacia, Sweden) to separate into neutral and acidic fractions. The acidic fraction was again desalted with a gel filtration column. Both the neutral and acidic fractions of each sample were chromatographed on a high performance TLC plate (MERCK, high performance TLC aluminum sheets silica gel 60, Germany). They were developed with C/M/W (60 : 25 : 4, v/v/v) for the neutral fraction and with C/M/0.2%-$CaCl_2$ (55 : 45 : 10, v/v/v) for the acidic fraction. Glycolipids developed on the plate were visualized in fluorography (7). An X-ray film (Kodak X-Omat) was exposed to the TLC at -80°C for 14 days.

DENSITOMETRIC ANALYSIS: Each sample of the acidic fraction was chromatographed on a high performance TLC plate (MERCK, silica gel 60, Germany), developed with C/M/0.2%-$CaCl_2$ (55 : 45 : 10, v/v/v). GSLs developed on the plate were stained by Resorcin-HCl reagent. Chromatograms were examined with a densitometer (Shimadzu dual-wave-length TLC scanner, CS 910) with a wave length of 580nm for the sample and 800nm for the reference. The percentage distribution of each band was calculated by integrating each pack.

RESULTS

GANGLIOSIDE PROFILE OF GLIOMA CELLS: Ganglioside components of glioma cell lines were analyzed by TLC. As reported elsewhere (4), either

GM3 or GM2, or both gangliosides were shown to be the predominant components in all cell lines studied. We arbitrarily classified glioma cells into 3 types based on the GM3/GM2 ratio; each cell line was referred to as either 1) GM3-dominant, 2) GM2-dominant, or 3) GM3, GM2-co-dominant. The 4 cell lines used in this experiment were representative of each ganglioside type. KG-1C belongs to GM3-dominant type, H4 and A172 to GM2-dominant type, and Hs683 to GM3, GM2-co-dominant type (Fig. 1). Every cell line has detectable amounts of sialylparagloboside as the third predominant component, of which antibody reactivity was also studied in H4 and Hs683 cells.

FACS ANALYSIS WITH ANTI-GANGLIOSIDE ANTIBODIES: The antibody reactivities against cell surface GM3 and GM2 were analyzed with FACS. The percent of immunofluorescent positive cells in KG-1C cells with anti-GM3 antibody was 19.9% at a sparse cell density, while that at a confluent one was 1.8% (Fig. 2). The chemical content of GM3 was consistent per cell basis as re-

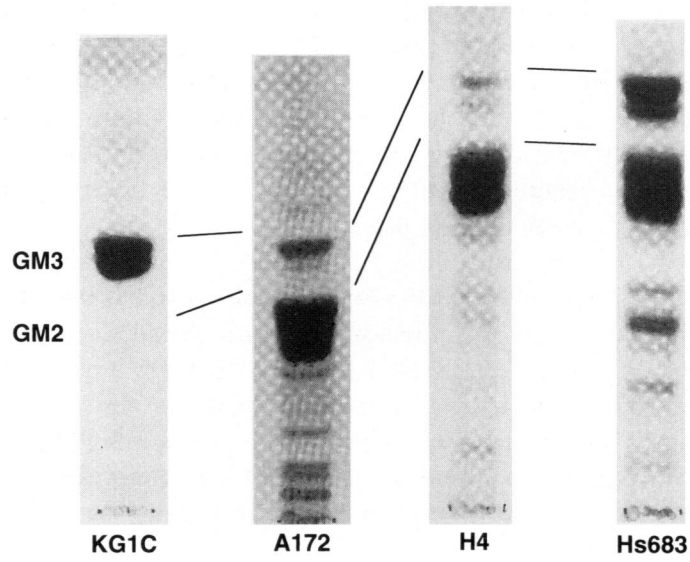

Fig. 1 Ganglioside profile of 4 glioma cell lines. Acidic glycolipids were developed on TLC. Dominant gangliosides were either GM3 (KG-1C) or GM2 (H4, A172) or both (Hs683), Sialylparagloboside seen below the band of GM2 was recognized in various amounts in each cell line.

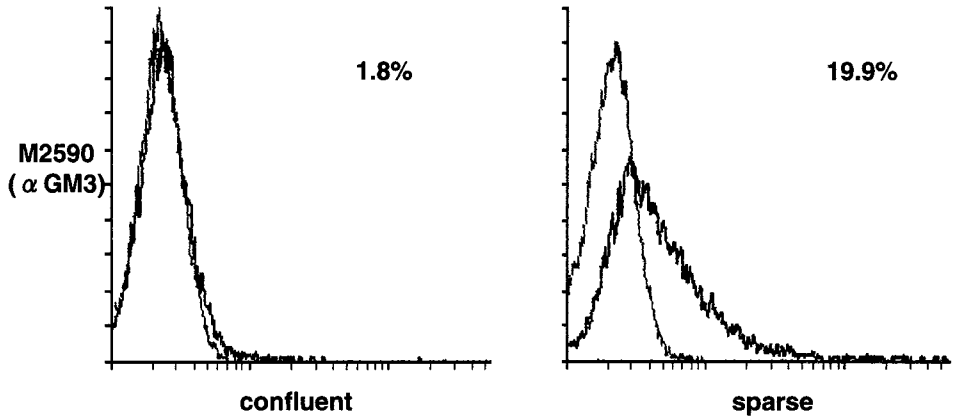

Fig. 2 FACS analysis of KG-1C cells using anti-GM3 antibody (M2590) at sparse or confluent cell densities. Sparse cells were more reactive with the antibody.

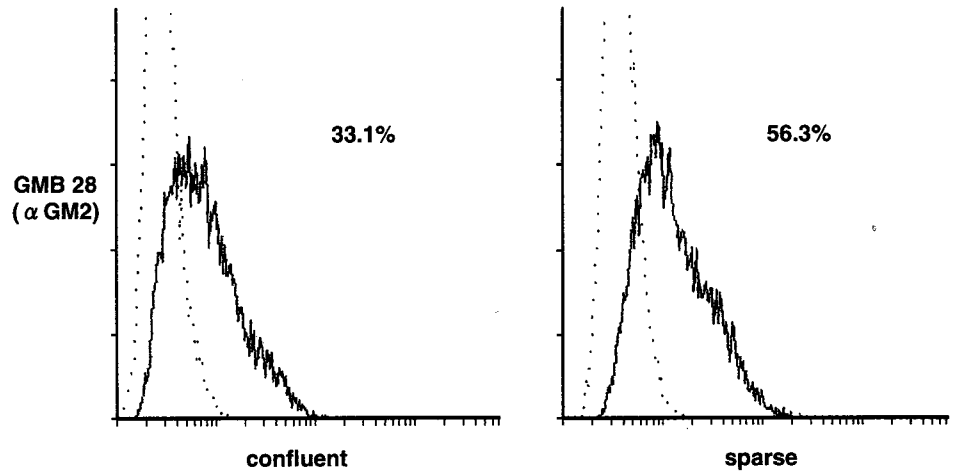

Fig. 3 FACS analysis of A172 cells using anti-GM2 antibody (GMB28) at sparse or confluent cell densities. Sparse cells were more reactive with the antibody.

ported previously (5) . Similarly, A172 cells showed 56.3% of anti-GM2-positive cells at a sparse density, which decreased to 33.1% at a confluent one (Fig. 3). H4 cells showed intense reactivity with anti-GM2 antibody, which resulted in no remarkable change in the percentage of fluorescent positive cells in different cell densities; however, anti-sialylparagloboside (LM1) antibody showed more reactivity with sparse cells (21.3%) than with confluent cells (5.7%) (Fig. 4). Hs683 cells showed uniformly increased immunofluorescence at a sparse density

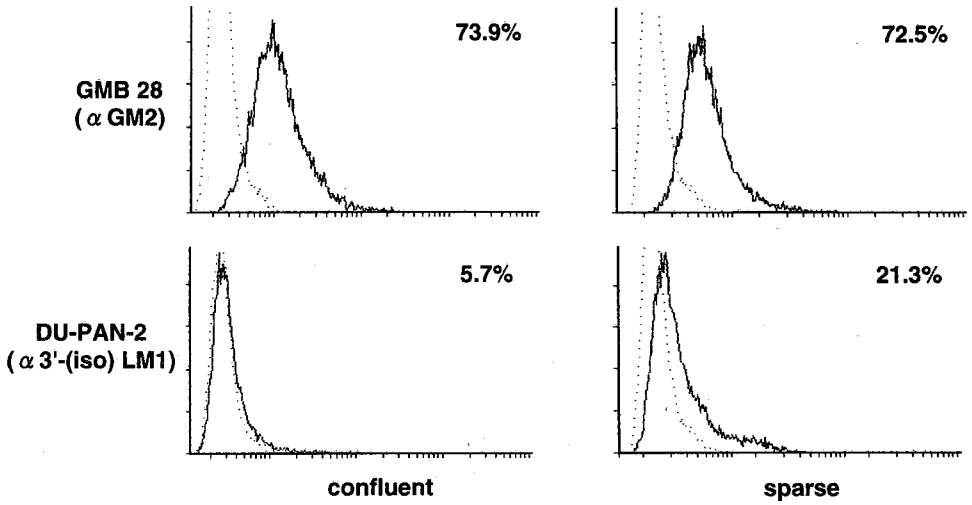

Fig. 4 FACS analysis of H4 cells using anti-GM2 (GMB28) and anti-sialylparagloboside (DU- PAN-2) antibodies. Sparse cells were more reactive with both antibodies.

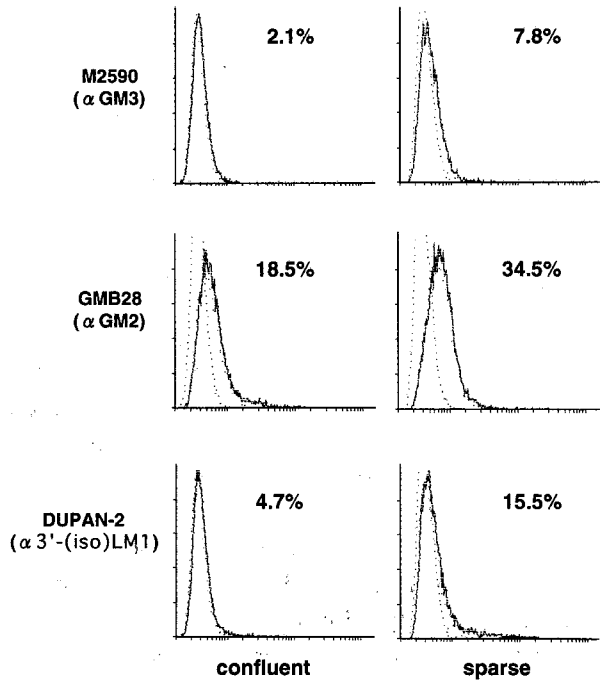

Fig. 5 FACS analysis of Hs683 cells using anti-GM3 (M2590), anti-GM2 (GMB28) and anti-sialylparagloboside (DU-PAN-2) antibodies. Sparse cells were more reactive with all antibodies examined.

with anti-GM3, -GM2, and-sialylparagloboside antibodies (Fig. 5).

METABOLIC LABELING OF GANGLIOSIDES: Radioactivities of tritium-labeled ganglioside from 1×10^6 cells counted with a scintillation counter were as follows: In all 4 cell lines examined, sparse cells were more actively labeled with tritium, which was statistically significant in Student's t-test (Table 1). The efficiency of labeling at sparse densities was approximately 2 fold in KG-1C, A172, and Hs683 cells, and nearly 4 fold in H4 cells as compared with that at confluent densities. Tritium-labeled gangliosides were then developed on TLC and were visualized by fluorography. As shown in Figure 6, the major labeled bands were GM3 and GM2, although different profiles were recognized between the sparse and the confluent culture, and between the labeled bands and the whole bands seen on a conventional TLC. Labeled gangliosides of KG-1C cells were composed almost solely of GM3 in confluent culture, while those in sparse culture were composed of GM3 and GM2. Hs683 showed more intense labeling of GM2 in sparse culture than in confluent culture, and other components including GM3 were also labeled in both cultures. Labeled gangliosides of H4 and A172 cells were revealed to be only GM2 in both sparse and confluent cultures. They showed more intense labeling of GM2 in sparse culture than in confluent culture. Labeling intensity was evaluated with densitometry of the exposed X-ray film, of which results were expressed as a percent distribution into GM3 and GM2 (Table 2). The chemical content of extracted GM3 and GM2 was also shown for comparison.

Table 1 *Radioactivities of tritium-labeled gangliosides at different cell densities*

	cell density[a]	
	sparse (dpm[b])	confluent (dpm)
cell lines		
KG-1C	420± 61[c]	248± 38
H4	8538± 686[d]	2240±221
A172	6925± 504[d]	3999±299
Hs683	29793±3617[d]	14159±881

a) The cells were cultured for 3 days in a culture dish with a diameter of 15 cm at a starting cell number of $1.5\text{-}2.0\times10^5$ for the sparse and $1.5\text{-}2.0\times10^6$ cells for the confluent culture.

b) Radioactivities of tritium-labeled gangliosides from 1×10^6 cells were counted in triplicate with a scintillation counter.

c) $p<0.05$

d) $p<0.005$

Table 2 *Labeling efficiency and chemical quantity of GM3 and GM2*

		KG1C		H4	
		sp.	conf.	sp.	conf.
metabolic label(%)[a]	GM3	64.46±9.32	83.98±12.68	4.7 ±0.38	undetectable
	GM2	35.54±5.14	16.02± 2.42	95.30±7.65	100.00
chemical content[b]	GM3	3.45		0.30	
	GM2	0.00		3.68	

		A172		Hs683	
		sp.	conf.	sp.	conf.
metabolic label(%)[a]	GM3	undetectable	undetectable	17.06± 2.07	11.19±0.70
	GM2	100.00	100.00	82.94±10.07	88.81±5.53
chemical content[b]	GM3	1.04		4.33	
	GM2	6.84		5.46	

a) Percentage distribution of labeled gangliosides was calculated from the results of densitometry of fluorograms.

b) Chemical content of gangliosides was expressed as lipid-bound sialic acid (μg)/10^7 cells.

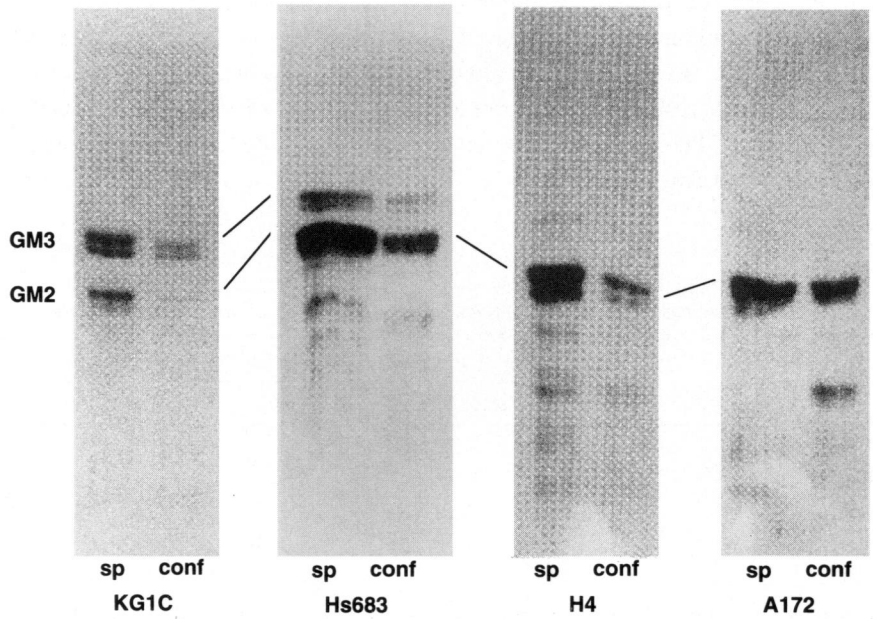

Fig. 6 Fluorography of gangliosides of 4 glioma cell lines. Cells at sparse or confluent density were incubated for 6 hr in the presence of [^{3}H] glucosamine hydrochloride, and were incorporated into the sialic acid residue of gangliosides. Gangliosides were extracted and their amount equivalent to 1×10^6 cells were developed on TLC. Those with a rapid turnover rate were labeled more strongly.

DISCUSSION

It has been long pointed out that the malignant transformation of cells would also manifest itself as an alteration of glycolipid profiles, usually in the form of the incomplete elongation of carbohydrate chains (8). The central nervous system is the organ most abundant with glycolipids, and their normal ganglioside profile is consisted of 4 major components; i. e., GM1, GD1a, GD1b and GT1b (9). All these gangliosides have the back bone structures of carbohydrate made up from 4 hexose linkage. In 10 glioma cell lines studied in our laboratory the dominant gangliosides were either GM3 or GM2 or both (4) ; the results supported the idea of an incomplete synthesis of carbohydrate chains in neoplastic cells in the central nervous system as well as other organs. One of our purposes of glycolipid study has been to evaluate the feasibility of targeting tumor-associated gangliosides with the use of antibodies to bring clinical benefits. The previous studies indicated that the simple presence of gangliosides in the cell membrane, or their chemical quantity would not necessarily determine the degree of antibody reactivities; the phenomenon called 'crypticity' of glycolipid antigens.

A variety of factors have been shown to influence the accessibility of external macromolecules including antibodies to carbohydrate heads of glycolipids in the cell membrane. One simple observation is enhancement of antibody binding by the pretreatment of cells with proteolytic enzymes such as trypsin (10), (11), (12), which explains a part of the crypticity phenomenon by steric hindrance from neighboring (glyco) proteins. Similarly, antibody accessibility toward a certain ganglioside was shown to be affected by the presence of more complex gangliosides in a cell (13). However, the concept of the physical burying of glycolipids as the cause of crypticity seems too simple to account for all cryptic phenomena. Peters *et al.* demonstrated in model membrane studies that factors other than steric interference by macromolecules can determine the course of a specific binding event (2). They concluded that simple, non-specific shielding by surface macromolecules may play a relatively minor role in glycolipid crypticity in cell membranes, and proposed that alternative possibilities should be considered from the point of glycolipid dynamics, interaction with other lipids, distribution, orientation and also specific associations. We happened to notice in KG-1C cells that the degree of antibody binding with GM3 ganglioside was clearly correlated with the cell density although the GM3 quantity was stable throughout the cell growth in culture. In the extensive study using KG-1C cells, we demonstrated that crypticity of GM3 was not due to steric hindrance from other cell surface macromolecules (5). In the present study, we picked up 4 representative cell lines including KG-1C cells with different ganglioside profiles. Those cell lines

contain either GM3 or GM2, or both gangliosides as their major components. In all 4 cell lines, stronger antibody reactivity was recognized in FACS analysis with sparse cells than with confluent cells. The only exception was GM2 of H4 cells, in which positive immunofluorescent cells were remarkably high regardless of the cell density (73.9% with confluent cells and 72.5% with sparse cells).

In the literature surveyed, cell density was reported to influence the glycolipid content or its profiles as well as its availability for external ligands. A cell density-related increase in glycolipids or elongation of carbohydrate chains was found in human skin fibroblasts, NIL hamster fibroblasts, and also glioma cells (14), (15), (16), (17). No glioma cell lines studied in this report showed any changes in glycolipid content or its type. We cannot account for this discrepancy between the reported results and ours at present. As for the changes in antibody reactivity along with the increase of cell density, Rösner *et al.*(14) observed increased antibody binding with GM3 in parallel with cell density, while Sakiyama *et al.* found a higher percent of immunofluorescent cells with anti-GM3 antibody at a sparse density than at a confluent one in several hamster and murine cell lines (15). Our results coincided with those of the latter. As often is the case in glycolipid research, here also, there are two, completely contradicting phenomena, which cannot be generalized by one uniform concept. The mechanisms behind the different availability of glycolipids for the antibodies must be disclosed in each experimental model.

One major mechanisms of receptor regulation is metabolic control: control of the biochemical pathways for receptor synthesis, and control of membrane turnover. If glycolipids could be regarded as cell surface receptors, metabolic control of glycolipids should become an important consideration. We first surveyed the metabolic background of glycolipids in an attempt to disclose different antibody reactivity at different cell densities. Tritium labeled gangliosides from 1×10^6 cells were counted for their DPM at different cell densities. In all 4 cell lines studied, gangliosides from sparse cells showed significantly higher counts (approximately 2 to 4 fold) than confluent cells, indicating more a rapid turnover rate of gangliosides in sparse cells. On looking at the fluorography of labeled-gangliosides, its profile in each cell line was considerably different from the TLC pattern of the whole extracted gangliosides. Rapid turnover should indicate rapid synthesis and transport of gangliosides to the cell membrane followed by rapid degradation by carbohydrate-hydrosis enzymes at the cell surface keeping the chemical content in these cell lines consistent at any cell density. Those gangliosides on sparse cells should then be presented on the cell surface in such a manner that degrading enzymes can access them more easily. This cell surface situation could also provide better access for antibodies toward gangliosides. In

subsequent experiments the direct link between rapid turnover rate and better access of antibodies should be clarified.

REFERENCES

1. HAKOMORI S. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu Rev Biochem 1981, 50: 733-764.
2. M. W. PETERS, C. J. SINGLETON, K. R. BARBER, and C. W. M. GRANT. Glycolipid crypticity in membranes-not a simple shielding effect of macromolecules. Biochim. Biophys. Acta 1983, 731: 475-482.
3. KANNAGI R, STROUP R, COCHRAN NA, URDAL DL, YOUNG Jr. WW, HAKOMORI S. Factors affecting expression of glycolipid tumor antigens: influence of ceramide composition and coexisting glycolipid on the antigenicity of gangliotriaosylceramide in murine lymphoma cells. Cancer Res 1983, 43: 4997-5005.
4. Yoshihiro MAEDA. Analysis of glycosphingolipid profile of human glioma cell lines and reactivities of anti-GM3 and-GM2 monoclonal antibodies. The Sapporo Medical Journal 1995, 64: 109-118.
5. Koshi TATEWAKI. Study on the regulation of crypticity of ganglioside GM3 on human glioma cell line KG1C depending on cell density. The Sapporo Medical Journal 1996, 65: 1-12.
6. KOBAYASHI T, HONKE K, KAMIO K, SAKAKIBARA N, GASA S, MIYANO N, TSUKAMOTO T, ISHIZUKA I, MIYAZAKI T and MAKITA A. Sulfolipids and glycolipid sulfotransferase activities in human renal cell carcinoma cells. Br J Cancer Res 1993, 67: 76-80.
7. BONNER WM and STEDMAN JD. Efficient fluorography of ^{3}H and ^{14}C on thin layers. Anal Biochem 1978, 89: 247-256.
8. HAKOMORI S. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res 1985, 45: 2405-2414.
9. ANDO S. Gangliosides in the nervous system. Neurochem Int 1983, 5: 507-537.
10. GLENNEY, J. R. Jr. and WALBORG, E. F. Jr. Biochim. Biophys. Acta 1980, 599: 493-504.
11. LANDA, C. A., DEFILPO, S. S., MACCIONI, H. J. F. and CAPUTTO, R. J Neurochem 1981, 37: 813-823.
12. TOMICH, J. M., MATHER, I. H. and KEENAN, T. W. Biochim. Biophys. Acta 1976, 433: 357-364.
13. LLOYD KO, GORDON CM, THAMPOE IJ, DIBENEDETTO C. Cell surface accessibility of individual gangliosides in malignant melanoma cells to antibodies is influenced by the total ganglioside composition of the cells. Cancer Res 1992, 52: 4948-4953.
14. RÖSNER H, GREIS C, RODEMANN HP. Density-dependent expression of ganglioside GM3 by human skin fibroblasts in an all-or-none fashion, as a possible modulator of cell growth in vitro. Exp Cell Res 1990, 190: 161-169.
15. SAKIYAMA, H. TAKAHASHI, T., HIRABAYASHI, Y and TANIGUCHI, M. Change in the topographical distribution of GM3 during cell spreading and growth: Immunostaining with monoclonal antibody against GM3. Cell Struct Funct 1987, 12: 93-105.
16. LIEPKALNS, V. A., ICARD, C., YATES, A. J., THOMPSON, D. K., HART, R. W. Effects of

cell density on lipids on human glioma and fetal neural cells. J Neurochem 1981, 36: 1959-1965.

17. YATES, A. J., LIEPKALNS, V. A., ICARD-LIEPKALNS, C., JUNGALWALA, F., STEPHENS, R. E., HART, R. W. Effects of cell density on the neutral glycolipid composition of cultured human brain and glioma cells. Neurochem Res 1982, 7: 1269-1276.