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Cell density-dependent changes of glycosphingolipid biosynthesis in cultured human skin fibroblasts

Željka Vukelić* and Svjetlana Kalanj-Bognar

Department of Chemistry and Biochemistry, Medical Faculty, University of Zagreb, Croatia

In this study, the glycosphingolipid biosynthesis was investigated in the sparse and the confluent cell populations of cultured human skin fibroblasts.

The human skin fibroblast cell populations were metabolically pulse labeled with ^{14}C-galactose (48 h). The amounts of ^{14}C-radioactivity (cpm) incorporated into extracted and purified total cellular glycosphingolipid fractions were counted by β-scintillation and the individual glycosphingolipid species were separated by high performance thin layer chromatography and visualized by autoradiography. The relative labeling (%) of individual newly synthesized glycosphingolipid species was detected by densitometric scanning of autoradiographic glycosphingolipid patterns.

The incorporation of ^{14}C-label into total glycosphingolipids per cell increased significantly as the cell-density increased, referring to five fold higher rate of glycosphingolipid biosynthesis *de novo* in cells at confluency vs. sparse populations. The total newly synthesized glycosphingolipid pattern (100%) of sparse cell populations showed a significant predominance of the gangliosides (70%) over the neutral glycosphingolipids (30%), with ganglioside GM2 as the major species followed by monohexosyl-ceramide. Oppositely, the newly synthesized neutral glycosphingolipids (67%) predominated over the gangliosides (33%) in cells at confluency (contact inhibition). Cells reaching confluency were characterized by: (a) a dramatic increase of absolute amount of all newly synthesized neutral glycosphingolipid species, particularly the most abundant monohexosyl-ceramide and trihexosyl-ceramide, but also of the ganglioside GM3; (b) a drastic decrease of absolute amount of newly synthesized ganglioside GM2. The specific shift in newly synthesized glycosphingolipid pattern in cells reaching confluency suggests a down-regulation of biosynthetic pathway primarily at the level of N-acetylgalactosaminyl-transferase. A possible involvement of glycosphingolipids in cell density-dependent regulation of cell growth through establishment of the direct intermolecular intermembrane interactions is discussed.

Keywords: glycosphingolipid biosynthesis *de novo*, cell density, normal human skin fibroblasts

Abbreviations: GGs, gangliosides; GSLs, glycosphingolipids; HPTLC, high-performance thin-layer chromatography. Gangliosides and the precursor glycosphingolipids are abbreviated according to the system of Svennerholm [25] and the recommendations of IUPAC-IUB Commission on Biochemical Nomenclature [26,27] as follows: GlcCer, Glcβ1Cer; LacCer, Galβ4Glcβ1Cer; GA2, Gg_3Cer, GalNAcβ4Galβ4Glcβ1Cer; GA1, Gg_4Cer, Galβ3GalNAcβ4Galβ4Glcβ1Cer; Gb_4Cer, Gb4, GalNAcβ3Galα4Galβ4Glcβ1Cer; Gb_5Cer, Galβ4GalNAcβ3Galα4Galβ4Glcβ1Cer; GM3, II^3-α-Neu5Ac-LacCer; GD3, II^3-α-$(Neu5Ac)_2$-LacCer; GT3, II^3-α-$(Neu5Ac)_3$-LacCer; GM2, II^3-α-Neu5Ac-Gg_3Cer; GD2, II^3-α-$(Neu5Ac)_2$-Gg_3Cer; GM1, II^3-α-Neu5Ac-Gg_4Cer; GD1a, IV^3-α-Neu5Ac, II^3-α-Neu5Ac-Gg_4Cer; GD1b, II^3-α-$(Neu5Ac)_2$-Gg_4Cer; CMH, monohexosyl-ceramide; CDH, dihexosyl-ceramide; CTH, trihexosyl-ceramide.

Introduction

Glycosphingolipids (GSLs) are found in essentially all eukaryotic cells as the ubiquitous building components of the outer leaflet of the plasma membrane contributing to the carbohydrate-rich glycocalyx that determines surface properties of cells [1,2]. It has been evidenced that they may regulate cell proliferation and induce differentiation acting as modulators of transmembrane signaling and mediators of cellular interactions [3,4]. Specific changes in GSL biosynthesis associated with cell cycle and "contact-inhibition" of cell growth at saturated cell density have been documented in several nontransformed cell lines [5–9]. Mentioned events are

*To whom correspondence should be addressed: Željka Vukelić, Department of Chemistry and Biochemistry, Medical Faculty, Šalata 3, 10000 Zagreb, Croatia. Tel.: + 385 1 45 66 919; Fax: + 385 1 45 66 709; E-mail address: zvukelic@mamef.mef.hr

thus in correlation with specific glycosphingolipid pattern of cellular membranes, due to increased activity of particular glycosyl-transferases involved in GSL biosynthesis. The experiments using specific anti-glycosphingolipid antibodies indirectly confirmed the hypothesis that activation of specific biosynthetic glycosyltransferase(s) either accompanies or is the part of growth control mechanism through contact inhibition due to increased cell density [1,10]. Also, the observation that exogenous GSLs incorporate into plasma membranes and behave like endogenous GSLs is another evidence of correlation between cyclic changes of GSL biosynthesis and cell growth cycles [11].

There have been only few publications so far, which refer to changes of GSL biosynthesis and/or metabolic turnover in correlation with increased cell density. Moreover, there have been no systematic data on mentioned phenomena in normal finite cell lines such as cultured skin fibroblasts of human origin. This study presents a systematic data describing specific cell density-dependent changes of glycosphingolipid biosynthesis *de novo* in normal cultured human skin fibroblasts.

Material and methods

Chemicals

For maintaining of cell cultures we used: RPMI culture medium; penicillin and streptomycin (Boehringer, Mannheim, Germany); fetal calf serum and trypsin (Dipro-Gibco, Karlsruhe, Germany); L-glutamin and ethylen-diamin-tetraacetate (EDTA, Sigma, St. Louis, USA); sterile flasks with 25 cm^2 growing area (Falcon-Becton Dickinson, Le Pont de Claix, France). For radioactive labeling of cell metabolism we used: D-1-[^{14}C]galactose (50–60 mCi/mmol, 250 μCi/ml, Amersham-Buchler, Braunschweig, Germany); Kodak X-Omat R roentgen films; diphenyloxazole (PPO) and 1-4-bis(5-phenyl-2-oxazolyl)benzene (POPOP, Sigma, St. Louis, USA). Extraction, purification and analysis of glycosphingolipids was performed using redestilled organic solvents of p.a. grade (Kemika, Zagreb, Croatia); Sephadex G-25 fine (Pharmacia, Uppsala, Sweden); HPTLC plates (Merck, Darmstadt, Germany).

Gangliosides standards

Standard ganglioside mixture "Cronassial" from bovine brain (21% GM1, 40% GD1a, 16% GD1b, 19% GT1b) was obtained from Fidia Research Laboratories (Abano Terme, Italy). Gangliosides GT3, GD3 and GM3 were extracted and purified from bovine liver (a generous gift from Dr. T. Ariga, Richmond, VA, USA). Gangliosides GD2 and GM2 were extracted and purified from human lung tissue in our laboratory. Ganglioside standard mixtures were applied onto HPTLC plates in parallel with GSL extracts from labeled human skin fibroblasts, and chromatographically separated individual ganglioside fractions were visualized by spraying with resorcinol-HCl reagent [12].

Skin fibroblasts

Two primary cultures of normal human skin fibroblasts were established from bioptic material (age: 1.5 years) taken during routine diagnostic procedure, and further subcultivated in split ratio 1 : 2 according to generally defined conditions [13]. Skin fibroblast cultures were maintained as a monolayer at 25 cm^2 growing area in culture medium RPMI supplemented with 10% fetal calf serum (FCS), in humid atmosphere containing 5% of CO_2 at 37°C. Experiments were performed on cell cultures at population doubling level 11.

Metabolic labeling of human skin fibroblasts

The procedure for pulse labeling was done according to van Echten and Sandhoff [14]. Subconfluent cell population (approx. 2×10^6 cells) was subcultivated into 6 culture flasks in split ratio of 1/12, 2/12 and 3/12, each in duplicate. In parallel, one subconfluent cell population was subcultivated in split ratio of 1 : 2 and left to reach the confluency corresponding to the relative cell density of 12/12. Sparse skin fibroblast cultures of different cell densities corresponding to 1/12, 2/12 and 3/12 of total cell number at confluency and confluent culture (12/12) were washed with RPMI and incubated in 2.5 ml of culture medium containing ^{14}C-galactose (2 μCi/ml) for 48 hours (pulse interval). Pulse treated cells were then counted and harvested for biochemical analysis. The experiment was performed twice.

Extraction and purification of glycosphingolipids

Sparse and confluent cultures of skin fibroblasts were homogenized in 1.4 ml of water (W) by freezing-thawing method, followed by thorough mixing and sonification. GSL extraction and purification was performed according to method of Svennerholm and Fredman [15] and modified according to Byrne et al. [16]. Briefly, chloroform (C)/methanol (M) solvent mixture (1 : 2, by vol.) was added to 1.4 ml of cell homogenate (final volume ratio of C : M : W was 1 : 2 : 0.7). After 24 hours of extraction at room temperature and centrifugation, the supernatant was dried under a N_2 stream. Dry lipid extract was subjected to mild alkaline hydrolysis in 1.5 ml of 0.5 M KOH in methanol, at 4°C overnight. Sample was then neutralized with 0.75 ml of 1.0 M acetic acid and dried under a N_2 stream. Dry GSL extract was further dissolved in 100–150 μl of C : M : W (60 : 30 : 4.5, by vol.) and gel-filtrated on 1 ml Sephadex G-25 column. Purified glycosphingolipids were finally eluted with 5 ml of C : M : W (60 : 30 : 4.5, by vol.).

Qualitative and quantitative analysis of newly synthesized glycosphingolipids

Total ^{14}C-radioactivity (cpm, counts per minute) incorporated into glycosphingolipids of pulse labeled skin fibroblasts was determined in GSL extracts by β-scintillation counter. Individual glycosphingolipids from purified GSL extracts were separated by high-performance thin layer chromatography

(HPTLC), using solvent mixture C : M : 12 mM aq.$MgCl_2$ (58 : 40 : 9, by vol.) as mobile phase. HPTLC plates were then covered with autoradiographic films being exposed at $-70^{\circ}C$ for 5 days. Autoradiograms revealed individual GSL fractions, into which ^{14}C-galactose was incorporated during 48 h of pulse interval. Relative quantification of individual ^{14}C-GSL fractions was performed by densitometric scanning of autoradiograms (LKB 2202 Laser Ultrascan, $\lambda = 620$ nm) after 3 days of exposure. The relative quantification performed by β-scintillation counting of radioactivity incorporated in individual GSL fractions scraped from the HPTLC plate showed almost identical results, confirming the linearity of densitometric detection. All given values are the mean values of two parallel experiments in which each measurement was performed in triplicate. Standard deviations did not exceed 10% of the mean values and are therefore omitted.

Assignment of detected glycosphingolipid fractions

All GSL fractions/species described in the section *Results* were monitored after separation on a HPTLC plate. Therefore, the fractions were tentatively assigned according to comparison of their TLC migration properties with the corresponding structurally well defined GSL species in the standard reference mixture, as introduced earlier by other authors [8,14,17–20].

Results

Absolute ^{14}C incorporation in total glycosphingolipid fraction of skin fibroblasts

Sparse cell populations of skin fibroblasts characterized by cell density corresponding to 1/12, 2/12 and 3/12 of confluent cell population, and confluent cell populations (12/12, approx. 2.0×10^6 cells/25 cm^2 of growth area) were labeled with ^{14}C-galactose during 48 hours (pulse interval). Table 1 gives data on amounts of total ^{14}C-radioactivity (cpm/10^6 cells) incorporated during pulse interval into total glycosphingolipid fraction (^{14}C-GSLs) of skin fibroblasts in sparse and confluent culture. Results showed that increase of cell density from 1/12 to 2/12 and 3/12 was accompanied by 2.1- and 3.3-fold increase of ^{14}C-incorporation into GSLs, respectively. Skin fibroblasts at confluency (12/12) incorporated 5.0-fold more ^{14}C-label comparing with cells in the sparse population of relative density 1/12. The incorporation of ^{14}C-label into GSLs per cell (cpm/cell), referring to a rate of GSL biosynthesis, generally increased in correlation with cell density as shown by maximal ^{14}C-incorporation in confluent cell populations. It seems that the most intensive increase in GSL biosynthesis accompanied the increase of cell density from 1/12 to 3/12 since the cell populations reaching the confluency from 3/12 to 12/12 showed less intensive increase of ^{14}C-label incorporation into GSLs.

Relative and absolute ^{14}C-labeling of individual glycosphingolipids. The ratio between gangliosides and neutral glycosphingolipids

Figure 1 shows autoradiograms of ^{14}C-GSLs extracted from sparse and confluent skin fibroblast cultures and separated by thin layer chromatography. Aliquots of purified GSL samples applied on the plate corresponded to: (I.a) equal amount of incorporated ^{14}C-radioactivity (approx. 2620 cpm per line) and (II.a) equal number of cells (approx. 1.67×10^5 per line, approx.

Table 1. Incorporation of ^{14}C-label into newly synthesized total GSLs (^{14}C-GSLs)* of human skin fibroblast (HSF) cell populations (CP) with different cell densities, during "pulse" metabolic labeling with ^{14}C-galactose

	HSF cell populations (PDL = 11, on 25 cm^2 growth area)			
	SPARSE CP			*CONFLUENT CP*
SR CP#	*1/12*	*2/12*	*3/12*	*12/12*
	pulse (48 h)			
Number of cells/flask (number of cells/mm^2)	1.674×10^5 (67)	3.303×10^5 (132)	5.099×10^5 (204)	1.953×10^6 (781)
Relative density (%)	100	197.2	304.5	1166.7
^{14}C-GSLs/CP (cpm)	2620.4	11077.1	26393.2	152039.0
Relative incorporation (%cpm)	100	422.7	1007.2	5802.1
^{14}C-GSLs/10^6 cells (cpm/10^6 cells)	15649.6	33541.5	51763.2	77849.0
Relative incorporation (%cpm)	100	214.3	330.8	497.5

#SR, split ratio of cell population (CP); subconfluent CP in 10th passage (approx. 2×10^6 cells) was subcultivated into 6 culture dishes, in split ratio of cell suspension: 1/12, 2/12, 3/12 (by vol.), each in duplicate; in parallel one subconfluent CP was subcultivated in split ratio of 1 : 2 and left to reach the confluency corresponding to the relative cell density of 12/12;
*Total GSLs were extracted and purified from HSF CPs after metabolic labeling, and incorporated radioactivity was counted by β-scintillation.
HSF, human skin fibroblasts.
PDL, population doubling level.

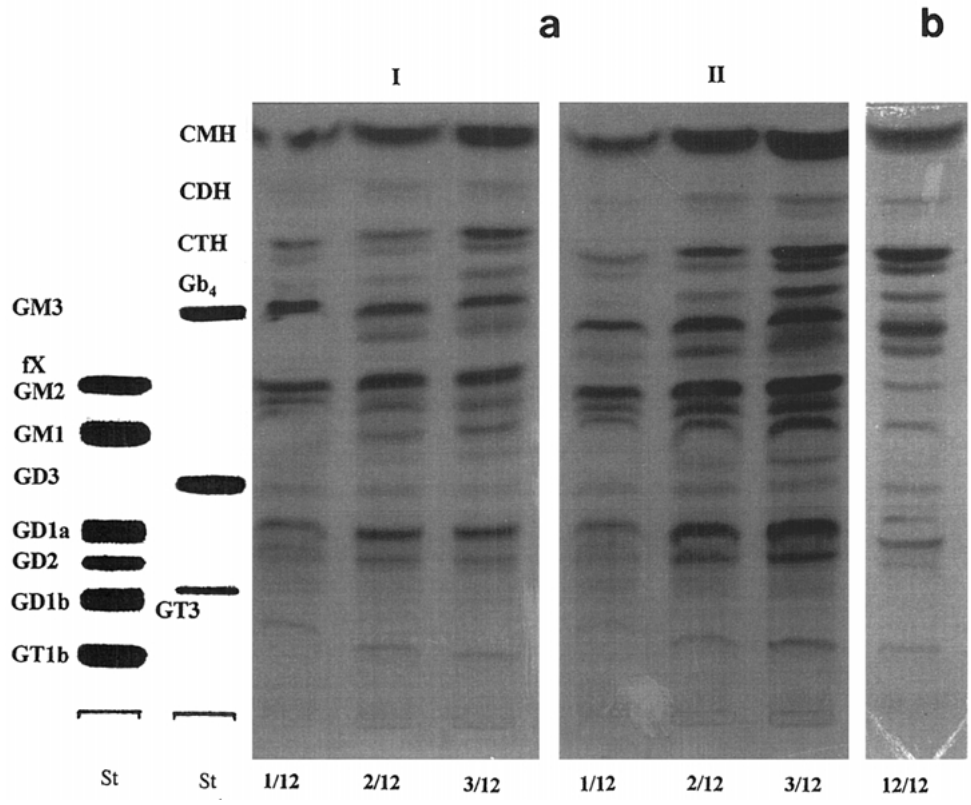

Figure 1. Autoradiographic patterns of the newly synthesized glycosphingolipids from the (a) sparse and the (b) confluent human skin fibroblast populations metabolically labeled (pulse, 48 h) with ^{14}C-galactose (2 μCi/ml). Extracted and purified GSL-fractions were separated by HPTLC (solvent system C : M : 12 mM $MgCl_2$, 58 : 40 : 9, by vol.). GSL-samples of the sparse HSF-CP correspond to: (I) the same radioactivity (approx. 2620 cpm); (II) the same number of cells (approx. 1.674×10^5). The accurate number of cells in each CP indicated as 1/12, 2/12, 3/12 and 12/12, is given in Table 1. HSF, human skin fibroblasts; CP, cell populations.

1/12). Figure 1b shows autoradiographic pattern of newly synthesized glycosphingolipids in confluent cell populations of human skin fibroblasts. The increase of cell density from 1/12 to 3/12 was accompanied by increased labeling of almost all individual GSL fractions. However, evident changes of quantitative proportions i.e. of relative labeling of individual GSLs in radioactive pattern, indicated quite different intensity of ^{14}C-incorporation into each GSL fraction (see Figure 3).

Figure 2 shows the relative labeling of individual glycosphingolipids in correlation to density of cell populations (1/12, 2/12, 3/12 and 12/12) as revealed by densitometric analysis of autoradiograms visualizing HPTLC-separated glycosphingolipid sample aliquots of equal radioactivity. The labeling of gangliosides vs. neutral GSLs was highly predominant in sparse cell populations (Figure 2A). On the other hand, an increase in cell density was accompanied by more intensive incorporation of radioactivity into neutral GSLs; predominant labeling of neutral GSLs vs. gangliosides was found in confluent cell populations. The ratio ^{14}C-gangliosides/^{14}C-neutral GSLs was 2.49 for cell populations of relative density 1/12 and 0.49 for confluent cell populations (12/12). The continuous increase of relative labeling of all detected individual neutral GSL species (with exception of CDH) correlated with increase in cell density from 1/12 to 12/12 (5.63-fold for Gb4/GA2, 5.15-fold for CTH, 1.52-fold for CMH) (Figure 2B). Consequently, relative labeling of majority of individual gangliosides continuously decreased especially GM2 (8.98-fold), GD1a (3.82-fold) and GD2 (3.90-fold); discontinuous changes in relative labeling were observed for GM3 and GD3 (Figure 2C). It should be pointed out that GM2 was recognized as the main species of the newly synthesized GSL-pattern of very sparse cell populations (1/12), maintaining its high proportion in other sparse populations (2/12 and 3/12), and extremely decreasing in confluent cell populations (12/12). One minor fraction migrating between gangliosides GM3 and GM2, assigned as fX, was detected by autoradiographic visualization of HPTLC-separated newly synthesized ^{14}C-glycosphingolipids of skin fibroblasts (Figure 1b). Only ^{14}C-GSL pattern of confluent cell populations (12/12) contained a detectable amount of fX, densitometrically quantified to account for 0.43% (not shown in Figure 2), while it was undetectable in very sparse (1/12) or found in traces in sparse cell populations (2/12, 3/12) (Figure 1).

Figure 3 shows the changes in absolute labeling (48 h of pulse interval; cpm/10^6 cells) of both neutral GSL and ganglioside individual fractions/species of human skin

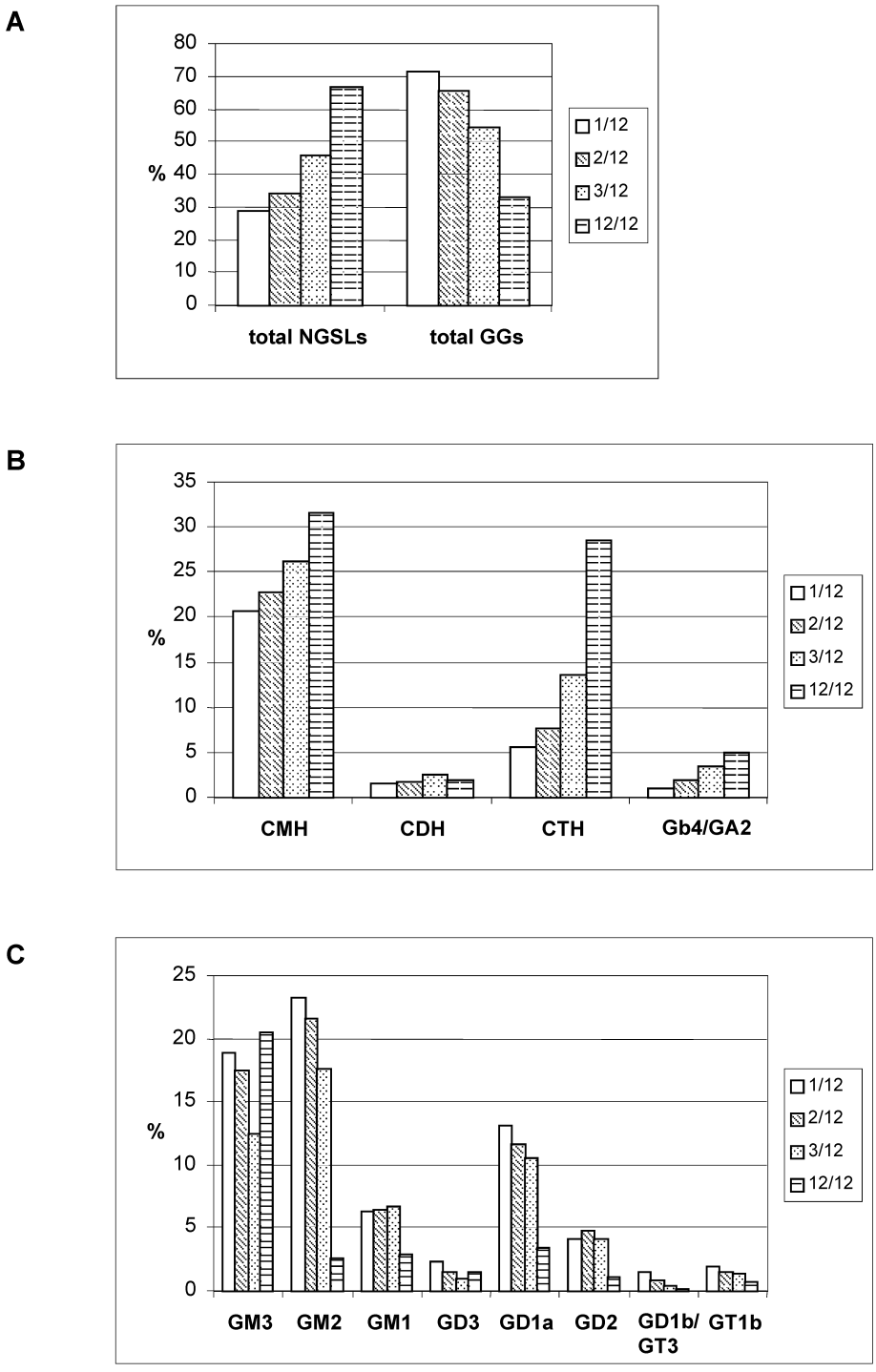

Figure 2. Relative incorporation of ^{14}C-label (%) into newly synthesized glycosphingolipid pattern of human skin fibroblasts in correlation to different cell densities during "pulse" interval of metabolic labeling with ^{14}C-galactose. Comparison of: (A) total neutral glycosphingolipids (NGSLs) and gangliosides (GGs); (B) individual neutral glycosphingolipid fractions; (C) individual ganglioside fractions. Relative densities of cell populations: sparse (1/12, 2/12, 3/12) and confluent (12/12).

fibroblasts with increase in cell density (1/12, 2/12, 3/12, 12/12). Absolute labeling i.e. ^{14}C incorporation into each individual GSL fraction (cpm/10^6 cells) was calculated by multiplying the relative proportion of a ^{14}C-GSL (proportion of autoradiogram's densitometric area) and the value of radioactivity incorporated into total ^{14}C-GSLs (cpm/10^6 cells). Increased labeling of all individual GSLs (except for constant GD1b/GT3 fraction) accompanied the increase of

cell density from 1/12 to 3/12, this increase being more intensive for neutral GSLs (particularly CTH and Gb_4Cer) than the gangliosides (Figure 3A). It seemed that labeling of neutral GSLs, GM3 and GD3 increased further (3/12 to 12/12) reaching maximal values in confluent cell populations (Figure 3B and C). On the contrary, labeling of all other gangliosides was lower in confluent (12/12) than in sparse cell populations (3/12) being the most prominent for GM2, GD2 and GD1a fractions (4.55-, 2.59-, 2.03-fold less, respectively) (Figure 3C). Absolute ^{14}C-labeling of the fraction fX, migrating between GM3 and GM2, was 335 cpm/10^6 cells in confluent cell populations (not shown in Figure 3), while none (1/12) or traces (2/12, 3/12) of ^{14}C-radioactivity were detected in sparse cell populations.

Discussion

Several studies have shown specific total (endogenous) as well as *de novo* biosynthetic GSL pattern i.e. composition and content of glycosphingolipids in confluent cultures of human skin fibroblasts [8,14,17–20]. Results on the composition and content of glycosphingolipids in normal confluent human skin fibroblasts obtained in our laboratory (data not shown) are completely in accordance with mentioned studies. As many of GSL species have not yet been structurally characterized, the tentative assignment of detected GSL species based on their TLC migration properties has been used in the mentioned studies as well as in this study.

According to available literature, there have been no systematic data on glycosphingolipid biosynthesis in correlation to cell density in normal finite cell lines. The majority of studies on glycosphingolipid metabolism were performed either on continuous cell lines, or fibroblast cultures derived from different species and tissues, or different *in vitro* age of cultures [5–9]. Biologic and growth characteristics of continuous cell lines are correlated to specific glycosphingolipid patterns and metabolism, which most probably do not reflect the real *in vivo* metabolism, composition and behavior of cellular glycosphingolipids. However, the results of those studies indicated that certain changes in biosynthesis and/or metabolic turnover of glycosphingolipids correlate with cell density and cellular interactions.

Our objective was to analyze whether the GSL biosynthesis *de novo* in normal finite human skin fibroblasts is in correlation with different cell population densities. The results showed that increased GSL biosynthesis in skin fibroblasts is correlated with increased cell density as shown by maximal ^{14}C-incorporation into total GSLs in confluent cell populations. Our results showing maximal GSL biosynthesis in confluent cell populations of human skin fibroblasts are in accordance with the same observations reported by Dawson et al. [9]. Based on data of our and other studies it may be generally assumed that different cell types show a manifold increase of net glycosphingolipid biosynthesis with increase in cell density reaching the confluency, while at the same time total concentration of glycosphingolipids is increased by 50–100%. Our data support the hypothesis that establishing of intercellular interactions and/or contact inhibition of growth is accompanied by an increase in both net biosynthesis and metabolic turnover of glycosphingolipids [2,4]. Here presented results referring to pulse labeling of individual glycosphingolipids of human skin fibroblasts clearly show that relative composition of newly synthesized GSL pattern is significantly different in sparse as compared with confluent cell populations, which indicates that the activity of particular biosynthetic glycosyl-transferases is differently expressed during increase of cell density. We observed that gangliosides GM2 followed by GD1a and GM1 represent the major species of newly synthesized ganglioside pattern of fibroblasts in low density populations, while in contact inhibited cells (confluency) their proportions are decreased due to increased GM3 fraction. Our results also showed a continuous increase of both absolute and relative labeling of detected neutral GSL-species as cell density increases. The phenomenon is observed to be particularly significant for more complex neutral GSL species i.e. Gb_4Cer/GA2 and CTH. The rate of their synthesis was more than 25-fold higher per cell at confluent vs. low density populations. Similar observations on increased proportions of neutral GSL-species in confluent cell populations were reported by several authors, for instance an increase of Gb_5Cer in NIL2 fibroblasts [21] and an increase of CTH (Gb_3Cer) in BHK fibroblasts [6,22] were found with increase in cell density.

Specific relative composition of newly synthesized GSL pattern was found to be significantly different in the low density cell populations (1/12–3/12 of confluency) vs. confluent populations of the human skin fibroblasts (12/12). In low density cell populations the proportion of gangliosides dominates over the neutral glycosphingolipids, with GM2 as the major GSL-species. An increase in cell density of sparse cell populations enables cells to establish the first cell-to-cell contacts. Our study showed that this event was accompanied by increased biosynthesis of all glycosphingolipids, but particularly by increase of neutral glycosphingolipids and ganglioside GM3 rather than other gangliosides. Hakomori described the increase of total cellular concentration of particularly GD3 and GM1 but also of GM3 in human diploid fibroblasts reaching the confluency [5], while Ohsawa showed increased total concentrations of gangliosides, especially increase of GD3 and GM1 but decrease in GM3 fraction in human fetal lung fibroblasts at early passages [8]. These results are not directly comparable with our data describing exclusively biosynthetic GSL pattern. However, it is clear that *de novo* synthesized neutral glycosphingolipids and GM3 serve not only as final functional biosynthetic products but also as precursors for biosynthesis of other gangliosides. We can speculate that changes of total concentrations of GD3, GM1 and GM3 as described in mentioned studies depend on pool of necessary biosynthetic precursors such as GlcCer

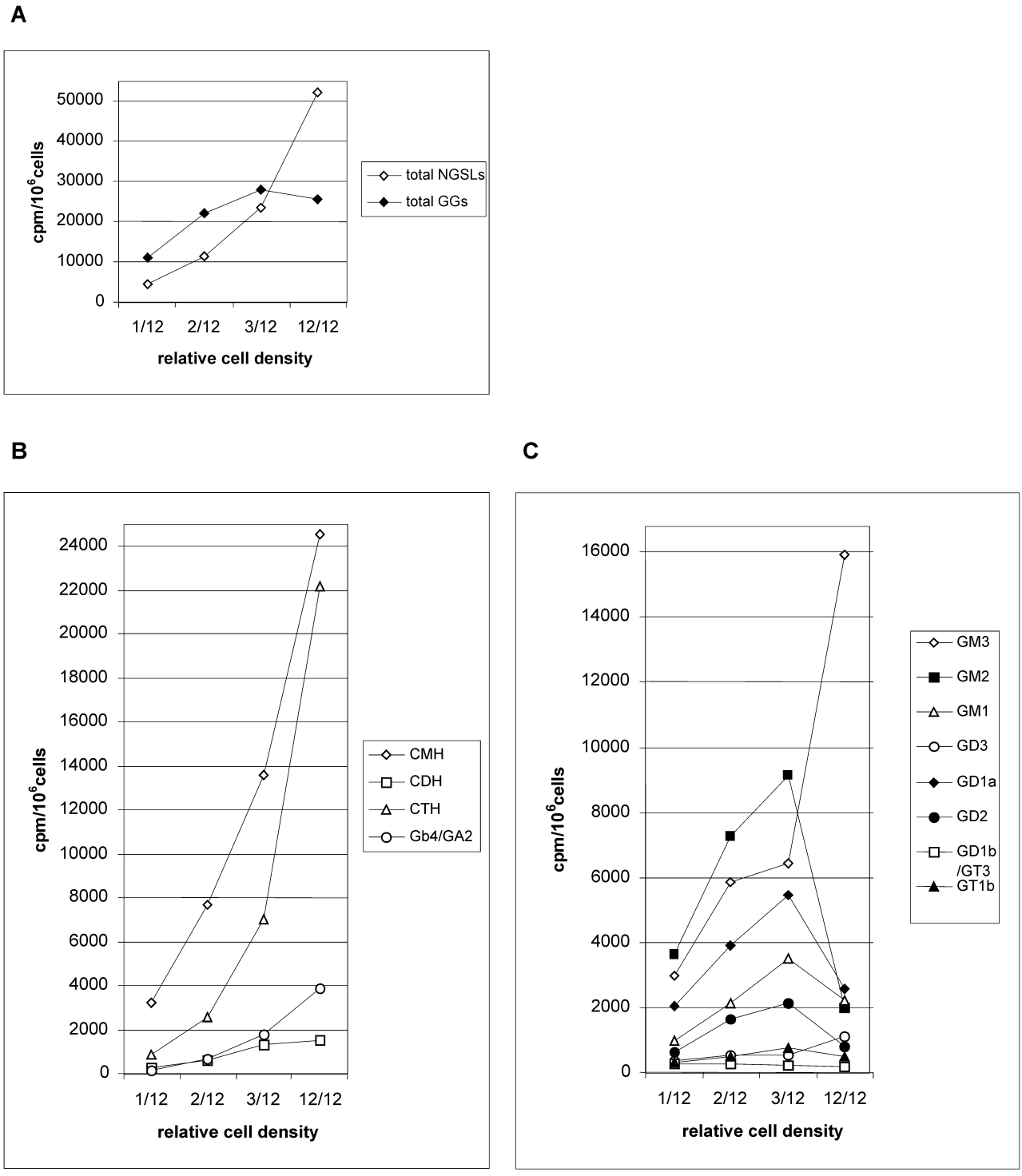

Figure 3. Changes in absolute labeling (cpm/10^6 cells) of newly synthesized glycosphingolipids from cultured skin fibroblasts with increase in cell population density during ''pulse'' interval of metabolic labeling with ^{14}C-galactose. Comparison of: (A) total neutral glycosphingolipids (NGSLs) and gangliosides (GGs); (B) individual neutral glycosphingolipid fractions; (C) individual ganglioside fractions. Relative densities of cell populations: sparse (1/12, 2/12, 3/12) and confluent (12/12).

(CMH), LacCer (CDH), GA2 (CTH) and GM3. Further, Yogeeswaran and Hakomori established that biosynthesis and total cellular content of GD1a is increased during first intercellular interactions in mouse 3T3 fibroblast cultures, which seemed to maintain their replicative ability [23]. This is in accordance with our finding showing the increase of newly synthesized ganglioside fractions GM2, GD1a, GM1 and GD2 during establishment of intercellular contacts (relative cell densities 1/12–3/12). However, it has to be noted that the experimental design described in Yogeswaran's paper is somewhat different from ours. As mentioned, study was performed on fibroblasts originating from mouse embryo and cells were cultured under different cultivation conditions. It is most probable that differential expression/activation of GSL biosynthetic enzymes (sialyl-transferases and glycosyl-transferases) during cell growth and proliferation is reflected in changes of newly synthesized GSL pattern. Our observation on increased proportion of GM2-species in sparse populations may be explained as a consequence of increased *N*-acetyl-galactosaminyl-transferase activity. Significant increase of newly synthesized GM3 in parallel with decrease of the GM2 and other more complex gangliosides in cells reaching confluency suggests down-regulation of biosynthetic pathway primarily at the level of *N*-acetyl-galactosaminyl-transferase and possibly up-regulation of sialyl-transferase I. Discussed findings of our study on human skin fibroblasts are in accordance with accepted hypothesis that glycosphingolipids, as a cell membrane components, are involved in the regulation of cell growth by direct cell-to-cell intermembrane molecular interactions [3,4]. Moreover, the ganglioside-neutral GSL intermolecular interactions, in particular GM3-LacCer, have been recognized as one of the first events involved in adhesion of the B16 melanoma cells to the endothelial cells, respectively [24].

In conclusion, glycosphingolipid biosynthesis *de novo* and consequent qualitative and quantitative composition of GSLs in normal human skin fibroblasts is specifically changed depending on population density. GSL biosynthesis *de novo* is significantly increased in contact inhibited cells at confluency in comparison with cells in sparse populations of human skin fibroblasts. Cell density dependent changes of newly synthesized GSL pattern are characterized by increased proportion of ganglioside species vs. neutral GSL-species in sparse cell populations. On the other hand, in spite of dramatic increase of newly synthesized ganglioside GM3, neutral GSLs predominate over the gangliosides in cells at confluency. It seems that increased expression of neutral glycosphingolipids and GM3 in plasma membrane of fibroblasts reaching confluency enable them to establish suitable intermembrane intermolecular interactions (GSL-GSL and/or GSL-protein) leading to inhibition of cellular growth in normal finite cell lines. The results of this study support the hypothesis that glycosphingolipids expressed at the cell surface might be involved in cell-density dependent regulation of cell growth.

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