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Changes of Sphingolipid Species in the Phenotype Conversion From Myofibroblasts to Lipocytes in Hepatic Stellate Cells

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Abstract Sphingolipids play a relevant role in cell–cell interaction, communication, and migration. We studied the sphingolipid content in the murine hepatic stellate cell line GRX, which expresses the myofibroblast phenotype, and can be induced in vitro to display the fat-storing phenotype. Lipid modifications along this induction were investigated by labeling sphingolipids with [^{14}C]galactose, [^{14}C]serine, or [^{14}C]choline, and determination of fatty acid composition of sphingomyelin. The total ganglioside content and the GM2 synthase activity were lower in myofibroblasts. Both phenotypes presented similar gangliosides of the a-pathway: GM2, GM1, and GD1a as well as their precursor GM3. Sphingomyelin and all the gangliosides were expressed as doublets; the upper/lower band ratio increased in lipocytes, containing more long-chain fatty acids in retinol-induced lipocytes as compared to the insulin/indomethacin induced ones. Time-course experiments indicated a transfer of metabolic precursors from phosphatidylcholine to sphingomyelin in the two phenotypes. Taken together, these results indicate that myofibroblast and lipocytes can use distinct ceramide pools for sphingolipid synthesis. Differential ganglioside expression and presence of the long-chain saturated fatty acids suggested that they may participate in formation of distinct membrane microdomains or rafts with specific functions on the two phenotypes of GRX-cells. J. Cell. Biochem. 88: 533–544, 2003.

Key words: sphingolipid; gangliosides; *N*-acetyl-galactosamine transferase; GRX cells; hepatic stellate cells

Sphingolipids are a family of cellular lipids consisting of ceramide as the hydrophobic moiety, and either a complex oligosaccharide or the phosphocholine as the hydrophilic moiety [Merrill et al., 1997]. They are predominantly located in the plasma membranes where they modulate the fluidity and the physicochemical properties of membranes. Their biosynthesis is a result of successive actions of enzymes that are resident in the endoplasmic reticulum and in the Golgi complex [Lloyd and Furukawa, 1998; Maccioni et al., 1999]. In this lipid family, the sialic acid containing glycosphingolipids or gangliosides are abundant in neuronal and glial cells, but they can also be found in smaller quantities in many extra-neural tissues [Ledeen, 1985]. The role of gangliosides in biological processes such as cell growth, differentiation, transformation, cell–cell interactions, and signal transduction have been extensively studied and reviewed by several authors [Hakamori, 1990, 2000; Rösner et al., 1992; Yates

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and Rampersaud, 1998; Mendez-Otero and Santiago, 2001].

Recent studies have proposed a model of cell membrane structure with functionally specific and laterally mobile microdomains, described as "caveolae" and "rafts," which are rich in sphingolipids and cholesterol and are important in controls of signal transduction and membrane trafficking [Simons and Ikonen, 1997; Brown and London, 1998]. One of the proposed models for raft structure states that a close packing of the long saturated acyl chains, found on both glycosphingolipids and sphingomyelin, plays a key role and helps these lipids to form liquid ordered phase domains in presence of cholesterol [Brown and London, 1998; Rietweld and Simons, 1998].

In both, healthy and diseased liver tissues, the functional role of gangliosides is unknown. Human, rat, and mouse livers express complex patterns of gangliosides, which belong mostly to the ganglio-a and ganglio-b series, with a predominance of their common precursor GM3, whose molecular species change during aging [Nilson and Svennerholm, 1982; Rokukawa et al., 1982; Nakamura et al., 1988; Riboni et al., 1992]. In rat liver, the GD1a was identified as a marker of a transition-phase of liver regeneration [Riboni et al., 1990]. Alterations of ganglioside content, composition, and biosynthesis were reported in human cirrhosis [Tanno et al., 1988], in rat biliary cirrhosis [Senn et al., 1991] and in response to drug administration [Cabello et al., 1994; Ruano et al., 1994]. A recent study on a series of animal species demonstrated species-specific differences of liver gangliosides [Saito et al., 2001]. All these observations were obtained using the total liver tissue. The major liver cell fractions are hepatocytes, which outnumber and have much a larger cell volume than all the other liver cells (>93% total liver volume). A small fraction of non-parenchymal cells (<1% total liver volume) is named hepatic stellate cells (Ito cells). They are located in the Disse's space, bearing long and branching processes that embrace two or more adjacent sinusoids, and provide a physical contact with all the hepatocytes with at least one stellate cell. Because they also have some of the characteristics of accessory cells of the nervous system, such as expression of glial fibrillary acidic protein (GFAP) and neural cell-adhesion molecule (N-CAM), and bear receptors for vasoactive agents, they may control the sinusoidal blood flow and be functional integrators of the liver tissue [Pinzani and Gentilini, 1999]. Their second function is related to their reactivity in liver tissue regeneration and repair. They maintain the homeostasis of the liver extracellular matrix and, under pathologic conditions, they have the major role in liver fibrosis and cirrhosis [Gressner and Bachem, 1995]. In these situations, they respond to and produce numerous inflammatory mediators and cytokines as well as the fibrotic extracellular matrix, and they orchestrate proliferation and differentiation of the liver stem cells [Grisham and Thorgeirsson, 1997; Knittel et al., 1999]. Under such conditions, stellate cells intensely proliferate and become mobile, and can migrate to sites of focal inflammatory reactions, or progressively form extensive fibrous septa inside the liver lobule [Boloukhère et al., 1993; Gressner, 1998]. On the other hand, stellate cells are the major storage sites for retinoids and other hydrophobic molecules that are accumulated in their intracellular lipid droplets. Their size and number reflect the systemic retinol status. The lipocyte phenotype-expressing cells (fat-storing cells) are only a small fraction of stellate cells in young animals, but they are numerous in older vitamin-A-replete animals, containing large lipid stores in the cytoplasm. Based on the cytoskeleton studies, we have proposed that the quiescent hepatic stellate cell progenitors can respond to stimuli by expression of distinct and somewhat opposed phenotypes, the myofibroblast and the lipocyte ones, with specific structural and functional properties [Guma et al., 2001].

Sphingolipids are expected to play a relevant role in cell–cell interactions and migrations of stellate cells expressing the myofibroblast phenotype in normal and pathologic situations, as well as during the lipid storage induction in the lipocyte phenotype that involves extensive membrane modifications. We have now addressed the question of the sphingolipid content of these cells, and in particular of gangliosides, in the experimental model of the GRX mouse hepatic stellate cell line. These cells express the myofibroblast phenotype under standard culture conditions, and can be induced in vitro to display the fat-storing phenotype by treatment with retinoids, or insulin supplemented with indomethacin. The retinol-mediated induction corresponds to hyperplasia of hepatic

lipocytes in a hypervitaminosis-A, while the treatment with indomethacin induces the phenotype switch by a yet unknown mechanism [Borojevic et al., 1985, 1990; Margis and Borojevic, 1989; Guaragna et al., 1991, 1992]. In order to characterize the modifications of cell metabolism along the induction of the lipocyte phenotype in GRX cells, we have investigated the metabolic labeling of sphingolipids with D-[U-^{14}C]galactose, L-[U-^{14}C]serine, and [methyl-^{14}C]choline. We also determined the fatty acid composition of sphingomyelin ceramide, and monitored the activity of *N*-acetyl-galactosamine transferase.

MATERIALS AND METHODS

Materials

D-[U-^{14}C]galactose (300 mCi/mmol), L-[U-^{14}C] serine (151 mCi/mmol), [methyl-^{14}C]-choline chloride (55 mCi/mmol) were purchased from Amersham Life Science, Buckinghamshire, UK. UDP-[6-^{3}H]*N*-acetyl-D-galactosamine (UDP-[^{3}H] GalNAc specific activity 10 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc., St. Louis, MO, USA. Standard lipids, all-trans-retinol, insulin, indomethacin and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich, Saint Louis, MO, USA. Silica-gel thin layer chromatography (TLC) plates were from Merck, Darmstadt, Germany. Fetal bovine serum (FBS) was obtained from Cultilab, Campinas SP, Brazil.

Cell Cultures

GRX cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, Brazil). Cells were routinely maintained in the standard medium: DMEM supplemented with 5% FBS and 2 mg/ml HEPES buffer, pH 7.4, under a humidified 5% CO_2 atmosphere at 37°C [Borojevic et al., 1985].

Induction of the Lipocyte Phenotype

Cells were plated in 25 cm^2 tissue culture flasks (5×10^5 cells/flask). After 24 h, the fat-storing phenotype was induced by incubation in the standard medium, supplemented with 5 μM all-trans-retinol, dissolved in ethanol (0.1% final concentration) or with 0.17 μM insulin and 0.13 mM indomethacin for 5 days [Borojevic et al., 1990].

Metabolic Labeling and Lipid Extraction

Subconfluent GRX cells were incubated in the medium described above, containing 1 μCi/ml D-[U-^{14}C]galactose or 1 μCi/ml L-[U-^{14}C]serine, during 24 h. Subsequently, cells were washed three times with cold phosphate buffered saline (PBS), scrapped from the plate and pelleted by brief centrifugation.

Lipids were extracted from the cell pellet with chloroform:methanol (C:M 2:1, v/v). In order to avoid a potential loss of gangliosides that may be distributed in the two phases obtained by the Folch method [Folch et al., 1957], the total lipid extract was directly freed from water-soluble contaminants by passing through a Sephadex G-25 column equilibrated in C:M:Water (60:30:4.5) [Rosales-Fritz et al., 1997]. When indicated, in order to hydrolyze the co-existing phospholipids, the lipid extract was submitted to a mild alkaline hydrolysis with 100 mM methanolic KOH for 2 h at 37°C, followed by reverse-phase chromatography using Sep-Pack C18 cartridges [Williams and McCluer, 1980; van-Echten et al., 1990].

In order to monitor kinetics of the [methyl-^{14}C]choline incorporation into phosphatidylcholine and shingomyelin (time-course experiments), cells were preincubated during 24 h with 0.2 μCi/ml [methyl-14C]choline. The cultures were then washed and further incubated for at least 30 h with standard medium. After various periods ranging up to 30 h, lipids were extracted by the method of Folch et al. [1957]. The chloroform phase was dried under nitrogen and the radioactive phospholipids were separated by TLC as described previously by Okazaki et al. [1989].

Gangliosides Contents

Aliquots of lipid extract from GRX cells corresponding to 800 μg protein were used to determine the contents of gangliosides, with the evaluation of *N*-acetyl-neuraminic acid (NANA), following the resorcinol method of Svennerholm [1957] modified by Miettinen and Takki-Luukkainem [1959]. The results were expressed as nmoles NANA/mg protein. Protein contents were estimated by the method of Lowry et al. [1951].

Chromatography and Fluorography

The purified lipid extract was evaporated under N_2 (~10,000 cpm) and run on TLC silica gel 60 plates (Merck) with two successive solvent systems: first, chloroform/methanol (4:1, v/v) and second, chloroform/methanol/0.25% aqueous $CaCl_2$ (60:36:8, v/v). The second migration was run in a developing tank [Nores et al., 1994]. The [^{14}C]serine-labeled lipid extract was also examined with chloroform/acetone/methanol/acetic acid/water (10:4:2:3:1, v/v) [Okazaki et al., 1989]. Radioactive sphingolipids were visualized by exposition of a radiographic film (Kodak X-Omat AR) at – 70°C, usually during 7 days, and their relative contribution was determined by densitometric scanning of the X-ray film in a CS 930 Shimadzu UV/vis densitometer. Standards were visualized by exposure to resorcinol-HCl (gangliosides) [Svennerholm, 1957; Lake and Goodwin, 1976] and by Coomassie Blue (ceramide monohexoside, ceramide dihexoside and sphingomyelin) [Nakamura and Handa, 1984].

Determination of Ceramide Fatty Acid Composition

Cells were washed with a buffered saline (PBS), harvested, and the lipids were extracted by the method of Folch et al. [1957]. After alkaline hydrolysis, the lipids were separated by TLC as above and the spots corresponding to sphingomyelin bands SM1 and SM2 were scraped. The bands from TLC were extracted with a mixture of methanol:chloroform:water (2:1:0.8, v/v) [Burdon and van Knnippenberg, 1986] and the solvent was dried under nitrogen. The residue was then submitted to transesterification according to Lepage and Roy [1986]. GC-MS was carried out by injection of 0.5 μL of the methyl fatty acid solution into a Omegawax 250 column (Supelco, USA) of 30 m length and 0.25 mm diameter. The GC-MS used was a GC-MS-QP5050 (Shimadzu, Japan) with the following chromatographic conditions: split 1:10, detector and injector temperatures of 280 and 300°C, respectively. The column temperature started with 120°C for 3 min, rising up to 180°C at 5°C/min, maintaining it for 2 min, increasing to 230°C at 2°C/min and maintaining it for 20 min at this temperature. The detector was a quadruple type with ionization by electron impact with a cyclic scan of 1 s and with a mass range m/z 50–500.

Determination of *N*-Acetyl-Galactosamine Transferase Activity

Transferase activity was determined following Daniotti et al. [1994]. Briefly, the activity of GM2 synthase was determined in the incubation system that contained 400 μM GM3, 100 μM UDP-[^{3}H]GalNAc (2,00,000 cpm), 20 mM $MnCl_2$, 3 mM CDP choline (to preserve UDP-[^{3}H]GalNAc from pyrophosphatases), 20 μg Triton CF54/Tween 80 (2:1, w/w), 100 mM sodium cacodylate–HCl buffer (pH 7.2). Incubations were done at 37°C for 90 min. In order to take in account incorporation into endogenous acceptors, incubations were done in the absence of exogenous acceptor, but in otherwise identical conditions. Reactions were stopped with 1 ml 5% (w/v) trichloroacetic acid/0.5% phosphotungstic acid, and the radioactivity incorporated into lipids was determined as previously described [Daniotti et al., 1997]. The results were expressed as pmol of sugar residue transferred/h/mg protein.

RESULTS

Ganglioside Contents and *N*-Acetyl Galactosamine Transferase Activity

The determination of total ganglioside content showed that the myofibroblastic GRX cells had approximately half the ganglioside content as compared to lipocytes (6.8 ± 1.3 nmol and 12.1 ± 1.7 nmol lipid-bound sialic acid/mg of protein, respectively).

We monitored the activity of the major step in ganglioside-a biosynthesis. The GM2 synthase activities were 505 pmol *N*-acetyl-galactosamine transferred/h/mg protein in myofibroblasts, and to 760 pmol *N*-acetyl-galactosamine transferred/h/mg protein in lipocytes. The lower synthase activity in myofibroblasts corresponded to the lower total ganglioside content, indicating that the control of this difference probably operates at the level of synthesis rather than of the turnover of gangliosides.

[^{14}C]Galactose Incorporation Into Glycosphingolipids of GRX Cells

Galactose incorporation into GRX cells resulted in labeling neutral lipids, phospholipids and proteins (data not shown), indicating its interconversion into precursors of the glucose-derived metabolic pathways and the

availability of labeled glucose in the studied system. GRX cells, expressing the myofibroblast or the lipocyte phenotype induced by retinol or insulin and indomethacin incorporated [^{14}C]galactose and the endogenous [^{14}C]glucose into neutral glycosphigolipids (ceramide monohexoside-CMH and ceramide dihexoside-CDH), as well as into gangliosides from the series-a (GM2, GM1, and GD1a) and their precursor GM3. The major radioactive incorporation was found in GD1a. All the gangliosides were expressed as doublets (Fig. 1) while only one band could be detected for neutral glycosphingolipids.

As shown in the Table I the faster band of each doublet had a higher radioactive incorporation. The ratio between the doublets (faster = b2/slower = b1 band) of each ganglioside increased with the lipocyte phenotype expression.

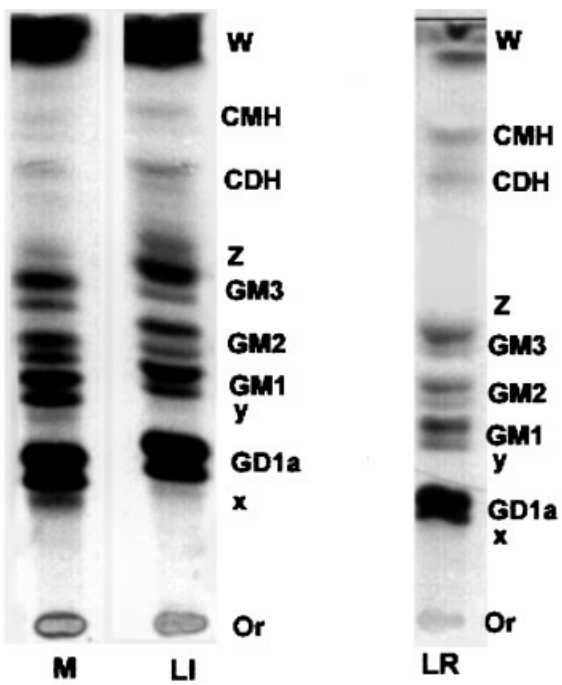

Fig. 1. Thin layer-autoradiography of D-[^{14}C]galactose-labeled endogenous glycosphingolipids from GRX cells expressing the myofibroblast (M), or the lipocyte phenotype induced by indomethacin and insulin (LI) or retinol (LR). Lipids were extracted, purified with a mild alkaline methanolysis and analyzed by TLC. The position of co-chromatographed glycolipid standards are indicated. Gangliosides were named as recommended by Svennerholm [1963]. x, y, z, and w are unidentified lipids. CDH, ceramide dihexoside; CMH, ceramide monohexoside; Or, origin. The presented results are representative of two independent experiments.

[^{14}C]Serine Incorporation Into Phospholipids and Gangliosides of GRX Cells

The lipid extracts of GRX cells (myofibroblasts and lipocytes induced by retinol or insulin and indomethacin) labeled with [U-^{14}C]serine were analyzed by TLC using a phospholipid solvent system. The radioactivity was incorporated in a large extent into glycerophospholipids (phosphatidylserine and phosphatidylethanolamine). In myofibroblasts it reached 40%, in insulin and indomethacin-induced lipocytes 46% and in retinol-induced lipocytes 61%. It was incorporated in somewhat lesser extent into sphingolipids such as sphingomyelin and gangliosides, reaching 36% in myofibroblasts, 35% in insulin and indomethacin-induced lipocytes, and in 21% retinol-induced lipocytes. The major difference was observed in the sphingomyelin content of retinol-induced lipocytes. (Fig. 2A and Table IIA).

When the TLC analyses of the same lipid cell extracts were carried out with a ganglioside solvent system, the major incorporation into gangliosides was observed in GD1a (Fig. 2B and Table IIB). Similar to gangliosides, sphingomyelin was present also in doublets (Fig. 2A). The relationship between the two SM bands (upper-b2/lower-b1 bands) also increased with the induction of the lipocyte phenotype expression (Table II) reaching higher values in retinol-induced lipocytes as compared to those induced by insulin and indomethacin.

Kinetics of [methyl-^{14}C]Choline Incorporation Into Phosphatidylcholine and Sphingomyelin

The higher ratio of the two sphingomyelin bands observed with serine incorporation in retinol-induced lipocytes, led us to use this lipocyte type for subsequent experiments The kinetics of [methyl-^{14}C]choline incorporation into phosphatidylcholine (PC) and sphingomyelin is shown in the Figure 3. The initial radioactivity incorporated into sphingomyelin (SM1 + SM2) was about 8% of the total incorporation into myofibroblasts and 5% into lipocytes, but after 30 h we observed 18 and 13%, respectively. In spite of these variations, the specific activity of SM during this period was constant (approximately 6.6 and 5 cpm/μg protein myofibroblasts and lipocytes, respectively), indicating that the SM synthesis/degradation ratio was close to 1.

TABLE I. Densitometric Analysis of [^{14}C]Galactose Incorporation Into Lipids of GRX Cells

			Lipocytes			
	Myofibroblast (M)	B2/B1 ratio	Ins/Indo (LI)	B2/B1 ratio	Retinol (LR)	B2/B1 ratio
Origin	2.1		1.7		0.6	
X	3.0		ND		ND	
GD1a (1)	15.4		11.6		13.7	
GD1a (2)	20.5	1.3	18.8	1.6	28.6	2.1
Y	1.2		ND		ND	
GM1 (1)	6.1		4.2		3.9	
GM1 (2)	7.8	1.3	6.9	1.7	8.1	2.1
GM2 (1)	3.5		2.6		2.2	
GM2 (2)	4.4	1.3	4.9	1.8	6.0	2.8
GM3 (1)	2.4		2.2		2.7	
GM3 (2)	5.6	2.3	7.7	3.6	7.9	3.0
Z	1.0		5.2		ND	
CDH	ND		4.5		6.1	
CMH	12.3		12.2		7.9	
W	14.6		17.3		12.1	

Results are expressed as % of total radioactivity incorporated. The ganglioside analysis was performed by TLC as described in Material and Methods. x, y, z, and w are unidentified lipids. CDH, ceramide dihexoside; CMH, ceramide monohexoside; Or, origin; ND, not detected by densitometric analysis.

In addition, the phosphatidylcholine/sphingomyelin label ratio decreased with the time, i.e., a decline of [^{14}C]-PC corresponded to an increase of [^{14}C]-SM, indicating a transfer of metabolic precursor moiety from phosphatidylcholine into sphingomyelin (Fig. 3, inserts).

Fatty Acid Composition of Sphingomyelin From GRX Cells

Since sphingolipids were present as doublets in all the analyses, we addressed the question of the fatty acid composition of ceramide species in GRX cells using the major sphingolipid species, the sphingomyelin. The two sphingomyelin bands were separated and their fatty acid composition determined by GC-MS (Table III). Striking differences were observed between the upper (SM2) and the lower band (SM1). Linoleic and long chain fatty acids were present only in SM2. Moreover, the lipocyte phenotype had an overall increase of myristic acid, particularly in the SM1, and the corresponding decrease of the palmitic acid, but broad differences were also observed between the retinol-induced lipocytes on one side and insulin-indomethacin induced ones on the other. In particular, in retinol-induced lipocyte the fatty acid composition of the upper band ceramide was characterized by a relatively high content of C22 and C24 fatty acids.

DISCUSSION

The experimental model of the permanent cell line GRX has let us to analyze and compare several parameters of hepatic stellate cell metabolism in the myofibroblast versus lipocyte phenotype, and along the induction of latter one by different pathways. Considering the wide differences in physiologic functions of stellate cells expressing these phenotypes, the differences among the overall composition of their sphingolipids were remarkably small, with essentially a quantitative modulation of sphingolipid species. Besides the general precursor GM3, all the observed gangliosides (GM2, GM1, and GD1a) belong to the a-pathway of synthesis.

The total ganglioside content was relatively low in myofibroblasts as compared to lipocytes. The former phenotype represents hepatic stellate cells that undergo activation under conditions of liver tissue response to injury and damage. The total ganglioside levels in human cirrhotic liver tissue are reported to be lower than in normal liver (31 and 53 μg NANA/mg protein, respectively) [Tanno et al., 1988], with a relative increase of GD1a, which was also the major ganglioside observed in GRX myofibroblasts. Both modifications may reflect the increasing participation of fibroblastoid cells in the composition of cirrhotic or fibrotic liver tissue. Our observations confirm that extensive proliferation and migration of myofibroblasts inside the regenerating or fibrosing liver tissue, in which gangliosides potentially may have a relevant role [Riboni et al., 1990], do not require an increase in their total quantity.

The induction of the lipocyte phenotype in GRX cells, either by retinol or by insulin/indomethacin, decreases the cell proliferation and

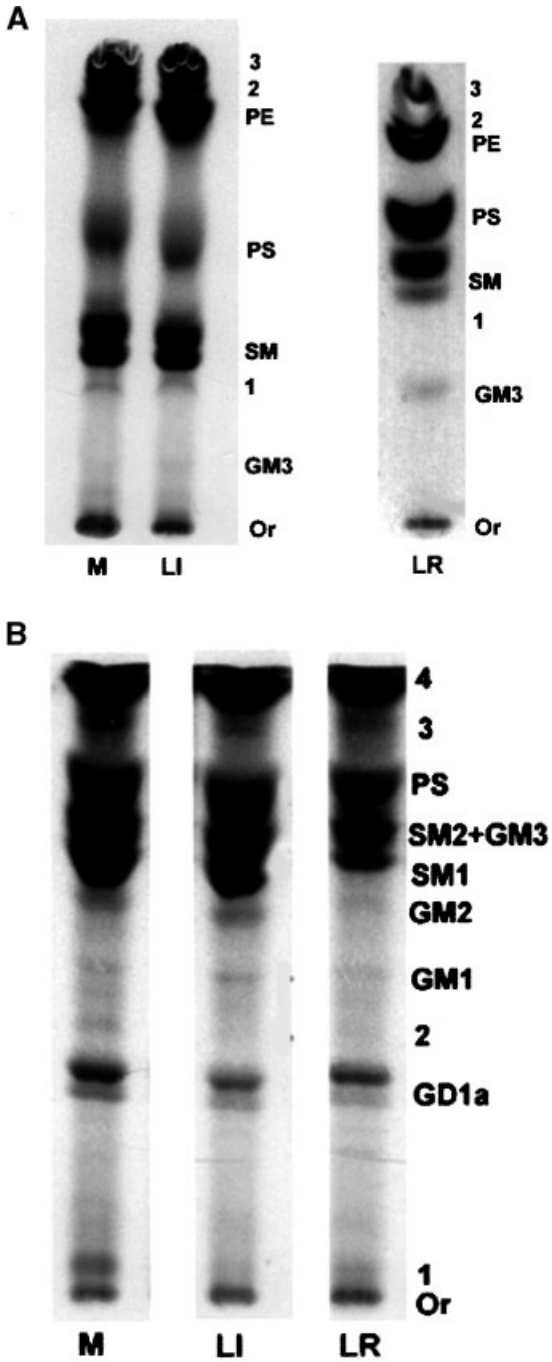

Fig. 2. Thin layer-autoradiography of L-[U-^{14}C]serine labeled endogenous lipids from GRX cells expressing the myofibroblast (M), or the lipocyte phenotype induced by indomethacin and insulin (LI) or retinol (LR). Lipids were extracted and purified with Sephadex G-25. Thin-layer chromatograms were done using a phospholipd solvent system (**A**) and ganglioside solvent system (**B**). The position of co-chromatographed lipid standards are indicated. The presented results are representative of two independent experiments. 1, 2, 3, 4, and 5 are unidentified lipids. SM, lower sphingomyelin band; SM2, upper sphingomyelin band; PS, phosphatidylserine; PE, phosphatidylethanolamine; GM3 and GD1a, gangliosides; Or, origin. Complex gangliosides remain at origin in the phospholipid solvent system.

cell density at confluence, modifies the pattern of extracellular matrix synthesis, as well as the cytoskeleton composition and organization [Borojevic et al., 1990; Pinheiro-Margis et al., 1992; Guma et al., 2001; Mermelstein et al., 2001]. As expected, the de novo synthesis of neutral and phospholipids is considerably increased and modified in lipocytes [Guaragna et al., 1991, 1992]. Accordingly, the total ganglioside content was also increased. The overall galactose and serine incorporations into gangliosides were different in retinol-induced lipocytes, which is mediated essentially by the retinoic acid pathway, as compared to the lipocyte induction via insulin. This may be a direct effect of retinoic acid on ganglioside metabolism, since it was shown to induce alterations of metabolic labeling of glycosphingolipids in other experimental models [Wiegant et al., 1987].

Similar to other cells types [Poulos et al., 1987; Kitano et al., 1996; Ziulkoski et al., 2001], GRX cells showed two sphingomyelin bands by TLC, named SM1 (lower) and SM2 (upper), and all the gangliosides were also expressed as doublets. The existence of two TLC bands for the same lipid class is due to structural differences that are normally consequent to differences in the fatty acid composition, but in glycolipids the multiple bands may be also due to the heterogeneity of sugars (*N*-acetyl-neuraminic acid/*N*-glycolyl-neuramic acid). The analysis of the fatty acid composition of the two SM bands favored the former proposal. Based upon the TLC behavior, the upper band of each sphingolipid doublet had a higher radioactive incorporation. The relationship between the upper and lower bands increased in the lipocyte phenotype, being higher and containing more long chain fatty acids in retinol-induced lipocytes as compared to the insulin and indomethacin-induced ones. Our recent studies have shown that the uptake of retinol coupled to the retinol-binding protein (RBP) in GRX lipocytes, but not in myofibroblasts, depended upon specific and saturable protein–protein interactions between the holo-RBP and a membrane receptor (R. Borojevic, unpublished data), and that the disruption of the lipocyte membrane organization disturbed its uptake. The presence of long chain fatty acids in sphingolipids may be relevant for the membrane properties, and it may be required for efficient retinol storage in lipocytes.

TABLE II. Densitometric Analysis of [^{14}C]Serine Incorporation Into Phospholipids and Gangliosides From GRX Cells

	Myofibroblasts	Lipocytes	
	(M)	Ins/Indo (LI)	Retinol (LR)
(A): Solvent system for phospholipids			
Origin	7.4	6.0	3.4
GM3	0.7	0.8	2.4
1	1.5	ND	ND
SM1	10.7	11.5	3.8
SM2	17.8	16.7	13.8
PS	16.9	14.7	38.1
PE	23.0	31.5	23.3
2	17.4	12.8	7.7
3	4.5	6.8	7.5
(B): Solvent system for gangliosides			
Origin	2.4	2.9	4.6
1	2.9	1.3	1.8
GD1a (1)	1.6	1.1	1.5
GD1a (2)	4.7	3.2	4.9
GM2	3.0	3.3	2.9
SM + GM3	37.0	31.8	23.7
PS	17.6	21.7	19.7
2	7.2	4.7	7.4
3	23.5	30.5	33.3

Results are expressed as % of total radioactivity incorporated. (A) A portion of lipids extracted from GRX cells developed with chloroform/acetone/methanol/acetic acid/water (10:4:2:3:1, by vol.); or (B) with two successive solvent system chloroform/methanol (4:1, by vol) and then chloroform/methanol/0.25% aqueous $CaCl_2$ (60:36:8, by vol.). SM1, lower SM band; SM2, upper SM band; PS, phosphatidylserine; PE, phosphatidylethanolamine; Or, origin; the gangliosides GM3 and GD1a. 1,2 and 3 are unidentified lipids.

Taken together, our findings indicate the presence of distinct sphingolipid precursors (ceramide containing different fatty acids) in stellate cells, and their differential incorporation into sphingolipid species. Polyunsaturated fatty acids were not present in sphingolipids, while they were abundant in diacylglycerol (DAG) and in some of the phospholipid species [Guaragna et al., 1992]. This is in agreement with the sphingolipid participation in formation of membrane rafts, in which long saturated chains are compatible with their tight packing with cholesterol. The fatty acid content of sphingolipids, neutral lipids and of different species of phospholipids was specific and characteristic of each lipid species, indicating tight controls of fatty acid synthesis and traffic in stellate cells.

The time-course experiments indicated a conversion of phosphatidylcholine to sphingomyelin and differences in synthesis in the two GRX phenotypes. The divergent kinetics of labeled precursor incorporation into the two sphingolipid bands in lipocytes may indicate a selective or preferential incorporation of different ceramide species into the studied sphingolipids. This may indicate distinct metabolic pathways that can distinguish and use different precursor pools, or the synthesis of sphingolipids in distinct compartments in which a composition of ceramide species is not similar. These issues are object of ongoing studies.

The de novo synthesis of sphingolipids begins by serine palmitoyltransferase producing 3-ketodihydrosphingosine and dihydrosphingosine, which is subsequently acylated. Dihydroceramide is desaturated and ceramide is converted to sphingomyelin or ceramide monohexoside (CMH) and then to more complex glycosphingolipids [Merrill et al., 1997]. The TLC study of metabolic [U-^{14}C]serine-labeled lipids carried out with the solvent system for phospholipids or gangliosides let us determine the relative importance of these synthesis pathways. The major incorporation was found in phospholipids (PS and PE), another significant part in the two sphingomyelin bands, and a minor part in gangliosides (Fig. 2 and Table II).

In conclusion, we report an increase in ganglioside content during the phenotypic differentiation of GRX cells to lipocytes, concomitant with a considerable change of the ceramide moiety, as shown by the determination of their fatty acid composition. The metabolic reasons

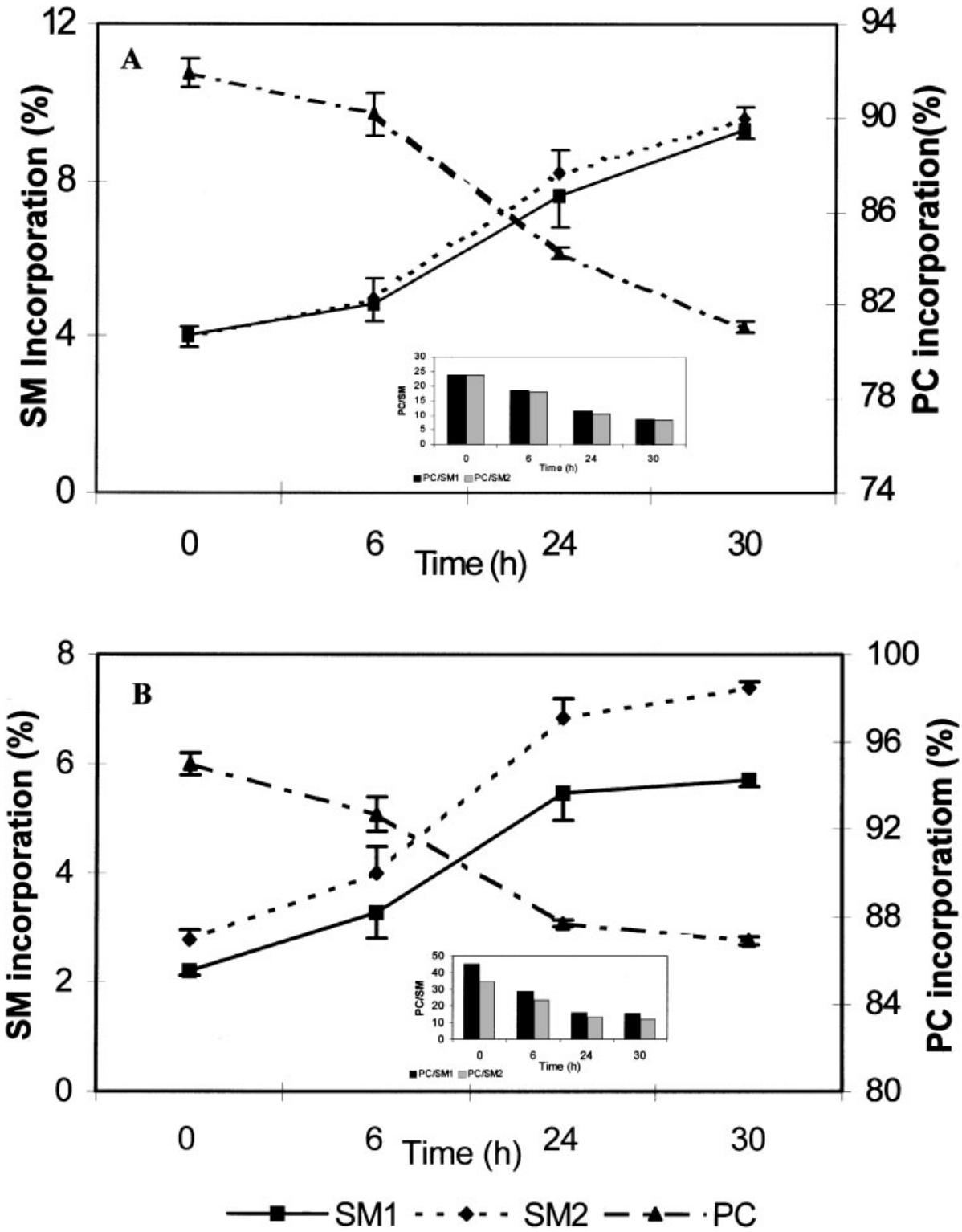

Fig. 3. Incorporation of [^{14}C] choline into lipids of GRX cells. Cells were labeled with [methyl-^{14}C]choline for 24 h, washed and incubated with cold complete medium for 0, 6, 24, or 30 h. After each incubation period, lipids were extracted and analyzed as described in Material and Methods. Results are expressed as mean ± SEM of triplicate determinations in a single experiment which was repeated twice with similar results. (**A**) Labeling pattern of myofibroblast GRX cells. (**B**) Labeling pattern of lipocyte GRX cells. The inserts represent the PC/SM ratio.

TABLE III. Fatty Acid Composition of the Lower (SM1) and Upper (SM2) Sphingomyelin Purified From GRX Express the Myofibroblast or the Lipocyte Phenotype

	Myofibroblasts		Lipocytes			
	(M)		Ins/Indo (LI)		Retinol (LR)	
Fatty acid	SM 1	SM 2	SM 1	SM2	SM 1	SM2
14:0	4.5	8.5	14.4	6.1	8.0	3.5
16:0	57.5	26.0	25.0	25.0	38.5	20.7
18:0	14.2	17.4	14.7	17.7	21.0	13.4
18:1	3.7	2.7	7.9	3.6	4.5	0.7
18:2	—	0.7	—	0.7	2.4	2.7
C22	—	5.8	—	5.8	2.4	14.5
C24 or C24:1	—	3.4	—	3.4	—	14.0

Results are expressed as % of total fatty acids. The fatty acids were extracted and analyzed by GC-MS as described in Materials and Methods. Unidentified fatty acids were also present in smaller quantities; they are not included in this table.

of these modifications remain to be elucidated. The two sphingolipid pools may have distinct function and cell distribution, affecting some of the functional properties displayed by the gangliosides in the plasma membrane. Ceramide structure governs the sphingolipid aggregation properties and surface dynamics of gangliosides, such as their capacity to undergo lateral phase separation within the membrane [Yohe et al., 1976; Masserini and Freire, 1986]. Consequently these properties may influence the interactions of gangliosides with external ligands and with other membrane components [Kannagi et al., 1982].

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REFERENCES

Boloukhère M, Baldo-Correa E, Borojevic R. 1993. Experimental schistosomiasis mansoni: Characterization of connective tissue cells in hepatic periovular granulomas. J Submicrosc Cytol Pathol 25:505–517.

Borojevic R, Monteiro ANA, Vinhas SA, Domont GB, Mourão PAS, Emonard H, Grimaldi G, Jr., Grimaud JÁ. 1985. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In Vitro Cell Dev Biol 21:382–390.

Borojevic R, Guaragna RM, Margis R, Dutra H. 1990. In vitro induction of the fat storing phenotype in a liver connective tissue cell line-GRX. In Vitro Cell Dev Biol 25:361–368.

Brown DA, London E. 1998. Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 14:111–136.

Burdon RH, van Knnippenberg PH. 1986. In: Laboratory techniques in biochemistry and molecular biology. 2nd edn. London; Elsevier. 253.

Cabello J, Ruano MJ, Cabezas JA, Hueso P. 1994. Cocaine exposure induces changes in the ganglioside content of rat liver. Biol Chem 375:817–819.

Daniotti JL, Landa CA, Maccioni HJF. 1994. Regulation of ganglioside composition and synthesis is different in developing chick retinal pigment epithelium and neural retina. J Neurochem 62:1131–1136.

Daniotti JL, Rosales-Fritz V, Kunda P, Nishi T, Maccioni HJF. 1997. Cloning, characterization, and developmental expression of α2,8 sialyltransferase (GD3 synthase, ST8Sia I) gene in chick brain and retina. Int J Dev Neuroscience 15:767–776.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for isolation of total lipids from animal tissue. J Biol Chem 226:497–509.

Gressner AM. 1998. The cell biology of liver fibrogenesis—An imbalance of proliferation, growth arrest and apoptosis of myofibroblasts. Cell Tissue Res 292:447–452.

Gressner AM, Bachem MG. 1995. Molecular mechanism of liver fibrogenesis—A homage to the role of activated fat-storing cells. Digestion 56:335–346.

Grisham JW, Thorgeirsson SS. 1997. In: Liver stem cells. Potten CS, editor. Stem cells. London; Academic Press. pp 231–282.

Guaragna RM, Trugo L, Borojevic R. 1991. Neutral lipid synthesis and accumulation during in vitro induction of the lipocyte phenotype in hepatic connective tissue cells. Biochim Biophys Acta 1085:29–84.

Guaragna RM, Trugo L, Borojevic R. 1992. Phospholipids modifications during conversion of hepatic myofibroblasts into lipocytes (Ito-cells). Biochim Biophys Acta 1128:237–243.

Guma FCR, Mello TG, Mermelstein CS, Fortuna VA, Wofchuck ST, Gottfried C, Guaragna RM, Costa ML, Borojevic R. 2001. Intermediate filaments modulations in an in vitro model of the hepatic stellate cell activation or conversion into the lipocyte phenotype. Biochem Cell Biol 79:409–417.

Hakomori S. 1990. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. J Biol Chem 265:18713–18716.

Hakomori S. 2000. Traveling for the glycosphingolipid path. Glycoconjugate J 17:627–647.

Kannagi R, Nudelman E, Hakamori S. 1982. Possible role of ceramide in defining structure and functoin of membrane glycolipids. Proc Natl Acad Sci USA 79:3470–3474.

Kitano Y, Iwamori Y, Kiguchi K, DiGiovani J, Takahashi T, Kasama K, Niwa T, Harii K, Iwamori M. 1996. Selective reduction in (α-hydroxypalmitic acid-containing sphingomyelin and concurrent increase in hydroxylated ceramides in murine skin tumors induced by na initiation-promotion regimen. Jpn J Cancer Res 87:437–441.

Knittel T, Kobolb D, Saile B, Grundmann A, Neubauer K, Piscaglia F, Ramadori G. 1999. Localization of liver myofibroblasts and hepatic stellate cells in normal and diseased rat livers: Distinct roles of (myo)-fibroblast subpopulations in hepatic tissue repair. Histochem Cell Biol 112:387–401.

Lake BD, Goodwin HJ. 1976. Lipids. In: Smith I, Seakins JWT, editors. Chromatographic and eletrophoretic. London; William Heinemann Medical Books Ltd. 345–366.

Ledeen RW. 1985. Gangliosides of the neuron. TINS 8:169–174.

Lepage G, Roy CC. 1986. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 27: 114–120.

Lloyd KO, Furukawa K. 1998. Biosynthesis and functions of gangliosides: Recent advances. Glycoconjugate J 15: 627–636.

Lowry OH, Rosenbrough AL, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Maccioni HJF, Daniotti JL, Martina JÁ. 1999. Organization of gangliosides synthesis in the Golgi apparatus. Biochim Biophys Acta 1437:101–118.

Margis R, Borojevic B. 1989. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). Biochim Biophys Acta 1011:1–5.

Masserini M, Freire E. 1986. Thermotropic characterization of phosphatidylcholine vesicles containing ganglioside GM1 with homogeneous ceramide chain lengh. Biochemistry 25:1043–1049.

Mendez-Otero R, Santiago ME. 2001. Functional role of a glycolipid in directional movements of neurons. An Acad Bras Cienc 73:221–229.

Mermelstein CS, Guma FCR, Mello TG, Fortuna VA, Guaragna RM, Costa ML, Borojevic R. 2001. Induction of the lipocyte phenotype in murine hepatic stellate cells: Reorganisatin of the actin cytoskeleton. Cell Tissue Res 306:75–83.

Merrill AH, Jr., Schmelz EM, Dillehay DL, Spiegel S, Shayman JA, Schroeder JJ, Riley RT, Voss KA, Wang E. 1997. Sphingolipids—The enigmatic lipid class: Biochemistry, phisiology, and pathophysiology. Toxicol Appl Pahamacol 142:208–225.

Miettinen T, Takki-Luukkainem IT. 1959. Use of butyl acetate in determination of sialic acid. Acta Chim Scand 13:856–858.

Nakamura K, Handa S. 1984. Coomassie brilliant blue staining of lipids on thin-layer plates. Anal Biochem 142:406–410.

Nakamura Y, Hashimoto Y, Yamakawa T, Suzuki A. 1988. Age-dependent changes in GM1 and GD1a expression in mouse liver. J Biochem (Tokyo) 103:396–398.

Nilson OL, Svennerholm L. 1982. Characterization and quantitative determination of gangliosides and neutral glycosphingolipids in human liver. J Lipid Res 23:327–334.

Nores GA, Mitzumari RK, Kremer DM. 1994. Chromatographic tank designed to obtain highly reproducible high-performance thin-layer chromatograms of gangliosides and neutral glycosphingolipds. J Chromatogr A 686:155–157.

Okazaki T, Bell RH, Hannum YA. 1989. Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. J Biol Chem 264:19076–19080.

Pinheiro-Margis M, Margis R, Borojevic R. 1992. Collagen synthesis in an established liver connective tissue cell line (GRX) during induction of the fat-storing phenotype. Exp Mol Pathol 56:108–118.

Pinzani M, Gentilini P. 1999. Biology of hepatic stellate cells and their possible relevance in the pathogenesis of portal hypertension in cirrhosis. Sem Liver Dis 19:397–410.

Poulos A, Johson DW, Beckman K, White JG, Easton C. 1987. Occurence of unsual molecular species of sphingomyelin containing 28–34 carbon polyenoic fatty acids in ram spermatozoa. Biochem J 284:961–964.

Riboni L, Ghidoni R, Benevento A, Tettamanti G. 1990. Content, pattern and metbolic processing of rat-liver gangliosides during liver regeneration. Eur J Biochem 194:377–382.

Riboni L, Acquotti D, Casellato R, Ghidoni R, Montagnolo G, Benevento A, Zecca L, Rubino F, Sonnino S. 1992. Changes of the human liver GM3 ganglioside molecular species during aging. Eur J Biochem 203:107–113.

Rietweld A, Simons K. 1998. The differential miscibility of lipids as the basis for the formation of functional lipid rafts. Biochim Biophys Acta 1376:476–479.

Rokukawa C, Kush Y, Ueno K, Handa S. 1982. Structural study on gangliosides froom rat liver and erythrocytes. J Biochem (Tokyo) 92:1481–1488.

Rosales-Fritz VM, Daniotti JL, Maccioni HJF. 1997. Chinese hamster ovary cells lacking GM1 and GD1a synthesize gangliosides upon transfection with human GM2 synthase. Biochim Biophys Acta 1354:153–158.

Rösner HM, Al-Aqtum M, Rahmann H. 1992. Gangliosides and neuronal diferentiation. Neurochem Int 20:339–351.

Ruano MJ, Martínez-Zorzano VS, Cabezas JA, Hueso P. 1994. Ganglioside content of rat liver after administration of ethanol and pentazocine or sucrose supplement diets. Arch Toxicol 68:576–581.

Saito M, Kitamura H, Sugiyama K. 2001. Liver gangliosides of various animals ranging from fish to mammalian species. Comp Biochem Physiol 129B:47–758.

Senn HJ, Geiser T, Fitzke E, Baumgartner U, Schölmerich J, Gerok W. 1991. Altered biosynthesis of gangliosides in developing biliary cirrhosis in the rat. J Hepatol 13:152–160.

Simons K, Ikonen E. 1997. Functional rafts in cell membranes. Nature 387:569–572.

Svennerholm L. 1957. Quantitative estimation of sialic acids a colorimetric resorcinol-hydrochloric acid method. Biochim Biophys Acta 24:604–611.

Svennerholm L. 1963. Chromatographic separation of human brain gangliosides. J Neurochem 10:613–623.

Tanno M, Yamada H, Shimada H, Oshahi M. 1988. Ganglioside varitions in human liver cirrhoris and hepatocelluar carcinoma as shown by two-dimensional thin-layer chromatography. Clin Biochem 21:333–339.

van-Echten G, Birk P, Brenner-Weiss G, Schmidt RR, Sandhoff K. 1990. Modulation of sphingolipid Biosynthesis in primay cultured neurons by long chain bases. J Biol Chem 256:9333–9339.

Wiegant H, Hellant R, Radsak K. 1987. Retinoic acid alters the metabolic 3H-labeling of glycosphingolipids. Biophys Biochem Res Commun 143:525–531.

Williams MA, McCluer RH. 1980. The use of Sep-Pak C18 cartridges during the isolation of gangliosides. J Neurochem 35:266–269.

Yates AJ, Rampersaud A. 1998. Sphingolipids as receptor modulators. An overiew. Ann NY Acad Sci 845:57–71.

Yohe HC, Roark DE, Rosenberg A. 1976. C20-sphingosine as a determining factor in aggregation of gangliosides. J Biol Chem 251:7083–7087.

Ziulkoski AL, Zimmer AR, Zanettini JS, Trugo LC, Guma FCR. 2001. Synthesis and transport of different sphingomyelin species in rat Sertoli cells. Mol Cel Biochemistry 219:57–64.