Similar content of phospholipids and gangliosides in normal and homozygous familial hypercholesterolemia fibroblasts

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Abstract The cellular content of total and individual phospholipids and gangliosides was measured in fibroblasts cultured from four normal subjects, three patients with lysosomal lipid storage diseases, and two subjects with homozygous familial hypercholesterolemia. Measurements were made on cells grown in medium containing fetal calf serum under conditions in which normal cells derive cholesterol for cell growth from low density lipoprotein present in the fetal calf serum, whereas familial hypercholesterolemia homozygote cells, which lack cell surface low density lipoprotein receptors, derive cholesterol from endogenous synthesis. No difference was observed in the cellular content of total or individual phospholipids and gangliosides in the normal and familial hypercholesterolemia homozygote cells. In contrast, cells from a patient with Niemann-Pick disease and a patient with Sandhoff disease showed elevations in the content of sphingomyelin and complex gangliosides, respectively.

Supplementary key words glycosphingolipids · 3-hydroxy-3-methylglutaryl coenzyme A reductase · low density lipoprotein

Most inborn errors of lipid metabolism are expressed in human fibroblasts in tissue culture (1). The culture technique has been particularly helpful in elucidating the genetic defect in familial hypercholesterolemia (FH), an autosomal dominant disorder in which the plasma level of a major cholesterol transport protein, low density lipoprotein (LDL), is elevated and premature atherosclerosis occurs (2). Fibroblasts from patients with one variety of the homozygous form of this disorder (receptor-negative FH homozygotes) lack a cell surface receptor that normally binds LDL. As a result of this primary defect, a fundamental difference exists in cholesterol metabolism between FH homozygote and normal fibroblasts (3). When normal fibroblasts are grown in culture medium containing LDL, the cells utilize the LDL receptor to derive cholesterol from the LDL in the medium. Under these conditions, normal cells maintain a low level of cholesterol synthesis through suppression of the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase) (3). On the other hand, the FH homozygote cells, lacking LDL receptors, are unable to derive cholesterol from LDL. As a result, even when grown in the presence of LDL, these mutant cells must synthesize the cholesterol required for their membranes and hence HMG CoA reductase activity is high (3).

Recently, Chatterjee, Sekerke, and Kwiterovich (4) reported that fibroblasts from one subject with receptor-negative homozygous FH exhibited marked differences in the cellular content of a variety of phospholipids, gangliosides, and neutral glycosphingolipids when compared with cells from one normal subject. In particular, cells from this FH homozygote were reported to have a 3-fold increase in the cellular content of total phospholipids, with a 2.5-fold increase in phosphatidylcholine (PC), a 2-fold increase in phosphatidylethanolamine (PE), and a 5-fold increase in sphingomyelin (SPH). When compared with cells from the one normal subject, the

Abbreviations: FH, familial hypercholesterolemia; LDL, low denisty lipoprotein; LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; G_{M1}, galactosyl-*N* - acetylgalactosaminyl - [*N* - acetylneuraminyl] - galactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide; G_{M3}, *N*-acetylneuraminylgalactosylglucosylceramide; G_{D3}, *N*-acetylneuraminyl-*N*-acetylneuraminylgalactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl - [*N* - acetylneuraminyl] - galactosylglucosylceramide.

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cells from the FH homozygote also were reported to exhibit a 4-fold increase in the content of total gangliosides, with a 5-fold increase in G_{M3} and a 4-fold increase in G_{D3}. The total cellular content of neutral glycosphingolipids also was reported to be elevated by almost 5-fold in the FH homozygote cells. These differences in the cellular lipid content between the normal and FH homozygote cells were observed when the fibroblasts were grown in fetal calf serum under conditions in which normal cells derive their cholesterol for cell growth from serum LDL and the FH homozygote cells derive their cholesterol from endogenously synthesized sterol.

The above findings are of considerable interest for several reasons. First, they suggest that a deficiency of cell surface LDL receptors might have widespread effects on the metabolism of phospholipids, gangliosides, and glycosphingolipids, in addition to the previously described effects on cholesterol metabolism. Second, they raise the possibility that gangliosides, which serve as membrane receptors for certain toxins and glycoprotein hormones (5), may play an essential role in the function of the LDL receptor. However, data from only one control subject were reported (4). Moreover, the contents of gangliosides and neutral glycosphingolipids are known to vary between different strains of cultured cells (6, 7). Therefore, we felt it important to carry out further studies before firm conclusions could be drawn about abnormal phospholipid, ganglioside, and neutral glycosphingolipid metabolism in homozygous FH.

In the present study we have measured the cellular content of phospholipids and gangliosides in cells from two patients with homozygous FH and compared these values with similar measurements made on cells from four normal subjects and three subjects with other disorders of lipid metabolism. All cells were grown under one standard set of conditions in the presence of fetal calf serum. The results show no difference in the cellular content of either gangliosides or phospholipids in cells from the two subjects with homozygous FH as compared with the normal cells despite a 30-fold higher level of HMG CoA reductase activity in the FH homozygote cells.

MATERIALS AND METHODS

Cells

Fibroblast strains were established from explants of skin biopsies (**Table 1**). Cells were grown in monolayer and used between the 12th and 20th generations as indicated in Table 1. All experiments were carried out with a standard format: cells from the stock flasks were dissociated with 0.05% trypsin–0.02% EDTA (day 0) and an innoculum of 2×10^5 cells was seeded into each of 15 dishes (100×20 cm) containing 7 ml of medium A (Eagle's minimum essential medium supplemented with penicillin [100 units/ml], streptomycin [100 μg/ml], 20 mM Tricine, pH 7.4, 24 mM $NaHCO_3$, 1% [v/v] nonessential amino acids, and 10% [v/v] fetal calf serum). On days 3 and 5, the medium was replaced with fresh medium A containing 10% fetal calf serum. On day 7, the monolayers from the 15 dishes were washed, harvested, and frozen as a single pellet in liquid nitrogen and kept at −196°C until use. For ganglioside analysis, the frozen pellets were mailed from Dallas to Bethesda in dry ice.

TABLE 1. Description of fibroblast strains on which lipid measurements were made

Designation of Fibroblast Strain	Clinical Diagnosis	Age (years)	Sex	Site of Skin Biopsy	Number of Cell Generations	HMG CoA Reductase Activity (pmol·min^{-1}·mg^{-1})
D.S.	Normal	Newborn	M	Foreskin	20	3.8
G.B.	Normal	2	M	Abdominal	16	3.9
M.T.	Normal	23	F	Deltoid	16	5.0
P.J.	Normal	25	F	Deltoid	16	9.1
GM 112[a]	Niemann-Pick Disease, Type A	10 months	M	Abdominal	14	4.9
GM 203[a]	Sandhoff Disease	1	M	Buttock	15	8.0
GM 806[a]	G_{M1} Gangliosidosis	2	F	Deltoid	13	11.0
M.C.-1[b]	FH Homozygote	6	F	Abdominal	20	115
M.C.-2[b]	FH Homozygote	8	F	Leg	12	184
W.D.	FH Homozygote	7	M	Deltoid	17	167

[a] Obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N. J. 08103.

[b] Two different skin biopsies were obtained on M.C. two years apart.

On day 7 of cell growth, cells were harvested for measurement of HMG CoA reductase activity as described in Methods. Each value represents the average of duplicate assays.

Phospholipid analysis

Each cell pellet was suspended in 1 ml of H_2O, and portions were removed for measurement of total protein content and for lipid extraction by the method of Folch, Lees, and Sloane Stanley (8). The lipid-containing lower phase was washed one time with pure upper phase solvent, evaporated to dryness, ashed (9), and the total content of inorganic phosphate was measured by the method of Chen, Toribara, and Warner (10). To determine the relative distribution of the phospholipid classes, the washed lipid-containing lower phase was evaporated to dryness and subjected to thin-layer chromatography on silica gel (11). The separated phospholipids, which were visualized by iodine vapor, showed the following R_f values: LPC, 0.09; SPH, 0.17; PC, 0.30; PI, 0.43; PS, 0.53; and PE, 0.69. Each phospholipid class was eluted from the silica gel with sequential extractions of chloroform–methanol 2:1, methanol, and methanol–water 9:1. After centrifugation to remove the silica gel, the pooled supernatants were evaporated to dryness, ashed, and the content of inorganic phosphate measured as described above. When authentic phospholipid standards were subjected to the above extraction and fractionation procedure, the recovery was greater than 90%. All calculations of phospholipid mass were made assuming that phosphorus accounts for 4% of total phospholipid weight.

Cholesterol analysis

The cellular content of free and esterified cholesterol was determined by gas–liquid chromatography as previously described (12).

Ganglioside analysis

Gangliosides were isolated from each pellet, separated by thin-layer chromatography, visualized with resorcinol reagent, and quantified by scanning the chromatograms as described previously (13).

Other assays

The activity of HMG CoA reductase was determined in detergent-solubilized extracts of fibroblasts as previously described (14). Protein was determined by the method of Lowry et al. (15).

Materials

Tissue culture supplies, thin-layer chromatographic supplies, and chemicals for assays were obtained from sources as previously reported (12–14).

RESULTS

Table 1 describes the fibroblast strains used in these studies. In addition to cells from two FH homozygotes and four normal subjects, fibroblasts from subjects with three different lysosomal lipid

TABLE 2. Content of phospholipids and cholesterol in fibroblast strains grown in the presence of fetal calf serum

Designation of Fibroblast Strain	Clinical Diagnosis	Total Phospholipids	Distribution of Phospholipid Classes (% of total)						Cholesterol	
			PC	PE	SPH	PI	PS	LPC	Free	Esterified
		μg/mg protein							*μg sterol/mg protein*	
D.S.[a]	Normal	150	57	23	11	5	4	ND[b]	13	2.4
		140	48	22	15	7	7	1	23	3.0
G.B.	Normal	164	65	24	5	4	2	ND	20	2.2
M.T.	Normal	180	60	26	6	4	4	ND	30	2.3
P.J.	Normal	160	62	21	8	5	4	ND	19	1.1
GM 112	Niemann-Pick Disease, Type A	290	46	22	18	6	7	1	31	3.6
GM 203	Sandhoff Disease	160	51	23	10	7	7	2	26	1.7
GM 806	G_{M1} Gangliosidosis	170	52	21	9	7	11	1	21	1.1
M.C.-1	FH Homozygote	160	49	24	9	10	6	2	16	1.6
M.C.-2	FH Homozygote	180	66	23	6	5	ND	ND	16	1.3
W.D.	FH Homozygote	160	64	23	8	3	2	ND	17	1.3

[a] The two sets of values for this cell strain represent the results of measurements on different sets of cultures that were grown and assayed two months apart. Both sets of data were obtained on cells during the 20th generation of growth.

[b] ND, not detected.

On day 7 of cell growth, cells were harvested for measurement of lipids as described in Methods. Each value represents the average of duplicate determinations.

TABLE 3. Ganglioside content of fibroblast strains grown in the presence of fetal calf serum

Designation of Fibroblast Strain	Clinical Diagnosis	Ganglioside Content (nmol of lipid-bound sialic acid/mg protein)					
		G_{M3}	G_{M2}	G_{M1}	G_{D3}	G_{D1a}	Total
D.S.	Normal	1.84	ND[a]	0.05	0.04	0.11	2.03
G.B.	Normal	0.76	0.06	0.08	0.10	0.23	1.22
M.T.	Normal	2.38	0.03	0.04	0.06	0.29	2.81
P.J.	Normal	1.30	0.03	0.09	0.04	0.12	1.57
GM 203	Sandhoff Disease	1.40	0.14	0.14	0.24	0.21	2.13
GM 806	G_{M1} Gangliosidosis	2.82	0.04	0.09	0.06	0.26	3.27
M.C.-1	FH Homozygote	2.62	0.04	0.06	0.02	0.15	2.89
W.D.	FH Homozygote	1.55	0.04	0.04	0.04	0.24	1.91

[a] ND, not detected.

On day 7 of cell growth, cells were harvested for measurement of gangliosides as described in Methods. Each value represents the average of duplicate measurements. No measurements were made on the cells from the subject with Niemann-Pick disease. The chromatographic patterns and ganglioside contents of the above fibroblast strains were similar to that reported previously for fibroblasts from a normal male (13).

storage diseases were studied: Niemann-Pick disease, type A (deficiency of sphingomyelinase); Sandhoff disease (deficiency of hexosaminidase A and B); and G_{M1} gangliosidosis (partial deficiency of beta-galactosidase). To confirm that these studies were carried out under conditions in which the genetic defect in the FH homozygote cells was expressed, we measured HMG CoA reductase activity in extracts of all of the cells at the time of harvest. The activity in the FH homozygote cells was approximately 30-fold higher than that in either the normal cells or in the cells with the lysosomal enzyme deficiencies (Table 1).

The total phospholipid content among the four normal fibroblast strains varied from 150 to 180 μg per mg protein (**Table 2**). The values for the two FH homozygotes (160–180 μg/mg protein) and those for the subjects with Sandoff disease and G_{M1} gangliosidoses (160 and 170 μg/mg protein) were within the normal range. On the other hand, in the cells from the subject with Niemann-Pick disease, the total phospholipid content was elevated nearly 2-fold (290 μg/mg protein). The distribution of the individual phospholipids was similar among all of the cell strains studied except for the cells from the subject with Neimann-Pick disease, which showed an elevation in the percentage of SPH and a corresponding reduction in the percentage of PC.

Among the normal cell strains, the content of free cholesterol varied from 13 to 30 μg per mg protein (**Table 2**). The values for the two FH homozygotes and the three lysosomal lipid storage diseases fell within this range. The content of esterified cholesterol was low in all of the cell strains with no significant differences noted.

Table 3 compares the total ganglioside content and distribution of individual gangliosides among the various cell strains. Among the normal fibroblasts, the total ganglioside content varied over a 2-fold range (1.22–2.81 nmol per mg protein). The values for the two FH homozygotes (2.89 and 1.91) were not significantly different from those in the normal cells. The highest total ganglioside content was seen in the G_{M1} gangliosidosis cells (3.27). The distribution of the gangliosides was similar in all of the cell strains with the exception that the Sandhoff disease cells exhibited an elevation in the content of gangliosides G_{M2}, G_{M1}, and G_{D3}.[2]

DISCUSSION

In contrast to a previous report (4), the current studies did not reveal a definite abnormality in the cellular content of either phospholipids or gangliosides in FH homozygote fibroblasts. We observed no differences in the cellular content of these lipid classes under conditions in which HMG CoA reductase activity (and hence cholesterol synthesis [3]) was 30-fold higher in the mutant FH homozygote cells as compared with the normal cells.

As positive controls for the adequacy of the lipid methods, we made measurements on fibroblasts from subjects with several lysosomal lipid storage diseases. As expected from previous studies, cells from the subject with Niemann-Pick disease showed

[2] Although neutral glycosphingolipids were not quantified, they were separated by thin-layer chromatography and visualized with orcinol reagent. As reported previously (7, 13), each cell strain contained mono-, di-, tri-, and tetrahexosylceramides. Except for an accumulation of tetrahexosylceramide (globoside) in the Sandhoff cells (7), there were no substantial differences observed among the various cell strains.

an elevation in the content of sphingomyelin (16), whereas cells from the subject with Sandhoff disease showed an elevation in the content of several glycosphingolipids, including G_{M2} and globoside (7).[2]

An important conclusion from this study is that the cellular content of various lipid classes can vary among normal human fibroblast strains grown under identical conditions. Such normal variation must be considered in the interpretation of studies comparing the cellular content of lipids in fibroblasts from normal and abnormal subjects.

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