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SPHINGOLIPID-INDUCED ENHANCEMENT OF RECEPTOR-MEDIATED UPTAKE OF LOW DENSITY LIPOPROTEINS IN NORMAL AND RECEPTOR-DEFICIENT HUMAN SKIN FIBROBLASTS

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(1) The receptor mediated endocytosis of homologous LDL by human skin fibroblasts can be significantly enhanced by prior incubation of the cells with sphingolipids. Gangliosides G_{M1} or G_{D1a}, their desialylated derivatives and sphingosine stimulate binding and uptake to LDL by up to 40% of normal values. The effect is observed in normal fibroblasts, LDL receptor deficient fibroblasts or in tunicamycin-treated cells with a reduced number of functional receptors but is dependent on the time of preincubation of the cells and the concentration of the sphingolipid in the medium. (2) Detailed studies on the ganglioside effect revealed, that cell bound gangliosides intensify the LDL-induced supression of [^{14}C]acetate incorporation into cholesterol. (3) The receptor dependence and relative receptor specificity of the sphingolipid effect is evident from the fact that (a) after complete suppression of receptor synthesis gangliosides fail to stimulate uptake of LDL, that (b) fatty acids or lipids not containing sphingosine are without effect and that (c) the receptor specific internalisation of α_2-macroglobulin or epidermal growth factor is not influenced by exogenous sphingolipids.

Introduction

The receptor mediated high affinity endocytosis of homologous low density lipoprotein (LDL) has been found to depend on the presence of specific receptors in the cell membrane as well as a recognition site on the apoprotein component of the LDL particle. Internalized LDL initiates a series of events that regulate cellular cholesterol metabolism including (a) supression of cholesterol synthesis (b) stimulation of cholesterol esterification and (c) inhibition of LDL receptor synthesis [1,2].

Reduction in the number, or modification of the structure and function of the LDL receptor result in a reduced interaction of the cells with LDL, in a corresponding failure to take up and degrade LDL and in a diminished ability to regulate the cellular cholesterol metabolism. This is indicated by the observation that (a) mutant fibroblasts in which the number of functional LDL receptor is reduced to 5–20% of normal, fail to bind and take up LDL [3], that (b) incubation of human skin fibroblasts in the presence of 25-hydroxycholesterol progressively reduces the ability of the cells to bind and internalize LDL [4] and that (c) tunicamycin treatment of cultured skin fibroblasts results in an inhibition of LDL uptake that is attributed to a defective *N*-glycosylation of the LDL receptor protein [5].

In previous studies [6–8] it has been shown that chemical modification of LDL or ganglioside incorporation into LDL particles modify the capability of LDL to be internalized by cultured skin fibroblasts and smooth muscle cells. The present report will show that preincubation of exogenous gangliosides and some other sphingolipids with cultured skin fibroblasts may enhance the metabolism of LDL both in normal cells and in cells with a hereditary or experimentally reduced number of functional LDL receptors.

Abbreviation: lecithin, 1,2-dioleyl-*sn*-glycero-3-phosphocholine.

Materials and Methods

Cow milk lactoperoxidase (160 U/mg) was purchased from Boehringer (Mannheim), ^{125}I carrier-free, [1-^{14}C]acetic acid, sodium salt (56 Ci/mol) and [9,10(n)-^{3}H]oleic acid (5.04 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, U.K.). Tunicamycin was a gift from Dr. R.L. Hamill, Lilly Research Laboratories (Indianapolis, In, U.S.A.). 25-Hydroxycholesterol was purchased from Straloids, Inc. (Wilton, NH, U.S.A.). Isolation, purification and desialylation of gangliosides were performed as described previously [9]. Gangliosides are designated as recommended by UIPAC-UIB lipid nomenclature ((1979) Lipids 12, 455). $G_{M1} = G_{Gtet}$ 1NeuAc; $G_{D1a} = G_{Gtet}$ 2NeuAc. Lecithin (1,2-dioleyl-*sn*-glycero-3-phosphocholine), cephalin (1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine), sphingomyelin (ceramide-1-phosphocholine, bovine brain), sphingosine (*trans*-4-sphingenine, base) and sulfatide (ceramide-1-*O*-D-galactosyl 3′-sulfate) were products of Serva GmbH (Heidelberg). α_2-Macroglobulin (Behringwerke, Marburg) and epidermal growth factor (Serva GmbH, Heidelberg) were labelled with ^{125}I according to Ref. 10.

Lipoproteins

Low density lipoproteins (*d* 1.02–1.055) were isolated and labelled with ^{125}I as described previously [7]. 95–97% of the radioactivity was precipitable with 10% trichloroacetic acid. Lipoprotein-deficient serum was prepared as described elsewhere [11].

Liposomes

Separation of liposomes was according to Ref. 12. Chloroform methanol (2 : 1, v/v) solutions of phospholipids (sphingomyelin, lecithin) and octylglucoside were mixed in equimolar ratio (20 μM) and the solvent evaporated under nitrogen. 1 ml of 0.155 M NaCl was added and the suspension was subjected to sonication using a Micro-Ultrasonic Cell Disruptor (H. Vetter, K.G., Wieserloch, F.R.G.). Samples were exposed to two 1 min bursts with a power setting of 3. Sphingomyelin and lecithin were sonicated at 45 and 20°C, respectively. The dispersions were centrifuged for 5 min at 3 000 rev./min in a laboratory centrifuge, the supernatant was carefully removed and used for experiments after determining the phospholipid content by phosphorous analysis.

Cell lines

Normal human skin fibroblasts were grown from the skin explant of a normal donor as described previously [9] and were used for incubation experiments between the third and eighth passages. Fibroblasts deficient in LDL receptor (GM 1 915) were obtained from the Institute for Medical Research (Camden, NJ, U.S.A.). Binding and incorporation of exogenous gangliosides and sphingolipids to cultured cells was performed by preincubating the cells for 24 h in the presence of the specified concentrations of sphingolipids or liposomes and subsequent repeated washings of cells with Hanks' solution.

Assays

The binding plus internalisation of [^{125}I]LDL were measured at 37°C according to the methods of Goldstein and Brown [13]. Incubation conditions are described in the corresponding tables and figures. ^{14}C and ^{3}H radioactivity associated with cellular cholesterol and cholesteryl oleate was measured as described previously [8]. Protein was determined by the method of Kaltwasser et al. [14]. Binding and uptake of α_2-macro [^{125}I]globulin and ^{125}I-labelled epidermal growth factor were determined according to Ref. 15.

Results

The experiemnts described are based on the following facts: (a) Exogenous gangliosides may be accumulated by cultured cells from the culture medium and are believed to become associated with the cell membrane [16].

(b) Gangliosides have been found to act as specific cell membrane receptors [17] or to cooperate specifically with glycoprotein receptors [18].

(c) At low concentrations (5 μg/ml medium [^{125}I]LDL) the receptor specific binding of LDL is rate limiting in the process by which normal cultured human skin fibroblasts internalize LDL [18].

(d) In a cell line with an inherited reduction in the number of LDL receptors the high affinity binding of LDL was suppressed to about 20% of normal cells.

Sphingolipid-enhanced endocytosis of LDL

When normal cells were incubated for 24 h at 37°C with the sphingolipids or glycerolipids listed in Table

TABLE I

EFFECT OF SPHINGOSINE, SPHINGOLIPIDS, GLYCEROPHOSPHOLIPIDS, FATTY ACIDS AND DETERGENTS ON RECEPTOR MEDIATED BINDING PLUS UPTAKE OF [^{125}I]LDL BY CULTURED SKIN FIBROBLASTS

The cells were preincubated for 24 h with the specified lipids and used for internalization experiments. Lipids were solubilized in alcohol, methanol or buffered saline (2 mg/ml). Controls received aliquots of alcohol, methanol or saline. Control experiments were performed with non-preincubated cells under otherwise identical conditions. Mean values (±S.D.) of four experiments are given.

Addition to medium (10 μM)	Binding + uptake (ng LDL per mg protein per 6 h)	% Activation
None	584 ± 10	100
Sphingosine	823 ± 21	141 [a]
Sphingo lipids		
Gangliosides (G_{M1})	771 ± 19	132 [a]
Sphingomyelin	712 ± 15	122 [a]
Sulfatide	692 ± 14	118 [a]
Glycerophospholipids		
L-α-Lecithin	598 ± 10	102
L-α-Cephalin	621 ± 19	106
Fatty acids		
Palmitic acid	595 ± 16	102
Stearic acid	582 ± 12	100
Eicosadienoic acid	578 ± 14	99
Detergents		
Octyl-β-D-glucoside	604 ± 18	103
Octyl-β-D-glucuronic acid	601 ± 15	102
Poly(ethylene glycol)	598 ± 16	102

[a] $P < 0.001$ as referred to control experiments.

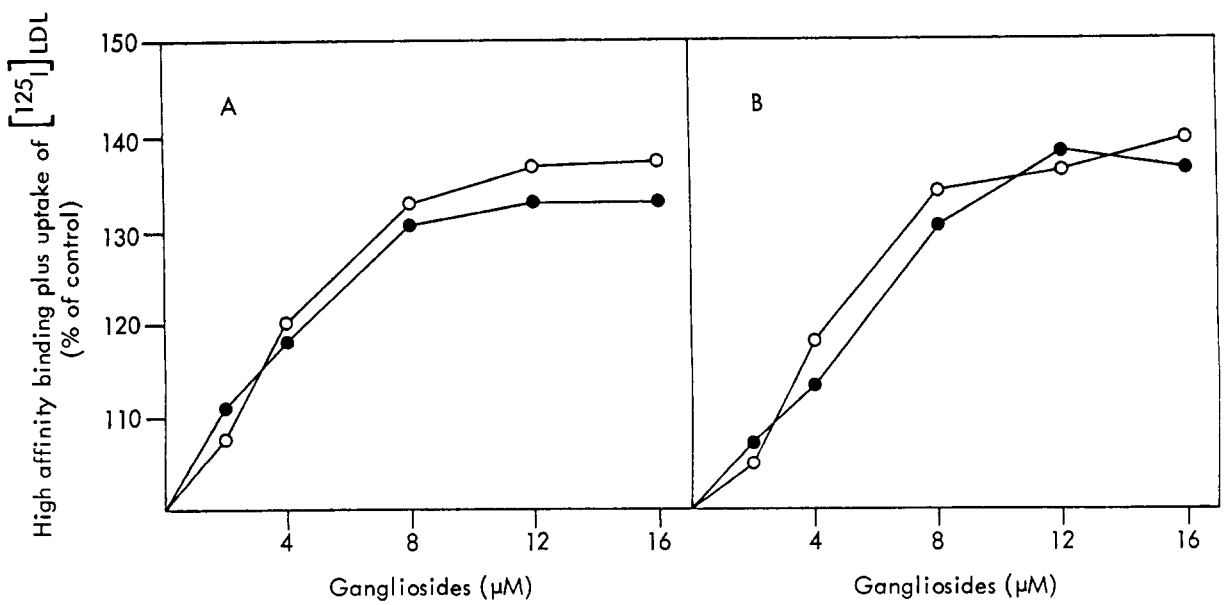

Fig. 1. The effect of increasing concentrations of G_{M1} (○) and G_{D1a} (●) gangliosides on high affinity binding plus uptake of [^{125}I]LDL by normal (A) and receptor-deficient (B) human skin fibroblasts. Cells grown to confluency were preincubated for 24 h in the presence of the indicated concentrations of gangliosides. Following four washes with Hanks' solution the cells were incubated for an additional 6 h with 5 μg/ml of labelled LDL either in the absence or presence of 300 μg/ml of nonlabelled LDL. High affinity binding plus internalization were calculated from the differences in the cell bound radioactivity after incubation the cells with and without nonlabelled LDL. Data are the mean values of five incubation experiments in duplicate.

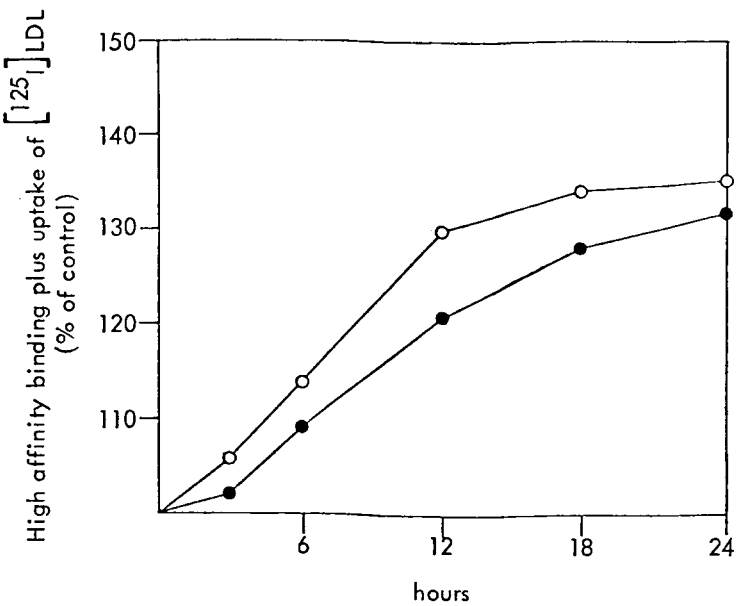

Fig. 2. High affinity binding plus internalization of [^{125}I]LDL by normal human skin fibroblasts as a function of the duration of cell preincubation with native (○) or desialylated (●) G_{M1} gangliosides. Cells grown to confluency were preincubated for the indicated periods of time with 10 μM native or desialylated G_{M1} ganglioside in the medium containing 10% lipoprotein-deficient serum. At the end of the treatment period, the cells were washed four times with Hanks' solution. For determination of high affinity binding and internalization cells were incubated for an additional 6 h with 5 μg/ml of [^{125}I]LDL either in the absence or presence of 300 μg/ml of nonradioactive LDL.

I, all compounds except for lecithin and cephalin increased the internalization of LDL by up to 40% as compared with non-preincubated normal cells. Since most of the sphingolipids exhibit a limited solubility the effect of gangliosides was studied in more detail. No further experiments were performed with the inefficient fatty acids and detergents (Table I).

The ganglioside-induced enhancement of internalization depends on the duration of preincubation the cells with gangliosides and reaches a maximum after 24 h (Fig. 2). The effect is not influenced by the number of sialic acid residues per ganglioside molecule nor does it show any requirement for sialic acid residues. Thus, the monosialoganglioside G_{M1} and the disialoganglioside G_{D1a} give a similar enhancement (Fig. 2) and desialylation of G_{M1} does not significantly alter its stimulating effect (Fig. 2). Desialylation converts G_{M1} to an neutral sphingolipid. The desialylated G_{D1a} was not examined.

The half saturation concentration for high affinity binding of LDL to normal cells is about 25 μg LDL/mg protein. Preincubation of cells with 10 μM G_{M1} lowers this value to about 17 μg LDL/mg protein. The higher affinity of ganglioside-preincubated cells to LDL is also indicated by a comparison of K_m values, found to be 25.4 μg LDL/ml for normal cells and 17.4 μg LDL/ml for cells preincubated with G_{M1}. However, no differences were found for the V values (3.1 and 2.99 μg LDL/6 h).

In receptor deficient cells G_{M1} also improves the high affinity binding and uptake of LDL at either LDL concentration (Fig. 3).

Pretreatment of fibroblasts with 1 μg tunicamycin/ml medium for 24 h inhibits LDL binding plus internalization to 50% of normal. The tunicamycin-mediated reduction of LDL endocytosis is partly restored when the cells are preincubated with 10 μM G_{M1} (Table II).

25-Hydroxycholesterol treatment of fibroblasts for 24 h effects a nearly complete inhibition of endocytosis, the residual uptake accounts for only 8% of normal fibroblasts. This inhibition is not, however, influenced by cell-associated gangliosides (Table II).

In fibroblasts, several macromolecules have been

TABLE II

EFFECT OF MEMBRANE ASSOCIATED GANGLIOSIDE ON HIGH AFFINITY BINDING PLUS INTERNALIZATION OF [^{125}I]LDL BY NORMAL HUMAN SKIN FIBROBLASTS PREINCUBATED WITH TUNICAMYCIN OR 25-HYDROXYCHOLESTEROL

Cells grown to confluency were preincubated for 24 h with the indicated concentrations of tunicamycin or 25-hydroxycholesterol either in the absence or presence of 10 μM G_{M1} ganglioside. Following washing with Hanks' solution of 3 ml of fresh medium containing 5 μg/ml of [^{125}I]LDL were added and incubation was continued for an additional 6 h. High affinity binding plus uptake were determined as described in Fig. 1. Data are the mean values (±S.E.) of three paired experiments.

Addition	[^{125}I]LDL bound plus internalized (ng/mg of cell protein)	
	Non-preincubated cells	Cells preincubated with G_{M1}
None	380 ± 26	507 ± 61
1 μg/ml tunicamycin	174 ± 20	262 ± 23
20 μg/ml 25-hydroxycholesterol	31 ± 4	35 ± 9

shown to enter cells by receptor mediated endocytosis [19]. In order to investigate the specificity of the ganglioside effect, binding and uptake experiments were performed with α_2-macroglobulin and epidermal growth factor. Preincubation of fibroblasts with 10 μm G_{M1} for 24 h virtually did not alter the uptake of these two macromolecules when compared with controls cells.

To ascertain that the inefficiency of glycerophospholipids (Table I) was not caused by their limited solubility a series of experiments was performed where the cultured cells were preincubated with liposomes containing sphingomyelin, lecithin or cephalin at a final concentration of 10 μM (see Methods). Determination of LDL binding and uptake by liposome pretreated cells gave exact confirmation of the results listed in Table I.

Ganglioside-influenced cholesterol metabolism

In normal fibroblasts LDL causes a reduction of cholesterol synthesis as indicated by a diminished incorporation of [^{14}C]acetate radioactivity into cholesterol, while in receptor deficient cells minor regulatory effects are observed. After preincubation with G_{M1} and desialylated G_{M1} for 24 h the suppression of cholesterol synthesis is more effective in both normal and receptor deficient cells, although desialylated gangliosides have a less pronounced action (Fig. 3).

Preincubation of normal cells with 15 μg native LDL/ml for 24 h brings about 40-fold increase in the rate of incorporation of [^{3}H]oleate into cholesterol ester, as compared with the results of an experiment in the absence of LDL. There is, however, only a limited stimulation of the LDL induced cholesterol esterification in normal and receptor deficient cells with membrane bound gangliosides (Table III).

TABLE III

STIMULATION OF CHOLESTEROL ESTER FORMATION BY MEMBRANE ASSOCIATED G_{M1} GANGLIOSIDES

Cells grown to confluency were preincubated for 24 h in the presence of 10 μm native or desialylated G_{M1} gangliosides and then washed four times with 3 ml of Hanks' solution. 3 ml of fresh medium containing 10% of lipoprotein-deficient serum and 20 μg/ml of LDL were added and incubation was continued for further 24 h. After wards the cells were replaced with 2 ml of fresh medium containing 4 nmol/ml (560 000 counts · min^{-1} · nmol^{-1}) of an albumin-bound [^{3}H]oleate and the incubation was continued for 3 h at 37°C. At the end of the incubation the cells were harvested for determination of the cellular content of cholesteryl [^{3}H]oleate. Each value represents the average ±S.E. of triplicate incubation.

Addition to medium	[^{3}H]Oleate incorporated into cholesteryl oleate (pmol per mg protein per h)			
	Non-preincubated		Preincubated with G_{M1}	
	Normal cells	Receptor deficient cells	Normal cells	Receptor deficient cells
None	31 ± 11	29 ± 10	28 ± 12	30 ± 8
Native LDL	1 332 ± 221	312 ± 28	1 564 ± 311	386 ± 62

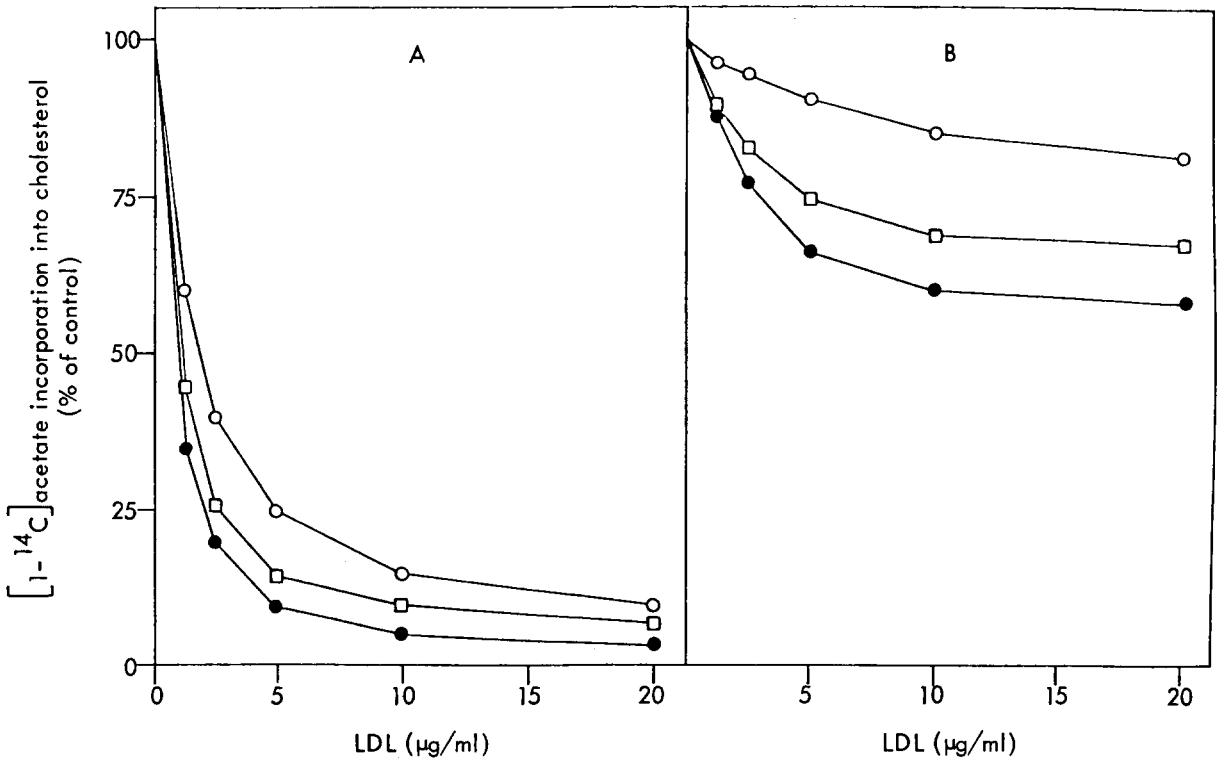

Fig. 3. Supression of cholesterol synthesis from [1-^{14}C]acetate in normal (A) and receptor-deficient (B) human skin fibroblasts by LDL. ○, Cells not preincubated with gangliosides; ●, cells preincubated in the presence of native G_{M1} ganglioside; □, cells preincubated with desialylated G_{M1} gangliosides. Cells were preincubated for 24 h with either 10 μM native or desialylated G_{M1} gangliosides. Following extensive washing with Hanks' solution 3 ml of fresh medium containing 10% lipoprotein deficient serum and the indicated concentration of LDL were added and the incubation was continued for an additional 24 h. Afterwards 10 μCi/ml of [1-^{14}C]acetate were added, the incubation was terminated after 3 h and the cells were harvested for protein and [^{14}C]-cholesterol determination. Each point represents the mean value of seven incubation experiments performed in duplicate.

Discussion

Sphingolipids are components of the cell surface membranes and are found to be associated with important membrane functions, such as Na^+ transport, choleratoxin binding and recognition [16]. Membrane sphingolipids are thought to anchored via the ceramide moiety in the fluid lipophilic matrix and with the polar head group exposed to the external environment [18]. Incorporation of exogenous gangliosides into the membrane of cultured cells [19] and their action as receptors [19] or as part of receptors [20] have been proven beyond doubt.

Our experiments suggest that in sphingolipid-treated cells the LDL receptor operates with higher efficiency. This could explained by the possibility that the gangliosides (or other sphingolipids) facilitate receptor migration to coated pits and enhance internalization. If this interpretation is correct, then the ganglioside treated cells should internalize LDL with a higher V. This is, however, not the case. The alternative explanation, that the receptor exerts an improved affinity possibly by cooperating with the exogenous gangliosides appears to be more feasible and is consistent with the observation that the K_m value for the LDL uptake is lowered, after the cells had bound gangliosides.

The relative specificity of the observed sphingolipid effect is indicated by the incapability of gangliosides to influence binding and uptake of α_2-macroglobulin and epidermal growth factor.

The ganglioside-induced enhancement of LDL

uptake requires a miminum number of available LDL receptors. On reduction of the receptor concentration to half the normal amount (tunicamycin experiments) or in receptor-deficient cells, a sufficient number of receptor sites still remain for interaction with gangliosides. An almost complete suppression of receptor synthesis, however, as induced by treatment of fibroblasts with 25-hydroxycholesterol abolishes the ganglioside effect.

Preincubation of cultured cells with either sphingolipids or sphingolipid containing liposomes resulted in an equal enhancement of LDL binding an uptake. However, Gatt and Bierman [12] reported that in cells preincubated with positively charged liposomes containing sphingomyelin and octadecylamine the activity of the LDL receptor was reduced, while the cholesterol synthesis was stimulated. The fact that the positive charge of the liposomes was found to be essential for these effects renders difficult a comparison with out experiments for the following reasons: (a) Desialylation converts G_{M1} to a neutral sphingolipid, that has been proven to be as effective as negatively charged gangliosides and (b) liposomes were prepared with the noncharged octylglucoside (see Methods).

Whether the gangliosides themselves or sphingolipid degradation products (e.g. sphingosine) are responsible for stimulating the LDL metabolism can not be decided from our experiments. The cellular synthesis of sphingoglycolipids proceeds via an initial sphingosine-dependent formation of ceramide to which the sugar residues are added through the function of highly specific glycosyltransferases [21]. The requirement for sphingosine of the initial step of synthesis may explain the high activation effect of exogenous sphingosine (Table I) that could be converted to a function specific sphingoglycolipid during the preincubation period. In cultured cells, there is some evidence, that a portion of the glycosyltransferase activities involved in sphingolipid synthesis is part of the plasma membrane [22].

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