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Modifications of LDL-receptor-mediated endocytosis rates in CEM lymphoblastic cells grown in lipoprotein-depleted fetal calf serum

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The efficiency of supplying cholesterol by the LDL endocytic pathway of lymphoblastic T CEM cells was compared when incubated in the presence of either fetal calf serum (FCS) or lipoprotein-depleted fetal calf serum (LDFCS). In the presence of FCS, there were 8600 ± 2000 LDL receptors/cell with a K_d of $(2.2 \pm 0.8) \cdot 10^{-8}$ M and a receptor cycling time of about 7 min; about 90% of the internalized LDL was degraded. LDL degradation produced 98% of total cellular cholesterol and only 2% came from endogenous synthesis. The absence of LDL in the culture medium of lymphoblastic CEM cells deeply modified certain metabolic and structural characteristics of the cells. Their cholesterol content decreased; the total number of LDL receptors increased 6-fold, whereas their affinity for the ligand decreased by the same factor ($K_d = (1.2 \pm 0.2) \cdot 10^{-7}$ M); the receptor cycling time increased 3-fold. Finally, LDL degraded by cholesterol-depleted CEM cells amounted to about 40% of that degraded by untreated CEM cells.

Introduction

Cholesterol forms an integral and large component of various cellular membranes and is usually found in larger amounts in plasma, lysosomal and Golgi membranes [1]. In the majority of cells, its concentration depends on the balance between the two cholesterol supply processes, i.e., endogenous synthesis under the control of HMG-CoA reductase, and exogenous cholesterol uptake via the LDL pathway involving endocytosis of the LDL-receptor complex [2,3]. Cholesterol has been shown to be responsible for suppressing HMG-CoA reductase activity (feedback control) [3]. It also regulates, in a coordinated action, two other cellular processes that stabilize the cellular cholesterol content. It activates acyl-CoA : cholesterol acyltransferase, and suppresses the synthesis of LDL receptors by lowering the concentration of receptor mRNA [3]. In lipoprotein-depleted medium, both the rate of endogenous cholesterol synthesis and the number of LDL-specific receptors increase [4–6]. Normal human monocyte-derived macrophages even exhibit an increased affinity for LDL [7].

Cholesterol is involved in the control of various membrane-associated properties, such as permeability, membrane-bound enzyme and receptor activities [8,9], immune responses and endocytosis [10–13]. It is also reported to intervene in some membrane-related events, such as fusion between virus and endosomal membrane in the case of the Semliki forest virus [14].

The present study deals with the relationship between the cellular cholesterol content and the binding capacity and recycling characteristics of the LDL receptor in human T lymphoblasts (CEM cells). We show in the present paper that the cholesterol supply in CEM cells is highly dependent on the LDL uptake. Depletion of cholesterol in the medium was associated with a decrease in cellular cholesterol content, which in turn was correlated with a very significant change in LDL receptor numbers, and decreases in the binding affinity, endocytosis rates and cholesterol uptake following LDL degradation.

Materials and Methods

Chemicals

[1-^{14}C]Acetic acid sodium salt (57 Ci/mol) was purchased from C.E.A. (Saclay, France). Na^{125}I (17

Abbreviations: LDL, low-density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34); FCS, fetal calf serum; LDFCS, lipoprotein-depleted fetal calf serum; BSA, bovine serum albumin.

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mCi/μg) came from Amersham, France; antibiotics, RPMI 1640 and fetal calf serum were from Gibco-France. Fat-free BSA and behenyl alcohol were Sigma products. Dibutyl phthalate was purchased from Kodak, France, and pronase (as *Streptomyces griseus* lyophilizate) from Boehringer Mannheim (France). Sterilizing filter units Millex-GV (0.22 μm) were provided by Millipore (Molsheim, France).

Cell line

The cell line from a human T leukemia [15] was obtained from Dr. Casellas (Sanofi Recherches, Montpellier, France). The cells were cultured at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and antibiotics (100 μg/ml streptomycin, 100 IU/ml penicillin) in an atmosphere containing 5%CO_2. The CEM cells doubled in number in about 24 h. For experiments, when the cell concentration reached $5 \cdot 10^5$ cells/ml, the medium was replaced with fresh medium containing 10% (v/v) FCS or 10% LDFCS and incubation was carried out for 48 h.

Cells cultured in the presence of FCS are designated as CEM (+) and cells cultured in LDFCS are designated as CEM (−).

LDL

Human LDL (density 1.019–1.063 g/ml) was prepared, as previously described in detail [16], from the plasma of healthy subjects by sequential ultracentrifugation, and was radioiodinated by the monochloride method [17]. ^{125}I-LDL (specific radioactivity 200–500 cpm/ng protein) was sterilized by ultrafiltration (Millex GV 0.22 μm), stored at 4°C and used within two weeks.

LDFCS was obtained by ultracentrifugation at 100000 × *g*, for 48 h in the presence of KCl salt solution at a density of 1.28 and then extensively dialyzed against 150 mM NaCl containing 0.04% EDTA (pH 7.4). The FCS cholesterol content was determined using the Iatroscan method [18]. Esterified cholesterol amounted to 421 ± 27 μg per ml of FCS, free cholesterol to 49 ± 2 μg per ml of FCS. The lipoprotein-depleted medium contained 31 ± 3 μg of esterified cholesterol per ml and no free cholesterol.

Determination of surface binding, uptake and degradation of ^{125}I-LDL

These determinations were done as previously described [16,19]. The surface binding assays were carried out at 4°C in the presence of medium A (phosphate-buffered saline containing 150 μM $CaCl_2$, 20 mg/ml fat-free BSA (pH 7.4)). Cells ($3 \cdot 10^6$) were washed three times in medium A, resuspended and incubated for 2 h at 4°C in the same medium containing ^{125}I-LDL at the indicated concentrations in the presence or absence of an excess of unlabeled LDL (50–100-fold) and treated as previously described [16,19]. Cell pellets were counted and dissolved in 0.5 ml of 0.1 M NaOH for protein assay according to the method of Lowry et al. [20]. All uptake (i.e., binding plus internalization) and degradation assays were carried out at 37°C in medium C (RPMI 1640, 30 mM Hepes, 150 μM $CaCl_2$, 5 mg/ml fat-free BSA, 10% (v/v) LDFCS, 100 IU/ml penicillin and 100 μg/ml streptomycin (pH 7.4)). Cells ($3 \cdot 10^6$) were incubated in this medium containing ^{125}I-LDL at the indicated concentrations in the presence or absence of an excess of unlabeled LDL and treated as previously described [16,19].

Evaluation of LDL receptor intracellular recycling time

Cells were incubated for 2 h at 4°C in medium C containing 50 μg/ml of ^{125}I-LDL in the presence or absence of unlabeled LDL. They were then incubated for different times (1–20 min) at 37°C. At the end of incubation, the cells were quickly cooled in ice and by adding 1 ml of cold medium B (the same as medium A, except for 5 mg/ml BSA). The cell pellets were washed three times with cold medium B and incubated in 500 μl of cold 150 mM NaCl containing 2 mg/ml pronase, for 1 h at 4°C [19]. The samples were centrifuged and the radioactivity in the medium and pellet were separately measured to determine the amounts of pronase-released and pronase-resistant radioactivity, respectively. An aliquot of homogenized cells in 0.5 ml of 0.1 M NaOH was used to determine the cellular protein content. During the short incubations (1–20 min) intracellular ^{125}I-LDL accumulated at a constant rate. The receptor recycling time was calculated from the ratio of ^{125}I-LDL internalized per min to that bound at the surface.

Analytical procedure

HMG-CoA reductase activity and [1-^{14}C]acetate incorporation into digitonin-precipitable sterols were determined as previously described [19]. Cellular lipids were extracted by the method of Folch et al. [21] and analyzed by the Iatroscan method, which combines thin-layer chromatography (TLC) and flame-ionization detection [18]. TLC on 1 mm-thick quartz rods coated with silica-based frit (chromarods S II) was developed first with chloroform/methanol/water (400:200:25, v/v) for 5 cm, and completed (10 cm) with hexane/diethyl ether/formic acid (450:50:1.5, v/v). Cholesterol and phospholipid spots were scanned in an Iatroscan device using behenyl alcohol as the standard solution. In one case, extracted cellular phospholipids were also determined by Bartlett's method [22].

Reactive and statistical data

The specific binding was calculated by subtracting ^{125}I-LDL bound in the presence of unlabeled LDL (nonspecific binding) from that measured in the absence of unlabeled LDL (total binding. The K_d values and

the number of sites/ cell were estima ed assuming a molecular weight of 500000 for the protein component of the LDL [23], and a cellular protein concentration of 1 mg per $(4.5 \pm 0.5) \cdot 10^6$ CEM (+) cells o. $(4.7 \pm 0.4) \cdot 10^6$ CEM (−) cells (mean of five analyses in duplicate, nonsignificant difference). The differences between means were tested for statistical significance using Student's *t*-test.

The amount of cholesterol supplied to cells from the degradation of LDL was computed from the rate of LDL protein degradation, as previously described [16], assuming a ratio of total cholesterol to protein equal to 2. The amount of cholesterol derived from endogenous synthesis was estimated, as previously [16], from sodium [1-^{14}C] acetate incorporated into sterols, according to the methods of Ho et al. [5] and Vial et al. [24]. Cell growth increased exponentially. The doubling time was estimated according to the formula: $T_2 = (\ln 2)/k$.

Results

Effects of lipoprotein-depleted medium on various characteristics of CEM cells

In one series of experiments, we successively analyzed the influence of the presence or the absence of plasma lipoproteins on several characteristics of CEM cells in culture, i.e., (i) on the overall behavior of CEM cells (growth rate), (ii) more specifically on the cell metabolism related to LDL (regulation of cholesterol biosynthesis) and (iii) on the cellular lipid content, which can modify LDL receptor function [13].

We measured the growth rate of CEM cells cultured for 72 h in medium containing either 10% (v/v) LDFCS or FCS (seven assays). During the first 48 h, the doubling time was not significantly different (28.6 ± 2.6 h for CEM (+) and 24.7 ± 3.6 h for CEM (−)). After 48 h, the doubling ti ne did not change for CEM (+) but for CEM (−) it increased to 50 ± 5 h. Thymidine incorporation into DNA (between 36 and 48 h of culturing) was of the same order in the two types of cell: 19022 ± 1000 dpm for CEM (+) and 27090 ± 6000 dpm for CEM (−) (mean of two experiments with eight assays, nonsignificant difference). CEM (+) and CEM (−) cells were pulse-labeled with [^{3}H]leucine (80 pM) for 4 h in the respective media. Radioactivity incorporated was measured by cold trichloroacetic acid precipitation of the cell lysate. The results showed that CEM (+) and CEM (−) incorporated 30920 ± 3160 dpm and 31700 ± 6220 dpm per $1 \cdot 10^6$ cells, respectively (mean of three experiments involving four assays ± S.E.), indicating that protein synthesis by cells in lipoprotein-depleted medium was not modified for at least 48 h of treatment. Our experiments were thus carried out on cells grown in FCS and then incubated for 48 h in LDFCS to remain in the logarithmic phase of multiplication.

The absence of lipoprotein in the medium resulted, as expected, in a 7–10-fold increase in cellular cholesterol synthesis (HMG-CoA reductase activity) and a 5-fold increase in the incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols (Table I).

In spite of this increase, the cellular cholesterol concentration decreased by 46% compared to the CEM (+) value (significant effects of LDFCS; $P < 0.01$). The quantities of phospholipids determined by the Iatroscan method or by Bartlett's method did not differ significantly between the two types of cell.

LDL receptor analysis

Lipoprotein binding at 4°C in CEM (+) and CEM (−) cells. The results of lipoprotein-specific binding at 4°C are given in Fig. 1 , which shows the Scatchard plot [25] of binding data on ^{125}I-LDL in CEM cells. For CEM (+) cells, we estimated the number of sites/cell at 8600 ± 2000 (mean ± S.E., $n = 3$). The mean apparent dissociation constant (K_d) calculated from three experiments was $(2.2 \pm 0.8) \cdot 10^{-8}$ M. The formation and dissociation of the ligand-receptor complex were measured versus time at 12.5°C. The pronase technique showed a blockage of endocytosis at this temperature. The K_d calculated from these two experimental parameters ($K_{on} = 6$ min, $K_{off} = 88.5$ min) is compatible with

TABLE I

Effects of culture medium on cholesterol biosynthesis

CEM lymphoblasts were routinely grown in 10% FCS and then incubated for 48 h in medium supplemented with FCS (CEM (+)) or LDFCS (CEM (−)). After incubation, the cells were washed and assayed for their HMG CoA reductase activity and cholesterol biosynthesis from [1-^{14}C]acetate. The lipid content of the cells was determined as described in Materials and Methods. Values shown are the means ± S.E. of results from three separate preparations of CEM (+) and CEM (−) cells with triplicate determinations.

	HMG-CoA reductase activity (pmol/mg per min)	[1-^{14}C]Acetate incorporated (nmol/h per 10^9 cells)	Lipids (μg/mg cell protein)		
			free cholesterol	esterified cholesterol	phospholipids
CEM (+)	9 ± 3	55 ± 15	9.6 ± 0.4	0.2 ± 0.03	73 ± 3
CEM (−)	95 ± 26 [a]	285 ± 48 [a]	5.2 ± 0.5 [a]	0.08 ± 0.02 [a]	62 ± 7

[a] Significant ($P < 0.01$) effect of LDFCS.

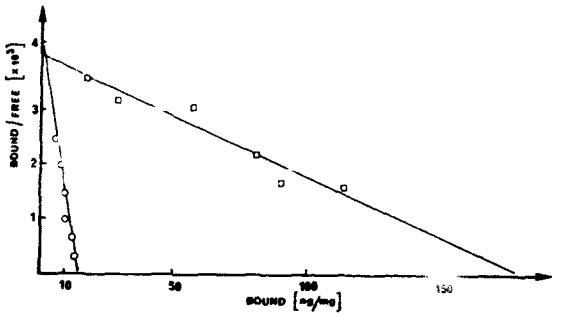

Fig. 1. Binding of human ^{125}I-LDL to CEM lymphoblast cells at 4°C, vs. ^{125}I-LDL concentration. CEM cells ($3 \cdot 10^6$) were incubated for 2 h at 4°C with 5–80 μg of labeled LDL (226 cpm/ng protein) in a final volume of 0.1 ml, either in the absence or presence of 500 μg of unlabeled LDL. The mean percentages (±S.E.) of nonspecific binding were 36.7±2% for CEM (−) cells and 52±7% for CEM (+) cells. The binding was determined as described in Materials and Methods, and Scatchard plots were drawn: ○, CEM (+) cell binding; □, CEM (−) cell binding. Points on the curves are mean values of duplicate determinations in a typical experiment, which was repeated three times. The K_d value and number of sites per cell were calculated assuming that the protein component of LDL has a molecular weight of 500000 and that 1 mg of cell protein corresponds to $4.5 \cdot 10^6$ cells. The differences between K_d values and site numbers were significant ($P < 0.01$).

that calculated by Scatchard method. With CEM (−) cells, the number of receptors increased about 6-fold (54600 ± 4000 receptors per cell, mean ± S.E., $n = 3$) as expected [2–7]. Surprisingly, however, the K_d also increased 6-fold $(1.2 \pm 0.2) \cdot 10^{-7}$ M, mean ± S.E., $n = 3$ and $P < 0.05$).

Uptake and degradation of ^{125}I-LDL. The time-course of uptake (i.e., binding plus internalization) and degradation of ^{125}I-LDL by CEM (+) and CEM (−) cells are shown in Fig. 2. At 10 μg/ml, ^{125}I-LDL, CEM (+) cell uptake reached a steady state in about 30 min and degradation increased regularly, in agreement with this phenomenon in other cell lines [2–7]. The results with CEM (−) cells were quite different. The uptake in CEM (−) cells did not occur in a steady state, even after 5 h, but increased linearly with time. The pronase technique showed that the quantities of ^{125}I-LDL bound to the cell surface (pronase-released) were constant after 1 h of incubation, whereas the quantities internalized (pronase-resistant) increased continuously (data not shown). At the same time, ^{125}I-LDL degradation was 60% lower in CEM (−) cells than in CEM (+) cells.

Uptake and degradation after 4.5 h were measured versus ^{125}I-LDL concentration (Fig. 3). The CEM (+) uptake (●) was saturated with about 15 μg/ml of labeled LDL. The CEM (−) uptake (■) was always higher than that of CEM (+), whatever the ^{125}I-LDL concentration. In contrast, CEM (−) degradation (□) was always lower than that of CEM (+) (○).

This first series of results appeared to show defective LDL metabolism in CEM (−) cells. To clarify this point, we studied the rate of internalization and receptor turnover.

Internalization and receptor turnover rates. In this case, the CEM (+) cells were incubated for 2 h at 4°C in medium C with 50 μg/ml ^{125}I-LDL, so that ^{125}I-LDL binding reached a steady-state. The cells were then incubated for short periods at 37°C and treated as described in Materials and Methods (Fig. 4). Under these conditions, the ^{125}I-LDL accessible to pronase remained constant (21.5 ± 2 ng/mg cell protein, mean of two experiments in duplicate), whereas the internalized LDL accumulated in the cells at a linear rate of 3.25 ± 0.7 ng/mg per min. Assuming, like Goldstein and Brown [26], that all internalized LDL has come from endocytosis of receptor-bound LDL, we have estimated that of the 21.5 ng of receptor-bound LDL, 3.25 ng entered the cell per minute. In other words, each receptor loaded with one molecule of LDL was internal-

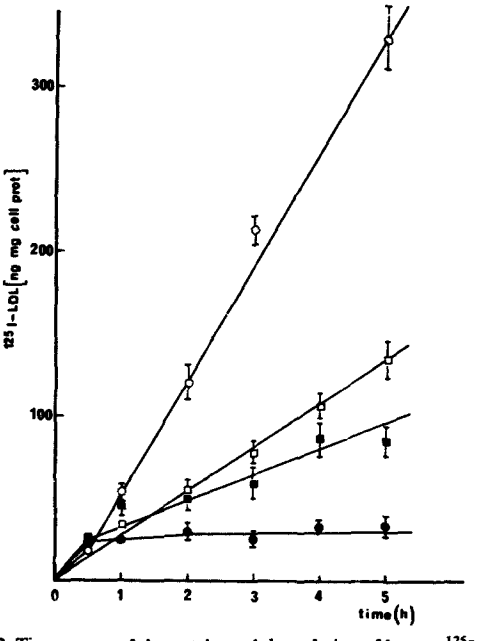

Fig. 2. Time-course of the uptake and degradation of human ^{125}I-LDL by CEM lymphoblast cells at 37°C. Cells ($3 \cdot 10^6$) in 1 ml of medium C were incubated with 10 μg of labeled human LDL (541 cpm/ng protein) in the presence or absence of a 100-fold excess of unlabeled LDL. At the indicated times the medium was collected and degradation by CEM (+) cells (○) or CEM (−) cells (□) was measured in terms of the amount of ^{125}I-labeled acid-soluble material, as described in Materials and Methods. The specific uptake, by CEM (+) cells (●) or CEM (−) cells (■), was calculated from the total cellular ^{125}I-content measured in the absence or presence of an excess of unlabeled LDL. Results are given as means ± S.E. of values from three experiments in triplicate with CEM (−) cells and two experiments with CEM (+) cells. The mean percentages (±S.E.) of nonspecific values were as follows: uptake 12±2% and degradation 8.6±0.9% for CEM (+) cells; uptake 9.5±1.5% and degradation 14.5±3% for CEM (−) cells.

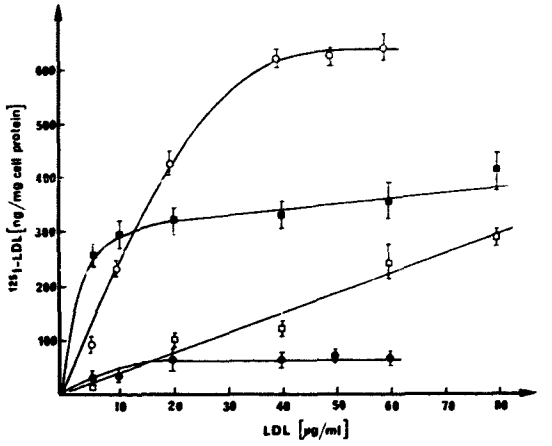

Fig. 3. ^{125}I-LDL uptake and degradation by CEM (+) or (−) lymphoblast cells at 37 °C vs. the ^{125}I-LDL concentration. CEM cells ($3 \cdot 10^6$) in 1 ml of medium C were incubated for 4.5 h with the indicated ^{125}I-LDL (287 cpm/ng) concentrations in the absence or presence of a 100-fold excess of unlabeled LDL. Uptake (full symbol) and degradation (open symbol) were measured with CEM (+) (●, ○) and CEM (−) (■, □) as described in Fig. 2. The nonspecific values for uptake and degradation were the same as in Fig. 2.

ized and took up a new molecule of LDL from the medium every 6.6 min. When the same experiment was performed with CEM (−) cells, the ^{125}I-LDL released by pronase was always higher (76.8 ± 5 ng/ml cell protein, mean of two experiments in duplicate), reflecting the increase in the number of receptors in these

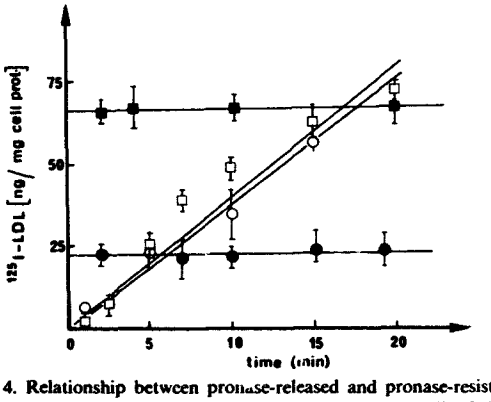

Fig. 4. Relationship between pronase-released and pronase-resistant ^{125}I-LDL at 37 °C during short incubation. CEM (+) cells ($3 \cdot 10^6$) were supplemented with 1 ml of medium C containing 50 μg/ml ^{125}I-LDL (100 cpm/ng) in the presence or absence of an excess of unlabeled LDL. They were first incubated for 2 h at 4 °C, and then at 37 °C. After the indicated incubation periods, cells were washed three times with cold medium B, and 500 μl of ice-cold 150 mM NaCl containing 2 mg/ml pronase was added. After incubation for 60 min at 4 °C, the medium was removed and radioactivity in cells and medium was determined to assess the amount of ^{125}I-LDL remaining cell-associated after pronase treatment (CEM (+) (○); CEM (−) (□)) and the amount of pronase-released radioactivity (CEM (+) (●); CEM (−) (■)).

cells. However, the difference between the quantities of ^{125}I-LDL bound by CEM (+) and CEM (−) cells was smaller than that observed during binding experiments at 4 °C. This could be due to the different measuring techniques used. However, the internalization rate was only 4.16 ± 0.5 ng/mg per min (mean of two experiments), which was not significantly different from CEM (+) cells. The receptors recycling time was 18.5 min in CEM (−) cells or about three times slower than in CEM (+) cells.

Discussion

We characterized the parameters of cholesterol homeostasis in cells cultured in lipoprotein-rich medium. By comparing these results with those obtained with the same cells cultured in lipoprotein-depleted medium, it was possible to study the role played by cholesterol in the endocytosis of LDL-specific receptors.

Cholesterol homeostasis in CEM cells grown in the presence of LDL

Our data indicate the main parameters controlling cholesterol homeostasis in CEM cells grown in cholesterol-rich medium. The ligand-receptor affinity value ($K_d = 2 \cdot 10^{-8}$ M) was close to that in data concerning different cell types. The uptake of LDL by CEM (+) reached a steady state at about 30 min (Fig. 2). This steady state reflects a balance between internalization and degradation: the experiments with short incubation times (Fig. 4) gave a mean internalization rate of 3.25 ng/mg per min when the LDL concentration was 50 μg/ml; under the same conditions, a degradation rate of 3.1 ng/mg per min can be computed from the rate (1/1 ng/mg per min) shown in Fig. 2, assuming that the amount of degraded LDL increases by a factor of 2.8 when the LDL concentration increases from 10 to 50 μg/ml (Fig. 3).

The receptor turnover rate of about 6.6 min (from Fig. 4) allowed a large LDL influx into the cells, so that the cholesterol supplied by LDL represented about 1.9 μg of cholesterol/24 h per 10^6 cells. Endogenous cholesterol biosynthesis was estimated at only 42.5 ng/24 h per 10^6 cells, corresponding to about 2% of the cholesterol requirements of CEM cells, as in normal lymphocytes [5,16]. It should be noted that the total cholesterol input (LDL supply + synthesis) per day was estimated to be of the same magnitude as the total cellular cholesterol content (1.95 ± 0.2 μg versus 2.1 ± 0.2 μg, respectively).

Cholesterol metabolism in CEM cells growing in LDFCS medium

After incubating CEM cells for 48 h in LDFCS medium, the number of LDL-specific receptors increased 6-fold, whereas HMG-CoA reductase activity,

in parallel with cholesterol synthesis, increased 7–10-fold (Table I). These results agree with the well-known consequences of LDL starvation [2–6]. However, in contrast with other results [7], some parameters of the endocytic pathway of LDL were disturbed: (i) as shown in Fig. 1, in CEM (−) cells, the affinity of LDL for its receptor decreased compared with CEM (+) (6-fold; mean of three experiments); (ii) in spite of a larger number of receptors on the cell surface, the internalization rate was the same for CEM (−) and CEM (+) (Fig. 4); (iii) the LDL degradation rate of CEM (−) was always lower than that of CEM; (iv) the uptake (Fig. 2) did not reach a steady-state, and an increasing amount of ^{125}I-LDL accumulated in the cells.

In the absence of LDL, endogenous cholesterol biosynthesis increased 5-fold, but this increase was not sufficient to balance the absence of an exogenous cholesterol supply. Under these conditions, the cellular cholesterol content decreased by 46% in 48 h, and after 48 h, cell division decreased, in agreement with other data on the role of cholesterol in cell division [27,28].

Mechanism of action

The above-mentioned results show that incubation of CEM cells in DLPFCS medium has modified some characteristics of the LDL endocytosis pathway.

(i) The number of receptors at the CEM cell surface was regulated by the serum LDL content, as previously observed in several cell types [4–6].

(ii) The affinity of these receptors for LDL decreased. This is a surprising result, since the effect of the LDL receptor increase was thus abolished by this affinity decrease. This is also in contradiction with the increase in the affinity of LDL for its receptor observed in monocyte-derived macrophages cultured in LDFCS [7]. Nevertheless, the latter cells were demonstrated to be relatively independent of exogenous cholesterol supply via receptor-mediated uptake of LDL [29], whereas LDL uptake accounted for 98% of the needs of cholesterol in CEM cells, and was not balanced by the endogenous synthesis in CEM (−). Thus, in cholesterol-depleted cells (CEM (−), and in the cholesterol auxotrophic cell line [13]), the cholesterol ratio in plasma membrane is clearly very low, probably inducing the low affinity of LDL for their receptors.

(iii) The internalization and degradation rates were decreased.

These results indicate an unusual behavior of LDL-receptor-mediated endocytosis in lymphoblastic CEM cells when the culture medium has been depleted of cholesterol.

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