Uptake and esterification of exogenous cholesterol by low-density-lipoprotein-receptor-negative human fibroblasts in culture

J. Peter SLOTTE,*† Stig EKMAN‡ and Sören BJÖRKERUD*

*Department of Pathology, University of Göteborg, Sahlgren Hospital, S-413 45 Göteborg, Sweden, and

‡Department of Biochemistry and Pharmacy, Åbo Akademi, SF-20500 Turku 50, Finland

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The incorporation and metabolism of both vesicle- and LDL (low-density lipoprotein)-derived [^3H]cholesterol by LDL-receptor-negative fibroblasts were studied. Independent of the cholesterol source, free [^3H]cholesterol was readily incorporated into the cells and was available for esterification. 7-Oxcholesterol stimulated both [^3H]cholesterol incorporation, by increasing the exchange rate, and the subsequent esterification of it irrespective of the source of exogenous [^3H]cholesterol. The 7-oxcholesterol-stimulated esterification of exogenously derived LDL free [^3H]cholesterol was progesterone-sensitive and energy-requiring.

The metabolism of LDL by normal human fibroblasts in culture has been extensively examined during the last decade (for a review, see Brown *et al.*, 1981). It has been shown that LDL binds with high affinity and specificity to membrane-bound receptors (Brown & Goldstein, 1974). The internalization of receptor-bound LDL results eventually in suppression of endogenous cholesterol synthesis as well as in an activation of microsomal cholesterol acyltransferase (acyl-CoA: cholesterol *O*-acyltransferase, EC 2.3.1.26; Goldstein *et al.*, 1974). Although this receptor-mediated internalization of LDL has been proposed as the main route for cellular uptake of cholesterol, it is known that LDL apoproteins and free cholesterol display different rates of cellular and tissue uptake both *in vitro* and *in vivo* (O'Malley *et al.*, 1981; Stender, 1982).

Fibroblasts from patients with FH (homozygotes) do not possess functional receptors for LDL (Goldstein & Brown, 1974). These FH fibroblasts cannot internalize LDL by the high-affinity pathway and are supposed to maintain their cholesterol balance mainly by endogenous synthesis of cholesterol. These cells should, however, be able to incorporate LDL free cholesterol

by physical exchange without concomitant LDL-particle incorporation. There has been a dispute about whether exchangeable free cholesterol is available for intracellular esterification in FH fibroblasts (Goldstein *et al.*, 1974; Shireman & Remsen, 1982).

The object of the present study was to examine the incorporation and metabolism of exogenous free [^3H]cholesterol by LDL-receptor-negative fibroblasts from a patient with FH. We have used both cholesterol/phosphatidylcholine vesicles (1:1 molar ratio) and normal human LDL as free-cholesterol donors to allow a comparison with results from previous studies (e.g. Slotte *et al.*, 1984; Shireman & Remsen, 1982).

Experimental

Cell culture

Human LDL-receptor-negative FH fibroblasts (GM 1915) were obtained from the Human Mutant Cell Repository (Camden, NJ, U.S.A.). This cell line has been shown to be LDL-receptor-negative by Dr. J. L. Goldstein of Dallas, TX, U.S.A. The cells were maintained as cell monolayers in 75 cm² Falcon tissue-culture flasks in a growth medium containing William's E-medium and Ham's F-12 (1:1, v/v; GIBCO Europe, U.K.) with 5% human donor whole blood serum and 50 µg of neomycin/ml. The cells were used between passages 7 and 15. Human lung fibroblasts were cultivated as described previously (Slotte & Björkerud, 1984).

Abbreviations used: LDL, low-density lipoproteins; FH, familial hypercholesterolaemia; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

† To whom reprint requests should be addressed. Present address: Department of Biochemistry and Pharmacy, Åbo Akademi, SF-20500 Turku 50, Finland.

Lipid vesicles

Lipid vesicles containing a 1:1 molar ratio of [^3H]cholesterol (47 Ci/mmol, diluted to 3.9 Ci/mol; Amersham International, Amersham, Bucks., U.K.) and egg phosphatidylcholine (Sigma Chemical Co., St. Louis, MO, U.S.A.) with 0.1% butylated hydroxytoluene as anti-oxidant were prepared by freeze-drying together and sonication in phosphate-buffered saline (pH 7.4) as described previously (Slotte *et al.*, 1984).

Isolation of LDL

LDL was isolated from fresh pooled human serum by preparative density (KBr)-gradient ultracentrifugation ($1.025 < d < 1.055$) in the presence of 0.02% NaN_3 , 1 mM-EDTA and 0.2 mM-phenylmethanesulphonyl fluoride. Isolated LDL was dialysed extensively against phosphate-buffered saline. The purity of the preparation was checked by analytical agarose-gel electrophoresis and Sepharose CL-4B (Pharmacia, Uppsala, Sweden) gel filtration. The preparation was sterilized by filtration (0.45 μm pore size) before use.

^3H -labelled LDL

[^3H]Cholesterol (30 μCi ; 47 Ci/mmol) was adsorbed on to the wall of a sterile glass vial by evaporating the solvent under N_2 . Then 5 mg of sterile LDL in phosphate-buffered saline was added to the vial in a volume of 5 ml, and the exchange process was allowed to proceed for 48 h at 4°C in the dark. After filtration (0.22 μm pore size), the recoveries of input radioactivity, LDL protein and LDL free cholesterol were determined. The specific radioactivity of LDL free [^3H]cholesterol was about 1.7 Ci/mol.

[^3H]Cholesterol uptake and esterification by cells

All experiments were performed in 60 mm-diameter Petri dishes with about 250 μg of cell protein per dish (5–6 days after seeding). Before incubation, the complete growth medium was replaced by an incubation medium (Ham's F-12) containing 50 μg of neomycin/ml and 20 mM-Hepes buffer (pH 7.4). Then 100 μg of vesicle [^3H]cholesterol or 75 μg of LDL protein was added to temperature-equilibrated dishes with an incubation volume of 3 ml. When the incubation medium contained 7-oxocholesterol or progesterone, the cells were preincubated for 1 h in the presence of these steroids before vesicle or LDL [^3H]cholesterol was added. The steroids were added to the incubation medium in ethanol (final ethanol concn. < 1%). Control cells received ethanol only. After completion of incubation, the cell monolayers were washed three times with chilled phosphate-buffered saline and detached by gentle

scraping in 1.0 ml of 0.05% sodium dodecyl sulphate solution. Samples of the solution were taken for protein determination, for measurement of total cellular radioactivity and for lipid extraction.

Analytical procedures

Free and esterified [^3H]cholesterol was extracted and separated as described previously (Slotte *et al.*, 1984). Cell protein was determined by the modified Lowry method (Markwell *et al.*, 1978). LDL cholesterol mass was determined by the enzymic method of Gamble *et al.* (1978).

Results and discussion

The movement of unesterified [^3H]cholesterol between uncharged lipid vesicles and normal human lung fibroblasts has been examined (Slotte *et al.*, 1984; Slotte & Björkerud, 1984). In the present study we have measured the incorporation of free [^3H]cholesterol from either cholesterol/phospholipid vesicles (1:1 molar ratio) or human LDL by cultured LDL-receptor-negative FH fibroblasts.

The incubation of FH fibroblasts with 33 μg of vesicle [^3H]cholesterol/ml led to a linear incorporation of [^3H]cholesterol into the cells during the 25 h incubation period (Fig. 1a). About 0.24 μg of vesicle [^3H]cholesterol was incorporated/h per mg of cell protein. Both the time course and the amount of vesicle [^3H]cholesterol incorporated by the FH fibroblasts are similar to the corresponding values found for normal human lung fibroblasts (Slotte & Björkerud, 1984; Slotte *et al.*, 1984). Since the same substrate was used with both cell types, it is obvious that the presence or absence of LDL receptors on the cell surface does not affect the rate of cholesterol exchange between lipid vesicles and cell membranes. This finding is consistent with the physico-chemical nature of the cholesterol-exchange process.

When FH fibroblasts were incubated with 25 μg of LDL protein/ml containing [^3H]cholesterol (free [^3H]cholesterol concn. about 9 μg /ml), about 0.23 μg of free [^3H]cholesterol was incorporated/h per mg of cell protein (Fig. 2a). Under similar experimental conditions normal human lung fibroblasts incorporated about 0.39 μg of LDL free [^3H]cholesterol/h per mg of cell protein. From these data it can be concluded that the LDL receptor is responsible for less than 40% of the LDL-free-cholesterol uptake by lung fibroblasts.

Addition of 7-oxocholesterol to the incubation medium led to an enhanced incorporation of exogenous vesicle [^3H]cholesterol (Fig. 1a). The same effect was also seen when LDL was the donor of exogenous [^3H]cholesterol (Fig. 2a). The in-

Fig. 1. Incorporation (a) and esterification (b) of vesicle [^3H]cholesterol by FH fibroblasts

Confluent FH fibroblasts (about 250 μg of cell protein per dish) were incubated in 3 ml of Ham's F-12 incubation medium with (●, ■) or without (○, □) 12.5 μM -7-oxocholesterol. Then 100 μg of vesicle [^3H]cholesterol was added and the incubation was stopped at various time intervals, as described in the Experimental section. The amounts of [^3H]cholesterol incorporated and [^3H]cholesteryl ester formed were calculated from the specific radioactivity of the vesicle [^3H]cholesterol. Values are the means of duplicates from two independent and representative incubations (range <10%).

Fig. 2. Incorporation (a) and esterification (b) of LDL free [^3H]cholesterol by FH fibroblasts

Confluent cells were incubated in 3 ml of Ham's F-12 incubation medium with (●, ■) or without (○, □) 12.5 μM -7-oxocholesterol. Then LDL (25 μg of protein/ml) was added and the incubation was stopped at various time intervals, as described in the Experimental section. The amounts of [^3H]cholesterol incorporated and [^3H]cholesteryl ester formed were calculated from the LDL free [^3H]cholesterol specific radioactivity. Values are the means of duplicates from a representative incubation (range <10%).

crease of [^3H]cholesterol incorporation is due to an increased rate of cholesterol exchange between extracellular [^3H]cholesterol donors and cellular plasma membranes. The increased exchange rate could be a result of the suppressed cholesterol synthesis and the subsequent decrease of the cellular cholesterol/phospholipid molar ratio that is caused by oxygenated derivatives of cholesterol such as 7-oxocholesterol and 25-hydroxycholesterol (Brown *et al.*, 1975; Kandutsch & Chen, 1977, 1978).

In their early studies on the metabolism of LDL by normal and LDL-receptor-negative fibroblasts, Goldstein *et al.* (1974) demonstrated that receptor-negative fibroblasts could esterify exogenous non-lipoprotein cholesterol provided that the LDL receptor could be by-passed. However, it has subsequently been claimed that FH fibroblasts (GM 1915) cannot esterify exogenous [^3H]chole-

sterol that is transferred to the cells from human LDL (Shireman & Remsen, 1982). The results shown in Figs. 1(b) and 2(b) demonstrate the esterification of vesicle- and LDL-derived [^3H]cholesterol respectively. The rate of esterification was rather low, but clearly observable, provided that the specific radioactivity of the exogenous [^3H]cholesterol was high enough.

The esterification of both vesicle- and LDL-derived [^3H]cholesterol was effectively stimulated by 7-oxocholesterol. Oxygenated steroids, such as 7-oxocholesterol and 25-hydroxycholesterol, have been shown to stimulate the activity of the microsomal enzyme cholesterol acyltransferase in different cell types (Drevon *et al.*, 1980; Bates *et al.*, 1983). Since the effects of 7-oxocholesterol on the activity of that enzyme have been measured with [^{14}C]oleic acid instead of [^3H]cholesterol, we found it necessary to characterize further the 7-oxocholesterol-stimulated [^3H]cholesterol esterifi-

Table 1. *Characteristics of incorporation and esterification of LDL free [³H]cholesterol by FH fibroblasts in the presence of 7-oxocholesterol*

The cells were incubated for 9 h in 3 ml of Ham's F-12 medium with 20 mM-Hepes buffer, 50 µg of neomycin/ml, 12.5 µM-7-oxocholesterol, 25 µg of LDL protein/ml and the indicated amounts of either progesterone or NaF. Values are means ± S.D. (*n* = 4). Significance was determined by Student's *t* test; ns, not significant.

Drug added	[³ H]Cholesterol incorporation (% of control)	[³ H]Cholesteryl ester formation (% of control)
—	100 ± 8	100 ± 5
Progesterone (80 µM)	106 ± 6 (ns)	22 ± 8 (<i>P</i> < 0.01)
NaF (0.02 M)	105 ± 13 (ns)	69 ± 3 (<i>P</i> < 0.01)

cation. Progesterone is known to be a potent inhibitor of cholesterol acyltransferase in many cell types (Goldstein *et al.*, 1978; Brown *et al.*, 1980; Lichtenstein & Brecher, 1983). Progesterone (80 µM) in the present study effectively counteracted the effect of 7-oxocholesterol on the esterification of LDL-derived [³H]cholesterol (Table 1). The 7-oxocholesterol-stimulated esterification of LDL [³H]cholesterol was also sensitive to the metabolic inhibitor NaF (at 0.02 M). These results suggest that the 7-oxocholesterol-stimulated esterification of exogenously derived [³H]cholesterol was catalysed by the energy-requiring cholesterol acyltransferase reaction. Characterization of the unstimulated esterification of [³H]cholesterol was complicated by the low esterification activity, and was not performed. Neither progesterone nor NaF had any significant effect on the 7-oxocholesterol-induced enhancement of the [³H]cholesterol incorporation by the FH fibroblasts.

The results of this study demonstrate the ability of FH fibroblasts to incorporate exogenous free cholesterol by physical exchange from both membrane structures (vesicles) and LDL. Irrespective of the source, incorporated free cholesterol was available for esterification in the FH fibroblasts, although the unstimulated esterification activity was rather low. The biological implication of these results is that, although FH fibroblasts do not possess high-affinity receptors for LDL, the cells can incorporate LDL free cholesterol by physical exchange. This cholesterol exchange contributes to the cellular cholesterol metabolism, since exchangeable cholesterol is available for esterification by cholesterol acyltransferase.

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