Selective uptake of cholesteryl esters of low-density lipoproteins is mediated by the lipoprotein-binding site in HepG2 cells and is followed by the hydrolysis of cholesteryl esters

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The study described in this paper shows that ^{125}I -labelled low-density lipoproteins (LDL) interact with high- and low-affinity binding sites on human hepatoma (HepG2) cells. The former site is the LDL receptor and the latter is the lipoprotein-binding site (LBS). The association of ^{125}I -labelled LDL and [^3H]cholesteryl ethers-LDL with HepG2 cells revealed a 4-fold selective uptake of cholesteryl esters (CE) in a 4 h incubation period, which correlated with the depletion of CE mass in LDL. This selective uptake was not observed when the cells were incubated in the presence of a 100-fold excess of high-density lipoprotein 3, conditions where only the LDL receptor is being monitored. Also, no reduction in uptake was observed in the presence of IgG-C7, an anti-LDL receptor) monoclonal antibody. Both findings indicate that the selective uptake occurs

through the LBS and that the LBS contributes more to the entry of CE from LDL into the cell than does the LDL receptor. The fates of CE entering the cell via the LDL receptor and the LBS were also followed. To achieve this, LDL were labelled with [^3H]cholesteryl oleate and the hydrolysis of [^3H]cholesteryl oleate was monitored. The results indicated that 45 % of the CE were hydrolysed after a 4 h incubation period, irrespective of the site of entry. Chloroquine (100 μM) was shown to inhibit hydrolysis, indicating that lysosomal enzymes were responsible for the hydrolysis of LDL-CE, whichever pathway was used. Thus our results reveal, for the first time, that the mass of CE entering the cell via the LBS is substantial and that hydrolysis of CE is by lysosomal enzyme activity. Overall, this suggests that the LBS has significant physiological importance.

INTRODUCTION

Lipoproteins are synthesized by the liver and the intestine. Once in the circulation, lipoproteins may remove cholesterol from cells or deliver cholesterol, in the form of cholesteryl esters (CE), to the cells. When this last phenomenon is accomplished without uptake of the complete lipoprotein particle, selective uptake of CE is suggested. Many studies emerging from different laboratories have shown that different cells can selectively take up CE from high-density lipoprotein (HDL) (reviewed in [1]). Two reports have revealed that the uptake of CE from HDL into the cell is followed by extra-lysosomal hydrolysis of the CE, which involves neutral CE hydrolase activity [2,3]. It has been reported that liver selectively takes up CE from low-density lipoproteins (LDL) [4], and it was shown that human hepatoma (HepG2) cells can selectively take up CE from intermediate-density lipoproteins (IDL) [5]. Rinninger et al. [6] reported that LDL-CE was selectively taken up by liver plasma membranes. More recently we have shown that HDLs and free apo-lipoprotein (apo)-A1 abolish the selective uptake of CE from IDL [7]. This suggests that the lipoprotein-binding site (LBS) of HepG2 cells is responsible for the selective uptake of CE from IDL.

The title LBS was first introduced by Bachorik et al. in 1978 [8] after they discovered that porcine liver membranes bound ^{125}I -labelled LDL and that the binding was equally inhibited by LDL and HDL. Later, Ose et al. [9] showed that the binding of ^{125}I -labelled HDL to rat hepatocytes could be displaced by LDL, very-low-density lipoproteins (VLDL) and HDLs. Similar results were also reported for cultured porcine [10] and rabbit hepato-

cytes [11]. This lack of specificity was not confined to liver tissue but was also observed in adipocytes [12], testicular and ovarian cells [13]. Earlier, our contribution to this research was to define the specificity of the LBS [14,15]. We showed that the LBS binds all classes of lipoproteins by their apo-lipoprotein moiety. The observation that IDL, LDL, HDL3, apo-E liposomes, apo-A1 liposomes and apo-C liposomes could interact with the LBS, whereas liposomes containing no proteins, or containing proteins unrelated to the family of apolipoproteins, did not interact with the LBS [5,15] led to this conclusion. Thus the metabolic function of the LBS was involved with the selective uptake of CE from IDL [7] but did not contribute to apolipoprotein degradation [5]. Our work on rat-liver membranes [16] has revealed that the LBS is not sensitive to heparinase 1, chondroitinase ABC or phospholipase C, it is moderately affected by phospholipase A2, but it is sensitive to heat and to protease activity. We have shown also that proteins solubilized from rat-liver membranes possessed LBS-binding activity when reconstituted into liposomes, suggesting that the LBS is a protein. The use of the ligand-blot technique to isolate the LBS and estimate its molecular mass was unsuccessful [17]. We rationalized that either the moderate affinity of the LBS for its ligands or the importance of membrane lipids in determining an active LBS protein structure could be responsible for this failure.

The first aim of the present study was to conduct a thorough analysis of the association of LDL with, or their degradation by, HepG2 cells in order to establish clearly if the selective uptake of CE from LDL occurs in these cells. LDL were particularly chosen since, in contrast to IDL, they are abundant in the circulation

Abbreviations used: apo, apolipoprotein; CE, cholesteryl esters; CEt, cholesteryl ethers; FBS, fetal bovine serum; FC, free cholesterol; FH, a homozygous, LDL-receptor-deficient, familial hypercholesterolaemia fibroblast strain; HDL, high-density lipoprotein; IDL, intermediate-density lipoproteins; LBS, lipoprotein-binding site; LDL, low-density lipoproteins; LPDS, lipoprotein-depleted serum; LRP, LDL-receptor-related protein; MEM, minimal essential medium; VLDL, very-low-density lipoproteins.

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and are positively associated with heart disease. Furthermore, LDL, in contrast to IDL, do not interact with receptors such as the LDL-receptor-related protein (LRP) or VLDL receptors, thus simplifying our investigation. Our second goal was to determine the role of the LBS in the selective-uptake pathway and to determine if the CE derived from selective uptake were hydrolysed.

MATERIALS AND METHODS

Materials

Human blood (3–5 days old) was from the Montreal Transfusion Centre of the Canadian Red Cross. The human hepatoma cell line (HepG2) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Fetal bovine serum (FBS) was from Gibco Laboratories (Burlington, Ontario, Canada). 1,2-[³H]cholesteryl oleyl ether (CET) (30–60 mCi/mmol), 1,2-[³H]cholesteryl oleate (CE) (30–60 mCi/mmol) and ¹²⁵I (as NaI, 100 mCi/ml) were from DuPont Canada (Mississauga, Ontario, Canada). [³H]CET and [³H]CE were found to be 99% pure by TLC. Fatty-acid-free or standard BSA (fraction V), trypsin, heparin, PMSF and silica-gel G TLC plates were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The IgG-C7 antibody was a gift from Dr. Ross Milne (Heart Institute, Ottawa, Ontario, Canada).

Preparation of lipoproteins

Blood cells were precipitated by low-speed centrifugation, and 0.01% (w/v) EDTA, 0.02% (w/v) sodium azide and 10 μ M PMSF were added to the plasma before isolation of lipoproteins by ultracentrifugation as described by Hatch and Lees [18]. LDL (density 1.025–1.05 g/ml) and HDL3 (density 1.125–1.21 g/ml) were prepared as described by Brissette et al. [15]. Apo-E was not detected in either lipoprotein. IDL were obtained as described by Brissette and Falstra [5].

Radiolabelling of LDL

LDL were iodinated by a modification [19] of the ICl method of McFarlane [20]. Na¹²⁵I (1 mCi) was added to 2.5 mg of LDL in the presence of ICl (30 nmol) in 0.5 M glycine/NaOH, pH 10. Free iodine was removed by gel filtration on Sephadex G-25 followed by an overnight dialysis in Tris-buffered saline. The specific radioactivity ranged from 36 000 to 331 000 c.p.m./ μ g of protein.

LDL were labelled with [³H]CET or [³H]CE. CET is a tracer for CE, and has the advantage of not being hydrolysed intracellularly and thus is an indicator of selective uptake of CE. [³H]CE was used for following the hydrolysis of CE. Lipoproteins were labelled by the method of [21]; briefly, the solvent from 25 μ Ci of [³H]CET or [³H]CE was evaporated under nitrogen and the residue was resuspended in 250 μ l of acetone. The acetone suspension was added dropwise to 3 ml of lipoprotein-depleted serum (LPDS) in a glass tube at room temperature, and the acetone was evaporated under a gentle stream of nitrogen. LDL (1 ml) at a concentration of 1 mg of protein/ml were added to the [³H]CET- or [³H]CE-labelled LPDS, and the mixture was incubated for 30 min at 4 °C. The labelled LDL were isolated by ultracentrifugation [14]. The specific radioactivity of CE-labelled LDL ranged from 7000 to 44 000 c.p.m./ μ g of LDL protein. In order to verify that the label was incorporated into LDL, labelled LDL (20 μ g/ml) were incubated with 100 μ g of *Escherichia coli* membrane (which is devoid of lipoprotein receptors) at 37 °C for 4 h. The membranes were recovered, and the radioactivity was

measured. No significant radioactivity was found in these membranes.

Cell culture

HepG2 cells were grown in 75 cm² flasks containing 15 ml of Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) FBS, penicillin (100 units/ml), streptomycin (100 μ g/ml) and glutamine (4 mM), as previously described [22]. At 3 days before the binding, association or protein-degradation studies, 4.5×10^5 cells were seeded in 3.8 cm² culture dishes (12-well dishes). For hydrolysis of CE, 20 cm² dishes were used and 3.9×10^6 cells were seeded. In all cases, the medium was changed every 2 days. A homozygous LDL-receptor-deficient, familial hypercholesterolaemia fibroblast strain (FH), obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.), was maintained and cultured as described for HepG2 cells except that 3×10^4 cells were seeded in 3.8 cm² tissue-culture dishes.

Binding assay

The binding assays were conducted on cells that were approx. 90% confluent. The cells were washed twice with PBS (1 ml). Cells were incubated for 4 h at 4 °C with ¹²⁵I-labelled LDL (0–50 μ g of protein/ml) in 125 μ l of MEM containing 4% fatty acid-free BSA and 50 mM Hepes, pH 7.4, in a total volume of 250 μ l (total binding). All lipoprotein fractions were maintained in PBS. Non-specific binding was determined by the addition of 1 mg of protein/ml unlabelled LDL. At the end of the incubation period, the cell monolayers were washed twice with PBS (1 ml) containing 1% BSA (PBS/BSA) followed by two washes with 1 ml of PBS. The cells were then solubilized in 1.5 ml of 0.1 M NaOH, the protein content was determined [23] using BSA as a standard, and the radioactivity was measured (Cobra II counter; Canberra-Packard).

Specific binding was calculated by subtracting the non-specific binding of ¹²⁵I-labelled LDL to HepG2 cells from the total binding counts. The curves generated by the specific-binding data were transformed into plots of the ratio of cell-bound to free ¹²⁵I-labelled LDL versus cell-bound ¹²⁵I-labelled LDL [24]. The ordinate of the Scatchard plots (bound/free) represents the amount of specifically bound ligand (μ g of protein/mg of cellular protein) divided by the concentration of unbound ligand in the reaction mixture (μ g of protein/ml). Concave-upward curvilinear Scatchard plots were resolved by the method of Rosenthal [25] or with Ligand software (Biosoft, Ferguson, MO, U.S.A.).

Cell association and degradation assays

The association of ¹²⁵I-labelled LDL with HepG2 cells was measured after incubation at 37 °C for 4 h, as described for the binding studies, but without Hepes. At the end of the incubation period, the dishes were put on ice, the medium was removed, and the cells were washed twice with 1 ml of PBS/BSA and twice with 1 ml of PBS. Association data were obtained by estimation of the radioactivity in the washed cells that had been resuspended in 0.1 M NaOH. The results are expressed as μ g of LDL protein/mg of HepG2 cell protein. The association of [³H]CET-LDL and of [³H]CE-LDL was determined by delipidation *in situ* with hexane/isopropanol (3:2, v/v) [22] followed by measurement of radioactivity using a beta-counter (Wallack; Fisher Scientific). In order to compare the association of LDL label in protein (¹²⁵I) or in CE (³H), the association data of [³H]CE-LDL or [³H]CET-LDL were estimated (μ g of LDL protein associated/mg of HepG2 cell protein). To achieve this, the specific radioactivity of

[^3H]Cet-LDL and [^3H]CE-LDL was established in c.p.m./ μg of LDL-protein. Selective uptake is demonstrated when the ratio of the association of [^3H]Cet-LDL/ ^{125}I -labelled LDL or [^3H]CE-LDL/ ^{125}I -labelled LDL is greater than unity.

The degradation of the protein of ^{125}I -labelled LDL was estimated from the medium. Essentially, trichloroacetic acid was added to the medium at a final concentration of 12% (w/v), and the protein content of the trichloroacetic acid fraction was measured. Controls without cells accounted for free ^3H . In order to determine the level of internalization and degradation of CE from LDL, [^3H]CE-LDL was incubated at 37°C for 4 h and [^3H]CE-LDL associated with HepG2 cell membranes was removed by incubation with heparin (2.5 mg/ml) for 2×10 min (heparin has been shown to abolish LDL binding [16]). The cells, on ice, were washed twice with 1 ml of PBS/BSA and twice with 1 ml of PBS. The lipids of the cell monolayer were extracted twice into 1 ml of hexane/isopropanol (3:2; v/v). The solvent was evaporated under a stream of nitrogen, the residue was resuspended in 100 μl of chloroform and applied on to a silica-gel G plate and separated by TLC using light petroleum/diethyl ether/acetic acid (90:10:1; by vol.) as eluant. The areas on the plate corresponding to free cholesterol (FC) and CE, identified by exposure to iodine vapour, were removed and the radioactivity was measured (beta-counter).

Effect of incubation with HepG2 cells on LDL composition

Subconfluent HepG2 cells in 20 cm^2 dishes were incubated at 37°C for 4 h with 300 μg of LDL protein/ml in MEM containing 4% (w/v) BSA. The conditioned medium was removed, adjusted to a density of 1.063 g/ml with NaBr and centrifuged to isolate LDL [15]. The LDL lipids were extracted according to the method of Folch et al. [26] and separated by TLC as described

above. FC and CE were recovered and quantified by the method of Franey and Amador [27].

RESULTS

Our goal was to determine accurately if there is a selective uptake of LDL-CE into HepG2 cells and eventually to elucidate the pathway. First, the binding of ^{125}I -labelled LDL to HepG2 cells was studied as a function of time in order to choose an incubation time reflecting equilibrium. Figure 1 shows that equilibrium was achieved after 2 h incubation at 4°C . With this knowledge, a 4 h incubation time was chosen for LDL saturation binding studies in which the specific binding of ^{125}I -labelled LDL added to the medium at various concentrations was analysed. Figure 2 shows that the specific binding of ^{125}I -labelled LDL to HepG2 cells

Figure 1 Kinetics of association of ^{125}I -labelled LDL with HepG2 cells in the absence or presence of human HDL3

^{125}I -labelled LDL at a concentration of 3.5 $\mu\text{g}/\text{ml}$ (■) or at the same concentration but including a 100-fold excess of HDL3 (▨) or at a concentration of 20 $\mu\text{g}/\text{ml}$ (□) were incubated at 4°C for 0.25, 1, 2, 3.5 or 5 h. The total binding was then determined. For each total-binding estimation, non-specific binding was measured by adding 1 mg/ml unlabelled LDL and the specific binding was calculated by subtracting the non-specific binding from the total binding. The error bars represent the S.E.M. of a minimum of three different experiments. 1, Statistically different ($P < 0.05$) from the results obtained with 3.5 $\mu\text{g}/\text{ml}$ LDL without HDL3. 2, Statistically different ($P < 0.05$) from the results obtained for the previous period of incubation. Abbreviation: prot, protein.

Figure 2 Specific binding of ^{125}I -labelled LDL to HepG2 cells in the absence or presence of either human HDL3 or EDTA

(A) Saturation curves of the specific binding of ^{125}I -labelled LDL to HepG2 cells in the absence (□) or presence (△) of a 100-fold excess of HDL3 protein relative to the amount of ^{125}I -labelled LDL in each well, or in the presence of 5 mM EDTA (○). To measure binding under these conditions, ^{125}I -labelled LDL (0–50 μg of protein/ml) were incubated at 4°C for 4 h with HepG2 cells. The non-specific binding was measured by adding 1 mg of protein/ml of unlabelled LDL, and the specific binding was calculated by subtracting the non-specific binding from the total binding. (B) Scatchard plots of the specific-binding data. These data are representative of at least three separate experiments each done in duplicate giving essentially the same results. Abbreviation: prot, protein. $1\text{E}-4$, 10^{-4} .

Table 1 Binding parameters of the two affinity components of the specific binding of ^{125}I -labelled LDL to HepG2 cells in the presence of either competitor lipoproteins or EDTA

The assay of ^{125}I -labelled LDL binding to HepG2 cells was carried out as described in the Materials and methods section. Briefly, ^{125}I -labelled LDL (0–50 μg of protein/ml) were incubated with HepG2 cells at 4 °C for 4 h. Unlabelled LDL (1 mg of protein/ml) was added and the specific binding was calculated by subtracting the non-specific binding from the total binding. K_d and B_{max} were determined from Scatchard plots of the specific binding data. Competitor lipoproteins were added to the binding assay as indicated (excess protein of competitor lipoproteins relative to ^{125}I -labelled LDL protein). Each value represents the mean $\pm$ S.E.M. The number of experiments is indicated in parentheses. N.D., not detected because the Scatchard plot became linear. N.M., not measurable because all specific binding was abolished.

Incubation conditions	High-affinity component (LDL receptor)		Low-affinity component (LBS)	
	K_d (μg of protein/ml)	B_{max} (μg of protein/mg of cell protein)	K_d (μg of protein/ml)	B_{max} (μg of protein/mg of cell protein)
Control (5)	1.4 ± 0.51	0.0084 ± 0.0019	79.4 ± 37.3	0.088 ± 0.021
100-Fold excess of HDL3 (3)	5.7 ± 2.1	0.016 ± 0.001	N.D.	N.D.
25-Fold excess of IDL (2)	N.M.	N.M.	N.M.	N.M.
5 mM EDTA (3)	N.D.	N.D.	80.6 ± 11.6	0.040 ± 0.005

(Figure 2A) yielded a curvilinear Scatchard plot representative of two binding sites: high- and low-affinity binding sites (Figure 2B). The binding parameters shown in Table 1 indicate that the affinity of ^{125}I -labelled LDL for the low-affinity binding site was 56 times lower than for the high-affinity site, and that its binding capacity was 10 times higher. When the binding of ^{125}I -labelled LDL was assessed in the presence of a 100-fold excess of human HDL3 or of 5 mM EDTA, less ^{125}I -labelled LDL was bound (Figure 2A) and linear Scatchard plots were obtained (Figure 2B). The binding parameters obtained in the presence of HDL3 (Table 1) were similar to the high-affinity binding parameters determined under control conditions (without HDL3), while those obtained in the presence of EDTA resembled the parameters of the low-affinity site. Figure 1 also shows that although less binding of ^{125}I -labelled LDL was observed in the presence of HDL3, as expected from the results shown in Figure 2, equilibrium was not modified by the inclusion of HDL3 in the binding assay. Similar results were obtained in the presence of EDTA (results not shown). Therefore it was concluded that a 100-fold excess of HDL3 abolished the binding of ^{125}I -labelled LDL to the low-affinity site whereas 5 mM EDTA inhibited binding to the high-affinity site. Interestingly, a 25-fold excess of IDL completely abolished the binding, indicating that both the high- and low-affinity sites can interact with IDL (Table 1). The sensitivity to EDTA and IDL and the insensitivity to HDL3 observed for the high-affinity site are indicators for the LDL receptor [28], whereas the insensitivity to EDTA and the sensitivity to HDL3 and IDL of the low-affinity site revealed the presence of the LBS [5,16]. Thus human LDL bind to both the LDL receptor and the LBS on HepG2 cells.

Our next step was to determine if the association of LDL with HepG2 cells was followed by selective uptake of CE from LDL by the cells. Selective uptake is suggested when more CE than apolipoprotein enters the cell and is established by the ratio of the two associations. To answer this question, either the protein moiety of LDL was labelled with ^{125}I or the LDL-CE were labelled with ^3H Cet. Neither labelling method modified the binding ability of the ligand (LDL) at 4 °C, a temperature at which no catabolism occurs following binding (results not shown). The association of each label with HepG2 cells was measured at 37 °C, a temperature at which events such as internalization or degradation can follow binding of LDL to the cell surface. Again, a 4 h incubation was chosen since ^{125}I -labelled LDL association was at equilibrium at this time point

Figure 3 Effect of human HDL3 on the specific association of ^{125}I -labelled LDL and ^3H Cet-LDL with HepG2 cells

Shown is the specific association of ^{125}I -labelled LDL to HepG2 cells in the absence ($\circ$) or presence ($\bullet$) of a 100-fold excess of human HDL3 protein relative to the amount of ^{125}I -labelled LDL in each well, and specific association of ^3H Cet-LDL determined under the same conditions in the absence ($\square$) or presence ($\blacksquare$) of a 100-fold excess of human HDL3. Association was measured as described in the Materials and methods section. Briefly, labelled LDL (0–50 μg of protein/ml) were incubated at 37 °C for 4 h with HepG2 cells. The non-specific association was measured by adding 1 mg of protein/ml of unlabelled LDL, and the specific association was calculated by subtracting the non-specific association from the total association. These data are representative of three separate experiments each done in duplicate and giving essentially the same results. Abbreviation: prot, protein.

(results not shown). The association curves of LDL, labelled in either protein or in CE shown in Figure 3, demonstrate that the apparent uptake of ^3H Cet-LDL is approx. 4-fold greater than the uptake of ^{125}I -labelled LDL. This may reflect either a true, selective uptake of CE from LDL or a large degradation of the apo-lipoproteins from LDL. To verify this, degradation of LDL protein was measured and its effect on selective uptake was evaluated. Table 2 shows that less LDL protein was degraded than was associated and that allowing for degradation of LDL protein in the calculation did not significantly change the estimation of selective uptake of CE into the cells. Determination of the selective uptake of CE by measurement of ^3H Cet association with the cell may reflect either a true transfer of CE

Table 2 Analysis of LDL association, degradation and selective uptake of CE in the absence or presence of a 100-fold excess of HDL3

HepG2 cells were incubated for 4 h at 37 °C with either 10 µg/ml of ¹²⁵I-labelled LDL or [³H]CEt-LDL and the association of LDL-protein or CEt with the cells was measured as described in the Materials and methods section. The degradation of LDL-protein was determined from the medium. In all cases, incubations were conducted in parallel with 1 mg/ml unlabelled LDL to determine the level of non-specific association or degradation. The measurements were also done on cells that had been incubated with 1 mg/ml HDL3, a quantity that represents a 100-fold excess of the ligand. The values in the Table represent the means ± S.E.M. of three experiments that were conducted in duplicate. ^a*P* < 0.025 from the results obtained in the absence of HDL3. ^b*P* < 0.005 from the results obtained in the absence of HDL3.

	—HDL3	HDL3 (100-fold excess)
Protein association (µg of LDL protein/mg of cell protein)	0.049 ± 0.0020	0.052 ± 0.0092
Protein degradation (µg of LDL protein/mg of cell protein)	0.028 ± 0.045	0.041 ± 0.015
CE association (µg of LDL protein/mg of cell protein)	0.57 ± 0.15	0.060 ± 0.015 ^a
Selective uptake of CE (CE association/protein association)	7.07 ± 0.98	1.12 ± 0.16 ^b
Selective uptake corrected for protein degradation (CE association/protein association and degradation)	5.23 ± 1.07	1.10 ± 0.40 ^a

Table 3 Effect of incubation with HepG2 cells on the composition of LDL

LDL (300 µg) were incubated with or without HepG2 cells (60-mm-diam. dish) at 37 °C for 3 h. LDL were isolated from the medium by ultracentrifugation. Each experiment was conducted in duplicate or triplicate. The protein, FC and CE contents of LDL samples were determined. Results are expressed as means ± S.E.M. of three experiments. ^aStatistically different (*P* < 0.05) from the controls without HepG2 cells.

	CE mass (µg)	FC mass (µg)	Protein mass (µg)	CE/protein	FC/protein
Without HepG2 cells	1050 ± 144	160.7 ± 25.4	362 ± 95.5	2.9	0.44
With HepG2 cells	507 ± 37 ^a	260 ± 31.0	375 ± 105.3	1.35	0.69

from LDL to the cell or an exchange between the [³H]CEt from LDL and the cell CE without a transfer of mass. An experiment was conducted to determine which of these two possibilities could account for our results; accordingly LDL were incubated with or without cells for 4 h at 37 °C, and the FC and CE concentrations of LDL contained in the medium at the end of the incubation period were determined. Table 3 shows that association with HepG2 cells substantially reduced the level of CE in LDL and thus the LDL-CE/protein ratio. To ensure that the loss of CE from LDL was not a result of the transfer of LDL-CE to other classes of lipoproteins synthesized and secreted by the cells, LDL were also incubated for 4 h in a 4-h-HepG2-cell-conditioned medium. We found no significant loss of CE from the LDL fraction under these conditions (results not shown). Thus it is clear that HepG2 cells selectively take up CE from LDL and that LDL are depleted in CE.

Next, we analysed the effect of a 100-fold excess of HDL3 on the association of LDL with HepG2 cells. Figure 3 shows that

Table 4 Association of LDL protein or LDL-CE with HepG2 cells under various conditions or with FH fibroblasts

Cells were pre-treated for 1 h with 10 mg/ml heparin and washed before the association assay, or were pre-treated with 40 µg/ml IgG-C7 for 1 h (the antibody was also present during the assay). Association was measured as described in the Materials and methods section, except that the concentration of LDL protein was 15 µg/ml. Each assay was done in duplicate or triplicate. The values shown are the means ± S.E.M. and the number of experiments are indicated in parentheses. ^aStatistically different (*P* < 0.05) from the control without HepG2 cells.

Cells	Conditions	LDL protein association (µg of LDL protein/ mg of cell protein)	LDL-CE association (µg of LDL protein/ mg of cell protein)	Selective uptake (CE/protein)
HepG2 cells	Control (5)	0.14 ± 0.012	0.35 ± 0.016	2.7 ± 0.22
	Heparin (5)	0.15 ± 0.011	0.47 ± 0.050	3.1 ± 0.32
	IgG-C7 (3)	0.069 ± 0.016 ^a	0.41 ± 0.043	6.5 ± 1.37 ^a
FH fibroblasts	Control (5)	0.077 ± 0.014 ^a	0.19 ± 0.049 ^a	2.5 ± 0.32

HDL3 significantly reduced the association of both ¹²⁵I-labelled LDL and [³H]CEt-LDL to a point where no selective uptake was observed. In Table 2 the association, degradation and selective uptake data between experiments conducted in the absence or in the presence of a 100-fold excess of HDL3 are compared. The results show that HDL3 had no significant effect on association or degradation of LDL protein but it significantly reduced the association of [³H]CEt-LDL to a level that completely eliminated the selective uptake of CE. Thus it can be deduced that the presence of HDL3 virtually abolished the selective uptake of CE from LDL. Moreover, as it is known that HDL3 impedes the binding of LDL to the LBS, the results suggest that LDL delivers CE to HepG2 cells by association with the LBS. Figure 3 therefore indicates that the LBS brings approx. five times more CE to the cell than does the LDL receptor. Noticeably, there was no major difference between LDL-protein degradation in the absence of or in the presence of HDL3 (Table 2), which indicates that the LBS does not participate significantly in the degradation of LDL proteins. This was predictable for a selective-uptake pathway. An experiment in which EDTA was used to demonstrate directly that selective uptake occurred via the LBS had to be abandoned because at 37 °C, EDTA detached HepG2 cells from the culture dishes.

The importance of the LBS in selective uptake of CE from LDL was confirmed by a direct approach using the monoclonal antibody IgG-C7 to impede or abolish the binding of LDL to the LDL receptor [29]. Table 4 shows that IgG-C7 diminished the association of LDL proteins by 51% but did not affect CE uptake. FH fibroblasts were also able selectively to take up CE from LDL with a similar CE/protein ratio to that found in HepG2 cells. It is, however, interesting to note that, overall, the association capability was only half that of HepG2 cells. These results show that the LDL receptor does not participate directly or indirectly in the selective uptake of CE from LDL. Along these lines, we also verified the possible implication of proteins, such as hepatic lipase, anchored to heparan sulphate chains of the membrane. This was done because it had been shown that hepatic lipase promotes the selective uptake of CE from HDL [30] and that lipoprotein lipase modulates the binding of lipoproteins to receptors such as the LRP [31]. Pre-treatment of the cells with heparin did not affect the association, as shown in Table 4, indicating that these enzymes are not involved in the CE selective-uptake process observed.

Table 5 LDL-CE hydrolysis by HepG2 cells

For cholesterol determination, 10 $\mu\text{g/ml}$ [^3H]CE-LDL were incubated at 37 °C for 4 h with HepG2 cells in the absence (total contribution) or in the presence of a 100-fold excess of human HDL3-protein, relative to the amount of [^3H]CE-LDL in each well (LDL-receptor contribution). The difference between the total and the LDL-receptor (LDLr) contributions represents the contribution of the LBS. In each case, unlabelled LDL were added (1 mg of protein/ml) and the specific association was calculated by subtracting the non-specific association from the total association. Following the incubation, cell lipids were extracted and separated by TLC, and the radioactivity incorporated into CE and FC was evaluated. CE hydrolysis was assessed as for the cholesterol determination except that cells were either not treated or were treated with 100 μM chloroquine. The results are the means $\pm$ S.E.M. The number of experiments are indicated in parentheses. ^aStatistically different ($P < 0.001$) from the respective total activity or LBS activity. ^bStatistically different ($P < 0.05$) from the hydrolysis observed in the absence of chloroquine.

	Cell cholesterol association (ng/mg of cell protein)		CE hydrolysis (%)	
	[^3H]CE	[^3H]FC	Without chloroquine	With chloroquine
Total activity	245.8 $\pm$ 32.0 (11)	217.7 $\pm$ 32.5 (11)	46.9 $\pm$ 3.5 (11)	9.7 $\pm$ 4.5 ^b (4)
LDLr activity	47.7 $\pm$ 18.1 ^a (17)	33.7 $\pm$ 8.1 ^a (17)	43.4 $\pm$ 4.1 (16)	2.9 $\pm$ 2.4 ^b (6)
LBS activity	219.8 $\pm$ 35.2 (9)	178.8 $\pm$ 33.1 (9)	38.8 $\pm$ 5.3 (9)	10.8 $\pm$ 4.5 ^b (4)

Our next step was to compare the pathway of CE entry into HepG2 cells via the LBS with entry by the LDL receptor pathway. As only the association of LDL with LDL receptors is measured in the presence of HDL3, the difference between the association determined in the absence and in the presence of HDL3 allows evaluation of the contribution of the LBS. The results given in Table 5 show that the LBS pathway contributes five times more than the LDL receptor pathway to entry of CE into the cell. For each pathway, however, the FC/CE ratio is approx. 0.75, which means that CE hydrolysis is about 45 %, and this was confirmed by conducting the experiment in the presence of IgG-C7 (results not shown). To determine if hydrolysis of CE entering the cell by the LBS occurred by a lysosomal process, such as for the LDL-receptor pathway [32], cells were incubated with 100 μM chloroquine, a lysosomotropic agent, and the percentage CE hydrolysis was measured. Table 5 shows that chloroquine had an important effect on CE hydrolysis; a 93 % reduction was observed in the CE contributed by the LDL receptor and a reduction of 72 % in the LBS-mediated CE uptake. These results indicate that CE hydrolysis occurs by a lysosomal pathway following LBS-mediated uptake.

DISCUSSION

To date, most of the studies analysing the interaction between human lipoproteins and receptors were done with human skin fibroblasts. Less work has been done with human hepatocytes for ethical and technical reasons. However, some interesting work on hepatic lipoprotein metabolism has been conducted with HepG2 cells, a hepatoma cell line that possesses many of the features of normal hepatocytes. It is important to understand the metabolism of lipoproteins by hepatic cells, since some aspects of the mechanism of cholesterol metabolism differ between fibroblasts and hepatocytes. Thus in contrast with peripheral tissues (fibroblasts), which rely on serum lipoproteins as a major source of cholesterol and regulate lipoprotein uptake by controlling LDL receptor levels, hepatic cholesterol homeostasis must take into account the dietary fat intake and the demand for cholesterol for synthesis of lipoproteins and bile acids. A further need for greater understanding of hepatic lipoprotein-receptor function is that lipoprotein clearance takes place mainly in the liver [33]. Important differences in regulation between fibroblasts and hepatocytes or hepatocyte-derived cells that have been reported are that normal hepatocytes and HepG2 cells are refractory to down-regulation of the LDL receptor by the presence of LDL in the culture medium [34] and that the LDL receptor mRNA

concentration in both fibroblasts and HepG2 cells responds similarly to culture and growth conditions, but that the translational and post-translational regulation differ [35].

One of our goals was to define more precisely the importance of the hepatic LBS in LDL-CE metabolism. In order to reduce the contribution of the LDL receptor as much as possible we chose to use HepG2 cells that were not incubated in LPDS, which, we believe, simulates the physiological conditions encountered by hepatic cells. The reason that LDL were chosen, and not IDL as used in earlier work [5,7], was that LDL are more abundant in plasma and consequently should be the physiological ligand of the LBS. Also, our consideration was based on the fact that the plasma concentration of CE from LDL is positively associated with higher risks of cardiovascular disease. Therefore the elucidation of a mechanism for depleting LDL of CE is physiologically important.

The thorough analysis of the association/degradation of LDL by HepG2 cells presented in this paper clearly showed that the LBS of HepG2 cells are able to selectively take up the CE from LDL. To our knowledge, this is the first report of direct selective uptake of CE from LDL by hepatocytes such as HepG2 cells. Furthermore, if CE uptake by the cell via the LBS and the LDL receptor pathways are compared, it becomes clear that the LBS contribution is by far the most important. This was supported by our demonstration that LDL were depleted in CE following incubation with HepG2 cells. Should we now reconsider the monopoly of the LDL receptor to internalize CE from LDL? However, entry of CE into the cell by way of the LBS may not be beneficial if the cell cannot further process the CE. Our results showed that the CE are indeed hydrolysed within the cell at approximately the same rate as the CE entering the cell by the LDL-receptor pathway. We acknowledge that the concentration of CE is only apparent, as it may also reflect the re-esterification of free cholesterol by acyl coA:cholesterol acyl transferase and/or the secretion of CE, presumably in lipoprotein form, into the medium. More information is required to elucidate these possible pathways.

The importance of lysosomal enzymes in CE hydrolysis was also investigated. We found that chloroquine inhibited both LDL-receptor- and LBS-mediated CE hydrolysis, which demonstrated that hydrolysis of CE derived from LDL occurred within lysosomes and was independent of the entry pathway. Although it has been known for many years that degradation of LDL entering the cell via the LDL receptor occurs within lysosomes [32], this is the first report that degradation of LDL entering the cell by the LBS pathway also takes place within lysosomes. This

clearly differentiates the LBS from the receptor involved in the selective uptake of CE from HDL, as CE hydrolysis following entry by the latter route is extralysosomal [2,3]. However, it was interesting to note that the inhibition of CE hydrolysis by chloroquine was greater for CE entering the cell by the LDL receptor than by the LBS pathway (93 % versus 72 % respectively). This may reflect either different kinetics of CE hydrolysis between the two pathways or extralysosomal hydrolysis of LDL-CE derived from the LBS pathway. This is currently being investigated in our laboratory.

In the past, our results [5,14,15] have indicated that the LBS may interact with all classes of lipoproteins. However, taking into account the moderate affinity of the LBS for lipoproteins, we propose that physiologically, the LBS would interact preferentially with LDL and HDL, the most abundant classes of plasma lipoproteins. Thus the hepatic LBS would play a very valuable role in depleting circulating LDL of CE. Also, the LBS could benefit the selective uptake of CE from HDLs when the HDL receptors are no longer sufficient for this task. Both of these pathways provide valuable means of reducing blood cholesterol and hence the incidence of heart disease.

In conclusion, this work shows that LDL are able to selectively deliver their CE to HepG2 cells. Additionally we have demonstrated that this selective uptake is mediated by the LBS, and that CE are hydrolysed in the cell by a lysosomal pathway. Altogether this reveals that the LBS may very well have an important physiological role.

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