Effect of reduced low-density lipoprotein receptor level on HepG2 cell cholesterol metabolism

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Low-density lipoproteins (LDL) are taken up by both LDL receptor (LDLr)-dependent and -independent pathways. In order to determine the importance of these pathways in the activity of the various enzymes that are important in maintaining the cellular cholesterol level in hepatic cells, we created HepG2 cells expressing lower levels of LDLr. Thus HepG2 cells were transfected with a constitutive expression vector (pRc/CMV) containing a fragment of *LDLr* cDNA inserted in an antisense manner. Stable transformants were obtained that showed significant reductions of 42, 72 and 85% of LDLr protein levels compared with the control, as demonstrated by immunoblotting and confirmed by the LDL binding assay. The best inactivation was achieved with the construct containing the first 0.7 kb of *LDLr* cDNA. Incubating the different HepG2 cell subtypes with LDL showed similar association of apolipoprotein B (apo B) or cholesteryl esters from LDL with the cells, indicating that the LDLr deficiency did not significantly affect LDL uptake by the

cell. However, apoB degradation was reduced significantly by 71–82% in the most LDLr-deficient HepG2 cells. We also found that 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA red) activity is significantly increased by 32–35% in HepG2 cells expressing very low levels of LDLr that also demonstrate a significant decrease of 20% in acyl-CoA:cholesterol acyltransferase (ACAT) activity. However, these effects are moderate compared with those observed when cells were incubated in lipoprotein-depleted medium, where a >900% increase in HMGCoA red activity and a loss of 60% of ACAT activity was observed. Thus, in HepG2 cells, different levels of LDLr affect LDL-apoB degradation, but have very little effect on LDL association, HMGCoA red and ACAT activities, revealing that LDLr is more important in the clearance of LDL-apoB than in HepG2 cell cholesterol homeostasis, a role that should be attributable to both LDLr-dependent and -independent pathways.

INTRODUCTION

Cholesterol is an important component of cell membranes and a precursor of steroid hormones and biliary acids. Cells may synthesize cholesterol or take it up in its lipoprotein form. Low-density lipoprotein (LDL) is positively associated with the incidence of heart disease, and therefore the catabolism of LDL has been well studied over the years. Above all, attention has been paid to the LDL receptor (LDLr). The LDLr is a glycoprotein of 160 kDa encoded by a 5.3 kb RNA [1] which recognizes apolipoprotein B (apo B) and apo E. The LDLr and its bound LDL take an endosomal pathway. In the lysosome, LDL-apo B is degraded into amino acids and its cholesteryl esters (CEs) are hydrolysed to form free cholesterol and fatty acids. This freshly arrived free cholesterol, or a derivative, reduces the number of LDLr at the cell surface to slow LDL uptake [2]. Also 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA red) [3], the key enzyme of cholesterol synthesis, is down-regulated, while acyl-CoA:cholesterol acyltransferase (ACAT) [4] is activated for storage of cholesterol in an esterified form. Important work on two well-known LDLr genetic defects, human familial hypercholesterolaemia (FH) [5] and Watanabe heritable hyperlipidaemia (WHHL) seen in the rabbit [6] provided convincing evidence that the absence of LDLr dramatically elevates blood LDL. Receptors other than LDLr are suggested by very many studies to be involved in the uptake of LDL, as has been demonstrated by the occurrence of LDL hepatic catabolism in FH and WHHL [7]. Also, the LDLr-independent pathway was

estimated to account for 42% of LDL-cholesterol clearance in humans [8] under normal physiological conditions. Moreover, fibroblasts and hepatocytes were shown to take up and degrade LDL by an LDLr-independent pathway which results in lysosomal degradation [9]. Such activity could be mediated by the ‘lipolysis-stimulated receptor’ [10], a receptor that binds and degrades LDL. However, it has to be kept in mind that this receptor is only detectable in the presence of non-esterified fatty acids. The ‘lipoprotein-binding site’ (LBS), which we have demonstrated on rat liver membranes [11] and HepG2 cells [12], binds LDL and should also be considered. Although this LBS is not responsible for the degradation of the protein moiety of LDL, it selectively takes up CEs from LDL [13], which are further hydrolysed in lysosomes, indicating that the LBS can be part of the LDLr-independent pathway.

LDLr deficiency is undoubtedly an excellent model to appreciate the function of LDLr and to ease the study of LDLr-independent pathways. To date, most of the information gathered on LDLr deficiency was obtained with human FH fibroblasts. Less work had been conducted on human hepatocytes for ethical and technical reasons. Analysis of the importance of the hepatic LDLr is relevant, since hepatic cholesterol homeostasis must take into account dietary fat intake and cholesterol requirements for lipoprotein and biliary acid synthesis. Moreover, the clearance of lipoproteins is known to occur mainly in the liver [14] and differences concerning the regulation of LDLr between fibroblasts and hepatocytes or hepatocyte-derived cells have already been reported [15,16].

Abbreviations used: apo, apolipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; B_{max} , maximal binding capacity; CE, cholesteryl ester; [³H]CET, [cholesteryl-1,2(n)-³H]hexadecyl ether; FBS, fetal bovine serum; FH, familial hypercholesterolaemia; HDL3, high-density lipoprotein-3; HMGCoA red, 3-hydroxy-3-methylglutaryl-CoA reductase; K_d , dissociation constant; LBS, lipoprotein-binding site; LDL, low-density lipoprotein; LDLr, LDL receptor; PDS, lipoprotein-depleted serum; MEM, minimal essential medium; WHHL, Watanabe heritable hyperlipidaemia.

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The aim of this study was to create HepG2 cells expressing lower levels of LDLr by using antisense technology, and to analyse the consequences of this LDLr gene-inactivation on the homeostasis of cell cholesterol. This effort was undertaken to increase understanding of the cell cholesterol homeostasis of hepatic cells in individuals with lower levels of LDLr, such as in those affected by various forms of FH. The principle of inhibition of expression by antisense RNA is to interfere with translation by the formation of an RNA duplex between the endogenous RNA and the antisense RNA. This technique was first used in 1984 by Izant and Weintraub [17] on the thymidine kinase gene of the herpes simplex virus. Since then, the technique has been used in many different systems and gene inactivation of 80–90 % has been obtained [18,19]. The results presented here demonstrate that HepG2 cells transfected with a constitutive expression vector containing LDLr cDNA fragments provided HepG2 cell subtypes expressing low or very low levels of LDLr. These cells bind and take up LDL at about the same level as normal HepG2 cells, but degrade less LDL-apoB. Also, in these new HepG2 cell subtypes, the small variations in the HMGCoA red and ACAT activities indicate that reducing the LDLr levels has a negligible effect on hepatic cell cholesterol metabolism.

MATERIAL and METHODS

Materials

Human blood (3–5 days old) was obtained from the Montreal Transfusion Center 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 purchased from Gibco Laboratories (Burlington, Ontario, Canada). ^{125}I (as sodium iodide, 100 mCi/ml), [cholesteryl-1,2(n)- ^3H]hexadecyl ether (^3H]CEt) and [9,10(n)- ^3H]oleic acid (2–10 Ci/mmol) were bought from Amersham (Oakville, Ontario, Canada), whereas 3-hydroxy-3-methyl-D,L-3-[glutaryl-3- ^{14}C]CoA (57.5 mCi/mmol) was bought from Sigma Chemical Company (St. Louis, MO, U.S.A.). Fatty acid-free or regular BSA (fraction V), trypsin, PMSF, Triton X-100, NADP $^+$ and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Company. Restriction enzymes and modification enzymes were obtained from Pharmacia (Montreal, Quebec, Canada). The plasmid pRc/CMV was purchased from Invitrogen (San Diego, CA, U.S.A.). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH, U.S.A.). IgG-C7 antibody was a gift from Dr. Ross Milne (Heart Institute, Ottawa, Canada).

Cell culturing

HepG2 cells were grown in 75-cm 2 flasks containing 15 ml of Eagle's minimal essential medium (MEM) supplemented, unless otherwise stated, with 10 % (v/v) FBS, penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and glutamine (4 mM), as previously described [20].

Preparation of stable transformants expressing an antisense *LDLr* RNA

pLDLR3 [1], a vector containing the full human *LDLr* cDNA sequence was digested with either *EcoRI*, *BglII* or *EcoRV*, and fragments were excised with *ApaI*. These fragments of *LDLr* cDNA were respectively 0.7 kb, 1.1 kb and 1.8 kb long, but they all share the same 5' end which is at the very beginning of the cDNA. In parallel, the pRc/CMV expression vector was digested with *NotI*, blunted and digested with *ApaI*. Ligation between each fragment and the vector was accomplished at 20 °C with T4

DNA ligase followed by transformation in *E. coli* DH5 alpha. Positive clones were checked for the orientation of the *LDLr* cDNA insert before they were transfected in the human hepatoma cell line (HepG2) using the standard calcium phosphate method [21]. HepG2 cells were also transfected with a mixture of the three constructs or with the expression vector alone. G418-resistant pools of cells were selected and propagated to study the LDLr expression level. The stable transformants resemble the native HepG2 cells in appearance and in growth rate. Analysis with these cells was done at a confluency of 90 %, in order to eliminate modulation of the expression of LDLr as a function of confluency [16]. The *LDLr* mRNA levels in these cells were measured by the technique described by Tam et al. [16].

LDLr protein estimation by immunoblotting

Cell proteins were obtained by following the method of Yoshimura et al. [22]. Proteins (100 μg) were separated by SDS/8 % -PAGE [23] followed by Western blotting. Cell proteins were transferred to nitrocellulose paper according to the method of Burnette [24]. The nitrocellulose paper was incubated in buffer containing the monoclonal antibody IgG-C7 [25] at a concentration of 3 $\mu\text{g}/\text{ml}$ for 2 h at room temperature. The nitrocellulose was washed and then incubated with rabbit ^{125}I -labelled anti-mouse IgG (10 $\mu\text{g}/\text{ml}$, 2×10^7 c.p.m.) for 1 h at room temperature. Thereafter, nitrocellulose paper was extensively washed and an autoradiogram was obtained by exposing the dried nitrocellulose paper to Kodak XAR film at -70 °C. The intensity of the autoradiogram signal was quantified by densitometry using the Imagequant system of Molecular Dynamics (Sunnyvale, CA, U.S.A.).

Preparation of lipoproteins

Human blood cells were removed by low-speed centrifugation and 0.01 % (w/v) EDTA, 0.02 % (w/v) sodium azide and 10 μM PMSF were added to the plasma before the isolation of lipoproteins, which was achieved by ultracentrifugation as described by Hatch and Lees [26]. Human LDL (density 1.025–1.063 g/ml) and human high-density lipoprotein-3 (HDL3; density 1.125–1.21 g/ml) were prepared as described by Brisette and Noel [11]. LDL was iodinated by a modification [27] of the iodine monochloride method of McFarlane [28] or was radiolabelled with ^3H]CEt according to the method of Roberts et al. [29] as described in Brisette et al. [13].

Binding assay

The cells in 12-well dishes were washed twice with 1 ml of PBS and were then incubated for 3 h at 4 °C with 0–50 μg of protein/ml of ^{125}I -labelled LDL in 125 μl of MEM containing 4 % (w/v) fatty-acid free BSA and 50 mM Hepes, pH 7.4, in a total volume of 250 μl (total binding). All lipoprotein fractions were maintained in PBS. Non-specific binding was determined by the addition of 1 mg of protein/ml of unlabelled LDL and represented less than 25 % of total LDL binding to HepG2 cells. At the end of the incubation the cell monolayers were washed twice with 1 ml of PBS containing 1 % (w/v) BSA (PBS-BSA), then twice with 1 ml of PBS. The cells were solubilized in 1.5 ml of 0.1 M NaOH, assayed for protein content and counted for radioactivity in a Cobra II counter (Canberra-Packard). The specific binding was calculated by subtracting the non-specific binding of ^{125}I -labelled LDL from the total binding. The curves generated by the specific-binding data were transformed into plots of the ratio of cell-bound to free ^{125}I -labelled LDL versus cell-bound ^{125}I -labelled LDL, according to the method of

Scatchard [30]. The ordinate of the Scatchard plots (bound/free) represents the amount of specifically bound ligand (μg of protein/mg cellular protein) divided by the concentration of unbound ligand in the reaction mixture (μg of protein/ml). The dissociation constants (K_d) were calculated from the slopes and the maximum binding capacity (B_{max}) was obtained from the x -axis intercept. Concave-upward curvilinear Scatchard plots were resolved by the method of Rosenthal [31] or using Ligand Software (Biosoft, Ferguson, MO, U.S.A.).

Cell association and degradation assays

HepG2 cell association of ^{125}I -LDL lasted for 4 h at 37 °C, as for the binding studies but without Hepes. At the end of the incubation, 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 from an estimate of the radioactivity of the washed cells resuspended in 0.1 M NaOH. The results are expressed in μg of LDL-protein/mg of HepG2 cell protein. To determine the association of ^3H JCet-LDL, in μg of LDL-protein/mg of HepG2 cell protein, *in situ* delipidation was performed with hexane/isopropanol (3:2, v/v) and this solution was counted in a beta-counter (Wallach, Fisher). The degradation of ^{125}I -LDL proteins was estimated from the medium. Essentially, trichloroacetic acid was used at a final concentration of 12% and degradation was estimated as the trichloroacetic acid-soluble fraction.

HMGCoA red activity

Three hours before the preparation of HepG2 cell microsomes, the medium was replaced with MEM containing either 10% FBS or 10% (v/v) lipoprotein-depleted serum (LPDS) supplemented with 50 $\mu\text{g}/\text{ml}$ LDL. In parallel, a series of cells was used that had been maintained for 48 h in MEM containing 10% (w/v) LPDS. The microsomes were prepared as previously described by Brown et al. [32] and HMGCoA red activity was determined essentially as previously reported [2]. Briefly, aliquots of the cell extracts (100 μg of proteins) were incubated for 2 h at 37 °C in a final volume of 0.2 ml containing 0.1 M potassium phosphate, pH 7.5, 20 mM glucose 6-phosphate, 2.5 mM NADP⁺, 1 unit of glucose 6-phosphate dehydrogenase, 5 mM dithiothreitol and 4.5 μM [^{14}C]HMGCoA (57.6 mCi/mmol). The reaction was stopped by addition of 20 μl of 6 M HCl. Aliquots were then incubated at 37 °C for 30 min in order to produce ^{14}C -mevalonolactone and they were extracted four times with 1 ml of diethyl ether. The ether extracts were evaporated to dryness and mevalonolactone was separated from other lipids by TLC using silica-gel G plates run in benzene/acetone (1:1, v/v). Mevalonolactone was located by using [^3H]mevalonolactone as an external standard. The mevalonolactone spot was recovered from the plate and quantified by liquid-scintillation counting in a beta-counter.

ACAT activity assay

Cells were prepared as specified in the section above. The ACAT activity was determined *in situ* according to the method described by Evans et al. [33] with some modifications. Each dish received 0.08 μCi (37 μM) of [^3H]oleic acid complexed with fatty acid-free BSA as in [34] and was incubated at 37 °C for 5 h. The cells were washed thrice with 0.15 M NaCl/50 mM Tris/HCl, pH 7.4, and the lipids were extracted *in situ* following two 30 min incubations with 1 ml of the hexane/isopropyl alcohol (3:2, v/v). Cells were digested overnight in 0.1 M NaOH for the determination of protein concentration. The lipid extracts were

dried under N_2 , resuspended in chloroform/methanol (2:1, v/v) and separated by TLC on silica-gel G plates run in petroleum ether/diethyl ether/acetic acid (84:15:1, by vol.). The plates were dried and the lipid spots were revealed by exposure to iodine vapour. Following complete disappearance of I_2 , spots corresponding to the CE positions were scraped from the plates and quantified by liquid-scintillation counting.

Other methods

Protein content was determined by the method of Lowry et al. [35] with BSA as standard. The data were analysed by analysis of variance or the Mann-Whitney test. Analysis of variance was used when groups had to be compared. The Mann-Whitney test was used under circumstances where the variation inside the group was more important than those observed between different groups.

RESULTS

Our primary goal was to create an HepG2 cell expressing very low levels of LDLr. To achieve this, expression vectors were constructed that could produce antisense *LDLr* RNA in the cell. Because we could not determine empirically the length of the antisense RNA that would be the most efficient, different portions of the *LDLr* cDNA were inserted in the vector. All of them however contained the 5' end of the cDNA. Thus, *ApaI-EcoRI* (0.7 kb), *ApaI-EcoRV* (1.1 kb) or *ApaI-BglII* (1.8 kb) fragments were inserted into the pRc/CMV vectors and pools of stable transformants were obtained. These cells were named according to the length of their cDNA insert, namely HepG2-0.7, HepG2-1.1 and HepG2-1.8 respectively. HepG2 cells were also transfected with the vector alone or with a mixture of the three constructs mentioned above. These were named respectively HepG2-0 and HepG2-all. Growth was not significantly different between these various pools of cells. Northern-blot analysis of the RNA of the different subtypes of HepG2 cells revealed lower levels of *LDLr* RNA in HepG2-0.7 and HepG2-all cells, while actin RNA levels were unchanged (results not shown). The level of LDLr in these different cells was determined by immunodetection of cell proteins separated by SDS/PAGE and transferred to nitrocellulose. Figure 1 shows that only normal HepG2

Figure 1 Immunoblot analysis of LDLr levels in different subtypes of HepG2 cells

Cells were solubilized with Triton X-100 and 100 μg of solubilized proteins were separated by SDS/8%-PAGE and transferred onto nitrocellulose membrane. The level of LDLr was then estimated by immunodetection with IgG-C7, a monoclonal anti-LDLr antibody, as described in the Materials and methods section.

Figure 2 Specific binding of ¹²⁵I-LDL to different subtypes of HepG2 cells

Saturation curves of the specific binding of ¹²⁵I-LDL to either normal HepG2 (◆), HepG2-0 (■), HepG2-0.7 (□), or HepG2-all (△) cells. To measure the binding on these cells, ¹²⁵I-LDLs (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. The curves represent the mean values of at least three experiments.

and HepG2-0 cells express a high level of LDLr. In contrast, the cells expressing antisense *LDLr* RNA have a lower level of LDLr. The decrease is more evident in HepG2-0.7 and HepG2-all cells. Indeed, quantification of the LDLr protein by densitometry indicates that, compared with normal HepG2 cells, LDLr is reduced significantly ($P < 0.01$) by 42, 72 and 85 % in the HepG2-1.1, HepG2-0.7 and HepG2-all cells respectively (average values of four different Western-blot analyses). HepG2-1.8 cells did not show a significant decrease in LDLr, while a reduction of 25 % was observed in HepG2-0 cells for obscure reasons.

These various subtypes of cells were analysed for their abilities to bind and degrade LDL. Figure 2 shows the binding of ¹²⁵I-

Figure 3 Specific binding of ¹²⁵I-LDL to the LDLr of the different subtypes of HepG2 cells

(A) Saturation curves of the specific binding of ¹²⁵I-LDL to either normal HepG2 (◆), HepG2-0 (■), HepG2-0.7 (□), HepG2-1.1 (●), HepG2-1.8 (○) or HepG2-all (△) cells. To measure the binding under these conditions, ¹²⁵I-LDLs (0–50 μg of protein/ml) were incubated at 4 °C for 4 h with cells in the presence of a 100-fold excess of HDL3 protein relative to the amount of ¹²⁵I-LDL in each well. 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. The curves represent the mean values of at least four different experiments. The SDs of the various points on the curves were calculated: we found highly significant differences between normal HepG2 cells and HepG2-all or HepG2-0.7 cells. (B) Scatchard plots of the specific-binding data.

labelled LDL to the different subtypes of HepG2 cells. It appears that HepG2-0.7 and HepG2-all cells bind less LDL than normal HepG2 and HepG2-0 cells. However, the curves were not

Table 1 Parameters of LDL binding to the different HepG2 cell subtypes

Binding assays of ¹²⁵I-LDL to the different subtypes of HepG2 cells were carried out as described in the Materials and methods section. Briefly, ¹²⁵I-labelled LDL (0–50 μg of protein/ml) was incubated with HepG2 cells at 4 °C for 3 h. Non-specific binding was measured by adding 1 mg of protein/ml of unlabelled LDL and specific binding was calculated by subtracting the non-specific from the total binding. The K_d and B_{max} were determined from the Scatchard plots of the specific-binding data. Each value represents the mean $\pm$ S.E.M. of the number experiments indicated in parentheses. *Statistically different ($P < 0.05$) from the results obtained with normal HepG2 cells.

Cell subtype	High-affinity binding (LDLr)		Low-affinity binding (LBS)	
	K_{d_1} (μg of protein/ml)	B_{max_1} (μg of protein/mg of cell protein)	K_{d_2} (μg of protein/ml)	B_{max_2} (μg of protein/mg of cell protein)
HepG2 (3)	6.4 $\pm$ 1.8	2.9 $\pm$ 1.1	96 $\pm$ 23.5	21.2 $\pm$ 9.1
HepG2-0 (4)	0.75 $\pm$ 0.25*	3.2 $\pm$ 0.71	47.3 $\pm$ 30.6	57.3 $\pm$ 41.3
HepG2-1.1 (3)	2.6 $\pm$ 0.79	9.6 $\pm$ 2.8	22.3 $\pm$ 10.5	43.1 $\pm$ 20.5
HepG2-all (4)	4.0 $\pm$ 1.6	5.3 $\pm$ 2.4	25.9 $\pm$ 19.2	19.0 $\pm$ 8.4

Table 2 Parameters of LDL binding to the LDLr of the different HepG2 cell subtypes

Binding assays of ^{125}I -labelled LDL to the different subtypes of HepG2 cells were carried out as described in the Materials and methods section. Briefly, ^{125}I -labelled LDL (0–50 μg of proteins/ml) was incubated with HepG2 cells at 4 °C for 3 h in the presence of a 100-fold excess of HDL3 relative to the amount of ^{125}I -labelled LDL in each well. Non-specific binding was measured by adding 1 mg of protein/ml of unlabelled LDL and specific binding was calculated by subtracting the non-specific from the total binding. K_d and B_{max} were determined from Scatchard plots of the specific-binding data. Each value represents the mean $\pm$ S.E.M. of the number of the experiments indicated in parentheses. *Statistically different ($P < 0.05$) from the results obtained with the normal HepG2 cells.

Cell subtype	K_d (μg of protein/ml)	B_{max} (μg of protein/mg of cell protein)
HepG2	4.1 ± 2.9 (6)	5.0 ± 2.3 (6)
HepG2-0	7.0 ± 3.3 (6)	5.6 ± 4.8 (6)
HepG2-1.8	5.5 ± 6.9 (5)	2.7 ± 1.9 (5)
HepG2-1.1	3.1 ± 2.1 (5)	3.3 ± 1.9 (5)
HepG2-0.7	$14.0 \pm 1.8^*$ (5)	$0.67 \pm 0.65^*$ (5)
HepG2-all	$17.5 \pm 2.6^*$ (4)	$0.68 \pm 1.07^*$ (4)

statistically different. As previously shown [13], LDL binds to both a high-affinity site (LDLr) and a lower-affinity site (LBS). The binding parameters of the curvilinear Scatchard plots are shown in Table 1. Under such conditions of binding, no significant differences were found between normal HepG2 cells and HepG2-0, HepG2-1.1 and HepG2-all cells with regard to the binding parameters of the low-affinity site (LBS). This was not surprising as no loss of LBS activity was anticipated. However, it was somewhat disturbing that no statistically different reduction of the high-affinity binding capacity (LDLr) was detectable between the normal HepG2 cell and its various subtypes. We rationalize that any binding assay with lipoproteins leads to some variation since the lipoprotein preparations may differ a little from one batch to another. Furthermore, the complexity of curvilinear Scatchard plots also adds to the variation. In order to simplify the quantification of the binding parameter of the high-affinity sites (LDLr), we used the strategy of inhibiting the binding of LDL to the low-affinity site (LBS). As LBSs interact with many classes of lipoproteins, the binding assay was conducted in the presence of HDL3 to abolish the binding of LDL to the LBS, as described by Brissette et al. [13]. This LDL binding study to LDLr is shown in Figure 3. Figure 3(A) represents the saturation curves of the specific binding of ^{125}I -LDL to the different subtypes of HepG2 cells. It is clear from these curves that the cells expressing antisense *LDLr* RNA bind less LDL, while the binding activity of HepG2-0 is not significantly different

from normal HepG2 cells. This decrease in LDL binding is most important in those cells which had shown the lowest level of LDLr protein by immunodetection. Scatchard plots of the specific binding of ^{125}I -LDL to the cells are shown in Figure 3(B). The intercepts with the x -axis (B_{max}) reveal that the amount of LDLr is clearly reduced in the cells expressing antisense *LDLr* RNA, and particularly in the HepG2-0.7 and HepG2-all cells. Table 2 shows the parameters of all binding studies individually assessed and reveals that HepG2-0.7 and HepG2-all cells have approximately 13 % of the LDLr found in normal cells. This decrease is statistically significant ($P < 0.01$). The HepG2-1.8 and HepG2-1.1 cells show LDLr levels at 54 and 66 % of the normal level respectively, however this reduction did not reach statistical significance. This exclusive-binding analysis of LDL to the LDLr (in the presence of HDL3) revealed a true inactivation of LDLr expression and agrees with the results obtained from immunoblotting analysis. Overall, HepG2-1.1 and HepG2-1.8 can be considered as low LDLr expressors and HepG2-0.7 and HepG2-all as very low LDLr expressors. Table 2 shows also a statistically significant ($P < 0.01$) increase in the K_d of HepG2-0.7 and HepG2-all. This result is somewhat surprising. It is possible that when the LDLr levels are very low, the saturation curve is also reflecting some traces of LBS that was not completely saturated with HDL3.

Knowing that we had cells expressing normal, low and very low levels of LDLr, it became interesting to analyse in a more detailed manner the metabolism of LDL by these cells. The experiments on LDL metabolism were conducted at 37 °C, a temperature that allows internalization and degradation of lipoproteins after binding to the receptors on plasma membranes. Firstly, we quantified the degradation of LDL-apo B. Table 3 shows that the cells expressing lower levels of LDLr degrade 71–82 % less apo B. This decrease in protein degradation is statistically significant ($P < 0.05$). However, when LDL cell-association was measured, no statistically significant differences were obtained whether the LDL were labelled in the proteins with ^{125}I or in the CEs with ^3H CEt. Furthermore, if CE association is compared with protein association, a CE-selective uptake phenomenon, which is of the order of 4-fold, becomes evident, since more CEs enter the cell than apo B. All this reveals that the LDLr is important for LDL-apo B degradation but that most LDL-CE uptake is carried out by a LDLr-independent pathway in HepG2 cells.

It then became important to determine whether LDLr deficiency had an effect on the activity of the enzymes known to control cell cholesterol levels. One would assume that if the LDLr-independent pathway does not play a significant role in bringing a bona fide regulatory pool of cholesterol into the cell,

Table 3 Association and degradation of LDL by different HepG2 cell subtypes

Cells were incubated for 4 h at 37 °C with either 10 μg /ml of ^{125}I -labelled LDL or ^3H CEt-LDL and cell association of LDL-protein or CEt was measured as described in the Materials and methods section. Degradation of LDL-protein was measured in the medium. In all cases, incubation was conducted in parallel with 1 mg of protein/ml of unlabelled LDL to determine the level of non-specific association. Each value represents the mean $\pm$ S.E.M. of the number of experiments indicated in parentheses. All experiments were conducted in duplicate. *Statistically different ($P < 0.05$) from the results obtained with normal HepG2 cells.

Cell type	LDL association (μg of LDL protein/mg of cell protein)		LDL degradation (μg of LDL protein/mg of cell protein)
	Protein	CE	Protein
HepG2	0.083 ± 0.015 (5)	0.352 ± 0.026 (5)	0.028 ± 0.0058 (5)
HepG2-0	0.08 ± 0.017 (3)	0.416 ± 0.052 (3)	0.020 ± 0.0072 (3)
HepG2-0.7	0.071 ± 0.007 (3)	0.373 ± 0.029 (3)	$0.008 \pm 0.0069^*$ (3)
HepG2-all	0.066 ± 0.007 (5)	0.408 ± 0.031 (5)	$0.005 \pm 0.0028^*$ (3)

Table 4 HMGCoA red activity of different HepG2 cell subtypes incubated in different culture media

HMGCoA red activity was determined as described in the Materials and methods section. Total protein (100 µg) was incubated with 4.6 µM of ^{14}C -labelled HMG-CoA at 37 °C for 2 h. The mevalonate formed was estimated by liquid-scintillation counting. The values are the means $\pm$ S.E.M. of the number of experiments indicated in parentheses. Statistically different, * $P < 0.05$, † $P < 0.001$, from the results obtained with normal HepG2 cell under the same conditions of culturing.

Cell subtype	HMG-CoA reductase activity (% of control)		
	MEM containing 10% FBS	MEM containing 10% LPDS	MEM containing 10% LPDS and 50 µg/ml LDL
HepG2	100.0 $\pm$ 4.2 (17)	912.5 $\pm$ 109.8 (6)	20.3 $\pm$ 6.2 (4)
HepG2-0	104.9 $\pm$ 2.5 (20)	936.3 $\pm$ 44.7 (6)	5.8 $\pm$ 0.7 (2)
HepG2-1.8	100.2 $\pm$ 4.9 (15)	1075 $\pm$ 98.6 (6)	5.2 $\pm$ 0.1 (2)
HepG2-1.1	108.1 $\pm$ 7.2 (14)	1200.3 $\pm$ 236 (6)	11.7 $\pm$ 6.3 (2)
HepG2-0.7	131.7 $\pm$ 8.9* (13)	1149.9 $\pm$ 164 (6)	5.8 $\pm$ 0.3 (4)
HepG2-all	134.6 $\pm$ 6.1† (19)	1101.7 $\pm$ 120.1 (6)	5.6 $\pm$ 0.2 (4)

then the activity of HMGC α red would be highly increased while ACAT activity would be lowered in cells deficient in LDLr. Table 4 shows that while the activity of HMGC α red is not affected in low LDLr expressor cells (HepG2-1.1 and 1.8), it is increased by 32–34 % in the very low LDLr expressor cells (HepG2-0.7 and HepG2-all) incubated in MEM containing 10 % FBS. This difference is significant ($P < 0.05$) and indicates that the inactivation of the LDLr has an impact on cholesterol synthesis only when LDLr expression is very low. It is noteworthy that this impact of LDLr lowering HMGC α red activity is weak compared with the increase in activity induced by the total absence of lipoproteins. Indeed, incubation of the cells in MEM containing 10 % LPDS induces increases of more than 900 % in HMGC α red activity in all HepG2 cell subtypes (Table 4). Under those conditions, no significant difference is detected between the various HepG2 cell subtypes. Table 4 also shows that the incubation of cells in MEM containing 10 % LPDS supplemented with 50 µg/ml of LDL dramatically reduces the reductase activity. Indeed, under these conditions, the different HepG2 cell subtypes display an HMGC α red activity that ranges between 5 and 20 % of the activity level measured in complete medium. No significant difference was observed under these conditions between the various HepG2 cell subtypes, indicating that LDL-cholesterol entry is not affected by LDLr inactivation. Overall, these results indicate that LDLr gene inactivation does not induce very important changes in cholesterol biosynthesis in HepG2 cells and that this receptor is not the unique pathway to provide a regulatory pool of cholesterol to HepG2 cells from LDL. In order to determine whether those

results were a consequence of cell transformation, a set of experiments was conducted with normal HepG2 cells that were incubated with LDL in the presence or absence of an anti-LDLr antibody (antibody IgG-C7 at a concentration of 100 µg/ml). Although LDL-protein degradation was reduced by 56 % with the antibody, HMGC α red activity was barely affected, confirming our results that HMGC α red activity is not totally dependent on LDLr activity (results not shown).

The ACAT activity of all HepG2 cell subtypes cultured in MEM containing 10 % FBS is shown in Table 5. Only in the very low LDLr expressor cells was the amount of CE formed from [^3H]oleate decreased significantly ($P < 0.05$), by 14 and 20 % compared with normal HepG2 cells. When the cells were provided with 50 µg of proteins/ml of LDL in MEM medium containing 10 % LPDS, 200–300 % increases in ACAT activity were measured, compared with those obtained in complete medium. Comparison of ACAT activity between the different cell subtypes under these conditions indicates that cells with very low levels of LDLr (HepG2-0.7 and HepG2-all) display a significant decrease of 35 % in ACAT activity. This somewhat more important loss in the presence of LDL compared with the medium containing 10 % FBS may be related to the fact that in the former case more cholesterol is provided to the cell. It is, however, worth noticing that a larger loss of activity (approximately 60 %) was found when cells were in the presence of medium containing 10 % LPDS (Table 5). Under these conditions no difference in ACAT activity was observed between all HepG2 cell subtypes. This was to be expected as no cholesterol substrate was provided to the cell. Overall, those results indicate that the entry of cholesterol

Table 5 ACAT activity of different HepG2 cell subtypes incubated in different culture media

ACAT activity was determined *in situ* as described in the Materials and methods section. Cells were incubated with 36.8 µM [^3H]oleic acid at 37 °C for 2 h, their lipids were extracted and the synthesized cholesterol esters were measured by liquid-scintillation counting. The values are the means $\pm$ S.E.M. of the number of experiments indicated in parentheses. All experiments were conducted in duplicate. Statistically different, * $P < 0.05$ † $P < 0.001$, from the results obtained with normal HepG2 cells under the same conditions of culturing.

Cell subtype	ACAT activity (% of control)		
	MEM containing 10% FBS	MEM containing 10% LPDS	MEM containing 10% LPDS and 50 µg/ml LDL
HepG2	100.0 $\pm$ 3.5 (19)	41.3 $\pm$ 2.4 (4)	261.8 $\pm$ 15.0 (4)
HepG2-0	107.8 $\pm$ 4.1 (14)	38.8 $\pm$ 1.5 (4)	269.0 $\pm$ 18.3 (4)
HepG2-1.8	101.1 $\pm$ 7.9 (14)	47.8 $\pm$ 4.4 (4)	311.0 $\pm$ 36.3 (4)
HepG2-1.1	112.1 $\pm$ 14.9 (11)	46.5 $\pm$ 6.2 (4)	293.0 $\pm$ 18.3 (4)
HepG2-0.7	80.9 $\pm$ 9.9* (11)	40.5 $\pm$ 5.1 (4)	170.8 $\pm$ 14.1* (4)
HepG2-all	86.8 $\pm$ 3.2† (12)	42.0 $\pm$ 3.5 (4)	173.8 $\pm$ 11.1† (4)

into the cells is important, even in the LDLr-deficient cells, and that both LDLr-dependent and -independent pathways are important for the regulation of ACAT activity.

DISCUSSION

Our goal was to define the role of the hepatic LDLr in cell cholesterol homeostasis. The best systems in which to assess the role of the human hepatic LDLr are primary cultures of hepatocytes from FH heterozygous and homozygous individuals, but those cells are difficult to obtain for practical and ethical reasons. Primary cultures of hepatocytes from a rabbit with WHHL [6] or LDLr-deficient transgenic mice [36] can also be considered. However they have disadvantages, as they come from species that are not close to humans and which have been shown, over the years, to differ in many aspects of their metabolism of lipoproteins. Moreover, primary cultures require complicated conditions of culture, extensive monitoring and have limitations, since cell de-differentiation may occur [37]. For these reasons we decided to investigate the role of the LDLr in cell cholesterol homeostasis in a more defined cell system, the HepG2 cell, and to reduce the level of LDLr using an antisense strategy. In the present paper we show for the first time that it is possible to create HepG2 cells that express low or very low levels of LDLr. Indeed, cells were obtained that have LDLr levels reduced by 42–85%. The efficiency of LDLr gene inactivation obtained in this study is very interesting and can be compared with that reported for other genes by other groups [18,19]. Inhibition of the LDLr activity had also been obtained indirectly with the monoclonal antibody IgG-C7 directed against the LDLr [38,39]. Although this inhibitory approach is adequate for binding studies (4 °C), we are far from being convinced that it is appropriate for association studies conducted at 37 °C, since we know that at this temperature new LDLr or recycled LDLr migrate to the plasma membrane and internalize again with a lipoprotein. One cannot be sure that the monoclonal antibody in the medium will be able to interact with a newly emerged LDLr on the cell surface before the arrival of LDL. This is supported by the fact that, in our hands, IgG-C7 is not able to reduce LDL-apo B degradation by much more than 56%. Our approach, with the constitutive expression of *LDLr* antisense mRNA designed for an immortalized cell, has three advantages. This system has produced cells that are more deficient in LDLr activity than the cells co-incubated with IgG-C7, it does not require a pre-incubation and/or a co-incubation with an antibody and it resembles more the FH hepatocytes, since LDLr are not inhibited but reduced in number.

Our results clearly show that inactivation of the LDLr has a major impact on apoB degradation, as expected from studies conducted in FH in man [40] and WHHL in rabbits [6]. They also reveal that although LDLrs have a statistically significant effect on HMGCoA red and ACAT activities, this effect is rather limited compared with the conditions under which no LDL enters the cell, such as when the cells are incubated in the presence of medium containing 10% LPDS. Thus we have to conclude that the LDLr-independent pathway not only ensures binding of LDL to the HepG2 cell, but also the accumulation of cholesterol in the cell, which is also recognized as a regulatory pool by the cellular enzymes involved in maintaining cell cholesterol homeostasis. To explain the moderate effect of LDLr removal on HMGCoA red and ACAT activities, two scenarios can be proposed. The first is that in our system, where levels equal to or above 15% of the normal LDLr level provide a sufficient regulatory pool of cholesterol to the cell in order to minimize the increase in HMGCoA red activity and the reduction

in ACAT activity. The second scenario is challenging but somewhat provocative, as it postulates that the HepG2 LDLr is crucial for LDL-apoB degradation, but not for a significant cholesterol uptake by the cell, a function that is devoted more to an LDLr-independent pathway. In other words, this means that the LDLr controls the amount of LDL particles in the blood, while an LDLr-independent pathway controls both the CE content of LDL and the homeostasis of cholesterol in the hepatic cell. This possibility is supported by the demonstration that in ovarian cells a HDL-CE-selective uptake pathway also induces an homeostatic response by the cell [41]. Among the mechanisms involved in cholesterol homeostasis in HepG2 cells deficient in LDLr, we believe that the selective uptake of CE from lipoproteins plays an important role. We [13] and others [42] have shown that apoB-containing lipoproteins can associate with HepG2 cells and selectively deliver CEs to the cells. Evidence for this was also obtained *in vivo* by Green and Pittman [43]. More precisely, we obtained results indicating that the LBS would be involved in this CE-selective uptake [12,13]. The selective uptake of free cholesterol from LDL described recently by Fielding and Fielding [44] also remains a possibility. Although our results suggest that the LDLr plays a minimal role in HepG2 cell cholesterol homeostasis, caution should be exercised before our conclusion is extended to normal hepatic cells. Indeed, even though the HepG2 cell is recognized as a good human hepatic-cell model, since it synthesizes apolipoproteins and lipoproteins [45], expresses lipoprotein receptors [13] and resists LDLr down-regulation as a normal hepatic cell [15,46], it is not a perfect model since it has a defect in lipid mobilization [47] and in bile acids synthesis and conjugation [48,49].

Our model allowed us to compare the respective role of two important pathways in cholesterol metabolism. Indeed, LDLr-deficient cells make it easy to identify and study the LDLr-independent pathway. Other investigations are, however, needed in order to define more precisely the importance of this pathway observed in HepG2 cells and in hepatocytes, and to confirm the validity of our system with low LDLr levels. Thus, experiments should be conducted with primary cultures of hepatocytes obtained from LDLr-deficient animal models. Our overall aim with this cell model is to examine the possibility of stimulating the LDLr-independent pathway to increase the clearance of CE from LDL and then to reduce the risk of heart diseases associated with this lipoprotein.

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