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Differences in hypolipidaemic effects of two statins on Hep G2 cells or human hepatocytes in primary culture

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1 The objective of this study was to compare in cultured human hepatocytes or Hep G2 cells, changes in the fate of unesterified low density lipoprotein (LDL)-cholesterol induced by crilvastatin, a new cholesterol lowering drug and a reference statin, simvastatin.

2 The experiments were carried out for 20 h, each well contained $4.2 \times 10^5/cm^2$ Hep G2 cells or $0.5 \times 10^5/cm^2$ human hepatocytes, 130 μM ursodeoxycholate, 0.68 μCi or 1.59 μCi unesterified human [^{14}C]-LDL-cholesterol, crilvastatin or simvastatin at 0 or 50 μM (both cell types) or 300 μM (Hep-G2 cells). Incubation with the two drugs resulted in increased amounts of unesterified [^{14}C]-LDL-cholesterol taken by the two cell types, compared to control.

3 Crilvastatin 50 μM led to significantly higher quantities of [^{14}C]-glyco- and [^{14}C]-tauro-conjugated bile salts, compared to simvastatin. Statins reduced the apo B100 level secreted by the two cell types (simvastatin) or human hepatocytes (crilvastatin). Crilvastatin enhanced both the level of apo A1 secreted by the Hep G2 cells and the level of APF, a high density lipoprotein (HDL) and biliary apoprotein.

4 Crilvastatin not only acts by stimulating LDL-cholesterol uptake by hepatocytes, but also by enhancing the catabolism of LDL-cholesterol in bile salts and probably by stimulating HDL and/or bile component secretion. Such a mechanism was not previously described for HMG CoA reductase inhibitors. Our results on APF show that this apoprotein could be considered also as an indicator of changes in bile and/or HDL compartments.

5 The human hepatocyte model appeared to be a suitable and relevant model in the pharmacological-metabolic experiments carried out in this study. It led to more consistent data than those obtained with Hep G2 cells.

Keywords: Cholesterol lowering drugs; cultured Hep G2 and human hepatocytes; lipoproteins; bile

Introduction

Hep G2 cell lines have been widely used to test lipoprotein metabolism, e.g. expression of lipoprotein receptors, uptake of lipoproteins as well as secretion of triglycerides, esterified and unesterified cholesterol, apolipoproteins A1, B100, C and E (Wang *et al.*, 1988; Javitt, 1990; Panini *et al.*, 1991). In addition, Hep G2 cells in primary culture are associated, showing bile canalicular spaces. A 140 kDa bile salt transporter has been described in Hep G2 cells (Chiu *et al.*, 1990); it results in the ability of cultured Hep G2 cells to synthesize and secrete bile salts, by a continuous efflux of chenodeoxycholate and cholate, the main human bile salts (Javitt *et al.*, 1989; Javitt, 1990). Thus this hepatoblastoma cell line appears to be suitable for the study of cholesterol homeostasis, particularly in the presence of cholesterol lowering drugs. Incubation of Hep G2 cells with inhibitors of hydroxy methyl glutaryl (HMG) CoA reductase results in a decrease in cholesterol synthesis (Nagata *et al.*, 1990; Panini *et al.*, 1991; Chao *et al.*, 1991). In addition, it has been hypothesized that Hep G2 cells incubated with an hypocholesterolaemic drug secrete low cholesterol-low density lipoproteins (LDL) and -high density lipoproteins (HDL) (Sato *et al.*, 1990). However, the oncogenic properties of Hep G2 cells restrict the extrapolation to non-cancerous human hepatocytes; the use of human hepatocytes is limited by difficulties in obtaining such cells and by the rapid degeneration of these cultured cells. Nevertheless, metabolic studies on cholesterol and lipoprotein carried out with healthy human hepatocytes have validated this human cell model (Kosykh *et al.*, 1985; Sviridov *et al.*, 1990).

We chose to study the cholesterol lowering effect of crilvastatin, a new inhibitor of HMG CoA reductase (Esnault-Dupuy *et al.*, 1988), in cultured Hep G2 cells and human hepatocytes. The drug has been shown to stimulate in the rat *in vivo* and in rat isolated hepatocytes, secretion of biliary sterols provided to a large extent by the metabolism of LDL-cholesterol (Clerc *et al.*, 1993; 1995b). Furthermore, added bile salt was shown to stimulate uptake and metabolism of LDL-cholesterol by rat isolated hepatocytes (Clerc *et al.*, 1995a). Ursodeoxycholate has been found to exert a hepatoprotective effect against the more hydrophobic bile salts, *in vivo* as well as in human cultured hepatocytes (Galle *et al.*, 1990). Thus, it seemed of interest to compare the effects of crilvastatin and a reference cholesterol lowering drug, simvastatin, on the LDL-cholesterol metabolism in bile salts of cultured Hep G2 cells or human hepatocytes in the presence of ursodeoxycholate. The cholesterol lowering effect of the two drugs was also evaluated by comparing the levels of the three main cholesterol-carrier apoproteins, apo B100, apo A1 and APF, a new 7.5 kDa hydrophobic biliary protein (Martigne *et al.*, 1989; Domingo *et al.*, 1990). APF has also been shown to be a minor apoprotein component of plasmatic nascent HDL, HDL_2 and HDL_3, and to have a partial sequence homology with apo A1 (Domingo *et al.*, 1992).

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This work is dedicated to the memory of Doctor Jacques Christian Hauton.

Methods

The experiments were carried out on cultured hepatoblastoma Hep G2 or human hepatocytes in primary culture. The cholesterol lowering effects of a new molecule, crilvastatin on the metabolism of LDL-unesterified cholesterol into bile salts, were compared to those of a reference statin, simvastatin. Previous results obtained with rat isolated hepatocytes showed the maximal metabolic effect of crilvastatin occurred at a concentration of 50 μM (Clerc *et al.*, 1995b).

Preparation of ^{14}C labelled unesterified cholesterol-LDL

Labelled LDL were obtained by incubation of human plasma with ^{14}C labelled unesterified cholesterol-liposomes.

Radiolabelled liposomes were prepared as previously described (Clerc *et al.*, 1993): 1.66 mg dioleoyl-phosphatidylcholine (Sigma, L'Isle d'Abeau Chesnes, France), 0.25 mg unlabelled cholesterol (Sigma) and 3.6 μCi 4-^{14}C-labelled cholesterol (NEN-Dupont de Nemours, Paris, France, specific activity: 50 mCi mmol^{-1}) were mixed in chloroform/ethanol/benzene. The solvent was carefully removed by evaporation under nitrogen. Trace amounts of solvent do not affect liposome structure. Dioleoyl-phosphatidylcholine was chosen in order to obtain liposomes of the smallest size (100 nm). After dispersion in 1 ml RPMI 1640 (Sigma) by 15 s pulses for 30 min in an ultrasonic device (Sonics-Bioblock), the resulting multilamellar liposomes were centrifuged at 100 000 $\times g$ for 20 min at 10°C (Beckman L 5.75 B ultracentrifuge, Palo Alto, CA, U.S.A. equipped with a fixed angle rotor type 60 Ti) to remove large particles. Characteristics and homogeneity of the liposomes were checked by filtration on A4 gel (Ultrogel, IBF, Villeneuve la Garenne, France) with Tris buffer 0.5 M pH 7.5. Liposomes were eluted in a single peak.

Normolipidaemic plasma (15 ml) from an overnight fasted male human volunteer was collected in EDTA (Sigma) (0.1 mg ml^{-1}) and incubated for 24 h at 10°C with 3 ml of ^{14}C-labelled cholesterol-liposomes in order to label plasma lipoproteins.

Liposomes were removed by ultracentrifugation and flotation at 120 000 $\times g$ at 10°C for 18 h in the density zone below 1.00 g ml^{-1}. LDL fraction was recovered after ultracentrifugation at 120 000 $\times g$ at 10°C for 22 h in a sucrose solution of density 1.063, as described previously (Hatch & Less, 1968). After labelling, LDL were dialysed against NaCl 0.9% for 24 h at 10°C to remove sucrose and then concentrated on Amicon filter (B15, Amicon, Paris, France). The final concentration was adjusted with RPMI 1640 medium. Purity of the ^{14}C LDL fraction was confirmed by SDS gel electrophoresis which showed only one fraction in the migration zone of LDL. Assays of apoproteins A1 and B100 revealed that the ^{14}C LDL fraction contained only apo B. To verify that no ^{14}C radioactivity was associated with the esterified cholesterol, thin layer chromatography of lipids was carried out on silica gel F 1500 (Schleicher and Schüll, Dassel, Germany) with heptane/diethyl ether/ice cold acetic acid 90/30/1 v/v as a developing solvent. Identification was achieved under iodine vapours and the spots were scraped and counted in 10 ml scintillation liquid (Hionic Fluor, Packard). This control experiment showed that [^{14}C]-cholesterol in the LDL was exclusively with the unesterified cholesterol fraction. The negligible mass of radioactive cholesterol did not modify the metabolism of LDL. Radioactive LDL accounted for 50% of total labelling of incubated ^{14}C liposomes.

Hep G2 cell growth

Hep G2 hepatoma cells derived from human liver carcinoma and their cell line were generously provided by Dr Guillouzo (Rennes, France). The cell growth was carried out in 25 cm^2 culture flasks (Falcon, Becton-Dickinson, Pont de Claix, France), containing a mixture of 50% of Opti-MEM medium (Gibco, Cergy Pontoise, France) and 50% Ham F12 medium (Gibco), enriched with 10% foetal calf serum (FDA, Axcell, Paris, France), 1% L-glutamine (Sigma), 1% penicillin/streptomycin mixture (Gibco) (10 000 u 10 mg^{-1}) and 1% non essential amino acid mixture (Sigma). The experiment was carried out when cells were confluent.

Isolation and culture of human hepatocytes

This work was carried out in cooperation with two services of digestive surgery, Conception's Hospital and the regional centre of cancer treatment, Marseille. The study received the agreement of both hospital services and the associated services of anatomy-pathology. Human liver tissues were obtained after surgery of non-invasive hepatocarcinoma from 2 male adult livers and 1 female adult liver. After surgery and sampling of biopsies by the anatomy-pathology service, the liver fragment normally destined to be destroyed was dissected in order to recover a peri-tumoral zone. This small fragment (4–5 g) was deposited in a survey chamber, the vessel of the afferent porte system was isolated and perfused through a non-recirculating 6-way system by two successive buffers. The first medium consisted of a buffer pH 7.65 (0.6 g Na_2HPO_4, 12 H_2O, 14.28 g HEPES (Sigma), 48 g NaCl, 1.2 g KCl in 6 l of water). It was perfused at 38°C for 30 min in order to warm the liver biopsy, previously kept in a cold ischemia. Then, a second perfusion was performed at 38°C for 10 min with type IV collagenase (Sigma). It consisted of a medium containing 3 g collagenase extemporaneously dissolved in 100 ml of the above buffer. The hepatic capsule was then disrupted and isolated cells were suspended at 25°C in a Leibovitz L15 medium (Gibco) containing 20% foetal calf serum (FDA, Axcell), 0.2% bovine serum albumin (Sigma), 0.1% fungizone (250 μg ml^{-1}) (Gibco), 1% kanamycin (0.5%) (Gibco) and 0.2% penicillin/streptomycin mixture (10 000 u 10 mg^{-1}) (Gibco). All these steps were carried out under sterile conditions. Cells were then rinsed three times by low speed centrifugation (500 $\times g$) in a mixture of a 199 medium (Gibco) and Opti MEM medium (Gibco), respectively in the proportions 1/3-2/3, containing 0.2% penicillin/streptomycin mixture (10 000 u 10 mg^{-1}) (Gibco), 0.1% fungizone (250 μg ml^{-1}) (Gibco), 1% kanamycin (0.5%) (Gibco), 0.2% bovine serum albumin (Sigma) and insulin (0.05 u ml^{-1}) (Novo, Boulogne, France). However, the first wash was done with the aforesaid medium enriched with 45% Percoll (Sigma), to remove non-surviving cells by gradient centrifugation (20 min, 800 $\times g$). Hepatocytes were seeded in 25 cm^2 flasks (Falcon), in the presence of the aforesaid medium enriched with 15% foetal calf serum (FDA, Axcell). Cultures were maintained for 4 days. Experiments were performed on the 5th day with ursodeoxycholate and simvastatin or crilvastatin.

Pharmacological experiments

At the beginning of the experiments carried out on Hep G2 cells and human hepatocytes, the culture medium was deprived of foetal calf serum, lipoproteins and antibiotics. Dispersion of different hydrophobic molecules (ursodeoxycholate, crilvastatin [Pan Medica, Ivry sur Seine, France], simvastatin [Merck, Paris, France]) was previously performed in 2 ml of RPMI 1640 (Sigma) by 15 s pulses for 30 min in an ultrasonic device. Each plate contained 4.2 $\times$ 10^5 cells (Hep G2 cells) or 0.5 $\times$ 10^5 cells (human hepatocytes), 130 μM ursodeoxycholate, 0.68 μCi or 1.59 μCi [^{14}C]-unesterified human LDL-cholesterol respectively with Hep G2 cells or human hepatocytes, and crilvastatin or simvastatin at 0 or 50 μM (both cell types) or 0, 50 or 300 μM (Hep G2 cells).

The duration of incubation was 20 h. The conditions of incubation are summarized in Table 1. Aliquots were taken at the beginning and at the end of the experiment. The cells were separated, rinsed 3 times in a PBS buffer and frozen at −80°C. The experiment was carried out in triplicate.

Assays

Protein content of the medium was measured by use of bovine serum albumin as a standard (Lowry *et al.*, 1951). Bile salts were assayed by the enzymatic method of 3α-hydroxysteroid dehydrogenase (Domingo *et al.*, 1972). Total cholesterol and triglycerides were measured respectively by enzymatic methods (Bucolo & David, 1973; Lie *et al.*, 1976). Total phospholipids were analysed by measuring inorganic phosphate (Amic *et al.*, 1972) after digestion in perchloric acid. The activities of the aspartate aminotransferase (AST) and alanine aminotransferase (ALT), γ-glutamyl transferase, alkaline phosphatase were measured respectively as described previously (Morgenstern *et al.*, 1965; Kessler *et al.*, 1975; Persijn & Van der Slik, 1976). Concentrations of apo A1 and apo B100 were assayed by immunoturbidimetry on a Behring nephelometer (Behringwerke AG, Marburg, Germany), with antibodies and standards from the manufacturer. Detection and quantification of APF in the culture medium of human hepatocytes was by enzyme-linked immunosorbent assay (ELISA) with murine monoclonal antibodies (from Balb/C mice) (Domingo *et al.*, 1992).

Distribution of the ^{14}C radioactivity in cells and incubation medium

Bile salts and cholesterol were extracted from the incubation medium in a 10 fold volume of a mixture isopropanol-water (90/10, v/v) after boiling for 20 min to precipitate proteins. We verified that non-labelled material precipitated with the proteins and that extraction of bile salts and cholesterol was complete. After centrifugation at $3000 \times g$ for 20 min, extracts were concentrated under nitrogen, bile salts (tauroconjugated, glycoconjugated, free cholate) and cholesterol were separated by thin layer chromatography on silica gel F 1500 (Schleicher and Schüll), ^{14}C radioactivity of the fractions was counted. To ensure against possible contamination between components during chromatography, previous tests were carried out to compare migrations of [^{14}C]-cholesterol, [^{14}C]-taurocholate, [^{14}C]-glycocholate standards and blank. The elution medium consisted of a mixture of isoamyle acetate, propionic acid, isopropranol and water (40/30/20/10, v/v). Fractions were detected under a 350 nm u.v. light after spraying silica gel with a reagent containing 0.4 ml of a 10% solution of $FeCl_3$ in ice-cold acetic acid diluted in 60 ml ice-cold acetic acid, 40 ml concentrated sulphuric acid and 100 ml absolute ethanol. The quantity (pmol/10^6 cells) of extrahepatic cholesterol metabolized into bile salts was calculated by dividing radioactivity of bile salts by the specific radioactivity of the extrahepatic cholesterol (d.p.m./pmol cholesterol) added in the medium at the beginning of the experiment (1 pmol cholesterol was metabolized into 1 pmol bile salt). Since bile salts are rapidly secreted by the hepatocytes, amounts of ^{14}C bile salts present in the cells were negligible and the corresponding results not presented. In addition, we verified that [^{14}C]-cholesterol was exclusively as an unesterified form, by using the thin layer chromatographic procedure described for estimation of [^{14}C]-unesterified LDL cholesterol.

Statistical analysis

Results are expressed as the arithmetical mean of each group with their standard errors. We previously verified the homogeneity of the data in all conditions. Thus, differences between two groups were compared by using analysis of variance (ANOVA) for factorial values (Figure 1) or for repeated values (Table 2, Figures 2 and 5) and the significations were determined by Fisher's post hoc least significance difference (PLSD) test and/or Scheffe F-test at a probability value of 95%. However, we restricted the statistical test to comparisons with only one variable. Other results (Figures 3 and 4) were compared by Student's *t* test.

Results

Cell viability and toxicological parameters

After 2, 8, 12, 16 and 20 h incubation of Hep G2 cells or human hepatocytes, trypan blue exclusion did not show significant changes in hepatocyte viability with or without the drugs. Cell morphological changes or alterations were not found by light microscope observations when drugs were added to the medium. In addition, the γ glutamyl transferase and alkaline phosphatase activities were unchanged by addition of the drugs in the two types of culture. Transaminase level, particularly ALT was significantly enhanced by simvastatin, in both incubation medium of Hep G2 cells or human hepatocytes (Figure 1).

Distribution of ^{14}C radioactivity in the incubation medium

Total ^{14}C radioactivity in the medium (Figure 2) In culture medium of Hep G2 cells and human hepatocytes, the level of radioactivity significantly decreased between 0 and 20 h in all groups, except in the presence of 50 μM crilvastatin. In this case, the radioactivity, carried by both cholesterol and newly synthesized bile salts, remained at a high level in the medium between 0 and 20 h.

Newly synthesized ^{14}C bile salts (Figures 3 and 4) Previous work had shown that bile salt secretion by hepatocytes in culture occured 20 h after seeding, in contrast to isolated he-

Table 1 Experimental design of cell incubations

Cell type	*Number of incubations*	*[^{14}C]-unesterified LDL-cholesterol* (μCi)	*Ursodeoxycholate* (μM)	*Crilvastatin* (μM)	*Simvastatin* (μM)	*Final volume of incubation medium* (ml/box)	*Cell density* (cell/cm^2)
HepG2	5	0.68	0	0	0	10	4.2×10^5
HepG2	5	0.68	130	0	0	10	4.2×10^5
HepG2	5	0.68	130	50	0	10	4.2×10^5
HepG2	5	0.68	130	300	0	10	4.2×10^5
HepG2	5	0.68	130	0	50	10	4.2×10^5
HepG2	5	0.68	130	0	300	10	4.2×10^5
Healthy hepatocytes	5	1.59	130	0	0	10	0.5×10^5
Healthy hepatocytes	5	1.59	130	50	0	10	0.5×10^5
Healthy hepatocytes	5	1.59	130	0	50	10	0.5×10^5

patocytes, which exhibited a bile salt secretion 10 min after isolation. Thus, we studied bile salt secretion after 20 h incubation. However, this secretion was at a low basal level, and there was no quantitative difference between the experimental groups. Comparisons between groups were therefore carried out by estimation of differences in the amounts of radiolabelled tracer incorporated into bile salts (subsequently converted to pmol of ^{14}C bile salts). Compared to control without drug, simvastatin incubation resulted in unchanged quantities of ^{14}C bile salts secreted in the medium. Compared to control and simvastatin, 50 μM crilvastatin significantly enhanced both the tauroconjugated- and the glycoconjugated-^{14}C bile salts neosynthesized by Hep G2 cells and human hepatocytes (except the glycoconjugated ^{14}C bile salts synthesized by HepG2 cells in the presence of 50 μM crilvastatin, compared to control).

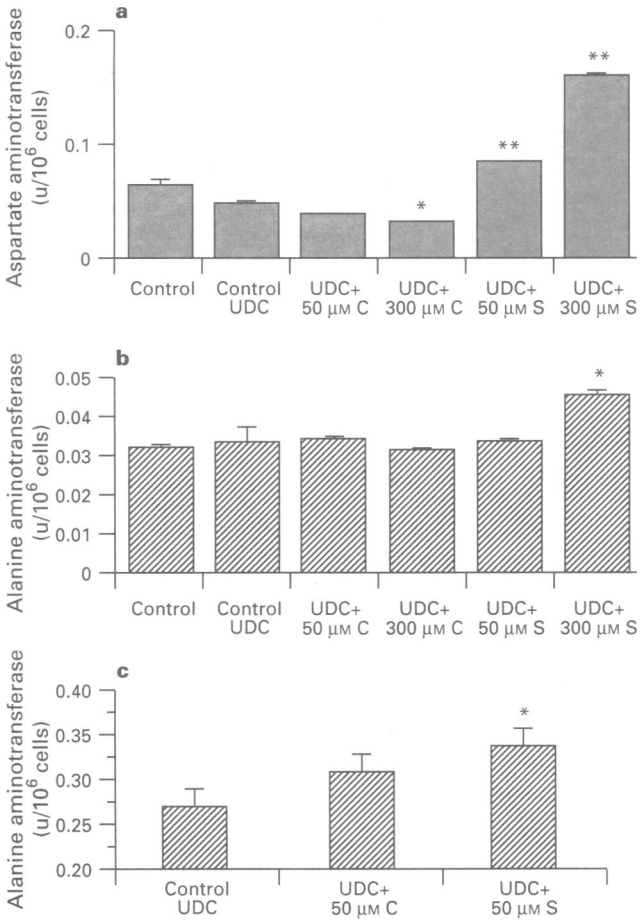

Figure 1 Transaminase levels. (a) The levels of aspartate aminotransferase in the medium after a 20 h incubation of Hep G2 cells with [^{14}C]-unesterified LDL-cholesterol (control) in presence of ursodeoxycholate (control UDC), with 50 μM crilvastatin (UDC + 50 μM C), 300 μM crilvastatin (UDC + 300 μM C), 50 μM simvastatin (UDC + 50 μM S) or 300 μM simvastatin (UDC + 300 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were determined by analysis of variance (ANOVA) for factorial values at a probability of 95% by Fisher's PLSD test or Scheffe F-test. *Difference significant by Fisher's test; **difference significant by the two tests (control UDC vs UDC + 300 μM C; control UDC vs UDC + 50 μM S; control UDC vs UDC + 300 μM C). Experiments were done in triplicate. (b) The levels of alanine aminotransferase in the medium after a 20 h incubation of Hep G2 cells with [^{14}C]-unesterified LDL-cholesterol (control) in presence of ursodeoxycholate (control UDC), with 50 μM crilvastatin (UDC + 50 μM C), 300 μM crilvastatin (UDC + 300 μM C), 50 μM simvastatin (UDC + 50 μM S) or 300 μM simvastatin (UDC + 300 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were determined by analysis of variance (ANOVA) for repeated values at a probability of 95% by Fisher's PLSD test. *Difference significant with Fisher's test (control UDC vs UDC + 300 μM S). Experiments were done in triplicate. (c) The levels of alanine aminotransferase in the medium after a 20 h incubation of human hepatocytes with [^{14}C]-unesterified LDL-cholesterol in the presence of ursodeoxycholate (control UDC), with 50 μM crilvastatin (UDC + 50 μM C) or 50 μM simvastatin (UDC + 50 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were determined by analysis of variance (ANOVA) for repeated values at a probability of 95% by Fisher's PLSD test. *Difference significant with Fisher's test (control UDC vs UDC + 50 μM S). Experiments were done in triplicate.

Secretion of apolipoproteins Apo A1, Apo B100 and APF in the medium

At the zero time point, there was a basal level of apo B, due to the addition of radiolabelled LDL in the medium. Apo A1 and APF were also present at the beginning of the experiment, since foetal calf serum, previously added for the first 4 days of culture contained these apolipoproteins. The quantities of apoproteins secreted by human hepatocytes were about 10 times higher than those secreted by Hep G2 cells. Compared with control, 50 μM crilvastatin significantly enhanced the level of apo A1 secreted by Hep G2 cells, reduced the apo A1 secretion by human hepatocytes. Simvastatin significantly enhanced the quantities of the apo A1 secreted by human hepatocytes. The level of secreted apo B100 was significantly reduced by 50 μM crilvastatin or simvastatin (human hepatocytes) or by 300 μM simvastatin (Hep G2 cells) (Table 2). Changes of APF level in the culture medium of human hepatocytes were comparable to

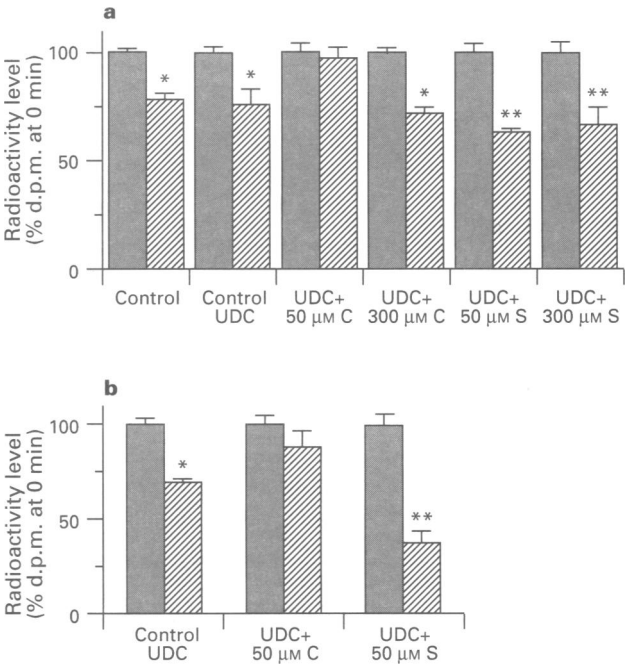

Figure 2 Distribution of ^{14}C radioactivity in the medium. Assays were done at the beginning of incubation (time 0, stippled columns) and after 20 h incubation (hatched columns). (a) Distribution of ^{14}C radioactivity in the medium of Hep G2 cells cultured with [^{14}C]-unesterified LDL-cholesterol (control) in presence of ursodeoxycholate (control UDC) with 50 μM crilvastatin (UDC + 50 μM C), 300 μM crilvastatin (UDC + 300 μM C), 50 μM simvastatin (UDC + 50 μM S) or 300 μM simvastatin (UDC + 300 μM S). (b) Distribution of ^{14}C radioactivity in the medium of human hepatocytes cultured with [^{14}C]-unesterified LDL-cholesterol in presence of ursodeoxycholate (control UDC) with 50 μM crilvastatin (UDC + 50 μM C) or 50 μM simvastatin (UCD + 50 μM S). For (a) and (b) results are expressed as means ± s.e.mean ($n = 5$). Differences were determined by analysis of variance (ANOVA) for repeated values at a probability of 95% by Fisher's PLSD test or Scheffe F-test. *Difference significant with Fisher's test; **difference significant with the two tests (time 0 vs time 20 h). Experiments were done in triplicate.

those observed in terms of ^{14}C radioactivity (Figure 5). In the presence of 50 μM crilvastatin, APF in the medium remained at a high level after 20 h incubation, it significantly decreased between 0 and 20 h in control conditions (without drug) and in the presence of 50 μM simvastatin.

Discussion

Freshly isolated hepatocytes could constitute a better model than cultivated hepatocytes in which to study the cholesterol lowering effect of statins (Alberts, 1988). Previous results showed that incubation of rat isolated hepatocytes in the presence of bile salts, systematically added to compensate eventual disturbances of bile salt metabolism, resulted in an active cholesterol metabolic process by the cells (Clerc *et al.*, 1995a,b). Thus, a preclinical model of rat hepatocyte was developed as an alternative to *in vivo* experiments, to mimic the effects of crilvastatin on its target cells and to define the optimal conditions of cell function (Clerc *et al.*, 1995b). The hepatoprotective effect of ursodeoxycholate (Galle *et al.*, 1990) was potentiated by crilvastatin, as shown by the decreased level

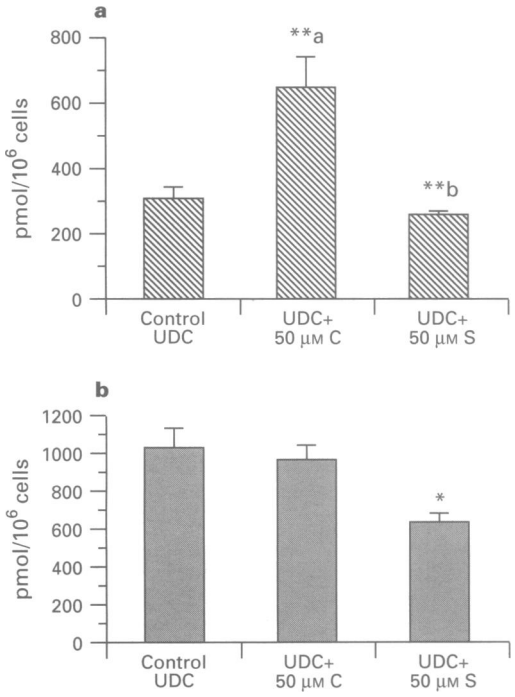

Figure 3 Quantities of ^{14}C bile salts synthesized from [^{14}C]-unesterified LDL-cholesterol and secreted in the culture medium of Hep G2 cells. (a) The levels of ^{14}C tauroconjugated bile salts in the medium after 20 h incubation with [^{14}C]-unesterified LDL-cholesterol in presence of ursodeoxycholate (control UDC) with 50 μM crilvastatin (UDC + 50 μM C) or 50 μM simvastatin (UDC + 50 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were analysed by Student's t test and are significant at $^{**}P < 0.01$ ([a] control UDC vs UDC + 50 μM C; [b] UDC + 50 μM C vs UDC + 50 μM S). Experiments were done in triplicate. (b) The levels of ^{14}C glycoconjugated bile salts in the medium after 20 h incubation with [^{14}C]-unesterified LDL-cholesterol in presence of ursodeoxycholate (control UDC) with 50 μM crilvastatin (UDC + 50 μM C) or 50 μM simvastatin (UDC + 50 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were analysed by Student's t test and are significant at $^{*}P < 0.05$ (control UDC vs UDC + 50 μM S). Experiments were done in triplicate.

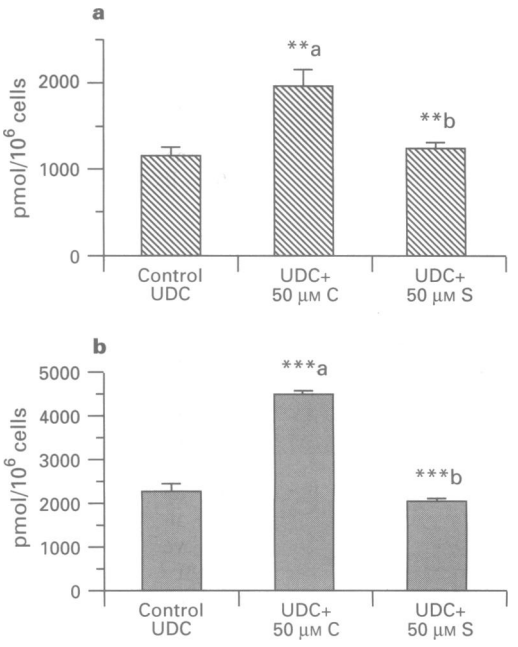

Figure 4 Quantities of ^{14}C bile salts synthesized from [^{14}C]-unesterified LDL-cholesterol and secreted in the culture medium of human hepatocytes. (a) The levels of ^{14}C tauroconjugated bile salts in the medium after 20 h incubation with [^{14}C]-unesterified LDL-cholesterol in presence of ursodeoxycholate (control UDC), with 50 μM crilvastatin (UCD + 50 μM C) or 50 μM simvastatin (UDC + 50 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were analysed by Student's t test and are significant at: $^{**}P < 0.01$ ([a] control UDC vs UDC + 50 μM C; [b] UDC + 50 μM C vs UDC + 50 μM S). Experiments were done in triplicate. (b) The levels of ^{14}C glycoconjugated bile salts in the medium after 20 h incubation with [^{14}C]-unesterified LDL-cholesterol in presence of ursodeoxycholate (control UDC) with 50 μM crilvastatin (UDC + 50 μM C) or 50 μM simvastatin (UDC + 50 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were analysed by Student's t test and are significant at: $^{***}P < 0.001$ ([a] UDC control vs UDC + 50μM C; [b] UDC ± 50 μM C vs UDC + 50 μM S). Experiments were done in triplicate.

Table 2 Apolipoprotein A1 and B100 levels recovered in the medium after 20 h of incubation

Apolipoprotein (μg per 10^6 cells)	*Cell type*	*Control*	*Control ursodeoxycholate*	*Ursodeoxycholate + 50 μM crilvastatin*	*Ursodeoxycholate + 300 μM crilvastatin*	*Ursodeoxycholate + 50 μM simvastatin*	*Ursodeoxycholate + 300 μM simvastatin*
Apo A1	HepG2	140 ± 5.8	127 ± 4.80	146 ± 7.50^{*a}	126 ± 4	130 ± 1.0	96.0 ± 4.0^{*b}
Apo A1	Human hepatocytes	ND	1210 ± 20	980 ± 60^{**a}	ND	1070 ± 20^{**b}	ND
Apo B100	HepG2	313.3 ± 3.3	302.5 ± 4.80	306 ± 6.80	296 ± 5.1	298.0 ± 2.0	274.0 ± 4.0^{*b}
Apo B100	Human hepatocytes	ND	4830 ± 80	3510 ± 320^{**a}	ND	3900 ± 50^{**b}	ND

Results are expressed as means ± s.e.mean ($n = 5$). Differences were determined by analysis of variance (ANOVA) for repeated values, by Fisher's PLSD test or Scheffe F-test at a probability of 95%. When the difference was significant by Fisher's test the symbol used is *, when it was significant by the two tests, the symbol is **([a]crilvistatin vs control ursodeoxycholate; [b]simvastatin vs control ursodeoxycholate). Experiments were done in triplicate. ND – not done.

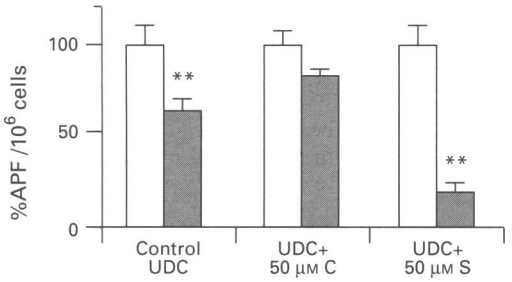

Figure 5 APF levels in the culture medium of human hepatocytes. Open columns: levels of APF in the medium at the beginning of incubation (time 0), stippled columns: after 20 h incubation. Human hepatocytes were incubated with [^{14}C]-unesterified LDL-cholesterol (control) in the presence of ursodeoxycholate (control UDC) with 50 μM crilvastatin (UDC + 50 μM C) or 50 μM simvastatin (UDC + 50 μM S). Results are expressed as means ± s.e.mean ($n = 5$). Differences were determined by analysis of variance (ANOVA) for repeated values at a probability of 95% by Fisher's PLSD test and Scheffe F-test. **Difference is significant with the two tests (UDC control at time 0 vs UDC control at time 20 h; UDC + 50 μM S at time 0 vs UDC + 50 μM S at time 20 h). Experiments were done in triplicate.

of AST in the incubation medium of Hep G2 cells. However, simvastatin at both 50 μM and 300 μM induced a significant increase in the transaminase levels in the incubation medium, an effect often described in man (Miserez *et al.*, 1994). Differences in AST observed between simvastatin and crilvastatin may be related to differences in drug metabolism. Crilvastatin has previously been shown to be without toxic effects in animals, likewise at chronic high doses (personal results). However, we cannot entirely exclude an interaction between the cell detoxification process and secretion of bile lipids or proteins. Simvastatin reduced the apo B100 level in the two cell types, crilvastatin reduced this level in human hepatocytes. In spite of a reduced synthesis of apoproteins in Hep G2 cells by mechanisms blocking the production of triglycerides (Dashti, 1991; Hahn *et al.*, 1992), the two statins are effective in reducing the quantitites of apo B100. This beneficial effect must be correlated with a decrease in the VLDL secretion by the cells, and an increase in LDL hepatic uptake, resulting from the enhanced expression of LDL receptors (Ma *et al.*, 1986). However, differences between crilvastatin and simvastatin can be explained by changes in the transport of the drugs, in relation to the difference in the chemical structure of the molecules. We previously advanced that crilvastatin, a hydrophobic molecule could be transported to a great extent by LDL and taken by the hepatocytes after recognition by the LDL receptor (Clerc *et al.*, 1995b). Such results were not observed with simvastatin. Crilvastatin increased the level of apo A1 secreted by Hep G2 cells; the opposite effect was observed in human hepatocytes. Such contrasting effects must be linked to a different apo A1 turnover by the two cell types, resulting from a lower lipoprotein expression and trafficking with Hep G2 cell, compared to human hepatocytes. The same explanation could be advanced to explain the differences for apo B100 between the two cell types. The level of APF after 20 h culture of human hepatocytes was higher in the presence of crilvastatin than of simvastatin. The enhanced level of APF in the medium induced by crilvastatin could be interpreted as a stimulant effect of the drug on the secretion of APF associated with lipid, implicated in biliary and-or HDL-like sources.

Stimulation of the synthesis of bile salts by crilvastatin, previously shown in the rats *in vivo* (Clerc *et al.*, 1993) and in rat isolated hepatocytes (Clerc *et al.*, 1995b), was clearly indicated in our experiments. In spite of the potential for Hep G2 cells to synthesize bile salts (Javitt *et al.*, 1989; Javitt, 1990), a defect in the catabolism of cholesterol into bile salts, particularly in the trihydroxy bile salt forms, was also described (Ostlund Farrants *et al.*, 1993). The level of radioactivity in the medium was comparable between 0 h and 20 h incubation of Hep G2 cells or human hepatocytes with 50 μM crilvastatin. The increased level of ^{14}C bile salts was related to the decreased level of ^{14}C cholesterol, resulting in comparable total amounts of ^{14}C in the medium. Our data show that crilvastatin stimulates synthesis of bile acids normally synthesized and secreted *in vivo*, i.e. glyco- and tauro-conjugated bile acids, this stimulating effect being more prominent in human hepatocytes than in Hep G2 cells. Such a difference between the two cell types could be due to a higher expression of LDL receptor and/or expression or activity of cholesterol 7-α hydroxylase in human hepatocyte, compared to Hep G2 cell. However, crilvastatin differs from simvastatin and other hypolipidaemic drugs in several respects. Unlike crilvastatin, simvastatin has an inhibitory effect on cholesterol 7-α hydroxylase activity (Leiss *et al.*, 1985; Björkhem, 1986). Bile salts may leave the peroxisomes as free acids prior to reconjugation with CoA (Russell & Setchell, 1992). Peroxisomal enzymatic proteins could have taurine and glycine as substrates and could be ultimately responsible for bile acid conjugation (Johnson *et al.*, 1991). The differences in the catabolism of LDL-cholesterol into bile salts under crilvastatin and simvastatin could be related to differences in the activity of peroxisomes. This hypothesis is supported by the fact that hypolipidaemic therapy such as bezafibrate leads to some disturbances of peroxisomes around the central veins (Lindauer *et al.*, 1994). Such disturbances finally result in alterations in the activity of cholesterol 7-α hydroxylase, the enzyme detected and localized in hepatocyte peroxisomes (Uegele *et al.*, 1991).

Our cell model validates previous results obtained in preclinical studies carried out with crilvastatin. It could give a suitable and interesting answer before the onset of clinical trials in man. The concentration, 50 μM, of the drugs used seems appropriate to test metabolic effects of cholesterol lowering drugs. Human hepatocytes appear to be useful for the study of cholesterol metabolism into bile salts and hepatic production of the three main apoproteins implicated in cholesterol homeostasis: apo B100, apo A1 and APF. In the presence of hypolipidaemic drugs such as crilvastatin, APF could be considered as an indicator of changes in both billary and/or HDL compartments. Thus this cell model could be used to elucidate two major questions. The first is to check drug activity by elucidating the precise effect of the drug on the subcellular target. The second is to test the efficiency of the drug in physiopathological conditions. Our human hepatocyte model led to more consistent data than those obtained with Hep G2 cells. Thus, suitable treatments of healthy cells in culture could result in a model of hypercholesterolaemia useful for subsequent studies of drug effects.

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