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The metabolism *in vitro* of human low-density lipoprotein by the human hepatoma cell line Hep G2

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The human hepatoma cell line Hep G2 was studied with respect to metabolism of human low-density lipoprotein (LDL). The Hep G2 cells bind, take up and degrade human LDL with a high-affinity saturable and with a low-affinity non-saturable component. The high-affinity binding possesses a K_D of 25 nM-LDL and a maximal amount of binding of about 70 ng of LDL-apoprotein/mg of cell protein. The high-affinity binding, uptake and degradation of LDL by Hep G2 cells is dependent on the extracellular Ca^{2+} concentration and is down-regulated by the presence of fairly high concentrations of extracellular LDL. Incubation of the Hep G2 cells with LDL results in suppression of the intracellular cholesterol synthesis. It is concluded that the human hepatoma cell line Hep G2 possesses specific LDL receptors similar to the LDL receptors demonstrated on extrahepatic tissue cells.

The level of serum cholesterol present in the LDL fraction is one of the main risk factors for atherosclerosis. During the last decade catabolism of LDL via high-affinity LDL receptor sites (B, E receptors) *in vivo* has been widely observed (see, for example, Shepherd *et al.*, 1979).

The role of the liver in the catabolism of serum LDL has been investigated by several groups of investigators, using various animal species. Some groups have tended to minimize the role of the liver in LDL catabolism (Sniderman *et al.*, 1974; Sigurdsson *et al.*, 1978), whereas others have suggested that the liver plays a very active role in removal of LDL from the circulation (Van Tol *et al.*, 1978; Kovanen *et al.*, 1979; Soltys & Portman, 1979; Kita *et al.*, 1981; Attie *et al.*, 1981; Pangburn *et al.*, 1981). Harders-Spengel *et al.* (1982) were able to show the presence of specific LDL-binding sites on adult human liver membranes. They suggest that liver LDL receptors are also involved in LDL catabolism in man.

The technique of the measurement of the binding of LDL to liver membrane fractions does not allow the measurement of the subsequent uptake and degradation of LDL and of intracellular metabolic regulation by LDL. Study of the metabolism of human LDL by human liver cells encounters some major problems: (i) the difficulty of obtaining pieces of fresh human liver routinely; (ii) the standardization of cell isolation; and (iii) the exposure of the hepatocytes during the isolation procedure to connective-tissue-disrupting enzymes such as collagenase that have been reported to influence binding of lipoproteins (Capuzzi *et al.*, 1979).

Therefore, in order to study the metabolism of human LDL by human liver cells, we decided to study the hepatoma cell line Hep G2 with respect to interaction with LDL. It is known from the literature that this cell line still displays a number of specific liver cell functions, such as synthesis of a number of plasma proteins (Knowles *et al.*, 1980) and of apolipoproteins (Zannis *et al.*, 1981). It is also known that the Hep G2 cells possess a functional receptor for asialoglycoproteins (Schwarz *et al.*, 1981). In the present study we show that Hep G2 cells exert high-affinity LDL receptor activity and subsequent handling of LDL, similar to that shown for various extrahepatic tissue cells (Goldstein & Brown, 1977).

Abbreviations used: LDL, low-density lipoprotein; DiI, 3,3,3′,3′-tetramethyl-1,1′-dioctanoylindocarbocyanine.

Materials and methods

Preparation of serum and lipoproteins

Human serum was prepared from freshly collected blood obtained from healthy donors.

Lipoprotein-depleted serum (LPDS) was obtained by ultracentrifugation of serum at a density of 1.21 g/ml followed by extensive dialysis of the infranatant against subsequently phosphate-buffered

saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 i.u./ml) and streptomycin (0.1 mg/ml).

LDL was isolated from freshly prepared serum by density-gradient ultracentrifugation by the method of Redgrave *et al.* (1975) followed by tube slicing. A narrow-density fraction (density 1.04–1.05 g/ml) was used. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed only the presence of apolipoprotein B (apo B). This LDL preparation was immediately used for iodination by the [^{125}I]-iodine monochloride method described by Bilheimer *et al.* (1972). After iodination LDL was dialysed against PBS for 4 h (4 × 500 vol.). Thereafter, it was stabilized by the addition of LPDS (15%, v/v) and further dialysed overnight against 500 vol. of DMEM supplemented with 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH 7.4), penicillin and streptomycin. The stabilized ^{125}I-labelled LDL was stored at 4°C. Its specific radioactivity ranged from 80 to 120 c.p.m./ng of LDL protein. Although stable for periods greater than 1 month under these conditions (Havekes, 1981), the iodinated LDL was used within 2 weeks. The trichloroacetic acid-soluble-free iodine-free fraction was less than 1%. The free iodine fraction of the ^{125}I-labelled LDL samples was less than 0.01%. When not labelled with [^{125}I]iodine, LDL was immediately stabilized with the addition of 15% LPDS and extensively dialysed against subsequently PBS and DMEM supplemented with penicillin and streptomycin.

Culturing of Hep G2 cells

The established Hep G2 cell line, derived from human liver tumour, was obtained from Dr. B. B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, U.S.A.). The cells were cultured at 37°C in 25 cm^2 flasks or Multiwell dishes (16 mm diameter; Costar) containing 0.1–0.2 ml of DMEM/cm^2 supplemented with 10% heat-inactivated foetal-bovine serum (Flow Laboratories, Irvine, Scotland, U.K.), penicillin and streptomycin under CO_2/air (1:19). The medium was renewed twice a week. Before the binding experiments the medium was replaced 18 h before assay with a similar medium containing 20% human LPDS or different amounts of serum as indicated. The concentration of LDL-bound apo B in serum was measured as described by Havekes *et al.* (1981).

Binding, internalization and degradation of ^{125}I-LDL by Hep G2 cells

At 18 h before the experiment the medium of the cell cultures (150–250 μg of cell protein/cm^2) grown in Multiwell dishes (16 mm diameter; Costar) was replaced with medium containing either 20% human LPDS or different amounts of human serum as indicated. When serum was present in the medium during these 18 preceding hours, the cells were, shortly before the experiment, washed and incubated three times with DMEM supplemented with bovine serum albumin (10 mg/ml) during a period of 30 min at 37°C. The experiment was started by replacing the medium by 0.3 ml of DMEM containing 20 mM-Hepes, pH 7.4, 20% LPDS and various amounts of ^{125}I-LDL in the presence or in the absence of either 300 μg of unlabelled LDL/ml or 3 mM-EDTA. Dishes without cells were used as controls. After 3 h of incubation at 37°C the medium was removed for determination of LDL degradation. The ^{125}I-LDL degradation was determined as 10% (w/v) trichloroacetic acid-soluble radioactivity (Goldstein *et al.*, 1976). In order to determine whether Hep G2 cells actively deiodinate ^{125}I-labelled LDL or its degradation products (Soltys & Portman, 1979; Attie *et al.*, 1981; Pangburn *et al.*, 1981) the radiolabelled free iodine fraction was extracted from a portion of the trichloroacetic acid-soluble fraction with chloroform after the addition of excess of unlabelled KI and H_2O_2 as described by Goldstein *et al.* (1976). In another portion of the trichloroacetic acid-soluble fraction the labelled free iodine was not removed. The difference in radioactivity between these trichloroacetic acid-soluble fractions with and without extraction of labelled free iodine represents the amount of deiodination of ^{125}I-LDL or ^{125}I-LDL degradation products by Hep G2 cells.

After removal of the incubation medium each cell dish was cooled to 0°C and washed exactly as described by Goldstein *et al.* (1976). When total cell-associated ^{125}I-LDL was measured the washed cells were dissolved in 0.2 M-NaOH. The radioactivity in the cell lysate represents the total amount of ^{125}I-labelled LDL that became cell-associated. A portion of the cell lysate was used for protein determination by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. When ^{125}I-LDL binding and internalization was measured separately, after washing the cells were incubated with heparin containing buffer for 1 h at 0°C (Goldstein *et al.*, 1976). The radioactivity released into the heparin buffer reflects the amount of ^{125}I-LDL bound to the plasma membrane. The radioactivity that remained cell-associated represents the amount of intracellular ^{125}I-LDL.

Measurement of intracellular cholesterol synthesis

Cells were grown in 25 cm^2 culture flasks as described above for 2 weeks, at which time there were about 2.5 × 10^6 cells per flask. Before addition of ^3H_2O, used for determination of the rate of cholesterol synthesis, the cells were pre-incubated in 2 ml of fresh DMEM buffered with 20 mM-Hepes and containing 10% human LPDS and 0–50 μg of protein/ml of human LDL for 18 h. Then, 0.48 mCi

of ^3H_2O was added as 0.125ml of Krebs–Henseleit solution (sterilized by passage through a Millex 0.2μm filter) to each of the bottles, and incubation was continued for 24h. The media were then poured off into 35ml glass tubes and extracted by the method of Bligh & Dyer (1959). The cells were detached with 1ml of 0.05% trypsin solution, and the resulting suspension was transferred to a 35ml glass tube. The culture flask was rinsed with 1ml of 0.9% NaCl (saline), which was combined with the cell suspension. The final cell suspensions were also extracted by the method of Bligh & Dyer (1959). The chloroform layers were collected and blown dry using a stream of N_2 in a hood. Incorporation of ^3H into non-esterified and esterified cholesterol was measured as described by Kempen *et al.* (1983).

Morphological studies of LDL interaction with Hep G2 cells

Fluorescent LDL was prepared by incubating LDL with DiI (Molecular Probes Inc., Junction City, OR, U.S.A.), as described by Pitas *et al.* (1981). In brief, to LDL (2mg/ml in 25% LPDS) was added DiI (dissolved in dimethyl sulphoxide) to a final concentration of 50μg/ml, and the mixture was incubated for 24h at 37°C. LDL was then re-isolated by density-gradient ultracentrifugation. The concentration of DiI was determined by extracting 0.1ml of LDL with 0.9ml of methanol, followed by centrifugation and measuring the absorbance at 550nm. As a standard, DiI in methanol (1–10μg/ml) was used. To the fluorescent LDL (which contained 30–35ng of DiI/μg of LDL-protein), human LPDS was added (10%, v/v) and the LDL was dialysed overnight against saline at 4°C.

Hep G2 cells were incubated overnight in 10% foetal-calf serum or in 10% LPDS in DMEM, washed once with the same medium and incubated in the same medium to which 50μg of DiI-labelled LDL/ml had been added. After incubation for 2h at 4°C, or for 15, 30 and 60min at 37°C, cells were washed three times with PBS/1% bovine serum albumin at 4°C and fixed with 4% *p*-formaldehyde in PBS for 30min at 4°C. Fluorescence microscopy was performed with incident light illumination, using the rhodamine filter package. In control experiments, a 10-fold excess of non-fluorescent LDL was used.

Results

Binding, internalization and degradation of ^{125}I-LDL by Hep G2 cells

In Fig. 1 binding, internalization and degradation of ^{125}I-LDL by cultures of Hep G2 cells pre-incubated for 18h in 20% LPDS are shown. The relationship between LDL concentration and respectively binding, internalization and degradation is curvilinear but the binding, internalization and degradation continued to increase with increasing LDL concentrations up to 250μg/ml. These data indicate high- and low-affinity binding of LDL to Hep G2 cells.

From the results in Fig. 1 it is also shown that after 3h of incubation the amount of LDL taken up and degraded by the Hep G2 cells exceeds the amount of LDL bound (Fig. 1). Furthermore, Fig. 1(*c*) shows that deiodination by Hep G2 cells of ^{125}I-labelled LDL or of its degradation products contributes about 30% of the total trichloroacetic acid-soluble radioactivity.

The high-affinity LDL receptor on fibroblasts is

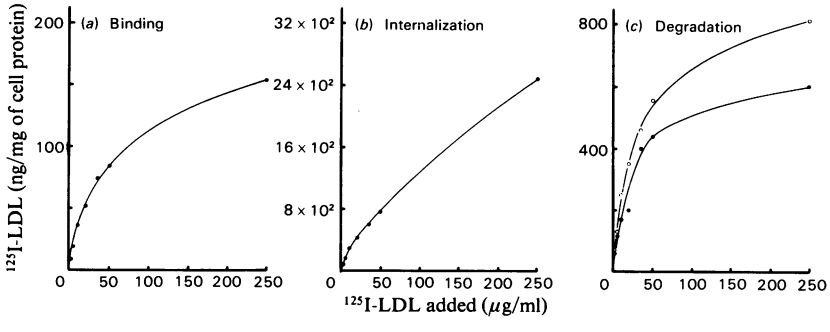

Fig. 1. *Binding (a), internalization (b) and degradation (c) of human LDL by confluent cultures of Hep G2 cells*
Cells were pre-incubated in medium supplemented with 20% LPDS. Binding, internalization and degradation were measured after 3h of incubation at 37°C with ^{125}I-labelled LDL as described in the Materials and methods section. The units on the horizontal axis represent μg of LDL protein/ml. The degradation was measured with (●) and without (○) extraction of free iodine from the trichloroacetic acid-soluble fraction. The difference between the results for the two methods represents the amount of deiodination by the Hep G2 cells.

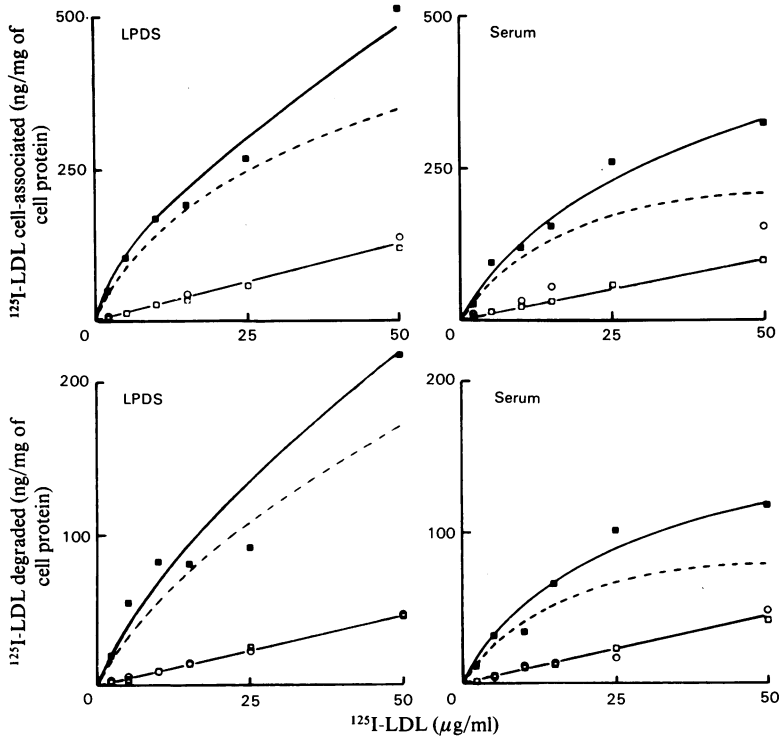

Fig. 2. *Effect of EDTA and of prior incubation with 20% human serum (0.65 g of LDL-bound apo B/litre) in place of 20% LPDS on cell-associated ^{125}I-LDL and on ^{125}I-LDL degradation by Hep G2 cells*

After reaching confluency the cells were incubated in 20% human serum or 20% human LPDS for 18 h. Thereafter the cells were washed as described in the Materials and methods section. During the experiment the cells were incubated with 20% LPDS containing increasing concentrations of ^{125}I-LDL (μg of LDL protein/ml) as indicated and in the presence or in the absence of 3 mM-EDTA or 300 μg of unlabelled LDL protein/ml. ■, Total amount of ^{125}I-LDL protein that became cell-associated or degraded; □ and ○, the amount of ^{125}I-LDL protein that became cell-associated or degraded in the presence of respectively 300 μg of unlabelled LDL protein/ml or 3 mM-EDTA. ----, the difference in cell-association or degradation in the absence and in the presence of 300 μg of unlabelled LDL protein/ml (high affinity cell-associated and degraded ^{125}I-LDL).

reported to be Ca^{2+}-dependent (Goldstein & Brown, 1977). Fig. 2 shows that the cell association and degradation of ^{125}I-LDL by Hep G2 cells is inhibited by the addition of 3 mM-EDTA. This inhibition is comparable with the inhibition by the addition of excess of unlabelled LDL.

The LDL receptor on extrahepatic cell types is regulated by the presence of LDL in the culture medium (Goldstein & Brown, 1977). To determine whether the high-affinity LDL receptor of the Hep G2 cells is similarly regulated, the Hep G2 cells were, before the binding experiment, pre-incubated in either 20% human LPDS or 20% human serum for 18 h.

Fig. 2 shows that the high-affinity receptor-mediated cell association and degradation of ^{125}I-LDL are only partly repressed when the cells are pre-incubated in medium containing 20% full human serum (0.65 g of LDL-apo B/litre) instead of 20% human LPDS. The cell association and degradation of ^{125}I-LDL by Hep G2 cells in the presence of an excess of unlabelled LDL is not sensitive to pre-incubating conditions. From Fig. 2 it seems that the repression of the high-affinity LDL receptor activity by pre-incubation with 20% human serum instead of 20% LPDS is only significant when higher concentrations of ^{125}I-LDL are used. Therefore, in order to investigate the regulation of the LDL receptor activity of Hep G2 cells in more detail the Hep G2 cells were pre-incubated with either 20% LPDS or 20 or 100% familial-hypercholesterolaemic serum (LDL-cholesterol and LDL-apo B concentrations are 11 mM and 1.6 g/litre respectively). Comparison of the results in Fig. 3 with the results from Fig. 2 shows that only after pre-incubation with fairly high concentrations of LDL the LDL receptor activity of Hep G2 cells is significantly repressed.

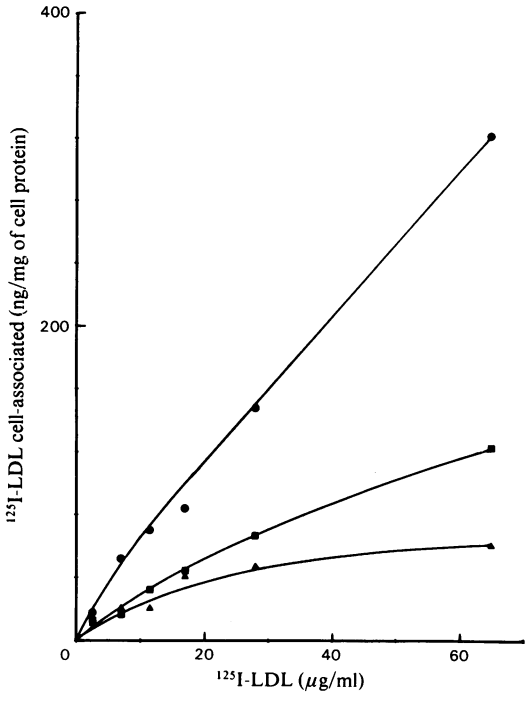

Fig. 3. *Effect of prior incubation of Hep G2 cells with 20 and 100% familial-hypercholesterolemic (FH) serum (LDL-cholesterol and apo B respectively 11 mM and 1.6 g/litre) on high-affinity cell-association of ^{125}I-LDL*

At 18h before the experiment the medium was replaced with medium containing 20% LPDS (●), 20% FH serum (■) or 100% FH serum (▲). Before the addition the LPDS and FH serum was dialysed against DMEM. After pre-incubation for 18h the cells were washed as described in the Materials and methods section. The experiment was started by adding medium containing 20% LDPS and various amounts of ^{125}I-LDL protein in the presence or in the absence of 300μg of unlabelled LDL protein/ml. The high-affinity cell-association is calculated from the difference in cell-association in the absence and in the presence of 300μg of unlabelled LDL protein/ml.

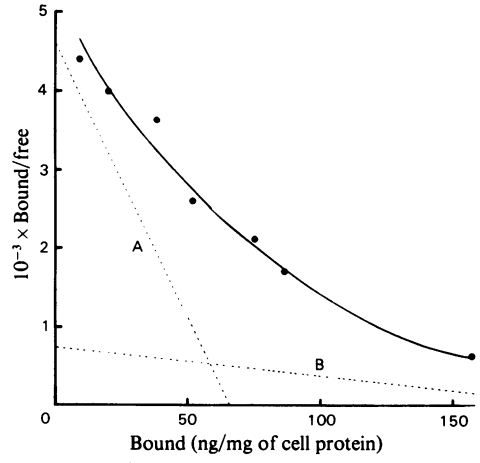

Fig. 4. *Scatchard plot of the binding of ^{125}I-LDL to Hep G2 cells*

Binding was measured as heparin-releasable ^{125}I-LDL after incubation of the cells with ^{125}I-LDL for 3h at 37°C in the absence of excess of unlabelled LDL. The steep broken line A represents the high-affinity component of the ^{125}I-LDL binding. Line B represents the low-affinity component of the ^{125}I-LDL binding.

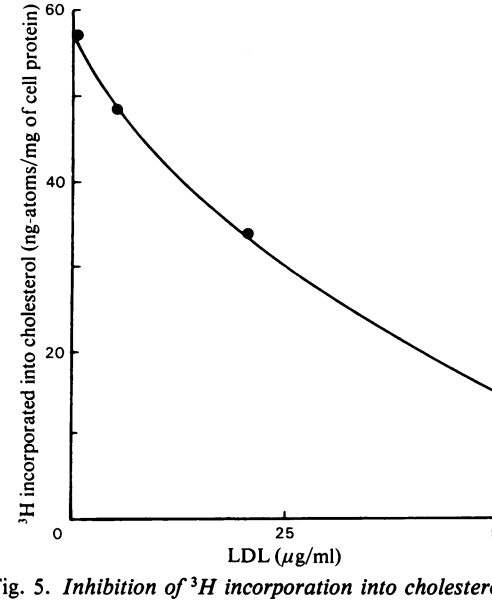

Fig. 5. *Inhibition of ^{3}H incorporation into cholesterol by LDL*

At 18h before the experiment the cells were pre-incubated in medium supplemented with 10% LPDS and increasing amount of LDL protein as indicated. Thereafter ^3H_2O was added and incubation was continued for 24h. The measurement of the incorporation of ^{3}H into cholesterol was carried out as described in the Materials and methods section. The rate of cholesterol synthesis is expressed as ng-atoms of ^{3}H incorporated into cholesterol per mg of cell protein during 24h.

In Fig. 4 the binding of ^{125}I-LDL to Hep G2 cells is analysed by a Scatchard (1949) plot. This plot shows that saturable high-affinity and non-saturable low-affinity binding of ^{125}I-LDL exist on Hep G2 cells. The apparent K_D for the high-affinity binding is 25nM-LDL (the molecular weight of LDL-apolipoprotein is assumed to be 6×10^5). The maximum amount of ^{125}I-LDL bound to the high-affinity receptor is about 70ng of ^{125}I-LDL apoprotein/mg of cell protein.

Suppression of intracellular cholesterol synthesis

Uptake and degradation of LDL by extrahepatic cells via the high-affinity LDL receptor results in

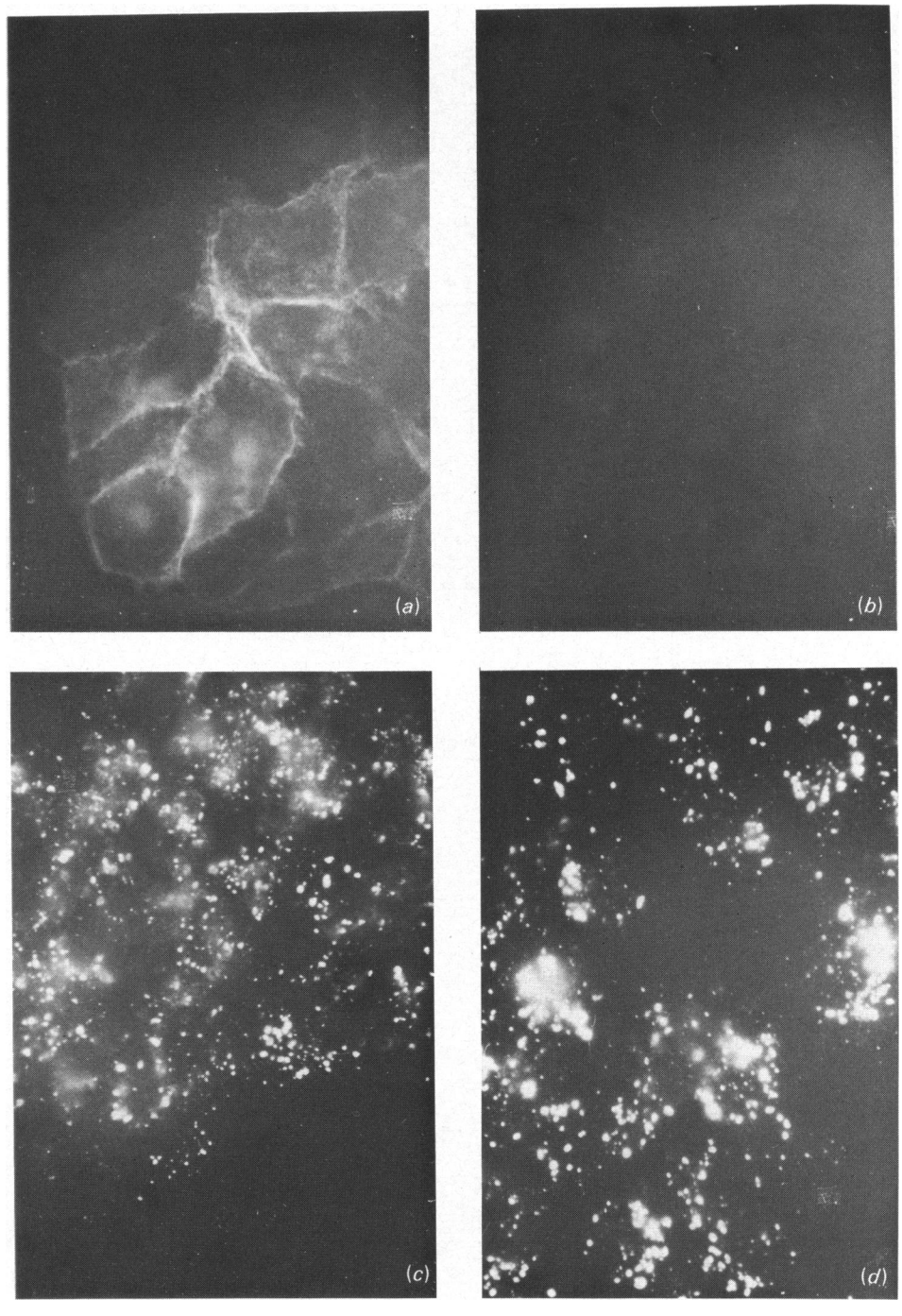

Fig. 6. *Visual detection of the binding and uptake of LDL by Hep G2 cells*
Cells were pre-incubated overnight in 10% LPDS in DMEM, washed once with the same medium and incubated in the same medium to which 50 μg of DiI-labelled LDL/ml had been added. The cells were incubated further either for 2 h at 4°C in the absence (*a*) or in the presence (*b*) of a 10-fold excess of non-fluorescent LDL or for 15 (*c*) or 60 (*d*) min at 37°C in the absence of excess non-fluorescent LDL.

suppression of the endogenous cholesterol synthesis (Goldstein & Brown, 1977). Fig. 5 shows that addition of LDL to the culture medium of Hep G2 cells also results in a concentration-dependent suppression of the intracellular cholesterol synthesis. At 50 μg of LDL/ml the incorporation of ^3H from ^3H_2O was decreased by about 70%.

Visual detection of LDL binding and uptake by Hep G2 cells

Hep G2 cells showed, after incubation with DiI-labelled LDL for 2h at 4°C, a diffuse membrane fluorescence (Fig. 6*a*). This fluorescence was slightly stronger in cells pre-incubated in LPDS than in cells pre-incubated in foetal-calf serum (results not shown). Cells incubated with DiI-labelled LDL in the presence of a 10-fold higher concentration of non-fluorescent LDL were barely visible (Fig. 6*b*). This indicates that the membrane fluorescence observed after incubation with DiI-labelled LDL is not due to staining by free DiI, but represents high-affinity saturable LDL binding. After incubation with DiI-labelled LDL at 37°C for 15 to 60 min the cells showed strong intracellular granular staining, which increased with incubation time, and diffuse surface fluorescence, at all time intervals studied (Fig. 6*c* and 6*d*).

Discussion

We have presented several lines of evidence indicating that Hep G2 cells bind, take up and degrade LDL in part by a high-affinity mechanism similar to the B,E-receptor present in extrahepatic tissue cells (Brown *et al.*, 1981): (i) binding, uptake and degradation as a function of LDL concentration proceeds, in part, via a saturable receptor; (ii) morphological studies with DiI-labelled LDL showed that the Hep G2 cells possess saturable LDL binding; (iii) the LDL bound is heparin releasable; (iv) the binding, uptake and degradation of LDL by Hep G2 cells is Ca^{2+}-dependent; and (v) incubation of Hep G2 cells with LDL results in inhibition of intracellular cholesterol synthesis.

Our findings show that the binding, internalization and degradation of LDL by Hep G2 cells proceeds via two components, one that is saturable with high affinity and one that is non-saturable with low affinity. The high-affinity component exhibited a K_D of 25 nM-LDL and a maximal amount of binding of about 70 ng of LDL-apolipoprotein per mg of cell protein. These values are in the same order of magnitude as found for the binding of LDL to extrahepatic LDL receptors (Brown *et al.*, 1979; Pitas *et al.*, 1979).

The low-affinity non-saturable component contributes markedly (about 30%) to the total interaction of LDL with Hep G2 cells. This agrees with studies performed on animal hepatocytes (Soltys & Portman, 1979; Attie *et al.*, 1981; Pangburn *et al.*, 1981) and with studies carried out on liver membranes (Brown *et al.*, 1979).

Degradation of LDL via the high-affinity receptor pathway in fibroblasts and monocytes is associated with a decreased cellular cholesterol synthesis (Goldstein & Brown, 1977; Havekes *et al.*, 1980; Ponec *et al.*, 1981). In the present paper we show that in Hep G2 cells the incorporation of ^3H from ^3H_2O into cholesterol is decreased by about 70% after 18h of incubation with 50 μg of LDL/ml. At this LDL concentration the relative suppression of the cellular cholesterol synthesis is comparable with that found by Pangburn *et al.* (1981) for pig hepatocytes and rather low compared with the LDL-mediated suppression of cholesterol synthesis in human fibroblasts (Goldstein & Brown, 1977) or human monocytes (Ponec *et al.*, 1981).

In extrahepatic tissue cells the number of LDL receptors expressed is subject to down-regulation by LDL (Goldstein & Brown, 1977). The Hep G2 cells only show a weak down-regulation of the high-affinity cell association of LDL after pre-incubation for 18h in 20% normal human serum compared with 20% LPDS. These results agree with the relatively weak LDL-mediated down-regulation of the LDL receptor activity in cultured pig hepatocytes (Pangburn *et al.*, 1981). We found that the high-affinity LDL receptor activity of Hep G2 cells is markedly, but still not completely, suppressed after pre-incubation of the Hep G2 cells in fairly high and even pathophysiological concentrations of LDL. This finding is comparable with the results reported by Fogelman *et al.* (1981) and Knight & Soutar (1982). They found that LDL receptors on human blood monocytes are not suppressed unless the LDL concentration in the medium is raised to fairly high levels.

In vivo, hepatocytes are in contact with physiological concentrations of LDL that are much higher than those sufficient to repress LDL receptors on cultured fibroblasts. Our results with the Hep G2 cells, used as a model for human hepatocytes, suggest that high-affinity LDL receptor activity of the liver is expressed *in vivo* irrespective of the presence of comparatively high LDL concentrations.

The presence of LDL receptor activity in livers *in vivo* has already been observed in dogs (Kovanen *et al.*, 1981) and rabbits (Chao *et al.*, 1982) after treatment with cholestyramine. Mahley *et al.* (1981) were unable to show the presence of specific LDL receptors on membranes of adult human livers. However, Brown *et al.* (1979) reported specific LDL receptor activity on hepatic membranes from human foetuses. It is possible that in human livers the LDL receptor activity decreases with increasing age compared with that found for immature and

adult dogs (Mahley *et al.*, 1981). In contrast with the results of Mahley *et al.* (1981), Harders-Spengel *et al.* (1982) were able to show the presence of LDL receptors on membrane fractions prepared from adult human liver biopsies. They suggest that LDL receptors are expressed in normal human livers and that hepatic LDL receptors contribute to the catabolism of LDL in man under physiological conditions.

From the results reported in the present paper, we conclude that the use of cultured Hep G2 cells offers a potentially helpful tool in studying the metabolism of LDL by the liver in man.

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