Characterization of the low-density-lipoprotein-receptor-independent interaction of β -very-low-density lipoprotein with rat and human parenchymal liver cells *in vitro*

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β -Migrating very-low-density lipoprotein (β -VLDL) is a cholesteryl-ester-enriched lipoprotein which under normal conditions is rapidly cleared by parenchymal liver cells. In this study the characteristics of the interaction of β -VLDL with rat parenchymal cells, Hep G2 cells and human parenchymal cells are evaluated. The binding of β -VLDL to these cells follows saturation kinetics ($B_{\max}$, respectively 117, 106 and 103 ng of β -VLDL apolipoprotein/mg of cell protein), with a relatively high affinity (K_d respectively for β -VLDL of 10.7, 5.1 and 8.4 μ g/ml). Competition studies of unlabelled β -VLDL, low-density lipoprotein (LDL) or acetylated LDL with the binding of radiolabelled β -VLDL indicate that a LDL-receptor-independent, Ca^{2+} -independent, specific recognition site for β -VLDL is present on rat and human parenchymal cells, whereas with Hep G2 cells or mouse macrophages β -VLDL recognition is performed by the LDL receptor. The binding of β -VLDL to Hep G2 cells was down-regulated by 89 % by prolonged exposure to β -VLDL, whereas for human parenchymal and rat parenchymal cells down-regulation of 44 % and 20 % respectively was observed. Studies with antibodies against the LDL receptor support the presence of a LDL-receptor-independent specific β -VLDL recognition site on rat and human parenchymal cells. It is concluded that a LDL-receptor-independent recognition site for β -VLDL is present on rat and human parenchymal liver cells. The presence of a LDL-receptor-independent recognition site on human parenchymal cells may mediate *in vivo* the uptake of β -VLDL during consumption of a cholesterol-rich diet, when LDL receptors are down-regulated, thus protecting against the extrahepatic accumulation of the atherogenic β -VLDL constituents.

INTRODUCTION

β -VLDL is an abnormal very-low-density lipoprotein (VLDL) that accumulates in the plasma from cholesterol-fed animals and from patients with type III hypercholesterolaemia [1–4]. It is enriched in cholesteryl esters and on electrophoresis it shows β -mobility. In blood monocytes and macrophages β -VLDL stimulates the synthesis and accumulation of cholesteryl esters, and this lipoprotein is thus considered to be specifically atherogenic [5–8].

The interaction of β -VLDL with macrophages has been described in a number of studies, and it has been shown that the uptake of this lipoprotein is mediated via a high-affinity receptor, which is related to the LDL (apo B,E) receptor [8–10]. This receptor requires Ca^{2+} for optimal binding of β -VLDL, whereas the cell association of radiolabelled β -VLDL can be competed for by both unlabelled β -VLDL and LDL. The observation that β -VLDL binding correlates directly with the amount of LDL receptors has led to the conclusion that in macrophages a so-called unusual LDL receptor is the predominant, if not the only, receptor responsible for β -VLDL binding. This receptor differs from the classical LDL receptor described in human fibroblasts in its low affinity for LDL and its relative resistance to down-regulation by extracellular cholesterol [8–10].

In vivo, it has been demonstrated that β -VLDL is rapidly cleared from the blood by the liver and that predominantly the liver parenchymal cells are responsible for this uptake [11,12].

Experiments *in vivo* have shown that in dogs the clearance of β -VLDL is retarded after cholesterol feeding or infusion with bile acids, and it was suggested that in dogs the LDL receptor is responsible for β -VLDL uptake by liver [11]. In rats, parenchymal liver cells hardly express active LDL receptors [13,14], and we suggested previously that β -VLDL in rats is therefore recognized by a so-called remnant receptor [12].

The existence of a remnant receptor in liver was supported by observations in patients with homozygous familial hypercholesterolaemia and WHHL rabbits in whom LDL receptors are genetically defective [15]. Both chylomicron remnants and large VLDL particles are rapidly cleared from the blood in these LDL-receptor-defective species [16,17], suggesting that a second receptor may mediate such clearance. Work by Herz *et al.* [18] suggested that a high-molecular-mass protein (the so-called LDL-receptor-related protein, LRP) might be an attractive candidate. Subsequently Kowal *et al.* [19,20], Lund *et al.* [21] and Beisiegel *et al.* [22] provided evidence that apo E might bind to LRP, although enrichment of β -VLDL *in vitro* with apo E was necessary to express binding.

The aim of the present study was to characterize the ability of β -VLDL to interact with isolated parenchymal cells, which form *in vivo* the site of rapid uptake [12,23]. The interaction of β -VLDL with rat parenchymal cells is thereby compared with data obtained simultaneously with Hep G2 and human parenchymal cells in order to verify the relevance of the animal data for the human situation.

Abbreviations used: VLDL, very-low-density lipoproteins; β -VLDL, β -migrating very-low-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; apo, apoprotein; LRP, LDL-receptor-related protein; PBS, phosphate-buffered saline; DMEM, Dulbecco's Modified Eagle's Medium; HSA, human serum albumin.

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METHODS

For the isolation of β -VLDL, male Wistar rats were maintained on a cholesterol-rich chow, which included 2% cholesterol, 5% olive oil and 0.5% cholic acid (Hope Farms, Woerden, The Netherlands). The rats were fasted for 20 h before blood was collected from the abdominal aorta. The sera obtained were centrifuged at 250 000 *g* for 22 h at 4 °C, by using a discontinuous KBr gradient [24]. The $d < 1.006$ fractions containing β -VLDL were obtained by tube slicing and dialysed against phosphate-buffered saline (0.15 M-NaCl/10 mM-phosphate, pH 7.4), containing 1 mM- Na_2EDTA (PBS-EDTA). Free cholesterol, cholesterol ester, triacylglycerol and phospholipid contents of β -VLDL were determined by enzymic procedures (Boehringer, Mannheim, Germany). Protein content was determined by the Lowry method [25] in the presence of 0.1% SDS. In order to determine the apoprotein compositions of the VLDL preparations, 10–30 μg samples were subjected to electrophoresis on SDS/5–17.5%-polyacrylamide gels. The gels were stained with 0.2% Coomassie Blue and the stained bands were identified and scanned. Both the chemical composition and the apolipoprotein composition were similar, as reported extensively previously [12].

β -VLDL was labelled with ^{125}I at pH 10.0 by a modification [26] of the ICI method [27]. Free ^{125}I was removed by Sephadex G-50 gel-filtration, followed by dialysis against PBS-EDTA. The distribution of radioactivity in the labelled β -VLDL preparation was $77.6 \pm 10.7\%$ in protein, $18.4 \pm 7.4\%$ in lipid and $3.9 \pm 1.4\%$ unbound, determined as described by Folch *et al.* [28]. The distribution of the protein-associated radioactivity over the various apoproteins was determined by electrophoresis of 5 μg of apolipoprotein from ^{125}I - β -VLDL (approx. 1.2×10^6 c.p.m.) on SDS/5–17.5%-polyacrylamide gel. The gel was stained with 0.2% Coomassie Blue and dried. The various protein bands were identified, cut out, and counted for radioactivity. It was found that $41.6 \pm 5.6\%$ of the protein-associated radioactivity was associated with apoproteins B100 and B48, $3.8 \pm 2.2\%$ with apoprotein AI, $14.0 \pm 7.2\%$ with apoprotein E and $40.6 \pm 8.2\%$ with apoprotein Cs.

LDL was obtained from sera from healthy fasted volunteers. These sera were centrifuged at 250 000 *g* in a discontinuous KBr gradient for 22 h at 4 °C (see above). The $1.024 < d < 1.055$ fraction was re-centrifuged by the same procedure. LDL was subsequently dialysed overnight against PBS-EDTA. The apo E content of LDL was less than 0.03% [29]. LDL was acetylated as described by Basu *et al.* [30]. In this procedure 1 ml of LDL, with a protein concentration between 2 and 10 mg/ml, was added to 1 ml of a saturated solution of sodium acetate (pH 7.4). This solution was placed in an ice/water bath. Under continuous stirring, acetic anhydride was added in 2 μl portions over a period of 1 h and in a total amount of 1.5 times the amount of protein used. The solution was then incubated for an additional 30 min and dialysed against PBS-EDTA at 4 °C.

Rat parenchymal cells

Rat parenchymal cells were isolated as described previously [29,31]. In short, a liver perfusion was performed during 10 min at 37 °C, with carbogen-saturated Hanks' buffer (pH 7.4), containing glucose (1.0 g/l) and Hepes (1.6 g/l; Merck, Darmstadt, Germany) at a flow rate of 14 ml/min. The perfusion was continued for 20 min with the same buffer to which collagenase (type I; Sigma, St. Louis, MO, U.S.A.) had been added to a final concentration of 0.05%. The liver tissue was then gently minced on ice and a crude cell suspension, containing parenchymal and non-parenchymal cells, was obtained. This cell suspension was filtered through a 50 μm -mesh nylon gauze and washed by four

successive centrifugations at 50 *g* for 1 min, after each of which the supernatant was discarded and the pellet was resuspended in Hanks' buffer containing 0.2% BSA (fraction V; Sigma). After the last centrifugation step the supernatant was aspirated. The cell pellet contained solely parenchymal cells and was used for the lipoprotein-binding studies.

For the studies with cultured cells, the rat parenchymal cells were maintained for 2 days in 25 mm multiwell culture dishes (Costar, Cambridge, MA, U.S.A.) at a density of 0.5×10^6 cells per ml and per well. The culture medium consisted of Williams E, supplemented with 10% (v/v) heat-inactivated fetal-calf serum, 2 mM-glutamine, 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml (Boehringer). At 20 h before the lipoprotein-binding assay the culture medium was replaced by Williams E containing 1% BSA.

Isolation and culture of human hepatocytes

Human hepatocytes were isolated from livers which were obtained through the Auxiliary Partial Liver Transplantation Program at the University Hospital Dijkzigt in Rotterdam, The Netherlands. Permission was given by the Medical Ethical Committee to use the remaining non-transplanted part of the donor liver for scientific research. The livers were taken from physically healthy organ donors, who died after brain haemorrhages or severe traumatic brain injury. During resection of the left lobe, the livers were perfused by portal-vein cannulation with Euro-Collins preservation solution at 4 °C [31a].

After resection, the left liver lobe was transported to the perfusion site within 45 min in a cold buffer (4 °C) containing 10 mM-Hepes, pH 7.4, 142 mM-NaCl, 6.7 mM-KCl and 0.5 mM-EGTA. Perfusion with 3 litres of this buffer at a rate of 40 ml/min per catheter was started after insertion of four polyethylene catheters (18 gauge) in the vascular orifices that were identified at the dissection surface. After the pre-perfusion the liver was perfused successively with 500 ml of a 10 mM-Hepes buffer, pH 7.6, containing 5 mM- CaCl_2 without recirculation, and with 200 ml of this buffer containing 0.05% and 0.1% collagenase, respectively, with recirculation for 20 min each. Liver tissue was dissociated in a Hanks buffer containing 2% BSA; cells were filtered through a 250 μm -pore filter, centrifuged (50 *g* for 30 s) and washed three times in cold Williams E culture medium (4 °C) to remove damaged and non-parenchymal cells. At this stage, the viability of the cells varied between 51 and 77%. Cells were seeded on 12-well cluster plates at a density of 0.5×10^6 cells/well and were maintained in Williams E medium supplemented with 10% heat-inactivated fetal-calf serum, 2 mM-L-glutamine, 20 m-units of insulin/ml, 1 nM-dexamethasone, 50 μg of kanamycin/ml, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37 °C in an atmosphere of air/ CO_2 (19:1) [32].

The medium was renewed 12 h after seeding to remove unattached non-viable cells and at every 24 h thereafter. Experiments were performed with cells that had been cultured for 48–96 h. The viability of the cells used in the experiments was greater than 95%.

The hepatocytes used in this study were non-dividing cells, able to accumulate taurocholic acid intracellularly to the same extent as cultured rat hepatocytes [33]. Furthermore the cells could synthesize and secrete VLDL, LDL, HDL (with VLDL as the major species, namely $68 \pm 9\%$), apo B, apo AI, apo AII, apo E, apo CII [34], albumin and plasminogen activator inhibitor 1 [35]. Also, the transport and metabolism of thyroid hormones were qualitatively comparable with these processes in rat hepatocytes [36]. The human hepatocytes possess a specific high-affinity site for human HDL with recognition properties similar to those described on rat hepatocytes [37].

Fig. 1. Binding of ^{125}I - β -VLDL to rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells as a function of β -VLDL concentration

The amount of ^{125}I - β -VLDL was varied and the cell binding in the absence ($\blacktriangledown$) and presence ($\circ$) of $300 \mu\text{g}$ of unlabelled β -VLDL/ml is determined. The specific binding ($\blacksquare$) is calculated by subtraction of the binding in the presence of excess unlabelled β -VLDL from the total binding. The plots represent data obtained with freshly isolated rat parenchymal cells in suspension, whereas, with Hep G2 and human parenchymal cells, cultured cells were utilized.

Hep G2 cells

Hep G2 cells were cultured in 25 cm^2 flasks, containing 5 ml of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Irvine, Scotland, U.K.), supplemented with $100 \mu\text{g}$ of streptomycin/ml, 100 i.u. of penicillin/ml and 10% heat-inactivated fetal-calf

serum. At 1 week before the lipoprotein-binding assays, the cells were treated with trypsin and transferred to 25 mm multiwell culture dishes (Costar) with a split ratio of 1:10. At 20 h before the experiments, the culture medium was replaced by DMEM containing 1% human serum albumin (HSA).

Mouse peritoneal macrophages

Peritoneal macrophages were obtained from 10-week-old male Swiss mice. The cells were harvested by lavage of the peritoneum with 2 ml of Hanks' buffer. The cells were washed once and the cell concentration was adjusted to 2×10^6 cell/ml. Samples (1 ml) were plated in 25 mm multiwell culture dishes (Costar). After 2 h the non-adherent cells were removed by washing, and 1 ml of culture medium was added. The culture medium was composed of 10% heat-inactivated rat lipoprotein-deficient serum, 2 mM-glutamine, $100 \mu\text{g}$ of streptomycin/ml, 100 i.u. of penicillin/ml and RPMI 1640 (Flow Laboratories). To induce a maximum expression of LDL receptors, the cells were cultured for 2 days.

Lipoprotein binding

The lipoprotein-binding assays were performed with the indicated amounts of ^{125}I - β -VLDL and, if indicated, unlabelled lipoproteins. These binding assays were performed for 3 h at 0°C . Incubations with freshly isolated rat parenchymal cells were carried out in plastic Eppendorf tubes, and the incubation volumes were adjusted to 0.5 ml with Ham's F10 medium (Flow Laboratories) containing 2% BSA. Incubations with the cultured cells were performed in 25 mm multiwell culture dishes (Costar), and the incubation volumes were adjusted to 0.7 ml with Williams' E medium containing 2% BSA (rat hepatocytes, mouse macrophages), with DMEM containing 2% HSA (Hep G2 cells), or with Williams' E medium containing 2% HSA (human parenchymal cells). The Ca^{2+} concentration was specifically varied by using EGTA. In contrast with EDTA, EGTA does not lead to release of cells from culture dishes, as in its absence or presence a similar amount of cell protein was recovered and no effect on the viability was noticed (as determined by Trypan Blue exclusion). After incubation, the cells were washed five times with 0.15 M-NaCl buffered with 0.05 M-Tris/HCl, pH 7.4, containing 0.2% BSA and twice with buffer without BSA. Finally, the cells were lysed with 1 ml of 0.1 M-NaOH and the radioactivity was counted. Protein contents of the samples were determined by the Lowry method [25].

Anti-LDL-receptor antibodies

Bovine LDL receptors were isolated as described by Russell *et al.* [38] and antibodies were raised in chickens. This antibody was kindly donated by Dr. P. Kroon (Merck Institute for Therapeutic Research, Nutley, NJ, U.S.A.). The IgG fraction was obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The antibody showed a single LDL-receptor band in a blot of β -octyl glucoside-solubilized bovine adrenal membranes. Immuno-electron-microscopic studies indicate that the antibody reacts readily with the LDL receptors from human liver cells [39] and with oestradiol-induced rat liver LDL receptors (M. F. Kleinherenbrink-Stins & Th. J. C. Van Berkel, unpublished work). Control IgG was also obtained from chickens. Cells were preincubated for 2 h at 0°C with the IgG fractions before the radiolabelled ligands were added.

RESULTS

High-affinity binding sites for β -VLDL

With freshly isolated and cultured rat parenchymal liver cells, Hep G2 cells and cultured human parenchymal cells, the extent of binding as a function of the ^{125}I - β -VLDL concentration was determined at 0°C (Fig. 1). The low-temperature incubation was chosen both to avoid internalization and to exclude the possibility

Fig. 2. Comparison of the ability of unlabelled lipoproteins to compete with the binding of ^{125}I - β -VLDL to mouse macrophages, rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells

Cells were incubated for 3 h at 0 °C with 1.0 μg of ^{125}I - β -VLDL/ml and with the indicated amounts of unlabelled β -VLDL (○), LDL (Δ) or acetyl (Ac)-LDL (■). The binding is expressed as the percentage of the radioactivity obtained in the absence of competitor. The plots represent data obtained with freshly isolated rat parenchymal cells in suspension, whereas with mouse macrophages, Hep G2 and human parenchymal cells, cultured cells were utilized. The 100% values for mouse macrophages, rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells were respectively 26.4 ± 2.7 , 8.6 ± 1.0 , 11.9 ± 0.6 and 22.0 ± 2.2 ng/mg of cell protein [means $\pm$ s.d. of triplicate determinations from representative experiments (two separate experiments for mouse macrophages and human parenchymal cells and three separate experiments for Hep G2 and rat parenchymal cells)].

that newly synthesized and secreted apo E could become attached to the added β -VLDL. It appears that with all cell types β -VLDL binds with a high affinity, and when the low-affinity component (determined in the presence of 300 μg of unlabelled β -VLDL/ml) is subtracted from the total binding, clear-cut saturation kinetics are observed. Analysis of the data in accordance with Scatchard [39a] indicated that the rat parenchymal cells bound ^{125}I - β -VLDL with a K_d of 10.7 $\mu\text{g}/\text{ml}$ (with cultured cells a K_d of 8.9 $\mu\text{g}/\text{ml}$ was obtained), and with Hep G2 cells and human parenchymal cells the K_d was 5.1 and 8.4 $\mu\text{g}/\text{ml}$ respectively.

The maximal amount of β -VLDL which becomes associated to the cells (B_{max}) was for rat parenchymal cells 117 ng/mg of cell protein (cultured cells 126 ng/mg of cell protein), and with Hep G2 cells and human parenchymal cells values of 106 and 103 ng/mg of cell protein respectively were found.

Nature of the recognition sites for β -VLDL

In order to analyse to what extent β -VLDL binds to the (unusual) LDL receptor or to a different recognition site, competition experiments were performed. Because Koo *et al.* [9] and Ellsworth *et al.* [10] reported that the macrophage

receptor which recognizes β -VLDL possesses an unusually low affinity for LDL, we performed competition experiments at 1 μg of ^{125}I - β -VLDL/ml with unlabelled competitor concentrations up to 500 $\mu\text{g}/\text{ml}$ (Figs. 2a–2d). In addition to the various liver cells (Figs. 2b–2d), we also analysed under the same conditions the specificity of the binding of β -VLDL to mouse macrophages (Fig. 2a).

In agreement with the data of Koo *et al.* [9] and Ellsworth *et al.* [10], we found that the binding of ^{125}I - β -VLDL to mouse macrophages can be completely competed for by unlabelled β -VLDL and LDL, whereas acetyl-LDL is hardly effective (Fig. 2a). With rat parenchymal cells, however, a completely different picture is obtained (Fig. 2b) and, although unlabelled β -VLDL is an effective competitor, LDL and acetyl-LDL do not compete at all. Similar data were obtained with rat parenchymal cells in culture. With Hep G2 cells it appears that a competition profile is obtained similar to that with mouse macrophages, whereas with cultured human parenchymal cells LDL appears to be less effective as a competitor than β -VLDL itself, as LDL is able to compete for β -VLDL binding by maximally 65% (Figs. 2c and 2d).

Fig. 3. Effect of varying the Ca²⁺ or EGTA concentration on the binding of ^{125}I - β -VLDL to rat parenchymal cells, Hep G2 cells and human parenchymal cells

The cells were incubated for 3 h at 0 °C with 5.0 μg of ^{125}I - β -VLDL/ml and with the indicated EGTA or the different Ca²⁺ concentrations. The plots represent data obtained with freshly isolated rat parenchymal cells in suspension, whereas with Hep G2 and human parenchymal cells, cultured cells were utilized. The 100% values are the cell binding observed at 2.0 mM-Ca²⁺, and for rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells were respectively 31.9 ± 0.2 , 45.3 ± 8.2 and 74.8 ± 5.2 ng/mg of cell protein [means $\pm$ s.d. of triplicate determinations from representative experiments (two separate experiments for human parenchymal cells and three separate experiments for Hep G2 and rat parenchymal cells)].

Dependency of ^{125}I - β -VLDL binding on Ca²⁺

The binding of ligands to the LDL receptor is strictly Ca²⁺-dependent [40], and the LDL-receptor-related protein also needs Ca²⁺ for binding of apo E-enriched β -VLDL [20]. In contrast, Jaeckle *et al.* [41] identified on highly purified endosomal membranes from rat liver a specific binding site for β -VLDL which is Ca²⁺-independent.

Fig. 4. β -VLDL-induced regulation of ^{125}I -LDL or ^{125}I - β -VLDL binding to cultured rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells

Cells were incubated with the indicated concentration of β -VLDL for 20 h at 37 °C. The cells were then washed and incubated twice for 10 min and 30 min at 37 °C to allow internalization of surface-bound β -VLDL. Total ^{125}I -LDL (10 $\mu\text{g}/\text{ml}$) and ^{125}I - β -VLDL (5.0 $\mu\text{g}/\text{ml}$) binding was then assessed by incubation for 3 h at 0 °C.

Comparison of the role of Ca²⁺ in the binding of ^{125}I - β -VLDL to rat parenchymal, Hep G2 and human parenchymal cells indicates that the binding of β -VLDL to Hep G2 cells is strictly Ca²⁺-dependent (Fig. 3b). For human parenchymal cells in the absence of Ca²⁺ a significant binding (40%) is still noticed (Fig. 3c). The binding of β -VLDL to rat parenchymal cells is essentially uninfluenced by modulating the extracellular Ca²⁺ concentration (Fig. 3a). Also, with rat parenchymal cells in culture the binding of β -VLDL was not influenced by Ca²⁺ (results not shown).

Regulatory characteristics of β -VLDL-binding site

A characteristic feature of the LDL receptor is its regulatory response to the cellular status of cholesterol [42]. In order to test

Fig. 5. Effect of a chicken IgG antibody against the bovine LDL receptor on the binding of ^{125}I -LDL or ^{125}I - β -VLDL to cultured rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells

Cells were preincubated with the indicated concentration of control IgG [IgG(control)] or IgG against bovine LDL receptors [IgG(LDL)] for 2 h at 0 °C; ^{125}I -LDL (10 $\mu\text{g/ml}$) or ^{125}I - β -VLDL (5.0 $\mu\text{g/ml}$) was then added to the cells, and the incubation was continued for 3 h at 0 °C. The data are expressed as percentages of labelled lipoprotein binding in the absence of IgG. The 100 % values for cultured rat parenchymal cells, Hep G2 cells and cultured human parenchymal cells were respectively for LDL 8.0 ± 0.6 , 57.7 ± 4.1 and 74.3 ± 4.2 ng/mg of cell protein and for β -VLDL 48.4 ± 3.3 , 61.1 ± 3.6 and 51.9 ± 3.7 ng/mg of cell protein [means $\pm$ S.D. of triplicate determinations from representative experiments (two separate experiments)].

the regulatory response of the β -VLDL-binding site, the effect of preincubation with increasing concentrations of β -VLDL was tested. For comparison, the effect on the binding of LDL was also analysed (Figs. 4a–4c).

The binding of β -VLDL to cultured parenchymal rat liver cells was only down-regulated by 20 %, whereas for LDL binding the percentage was 30 % (Fig. 4a). With Hep G2 cells, however, β -VLDL appears to be very effective as a feedback-regulator of the binding of both β -VLDL and LDL (by 74 and 83 % respectively; Fig. 4b). For human parenchymal cells the binding site for LDL (LDL receptor) can be down-regulated by β -VLDL by 89 %, whereas for binding of β -VLDL a 44 % decrease is noticed when cells are preincubated with 200 μg of β -VLDL/ml (Fig. 4c).

Influence of anti-LDL-receptor antibodies on β -VLDL binding

The binding of iodinated LDL or β -VLDL to rat parenchymal cells was lowered by preincubation with antibodies against the LDL receptor by approx. 25 % for both (Figs. 5a and 5b). With Hep G2 cells it is clear that the antibody is able to lower the binding of LDL to a great extent (by 89 %), and also the binding of β -VLDL is greatly diminished (by 59 %) (Figs. 5c and 5d). For cultured human parenchymal cells intermediate values are found (Figs. 5e and 5f), whereby the LDL binding is lowered by 81 %, whereas the binding of β -VLDL under the same condition was diminished by 34 %.

DISCUSSION

β -VLDL is a cholesterol (ester)-enriched lipoprotein which can be isolated from cholesterol-fed animals [11,12] or from patients with type III hypercholesterolaemia [1–4]. In accordance with Ellsworth *et al.* [10], we observed, as described previously [12,23], that β -VLDL from cholesterol-fed rats forms a homogeneous single band with β -mobility. The atherogenicity of β -VLDL is related to the ability to cause deposition of cholesteryl esters in macrophages, both *in vivo* and *in vitro* [5–8]. However, upon injection of β -VLDL into rats, the particles are rapidly cleared from the blood ($t_{1/2}$ 2 min) by the liver [11,12,23]. Recently we showed, in accordance with autoradiographic data [43], that the rapid liver uptake is carried out by parenchymal cells [12,23]. Already 2 min after injection of [^3H]cholesteryl-ester-labelled β -VLDL an association with small vesicles is observed, and after 10 min the highest specific activity is already localized in the lysosomal fraction [23]. The kinetics of serum decay, parenchymal-cell association and uptake *in vivo* are very comparable with those of ligands for specific parenchymal-cell receptors, i.e. asialofetuin for the asialoglycoprotein receptor [44]. In the present paper we characterized the interaction site for β -VLDL on rat parenchymal cells, and the relevance of animal studies to the human situation was verified by a direct comparison with Hep G2 cells, a cell line which is supposed to reflect

functions of human parenchymal cells [45,46], and cultured human parenchymal cells.

The binding of β -VLDL to rat parenchymal cells, Hep G2 cells and human parenchymal cells follows clear-cut saturation kinetics ($B_{\max}$, 103–126 ng/mg of cell protein), with a relatively high affinity (K_d , 5.1–10.7 μ g/ml). The following four points provide evidence that a LDL-receptor-independent recognition site for β -VLDL is present on rat and human parenchymal cells.

1. Competition studies provide evidence that on mouse macrophages the recognition of β -VLDL is presumably exerted by the (unusual) LDL receptor [9,10]. The data presented are in perfect accordance with the data published previously by Koo *et al.* [9] and Ellsworth *et al.* [10], and actually our conditions for performing competition studies are based on their original finding that β -VLDL is recognized by macrophages by the LDL receptor which possesses a low competitive affinity for LDL. Also, for Hep G2 cells we found that the binding of 125 I- β -VLDL (1 μ g/ml) is completely inhibited by an excess of LDL. With human parenchymal cells 65% inhibition by a 500-fold excess of LDL is found, whereas with rat parenchymal cells LDL is essentially ineffective. These data indicate the presence of a LDL-receptor-independent recognition site for β -VLDL on human and rat parenchymal liver cells. The inability of human LDL to compete for rat β -VLDL binding to rat parenchymal cells is not likely to be caused by species differences, because human LDL competes completely in the mouse macrophage system, and human LDL is also avidly recognized by the oestrogen-induced LDL receptor on rat parenchymal cells [47]. Also, differences are not accounted for by using freshly isolated cells rather than cultured cells, because with rat parenchymal cells both conditions led to identical results.

2. A characteristic feature of the LDL receptor and also the LDL-receptor-related protein is that the binding of ligands is Ca^{2+} -dependent [20,40]. With Hep G2 cells it appears that the binding of β -VLDL is Ca^{2+} -dependent. For human parenchymal cells 60% of the total binding is Ca^{2+} -dependent, whereas with rat parenchymal cells no effect of Ca^{2+} is found. So these data confirm the competition studies and indicate that the LDL-receptor-independent recognition site on rat and human parenchymal cells does not need the presence of Ca^{2+} for recognition of β -VLDL.

3. The LDL receptor is down-regulated by prolonged exposure to LDL or β -VLDL [42,48]. With Hep G2 cells we found that the binding of either LDL or β -VLDL to the cells was subject to down-regulation by β -VLDL. With human parenchymal cells the binding of LDL was down-regulated by β -VLDL by 89%, whereas the binding of β -VLDL was diminished by 44%. With rat parenchymal cells the binding of LDL or β -VLDL was only diminished by preincubation with β -VLDL by maximally 20–30%. These data indicate that the LDL-receptor-independent recognition site on rat and human parenchymal cells is apparently not subject to down-regulation in conditions under which the LDL receptor is greatly influenced.

4. As a last point, the effect of chicken antibodies against the LDL receptor on the binding of LDL and β -VLDL to the liver cells shows that with Hep G2 cells the binding of both LDL and β -VLDL is greatly inhibited by the antibodies. The interaction of β -VLDL with the cultured human parenchymal cells is inhibited to a lesser extent than for the Hep G2 cells, whereas with rat parenchymal cells the antibody only slightly affects the binding of both LDL and β -VLDL. The inability of the antibody to affect β -VLDL binding to rat parenchymal cells is not due to species specificity, because, as shown by immuno-electron microscopy, the antibody readily interacts with both human liver cells [39] and with the oestradiol-induced rat LDL receptors (M. F. Kleinherenbrink-Stins & Th. J. C. Van Berkel, unpublished

work). In general, the antibody data thus sustain the previous conclusions.

The characteristics of the β -VLDL interaction with rat parenchymal cells (absence of competition with a 500-fold excess of LDL, Ca^{2+} -independent binding, absence of down-regulation, slight effect of antibodies against the LDL receptor) indicate that the high-affinity binding of β -VLDL is not exerted by the classical or unusual LDL receptor [9,10]. This conclusion is in accordance with the difficulty of showing the presence of a LDL-specific recognition site in liver from untreated rats [29,40]. In rats the recognition of β -VLDL is thus performed by a specific β -VLDL receptor, which might be identical with the chylomicron-remnants recognition site and thus be called remnant receptor.

Jaeckle and co-workers [41] described a specific EDTA-resistant β -VLDL-binding site on endosomes from rat liver. The binding of β -VLDL to this site could not be competed for by LDL, although antisera to the LDL receptor blocked the interaction. Cooper and associates [49,50] have described saturable binding of chylomicron remnants to crude membranes and plasma membranes from rat liver which is mainly resistant to 10 mM-EDTA and immunologically unrelated to the LDL receptor. It is likely that the specific β -VLDL-binding site on isolated or cultured rat parenchymal cells described here is thus similar to the chylomicron-remnant binding site ('remnant receptor').

It can be questioned if the remnant receptor is identical with LRP, as described by Herz *et al.* [18]. A comparison of the binding characteristics of rat parenchymal cells and the LRP for β -VLDL points to some distinctive differences, however. As recently published by Lund *et al.* [21] and Kowal *et al.* [20], it appears that native β -VLDL binds poorly to LRP in ligand blots. An additional enrichment by added apo E appears to be needed for optimal interaction [20]. The kinetics of association of β -VLDL to parenchymal cells *in vivo* [12,23] and the properties of cell binding *in vitro* (performed at 0 °C) do not indicate that additional apo E is needed for an optimal interaction of β -VLDL with parenchymal cells. For LRP it was reported that Ca^{2+} is obligatory for binding of apo E-enriched β -VLDL [20]. Our data indicate that the LDL-receptor-independent β -VLDL-interaction site on parenchymal cells is independent of added Ca^{2+} . This evidence might be used to indicate that a protein other than LRP is mediating the binding of β -VLDL. Upon injection of β -VLDL into rats *in vivo*, the particles become nearly exclusively associated with rat parenchymal liver cells. Since LRP is widely distributed among cells in the body [18], its cellular distribution does not reflect the specificity of β -VLDL association with rat parenchymal cells [12,23].

In the present work, an attempt was undertaken to verify the relevance of the findings with rats to the human situation. Hep G2 cells have been widely used as a model for human hepatocytes [45,46], and therefore we also performed simultaneously experiments with this cell line. It appears that with Hep G2 cells the high-affinity recognition of β -VLDL was completely competed for by LDL, and was Ca^{2+} -dependent and down-regulated similarly to the binding of LDL to this cell line. Furthermore, antibodies against the LDL receptor greatly diminished the binding of β -VLDL. All these properties point to recognition of β -VLDL by the LDL receptor, and no evidence for a remnant receptor on Hep G2 cells could be obtained.

For human hepatocytes (parenchymal cells) the data are consistent with the conclusion that β -VLDL interacts with both the LDL receptor and the remnant receptor. It is likely that their relative importance will be dependent on the metabolic conditions of the cells, because the LDL receptor is subject to down-regulation by LDL [51] or β -VLDL. The noticed differences in interaction of β -VLDL with Hep G2 cells and rat and human

hepatocytes suggest that direct studies with human liver cells are needed to allow conclusions on the interaction of lipoproteins with the liver in the human situation. The LDL-receptor-independent recognition site for β -VLDL thus identified on human parenchymal cells may mediate *in vivo* the uptake of cholesteryl-ester-rich VLDL during a cholesterol-rich diet, when LDL receptors are down-regulated, or in LDL-receptor-deficient patients [52], thus protecting against the extrahepatic accumulation of the atherogenic β -VLDL constituents.

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