Interaction *in vivo* and *in vitro* of apolipoprotein E-free high-density lipoprotein with parenchymal, endothelial and Kupffer cells from rat liver

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The interaction of apolipoprotein (apo) E-free high-density lipoprotein (HDL) with parenchymal, endothelial and Kupffer cells from liver was characterized. At 10 min after injection of radiolabelled HDL into rats, $1.0 \pm 0.1\%$ of the radioactivity was associated with the liver. Subfractionation of the liver into parenchymal, endothelial and Kupffer cells, by a low-temperature cell-isolation procedure, indicated that $77.8 \pm 2.4\%$ of the total liver-associated radioactivity was recovered with parenchymal cells, $10.8 \pm 0.8\%$ with endothelial cells and $11.3 \pm 1.7\%$ with Kupffer cells. It can be concluded that inside the liver a substantial part of HDL becomes associated with endothelial and Kupffer cells in addition to parenchymal cells. With freshly isolated parenchymal, endothelial and Kupffer cells the binding properties for apo E-free HDL were determined. For parenchymal, endothelial and Kupffer cells, evidence was obtained for a saturable, specific, high-affinity binding site with K_d and B_{max} values respectively in the ranges 10–20 μg of HDL/ml and 25–50 ng of HDL/mg of cell protein. In all three cell types nitrosylated HDL and low-density lipoproteins did not compete for the binding of native HDL, indicating that lipids and apo B are not involved in specific apo E-free HDL binding. Very-low-density lipoproteins (VLDL), however, did compete for HDL binding. The competition of VLDL with apo E-free HDL could not be explained by label exchange or by transfer of radioactive lipids or apolipoproteins between HDL and VLDL, and it is therefore suggested that competition is exerted by the presence of apo Cs in VLDL. The results presented here provide evidence for a high-affinity recognition site for HDL on parenchymal, liver endothelial and Kupffer cells, with identical recognition properties on the three cell types. HDL is expected to deliver cholesterol from peripheral cells, including endothelial and Kupffer cells, to the liver hepatocytes, where cholesterol can be converted into bile acids and thereby irreversibly removed from the circulation. The observed identical recognition properties of the HDL high-affinity site on liver parenchymal, endothelial and Kupffer cells suggest that one receptor may mediate both cholesterol efflux and cholesterol influx, and that the regulation of this bidirectional cholesterol (ester) flux lies beyond the initial binding of HDL to the receptor.

INTRODUCTION

The function of HDL is accepted to be coupled to the removal of cholesterol from peripheral tissues [1,2]. With fibroblasts [1], arterial smooth-muscle cells [1], aortic endothelial cells [2,3] and peritoneal macrophages [4], evidence has been provided that HDL must be bound to a high-affinity site before it can serve as a cholesterol acceptor. Up-regulation of the HDL-binding sites can be induced by loading the cells with cholesterol/albumin complexes [3,5] or acetylated LDL [2,4].

After injection of acetylated LDL into rats, most of this particle is taken up extremely rapidly by liver endothelial cells [6,7]. In order to function as an adequate scavenger site, it can be argued that these liver cells, which function *in vivo* as uptake site for modified LDL, must possess an active mechanism to release cholesterol [8].

According to the generally accepted concept of Glomset [9], HDL is expected to deliver cholesterol from peripheral cells to the liver hepatocytes, where cholesterol can be converted into bile acids and thereby irreversibly removed from the circulation. Similarly as for peripheral cells, a high-affinity site for HDL has been identified on hepatocytes of various species [10–18] and on liver membranes [19–22]. However, as compared with the evidence for the peripheral recognition site, variable properties are reported for the HDL recognition site on hepatocytes. In early studies [10,11,14–17], including our own [12,13], apo E-containing HDL was applied and cross-competition with other apo E-containing lipoproteins was detected, indicating a lack of specificity for HDL. In order to obtain HDL completely free from apo E, it was necessary to use human HDL, because all rat HDL preparations appeared to contain low amounts of apo E. Although it can be questioned to what extent

homologous HDL should possess the same affinity for the high-affinity recognition sites, our preliminary experiments with human liver cells and human apo E-free HDL indicate that no differences can be detected. Earlier studies with apo E-deficient HDL and parenchymal cells provided evidence that apo A-I might be the ligand for the high-affinity recognition site [18]. However, Bachorik *et al.* [15] showed with pig hepatocytes also cross-competition for apo E-free HDL with LDL, and Chacko [21] obtained evidence with rat liver membranes for binding sites not competed for by LDL.

More recently, modification of HDL with tetra-nitromethane was shown to lead to a loss of its ability to bind to the high-affinity binding sites on liver membrane preparations [22].

In the present paper we investigated the characteristics of the interaction of human apo E-free HDL with isolated parenchymal, Kupffer and endothelial cells from rat liver and compared directly the properties of the cellular recognition sites involved in the multidirectional cholesterol flux in the liver. Furthermore, a low-temperature cell-isolation procedure [7] was used to determine the relative importance of these cell types in the initial liver recognition of apo E-free HDL *in vivo*.

MATERIALS AND METHODS

Materials

Trypsin was purchased from Flow Laboratories. Nycodenz was obtained from Nycomed and Co. Oslo, Norway. Collagenase type I and albumin (fraction V) were purchased from Sigma. ^{125}I (carrier-free) in NaOH was purchased from Amersham International. Pronase was obtained from Boehringer Mannheim. Tetra-nitromethane (98% pure) was purchased from Aldrich Chemical Co. and Ham's F-10 medium was from Gibco-Europe, Hoofddorp, The Netherlands. Throughout this study 12-week-old male Wistar rats were used.

HDL, LDL and VLDL: isolation and iodination

Human VLDL ($d < 1.006$) was isolated by differential ultracentrifugation as described by Redgrave *et al.* [23]. Human LDL ($1.024 < d < 1.055$) and human HDL ($1.063 < d < 1.21$) were isolated from serum by two repetitive centrifugations by the method of Redgrave *et al.* [23] as described previously [24]. The HDL was passed over a Sepharose-heparin column [25], and the apo E-free fraction was checked for the presence of apo E and albumin by SDS/10%-polyacrylamide-gel electrophoresis, followed by Coomassie Blue staining and densitometric scanning. HDL was radiolabelled with ^{125}I by the ICl method of McFarlane [26], as modified by Bilheimer *et al.* [27].

In the lipoprotein preparations $2 \pm 1\%$ of the radioactivity was trichloroacetic acid-soluble, and $4 \pm 1\%$ of the radioactivity was present in lipids. The distribution of the label between the apolipoproteins of HDL was $44 \pm 2\%$ in apo A-I, $44 \pm 3\%$ in apo A-II and $6 \pm 1\%$ in apo Cs as determined after separation of the apolipoproteins by SDS/polyacrylamide-gel electrophoresis, with 5–22.5%-acrylamide gels. The gels were stained, dried and cut into pieces and counted for radioactivity.

Nitrosylation of HDL

Nitrosylation was performed exactly as described by Brinton *et al.* [28].

Serum clearance and liver association *in vivo*

Rats were anaesthetized by intraperitoneal injection of 28 mg of Nembutal. The abdomen was opened and 100 μg of radiolabelled HDL was injected into the inferior vena cava at the level of the renal veins. Blood sampling and lobule excising were performed at the indicated times, as described previously [29].

Cell-isolation procedure at 8 °C

Rats were anaesthetized and injected with 100 μg of ^{125}I -HDL as described above. At 10 min after injection, the vena porta was cannulated and liver perfusion at 8 °C was started as described previously [29]. As found previously with other protein substrates, no loss of cell-bound radioactivity or formation of acid-soluble radioactivity occurred from the ^{125}I -labelled HDL during the low-temperature cell-isolation procedures, leading to a quantitative recovery of the radioactivity associated with the total liver in the subsequently isolated cells. The percentage of recovery is indicated in the Results section. As reported previously [7], about 1–2 mg of protein of pure endothelial cells and 1 mg of purified Kupffer cells are obtained with this low-temperature method, representing a yield of 1.5–3% and 2% respectively.

Cell-isolation procedure at 37 °C

The three types of liver cells (i.e. parenchymal, endothelial and Kupffer cells) were isolated and purified from rat livers by perfusion of the liver *in situ* with 0.05% collagenase, as described previously [7], except that metrizamide is replaced by Nycodenz. Cell viability of the three cell fractions was checked by Trypan Blue exclusion, and was in all three cell fractions higher than 95%. Purity of the cell fractions was examined by peroxidase staining with 3,3'-diaminobenzidine, as described previously [7].

HDL uptake and binding *in vitro*

Incubation of freshly isolated liver cells with the indicated amounts of ^{125}I -HDL was performed in Ham's F-10 medium, containing 2% of bovine serum albumin (fraction V) and 1.6 g of Hepes/l, pH 7.4. The incubations were carried out in plastic tubes in a total volume of either 0.5 ml or 3.5 ml with a total incubation time as indicated on the abscissae of the Figures or in the legends to the Figures. At the indicated times, 0.5 ml samples were withdrawn and the cell-associated radioactivity per mg of cell protein was determined as described previously [12]. In corresponding blanks the HDL was incubated in the absence of cells. Binding of ^{125}I -HDL was determined by incubating the washed cell pellet with 0.5 ml of trypsin 4 mg/ml for 45 min on ice in a refrigerated room, by the method of Oram *et al.* [5]. After incubation, the cells were pelleted twice by centrifugation (200 g for 2 min), and the two supernatants were collected and used for determination of cell-bound reactivity.

Determination of possible label exchange

Determination of the possible label exchange from apo E-free HDL to VLDL was performed by ultracentrifugation [30]. After incubation for 10 min of 5 μg of ^{125}I -HDL with 50 μg of VLDL in Ham's F-10 medium with 2% bovine serum albumin at 37 °C, the medium was cooled to 4 °C. The medium was adjusted to a density of 1.006 g/ml with solid KBr and centrifuged for

4 h at 200 000 *g*. It was found that $2.5 \pm 0.2\%$ ($n = 3$) of the iodine label was recovered in the upper part of the centrifuge tube, indicating minor transfer or exchange of radiolabelled apolipoproteins from HDL (recovered in the bottom of the tube) to VLDL under the competition conditions.

RESULTS

Serum decay and liver association of human apo E-free ^{125}I -HDL

The decay in serum (Fig. 1) of apo E-free ^{125}I -HDL in rats is very slow; 10 min after injection only $1.0 \pm 0.1\%$ ($n = 4$) of the label becomes associated with the liver (values determined after liver perfusion). This percentage remains relatively constant up to 60 min after injection. Although nitrosylation of tyrosine residues is known to block competition of apo E-free HDL with labelled native HDL with isolate cells [31,32] or liver plasma membranes [22], it does not behave similarly when the total liver association is considered, and we even found an increased liver association of radiolabelled nitrosylated HDL. Apparently, nitrosylated HDL can be recognized in the liver by a pathway different from that for native HDL. This makes it impossible to investigate specific uptake of apo E-free HDL *in vivo* by comparing native HDL with nitrosylated HDL.

Cellular distribution of apo E-free HDL in rat liver

In order to quantify the relative importance of the liver endothelial, Kupffer and parenchymal cells in the initial recognition of apo E-free HDL, we investigated the total cell association of HDL to the various liver cells at 10 min after injection. The amount of the apo E-free HDL associated with the total liver was $5.0 \pm 0.5\%$

Fig. 1. Liver association and serum decay of apo E-free HDL and nitrosylated apo E-free HDL

Apo E-free ^{125}I -HDL (100 μg of apolipoprotein or nitrosylated HDL) was injected into anaesthetized rats, and the liver association and serum decay were determined. The liver was not perfused, and the broken line represents the maximal contribution of the serum value to the liver uptake [12]. $\circ$, Serum values of native HDL; $\triangle$, serum values of nitrosylated HDL; $\bullet$, liver association of native HDL; $\blacktriangle$, liver association of nitrosylated HDL.

($\times 10^{-4}$) of the injected dose/mg of cell protein (Fig. 2). The uptake of HDL by parenchymal cells, which contribute 92.5% of the total liver mass and are therefore the quantitatively most important cell type of the liver, was $3.0 \pm 0.1\%$ ($\times 10^{-4}$) of the injected dose/mg of cell protein. From the difference between these two values, it is clear that the non-parenchymal cells are also quantitatively important in the cell association of apo E-free HDL by the liver. The cell association of HDL to endothelial cells, which contribute 3.3% of the total liver mass, was $14.6 \pm 1.8\%$ ($\times 10^{-4}$) of the injected dose/mg of cell protein, and the uptake of HDL by Kupffer cells, 2.5% of the total liver mass, was $20.3 \pm 0.1\%$ ($\times 10^{-4}$) of the injected dose/mg of cell protein ($n = 4$). By taking into account the amount of protein contributed by each cell type to total liver [7], the recovery of radioactivity after cell isolation relative to that originally associated with the liver can be calculated, and this value is $94 \pm 2\%$ ($\pm \text{S.E.M.}$, $n = 4$).

To determine whether impurities or denatured proteins in the apo E-free ^{125}I -HDL preparation may influence the liver uptake and relative cellular distribution, we injected iodinated HDL into rats, let it circulate for 1 h, isolated the serum and injected it directly into a second rat. After 10 min we determined the cellular distribution.

Fig. 2. Distribution of apo E-free ^{125}I -HDL between Kupffer, endothelial and parenchymal liver cells, 10 min after injection of apo E-free ^{125}I -HDL

Cells were isolated with collagenase and Pronase at 8 $^{\circ}\text{C}$. Each value represents the mean $\pm \text{S.E.M.}$ for four cell isolations. Values all expressed as % of injected dose ($\times 10^{-4}$) per mg of cell protein.

Table 1. Relative contribution of the different liver cell types to the total liver uptake of apo E-free HDL

The amount of radioactivity per mg of cell protein in the isolated cell fractions was multiplied by the amount of protein that each cell type contributes to total liver protein. The values are expressed as percentages of the total liver uptake of apo E-free HDL, and are means $\pm$ S.E.M. for four determinations.

Cell type	(% of the total Radioactivity liver-associated)
Parenchymal	77.8 $\pm$ 2.4
Endothelial	10.8 $\pm$ 0.8
Kupffer	11.3 $\pm$ 1.7

The values obtained for endothelial cells, Kupffer cells and parenchymal cells of the screened HDL preparation were respectively $14.4 \pm 2.8\%$, $19.3 \pm 2.0\%$ and $2.9 \pm 0.2\%$ ($\times 10^{-4}$) of the injected dose/mg of cell protein ($n = 2$). These values are identical with those obtained with the unscreened preparations.

Table 1 quantifies the relative contribution of the different liver cell types to the total uptake of apo E-free HDL by rat liver. The amount of apoprotein per mg of cell protein in the isolation cell fractions was multiplied by the mg of protein that each cell type contributes to total liver protein. The values are expressed as percentages of total liver-associated radioactivity.

Effect of nitrosylation on the cell association of apo E-free HDL to parenchymal liver cells *in vitro* at 37 °C

The time-dependent cell-association of HDL and nitrosylated HDL with parenchymal cells is shown in Fig. 3. The cell association is rapid and reaches an equilibrium at about 1 h. After incubation for 2 h, only 10% of the total cell-associated HDL appears to be degraded. Internalization of HDL is, however, relatively slow, and pulse-chase experiments indicate that both the slow internalization and retroendocytosis may explain the apparent equilibrium. When 10 μ g of nitrosylated apo E-free HDL is incubated with parenchymal cells, at least 50% of the binding ability vanishes as compared with the binding of 10 μ g of apo E-free HDL (Fig. 3). This indicates that there is a specific binding site for apo E-free HDL on parenchymal cells. A similar conclusion cannot be derived from similar experiments with endothelial or Kupffer cells, because these cell types show a much higher cell association of nitrosylated HDL as compared with native HDL (results not shown).

Comparison of the ability of unlabelled nitrosylated HDL and native lipoproteins to compete with the cell association of 125 I-labelled human apo E-free HDL by parenchymal, Kupffer and endothelial liver cells

To investigate the specificity of the binding of apo E-free HDL to parenchymal, Kupffer and endothelial cells, we studied the competition of radiolabelled apo E-free HDL with an excess of unlabelled nitrosylated HDL or unlabelled HDL. We incubated the cells with 5 μ g of 125 I-HDL and competitors for 10 min at 37 °C and determined the cell-associated radioactivity as described in the Materials and Methods section. A short incubation

Fig. 3. Time course of the association *in vitro* of 125 I-HDL (○) and nitrosylated 125 I-HDL (●) with parenchymal cells

The cells were incubated with 10 μ g of 125 I-HDL or nitrosylated 125 I-HDL/ml at 37 °C.

time was chosen in order to minimize possible exchange of apolipoproteins, and 37 °C instead of a low temperature was used because especially the interaction of HDL with cells is temperature-dependent [12,32]. Fig. 4 shows that an excess of unlabelled HDL decreases the amount of cell-bound radioactivity by 60–65% in all three cell types, whereas an excess of unlabelled nitrosylated HDL does not compete for the binding of labelled HDL at all. In order to evaluate the apolipoprotein specificity of the high-affinity sites on the various cell types, the ability of LDL and VLDL to compete for radiolabelled apo E-free HDL was also determined. The results of these experiments (also shown in Fig. 4) show that VLDL is able to inhibit cell-association of HDL effectively in all three cell types, but LDL is much less effective. Label exchange cannot account for the fact that VLDL is able to compete for radiolabelled apo E-free HDL, because after incubation for 10 min at 37 °C only 2.5% of the label is found in VLDL (see the Materials and methods section).

Amount of cell binding as a function of the extracellular HDL concentration

The binding of lipoproteins to cells is often studied by an incubation at lower temperatures. However, our initial studies [12] already indicated that, with liver cells, the cell association is temperature-dependent. This finding has been further evaluated by other groups [32]. Therefore, we determined the cell binding of apo E-free HDL after an incubation period of 24 h at 37 °C. Because liver cells are able to internalize receptor-bound ligands rapidly [24], the amount of cell-bound ligand was not determined by a short incubation with trypsin at 37 °C [33], but the recently reported low temperature/high trypsin method was applied instead [5]. It was monitored that cell viability was maintained during this procedure (the cells still excluded Trypan Blue). Furthermore, the procedure removes all cell-bound radioactivity, exposed at the

Fig. 4. Comparison of the ability of unlabelled lipoproteins to compete with the cell association of ^{125}I -labelled apo E-free HDL to parenchymal (a), endothelial (b) and Kupffer (c) cells

Cells were incubated for 10 min at 37°C with $5\ \mu\text{g}$ of ^{125}I -labelled apo E-free HDL/ml and with the indicated amounts of unlabelled human LDL (Δ), human VLDL ($\blacktriangle$), human HDL ($\circ$) and human nitrosylated apo E-free HDL ($\bullet$). ^{125}I -labelled HDL apolipoprotein association is expressed as the percentage of the radioactivity obtained in the absence of unlabelled lipoprotein.

Fig. 5. Relation of the concentration of apo E-free HDL to the extent of cell binding for liver parenchymal cells (a), liver endothelial cells (b) and Kupffer cells (c)

Cells were incubated for 2 h at 37°C with various amounts of ^{125}I -HDL. After the cells were washed three times at 4°C with phosphate-buffered saline 0.2% bovine serum albumin, they were incubated with 4 mg of trypsin/ml on ice for 45 min. After incubation, the cell-bound radioactivity was determined in the supernatant. The non-specific binding ($\circ$), determined in the presence of a 20-fold excess of apo E-free HDL, was subtracted from the total binding ($\bullet$), resulting in the broken line, which represents the specific binding of ^{125}I -HDL to the three cell types.

outside of the cell membrane, because after an initial incubation at 4°C all the cell-associated radioactivity is removed by the low-temperature trypsin method (results not shown). Figs. 5(a), 5(b) and 5(c) indicate the amount of cell binding as a function of the extracellular HDL concentration for parenchymal, endothelial and Kupffer cells. Similar experiments were performed with a 20-fold excess of apo E-free HDL. The residual association represents the non-specific binding component. The broken line represents the specific binding of ^{125}I -HDL to the three cell types, obtained by subtracting the non-specific binding component. The data suggest a high-affinity binding site for apo E-free HDL on all three cell types and, when replotted in accordance with Scatchard [34,35], the specific binding component indicates K_d

values of 10–20 μg of HDL/ml and B_{max} values of 25–50 ng of HDL/mg of cell protein.

DISCUSSION

In previous studies [12,13] we found that, after intravenous injection of apolipoprotein-labelled HDL *in vivo*, protein-bound radioactivity is found in both isolated parenchymal cells and non-parenchymal liver cells, with a 4.5-fold higher specific activity in the non-parenchymal cells. These results already suggested that, in addition to parenchymal cells, non-parenchymal cells are also quantitatively important for HDL catabolism in the liver. However, these findings were based on studies with HDL containing apo E (rat HDL), and therefore the contribution of a specific HDL high-affinity site could not be evaluated, because apo E-containing HDL can also bind to the remnant [36] and/or the LDL receptor [19].

The aim of the present study was to evaluate the presence of specific high-affinity sites for apo E-free HDL on liver parenchymal, endothelial and Kupffer cells. The data obtained *in vivo* indicate that all three cell types bind HDL, whereas 22% of the total liver-associated radioactivity becomes associated with endothelial and Kupffer cells. Evaluation *in vivo* of the role of the apolipoproteins in the recognition process was not possible, because nitrosylated HDL showed an avid interaction with the liver (especially with endothelial and Kupffer cells).

With freshly isolated cells, however, we obtained evidence for a saturable, specific, high-affinity binding site on freshly isolated Kupffer, liver endothelial and parenchymal cells. This site mediates the binding of apolipoprotein E-free HDL *in vitro*, with K_d and B_{max} values respectively in the ranges 10–20 μg of HDL/ml and 25–50 ng of HDL/mg of cell protein. These values are comparable with the results obtained with other cell types [1–5] *in vitro*.

By analogy with fibroblasts, aortic endothelial cells and macrophages, we found with liver cells that nitrosylation of HDL abolished its ability to compete for native apo E-free HDL binding, indicating that the lipid moiety is not responsible for the specific recognition process [37]. The ability of VLDL to compete with the cell association of HDL is probably caused by the presence of apo Cs. Also with testis [21], ovarian [38] and kidney [30] membranes a displacement with an excess of VLDL has been noticed, although LDL does not compete. The possibility that exchange or transfer of radioactive lipids or apolipoproteins made a substantial contribution to this observation is minimized by the choice of a very short incubation time (10 min at 37 °C). Furthermore, we performed similar studies at 4 °C, and comparable competition characteristics were obtained (results not shown). Finally, we re-isolated VLDL from the vials after incubation for 10 min at 37 °C and recovered only 2.5% of the radioactivity originally present in HDL in the $d < 1.006$ fraction, whereas under the same conditions 50–60% inhibition of the cell-association of radioactive HDL is found.

The absence of a significant effect of unlabelled LDL on the cell association of ^{125}I -HDL with rat hepatocytes contrasts with studies with pig hepatocytes [15], in which it was shown that LDL competes completely with apo E-free HDL binding. Preliminary observations in our laboratory indicate that human hepatocytes in this re-

spect are more similar to rat hepatocytes than to pig hepatocytes.

In previous work we established that, *in vivo*, acetylated LDL is specifically taken up by liver endothelial cells [6,7]. By analogy with the peritoneal-macrophage system, as described by Schmitz *et al.* [4], we can expect that the cholesterol (esters) internalized by the endothelial cells must be redistributed within the liver from endothelial to parenchymal cells. Recently we established that such a process occurs with the fatty acids from the cholesterol esters, and that albumin exerts a transport function [39]. For the cholesterol moiety a similar mechanism has been proposed [8], which may utilize HDL. In the cholesterol transport system inside the liver, HDL is used as acceptor for cellular cholesterol (from endothelial cells) and as donor of cholesterol (for parenchymal cells). Our present simultaneous characterization of the high-affinity recognition site for HDL on parenchymal and endothelial cells indicates that the putative receptor exerts identical recognition properties. From our results it is therefore likely that one receptor mediates both cholesterol efflux and cholesterol influx. The discriminating mechanism in the regulation of the bidirectional cholesterol (ester) flux thus lies probably beyond the initial binding of HDL to the receptor, and may depend on the relative cholesterol load of the cell (membrane).

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