INTERACTION OF LIPOPROTEIN WITH LIVER CELLS

Monique Kleinherenbrink-Stins

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WITH LIVER CELLS

STELLINGEN

behorende bij het proefschrift van
Monique Kleinherenbrink-Stins
Interaction of lipoproteins with liver cells
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1. Het waargenomen transport van LDL gemerkt met tyramine-cellobiose naar de gal wijst uit dat tyramine-cellobiose niet onder alle condities kan worden gebruikt als maatstaf voor de totale hoeveelheid door het betreffende weefsel gemetaboliseerde ligand.
   Dit proefschrift

2. Humane leverendotheelcellen bezitten een scavengerreceptor.
   Dit proefschrift

3. Nitrosylering van HDL kan niet gebruikt worden om de door de HDL receptor gemedieerde opname te analyseren in cellen welke een scavenger receptor bezitten.
   J.E. Nestler et al. (1985) J. Biol. Chem. 260; 7316-7321
   Dit proefschrift

4. De rol die de grootte van het colloidale gouddeeltje speelt in de opname van goudligandcomplexen wordt veelal niet in beschouwing genomen.
   D. Handley et al. (1981) J. Cell Biol. 90; 778-787
   D. Handley et al. (1983) Eur. J. Cell Biol. 30; 266-271
   G. Renaud et al. (1989) Hepatology 9; 380-392

5. De door Bachorik et al. gevonden competitie van LDL met de associatie van apo E-vrij HDL met varkenshepatocyten kan mogelijk verklaard worden door onzuiverheden in het gebruikte varkens-HDL.
   P.S. Bachorik et al. (1985) Arteriosclerosis 5; 142-152

6. De door Babaev et al. getoonde verschillen betreffende de de opname van LDL en malondialdehyde gemodificeerd LDL door humane hepatocyten rechtvaardigen niet de conclusie dat LDL specifiek wordt opgenomen.
   V.R. Babaev et al. (1989) Hepatology 10; 56-60

7. Lysosomale enzymen zijn niet zo lysosomaal.

8. Gezien de schade welke aan de lever optreedt bij warme reperfusie, volgend op een lange periode van koude ischemie, is het niet verklaarbaar dat deze levers succesvol voor transplantatiedoeleinden kunnen worden gebruikt.
   J.C. Caldwell et al. (1989) Hepatology 10; 292-299

9. De verwarring rond het al dan niet aanwezig zijn van Factor VIII en de Van Willebrandfactor in lever endotheelcellen berust niet alleen op een spraakverwarring rond de nomenclatuur, maar ook op onduidelijkheid tegen welke epitoop van het complex de door de betreffende onderzoekers gebruikte antilichamen gericht zijn.
   H.V. van der Stel et al. (1983) Nature 303; 530-532
   J.H. Reinders et al. (1988) Haemostasis 18; 246-261
   R.L. Harrison et al. (1989) Liver 9; 242-249

10. Er kan niet zonder meer gesteld worden dat (matig) alcohol gebruik goed is voor hart en bloedvaten.

11. De toenemende magnetische beveiligingen van warenhuizen zal de invoering van de chipkaart bevorderen.

12. Ter bevordering van het openbaar vervoer dient niet alleen de frequentie van zowel het trein- als busverkeer opgevoerd te worden, maar dienen vooral de aansluitingen tussen beide verbeterd worden.

13. Anticipatie op emancipatie houdt mede in het aanbrengen van föhnstopkontakten naast scheerstopkontakten in hotels en congrescentra.

14. De rol van het ministerie van Binnenlandse Zaken als "subsidiegever" voor het afronden van promotie-onderzoeken mag helaas niet worden onderschat.

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INTERACTION OF LIPOPROTEINS WITH LIVER CELLS

Proefschrift

ter verkrijging van de graad van Doctor aan de Rijksuniversiteit te Leiden, op gezag van de Rector Magnificus Dr. J.J.M. Beenakker, hoogleraar in de faculteit der wiskunde en natuurwetenschappen, volgens besluit van het college van dekanen te verdedigen op donderdag 1 februari 1990 te klokke 14.15 uur

door

MONIQUE FABIENNE KLEINHERENBRINK-STINS
geboren te Den Helder in 1957

Aan mijn ouders

Aan Onno

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GENERAL INTRODUCTION

1.1 The liver and lipoproteins

The liver plays an important role in the processing of both physiological and exogenous substances which are present in the blood circulation. This liver function includes the uptake of nutrient components from the blood and their subsequent metabolism, storage and redistribution to blood and bile as well biotransformation of xenobiotic components, drugs and endogenous metabolites. The liver plays also an important role in the synthesis and metabolism of lipoproteins. Lipoproteins are transport vehicles for lipophilic substances such as triglycerides, cholesterol and cholesterol esters. The synthesis and uptake of lipoproteins by the liver can be considered as a highly complex multicompartiment system involving various liver cell types. The main subject of this thesis is to determine the various liver cells involved in the uptake of lipoproteins and to characterize the nature of this interaction. Therefore, an introductory description of some aspects of the main liver cell types will be given. Then the various types of lipoproteins and their main sites of uptake will be described in general. When known, the cell types involved in the uptake of these lipoproteins will be indicated. The presence and regulation of receptors for low density lipoprotein and high density lipoprotein inside the liver, will be discussed in more detail.

1.2 The liver cells

The major cell types in the liver are: parenchymal, endothelial, Kupffer, fat storing and pit cells. Liver parenchymal cells or hepatocytes are the most prominent class of liver cells in terms of both number and volume. They are arranged in one- layer cords, separated by the sinusoids, radiating from branches of the portal veins to branches of the central veins. The hepatocyte is believed to be responsible for most of the aforementioned main liver functions. The sinusoids are lined by endothelial cells. Liver endothelial cells contain fenestrations (163) and in this aspect they differ morphologically from vascular endothelial cells. These fenestrations have a diameter of about 0.1 μm. This allows the hepatocytes to have a direct contact with blood plasma, but an interaction with particles larger than 0.1 μm, such as chylomicrons, is prevented. Kupffer cells are situated in the sinusoids preferentially around branches of the portal veins (Wisse, 1977). *In* vivo and *in vitro* Kupffer cells are capable of phagocytosing large particles such as colloidal carbon, colloidal gold, latex etc (122). Fat storing cells or stellate cells are localized underneath the sinusoidal lining. Fat storing cells play a role in the synthesis of extracellular matrix components (41, 133, 135) and in vitamin A metabolism, and storage (74). Pit cells are located on or embedded in the endothelial lining. The function of pit cells is not yet clear. Since pit cells have a natural cytotoxic activity against tumors, they may play a role in the hepatic defence against metastatic tumors (18).

In rat, the parenchymal cells account for 77.8% of the total liver volume (17). The endothelial, Kupffer and fat storing cells account respectively for 2.8%, 2.1% and 1.4%

of total rat liver volume. Extracellular spaces (sinusoids, space of Disse, biliary space) form a large part of the remaining volume (about 15%). A minor proportion of the liver consists of vascular epithelial cells, bile duct cells, connective tissue cells, pit cells and smooth muscle cells. When these data are expressed as percentages of the total liver cell volume and assuming an equal protein to volume ratio of the liver cells, it can be calculated that parenchymal cells contribute 92.5%, endothelial cells 3.3%, and Kupffer cells 2.5%, to the total liver protein mass. The sinusoidal liver cells are much smaller in size than parenchymal cells but they contribute considerably to the total number of liver cells (33-35%) (62).

1.3 Lipoproteins

Lipophilic substances are necessary components for maintaining cell structures and metabolism. Since they are insoluble in water lipophilic substances are transported in vehicles called lipoproteins (see for reviews 59 and 128). These vehicles have a micellar structure in which triglycerides and cholesteryl esters form a hydrophobic core, surrounded by a surface coat of phospholipids, free cholesterol and proteins, the so called apoproteins. Apoproteins can function as ligands for the lipoprotein receptors but also as cofactors for enzymes involved in lipoprotein metabolism (Table I). Lipoproteins have characteristic sizes, densities, compositions and apoproteins. The nomenclature of the lipoproteins has been based on their density determined by density gradient ultracentrifugation (56, 123). Four major different lipoprotein classes can be distinguished: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (58). Major apoproteins are apoprotein (apo) AI, apo B, apo C and apo E (138).

1.3.1 Chylomicrons

Chylomicrons are the largest of the lipoproteins (75 - 1200 nm diameter) and are synthesized in the intestine (see for reviews 147 and 148). After uptake of dietary fat (monoglycerides, fatty acids, lysophospholipids, cholesterol etc.) by the enterocytes of the intestine, these fatty components are incorporated into chylomicrons and subsequently secreted into the mesenteric lymph. The size and the composition of the chylomicrons are strongly dependent on the nutritional status. Chylomicrons consist mainly of triglyceride and the apoproteins present are mainly apo B48, apo AI, apo AII, and apo AIV. Chylomicrons are transported from lymph to plasma, where they acquire apo C's, and apo E (81). During circulation (Fig. 1), the triglyceride content of the chylomicrons is hydrolyzed by the enzyme lipoprotein lipase (LPL), which can be activated by apo CII (43). LPL is present on capillary endothelium of extrahepatic tissue, skeletal muscle, cardiac muscle and adipose (fat) tissue (13). The fatty acids liberated during the lipolysis are used as an energy source by various cells or taken up by adipocytes

Table I. Summary of plasma apolipoproteins (from Gotto 1988)

Name	Lipoprotein	Molecular weight	Function
A-I	HDL Chylomicrons*	28,000	structural, LCAT activator
A-II	HDL Chylomicrons	16,000	structural
A-IV	HDL Chylomicrons*	46,000	unknown
B-100	LDL, VLDL	550,000	structural,secretion VLDL, binds LDL receptor (B,E)
B-48	Chylomicrons	250,000	structural,secretion from intestine.
C-I	HDL, VLDL Chylomicrons	6,000	LCAT activator
C-II	HDL, VLDL Chylomicrons	7,000	LPL activator
C-III	HDL, VLDL Chylomicrons	7,000	surface stabilization (negative charge)
D	HDL chylomicrons*	21,000	cholesterol ester exchange
E	HDL, VLDL chylomicrons*	34,000	binds to apo E and BE receptor

LCAT = lecithin cholesterol acyl transferase
LPL = lipoprotein lipase
* = only on nascent chylomicrons

and stored as triglycerides. Upon lipolysis, the volume of the chylomicrons decreases, a part of its surface coat becomes redundant and splits off in the form of bilayer discs. These discs are thought to be precursors of plasma HDL. The resulting smaller chylomicron remnants are enriched in apo E and have lost most of their apo C's. These remnants are rapidly cleared by the liver and mainly by parenchymal cells (see for a review 44, 75), but also nonparenchymal cells are involved (63, 153). The size of the chylomicron remnants may be an important factor in determining the uptake by the liver parenchymal cells since the diameter of the fenestrae (approximately 0.1 μm) in the liver endothelial cells prevents the interaction of the larger, not ultimate remnant particles.

The main receptor involved in the clearance is probably the remnant or apo E receptor (1, 2, 97, 152). However recently, it was claimed, that in rats chylomicrons are removed by the LDL receptor (102, 162). The evidence indicating involvement of the remnant or apo E receptor is strengthened by the following findings. In humans and Watanabe rabbits with absent, defective or reduced number of LDL receptors, chylomicron remnant removal is unaffected and the capacity of the liver to take up chylomicron remnants is unchanged (86). Chylomicron remnants also bind to a protein with a molecular weight different from the LDL receptor (12).

1.3.2 Very low density lipoproteins and low density lipoproteins

Very low density lipoproteins (VLDL) are particles with sizes ranging from 30 to 90 nm diameter. They are primarily synthesized by the liver and to a lower extent by the intestine. Their main constituents are triglycerides and cholesterol (esters) (Table II). Following secretion by the liver, they are metabolized in plasma in a way analogous to chylomicrons (Fig. 1). The main apoprotein on VLDL is apo B 100, but also apo E and apo C's are present. During the sequential delipidation process by action of lipoprotein lipase, mainly on large VLDL and liver lipase on smaller VLDL remnants, they loose apo C's (see for a review 36). In rats (155) the resulting VLDL remnants are removed by the liver, probably by the apo E receptor (150, 155) and only a small portion is catabolized to LDL. In humans the VLDL are catabolized through a transient intermediate density lipoprotein to cholesterol rich LDL (20 nm diameter), which contains a sole apo B100.

Low density lipoproteins (LDL) are the major cholesterol carrying lipoprotein in human plasma and are an important risk factor with regard to atherosclerosis. In rabbits (120), hamsters (141), swine (118) and rats (14, 26, 70, 119, 127, 145) the liver is the major site for autologous and or human LDL uptake. The major cells implicated in the uptake of LDL in rat are, the liver parenchymal cells (31, 70) and Kupffer cells (70). In humans, the importance of LDL receptors in maintaining normal plasma cholesterol levels is illustrated in patients with a reduction in number of functional LDL receptors (see for review 24). In such patients a marked hypercholesterolemia and premature atherosclerosis are observed. The importance of the LDL receptors in the liver is very well illustrated by the fact that liver transplantation in a child with a lack of functional LDL receptors (familial homozygous hypercholesterolemia) resulted in a normalisation of plasma cholesterol levels (15).

1.3.3 High density lipoproteins

High density lipoproteins (HDL) are relatively small particles (8-12 nm diameter), rich in protein, cholesterol (esters) and phospholipid (Table II). They form a heterogeneous group of particles, due to their varying apoprotein compositions and different origins, as will be further discussed below.

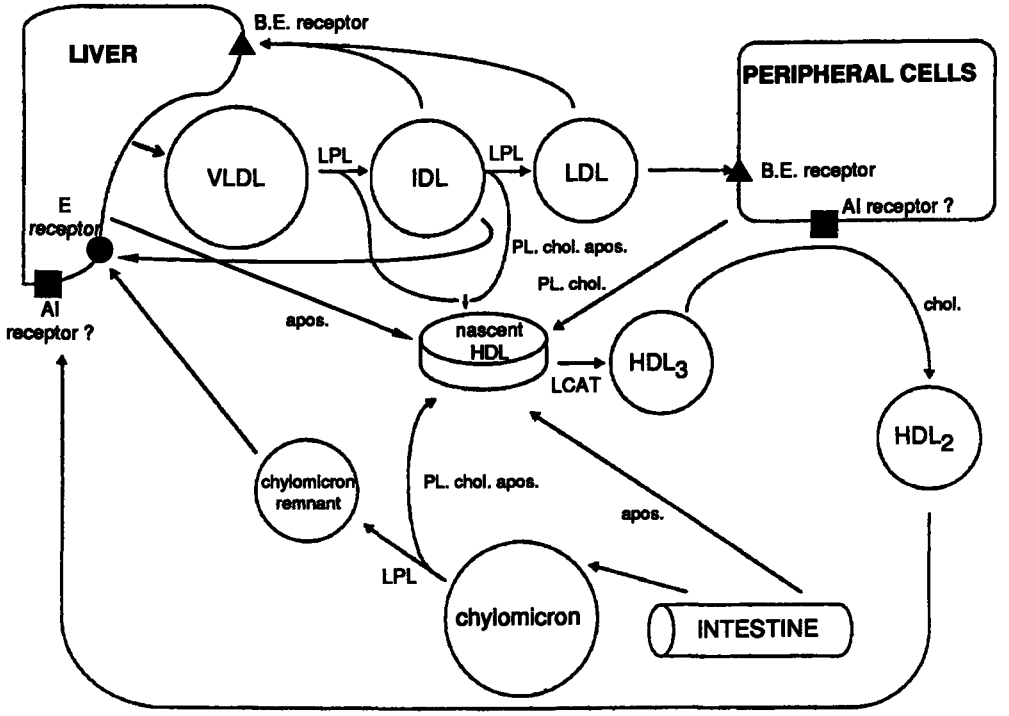

Fig. 1. Diagrammatic representation of lipoprotein metabolism (from Brindley and Salter 1988).

Chylomicrons are secreted by the intestine and on entering the plasma are acted on by the enzyme lipoprotein lipase (LPL). On hydrolysis of the bulk of the triacylglycerol content they are transformed into chylomicron remnants. The remnants are removed from the circulation after interaction with specific apo E receptors on hepatocytes.

VLDL is secreted by the liver and again triacylglycerol is hydrolysed by LPL resulting in the formation of IDL. IDL may be removed from the circulation after interaction with E receptors on hepatocytes. IDL which is not removed is further metabolized to become LDL which is then removed from the circulation after interaction with B.E receptors either on liver cells or on peripheral cells.

HDL apo-lipoproteins are secreted by both the liver and the intestine. These interact with phospholipid (PL), cholesterol (chol) and other apoproteins (apos), shed from the surface of the chylomicrons and VLDL during the action of LPL, to form discoidal "nascent" HDL. Free cholesterol is esterified by the enzyme lecithin cholesterol acyl transferase (LCAT) and as this moves into the centre of the particles they are transformed into spherical HDL_3. HDL_3, perhaps through interaction with apo-AI receptors on peripheral cells, pick up more cholesterol and are transformed into HDL_2. HDL_2, through the interaction with an apo AI receptor on hepatocytes, may deliver cholesterol to the liver.

Nascent discoid shaped HDL particles are synthesized by the liver (67, 99) and the intestine (50, 61). HDL is also formed during the metabolism of VLDL and chylomicrons (see for a review 40). In plasma, nascent HDL adopts a spherical configuration by the action of the enzyme lecithin-cholesterol acyl transferase (LCAT). The apoproteins present in the HDL fraction are apo AI, AII, AIV, C's and E. The nascent HDL is first transformed to HDL_3 and subsequently to HDL_2. HDL_2 and HDL_3 are the major HDL density fractions present in most species. HDL_2 contains one more apo AI than HDL_3 and about twice as much cholesterol (40). HDL can also be further subdivided in populations containing or lacking one or more species of apoproteins. The above described differences in origin, density and apoprotein composition can implicate a specific functional significance. However, the role of these different HDL subpopulations in total HDL and cholesterol metabolism is still under debate.

Both nascent and mature HDL are capable of removing cholesterol from peripheral tissue and from other lipoproteins. In many species, including humans and rabbits, the acquired cholesterylester can be rapidly transferred to other lipoproteins. In rats, the extent of exchange is limited, since this animal lacks cholesteryl ester transfer protein. This plasma protein is responsible for cholesteryl ester transfer between lipoprotein particles (10, 112).

The tissues responsible for the catabolism of HDL were defined for apo AI and apo AIV. In rats, the liver and kidneys are the principal site for uptake of apo AI (52, 154) and apo AIV (33). The cells involved in the uptake of apo AI are the liver parenchymal cells (52) and the proximal tubulus cells in the kidney (33, 52). The liver also takes up HDL cholesterolesters (3, 53, 54, 144, 156). The uptake of the cholesterol moiety by the liver exceeds that of apoprotein AI and possibly the internalization of these components occurs independently (3, 53, 54). From *in vitro* studies with hepatocytes, fibroblasts and adrenal cells from rats it was concluded that the selective delivery of HDL cholesterolesters to hepatocytes was by a nonendocytic mechanism (121).

Epidemiological studies revealed that, in contrast to LDL, HDL cholesterol levels are negatively correlated with the prevalence of atherosclerosis and HDL is therefore considered as a protective factor. Glomset (55) proposed that this protective function is due to the capability of HDL to remove cholesterol from peripheral tissue and deliver this to the liver. Subsequently the liver is capable of removing cholesterol irreversibly from the circulation via the biliary pathway (Fig. 2).

The supposed role of HDL in reverse cholesterol transport has been mainly demonstrated in *in vitro* systems. HDL was capable of removing excess cholesterol from mouse macrophages (6, 130), smooth muscle cells and fibroblasts (137) and HDL was capable of delivering cholesterol to hepatocytes (54). Direct support for this role of HDL *in vivo* was obtained by Miller (100) and Badimon (9). Miller showed that, *in vivo* delivery of cholesterol to rabbit tissues, induced an increase of cholesterol in the HDL frac-

tion, a similar change as observed after *in vitro* incubation of HDL with cholesterol loaded acrophages. Badimon (9) demonstrated that the administration of HDL fractions to cholesterol fed rabbits inhibited the extent of fatty streak formation *in-vivo.* Fatty streaks are cholesterolester accumulations in smooth muscle cells and macrophages, located in the intima of the arterial wall. The appearance of these fatty streaks is typical for a progressive stage of developing atherosclerosis.

1.4 Lipoprotein receptors

Several receptors are involved in the binding and uptake of lipoproteins. The receptors or binding sites, which have been identified are the remnant or apo E receptor, the LDL (B/E) receptor, the scavenger receptor, and HDL receptor(s). These distinct receptors show a specificity for apoprotein ligands, and differ in regulatory responses.

1.4.1 Structure, function and regulation of the LDL receptor

The LDL receptor was first purified from adrenal gland in the laboratories of Brown and Goldstein by Schneider (132). The receptor is a single chain, transmembrane cell surface glycoprotein with an apparent molecular weight of 164,000 (see for a review 24). The mature receptor consists of 839 amino acids. A large domain of 750 amino acids is exposed to the plasma on the extracellular surface followed by a small hydrophobic sequence of 22 amino acids that forms the transmembrane part. A short carboxy terminated 50 amino acids part of the LDL receptor is localized in the cytosol. The extracellular domain is capable of binding apoprotein B and apoprotein E. The binding of LDL is calcium dependent and lysine and arginine residues of apo B are important in the recognition of LDL by its receptor (160). The cytoplasmic domain plays a role in clustering in coated pits and internalization of the receptor (93).

Studies on the regulation of the LDL receptor have been mostly performed with cultured human fibroblasts (see for a review 24). After binding to its receptor, LDL is internalized via coated pits. Within the cell, LDL dissociates from its receptor. The LDL receptor recycles back to the cell surface within 20 minutes. The cholesterolesters of LDL are hydrolyzed inside the lysosomes. High LDL uptake suppresses the transcription of HMG-CoA reductase, the key enzyme in the biosynthesis of cholesterol and it activates the enzyme acyl cholesterol acyl transferase (ACAT). Therefore excess cholesterol can be stored as cholesterolester droplets in the cytoplasm. High LDL uptake also decreases the *de novo* synthesis of the LDL receptors by lowering the concentration of receptor messenger RNA's. The LDL receptor numbers are adjusted to provide cholesterol for metabolic needs and no massive intracellular cholesterol ester deposition will occur.

1.4.2 Function and regulation of the LDL receptor in liver

The major site of LDL uptake *in vivo* is the liver (14, 26, 70, 118, 119, 127, 141, 145). Receptors for LDL were identified on liver tissue of various species, including rats (69, 90, 161), rabbits (139), dogs (91), swine (7, 116) and humans (27, 68, 79, 80). The quantitative expression of LDL receptors on liver membrane preparations of various animals correlates well with the capacity of livers to take up LDL *in vivo* (30, 161). A direct relationship exists between the amount of hepatic LDL receptors and plasma cholesterol levels in humans (15, 68, 106).

In rat the amount of LDL receptors is low. Ethinyl estradiol (EE) treatment induces LDL receptors in the liver profoundly (90). In rabbits, it was demonstrated that EE treatment resulted in *de novo* synthesis of hepatic LDL receptors (95). These receptors have the same characteristics as the fibroblast LDL receptor (90) and recognize both human and rat LDL (26, 30). Cooper (32) demonstrated that EE induced LDL receptors are of a similar molecular weight and immunologically related to the uninduced rat liver LDL receptor and that of other species.

The uptake of LDL by normal rat liver is largely receptor mediated (26, 70). Only Attie (4) reported that a large portion of LDL uptake was non-receptor mediated and suggested that in the hepatocyte two LDL pathways were present, a saturable and a non saturable one.

The structural and functional characteristics of the LDL receptor in the liver are generally comparable to the LDL receptor as first described by Brown and Goldstein for fibroblasts. On immuno- and ligand blots, the same apparent molecular weight was demonstrated for human, rabbit, rat, and dog liver, Hep-G2 cells, and HeLa cells (11, 34, 51, 73, 158). The receptor recognizes apoprotein B and apoprotein E with high affinity, but not reductively methylated LDL. It is inhibited by EDTA, sensitive to pronase treatment and fails to bind HDL (90).

As described above, the same LDL receptor as described for fibroblasts is present in liver or liver cells from various animals, nevertheless some controversy exists. Hoeg (79) reported a lower molecular weight of the LDL receptor and no effect of EDTA and pronase on LDL binding, consequently he concluded that the LDL receptor in human liver was distinct from the fibroblast LDL receptor. Mahley (97) reported an absence of LDL receptors in human liver.

The mechanism of regulation of the LDL receptor in liver is considered to be comparable to that in fibroblasts, although quantitative differences exist (69, 71). In hepatocytes of rabbits (5, 139, 140), pigs (116) and Hep-G2 cells (35, 70), binding of LDL is coupled to degradation of the apoprotein and the liberated cholesterol is able to regulate the intracellular cholesterol metabolism. In rats, controversial reports about the effects of LDL uptake have been published. No inhibiting effect of LDL on HMG-CoA reductase or stimululation of ACAT has been reported by Breslow (19) and Attie (4).

However, both Calandra (25) and Edwards (39) found an inhibiting effect of LDL uptake on HMG-CoA reductase activity.

1.4.3 The scavenger receptor

A specific recognition site for a modified form of LDL (AcLDL) was first described by Goldstein (57) for macrophages (see also 23). The apparent molecular weight of the scavenger receptor was reported to be 260 kDa (157) or 220 kDa (89) or 250 kDa (38). The bovine scavenger receptor (89) was shown to be a trimer of a 77 kDa glycoprotein.

Ligands bound to the scavenger receptor are internalized through coated pits (117, 104) and transported to the lysosomes for degradation. Excess cholesterol, internalized by the scavenger receptor, is esterified by ACAT in the cytoplasm. Because scavenger receptor activity can not be down regulated, continued uptake of AcLDL results, at least in macrophages, in massive cholesterolester accumulation and appearance of large fat droplets inside the cells. These cells are then named foam cells. The recognition of AcLDL by the scavenger receptor is probably based on the negative charge of the particle. Other modifications of LDL that also result in recognition by a scavenger receptor and subsequent cholesterol accumulation include malondialdehyde treatment (46), biological modification (76), enzymatic modification (142) and treatment with cigarette smoke extract (166). Similar modifications probably occur *in vivo* (46, 76, 142). Further evidence for the existence of modified LDL *in vivo* is based upon the demonstration of oxidized LDL in atherosclerotic plaques from rabbits (66, 115). However, when present in the blood circulation, these modified lipoproteins are rapidly cleared (16, 84, 96, 104, 151). These modified lipoproteins are mainly taken up by the liver (96) and the (liver) sinusoidal cells are involved in their clearance (16, 103, 104, 117). The rapid uptake of modified LDL by the liver is probably a mechanism to prevent peripheral cells from exposure to these very atherogenic particles. This may explain why it has not been possible so far to demonstrate directly the existence of modified forms of LDL in the plasma.

1.4.4 HDL binding site and reverse cholesterol transport

The high density lipoprotein density range is composed of a heterogeneous group of particles with varying apoprotein compositions. HDL particles containing apo E may interact with the LDL receptor, the remnant receptor (98) and the postulated HDL receptor.

In humans, apo E containing HDL represents a minor fraction of the total HDL population. Only in persons who are lacking apo B containing proteins, an enrichment of apo E containing HDL is found in plasma. Apo E HDL then serves as the major cholesterol delivering lipoprotein to cells which have LDL or remnant receptors (82). High

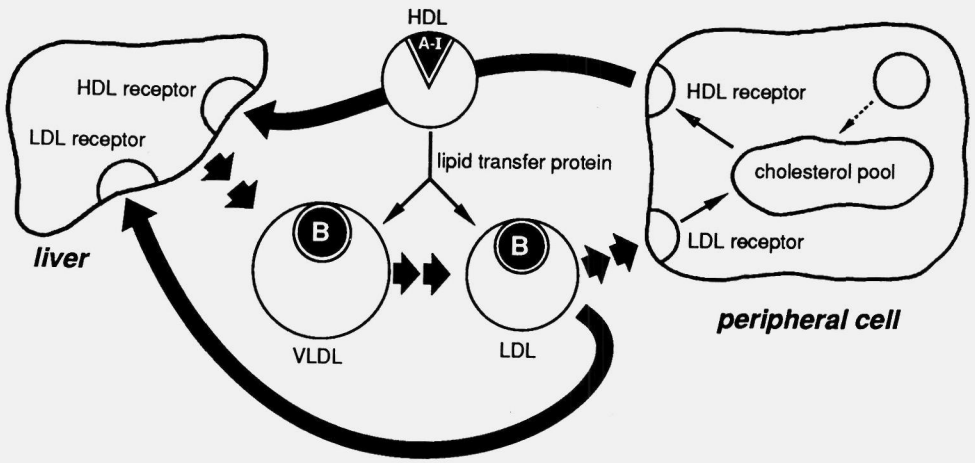

Fig. 2. Schematic overview of reverse cholesterol transport (from Brewer 1988)

VLDL secreted by the liver is ultimately converted to LDL, the major cholesterol carrying lipoproteins in plasma. LDL interact with the high-affinity LDL receptors on the liver and peripheral cells. It has been proposed that the excess cholesterol in peripheral cells is removed and transported to the liver by HDL. HDL facilitate the removal of cholesterol from the cell by interaction with the putative HDL receptor. Cholesterol within HDL may be directly transported back to the liver, where HDL interact with the hepatic HDL receptor, or the cholesterol may be exchanged into VLDL or LDL and tranported back to the liver via the apo B-containing lipoproteins.

affinity binding sites for apo E free HDL, were shown to be present in rat liver (8, 43, 140), rat adrenocortical cells (64), and in canine adipose tissue (47, 49). A role for apo AI in specific binding of HDL was indicated for several tissues (54), including rat liver parenchymal cells (29, 126), rat adrenal cells (42) canine adipose cells (48), human fibroblasts, smooth muscle cells (60) and human macrophages (129). In general, the binding of HDL appeared to be reversible, Ca^{2+} independent and not influenced by pronase treatment (8, 64, 78, 114, 140).

The receptor protein involved in the binding of apo E free HDL by various cells has not been clearly identified. Fidge (42) isolated a tentative HDL binding protein with an apparent molecular weight of 78,000 D from sheep adrenocortical membranes. HDL binding proteins with a molecular weight of approximately 110 KDa were reported for both fibroblasts, smooth muscle cells (60) and macrophages (131). Schmitz reported that HDL binding proteins consisted of subunits of 16, 20 and 80 kDa. A binding protein of 120 kD (50 and 30 kD subunits) was purified from placental membranes (85). From both rat and human liver membranes, two HDL binding proteins were isolated, one with a molecular weight of 120 kD and one of 100 kD (141). All the isolated proteins were capable of binding apo AI and some did also bind apo AII, but none did bind apo E.

In various tissues HDL cholesterolesters were taken up to a greater extent than the major surface component apo AI (53, 54, 94, 144). On the other hand, for fibroblasts, smooth muscle cells and macrophages, it was demonstrated that HDL is capable of taking up excess cellular cholesterol (108, 110, 129, 143).

The intracellular events which occur after binding of HDL to its receptor and determine whether cholesterol is delivered to or taken up from cells are unclear at the moment. Two mechanisms were proposed in wich HDL can serve as an acceptor of excess cholesterol from cells. One for fibroblasts and smooth muscle cells (111) and another for macrophages (see for a review 130). Schmitz (130) proposed that apo E free HDL, binds to the HDL receptor, present on cholesterol loaded macrophages. HDL is then internalized as an integral particle, it comes in close contact with lipid droplets, takes up unesterified cholesterol and subsequently leaves the cell. Oram (111) proposed that apo E free HDL binds to membranes of fibroblasts and smooth muscle cells and can directly serve as an acceptor of cellular cholesterol. Recent studies by Slotte (137) with cultured fibroblasts and aortic endothelial cells revealed that the binding of HDL to its receptor initiated an intracellular translocation of cholesterol to the membrane. At this site HDL can serve as an acceptor of cholesterol and subsequently leaves the membrane.

In vitro, the binding of HDL to fibroblasts and adipose cells can be increased by cholesterol loading of the cells (21, 49, 109). The direction of the cholesterol flux between HDL and cells was dependent on the cholesterol load of the cells (6). It is possible that the interaction of HDL with all these cell types is mediated by a common receptor and that the post receptor binding events may be different for the various cell types involved.

1.5 Scope of this thesis

The central theme for this study was to determine the various liver cells involved in the uptake of LDL and HDL, to further clarify the nature of the interaction and to characterize the intracellular pathway. Furthermore it was important to establish the relevance of the data obtained in the rat for the human situation.

At the start of this study, the involvement of parenchymal rat liver cell types in the uptake of LDL had already been determined biochemically, but the involvement of Kupffer cells was still questioned. Also, the intracellular pathway(s) of LDL processing was (were) still not clearly defined. With regard to the human situation, no data were available on the relative contribution of parenchymal and Kupffer cells to LDL metabolism nor of the involvement of liver endothelial cells in the clearance of acetylated LDL.

The existence of binding sites for HDL in rat liver was implicated but no data were available on the specificity of this interaction and on the relative contribution of the liver endothelial, Kupffer and parenchymal cells. The data available for human liver cells, mostly concerned the interaction of HDL with the hepatoma cell line Hep-G2 and the relevance of this cell line with regard to HDL metabolism was uncertain.

The interaction of lipoproteins with the liver can be considered as a complex multi-compartiment process involving various liver cell types and their intracellular compartments. Therefore, a variety of complementary light microscopical, electron microscopical, biochemical and cell isolation techniques were used to visualize and to characterize the interaction of lipoproteins with the various human and rat liver cells. Further progress requires, in our point of view, a combined attention for the topographical as well as the biochemical aspects of these processes.

RESULTS AND GENERAL DISCUSSION

2.1 Selection of methods

To visualize the interaction of the different types of lipoproteins at the light microscopical level, the fluorescent phospholipid analog 1'1'-dioctadecyl-3,3,3',3',-tetramethyl indocarbocyanine perchlorate (DiI) was used to label lipoproteins. DiI can be noncovalently incorporated into lipoproteins. Earlier studies indicated that incorporation of DiI did not alter the binding characteristics (125). Following internalization the lipophilic probe accumulates progressively inside the cells. Therefore, DiI is a valuable marker to determine cell types involved in the metabolism of lipoproteins.

To study the intracellular pathway of LDL at the ultrastructural level, an immunocytochemical approach was chosen. In order to maintain antigenicity of proteins, liver tissue was first lightly chemically fixed, followed by an additional physical fixation by freezing in liquid nitrogen. The frozen tissue was sectioned at -80 to -100^{o}C and subsequently the sections, were labeled "on grid".

In contrast to conventional electron microscopical techniques where tissue is embedded in hydrophobic plastics, this procedure allows the tissue to remain in the water phase. As a result, the antigens present in the section are optimally accessible to the antibodies.

Uptake of human LDL by cells from rat liver could be visualized specifically by using antibodies recognizing human apo B, but not rat apo B. This visualization method has the advantage that it does not require prior labeling of the lipoproteins.

In our attempts to visualize the interaction of HDL *in vivo* with the liver cells we applied a prelabeling technique, using 10 and 17 nm gold conjugates, for coupling. We found evidence that the size of the colloidal gold used for labeling of HDL influenced the actual cell type involved in the uptake; HDL-gold (10 nm) was mainly taken up by liver endothelial cells, whereas HDL-gold (17nm) was taken up primarily by Kupffer cells. No uptake of HDL-gold conjugates (17 an 10 nm) was found by liver parenchymal cells (87). HDL free of apo E exhibited a low association with liver parenchymal, endothelial and Kupffer cells (see appendix paper 5). Hardly any degradation of HDL apoproteins was found in these experiments, whereas the HDL gold conjugates were massively taken up and localized in lysosomal structures. Therefore, we further abandoned methods using this kind of prelabeling. Recently, also Fodor (45) and Renaud (124) described that prelabeling of lipoproteins with colloidal gold particles induced artifacts.

Immunocytochemical techniques that were useful for visualization of LDL (see chapter 4), were not directly applicable to visualize the interaction of HDL with the liver cells at the light and electron microscopical level. This was due to the low level of interaction of HDL with liver and to the little specific labeling of the available antibodies against apo AI on liver tissue sections.

Biochemical methods were applied to determine the rat liver cells involved in the interaction of apo E free HDL. This was studied *in vivo* after injection of ^{125}I labeled HDL,

using a cell isolation method at 4°C. The specificity and the characteristics of the interaction of apo E free HDL with the various rat and human liver cells were further analyzed *in vitro*, with liver cells isolated at 37°C.

Specific amino acid residues are known to be involved in the recognition of lipoproteins by their receptors. To verify the involvement of these amino acid residues in the recognition of LDL and HDL apoproteins by their receptors, chemical modifications directed against these residues were used. Tyrosine residues of HDL were modified using nitrosylation (TNM-HDL) (22, 29) and lysine residues of LDL were modified by reductive methylation (MeLDL) (160) or hydroxy acetaldehyde treatment (HOET-LDL) (136). Acetylation of LDL was used to induce a more negative charge of LDL apoproteins and thereby a recognition by the scavenger receptor.

The biliary excretion of LDL constituents was studied *in vivo*, by using rats permanently equipped with catheters in duodenum, bile duct and heart (92). In these rats, labeled lipoproteins can be administered and excretion can be studied while rats are conscious, non- stressed and under physiological conditions.

2.2 Visualization of the interaction of LDL with rat liver cells

The involvement of various liver cells in the uptake of LDL was studied *in vivo* in untreated and ethinyl estradiol (EE) treated rats (see chapter 4). EE treatment induces LDL receptors on parenchymal cells.

In untreated rats, after injection of DiI-LDL, Kupffer cells became brightly fluorescent, whereas fluorescence in parenchymal cells was low. After EE treatment, Kupffer cells were labeled to about the same extent as in untreated rats, but the parenchymal cells became brightly fluorescent. This shows clearly that in untreated and in EE treated rats Kupffer cells are involved in the metabolism of LDL.

The fluorescent labeling of the parenchymal cells of the EE treated rats at 10 minutes after injection was strongly concentrated at the apical side, while the area's around bile canaliculi were brightly labeled after 30 to 60 minutes. The organelles involved in LDL uptake were investigated at the ultrastructural level in the EE treated rats. This revealed that for parenchymal cells LDL-apo B was first associated with the microvilli, followed by uptake into endocytotic vesicles and then transported to multivesicular bodies in the bile-canalicular area. LDL-apo B was also observed in small vesicles around and even inside the bile canaliculi suggesting secretion of immunoreactive apo B (fragments) into the bile. Very little label was found in lysosomes. Treatment of the animals with inhibitors of the lysosomal degradation pathway led to the appearance of immunoreactive material in this compartment, indicating that lysosomes were also involved in the processing of LDL in parenchymal cells. LDL taken up by Kupffer cells was also handled lysosomally.

The morphological observation that immunoreactive (fragments of) apo B were present inside bile canaliculi, initiated studies on the quantification of this pathway. For this reason catheterized rats were used. During the first 3 hours after administration of ^{125}I-tyramine-cellobiose labeled LDL (^{125}I-Tc-LDL), 5% of the injected dose was found in bile of untreated rats, while 25% was found in bile of EE treated rats. Methylation of ^{125}I-Tc-LDL to prevent recognition of LDL by the LDL receptor, strongly decreased the appearance of radioactivity in bile. The radioactivity in bile was TCA precipitable, indicating that protein (fragments) were excreted. On western blots it was demonstrated that the excreted proteins were immuno reactive apo B fragments with a molecular weight of 120 to 200 kD. It can be concluded that a substantial portion of apo B, administered as LDL, is taken up via an LDL receptor mediated pathway and excreted in bile. Cholesterolesters in LDL are not excreted in bile simultaneously with apo B (105). The delay in cholesterol excretion is probably due to a further intracellular processing (hydrolysis, conversion to bile acids and or sorting) of cholesterolesters before excretion while apo B follows a more direct route. The multivesicular structures are possibly involved in the sorting of apo B and cholesterol for excretion or intracellular usage.

The physiological meaning of the pathway of apo B to the bile and the role of apo B (fragments) in the bile are still speculative. To obtain unequivocal evidence, further experimentation with the catheterized rat as a physiological model to study biliary appearance is nessecary.

2.3 Interaction of HDL with rat liver cells.

It is generally believed that the function of HDL is coupled to removal of cholesterol from peripheral tissues (21, 110). According to the concept of Glomset (5), HDL can subsequently deliver cholesterol to the liver parenchymal cells, where it can be irreversibly removed from the circulation. Several reports demonstrated the existence of high affinity binding sites for HDL on liver parenchymal cells (8, 113, 114, 126, 140, 149, 150, 159) and on liver membranes (28, 29, 90). However the relative importance of the various cell types and the characteristics of the HDL recognition sites were unknown.

In this study, the liver cell types involved in the uptake of HDL and the characteristics of an HDL binding site on these cells was determined (see chapter 5). In order to exclude interaction of the particles by other lipoprotein receptors, it was necessary to obtain apo E free HDL. After injection of iodinated apo E free HDL into rats, the total liver association of HDL apoproteins did not reach values higher than 1% of the injected dose. A cell isolation at low temperature revealed that parenchymal, endothelial and Kupffer cells took part in the interaction of HDL with the liver. The parenchymal cells contributed about 78% to the total liver associated radioactivity, while the contribution of liver endothelial and Kupffer cells was about 11% each. With freshly isolated cells it was found that all three cell types possessed a saturable high affinity binding site with

similar properties. The dissociation constant K_d varied between 10-20 μg HDL/ml and the maximum binding capacity B_{max} was between 25-50 ng HDL/mg cell protein. These binding affinity and capacity are comparable with the binding properties of apo E free HDL on peripheral cell types. Studies on the specificity of the HDL interaction indicated that TNM-HDL and LDL did not compete for the binding of native HDL, whereas VLDL did compete. The competition of VLDL could not be explained by label exchange or by transfer of radioactive components from HDL to VLDL. This suggests that, besides apo AI, possibly apo C's, but not apo B or lipids are involved in the recognition of HDL by rat liver parenchymal, endothelial and Kupffer cells.

The observed identical recognition properties for the HDL binding site on all three liver cell types and their apparent similarity for other cell types, suggests that one receptor may be involved in the regulation of the HDL mediated cholesterol flux. The regulation of the direction of this flux lies probably beyond the initial binding of HDL to its binding site. These differences in direction of the cholesterol flux may be intrinsic to specific phenotypes of cells or related to the relative cholesterol load of the cell.

2.4 Interaction of nitrosylated HDL with rat liver cells

High affinity binding sites for HDL were identified on various cells and on membrane preparations, including liver membranes (see chapter 2.3). For several peripheral cells and liver cells it was shown that tyrosine residues, present in apoprotein AI are involved in the recognition of HDL (see chapter 1.2.3 and 1.3.4). Nitrosylation with tetranitromethane abolished the high affinity interaction of HDL with the cells. In order to asses *in vivo* the presence of specific recognition sites for HDL in the liver, apo E free HDL was modified with tetranitromethane (TNM-HDL) (see chapter 6). The plasma decay of TNM-HDL was compared with that of untreated HDL. Unexpectedly, the decay of TNM-HDL was, irrespective of the conditions used for modification, greatly enhanced. Concomitantly, the liver association of TNM-HDL was increased. Using a cold cell isolation method it became evident that the liver endothelial cells were responsible for this increased uptake of TNM-HDL from the circulation. The enhanced uptake by the liver endothelial cells could not be ascribed to cross-linking of the apoproteins because no enlargement of the particle was revealed by electron-microscopical methods. The increased uptake was probably caused by the negative charge resulting from the modification of the particle.

In-vitro studies with isolated liver endothelial cells show that association of TNM-HDL with the cells could be displaced by acetylated LDL (AcLDL) but not by native LDL or HDL. These data indicated the involvement of an scavenger receptor in the clearance of TNM-HDL. Further evidence was provided by the finding that polyinosinic acid and fucoidin, both known inhibitors of the scavenger receptor (23), also blocked the association of TNM-HDL with the liver endothelial cells.

It can be concluded that modification of HDL by nitrosylation induces an increased association with the liver as the result of recognition by a scavenger receptor. This restricts the usefulness of TNM-HDL to assess the non-receptor dependent uptake of HDL *in vivo*. The use of TNM-HDL *in vitro* is restricted to cell types that are devoid of scavenger receptors.

2.5 Interaction of HDL with human liver parenchymal cells.

Until now, no information was available on the interaction of HDL with human liver cells. In order to obtain relevant information for the human situation, the interaction of apo E free HDL with human parenchymal liver cells was characterized in analogy with the experiments with rat hepatocytes. With primary hepatocyte cultures a saturable high affinity site was found. The apparent K_m was 20 μg HDL/ml medium. The binding of HDL to human liver cells was competed for by HDL and VLDL but not by TNM-HDL or LDL. These results indicated that human hepatocytes do possess a specific binding site for HDL with properties comparable to the binding site for HDL on rat liver cells.

It was further investigated to what extent binding of HDL was coupled to cellular uptake. No evidence was obtained that ^{125}I labeled HDL apoproteins were internalized and degraded in the lysosomes. When incubated under the same conditions, ^{125}I-LDL internalization and degradation was readily demonstrated. Visualization of the interaction of fluorescently labeled HDL (DiI-HDL) and LDL (DiI-LDL) showed that the nature of the interaction with the cells was clearly different. After three hours incubation at 37°C, DiI-HDL fluorescence was still found to be mainly associated with the membrane, whereas DiI-LDL fluorescence was mainly intracellularly localized. These observations, together with the biochemical data suggest that both the apoprotein and the phospholipid of HDL may remain at the plasma membrane during the interaction of HDL with the hepatocytes. Taken into consideration the finding that the selective liver uptake of the cholesterol moiety of double labeled (^{125}I-apoprotein and ^{14}C-cholesterolester) HDL in the rat (54, 121), it is possible that the cholesterol (-ester) moiety of HDL will be separated from the HDL apoproteins at the plasma membrane.

2.6 Visualization of uptake of LDL and AcLDL by human liver parenchymal, Kupffer and endothelial cells

The interaction of fluorescently (DiI) labeled LDL, MeLDL, HOET-LDL, apo E free HDL and apo E free TNM-HDL with human liver cells was studied in primary hepatocyte cultures and *in situ*, using perfused liver tissue blocks.

Liver parenchymal and Kupffer cells showed a specific time dependent interaction with DiI-LDL. A bright, punctate fluorescence of DiI-LDL in cells was observed. The intensity of the fluorescence in parenchymal cells was strongly dependent on the liver donor, whereas the DiI-LDL uptake by Kupffer cells varied only slightly. This suggests

that the LDL receptor on Kupffer cells is regulated differently from the receptor on the parenchymal cells.

Immunocytochemistry on liver sections with anti-LDL receptor antibodies confirmed the presence of LDL receptors on Kupffer and parenchymal cells. The immunostaining revealed donor dependent variation in abundancy of the LDL receptor on the apical surface of the parenchymal cells. These differences were related to the uptake of DiI-LDL by parenchymal cells in tissue blocks from the same liver donors. LDL receptors were also demonstrated around bile canaliculi. Since apo B is present in human bile (134) and an LDL- receptor dependent and bile directed pathway of apo B was demonstrated in rats (chapter 4) it seems likely that transport of apo B (fragments) to the bile is coupled to recognition by its receptor in human as well as in rat liver.

DiI labeled acetylated LDL (AcLDL) was clearly taken up by liver endothelial cells. This uptake was specific since it was inhibited by polyinosinic acid. This demonstrates that human liver endothelial cells possess substantial numbers of scavenger receptors. No evidence for uptake of DiI-AcLDL by the parenchymal cells was obtained and a only minor subpopulation of the Kupffer cells was involved in the uptake of DiI-AcLDL.

The cellular localization of the receptors and the uptake of the different lipoproteins by the human liver parenchymal, endothelial and Kupffer cells is comparable to the situation in the rat. This may indicate that studies concerning interactions of lipoproteins with liver cells, initially performed with rats, may be extrapolated to the human situation.

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LIGHT- AND IMMUNO ELECTRON MICROSCOPICAL VISUALIZATION OF IN VIVO ENDOCYTOSIS OF LOW DENSITY LIPOPROTEIN BY HEPATOCYTES AND KUPFFER CELLS IN RAT LIVER.

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ABSTRACT

The *in vivo* interaction of LDL with the liver was investigated by visualizing the endocytic route using light- and immuno-electron microscopical methods in control and ethinyl estradiol (EE) treated rats. The fluorescent dye Dioctadecyl Indocarbocyanine perchlorate (DiI) allowed the visualization of LDL at the light microscopical level. Cryo-immunocytochemistry using antibodies against apolipoprotein B (apo B) was applied at the electron microscopical level. In untreated as well in EE treated rats, DiI-LDL was taken up by Kupffer cells (KC) to a substantial extent. Parenchymal cell (PC) uptake was strongly increased after EE treatment and at 10 min after injection, LDL was found to be attached to the microvilli of the PC's and some LDL was already localized in multivesicular structures. At later time points, substantial labeling in multivesicular structures, vesicles near bile canaliculi and also inside bile canaliculi was observed. A low amount of labeling was found in lysosomes. In untreated rats, label was also observed in the aforementioned structures, but at a much lower level.

The biliary appearance of LDL was quantified in rats equipped with permanent catheters in the bile duct, duodenum and heart. After administration of ^{125}I-tyraminecellobiose-LDL (^{125}I-Tc-LDL) in control rats, about 5% of the injected dose was secreted into the bile during the first 3 hours after injection. This value was about 25% for EE treated rats. The radioactivity secreted into the bile was trichloroacetic acid precipitable and high molecular weight bands were immunoreactive for apo B as revealed by western blotting. The described events were not observed when methylated ^{125}I-Tc-LDL was administered.

It is concluded that, in rat liver, a significant portion of apo B derived from LDL is directly transported to the bile. Since this pathway is enhanced in EE treated rats it appears to be a route specific for liver parenchymal cells, dependent on uptake via the LDL receptor.

INTRODUCTION

The mechanism of binding, uptake and processing of low density lipoprotein (LDL) has been extensively studied in human fibroblasts and in several other extrahepatic tissues (11, 12). *In vivo* the major part of LDL is cleared from the circulation by the liver in man as well as in various animals (3, 5, 6, 37, 38, 39, 41, 47, 51).

Hepatic cellular distribution studies revealed that for control rats both parenchymal and non-parenchymal cells were responsible for the uptake of LDL by (17, 56). Ethinyl estradiol (EE) treatment induces LDL receptors in liver (27) and selectively increases the uptake of LDL by parenchymal cells (17, 56). Autoradiographic studies of Chao *et*

al. (7) demonstrated that the parenchymal cells were the principal site of uptake of ^{125}I-LDL in EE treated rats. Sinusoidal cells appeared to be involved to a minor extent; due to a low resolution of ^{125}I-autoradiography no differentiation could be made between the involvement of Kupffer and endothelial cells. Handley *et al.* (15, 16) used gold-LDL conjugates and demonstrated an involvement of non-parenchymal cells of EE treated rats. The uptake of these large conjugates by the Kupffer cells was interpreted as the result of non-specific phagocytosis (16). Recently we reported that the size of the colloidal gold strongly determined by which cell type lipoprotein-gold complexes were taken up (24). Fodor (10) indicated that the intracellular destination of transferrin was altered due to coupling to gold particles. Recently Renaud *et al.* (44) observed a considerable dissociation of double labeled colloidal gold-LDL complexes *in vivo* before internalization. Once internalized, colloidal gold can become also dissociated from the LDL and the localization of the gold particle might be not representative for the pathway of the ligand.

Cellular distribution studies (17, 56) and morphological studies (7, 15, 16) apparently led to conflicting conclusions concerning the relative importance of the parenchymal cells and sinusoidal cells in the uptake of LDL. Therefore, in the present study we determined the liver cells involved in the uptake of human LDL at the light- (LM) and the electron-microscopical (EM) level, in both untreated and EE treated rats. To avoid the aforementioned problems, we used the fluorescent molecule 1.1'dioctadecyl 3,3,3',3' tetramethyl indocarbocyanine perchlorate (DiI) at the LM level and the intracellular pathway of LDL was ultrastructurally visualized at the EM level by an immunocytochemical approach on ultrathin cryosections. Antibodies against human apolipoprotein B (apo B), the major protein of LDL, were used in combination with antibodies against lysosomal marker enzymes to characterize lysosomal compartments.

At the ultrastructural level we found substantial labeling of apo B in multivesicular structures, in area's near bile canaliculi and also inside bile canaliculi of EE treated rats. In order to quantify the morphological findings of the biliary appearance of apo B, unrestrained rats, equipped with permanent catheters in bile duct, duodenum and heart (28) were used.

MATERIAL AND METHODS

Materials

Bovine serum albumin (fraction V), 17α-ethinylestradiol, chloroquine, tyramine, cellobiose, and iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) were purchased from Sigma (St Louis, MO, USA). Propylene glycol, gelatin and glycine were obtained from Merck (Darmstadt, West Germany), 1,1' dioctadecyl 3,3,3',3' tetramethyl indocar-

bocyanine perchlorate C18 (DiI) from Molecular Probes (Eugene , Oregon, USA). Fluoresbrite latex particles (0.55 μm, YG) were obtained from Polysciences (Warrington, USA), tylose (MH 300) from Fluka (Buchs, Switzerland) and antibodies and protein A coupled to colloidal gold (5 or 10 nm) from Janssen Lifesciences (Beerse, Belgium). Test kits for total phospholipid, cholesterol (CHOD-PAP) and triglycerides (GPO-PAP) were obtained from Boehringer (Mannheim, West Germany). Rainbow molecular weight markers and radiolabeled sodium (^{125}I) iodine (carrier free) were purchased from Amersham International, (Amersham, UK). All other materials were of reagent grade.

Lipoprotein isolation, labeling and modification

Human LDL ($1.024 < d < 1.055$) was isolated by density gradient centrifugation according to Redgrave (43). Reductive methylation was done as described by Weisgraber *et al.* (59). About 80% of the lysyl residues from human LDL were methylated as determined by the trinitrosulfonic acid colorimetric method (14). Protein determinations were performed according to the method of Lowry *et al.* (30).

For the visualization studies at the light microscopical level, LDL or methylated LDL (MeLDL) were labeled with DiI according to Pitas (36); the density of the mixture was subsequently raised to 1.21 with potassium bromide and the LDL was re-isolated by density gradient centrifugation (43).

Radio-iodination of freshly isolated LDL was done with tyramine cellobiose according to the method described by Pittman *et al.* (40).

Animals

For the visualization studies on *in vivo* endocytosed LDL, 3-month-old male Wag/Rij rats were used. Native or DiI labeled lipoproteins were injected (50 μg/ml plasma) into the jugular veins of overnight fasted rats, under light halothane anaesthesia and were allowed to circulate for the indicated time periods. Rat livers were rinsed with phosphate buffered saline (PBS) and subsequently fixed with 4% paraformaldehyde (PF) and 0.1% glutaraldehyde (GA) in PBS by *in situ* liver perfusion via the portal vein. To identify Kupffer cells at the LM level, 3.5 min before the start of the perfusion fluorescent latex particles were injected via the inferior vena cava in a final concentration of 0.1% solids (42).

For the studies on the *in vivo* liver association and biliary appearance, 3-month-old male Wistar rats were used. Rats were equipped with permanent catheters in the bile duct, duodenum and heart as described previously by Kuipers *et al.* (28). The catheters of the bile duct and the duodenum were immediately connected to each other in order to maintain an intact entero-hepatic circulation of bile acids. The rats were allowed to recover from the operation for 7 days. Lipoproteins were introduced (50 μg/ml plasma) via the heart catheter.

Table I. Effects of estradiol treatment and overnight fasting on plasma cholesterol, trigycerides and phospholipid concentrations.

Values of the estradiol (EE) treated rats were determined after overnight fasting of the animals prior to the experiment. Values (mean ± SD) were determined on pooled serumfractions of two rats. Significant differences ($p < 0.05$; Mann Withney U test) are indicated between the different numbered groups: a = 1 and 2; b = 2 and 3; c = 1 and 4; d = 2 and 3; e = 2 and 4; f = 3 and 4.

Rats (number)	Cholesterol mg/ml	Trigyceride mg/ml	Phospholipid mg/ml
1 Untreated (14)	71 ± 8^{abc}	88 ± 14^{c}	124 ± 11^{abc}
2 Fasted (6)	53 ± 0.2^{ade}	53 ± 11	96 ± 11^{ade}
3 EE (1 day) (5)	22 ± 0.5^{bd}	57 ± 14	56 ± 2^{bdf}
4 EE (3 days) (10)	5 ± 5^{ce}	35 ± 7^{c}	37 ± 9^{cef}

When indicated, rats were pretreated subcutaneously with 5 mg per kg body weight of 17α ethinyl-estradiol (EE) dissolved in propylene glycol for three successive days (6). Control rats received equal volumes of the solvent using the same schedule for administration. Plasma samples were drawn for determination of cholesterol (CH), phospholipid (PL) and triglycerides (TG) (Table I). A decrease in plasma lipid levels was already induced by overnight fasting of the animals. EE treatment for 1 day resulted in a further decrease in plasma CH and PL. A maximal decrease of plasma CH, PL and TG was obtained after 3 days EE treatment. Prolonged treatment of 5 days did not result in a further decrease.

Rats were treated with leupeptin according to Van Berkel *et al.* (58) or with chloroquine according to Hornick *et al.* (19) when indicated.

To determine liver association of radiolabeled LDL, non-catheterized Wistar rats were anaesthetized with halothane and lipoproteins were injected into the jugular vein. Liver biopsies were taken as described by Seifert *et al.* (48). At the same time points, blood samples were taken. Values of liver association were calculated as described by Van Berkel *et al.* (58). Plasma volume was estimated according to Bijsterbosch *et al.* (4).

Bile Sampling

From the catheterized rats, the bile duct catheter was connected to a fraction collector and bile was collected into preweighed test tubes at room temperature, or, when indicated, on ice in the presence of 1 mM para-methylsulfonylfluoride (PMSF). The radiolabeled lipoproteins were introduced via the heart catheter. Bile fractions were taken every hour up to 48 hours. Fresh bile samples, collected on ice were fractionated into a micellar and vesicular fraction according to Groen *et al.* (13).

Antibodies

Antibodies against human apolipoprotein B (apo B), raised in rabbits, were kindly donated by dr L. Havekes (Gaubius Institute TNO, Leiden, The Netherlands) (18). The antibody was tested for cross reactivity with other lipoproteins using the double radial immunodiffusion method as described by Crowle (9) (Fig. 1) and by dot immunoblot assay (DIBA) on nitrocellulose sheets (21) (not shown). The antibody against human apo B reacts with LDL, VLDL, methylated LDL and acetylated LDL from human origin, but not with several HDL fractions or lipoprotein deficient serum derived from humans. The antibody does not crossreact with rat lipoproteins or with rat lipoprotein deficient serum.

Rabbit antibodies against acid phosphatase and α-glucosidase were kindly given by dr R. Willemsen (Dept. of Cell Biology, Erasmus university, Rotterdam, The Netherlands). Their specificity has been described respectively by Parenti *et al.* (35) and Reuser *et al.* (45).

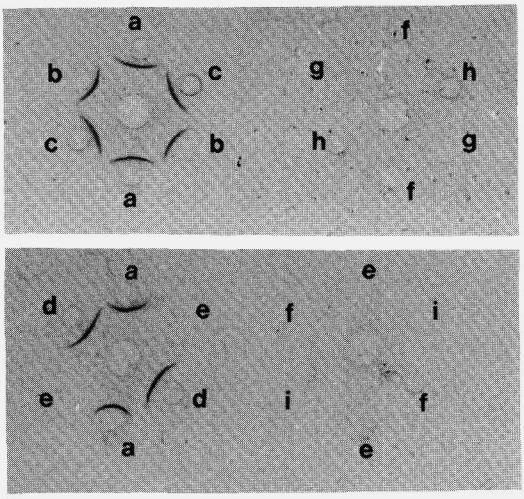


Figure 1. Characterization of the specificity of antibodies against different lipoproteins by the double diffusion technique.

a = human LDL, b = human methylated LDL, c = human VLDL, d = human acetylated LDL, e = human HDL, f = rat LDL, g = rat VLDL, h = rat HDL, i = rat lipoprotein deficient serum.

LM and EM studies

Fixed liver tissue was dissected and 200 μm vibratome slices were immersed in 2.3 M sucrose. Small pieces of tissue were then placed on a specimen holder and frozen in liquid nitrogen. Semithin and ultrathin cryosections were prepared as described by Tokuyasu (53) with a Reichert FC4 cryomicrotome. Ultrathin sections were placed on carbon coated nickle grids for immunolabeling. Semithin LM-sections were placed on glass coverslips. Using this method the antigenicity is well preserved and the antigens are optimally accessible to the antibodies in comparison to conventional electronmicroscopical techniques. Semithin sections were viewed in a Leitz ortholux microscope.

Standard Rhodamine and FITC emission and excitation filter combinations were used, respectively for viewing DiI- and latex fluorescence. The pattern of labeling was related to the tissue structure by comparing the same field with phase contrast illumination. Ultrathin sections were viewed in a Philips EM 401 microscope.

Immunolabelings procedure

The antisera and gold probes were diluted in PBS containing 0.1% gelatin, 0.5% BSA and 0.1% Tween 20. The dilutions used were: anti-apo B, 1 : 500; anti-acid phosphatase, 1 : 200; anti-α-glucosidase, 1 : 100; goat anti-rabbit IgG-gold, 1 : 80; protein A-gold, 1 : 50. For the washing steps the same medium was used unless otherwise indicated.

Cryosections were incubated with 0.1 M glycine in PBS pH 7.4, washed, incubated with the primary antibody, washed, incubated with secondary antibody coupled to colloidal gold, washed, washed again with aqua dest, stained with uranylacetate and covered with 1% tylose. In case of a double labeling procedure the first primary antibody was labeled with a secondary antibody coupled to colloidal gold particles (5 or 10 nm), washed, incubated with an excess protein A before incubating with the second primary antibody and finally labeled with protein A-gold, distinct in size from the first step, as described by Slot *et al.* (50). In control sections, the primary antibodies were omitted from the procedure or preimmune rabbit serum was used.

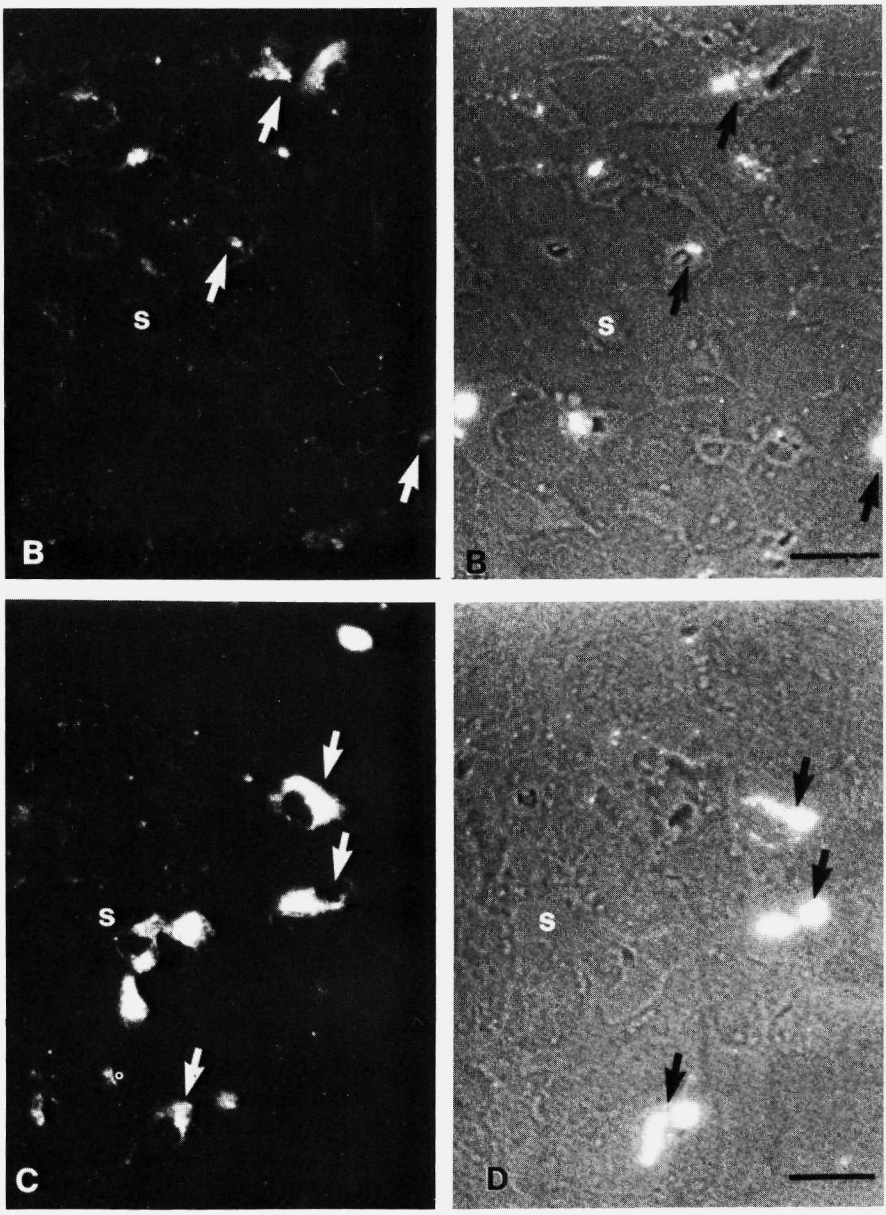
Gelelectrophoresis and western blotting

Biliary proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemlli (29). The gels were stained with silver according to Merril *et al.* (31). Proteins were transferred from polyacrylamide gels to nitrocellulose sheets according to Towbin *et al.* (54) using an LKB NOVA blot apparatus for 1.5 hour with 1.5 mA/cm^2. As a transfer medium 39 mM glycine, 48 mM trishydroxymethyl-aminomethane (TRIS), 0.03% SDS and 20% methanol was used. Immunostaining was done with rabbit anti-human apo B (9).

RESULTS

Lightmicroscopical visualization of LDL uptake by the liver.

To determine the cell types involved in the uptake of LDL, DiI labeled LDL was injected into untreated and EE treated rats. Uptake of DiI-LDL resulted in a discrete and bright fluorescence inside cells. The observed signal was not due to unspecific adherence of DiI, since fluorescence was not observed when the rats were injected with DiI-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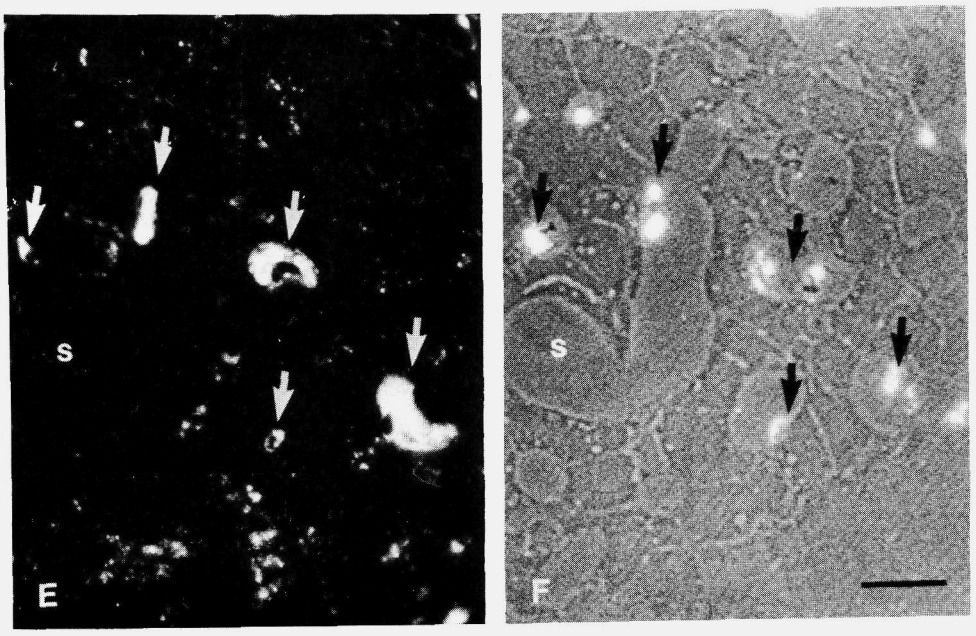


Figure 2. Cryosections of untreated rat livers showing the labeling with DiI-LDL at various times after injection.

Same fields have been photographed for DiI fluorescence (a,c,e) and for latex fluorescence in combination with normal illumination (b,d,f). Kupffer cells were identified by their phagocytic uptake of fluorescent latex particles (0.55μm). White arrows indicate DiI uptake by Kupffer cells, black arrows indicate latex uptake by Kupffer cells, s = sinusoid. Bar = 10 μm.

A: Fluorescence of DiI- LDL at 10 min after injection. **B:** Fluorescence of latex from the same section as in A. **C:** Fluorescence of DiI-LDL in Kupffer cells 30 min after injection. **D:** Fluorescence of latex in the same section as in C. **E:** Fluorescence of DiI-LDL in Kupffer and some in parenchymal cells 60 min after injection. **F:** Fluorescence of latex in the same section as in E.

labeled methylated LDL. This indicated that the uptake of DiI-LDL was mediated by the LDL receptor in untreated as well in EE treated rats.

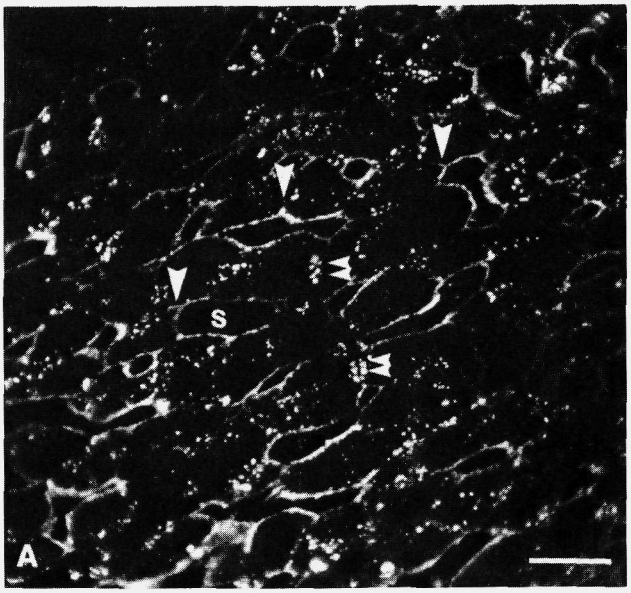
In untreated rats, at 10 minutes after injection of DiI-LDL, a slight, but discrete fluorescence in Kupffer cells, as identified by their uptake of fluorescent latex, and a very weak perisinusoidal fluorescence was observed (Fig. 2a, b). At 30 minutes, most Kupffer cells were brightly labeled and a slight fluorescent signal was found inside parenchymal cells (Fig. 2c, d). At 1 hour, Kupffer cells were still brightly labeled and also parenchymal cells showed some fluorescence, mainly in area's around bile canaliculi (Fig. 2e, f).

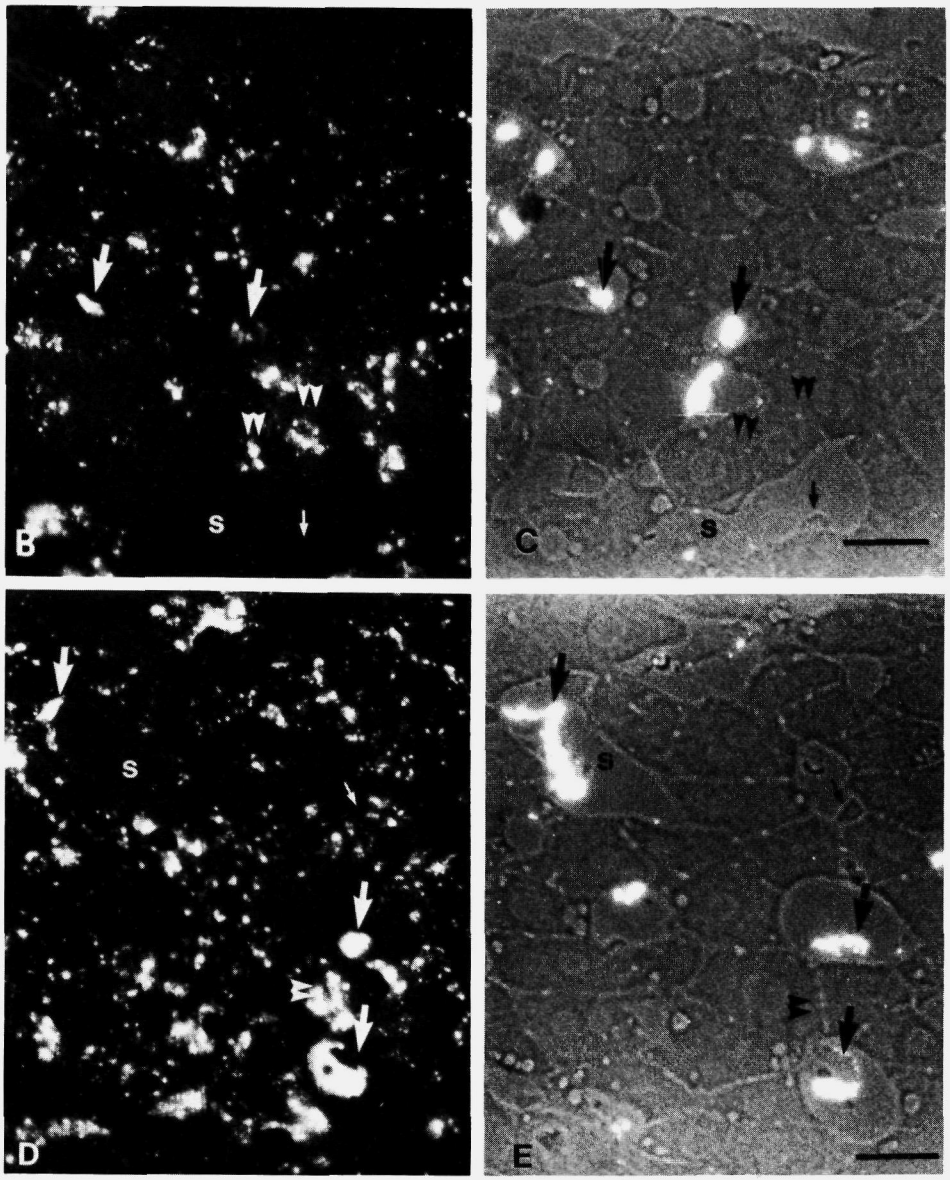
In EE treated rats, the intensity of fluorescence in Kupffer cells was, at all time points after injection of DiI-LDL, not altered as compared to control animals. However a striking increase in the labeling of the parenchymal cells was observed. At 10 minutes after injection a strong fluorescence of DiI-LDL was observed along the sinusoids, associated with the apical side of the parenchymal cells and the upper lateral side between two adjacent hepatocytes (Fig. 3a). Intracellularly, punctate labeling near bile canaliculi. was observed. During the next 60 minutes the plasma membrane bound signal of

Figure 3. Cryosections of estradiol treated rat livers showing the labeling with DiI-LDL at various times after injection.

Same fields have been photographed for DiI fluorescence (a,c,e) and for latex fluorescence in combination with normal illumination (b,d,f). Kupffer cells were identified by their fagocytic uptake of fluorescent latex particles. White arrows indicate DiI uptake by Kupffer cells, black arrows indicate latex uptake by Kupffer cells. Large single arrow heads indicate fluorescence at the lateral side between two adjacent hepatocytes, double arrow heads indicate DiI fluorescense near bile canaliculi, s = sinusoid. Thin arrows indicate endothelial cells. Bar = 10 μm (Fig 3b-e, see next page).

A: Fluorescense of DiI- LDL along the apical and lateral side of the parenchymal cells and near bile canaliculi, at 10 minutes after injection of DiI-LDL. **B:** Punctate fluorescence of DiI in parenchymal cells, around bile canaliculi and Kupffer cells, at 30 minutes after injection of DiI-LDL. **C:** Same section as in C, showing latex fluorescence. **D:** Fluorescence of DiI in parenchymal cells around bile canaliculi and Kupffer cells at 60 minutes after injection of DiI-LDL. **E:** Same section as in E, showing latex fluorescence.

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DiI-LDL on parenchymal cells disappeared and the labeling inside the parenchymal cells increased strongly. Especially around the bile canaliculi a bright punctate labeling was present (Fig. 3b-e). At all described time points no fluorescence associated with liver endothelial cells was noticed.

For rats treated with estradiol for 1 day or 5 days, similar patterns of fluorescence were observed as when the rats were treated for 3 days. Lobular heterogeneity was not evident.

Electron microscopical visualization of LDL uptake by the liver.

To identify the intracellular structures involved in the uptake of LDL, the studies were extended to the electron microscopical level. At all the time points studied, few gold particles were observed over Kupffer cells in untreated as well as in EE treated rats. These amounts were too low to allow adequate interpretation. This low amount of labeling was possibly due to a rapid internalization of LDL and a lysosomal breakdown of apo B, thereby preventing recognition by the antibody, as will discussed later in this chapter. No specific label was observed over endothelial cells.

The immunogold labeling of apo B in the untreated rats, injected with LDL was very low, at any of the time points studied. At 10 minutes after injection of LDL only a few gold particles representing immunoreactive apo B were noticed in the space of Disse (Fig. 4a): no label could be detected in the bile canalicular regions. Up to 60 minutes after injection, few gold particles were present at the apical side of the parenchymal cells. Occasionally label in the bile canalicular regions was observed, associated with small vesicular structures and electron lucent multivesicular structures (not shown).

In EE treated rats, immunogold label was abundantly present and its distribution pattern could be readily followed at the different time points after LDL injection. Ten minutes after injection, most of the gold particles were localized in the space of Disse, in close association with the microvilli located at the apical and lateral side of the parenchymal cell (Fig. 5a). Some gold particles were apparently present in coated structures at the basis of the microvilli and in endocytic vesicles just beneath the plasmamembrane. Further inside the cell, gold particles were also localized in electron lucent vacuoles, small vesicles, undefined cytoplasmic structures and multivesicular structures near bile canaliculi. Also some particles were observed inside bile canaliculi and at the basis of the microvilli suggesting transcytosis to the bile (Fig. 5b). In none of the examined sections was label inside lysosomal structures observed.

At 20 minutes to 1 hour after injection of LDL, the labeling density at the sinusoidal surface of the parenchymal cells was decreased and an increase of label in endocytic structures, multivesicular structures, small vesicular structures near bile canaliculi and inside bile canaliculi was observed. A portion of the labeled multivesicular struc-

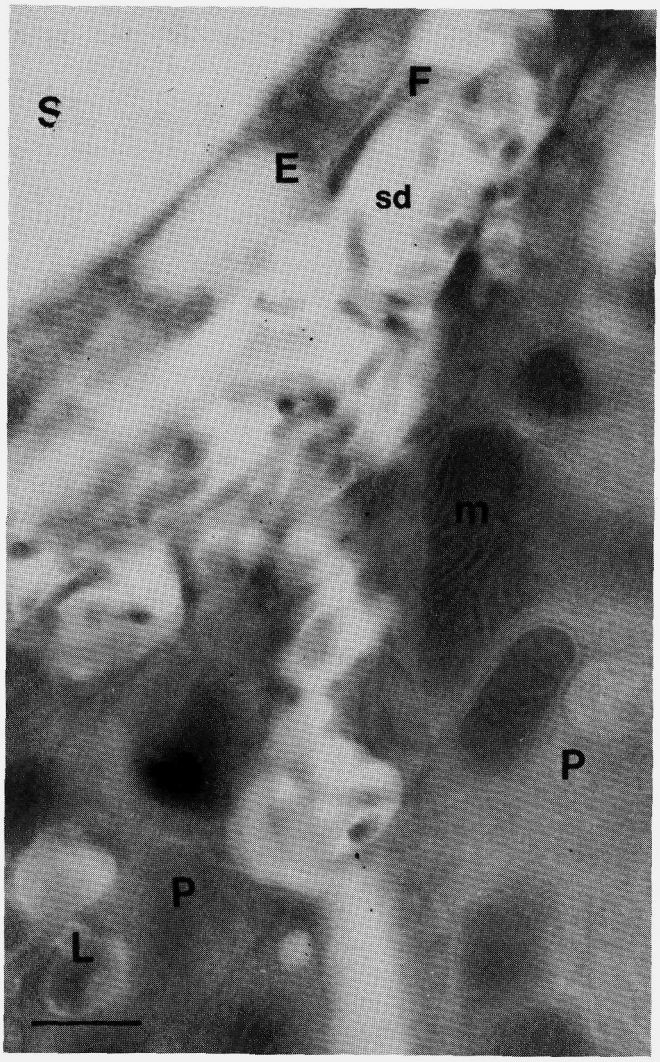


Figure 4. Immunolabeling with anti-apo B (GAR 10nm) of a cryosection of an untreated rat liver, 10 minutes after injection of LDL.

P = parenchymal cel, sd = space of Disse, F = fat storing cell, E = endothelial cell, S = sinusoid, m = Mitochondrium, L = lysosome. Magnification = 27,500 x; bar = 0.5 μm. **A**: A few gold particles are noticed in the space of Disse.

tures contained appendages, possibly representing an LDL-receptor enriched compartment, as described by Jost-Vu *et al.* (22) and Belcher *et al.* (2). Occasionally label was present inside more electron dense, acid phosphatase positive, lysosomes. The low amount of label over lysosomal structures was not the result of a loss of antigenicity of apo B in an acidic environment as was tested *in vitro* using a dot blot assay (results not shown).

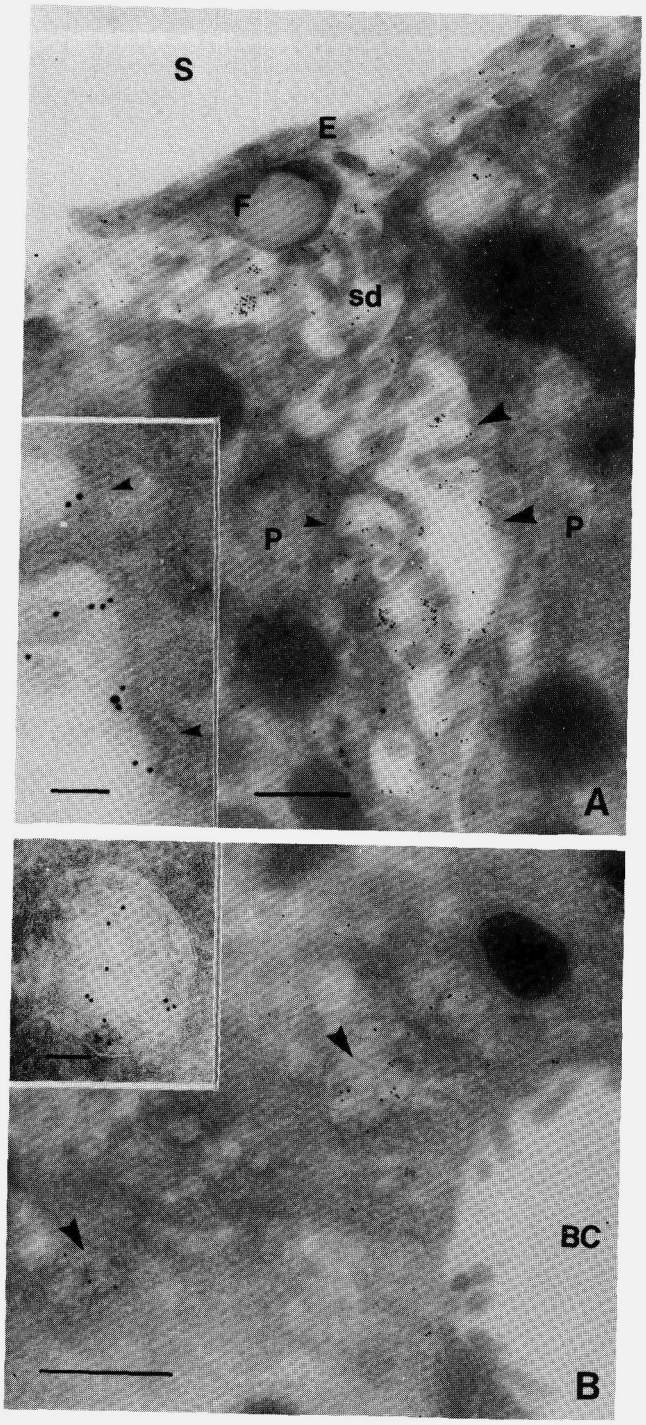


Figure 5. Immunolabeling with anti-apo B (GAR 10nm)of a cryosection of an estradiol treated rat liver, 10 minutes after injection of LDL.

P = parenchymal cel, sd = space of Disse, F = fat storing cell, E = endothelial cell, S = sinusoid, BC = bile canaliculus, m = mitochondrium. Arrow heads indicate label located near coated pits (see inset A) and multivesicular body (see inset B). Magnification (A) = 26,356 x; inset (A) = 79,068 x; (B) = 37,125 x; inset (B) = 61,776 x; bar = 0.5 μm, in inset bar = 0.1 μm.

A: Numerous gold particles are located in the space of Disse and in the area between two adjacent hapatocytes. Inset: label near coated structures.

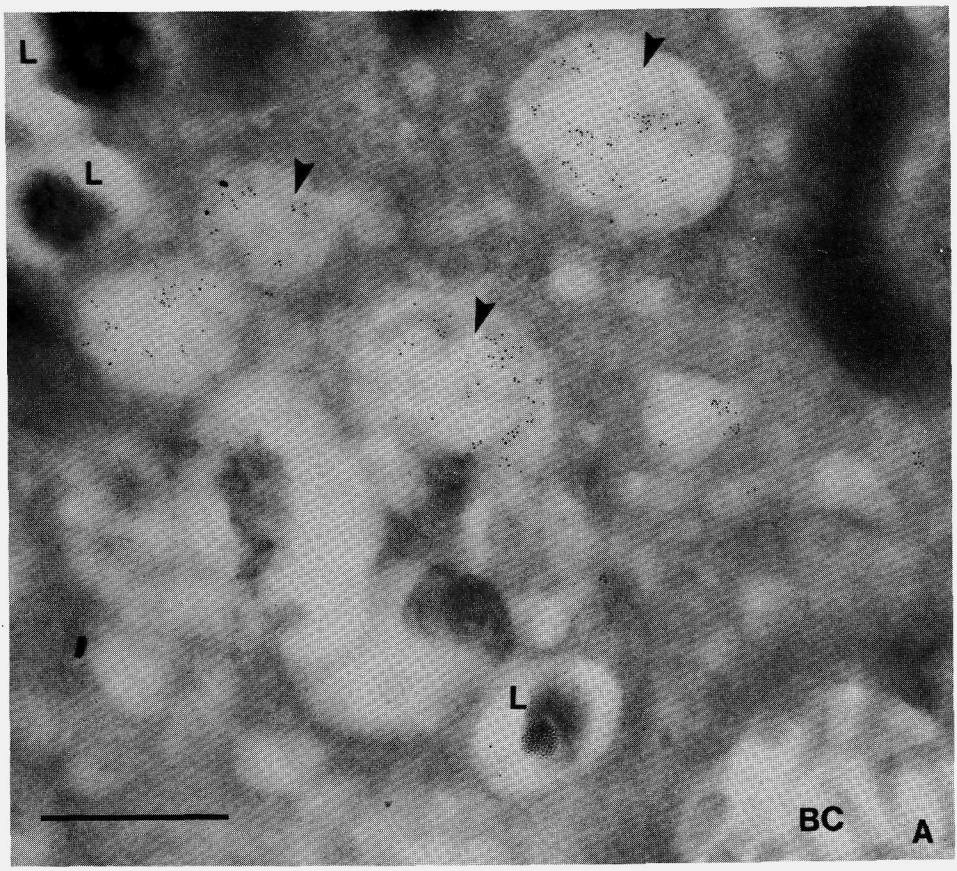
B: Gold label is located in multivesicular structures and near the bile canaliculus. Inset shows a multivesicular body. Inset: label in multivesicular bodies.

Effect of lysosomal inhibitors on apo B labeling in parenchymal and non-parenchymal cells.

To investigate whether a rapid lysosomal breakdown of the endocytosed LDL could have prevented immuno-labeling of apo B inside lysosomes, EE treated rats were also treated with chloroquine or leupeptin prior to LDL administration.

The effect of chloroquine treatment was studied 20 minutes after injection of LDL. Chloroquine treatment resulted in an increased number and a higher apo B immuno-labeling of electron lucent structures in parenchymal cells (Fig. 6a). After immunogold labeling for lysosomal enzymes, these structures were negative for acid phosphatase but some of them were shown to be positive for α-glucosidase. In analogy with the results of Hornick *et al.* (19, 20), who demonstrated the prelysosomal nature of similar enlarged multivesicular structures, they must be identified as late, CURL type endosomes. Chloroquine treatment resulted in an impediment with the conversion of these multivesicular structures to lysosomes. Therefore, the total number of these organelles

Figure 6a. For legend see next page



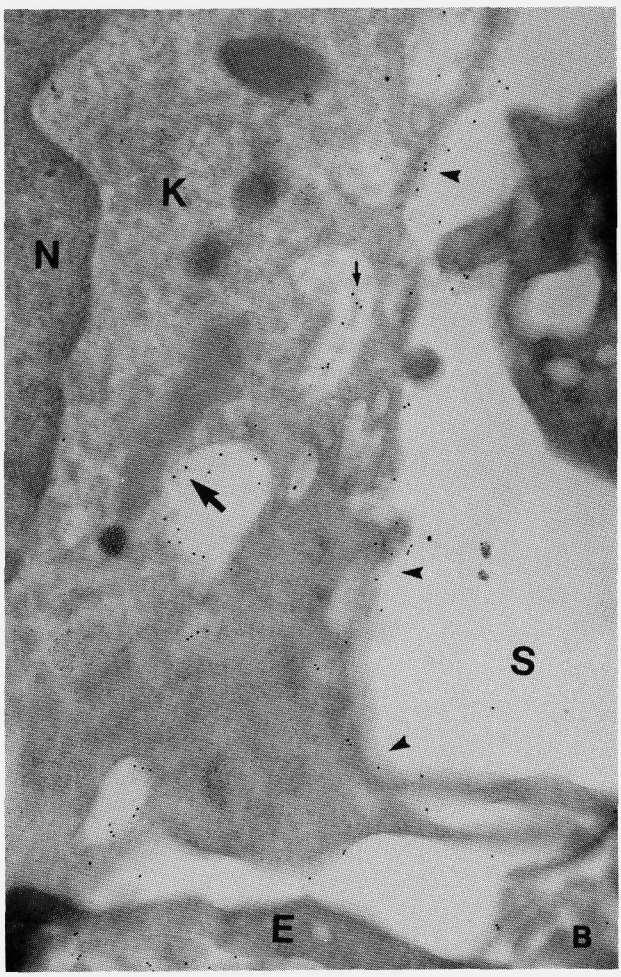


Figure 6. Immunolabeling with anti-apo B (GAR 5nm or GAR 10nm) of a cryosection of an estradiol and chloroquine treated rat liver 20 minutes after injection of LDL.

K = Kupffer cell, N = nucleus, E = endothelial cell, S = sinusoid, BC = bile canaliculus, L = Lysosome. Magnification (A) = 47,500 x; Magnification (B) = 34,200 x; bar = 0.5 μm. Arrow heads indicate label associated with the plasma membrane. Arrows indicate label inside vacuoles.

A: Numerous gold particles (5 nm) are localized into electron lucent vacuoles representing multivesicular structures. Ferritin aggregate is present in one of the lysosomes. **B:** Kupffer cell is labeled (10 nm) on the extracellular plasmamembrane (arrowheads) and inside vacuoles associated with the membrane (large arrows) and with fuzzy material (small arrow).

was increased. A small increase in apo B label in electron dense, acid phosphatase positive, vacuoles was also observed.

The effect of leupeptin treatment was studied at 60 minutes after LDL injection. At this time point, some immunolabel was still present on the hepatocellular membrane (Fig. 7a, b). An increase of intracellular label was observed in the pericanalicular structures (Fig. 7d), including multivesicular structures and more electron dense lysosomes. After immunogold labeling for lysosomal enzymes, these stuctures were partially positive for α-glucosidase, the more electron dense these structures were, the more acid phosphatase immunolabeling was observed. This indicates that lysosomes are also involved in the intracellular handling of LDL in parenchymal cells.

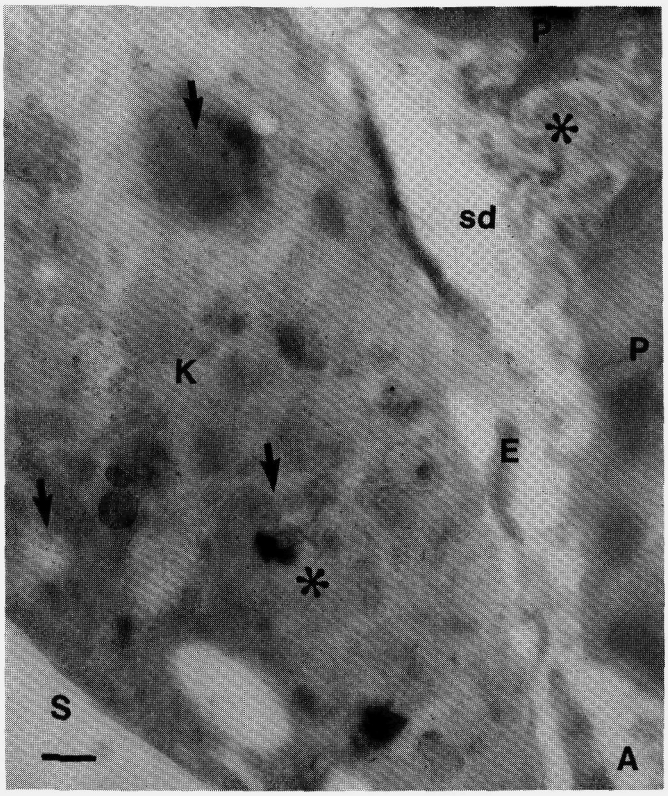
In Kupffer cells, chloroquine treatment resulted in an increased labeling of Kupffer cells. Gold particles were localized, along the exterior cell membrane and inside electron lucent vacuoles, in which label was often attached to the membrane, or associated with fuzzy material (Fig. 6a). After leupeptin treatment immuno- reactive apo B could be observed in secundary lysosomes of Kupffer cells (Fig. 7a, c). In addition to the results obtained at the light microscopical level, these data confirm that LDL is taken up by Kupffer cells via receptor mediated endocytosis and degraded inside lysosomes. Treatment with lysosomal inhibitors did not result in any immuno labeling of the liver endothelial cells, which is further evidence that these cells were not involved in the uptake of LDL.

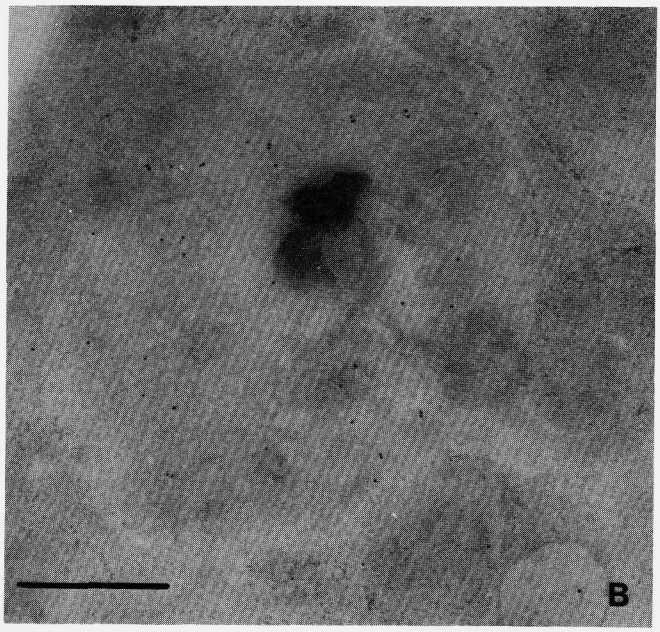
Figure 7. Immunolabeling with anti-apo B (GAR 10nm) of a cryosection of an estradiol and leupeptin treated rat liver 60 minutes after injection of LDL.

K = Kupffer cell. E = endothelial cell, P = parenchymal cell, S = sinusoid, L = lysosome, sd = space of Disse. Magnification (A) = 13,640 x; (B) = 38,720 x; (C) = 38,720 x; (D) 37,500 x; bar = 0.5 μm (Figure b-d, see next pages).

A-C: Labeling is present inside lysosomes of Kupffer cells (a, c) and on the microvilli of the hepatocytes (a, b). Parts of the figure are enlarged, as indicated with an asterisk.

D: Parenchymal cell labeled in the bile canalicular region, gold particles are present in a lysosome.





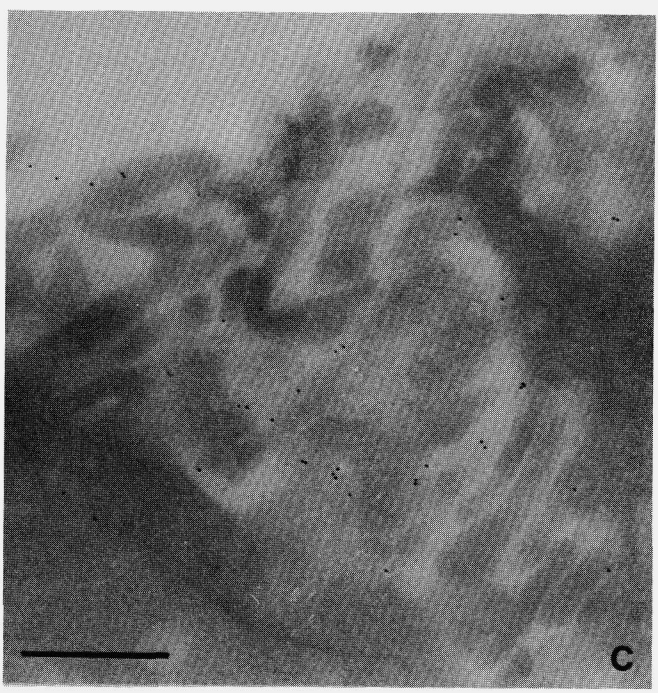


Figure 7b, c.
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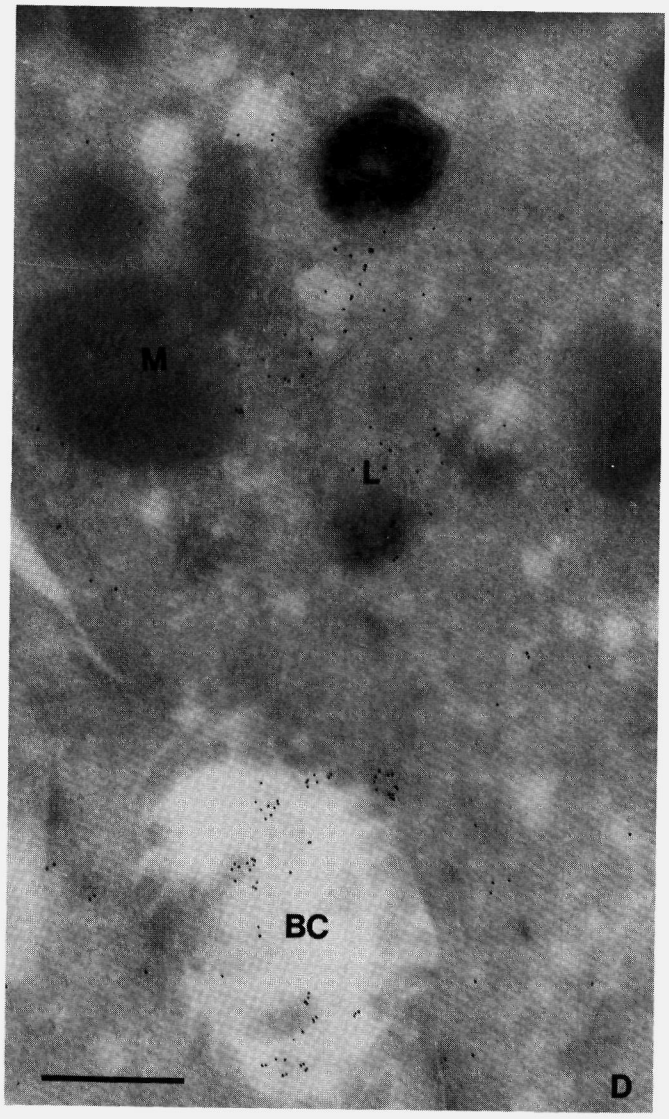

Figure 7d.
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In vivo serum decay, liver association and biliary secretion of ^{125}I-Tc labeled LDL.
Serum decay and liver association.

The serum decay of ^{125}I tyramine cellobiose labeled LDL (^{125}I-Tc-LDL) in untreated and EE treated rats is shown in Fig. 8. EE treatment of rats markedly increased the serum decay of LDL.

The association of ^{125}I-Tc-LDL with livers of untreated and EE treated rats is shown in Fig. 9. In control rats, the liver- associated radioactivity increased with time and reached a level of about 10% of the injected dose at 24 hours after injection. The asso-

ciation of ^{125}I-Tc-LDL with the livers of EE treated rats in the first 30 minutes rapidly reached a level of 40% of the injected dose (Fig. 9). After 2 hours, the amount of radioactivity associated with the liver decreased slowly to a value of 11% at 24 hours after LDL injection and up to 48 hours after injection this level is retained. Since tyramine cellobiose is trapped inside lysosomes, the values at 24 hour and later approach the absolute amounts of LDL catabolized in the lysosomes.

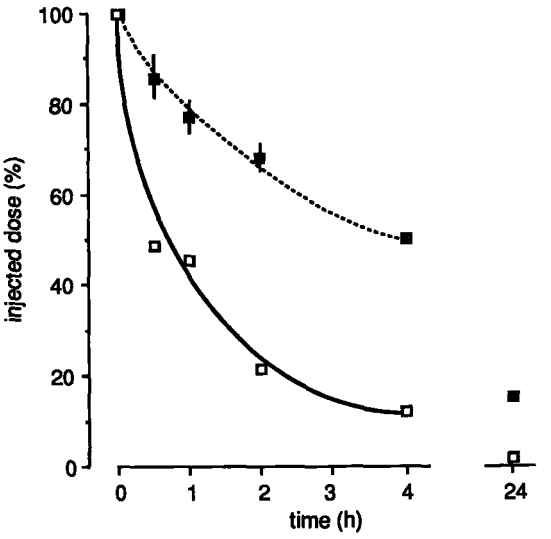

Figure 8. Serum decay of ^{125}I-tyraminecellobiose labeled LDL in control and estradiol treated rats. ^{125}I-Tc-LDL was injected into untreated (■) and estradiol (□) treated rats. At the indicated time points serum radioactivity was measured. Values are expressed as percentage of the injected dose ± s.e.m. For the estradiol treated condition typical data are given corresponding to the data obtained with the same lipoprotein preparation as in figure 9 and 10a.

Characterization of the biliary appearance of radio-labeled LDL

Figure 10a shows the biliary appearance of radioactivity up to 48 hours after injection of ^{125}I-Tc-LDL and ^{125}I-Tc-MeLDL in both control and EE treated rats. In EE treated rats, a considerable peak of radioactivity was excreted into bile around 2 hours after injection of LDL. This peak in biliary secretion in EE treated animals was 7 to 10 times higher than that in control rats. This high peak of radioactivity in bile is not observed when methylated ^{125}I-Tc-LDL is injected into EE treated rats, which indicates that the secretion is coupled to recognition by the LDL receptor. Cumulative over 24 hours, 42% ± 6.8% of the injected radioactivity in the EE treated rats and 22.3 ± 5.6% of the injected radioactivity in the control rats were secreted into bile (Fig. 10b).

The radioactivity, secreted into the bile, was trichloroacetic acid precipitable for more than 70%. In order to verify if intact apo B protein was secreted into bile, bile samples of both untreated and EE treated animals were subjected to SDS-polyacrylamide gel electrophoresis and stained with silver (not shown). This revealed several proteins

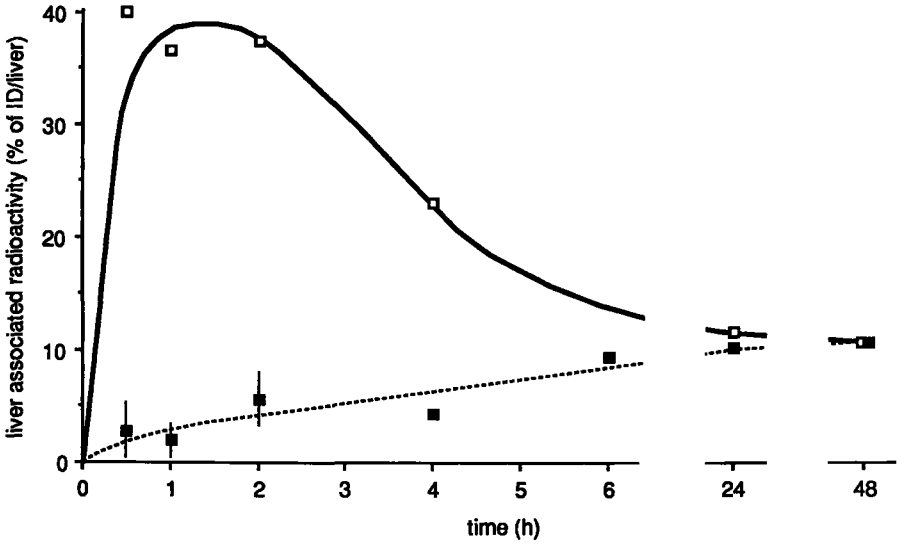

Figure 9. Association of ^{125}I-tyraminecellobiose labeled LDL with the liver in control and estradiol treated rats at different time points after injection.

^{125}I-Tc-LDL was injected into control (■) and estradiol (□) treated rats. At the indicated time points liver biopsies were taken and liver associated radioactivity was measured.

Values are expressed as percentage of the injected dose ± s.e.m. For the estradiol treated condition typical data (based on 4 rats) are given, corresponding to the data in figure 10.

with molecular weights (MW) similar to albumin, secretory component and, IgA, all known to be present in bile (25). In addition, proteins of 26 kD and 30 kD and proteins near the front were present in EE treated animals. No bands were found at the position of intact apo B (MW = 650.000).

When bile fractions were subjected to western blotting for human apo B, several immuno reactive bands were identified in samples of EE treated rats. Bile collected on ice and in the presence of the protease inhibitor PMSF, showed several reactive bands with MW ranging from 120 -200 kD (Fig. 11). When bile was collected at room temperature, bands with a lower MW ranging from 40 kD to 180 kD were reactive with the antibody (not shown). In both cases no protein with a molecular weight of apo B was seen.

To assess whether the radioactivity in bile was associated with specific fractions, bile collected at 0°C in the first 4 hours after LDL injection was subfractionated by density gradient centrifugation (Fig. 12). Radioactivity was mainly associated with the high density bottom fraction. Further, no differences in the relative distribution of radioactivity in the micellar and vesicular fraction were observed between bile from control and that from EE treated rats.

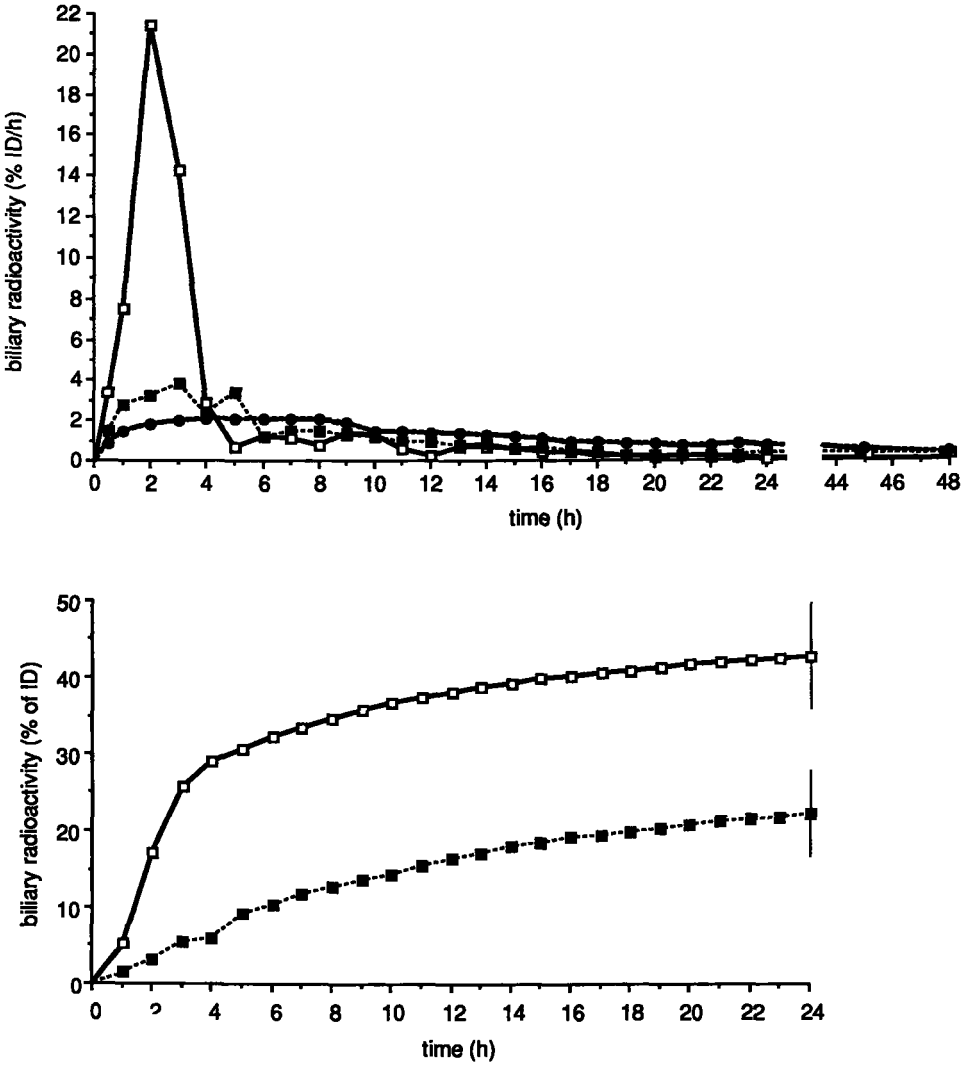

Figure 10. Biliary excretion of ^{125}I - radioactivity during 48 hours after injection.

^{125}I-Tc-LDL(■□)or ^{125}I-Tc-MeLDL (●) was injected into unrestrained control (---) and estradiol (——) treated rats equipped with permanent heart and bile cathethers. Bile was collected for 48 hours.

A: Values are expressed as a percentage of the injected dose recovered from the bile per hour.

B: Cumulative values are expressed as the mean percent of the injected dose recovered from the bile from 4 rats. In the end points the standard error of the mean is indicated.

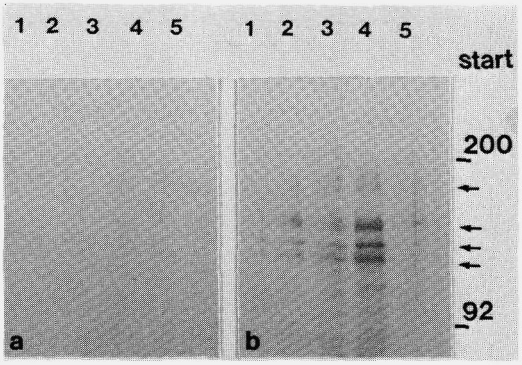

Figure 11. Western blot of bile samples from control and estradiol treated rats.

Bile samples from control (a) and estradiol treated (b) rats were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and subsequently incubated with anti apo B followed by anti rabbit-peroxidase.

A, B: Lane 1; bile sampled before injection. Lane 2, 3, 4, 5: bile fraction 15 min., 1, 2.5 and 6 hours after injection of ^{125}I-Tc-LDL.

DISCUSSION

Light microscopical visualization

The light microscopical studies were desingned to determine the relative importance of the various liver cell types in the uptake of LDL. Incorporation of the phospholipid analog DiI into LDL does not affect the recognition by the LDL receptor, as shown by Reynolds and St Clair (46). Following internalization, the lipophilic probe accumulates inside the cells. DiI can, therefore, be adequately used to determine which cells are involved in the recognition and catabolism of lipoproteins and to what extent.

The results obtained, showed that in untreated rats, Kupffer cells strongly participated in the uptake of human LDL from the circulation. This confirms quantitative biochemical findings on isolated cell fractions from liver, demonstrating the primary involvement of Kupffer cells in the uptake of LDL in untreated rats (17).

EE treatment of rats strikingly changed the relative intensity of DiI-LDL fluorescence in Kupffer versus parenchymal cells. The high level of DiI-LDL fluorescence in parenchymal cells agrees with biochemical data, concerning a selective induction of LDL receptors on parenchymal cells (17). After EE treatment, the relative contribution of these cells to the catabolism of LDL is largely increased. No evidence for any involvement of endothelial cells in livers of either untreated or EE treated rats was found.

Electron microscopical visualization

The intracellular compartments involved in the processing of LDL were visualized using cryo-immuno electron microscopy. Shortly after injection of LDL in the EE treated rats a large amount is bound to the plasma membrane of the hepatocytes, which is quickly internalized and transferred to bile canalicular regions. Although it is generally accepted that the LDL receptor present in (EE treated) liver is identical to the LDL re-

ceptor described for fibroblasts (8, 60), the intracellular behaviour of the LDL receptor in liver is at least partially different. Our results suggest that, at least in EE treated rats, LDL binding to its receptor leads to a rapid internalization and a partial targeting of LDL and possibly its receptor to the bile. Unexpectedly high amounts of immunoreactive apo B observed in the vicinity of and inside bile canaliculi of EE treated rats, wich shows that a significant part of apo B is transported to the bile.

The low amount of label in lysosomes was also demonstrated by Chao *et al.* (7). Only when leupeptin, as an inhibitor of lysosomal breakdown was administered, a slight increase in immunolabel was observed inside lysosomes of control and EE treated rats, indicating that some apo B is processed through these organelles. Chloroquine treatment, however, resulted in a large increase of apo B label in the endosomal compartment, as was also found by Hornick (19) in (^{125}I) autoradiographic studies. The known interference of chloroquine with the conversion of endosomal to lysosomal compartments, explains the low labeling of lysosomes after this treatment.

Pretreatment of rats with chloroquine or leupeptin allowed the visualization of intracellular pathway of apo B in Kupffer cells. This indicates that LDL is internalized and rapidly catabolized by these cells inside the lysosomes. Such a rapid degradation is in agreement with earlier data that non-parenchymal cells exhibit a much larger capacity to degrade LDL than parenchymal cells (55) and that these cell types contain high concentrations of lysosomal enzymes (26, 57).

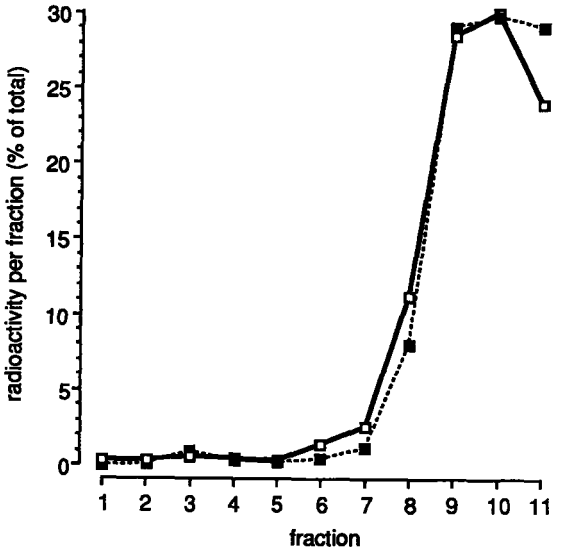

Figure 12. Distribution of ^{125}I - radioactivity over bile fractions after density gradient centrifugation.
^{125}I-Tc-LDL was injected into control (■) and estradiol (□) treated catheterized rats. Bile fractions were collected up to 4 hours after injection and subfractionated. Values are expressed as percentages per fraction of the total recovered radioactivity. The average for the total radioactivity recovered for the estradiol treated rats was 104,729 cpm and for the untreated rats this was 38,324 cpm.

Catheterized rats

In order to evaluate the morphological observations, concerning the appearance of immunoreactive apo B inside bile canaliculi, catheterized rats were used in which the biliary appearance of apo B could be quantified under non-stressed conditions.

The serum decay of ^{125}I-Tc-LDL in control rats was comparable to the data presented in literature obtained with ^{14}C-sucrose LDL (3, 5, 7, 17) and ^{14}C-cholesteryl oleate LDL (34). In the EE treated rats similar serum decay data were obtained as reported by others (3, 7, 34, 47).

The high association of 125I-Tc-LDL with the liver of EE treated animals in the first hour is in agreement with the strong fluorescence of DiI-LDL in the parenchymal cells. However, this initial high association of ^{125}I-Tc-LDL was followed, unexpectedly, by a decrease to a similar level as in untreated rats. During this period, the amount of ^{125}I-radioactivity secreted into the bile accounted for most of the decrease in liver associated radioactivity. The association of ^{125}I-Tc-LDL with the liver of both control and EE treated rats reached a steady level of about 10% of the injected dose at 24 hours after injection up to 48 hours. Since tyramine cellobiose is trapped inside the lysosomes upon degradation of the ligand, it shows that a similar percentage of injected LDL is handled via the lysosomes in untreated as well in EE treated animals. Some authors reported higher values for the association of LDL with the EE treated livers, but most of these studies were performed with ^{14}C-sucrose labeled LDL (3, 47). Sucrose labeled derivatives may also be trapped if uptake occurs by pathways different from the lysosomal pathway (1) leading to higher cumulative values. Apparently, a part of Tc-LDL is surpassing the lysosomes and is directly excreted into the bile, as was also indicated by the localization of immunogold label.

Tyramine cellobiose radioactivity was secreted into the bile of rats, the excretion was greatly enhanced after EE treatment. Since no label is found in bile after injection of methylated LDL it is concluded that secretion into the bile is specifically coupled to the uptake of LDL via the LDL receptor. The appearance of some radioactivity derived from radiolabeled LDL into the bile (52) and gut contents and faeces, the latter two also originating from the bile (5, 38), was reported earlier. This was mainly attributed to a leakage of the used label (5, 38) but the authors did not give a mechanistic explanation for this finding. The results now obtained with immunogold labeling demonstrate that there is a specific intracellular route and no paracellular "leakage" of LDL to the bile. The presence of TCA-precipitable radioactivity and of large (120-200kD) immunoreactive apo B fragments in bile shows that biliary secretion is not the result of an artifact, due to the use of tyramine cellobiose as a label for LDL.

A large number of proteins, including apo B (fragments), is present in bile of man and rat and some of these proteins originate from plasma (23, 32, 33, 49). Some apo B fragments of 100 kD were reported in bile after *in vitro* perfusion of rat livers with LDL

(23). The function of proteins in bile is largely unknown but a role in the solubilisation of lipophilic constituents in bile has been suggested by Sewell (49). Since no association of radioactivity with specific bile fractions containing lipophilic constituents was observed, a role for apo B in this respect is still questionable. Addition of protease inhibitors to the bile during collection prevented the appearance of apo B fragments of lower molecular weight, indicating that a further proteolysis occurs in bile.

Conclusions

In conclusion it can be stated that in untreated rats, LDL is strongly processed by Kupffer cells. In the EE treated animals the hepatocytes are primarily involved in the processing of LDL. Lysosomes are mainly involved in the processing of LDL in Kupffer cells and only partially in parenchymal cells. In the parenchymal cells, only some lysosomal processing occurs and a substantial portion of apo B is directly transported to the bile for excretion, a mechanism that is coupled to uptake by the LDL receptor. EE treatment enhances the relative contribution of excretion of apo B into the bile. Inside the bile, apo B can be proteolytically processed into smaller fragments. The relative (patho) physiological significance of this bile directed pathway of apo B, that prevents lysosomal processing of at least some LDL constituents remains to be established.

Acknowledgements

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INTERACTION in vivo AND in vitro OF APO E-FREE HDL WITH PARENCHYMAL, ENDOTHELIAL AND KUPFFER CELLS FROM RAT LIVER

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ABSTRACT

The interaction of apolipoprotein E-free high density lipoproteins (HDL) with parenchymal, endothelial and Kupffer cells from liver was characterized. Ten minutes after injection of radiolabeled HDL into rats 1.0 ± 0.1% of the radioactivity was associated with the liver. A subfractionation of the liver into parenchymal, endothelial and Kupffer cells, by a low-temperature cell isolation procedure, indicated that 77.8 ± 2.4 % of the total liver associated radioactivity was recovered with parenchymal cells, 10.8 M ± 0.8 % with endothelial cells and 11.3 ± 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 is obtained for a saturable, specific high affinity binding site with K_d and B_{max} values respectively, in the range of 10-20 μg HDL/ml and 25-50 ng HDL/mg cell protein. In all three cell types nitrosylated HDL and LDL 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 transfer of radioactive lipids or apolipoproteins between HDL and VLDL and it is therefore suggested that competition is exerted by the presence of apo C's in VLDL. The results presented in this paper 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.

Upregulation of the HDL binding sites can be induced by loading the cells with cholesterol either derived from 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 (9), 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). 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 tetranitromethane 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 & 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, England. Pronase was obtained from Boehringer Mannheim. Tetranitromethane (98% pure) was purchased from Aldrich Chemical Co. and Ham's F-10 medium from Gibco-Europe, Hoofddorp, The Netherlands. Throughout this study 12-week-old male Wistar rats were used.

HDL, LDL, VLDL, isolation and iodination

Human VLDL ($d < 1.006$) was isolated by differential ultracentrifugation according to 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 according to Redgrave (23) as described before (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 10% SDS-polyacrylamide gel electrophoresis, followed by Coomassie blue staining and densitometric scanning. HDL was radiolabeled with ^{125}I by the ICl method of McFarlane (26) as modified by Bilheimer et al (27).
In the lipoprotein preparations 2 ± 1% of the radioactivity was trichloroacetic acid soluble and 4 ± 1% of the radioactivity was present in lipids. The distribution of the label between the apolipoproteins of HDL was 44 ± 2% in apolipoprotein A-I, 44 ± 3% in apolipoprotein A-II and 6 ± 1% in apolipoprotein C's as determined after separation of the apolipoproteins on SDS-polyacrylamide gelelectrophoresis, using 5 - 22.5% 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 anesthetized by intraperitoneal injection of 28 mg Nembutal. The abdomen was opened and 100 μg of radiolabeled 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 anesthetized and injected with 100 μg ^{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 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 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 in situ perfusion of the liver 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 DAB, 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/l Hepes, 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 at the abcissae of the Figures or in the legends to the Figures. At the indicated time 0.5 ml samples were withdrawn and the cell-associated radioactivity per mg 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 4 mg/ml trypsin for 45 min on ice in a refrigerated room according to 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 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 ± 0.2% (n=3) of the iodine label was recovered in the upper part of the centrifuge tube, indicating minor transfer or exchange of radiolabeled 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 serum decay (Fig. 1) of apo E-free ^{125}I-HDL in rats is found to be very slow. Ten minutes after injection only 1.0 ± 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 labeled native HDL with isolated 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 radiolabeled nitrosylated HDL. Apparently, nitrosylated HDL can be recognized in the liver by a pathway different from that of native HDL. This makes it impossible to investigate specific uptake of apo E-free HDL *in vivo* by comparing native HDL with nitrosylated HDL.

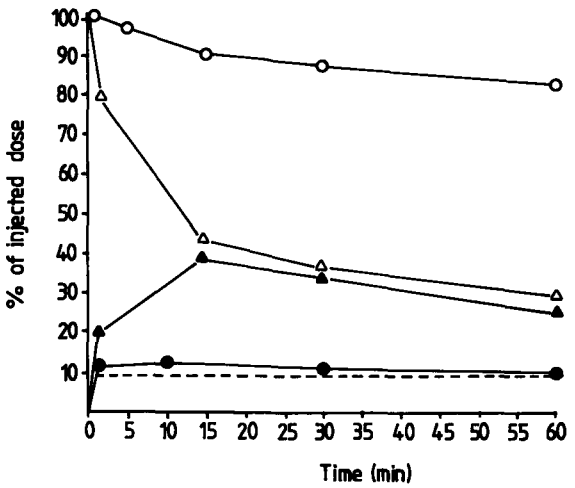

Figure 1. Liver association and serum decay of apo E-free ^{125}I-HDL and nitrosylated apo E-free ^{125}I-HDL.

Apo E-free ^{125}I-HDL (100 μg of apolipoprotein or nitrosylated HDL) was injected into anesthetized rats and the liver association and serum decay was determined. The liver was not perfused and the dotted line represents the maximal contribution of the serum value to the liver uptake (12). serum values of native HDL; serum values of nitrosylated HDL liver association of native HDL; liver association of 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 ten minutes after injection. The amount of the apo E-

free HDL associated with the total liver was 5.0 ± 0.5% (x 10^4) of the injected dose/mg 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% ± 0.1% (x 10^4) of the injected dose/mg cell protein. From the difference of 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 ± 1.8% (x 10^4) of the injected dose/mg cell protein and the uptake of HDL by Kupffer cells, 2.5% of the total liver mass, was 20.3 ± 0.1% (x 10^4) of the injected dose/mg 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 ± 2% (S.E.M., n = 4).

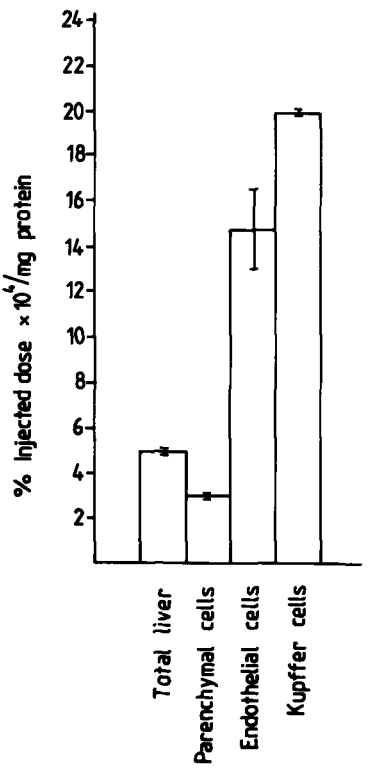

Figure 2. Distribution of apo E-free ^{125}I-HDL between Kupffer, endothelial, and parenchymal liver cells, 10 min after injection of apo E-free ^{125}I-HDL.

Cells were isolated with collagenase and pronase at 8°C. Each value represents the average of 4 cell isolations S.E.M. Values all expressed as % injected dose x 10^4 per mg cell protein.

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 hour, isolated the serum and injected it directly into a second rat. After 10 minutes we determined the cellular distribution. The values obtained for resp. endothelial cells, Kupffer cells and parenchymal cells of the screened HDL-preparation were 14.4 ± 2.8 %, 19.3 ± 2.0 % and 2.9 ± 0.2 % (x 10^4) of the injected dose/mg cell protein (n = 2). These values are identical as the values obtained with the unscreened preparations.

Table I 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 cell protein in the isolated cell fractions was multiplied with the amount of mg of protein that each cell type contributes to total liver protein. The values are expressed as percentage of total liver associated radioactivity.

Cell type	% of the total liver-associated radioactivity
Parenchymal cells	77.8 ± 2.4 %
Endothelial cells	10.8 ± 0.8 %
Kupffer cells	11.3 ± 1.7 %

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

The amount of radioactivity per mg cell protein in the isolated cell fractions was multiplied with the amount of protein that each cell type contributes to total liver protein. The values are expressed as percentage of the total liver uptake of apo E-free HDL and are the means ± S.E.M. of four determinations.

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 to parenchymal cells is shown in Fig. 3. The cell-association is rapid and reaches an equilibrium at about 1 h. After two hours of incubation 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 μg 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 μg 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 to native HDL (results not shown).

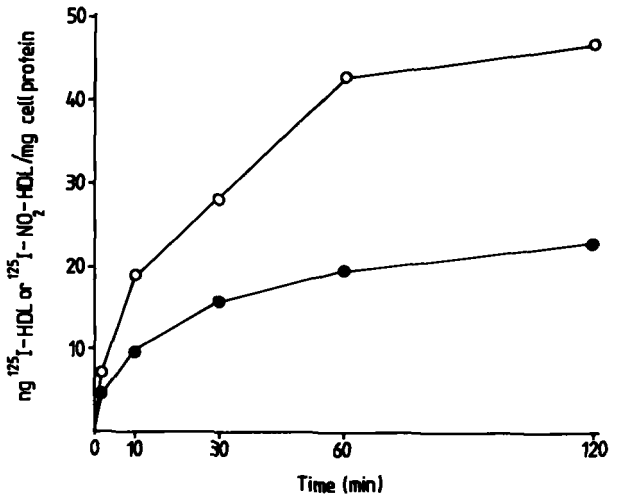

Figure 3. Time course of the in vitro association of ^{125}I-HDL (○) and nitrosylated ^{125}I-HDL (●) to parenchymal cells.

The cells were incubated with 10 μg/ml of ^{125}I-HDL or nitrosylated ^{125}I-HDL at 37°C

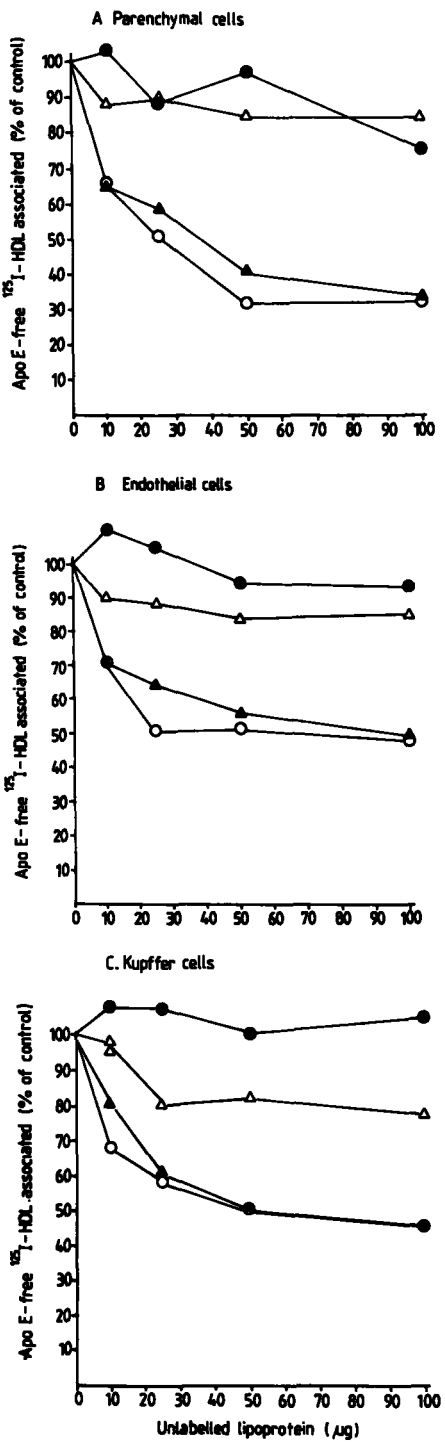

Figure 4. Comparison of the ability of unlabeled lipoproteins to compete with the cell association of 125I-labeled apo E free HDL to parenchymal (a), endothelial (b) and Kupffer cells

Cells were incubated for 10 min at 37°C with 5 μg of ^{125}I-labeled apo E-free HDL/ml and with the indicated amounts of unlabeled human LDL (△), human VLDL (▲), human HDL (0) and human nitrosylated apo E-free HDL (●). ^{125}I-labeled HDL apolipoprotein association is expressed as the percentage of the radioactivity obtained in the absence of unlabeled lipoprotein.

Comparison of the ability of unlabeled nitrosylated HDL and native lipoproteins to compete with the cell association of ^{125}I-labeled 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 radiolabeled apo E-free HDL with an excess of unlabeled nitrosylated HDL or unlabeled HDL. We incubated the cells with 5 μg ^{125}I-HDL and competitors for ten minutes at 37°C and determined the cell-associated radioactivity as described in the Materials and methods section. A short incubation time was chosen in order to minimize possible exchange of apolipoproteins, while 37°C, instead of a low temperature was applied because especially the interaction of HDL with cells is temperature-dependent (12,32). Fig. 4 shows that an excess of unlabeled HDL decreases the amount of cell-bound radioactivity by 60-65% in all three cell types, whereas an excess of unlabeled nitrosylated HDL does not compete for the binding of labeled 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 radiolabeled 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 radiolabeled apo E-free HDL, because after incubation for 10 min at 37°C only 2.5% of the label is found in VLDL (see 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 2 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

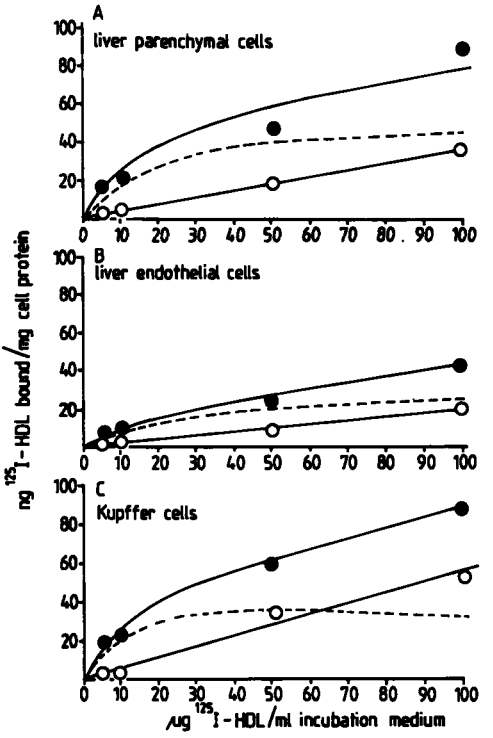

Figure 5. Relation of the concentration of apo E-free HDL to the extent of cell-binding for liver parenchymal cells (A), liver endothelial cells (B) and Kupffer cells (C).

Cells were incubated 2 hours at 37°C with varying amounts of ^{125}I-HDL. After washing the cells three times at 4°C with PBS + 0.2% BSA, they were incubated with 4 mg/ml Trypsin on ice for 45 minutes. After incubation the cell-bound radioactivity could be determined in the supernatant. The aspecific binding (○), determined in the presence of a 20-fold excess amount of apo E-free HDL was subtracted from the total binding (●), resulting in the dotted line which represents the specific binding of ^{125}I-HDL to the three cell types

viability was maintained during this procedure (the cells still excluded trypan blue). Furthermore the procedure removes all cell-bound radioactivity, exposed at the 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. 5a-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 amount 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 cell protein.

DISCUSSION

In previous studies (12,13) we found that after intravenous injection of apolipoprotein labeled 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 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 range of 10 - 20 μg of HDL/ml and 25 - 50 ng of HDL/mg cell protein. These values are comparable to 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 C's. 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, while under the same condition a 50 - 60% inhibition of the cell-association of radioactive HDL is noticed.

The absence of a significant effect of unlabeled 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 lab indicate that human hepatocytes in this respect 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 indicate 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 upon the relative cholesterol load of the cell (membrane).

Acknowledgements

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NITROSYLATED HIGH DENSITY LIPOPROTEIN IS RECOGNIZED BY A SCAVENGER RECEPTOR IN RAT LIVER

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ABSTRACT

In order to assess the presence of specific recognition sites for high density lipoprotein (HDL) *in vivo*, HDL was nitrosylated with tetranitromethane and the decay and liver uptake were compared with that of native HDL. The association of intravenously injected nitrosylated HDL (TNM-HDL) with liver was greatly increased as compared to native HDL. Using a cold cell isolation method, it became evident that the liver endothelial cells were responsible for the increased uptake of the modified HDL. The involvement of the endothelial cells in the uptake of TNM-HDL from the circulation could also be demonstrated morphologically by using the fluorescent dye dioctadecyl-tetramethyl-indocarbocyanine perchlorate (DiI) to label HDL. *In vitro* competition studies with isolated liver endothelial cells indicated that unlabeled modified HDL and acetylated LDL displaced iodine-labeled TNM-HDL, while no competition was seen with LDL and a slight displacement was seen with unlabeled native HDL. Non-lipoprotein competitors of the scavenger receptor such as fucoidin and polyinosinic acid blocked the interaction of TNM-HDL with the liver endothelial cells. Also the degradation of TNM-HDL was blocked by low concentrations of chloroquine. It can be concluded that a scavenger receptor on liver endothelial cells is involved in the clearance of tetranitromethane-modified HDL, which excludes the possibility of using TNM-HDL *in vivo* to assess the non-receptor-dependent uptake of HDL. The use of nitrosylated HDL *in vitro* as a low affinity control is limited to cell types that do not possess scavenger receptors, because cell types with scavenger receptors will recognize and internalize TNM-HDL by a high affinity scavenger pathway.

INTRODUCTION

High affinity binding sites for high density lipoproteins (HDL) have been detected on liver cells (1-9) and liver membrane preparations (10-13) of various species and peripheral cell types (14-18). In peripheral cells the amount of high affinity sites can be up-regulated by loading the cells with cholesterol, either in the form of cholesterol-albumin complexes (13, 16, 18) or as acetylated LDL (15, 17). According to the concept of Glomset (19), HDL functions as a cholesterol acceptor of the peripheral cells and will deliver its cholesterol mainly to the liver. Several studies concerning the identification of the apolipoprotein involved in the recognition of HDL by different cell types indicated a role for apo A-I (20, 21).

Recent studies have shown that treatment of human apo E-free HDL with tetranitromethane (TNM) inhibits binding of the HDL to the high affinity HDL binding sites of fibroblasts (22), hepatic membranes (23, 24), and ovarian cells (25). It is known that te-

tranitromethane treatment affects the tyrosine residues on proteins (26). Two types of conditions have been used to modify HDL. Nestler, Chacko, and Strauss (25) used a 0.5-10 x molar excess of TNM over HDL, while Brinton et al. (22) used varying concentrations of tetranitromethane (TNM) (0.03-30 mM). In the present investigation we tested various nitrosylation conditions in order to use the nitrosylated HDL *in vivo* to assess the role of the high affinity site in the uptake of HDL by the various liver cell types. A comparison of the uptake of native HDL with that of nitrosylated HDL should then indicate the relative importance of the tentative HDL receptor in a similar way as used earlier for LDL and methylated LDL (27, 28). However, it appeared that irrespective of the nitrosylation condition the TNM-HDL disappeared more rapidly from the circulation than untreated HDL.

In the present study we determined which hepatic cells were responsible for the increased interaction of TNM-HDL with the liver. Low temperature cell isolation techniques were used (29). Furthermore, the tentative receptor responsible for the avid interaction is indicated. We also visualized the cell type responsible for the *in vivo* clearance of TNM-HDL by the liver and therefore used the fluorescent molecule 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) (30). DiI is a highly lipophilic molecule that can be noncovalently incorporated into lipoproteins and thus has no effect on surface charge. When the fluorescent-labeled lipoprotein is internalized and degraded within cells, the lipophilic fluorenscent probe remains in the lysosomal membrane of the individual cells. DiI has been used to visualize the uptake of lipoproteins by macrophages, fibroblasts and arterial foam cells (30, 31), hepatocytes (32), endothelial cells (33, 34), and vascular endothelium (35).

MATERIALS AND METHODS

Materials

Collagenase (type I), bovine serum albumin (fraction V), chloroquine, and polyinosinic acid were purchased from Sigma (St. Louis, MO). Tetranitromethane (TNM) was obtained from Aldrich Chemical Company (Brussels, Belgium), metrizamide from Nyegaard & Co. (Oslo, Norway), Na ^{125}I from Amersham International (Amersham, UK), 1,1'-dioctadecyl-3,3,3',3'-indocarbocyanine perchlorate C18 (DiI) from Molecular Probes (Eugene, OR), fucoidine from Kochlight (Haverhill, UK), and Ham's F-10 medium from Gibco-Europe, Hoofddorp, The Netherlands. All other chemicals and solvents were of reagent grade.

Animals

Throughout the study 3-month-old Wistar rats were used; they had free access to food and water. Prior to the experiments, the rats were anesthetized with nembutal given intraperitoneally. Radiolabeled lipoproteins were injected into the inferior vena cava. DiI-labeled lipoproteins were injected into the jugular vein of overnightfasted rats after halothane anesthesia. The injected dose was calculated on the basis of a plasma volume of 3.75 ml per 100 g body weight for our rats (29).

Methods

For the determinations of serum decay and liver association, serum and liver samples were taken at the several time intervals, as described by Van Berkel et al. (36, 37). The cellular distribution in the liver of the injected lipoproteins was determined by isolation of liver cells at low temperature. Parenchymal cells and nonparenchymal cells were isolated by a cold collagenase method and nonparenchymal cells were further separated into endothelial and Kupffer cells by centrifugal elutriation (29, 38).

For *in vitro* binding studies, the different types of liver cells were isolated using collagenase at 37°C (3, 4, 29). The purity and integrity of the parenchymal cells (99% pure) were judged by light microscopy and trypan blue exclusion. The purity of the Kupffer and endothelial cell preparations was greater than 95%, as determined using an endogenous peroxidase stain (39).

Lipoprotein isolation, labeling and modification

Human HDL ($1.06 < d < 1.21$ g/ml) were isolated using the method of Redgrave, Roberts, and West (40). The HDL fraction was subjected to a second identical centrifugation to remove albumin and was further processed by heparin-Sepharose chromatography to remove apo-E (41). The absence of apo-E was checked by SDS-PAGE under reducing conditions according to Laemmli (42).

Native human HDL was radiolabeled with ^{125}I using the method of McFarlane (43) as modified by Bilheimer, Eisenberg, and Levy (44). The specific activities of the ^{125}I-labeled HDL ranged from 100 to 200 cpm/ng lipoprotein.
Distribution of label in HDL was 44% ± 2% in apo A-I; 44% ± 2% in apo A-II; and 6% in apo Cs; 2% was TCA-soluble and 4% was present in lipids.

For nitrosylation of radiolabeled HDL, a 0.5- to 10-fold molar excess of TNM to HDL (HDL:TNM = 1:x) was used, based on the presence of seven tyrosine residues per molecule of apo A-I as described by Nestler et al. (25). HDL was also nitrosylated using various molar solutions of TNM (TNM-HDL) as described by Brinton et al. (22).

Low density lipoprotein (LDL) was acetylated (AcLDL) with acetic acid anhydride as described by Basu et al. (45). Native and modified HDL were subjected to SDS-PAGE (42) and to agarose gel electrophoresis, according to the method of Demacker (46).

HDL and TNM-modified HDL were labeled with DiI according to Pitas et al. (30), the density of the incubation mixture was subsequently raised to 1.21 g/ml with KBr, and the lipoproteins were reisolated by ultracentrifugation (39). The DiI-labeled lipoproteins were then made free of apo-E as described above.

In vitro experiments

For competition studies, freshly isolated liver cells were incubated with different amounts of unlabeled human HDL, LDL, modified HDL, or AcLDL in the presence of an indicated amount of radiolabeled native or modified HDL or AcLDL in Ham's F-10 medium, 1% BSA for the indicated periods at 37°C. Samples were centrifuged for 2 min at 600 g. The pellets were resuspended in 50 mM Tris-HCl (pH 7.4) 0.15 M NaCl, 1% BSA, and washed twice. A final washing was performed in a similar medium without BSA. Degradation of lipoproteins was measured as previously described by Van Berkel et al. (4). The degradation values represent radioactivity present in the acid-soluble water phase. Inhibitors of cellular association or degradation were added prior to the lipoproteins. Protein determinations were according to the method of Lowry et al. (47) using BSA as a standard. All experiments were repeated at least two times with different cell and lipoprotein preparations.

Light and electron microscopy

For the visualization studies, DiI-lipoproteins (5-15 μg/ml plasma) were injected into the jugular vein and allowed to circulate for the indicated periods.

At the indicated time points, rat livers were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) by a total body perfusion-fixation via the heart. Tissues were dissected and processed for light microscopical cryomicrotomy as described by Tokuyasu (48). Cryosectioning was performed on a Reichert FC4 cryomicrotome. Sections (thickness: 4 μm) were picked up with a droplet of sucrose, placed on glass slides, and mounted with glycerol. Photographs were taken with a Leitz Orthoplux Microscope, using standard Rhodamine emission and excitation filter combination and Kodak 1600 Ektachrome films. For the electron microscopy, lipoprotein samples were negatively stained with phosphotungstate according to Forte and Nordhausen (49).

RESULTS

In vivo biochemical studies

The disappearance of native apo E-free HDL from serum proceeded at a low rate and less than 2% of the iodine radioactivity was found in liver up to 60 min after injection. In contrast to native HDL, nitrosylated HDL injected into rats was cleared much faster from the circulation. Within 60 min, more than 60% of the injected dose was cleared from the circulation. The association of nitrosylated HDL with the liver occurred very rapidly and at 15 min after injection, up to 37% of the injected dose was recovered in the liver. Thereafter, the amount of label associated with the liver slowly decreased (Fig. 1).

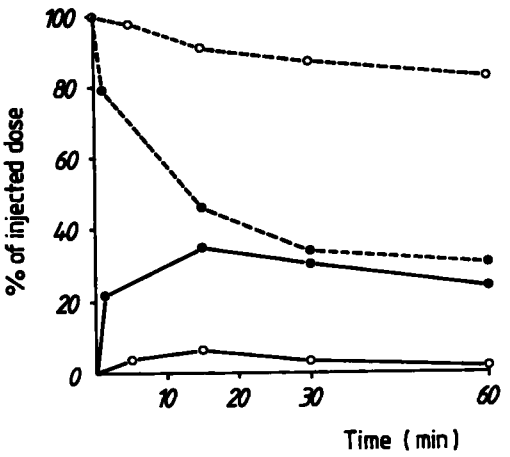


Figure 1. Serum decay and uptake by liver of apo E-free ^{125}I-labeled HDL and apoE-free ^{125}I-labeled TNM-HDL.

Apo E-free ^{125}I-labeled HDL or apo E-free ^{125}I-labeled TNM-HDL (100 μg of apolipoprotein) was injected into anesthetized rats and the uptake by liver and serum decay were determined. The liver was not perfused and the values are corrected for the estimated contribution of the radioactivity in serum to the liver values (36, 37). HDL was modified using HDL-TNM 1:10; (○- -○), serum values of native HDL; (●- - -●), serum values of TNM-HDL; (○—○), uptake by liver of native (●—●), uptake by liver of TNM-HDL.

The excess of tetranitromethane (TNM) over HDL used for the determination of the serum decay and liver uptake with time was tenfold, an excess similar to that used by Nestler et al. (25). Figure 2 shows the nature of the modification in HDL which occurs under various nitrosylation conditions. SDS-PAGE showed that apo A-I was the most prominent apolipoprotein in native HDL.

Modification with HDL-TNM ratios ranging from 1:7.5 to 1:2.5 resulted in a protein band with a slightly higher molecular weight than apo A-I. Also, a distinct protein band with a molecular weight of 90,000 representing cross-linked apo A-I was observed. Upon modification with a ratio 1:10, apo A-I was virtually absent and a broad band with a molecular weight range from 65,000 to 95,000 was seen in the gel in accordance with the data of Chacko (13) (not shown). The modification with 3 mM TNM gave results on

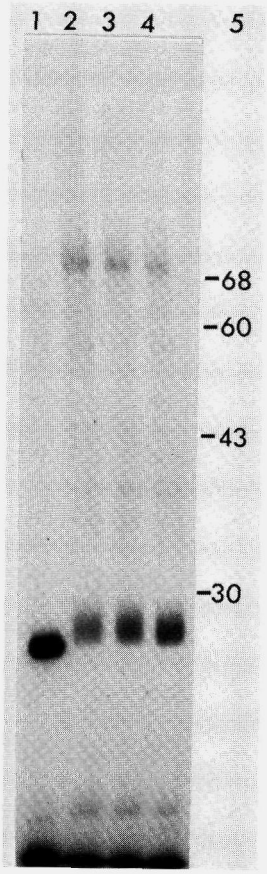


Figure 2. Autoradiographic analysis of iodinated HDL and iodinated TNM-HDL in 10% SDS gels.

HDL was incubated with various amounts of tetranitromethane. The ratios of HDL-TNM used in modification are: 1:0 (lane 1); 1:7.5 (lane 2); 1:5 (lane 3); 1:2.5 (lane 4); molecular weight markers (lane 5). Twenty five μg of protein was added to each well and chromatographed as described.

SDS-PAGE similar to those with a 1:10 HDL-TNM, which is comparable with the results of Chacko (13) and Brinton et al. (22). Apparently, the nitrosylation conditions that are effective in blocking the interaction with the high affinity site for HDL (either HDL-TNM of 1:10 or 3 mM TNM) lead to extensive cross-linking and the apolipoproteins are converted completely into high molecular weight polymers.

On agarose gel electrophoresis the extensively nitrosylated HDL moved ahead of native HDL which indicates an increased negative charge. In accordance with the data of Chacko et al. (13, 50) and Brinton et al. (22), the modifiation with tetranitromethane (TNM) did not result in an increase in size or difference in shape of the lipoprotein particles as judged by electron microscopy. An average particle size of 11.7 ± 1.8 nm and 11.7 ±1.9 nm was found for the native and modified apoE-free human HDL, respectively (Fig. 3). Furthermore, no aggregates or other indications for cross-linking of the lipoproteins to each other were observed.

In order to identify the cell type(s) responsible for the increased liver uptake of nitrosylated HDL, we injected the radiolabeled modified HDL into rats and isolated parenchymal, endothelial, and Kupffer cells from the liver by a low temperature method. Figure 4 illustrates that, irrespective of the nitrosylation conditions, the modified HDL showed an equal or

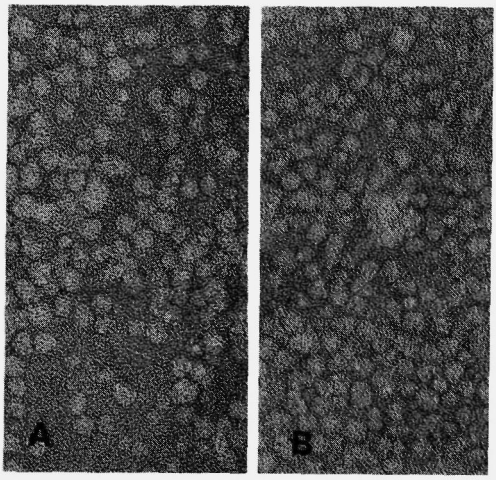
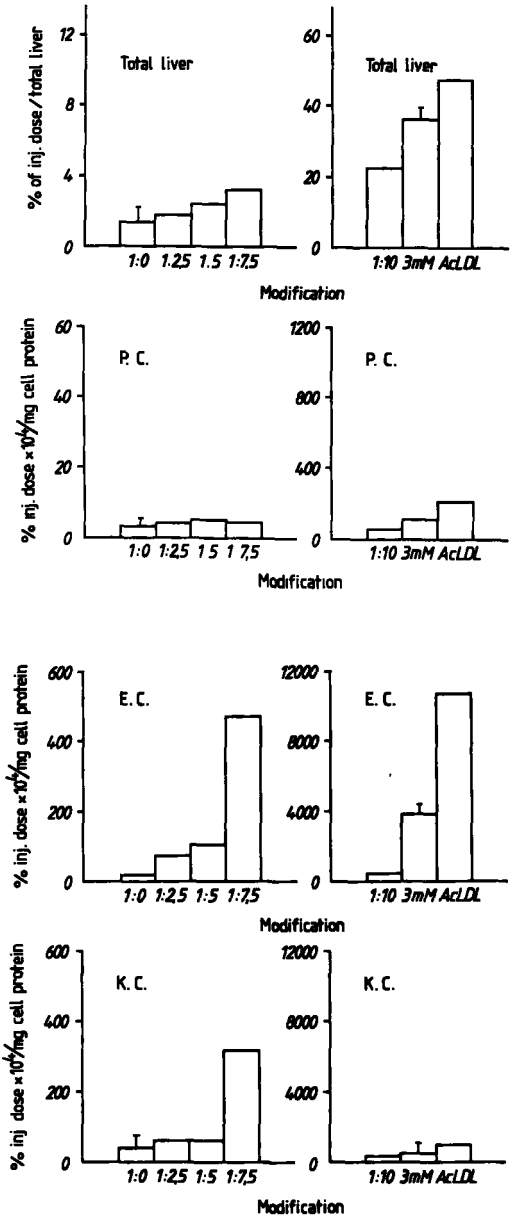


Figure 3. Electron microscopical analysis of HDL (A) and of TNM-modified HDL (B).

HDL was treated with 3 mM TNM, negatively stained, and analyzed by electron microscopy as indicated in Materials and Methods; magnification x 281,600.



increased association to liver as compared to native HDL. The liver endothelial cells especially showed an increased uptake of nitrosylated HDL, even when nitrosylation at an HDL-TNM value of 1:2.5 was used. When the relative uptake of nitrosylated HDL in the various cell types is compared to that of acetylated LDL, a striking similarity is evident.

From these data, taking into account the amount of protein contributed by each cell type to the total liver, one can calculate the contribution of the various liver cell types to the total liver uptake of nitrosylated HDL (Table 1). The similarity in cellular uptake of nitrosylated HDL and acetylacted LDL may indicate that the same receptor is responsible for the uptake by the liver. To test this hypothesis, cross-competition experiments were performed with isolated liver endothelial cells.

Figure 4. Uptake by liver and cellular distribution after in vivo injection of native apoE-free ^{125}I-labeled HDL, apoE-free ^{125}I-labeled TNM-HDL with varying degrees of nitrosylation, and ^{125}I-labeled AcLDL.

Modification conditions for nitrosylation were varied with the following molar ratio's of HDL-tetranitromethane 1:2.5, 1:5, 1:7.5, and 1:10 according to Chacko (13). In addition, HDL was modified with 3 mM tetranitrome-thane according to Brinton et al. (22). PC, parenchymal cells; KC, Kupffer cells; EC, endothelial cells.

In vitro studies

The association of iodinated TNM-HDL to endothelial cells *in vitro* was effectively inhibited in the presence of an excess of unlabeled TNM-HDL and AcLDL, but not by the presence of LDL. HDL showed an intermediate behavior (Fig. 5). When iodinated AcLDL was used as a ligand for the scavenger receptor, it appeared however that unlabeled TNM-HDL competed only slightly compared to AcLDL (Fig. 6). The scavenger receptor is reported to be inhibited also by the polyanions fucoidin and polyinosinic acid (51). Figure 7 shows that fucoidin and polyinosinic acid are effective inhibitors of the association of TNM-HDL to endothelial cells. Addition of 10 μm of either of these polyanions decreased cell association at least 85%. Comparison of the time course of cell-association (Fig. 8) indicates that nitrosylated HDL associated with isolated endothelial cells to a much greater degree than native HDL. The association of TNM-HDL with the liver endothelial cells increased to 1350 ng/mg cell protein after 2 hr of incubation. The actual uptake was even higher since 900 ng TNM-HDL/mg cell had been degraded and excreted during

TABLE 1. Relative contribution of different liver cell types to the total liver uptake of native HDL, nitrosylated HDL[a], and acetylated LDL

Data are given as percentages based upon the amount of radioactivity/mg cell protein and multiplied by the amount of protein that each cell type contributes to total liver protein.

[a] Using 3 mM tetranitromethane.

	Parenchymal Cells	Kupffer Cells	Endothelial Cells
	%	%	%
HDL	74	13	13
TNM-HDL	41	4	55
AcLDL	33	5	61

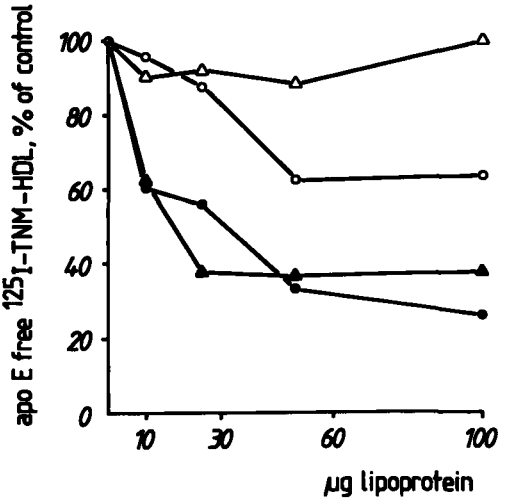


Figure 5. Comparison of the ability of unlabeled lipoproteins to compete with the cell association of ^{125}I-labeled apoE-free TNM-HDL to liver endothelial cells.

Cells were incubated *in vitro* for 10 min at 37°C with 5 μg of ^{125}I-labeled apo E-free TNM-HDL and with the indicated amounts of AcLDL (▲), LDL (△), HDL (○), and TNM-HDL (●). ^{125}I-labeled TNM-HDL apolipoprotein association is expressed as the percentage of radioactivity obtained in the absence of unlabeled lipoprotein.

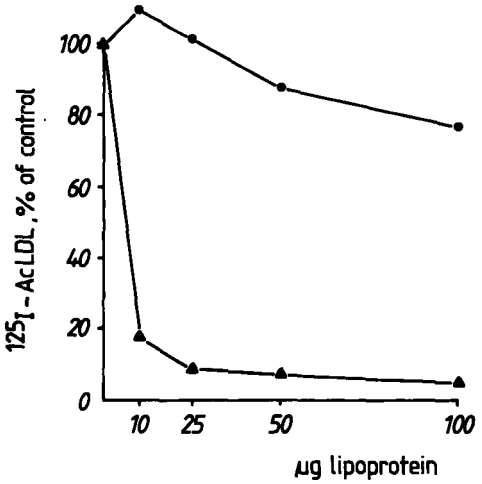
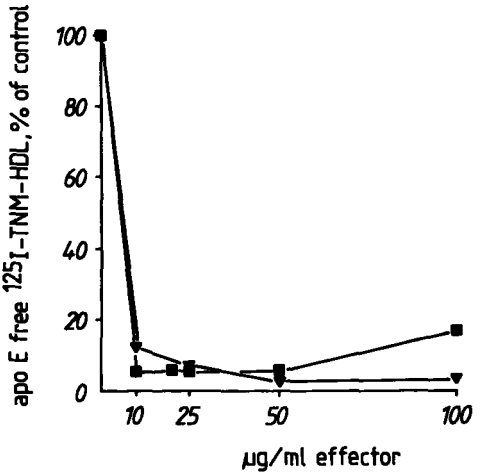


Figure 6. Comparison of the ability of unlabeled apo E-free TNM-HDL (●) and AcLDL (▲) to compete with the degradation of ^{125}I-labeled acetylated LDL by liver endothelial cells.

Cells were incubated for 2 hr at 37°C with 1 µg of AcLDL and the indicated amounts of unlabeled lipoproteins. ^{125}I-labeled apolipoprotein degradation is expressed as the percentage of radioactivity obtained in the absence of unlabeled lipoprotein.



graded and excreted during this period. For native HDL a value of 50 ng/mg cell protein was obtained after 2 hr, with little or no degradation (not shown). During the whole time course, unlabeled acetylated LDL was very effective in blocking the interaction of nitrosylated HDL with the endothelial liver cells. The degradation of TNM-HDL was inhibited by low concentrations of chloroquine (Fig. 9) implying an involvement of the lysosomes.

In vivo visualization studies

To establish morphologically the involvement of liver endothelial cells in the clearance of TNM-HDL by a method independent from the cell isolation procedure, the cellular site of hepatic uptake of native and TNM-HDL was also studied with DiI-labeled lipoproteins.

In cryo sections of livers from rats injected with DiI-labeled TNM-HDL that was allowed to circulate for 10 min, we found a punctate fluorescent labeling (Fig. 10), clearly outlining the sinusoids, which supports suggestions of an involvement of the liver endothelial cells in the clearance of TNM-HDL. Kupffer cells also showed some labeling (Fig. 10). When the labeled lipoprotein was injected and allowed to circulate for 1 hr (not shown), the labeling pattern appeared identical to that observed after

Figure 7. The effect of increasing concentrations of fucoidine (■) and polyinosinic acid (▼) on the cell association of TNM-HDL.

The cells were incubated for 2 hr at 37°C with 5µg ^{125}I-labeled TNM-HDL in the presence of the indicated amounts of fucoidine or polyinosinic acid. ^{125}I-labeled apolipoprotein association is expressed as the percentage of radioactivity obtained in the absence of effector.

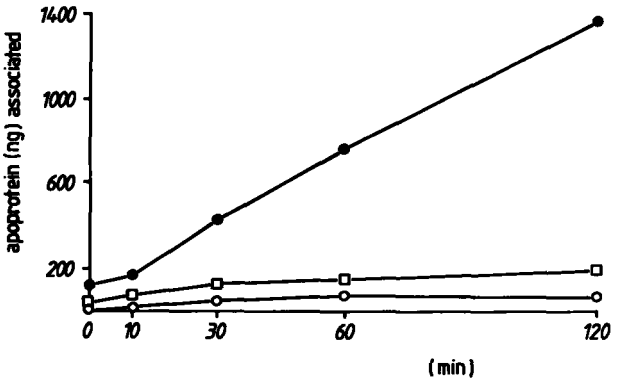


Figure 8. Time course of the in vitro association of apo E-free ^{125}I-labeled TNM-HDL in the absence (●) or presence (□) of an excess of 100 μg/ml AcLDL compared to the time course of the association of apoE-free ^{125}I-labeled HDL (○) with liver endothelial cells.

The cells were incubated with 10 μg/ml of apo E-free ^{125}I-labeled HDL or apo E-free ^{125}I-labeled TNM-HDL at 37°C and the values are expressed as ng apolipoprotein associated to the cells per mg cell protein.

10 min. The labeling of the liver with DiI-TNM-HDL showed a striking similarity with the results of Pitas et al. (34) and Netland et al. (35) after intravenous injection of DiI-AcLDL. The endothelial cells could be distinguished from the Kupffer cells by their abundance and their uniform distribution and because they were situated along the lining of the sinusoids, whereas the Kupffer cells had a different distribution and were less abundant.

When DiI-labeled native HDL was injected, no punctate labeling along the sinusoids was noticed after 10 min or 1 hr of circulation (not shown). These results are consistent with the slow rat of uptake of the native HDL and also serve as a control for the fluorescence exhibited with nitrosylated HDL.

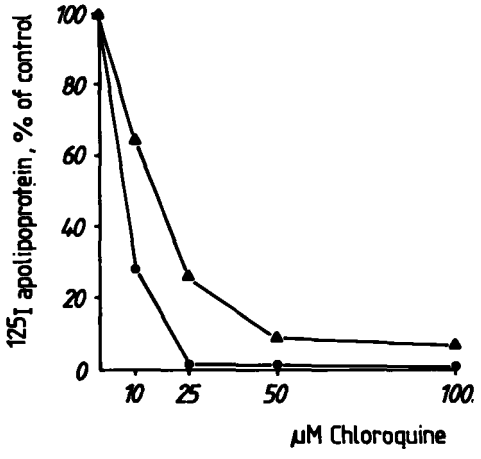


Figure 9. The effect of increasing concentrations of chloroquine on the degradation of apo E-free ^{125}I-labeled TNM-HDL (●) and ^{125}I-labeled AcLDL (▲) by isolated liver endothelial cells.

The cells were incubated for 2 hr at 37°C with 10 μg of apo E-free ^{125}I-labeled TNM-HDL or ^{125}I-labeled AcLDL and the indicated amounts of chloroquine. ^{125}I-labeled apolipoprotein degradation is expressed as the percentage of radioactivity obtained in the absence of chloroquine.

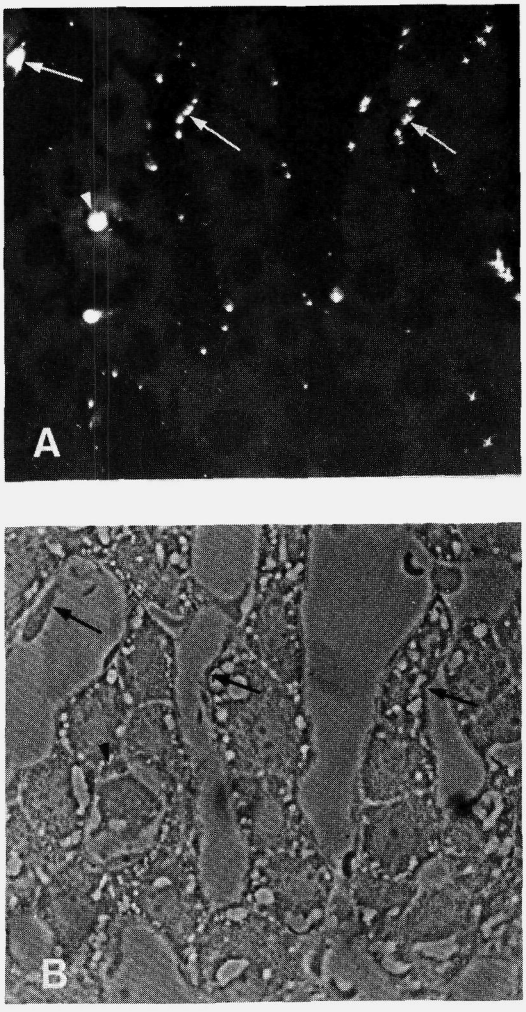


Figure 10. Liver association of DiI-labeled TNM-HDL at 10 min after intravenous injection.

A: Fluorescence of DiI-TNM-HDL in endothelial liver cells is indicated by arrows, while Kupffer cells are indicated by an arrow head; magnification x 992.

B: Same section as shown in A using phase contrast; magnification x 992.

DISCUSSION

Chemical modification of HDL with tetranitromethane prevents the competition of unlabeled HDL with iodinated HDL (13, 22-25). The inability of nitrosylated HDL to compete with the native iodinated HDL has been shown with fibroblasts (22), HepG2 cells (23), ovarian cells (25), and liver membranes (13, 24). In addition, it was recently shown that ^{125}I-labeled nitrosylated HDL did not bind to liver membranes with high affinity or with saturable kinetics (13). The mechanism of the effect of nitrosylation is suggested to be the consequence of modification of tyrosine residues (52) or cross-linking of apolipoproteins to one another (13). Because TNM-mediated cross-linking is also believed to alter the secondary structure of the protein, the TNM treatment itself can affect the accessibility of many amino acid residues at some distance from the cross-linking site (22). Electron microscopy studies revealed that there was no interparticle cross-linking (13, 22) and it was shown by Chacko et al. (50) that the inactivation of the high affinity binding of HDL to liver membranes by TNM treatment is not the result of cross-linking of apoprotein to phospholipids. In the present experiments we determined the effect of gradual nitrosylation upon the *in vivo* interaction of apoE-free HDL with liver parenchymal, endothelial, and Kupffer cells. The data indicate that nitrosylation of HDL leads to an increased decay and increased liver association. It appears

specifically that liver endothelial cells possess a recognition mechanism whereby even slightly nitrosylated HDL is readily recognized. The nitrosylation grade used to block the high affinity HDL recognition (1:10) (13) or 3 mM (22) leads, respectively, to a 10- and 20-fold increased liver uptake of HDL. Our *in vitro* competition experiments showed that, in addition to nitrosylated HDL itself, acetylated LDL was also a competitor for the association of nitrosylated HDL to endothelial cells, while LDL was ineffective. The decrease in cellular association of TNM-HDL by AcLDL and non-lipoprotein competitors such as fucoidin and polyinosinic acid indicate that the association of TNM-HDL with the liver endothelial cells is mediated by the scavenger receptor. The partial inhibition of TNM-HDL association by HDL indicates that a minor portion of the uptake of TNM-HDL may be mediated by a receptor other than the scavenger receptor.

High affinity recognition appears to be coupled to uptake and degradation of the TNM-HDL in the lysosomes because chloroquine appears to be an effective inhibitor of the degradation. Such an intracellular route is comparable to evidence obtained with other substrates for the scavenger receptor (53).

However, in experiments testing the ability of TNM-HDL to inhibit the degradation of acetylated ^{125}I-labeled LDL, TNM-HDL was a poor competitor. A similar poor competition was recently described for endothelial cell-modified LDL or enzymatically modified LDL, although a clear-cut scavenger receptor interaction of these substrates is established (54). This difference in cross-competition may either be explained by a difference in affinity of the scavenger receptor for AcLDL as compared to TNM-HDL or by the existence of more than one class of scavenger receptor as suggested in recent publications (54-56).

The charge of the nitrosylated HDL is affected by the degree of nitrosylation and results in a more negative charge than for native HDL as also shown by Chacko (13) and Brinton et al. (22). The increased negative charge may be responsible for the induced recognition by the scavenger receptor (51). Scavenger receptor-mediated uptake of modified lipoproteins is dependent on the grade of modification as originally shown with malondialdehyde-modified LDL (57). We could not demonstrate a threshold modification degree with TNM in which the tyrosine-induced receptor recognition was abolished and no scavenger receptor activity was induced. A shift in configuration in the apolipoprotein, as a result of the introduction of negatively charged groups - as in the case in nitrosylation - may, with HDL, unavoidably express parts of the apolipoprotein that are recognized by the scavenger receptor. Evidence for such a mechanism was presented by Haberland and Fogelman (58) with maleylated serum albumin.

Chemically modified LDL has been used extensively *in vivo* to characterize the tissue sites for the receptor-dependent uptake (27, 28). Furthermore, the regulation of the LDL-receptor-dependent uptake was studied by comparing the *in vivo* fate of native and either arginine- or lysine-modified LDL (59, 60). The present experiments show

that a similar approach to study the HDL-receptor-dependent uptake and degradation *in vivo* is not possible with nitrosylated HDL because even the lowest degree of nitrosylation induces recognition by a scavenger receptor. This property not only limits the possibility of using nitrosylated HDL as an experimental tool *in vivo*, but also may influence the interpretation of *in vitro* data. For instance, Nestler et al. (25) showed that in the rat ovarian cell steroidogenenis is stimulated by nitrosylated HDL to a degree at least equal to that of native HDL. They concluded that high affinity binding is not an essential event in the HDL pathway and that HDL can deliver its sterol through low-affinity cellular associations. However, Pitas et al. (34) showed that at least ovarian endothelial cells do possess scavenger receptors, and it may be possible that

Although nitrosylated HDl does not follow the high affinity pathway of HDL to deliver its cholesterol intracellularly, it utilizes the scavenger receptor. Because scavenger receptors are present on macrophages and endothelial cells in spleen, bone marrow, adrenal, and ovary (34), this property may be relevant for a variety of cell types.

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CHARACTERIZATION in vitro OF THE INTERACTION OF HUMAN APO E-FREE HIGH DENSITY LIPOPROTEIN WITH HUMAN HEPATOCYTES

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ABSTRACT

The interaction of iodinated apo E-free HDL with cultured human hepatocytes was characterized. Evidence is presented for a saturable, Ca^{2+}-independent high affinity binding site with an apparent k_m value of 20 ug/ml of apolipoprotein. Nitrosylated HDL and LDL did not compete effectively with the binding of native HDL. VLDL, however, did compete with HDL binding. It is suggested that competition is exerted by the presence of apo C's in VLDL.

Inhibitors of the lysosomal pathway of protein degradation (NH_4Cl and chloroquine) had no significant effect on the degradation of apo E-free HDL by human hepatocytes while under similar conditions LDL degradation was inhibited by more than 80%. In the presence of 10 M monensin the cell-association of HDL was unaffected while the degradation was inhibited by 30%. Under similar conditions the cell-association of LDL was inhibited by 40% and the degradation by 90%. Incubation of human hepatocytes with fluorescently labeled HDL (DiI-HDL) reveals mainly strong punctate membrane-bound fluorescence, and hardly any labeling of small vacuoles in the cytosol of hepatocytes is noticed whereas with DiI-LDL strong intracellular fluorescence concentrated in small vacuoles is observed.

It is concluded that human hepatocytes possess a specific high affinity binding site for human HDL with recognition properties similar to those described earlier on rat hepatocytes. In contrast to the interaction of human LDL with human hepatocytes, no evidence could be obtained that the binding of HDL is actively coupled to uptake and lysosomal degradation, indicating that binding of LDL and HDL to human hepatocytes may be coupled to intracellular pathways in different ways.

INTRODUCTION

According to the generally accepted concept of Glomset (1), HDL delivers cholesterol from peripheral cells (2,3) to hepatocytes, where cholesterol can be converted into bile acids and thereby irreversibly removed from the circulation. With fibroblasts (2), arterial smooth muscle cells (2), aortic endothelial cells (3,4) and peritoneal macrophages (5), evidence has been provided that HDL should 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 (4,6) or acetylated LDL (3,5). Similarly as for peripheral cells, a high-affinity binding site for HDL has been identified on hepatocytes of various species (7-15) and on liver membranes (16-19) including human liver membranes (20). Recently, we characterized the high-affinity recognition sites for HDL on parenchymal, endothelial and Kupffer cells from rat liver

in vitro (21). We found identical recognition properties for the high affinity binding site in all three cell types, suggesting that one receptor may mediate both cholesterol efflux and cholesterol influx.

In order to study HDL interaction with human liver cells, several investigators used the human liver tumor cell line Hep G2 as a model. Dashti *et al.* (22) reported high-affinity as well as low-affinity binding sites for ^{125}I-HDL on Hep G2 cells and indicated that HDL was taken up and degraded via a lysosomal pathway. However in these studies apo E containing HDL was used and therefore an interaction with the remnant (23) and/or LDL receptor (16) can not be ruled out.

Recently Kambouris *et al.* (24) demonstrated a specific HDL-binding protein on Hep G2 cells with an apparent molecular mass of 80 kDa. However, with human liver membranes Mendel *et al.* (25) found with radiation inactivation no evidence for a specific HDL binding protein. Rinninger and Pittman (26) showed that cholesterol loading of Hep G2 cells resulted in a down-regulation of the selective uptake of cholesteryl esters in HDL although such a regulation was not found with primary cultures of rat hepatocytes (27). Apparently the interaction of HDL with Hep G2 cells differs from the interaction of HDL with rat hepatocytes and may also not be representative for human liver cells or membranes derived from human liver.

In view of these uncertainties we decided to study the interaction of apo-E free HDL with human hepatocytes. In this paper, we report that human hepatocytes do possess a specific high affinity binding site for HDL and that the processing of apo E-free HDL by human hepatocytes is clearly different from the processing of LDL.

MATERIALS AND METHODS

Materials

Collagenase (type I), bovine serum albumin (fraction V), human serum albumin (fraction V), dexamethasone and chloroquine were purchased from Sigma (St.Louis, MO, U.S.A.). Tetranitromethane (TNM, 98%) was obtained from Aldrich Chemical Company Inc. (Brussels, Belgium). Monensin was obtained from Calbiochem Behring Corp. (La Jolla, U.S.A.). ^{125}I (carrier-free) was NaOH is purchased from Amersham, UK and 1.1' dioctadecyl 3.3.3'.3' indocarbocyanine perchlorate C_{18} (DiI) from Molecular Probes (Eugene, Oregon, U.S.A.).

Williams' E medium and kanamycine was obtained from Flow laboratories (Irvine, Scotland, UK). Penicillin, streptomycin and fetal bovine serum from Boehringer Mannheim (FRG). Multi-well cell culture dishes were obtained from Costar (Cambridge, MA, U.S.A.).

HDL, LDL, VLDL isolation and iodination.

Human VLDL ($d < 1.006$) was isolated by differential ultracentrifugation according to Redgrave *et al.* (28). Human LDL ($1.024 < d < 1.055$) and human HDL ($1.063 < d < 1.21$) were isolated from serum by two repetitive centrifugations according to Redgrave *et al.* (28) as described before (29). The HDL was passed over a sepharose-heparin column (30) and the apo E-free fraction was checked for the presence of apo E and albumin by 10% SDS-polyacrylamide gel electrophoresis, followed by Coomassie-blue staining and densitometric scanning. HDL was radiolabeled with ^{125}I by the ICl method of McFarlane (31) as modified by Bilheimer *et al.* (32).

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 apolipoprotein A-I, $44 \pm 3\%$ in apolipoprotein A-II and $6 \pm 1\%$ in apolipoprotein C's as determined after separation of the apolipoproteins on SDS-polyacrylamide gelelectrophoresis, using 5-22.5% gels. The gels were stained, dried and cut into pieces and counted for radioactivity.

The nitrosylation of HDL.

Nitrosylation was performed exactly as described by Brinton *et al.* (33).

Isolation and culturing of human hepatocytes.

Human hepatoyctes were isolated from livers which were obtained through the Auxiliary Partial Liver Transplantation Program which is carried out at the University Hospital Dijkzigt in Rotterdam, The Netherlands. Permission was given by the Medical Ethical Committee to use the remaining, not transplanted, part of the donor liver, for scientific research. The livers were taken from physically healthy organ donors, who died after brain hemorrhages or severe traumatic brain injury. During resection of the left lobe, the liver was perfused by portal vein cannulation with Euro-Collins (4^oC).

After resection, the left liver lobe was transported to the perfusion site within 45 min in a cold buffer (4^oC) containing 10 mM Hepes, pH 7.4, 142 mM NaCl, 16.7 mM KCl and 0.5 mM EGTA. Perfusion with 3 liter 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 can be identified at the dissection surface. After the pre-perfusion the liver was perfused successively with 500 ml of a 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% bovine serum albumin, cells were filtered through a 250 m filter, centrifuged (50 g for 30 sec.) and washed three times in a cold culture medium (4^oC) to remove damaged and non-parenchymal cells. Cells were seeded on 12-well cluster plates at a density of 0.5×10^6 cells per well and were maintained in Wil-

liams' E medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 mU/ml insuline, 1 nM dexamethasone, 50 μg/ml kanamycine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO2/95% air atmosphere (34).

The medium was renewed 12 hr after seeding in order to remove unattached nonviable cells and every 24 hr thereafter. Experiments were performed with cells cultured for 48 to 96 hr.

HDL association and degradation by hepatocytes in culture.

24 hr before the experiment the culture medium, containing fetal bovine serum, was replaced with culture medium containing 2% human serum albumin. The experiments were started by replacing the medium with medium containing ^{125}I-HDL or ^{125}I-LDL, 2% human serum albumin and additives. After incubation, the dishes were placed on ice and cell association and degradation was measured exactly as described before (10). Briefly, the amount of degradation of ^{125}I-HDL or ^{125}I-LDL was measured as trichloroacetic acid-soluble radioactivty present in the incubation medium excluding free ^{125}I label.

After discarding the medium, the cells were washed 5 times with 0.15 M NaCl, 50 mM Tris-HCl and 5 mM $CaCl_2$ (Buffer A) containing bovine serum albumin (0.2%) followed by two washes with Buffer A without albumin. Thereafter the cells were dissolved in 0.1 M NaOH. This solution was counted for radioactivity and assayed for protein content (35).

Morphological studies of apo E-free HDL and LDL interaction with human hepatocytes in culture.

Fluorescent DiI-labeled HDL and LDL was prepared as described by Pitas *et al.*(36). Human hepatocytes cultured in wells containing microscope cover slips were incubated for 3 hr with DiI-LDL or apo E-free DiI-HDL in 1% human serum albumin in Williams' E medium at 37°C. Cells were washed three times at 4°C with Hanks' medium containing 0.2% bovine serum albumin and fixed with 4% paraformaldehyde in phosphate buffered saline for 30 min at 4°C. Cover slips were mounted on glass slides with glycerol. Photographs were taken with a Leitz Ortholux Microscope, using standard Rhodamine emisson and excitation filter combination and Kodak 1600 Ektachrome films.

Determination of possible label exchange.

Determination of the possible label exchange from apo E-free HDL to VLDL was performed by ultracentrifugation (37). After incubation for 10 min of 5 μg of ^{125}I-HDL in Ham's F-10 medium with VLDL in 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±0.2% (n=3) of the iodine label recovered in the upper part of the centrifuge tube, indicating minor transfer or exchange of radiolabeled apolipoproteins from HDL (recovered in the bottom of the tube) to VLDL under the conditions used for competition studies.

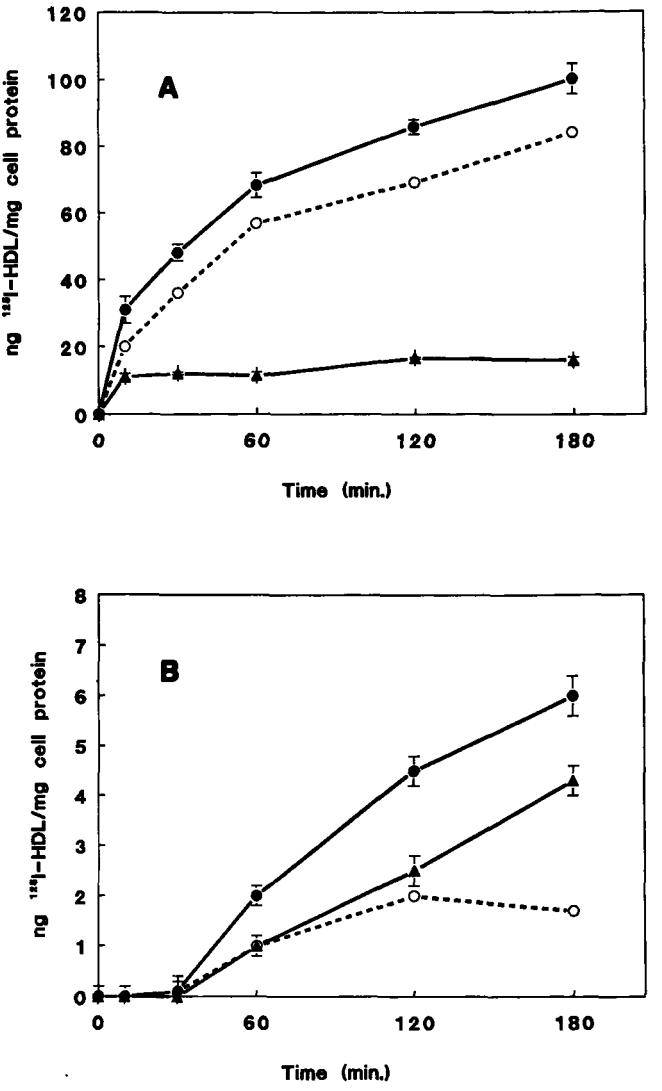

Figure 1. Time course of the association (A) and degradation (B) of apo E-free ^{125}I-HDL by human hepatocytes.

Cells where incubated for the indicated time at 37°C with 10 μg apo E-free ^{125}I-HDL/ml. The association (A, ▲) or degradation (B, ▲), determined in the presence of a 20-fold excess of apo E-free HDL, is substracted from the total association (A, ●) or degradation (B, ●), resulting in the broken line, which represents the high affinity association or degradation of ^{125}I-HDL. The results are given ± s.e.m. (n=3) indicated by bars from isolation number 2 (see table 1).

RESULTS

The time-dependent cell-association and -degradation of apo E-free HDL.

The time-dependent association of HDL with cultured human hepatocytes at 37°C is shown in figure 1. The high affinity component is assessed by comparison with an incubation in the presence of an excess (20-fold) of unlabeled apo E-free HDL. The high affinity cell-association is initially rapid and levels off after 60 min of incubation. With hepatocytes from eight different donors the high affinity association at 3 hours of incubation varied between 29 and 171 ng HDL/mg cell protein (Table 1).

Indication for degradation of HDL under these conditions is revealed from figure 1b. It appears that the amount of HDL that is degraded to TCA-soluble products is quite low as compared to the amount of cell-associated HDL. The degradation of apo E-free ^{125}I-HDL to TCA-soluble products after 3 h of incubation at 37°C was highly variable between cells from different human donors, as shown in table I.

Isolation number	High affinity association	High affinity degradation
1	69.5 ± 6	n.d.
2	81 ± 2.5	1.7 ± 0.4
3	67.7	8.7
4	68 ± 1.1	6.0 ± 1.3
5	28.6 ± 1.7	16.2 ± 1
6	171 ± 1.8	27.2 ± 1
7	84.3 ± 6.5	n.d.

Table I. High affinity association and degradation of ^{125}I-labeled apo E-free HDL by human hepatocytes from different donors.

Cells were isolated, incubated for 3 hours at 37°C with 10 μg/ml apo E-free ^{125}I-HDL in the absence or presence of 200 μg/ml unlabeled apo E-free HDL. High affinity association and degradation is the difference between the incubation in the absence and presence of an excess unlabeled HDL. Results are means ± s.e.m from at least 3 experiments, or means from 2 experiments (n.d.: not detectable).

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

In earlier studies (9) we showed that, with rat liver cells, the cell association of HDL is temperature-dependent. Hoeg *et al.* (20) showed that binding of apo E-free HDL to human liver membrane preparations is also a temperature-dependent process. Therefore, we determined the cell binding of apo E-free HDL as a function of the extracellular HDL concentration after a 10 min incubation at 37°C (Fig. 2). Degradation of added apo E-free HDL during a 10 min incubation is minimal and 95-100% of the cell-associated radioactivity is precipitated by trichloroacetic acid. The high affinity binding component was assessed by comparison with an incubation with an excess (20-fold) of unlabeled apo E-free HDL. The data indicate that a saturable high-affinity binding site for apo E-free HDL is present on human hepatocytes. Replotting of the data in a double

reciprocal plot, using Michaelis-Menten kinetics, reveals an apparent K_m value of 20 μg HDL/ml (see inset Fig. 2).

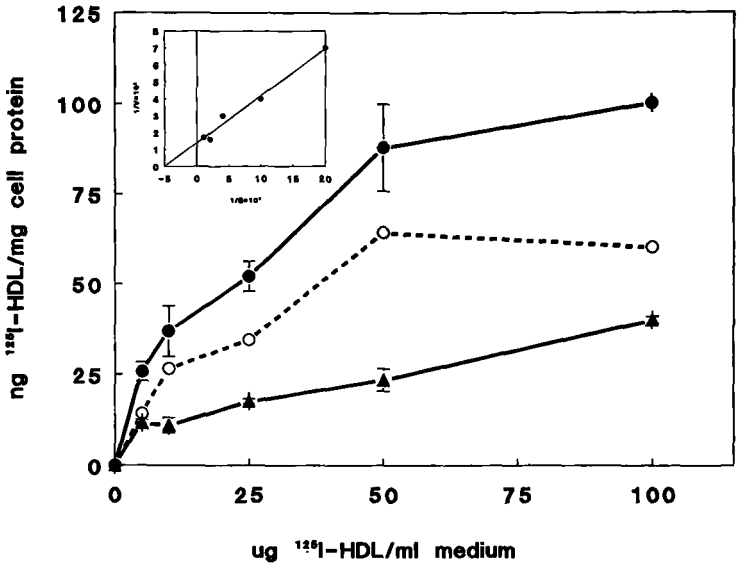

Figure 2. Relation of the concentration of apo E-free ^{125}I-HDL to the extent of binding by human hepatocytes.

Cells were incubated for 10 min at 37°C with 10 μg apo E-free ^{125}I-HDL/ml. After incubation the cell bound radioactivity was determined as described before (10). The non-specific binding (▲), determined in the presence of a 20-fold excess of apo E-free HDL, was substracted from the total binding (●), resulting in the broken line (○), which represents the specific binding of ^{125}I-HDL to human hepatocytes. Values are given ± s.e.m. (n = 3) indicated by bars. The inset shows the double reciprocal plot of the high-affinity binding of apo E-free HDL.

Comparison of the ability of unlabeled nitrosylated HDL and native lipoproteins to compete with the binding of ^{125}I-labeled apo E-free HDL to human hepatocytes.

To investigate the specificity of the binding of apo E-free HDL to cultured human hepatocytes, we studied the competition of radiolabeled apo E-free HDL with an excess of unlabeled nitrosylated HDL or unlabeled apo E-free HDL. We incubated the cells with 5 μg of ^{125}I-HDL and competitors for 10 min at 37°C and determined the cell-associated radioactivity as described in Materials and Methods. A short incubation 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 (9,20). Figure 3 shows that an excess of unlabeled HDL decreases the amount of cell-associated radioactivity by 65%, whereas an excess of unlabeled nitrosylated HDL hardly competes for the binding with labeled HDL. In order to evaluate the apolipoprotein specificity of the high affinity sites on cultured human hepatocytes, the ability of LDL and VLDL to compete with radiolabeled apo E-free HDL is also determined. The results of these experiments (also shown in Fig. 3) show that VLDL is able to effectively inhibit binding of HDL to human hepatocytes, but that LDL is much less effective. Label exchange cannot account for the fact that VLDL is able to compete for radiolabeled apo E-free HDL because after incubation for 10 min 37°C only 2.5% of the label is found in VLDL (see Materials and Methods)

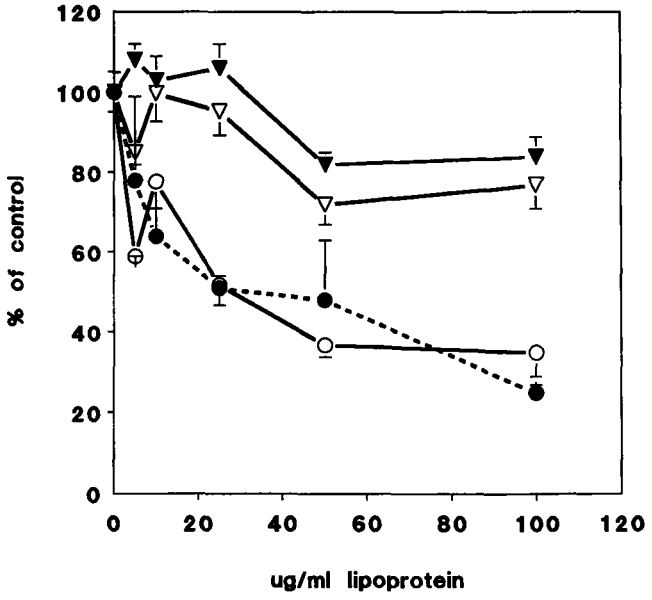

Figure 3. Comparison of the ability of unlabeled lipoproteins to compete with the cell association of apo E-free ^{125}I-HDL to human hepatocytes.

Cells were incubated for 10 min at 37°C with 5 μg of apo E-free ^{125}I-HDL/ml and with the indicated amounts of unlabeled LDL (▽), VLDL (●), HDL (○) and nitrosylated apo E-free HDL (▼). ^{125}I-labeled HDL binding is expressed as the percentage of the radioactivity obtained in the absence of unlabeled lipoprotein. The values are given ± s.e.m. (n=3) indicated by the bars.

The effect of [Mg-EGTA] and [Ca^{2+}] upon the cell binding of apo E-free HDL.

The effect of [Mg-EGTA] and [Ca^{2+}] upon the cell binding of apo E-free HDL is investigated (Fig. 4). We used EGTA to chelate specifically Ca^{2+} in the presence of Mg^{2+}. Treatment of cells with EDTA gave comparable results (not shown) but caused a considerable release of cell protein from the culture dishes. Figure 4 shows that binding of apo E-free HDL to human hepatocytes is Ca^{2+}-independent. The 100% value is taken at the point where no free Ca^{2+} is present in the medium.

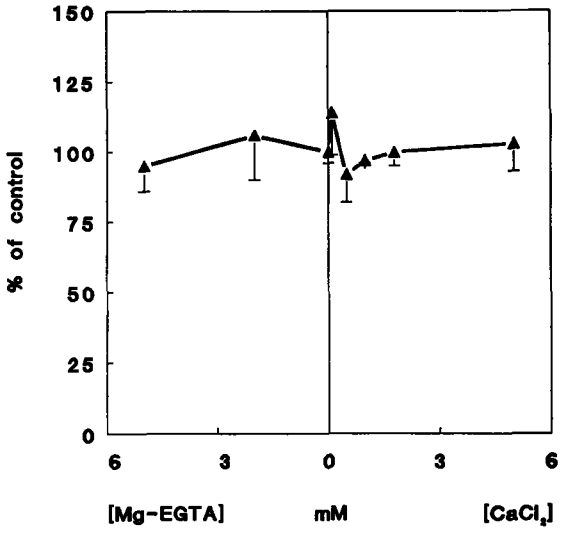

Figure 4. The effect of Mg-EGTA and Ca^{2+} on the cell association of apo E-free ^{125}I-HDL to human hepatocytes.

Cells were incubated for 10 min at 37°C with 10 μg/ml apo E-free ^{125}I-HDL in the presence of the indicated amounts Mg-EGTA or Ca^{2+}. The results are given as mean percentage of the binding at the point where no free Ca^{2+} is present in the medium. Values are given ± s.e.m.

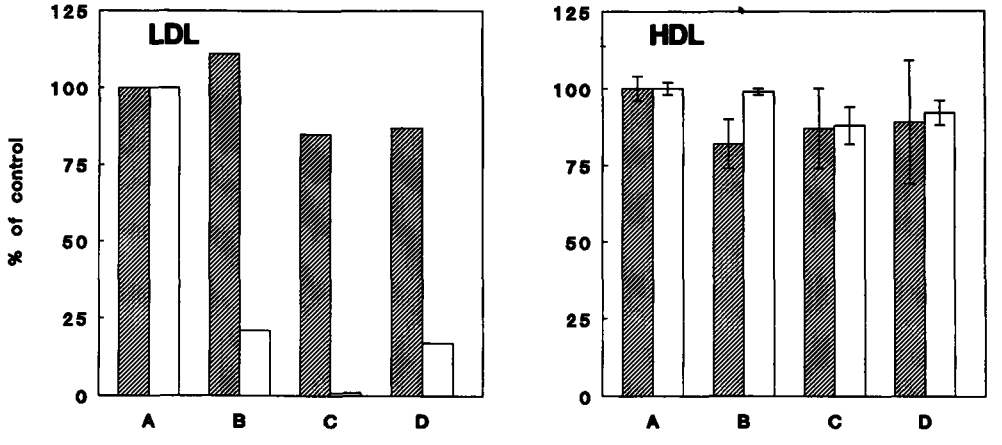

Figure 5. The effect of chloroquine or NH_4Cl on the association and degradation of ^{125}I-LDL or apo E-free ^{125}I-HDL by human hepatocytes.

Cells were incubated for 3h at 37°C with 10 μg/ml of ^{125}I-LDL (5A) or ^{125}I-HDL (5B) in the absence or presence of 50 μM chloroquine (B) or 100 μM chloroquine (C) or 10 mM NH_4Cl (D), and assayed for cell-association (closed bars) and degradation (open bars). Values are given as means ± s.e.m. as indicated by bars from at least 3 experiments or means from 2 experiments (LDL).

The effect of chloroquine, NH_4Cl or monensin on the processing of apo E-free HDL and LDL by human hepatocytes.

Chloroquine and NH_4Cl are unrelated compounds which can inhibit the lysosomal pathway of protein degradation. In figure 5a it is shown that 100 μM chloroquine inhibits LDL degradation completely, while 50 M chloroquine and 10mM NH_4Cl cause approximately 80% inhibition. The degradation of apo E-free HDL, however, was not significantly affected by chloroquine and NH_4Cl (Fig. 5b).

Monensin is a monovalent ionophore which has the ability to disrupt secretory and endocytic activities of cells by disturbing proton gradients (38). We used this compound to obtain more information about possible intracellular routes of apo E-free HDL or its putative receptor. LDL is used as a positive control because it is known that the recycling of LDL-receptors to the cell membrane is largely blocked by monensin, leading to a low level of cell-association and degradation (39). In figure 6 it is shown that at 3 hr of incubation the association of LDL to human hepatocytes is 60% of the control value in the presence of monensin while the association of apo E-free HDL is not significantly altered. The degradation of LDL in the presence of monensin is inhibited by 88% while with apo E-free HDL an inhibition of 32% is found.

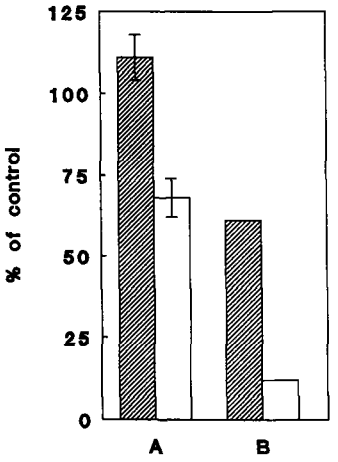

Figure 6. The effect of monensin on the association and degradation of apo E-free ^{125}I-HDL or ^{125}I-LDL by human hepatocytes.

Cells were incubated for 3h at 37°C with 10 μg/ml ^{125}I-HDL (A) or ^{125}I-LDL (B) in the presence of 10 μM monensin and compared with incubations without addition (100% value). The closed bars are representing the association and the open bars are representing the degradation. Values are given as means ± s.e.m. as indicated by bars from at least 3 experiments or means from 2 experiments (LDL).

Morphological comparison of the binding and processing of LDL and apo E-free HDL by human hepatocytes.

Human hepatocytes show, after incubation with DiI-labeled LDL (DiI-LDL) for 3 hr at 37°C, membrane bound fluorescence and a strong intracellular signal concentrated in small vacuoles (Fig. 7a). In contrast, an incubation of human hepatocytes with DiI-labeled HDL (DiI-HDL) under identical conditions leads to strong punctate membrane-

bound fluorescence, and hardly any labeling of small vacuoles in the cytosol of the hepatocytes is noticed (Fig. 7b). The punctate staining pattern with DiI-LDL (mainly intracellular) or DiI-HDL (mainly membrane-associated) can be prevented by incubation in the presence of a 20-fold excess of non-fluorescent LDL resp. apo E-free HDL, while the non-punctate fluorescence (visualized as large intracellular spots) represent autofluorescence of intracellular lipids. These results suggest that the cell-association of DiI-LDL is followed by cellular uptake while DiI-HDL apparently remains associated to a higher extent to the (vicinity of the) cell membrane.

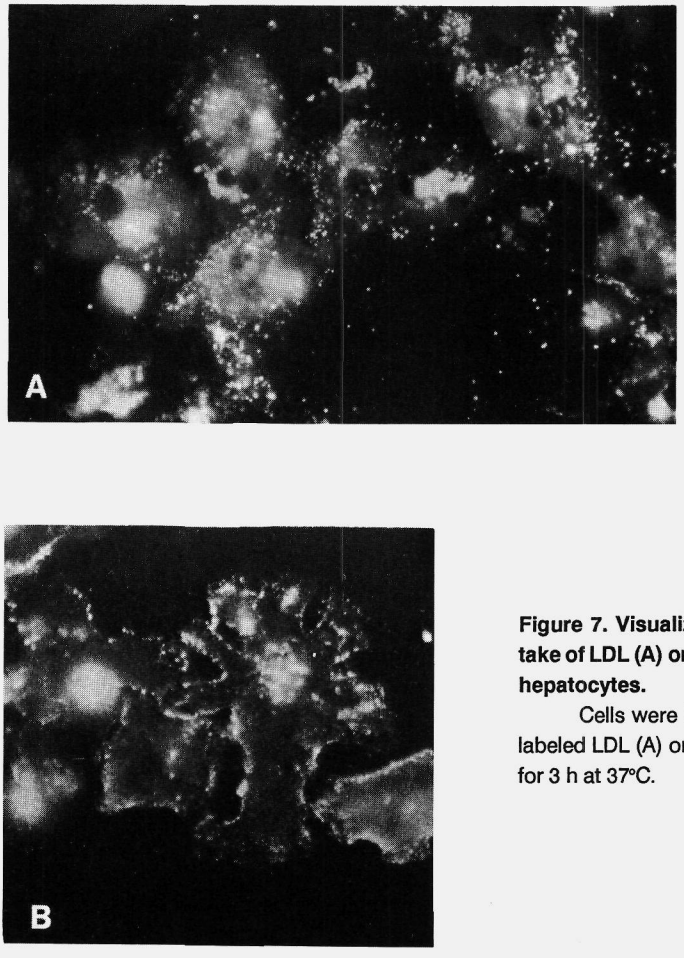

Figure 7. Visualization of the binding and uptake of LDL (A) or apo E-free HDL (B) by human hepatocytes.

Cells were incubated with 50 μg/ml of DiI-labeled LDL (A) or DiI-labeled apo E-free HDL (B) for 3 h at 37°C.

DISCUSSION

A high affinity binding site for HDL has been identified on hepatocytes of various species (7-15, 21) and on liver membranes (16-19), including human liver membranes (20). Recently Tozuka *et al.* (40) identified on human liver membranes proteins with MW's of 100 and 120 kD which interact with HDL on ligand blotting. However, Mendel *et al.* (25) were unable to identify a high molecular weight protein that accounts for the HDL binding activity in human liver membranes. Up till now, to our knowledge no studies have been performed on the interaction of human apo E-free HDL with human hepatocytes. The aim of the present study was to evaluate the presence of specific high affinity sites for human apo E-free HDL on human liver parenchymal cells and to obtain information about the processing of apo E-free HDL after its binding to a possible high affinity site.

The high affinity association of apo E-free ^{125}I-HDL with human hepatocytes satisfies several criteria for specific binding. The binding is time dependent, saturable and can be inhibited by excess HDL but not by nitrosylated-HDL or LDL. The specific binding of apo E-free HDL has an apparent K_m value of approx. 20 μg HDL/ml. These results are comparable with the results obtained for other cell types (2-6) and hepatocytes from other species (7-15) including our own recent studies on the interaction of human apo E-free HDL with rat hepatocytes (21).

The inability of nitrosylated-HDL and LDL to compete effectively with native apo E-free HDL for binding, indicates that the lipid moiety of HDL is not responsible for the specific recognition process. However, VLDL does compete for apo E-free HDL binding, probably caused by the presence of apo C's. Also with rat hepatocytes (21), testis (18), ovarian (41) and kidney (37) membranes a competition of HDL binding by an excess of VLDL has been noticed. The possibility that exchange or transfer of radioactive lipids or apolipoproteins made a substantial contribution to this observation is unlikely because similar studies performed at 4^{o}C lead to comparable competition characteristics (results not shown), while for the studies at 37^{o}C we used a short incubation time (10 min). Furthermore, we re-isolated VLDL from the vials after incubation and recovered only 2.5% of the radioactivity, originally present in HDL in the $d < 1.006$ fraction while under these conditions more than 60% inhibition of the cell-association of radioactive HDL was found.

As reported earlier (21), the absence of a significant effect of unlabeled LDL on the cell association of ^{125}I-HDL with rat hepatocytes contrasts with studies with pig hepatocytes, in which it was shown that LDL competes for the binding of apo E-free HDL (12). In this study we provide evidence for a specific high affinity binding site for apo E-free HDL on human hepatocytes which resembles more the site on rat hepatocytes than that on pig hepatocytes.

The amount of apo E-free HDL that is degraded by human hepatocytes to TCA-soluble products is low as compared to the amount of cell associated HDL and substantially varies between cell isolations from different donors (Table 1). This variability may be due to biological variations, nutrition and/or medication. Although we cannot exclude that this variation is due to the isolation procedure it seems more likely that it is caused by the aforementioned variations of the liver donors, because with isolated rat hepatocytes the degradation of HDL after 2 hours of incubation at 37°C appears to be 10% of the amount of cell-associated HDL with only small variations (unpublished).

In one case (isolation 6) in which a relatively high association was found, light microscopy revealed large lipid droplets in the hepatocytes. It is known that this donor received a high glucose cum insulin infusion prior to the operation. Although no biochemical evidence was found for a relation between these two observations, it may be relevant for future investigations. In 3 cases, degradation of apo E-free HDL by human hepatocytes was not detectable after 3 hours of incubation at 37°C, although the association and degradation of LDL was comparable in all cell preparations. Other investigators did not find any degradation of HDL by rat liver sinusoidal cells (42), human fibroblasts or rat hepatoma cells (43). Ose et al. (44), Wandel *et al.* (14) and Dashti *et al.* (22) observed binding, internalization and degradation of HDL, but this HDL was not apo E-free and therefore interactions with the remnant (23) and/or LDL receptor (16) might have occured.

In hepatocytes from donors with detectable degradation of apo E-free HDL, chloroquine and ammmoniumchloride were used to investigate whether degradation of apo E-free HDL is influenced by these lysosomotropic agents. The effect of these agents on the degradation of LDL was used as a control. We found no significant effect of these compounds on the degradation or association of apo E-free HDL with human hepatocytes. Experiments using the fluorescent dye Dioctadecyl-Indocarbocyanine-perchlorate (DiI) reveal that HDL fluorescence as compared to LDL is mainly associated with the vicinity of the cell membrane and hardly any specific labeling of small intracellular vacuoles is observed. In contrast, DiI-labeled LDL shows under the same conditions strong intracellular fluorescence concentrated in small vacuoles. Also monensin did not influence the association of HDL with human hepatocytes while the degradation was inhibited by 30%. With LDL, incubation of monensin leads to a 40% inhibition of the association and 90% inhibition of the degradation. As shown by Basu *et al.* (39) for fibroblasts, the recycling of internalized LDL receptors is blocked by monensin.

It seems reasonable to conclude that the specific high affinity site for HDL on human hepatocytes is, in contrast to the LDL-receptor on these cells, not actively coupled to uptake and delivery of its ligand to the lysosomes. It seems possible that the prolonged residence time of HDL in the vicinity of the cell membrane may allow specific cholesterol(ester) influx into human hepatocytes in a way comparable to that by which

HDL sequesters cellular cholesterol from extrahepatic cells (6). The presence of a specific high affinity site for HDL on human hepatocytes with similar properties as in rats may thus indicate that the supposed function of HDL in reverse cholesterol transport to rat hepatocytes may be similarly operative in man.

Acknowledgements

Human liver tissue was obtained through the Auxiliary Partial Liver Transplantation Program carried out at the Department of Surgery of the University Hospital Dijkzigt in Rotterdam, The Netherlands. Consent was given by the Medical Ethical Committee of the University Hospital Dijkzigt in Rotterdam. Cells used in the experiments described in this paper were obtained through the Human Liver Cell Foundation, which was established to make optimal use of human liver tissue, to exchange ideas, to stimulate collaboration, and to provide members with isolated liver cells. Mrs. M.I. Wieriks is thanked for typing the manuscript.

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VISUALIZATION OF THE INTERACTION OF NATIVE AND MODIFIED LIPOPROTEINS WITH PARENCHYMAL, ENDOTHELIAL AND KUPFFER CELLS FROM HUMAN LIVER

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Submitted for publication

ABSTRACT

The interaction of LDL, acetylated LDL (AcLDL) and apo E free HDL with parenchymal, endothelial and Kupffer cells of human liver was visualized. For this purpose, the fluorescent phospholipid analog 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) was used to label the lipoproteins. The involvement of both parenchymal and non-parenchymal cells in the uptake of DiI labeled LDL and AcLDL was studied using *in vitro* perfusion of human liver tissue blocks. In addition, primary hepatocyte cultures were used to visualize the interaction with DiI labeled apo E free HDL and (modified) LDL.

DiI-LDL showed a time and concentration dependent interaction with both hepatocytes and Kupffer cells, although the intensity of the interaction with parenchymal cells varied strongly between the liver donors. Uptake of DiI-LDL by both cell types was strongly inhibited by the presence of an excess unlabeled LDL in the (perfusion) medium. Methylation and hydroxyacetaldehyde treatment of LDL prevented the uptake of LDL. This indicated that the uptake of LDL by Kupffer and parenchymal cells was mediated by the LDL receptor.

DiI-AcLDL was *in situ* mainly taken up by liver endothelial cells and by a minor population of Kupffer cells. Polyinosinic acid, a known inhibitor of the scavenger receptor, prevented the uptake by liver endothelial cells. Therefore human liver endothelial cells express active scavenger receptors on their surface.

Apo E free DiI-HDL was found to be associated with the membrane of cultured liver parenchymal cells, but was not taken up intracellularly, indicative for a cholesterol exchange process to occur extracellularly at the plasmamembrane. The cellular localization of lipoprotein receptors and uptake of the various classes of lipoproteins is comparable with the situation in rats.

INTRODUCTION

Hepatic lipoprotein receptors play an important role in the removal of native and modified lipoproteins and in the regulation of plasma cholesterol levels. The presence and function of these receptors in humans is important in the prevention of atherosclerosis. It is, therefore, of significance to clarify their cellular localization.

Receptors for LDL were identified in liver of several species, including rat (19, 29), rabbit (54) and swine (3, 40). In rat, both hepatocytes and Kupffer cells are involved in the receptor mediated uptake of LDL (19, 32, 34). Also evidence is provided for the presence of LDL receptors on human liver membranes (18, 22, 24), on cultured adult and fetal hepatocytes (9, 26). Evidence for a relationship between the presence of he-

ERRATA

Page 129.
Table I must be completed with:

Perfusion	DiI-AcLDL		DiI-LDL		LDL receptor	
	EC	KC	PC	KC	PC	KC
H	nd	nd	+++	+++	++	+++
I	nd	nd	+++	++++	++	++

Page 134.
Table II must be completed with:

Isolation number	DiI-LDL	DiI-HDL
VI	++	nd

Page 139-141.
Ref. 36 (Oram et al.) up to ref. 62 (Weisgraber et al.) must be numbered 37 up to 63.

Page 123, 2nd sentence.
Reference Hardens-Spengel must be (18) and Nanjee and Miller must be (36)

patic LDL receptors and plasma cholesterol levels was provided by Harders-Spengel *et al.* (19), Bilheimer *et al.* (6), Soutar *et al.* (55) and Nanjee and Miller (37). No data were available concerning the relative role of Kupffer cells and hepatocytes in the uptake of LDL.

HDL is supposed to play a role in reverse cholesterol transport. According to the concept of Glomset (17), HDL is expected to receive cholesterol from peripheral cells for subsequent transport and irreversible delivery to hepatocytes. Binding sites for HDL were identified on hepatocytes of rats (35, 38, 39, 54, 57, 58), rabbits (54), pigs (4) and on liver membranes (11, 12) including those of humans (23). Also on non-parenchymal cells of rat liver binding sites for HDL have been identified (51, 57, 61).

The scavenger receptor in rat liver is mainly localized on the liver endothelial cells and recognizes acetylated low density lipoprotein (AcLDL) (14, 32, 59) and biologically modified LDL (32, 33). The presence and cellular localization of the scavenger receptor in the human liver were speculative.

In the present studied we investigated the interaction of LDL, AcLDL and apo E free HDL with the human liver cells at the light microscopical level. The fluorescent phospholipid analog 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) was used to label the lipoproteins. The interaction of DiI labeled LDL and AcLDL with parenchymal and non-parenchymal cells was studied using perfused human liver tissue blocks. The identification of the different cell types involved was facilitated by the use of monoclonal antibodies reacting specifically with various liver cell types. Kupffer cells were also identified by their capacity to phagocytose latex particles. The interaction of DiI labeled apo E free HDL and LDL was further studied using cultured hepatocytes.

MATERIAL AND METHODS

Materials

Human serum albumin (HSA) (fraction V), bovine serum albumin (BSA) (fraction V), dexamethasone, levamisole and naphtol A.S.M.X. phosphate were purchased from Sigma (St Louis, MO, USA). Dulbecco's modification of Eagles medium (DME), was obtained from Flow laboratories (Irvine, Schotland, UK). Multiwell cell culture dishes were obtained from Costar (Cambridge, MA, USA). 1,1'-dioctadecyl-3,3,3',3'-indocarbocyanine perchlorate (DiI) from Molecular probes (Eugene, Oregon USA) and tetra-nitromethane 98% (TNM) from Aldrich Company Inc (Brussels, Belgium). Fluoresbrite latex particles (0.6 μm, YG) were obtained from Polysciences (Warrington, PA, USA).

The monoclonal antibodies against RFD-7 were obtained from the Royal Hospital London (London, UK), antibodies against desmine and cytokeratine CK18 (RGE-53)

from Sigma (St Louis, MO, USA). Rabbit anti-mouse IgG and mouse anti-alkaline phosphatase-alkaline phosphatase complexes (APAAP) were obtained from Dakopatts (Glostrup, Denmark).

Lipoprotein isolation labeling and modification

Human LDL ($1.024 < d < 1.055$), human HDL ($1.063 < d < 1.21$) and heavy HDL ($1.16 < d < 1.21$) were isolated from serum by density gradient centrifugation according to Redgrave (48).

Reductive methylation was done as described by Weisgraber *et al.* (63). Hydroxy acetaldehyde treatment was done as described by Slater *et al.* (52) and nitrosylation as described by Brinton *et al.* (8).

LDL, methylated LDL (MeLDL), hydroxy acetaldehyde treated LDL (HOET-LDL), HDL and nitrosylated HDL (TNM-HDL) were labeled with DiI according to Pitas (41), the density of the DiI and lipoprotein mixture was subsequently raised to 1.21 and the lipoproteins were re-isolated according to Redgrave (48). Unlabeled and DiI labeled HDL and TNM-HDL were made apo E free by heparin sepharose chromatography (62).

Liver tissue and general histologic examination

Human livers were obtained from patients undergoing hepatic resection for liver tumors at the Academic Medical Centre (Amsterdam, The Netherlands) and from auxiliary liver transplant donors at the University Hospital Dijkzigt (Rotterdam, The Netherlands), under protocols of the Medical Ethical Commisions. The age of the patients and donors varied between 12 and 69 years. Livers from patients were obtained after surgery, free of discrete tumor nodules and micrometastasis. The liver parts were immersed in saline and kept on ice during transport to the laboratory. Livers obtained via the auxiliary liver transplant program were, after hepatectomy, perfused with Eurocollins solution and kept on ice until transplantation. Part of the liver, not used for transplantation, was used for cell isolation and liver perfusion studies. The cold ischemic periods were 2 to 3 hours for resection donors, and 3 to 18 hours for transplant donors. Specimens for routine histologic examination were fixed by immersion in 4% formaldehyde solution. Sections of paraffine embedded tissue were stained with haematoxylin-phloxine saffran (HPS), periodic-acid-Schiff (PAS), and a PAS stain after diastase treatment. Formaldehyde fixed cryo sections were stained for lipid with an Oil red O or with Sudan Black.

Immunohistochemistry on liver sections

Cryosections of human livers or liver cells cultured on glass cover slips were immunolabeled with mouse antibodies against tissue macrophages RFD-7 (44) for Kupf-

fer cells (dilution 1 : 10), desmin (13) for fat storing cells (dilution 1 : 10), cytokeratin (46) for parenchymal cells (dilution 1 : 10), and factor VIII/ von Willebrand factor complex (FVIII/WF) (54) for liver endothelial cells (dilution 1 : 50). Liver sections were also incubated with chicken antibodies, against the bovine LDL receptor (1 : 50). The latter antibody was kindly donated by Dr. P. Kroon, (Merck Institute for Therapeutic Research NJ, USA). The anti-FVIII/WF (CLB-RAg 35) was kindly donated by Dr. J. van Maurik (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Mouse monoclonal antibodies were labeled with the indirect alkaline phosphatase-anti-alkaline phosphatase (APAAP) method (Cordell 1984). In short, samples were incubated with the first antibody, rinsed, incubated with rabbit anti-mouse IgG (dilution 1 : 150), rinsed and incubated with the APAAP complex (dilution 1 : 100) and developed in 1 mM Naphtol A.S.M.X. phosphate in 0.1 M TRIS, pH 8.2. Sections were rinsed with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA). After the APAAP step sections were rinsed in PBS without BSA. Endogenous alkaline phosphatase activity was inhibited by including 1 mM Levamisole in the developer solution. Chicken antibodies were labeled with a monoclonal mouse antibody against chicken IgG (dilution 1 : 2000) (25) followed by the aforementioned APAAP method. The mouse anti chicken antibody was kindly donated by Dr. G. Koch (Central Veterinary Institute Lelystad, The Netherlands).

In-vitro liver perfusion studies

Liver specimens were dissected, cannulated and rinsed with HANKS balanced salt solution at 4°C and perfused with the different fluorescent DiI labeled lipoproteins for 30 or 60 min. in a concentration of 10-20 μg/ml Dulbecco's modifcation of Eagles medium (DME) containing 1% HSA, saturated with air. During the last five minutes of the perfusion, fluorescent latex was included in the medium. When indicated the perfused liver specimens were rinsed with saline and fixed in 4% PBS buffered paraformaldehyde or divided into pieces and snap frozen in liquid nitrogen.

Cryosections (6 μm) of the unfixed liver pieces were cut on a Minitome cryostate microtome. Fixed liver tissue samples were immersed with 2.3 M sucrose, frozen in liquid nitrogen and semithin sections (5 μm) were cut on a Reichert FC 4 cryomicrotome. Sections were immunolabeled when indicated, mounted with glycerol and viewed with a Leitz ortholux microscope with standard rhodamine excitation and emission filters for DiI fluorescence. Latex fluorescence was viewed with a broad band FITC filter block. Latex fluorescence did not show overlap with the DiI fluorescence and vice versa. Photographs were taken with a fixed exposure time in order not to cover up differences in fluorescence by automatic exposure.

Isolation and culture of hepatocytes.

Human hepatocytes were mainly isolated from liver parts obtained via the auxiliary partial liver transplantation program. Isolation was started by cannulation of blood vessels visible at the dissection surface of the liver part . The liver tissue was perfused with 10 mM HEPES buffer pH 7.4, containing 142 mM NaCl, 16.7 mM KCl and 0.5 mM EGTA, at a rate of 40 ml/min at 37°C for 75 minutes. The perfusion was continued with 500 ml HEPES buffer pH 7.6 containing 5 mM $CaCl_2$ and subsequently with this buffer containing respectively 0.05% and 0.1% collagenase, under recirculating conditions, for 20 minutes each. Liver was dissociated in HANKS buffer containing 2% BSA. Cells were filtered to remove undissociated fragments, centrifuged for 30 seconds at 50 g and washed three times in cold culture medium to remove damaged and nonparenchymal liver cells. Cells were seeded on collagen coated (30) round glass cover slips in 24 wells plates. Cells were maintained as described by Princen *et al.* (45). Culture medium was changed 12 hours after seeding and every 24 hours thereafter. Experiments were done with cells cultured for two days after a standard preincubation for 20 hours with medium containing 2% HSA. When indicated, cells were preincubated with HDL fractions (20% v/v) or with LDL (20 μg/ml). Cells were washed twice with DMEM containing 1% HSA and incubated at 37°C with DiI labeled lipoproteins in a concentration of 10-50 μg/ml for different time periods in the presence or absence of 15 fold excess unlabeled lipoprotein. After incubation, cells were washed twice in DMEM containing 1% BSA, once with PBS and fixed with 2% gluteraldehyde in phosphate buffered saline (PBS). Coverslips were mounted with glycerol and viewed for fluorescence.

RESULTS

Histology and identification of cells

Livers from donors and from resection patients showed a normal histology. In the livers of resection patients, less glycogen was present due to fasting prior to surgery and no morphologic evidence for extrahepatic biliary obstruction was observed. Fig 1a illustrates the morphology. Oil red O staining revealed the presence of some small fat droplets in parenchymal cells (not shown). Two times (out of 16 livers) a light macrovesicular steatosis was observed. Kupffer cells varied in abundancy between the different liver donors. Some of them showed strong fagocytic activity. Endothelial cells lined the sinusoids and fat storing cells were present but not conspicuously. Occasionally small white blood cell infiltrates were observed.

The identification of the non-parenchymal cells was facilitated by the use of antibodies, specifically reacting with the different liver cell types. The different pattern after immunostaining with anti-FVIII/WF for liver endothelial cells and RFD-7 for Kupffer cells

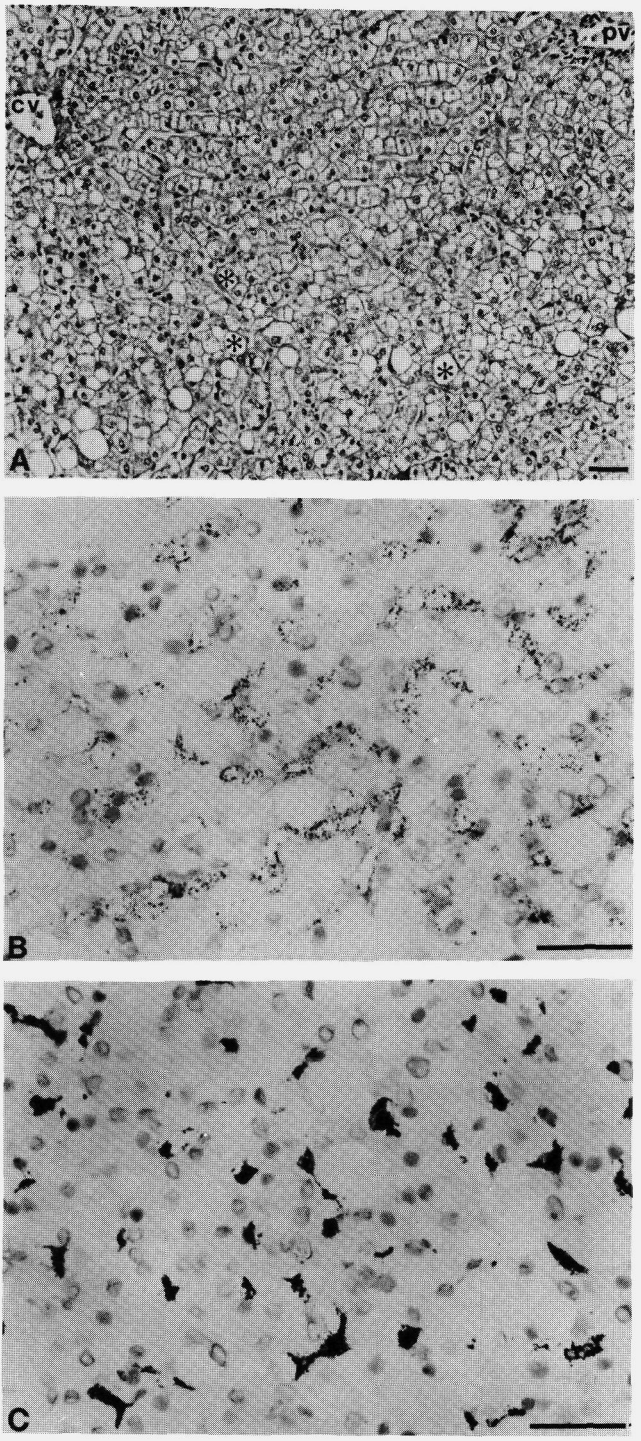

Figure 1. Sections of human livers; overview (A) and localization of endothelial (B) and Kupffer cells (C).

Magnification (A) = 105 x, magnification (B, C) = 345 x, bar indicates 50 μm.

A: Formaldehyde fixed section of human liver (HPS-stain). PV = portal vein, CV = central vein, asterisks indicate parenchymal cells with a steatotic appearance.

B: Cryostate section of human liver after incubation with anti-FVIII/WF for liver endothelial cells.

C: Cryostate section of human liver after incubation with anti-RFD-7 for Kupffer cells.

is shown in Fig 1b and 1c.
The immunostaining was compared to the fluorescence after perfusion with the different DiI-lipoproteins.

Uptake of lipoproteins by in vitro perfused liver cells

The interaction of DiI-labeled lipoproteins with the non-parenchymal cells was investigated in perfused liver tissue blocks. In most livers (see table I), DiI-AcLDL was clearly taken up by the sinusoidal endothelial cells (Fig 2), as identified by their morpho-

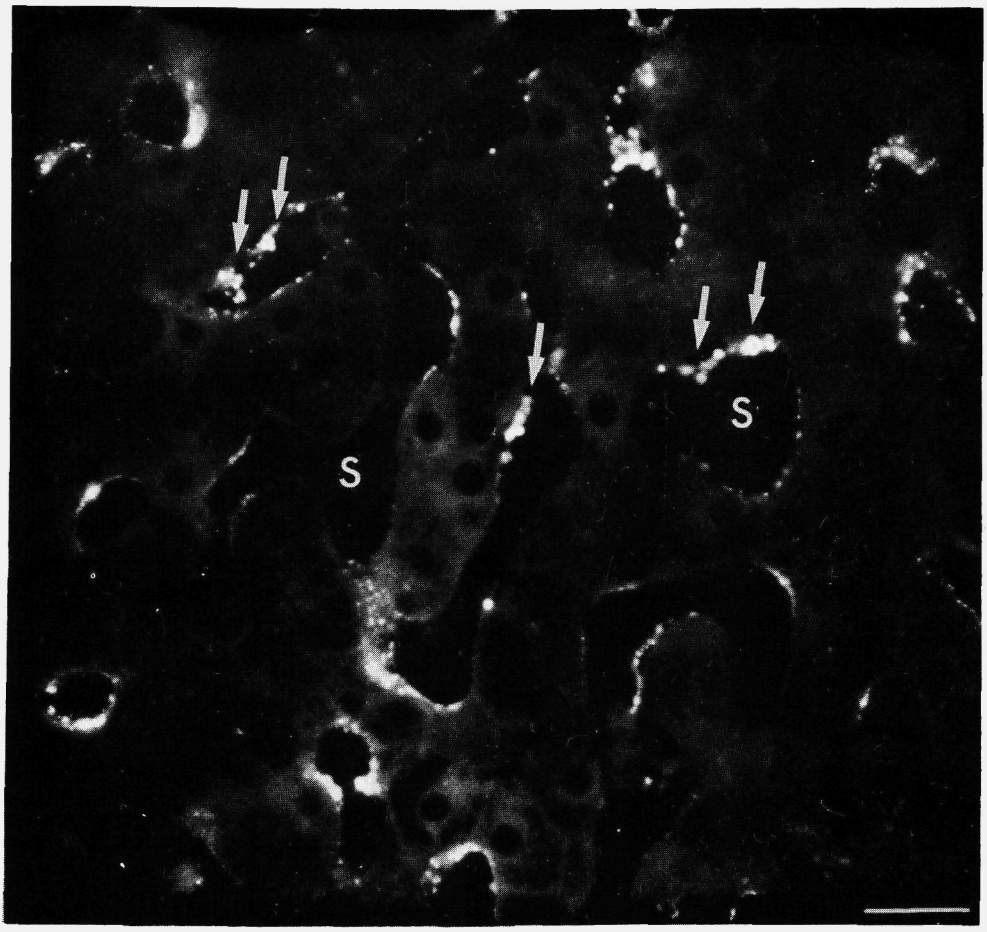

Figure 2. Perfusion of liver tissue (liver A) with DiI-AcLDL for the 30 minutes at 37°C.
S = sinusoid. Magnification = 1390 x , bar indicates 10 μm.
A: Liver endothelial cells (arrows) show a clear punctate DiI fluorescence.

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Page 129.
Table I must be completed with:

Perfusion	DiI-AcLDL		DiI-LDL		LDL receptor	
	EC	KC	PC	KC	PC	KC
H	nd	nd	+++	+++	++	+++
I	nd	nd	+++	++++	++	++

Page 134.
Table II must be completed with:

Isolation number	DiI-LDL	DiI-HDL
VI	++	nd

Page 139-141.
Ref. 36 (Oram et al.) up to ref. 62 (Weisgraber et al.) must be numbered 37 up to 63.

Page 123, 2nd sentence.
Reference Hardens-Spengel must be (18) and Nanjee and Miller must be (36)

logy and reactivity towards anti-FVIII/WF (Fig 1b). Polyinosinic acid, a known competitive inhibitor of the scavenger receptor prevented uptake of DiI-AcLDL. Some Kupffer cells participated in the uptake of DiI-AcLDL. No involvement of parenchymal cells was observed, however in one liver DiI-AcLDL was taken up by parenchymal cells with a steatotic appearance. A minor portion of the cultured hepatocytes isolated from the same liver donor also took up DiI-AcLDL. These hepatocytes contained also more fat droplets than other hepatocytes wich did not take up AcLDL (not shown).

Table I. Relative uptake of DiI-labeled LDL and AcLDL by various liver cells and relative reactivity towards anti-LDL receptor antibodies.

The relative fluorescence intensity of the uptake of DiI-LDL and DiI-AcLDL by the indicated cells from different donors is indicated using + indicating presence of fluorescence or − indicating absence of specific fluorescence. The relative intensity of the anti- LDL receptor immunoreaction on Kupffer cells and on the apical side of parenchymal cells is also indicated. EC = endothelial cells, KC = Kupffer cells, PC = paarenchymal cells. nd = not determined.

Perfusion	DiI-AcLDL		DiI-LDL		LDL receptor	
	EC	KC	PC	KC	PC	KC
A	+++	−	+	+++	±	+++
B	+++	−	±	++	nd	nd
C	+	nd	±	+++	±	+++
D	++	−	nd	nd	nd	nd
E	nd	nd	±	++	±	++
F	±	±	±	+	++	+++
G	++	±	+	++	nd	nd

DiI-LDL was taken up by Kupffer cells of all the livers tested (Fig 3a, b) and its labeling pattern resembled that of anti-RFD-7 (Fig 1c). Perfusion for 30 minutes resulted in a bright punctate labeling. The intensity of the labeling increased further upon prolonged perfusion, but no separate vacuoles could be distinguished at 60 minutes. The interaction of DiI-LDL with the parenchymal cells was highly variable between the various liver donors. In some cases parenchymal cells showed a slight interaction at the sinusoidal membrane and some punctate uptake in the hepatocytes (Fig 3b) but other livers showed at 30 minutes already a strong interaction with DiI-LDL, which was greatly increased upon further perfusion (Fig 3a). The uptake of DiI-LDL by both Kupffer and parenchymal cells was decreased to below the detection limit by addition of an excess

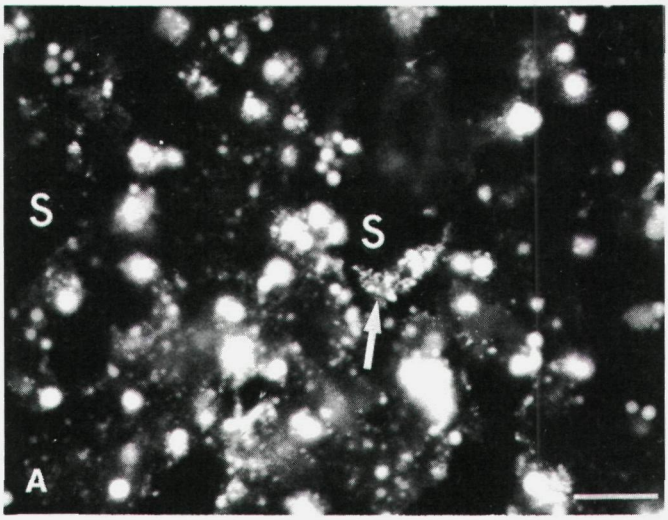
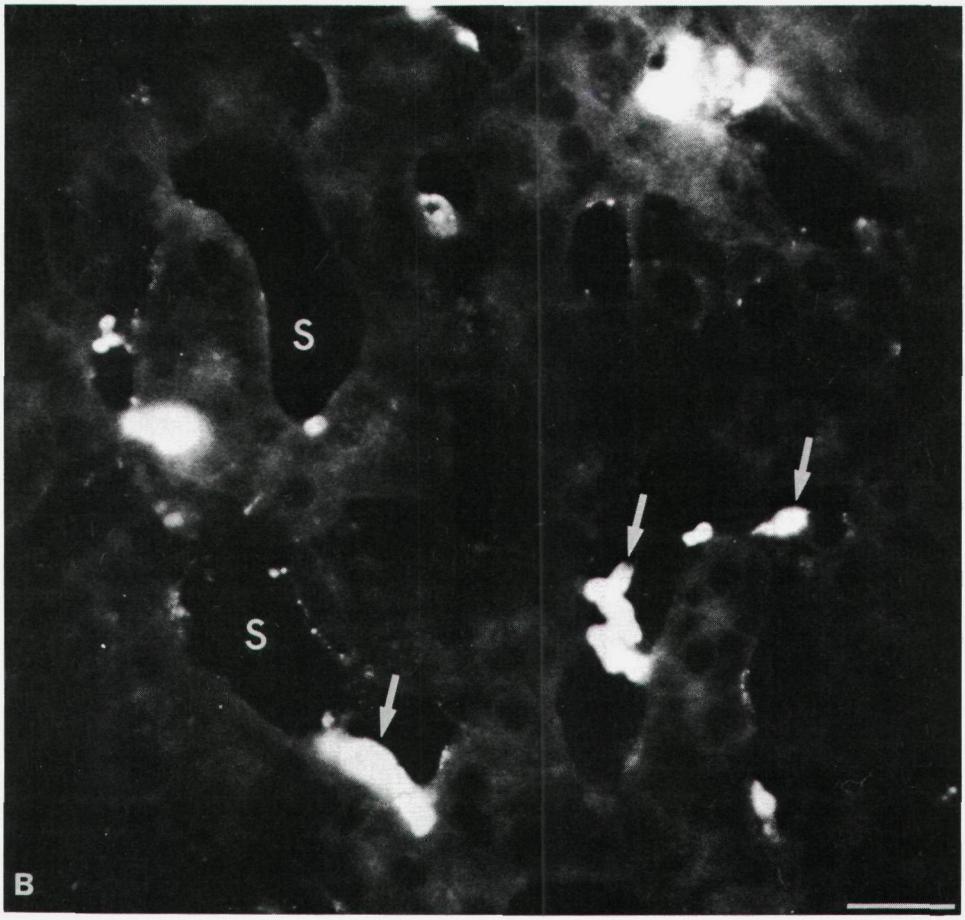


Figure 3. Perfusion of liver tissue with DiI-LDL for the indicated periods at 37°C.

S = sinusoid. Magnification (A) = 1134 x, magnification (B) = 1390 x, bar indicates 10 μm.

A: Liver (liver H) perfused for 30 minutes. Parenchymal cells and a Kupffer cell (arrow) show DiI fluorescence.

B: Liver (liver A) perfused for 60 minutes. Kupffer cells (arrows) show a bright DiI fluorescence.



unlabeled LDL to the perfusion medium and also by modification of LDL by methylation or hydroxy acetaldehyde treatment (not shown). However, sometimes DiI-MeLDL was taken up by some small groups of parenchymal cells and by few (less than 5%) of the Kupffer cells.

Immunocytochemical detection of LDL receptor in liver sections.

Incubation of liver sections with an antibody against the LDL receptor resulted in a strong labeling of the Kupffer cells, (Fig 4a) a pattern similar to that of RFD-7 labeling and if DiI-LDL uptake by the Kupffer cells.

The intensity of the anti-LDL receptor immunolabeling at the apical side of the hepatocytes (Fig 4b) varied between the donors, but could be very well related to the intensity of the DiI-LDL uptake by the hepatocytes of these livers (Table I). In some livers, immunostaining at the basal membrane and around bile canaliculi was observed (Fig 4c).

Isolation and primary cultures of hepatocytes

The viability of the freshly isolated hepatocytes varied between 51% and 77% for the different donors. Twelve hours after seeding, the cultures were washed and the majority of the non-attached non-viable cells was removed. After culturing for two days, clusters of well spread parenchymal cells were present but also single spread parenchymal cells. A few small non-parenchymal cells were also present in the culture.

Lipoprotein uptake by cultured hepatocytes

Hepatocyte cultures which were not incubated with fluorescently labeled lipoproteins did show autofluorescence (Fig 5a, b). The intensity of this autofluorescence was strongly dependent of the liver donor. Vacuoles, mostly lipid vacuoles, showed non-fading diffuse fluorescence, visible at both rhodamine and FITC excitation and emission wavelength combinations. Occasionally the lipid droplets showed rapidly fading fluorescence at 325 nm, indicative for the presence of vitamin A.

Incubation of the hepatocyte cultures with DiI-LDL (10 to 50 μg/ml) up to 3 hours resulted in an increase of punctate fluorescence in the hepatocytes (Fig 5c, d). The intensity of the uptake differed between the cells and the cell preparations (Table II). In general, always a time dependent and LDL concentration dependent increase was found. Addition of an excess of unlabeled LDL resulted in a strong decrease of the DiI-fluorescence in cells (Fig 6a, b). Modification of LDL by methylation or by hydroxy-acetaldehyde treatment, which prevents recognition by the LDL receptor caused an almost complete inhibition of DiI-LDL association (Fig 6c, d).

Preincubation of hepatocyte cultures with LDL (50 μg/ml) for 20 hours, resulted in a de-

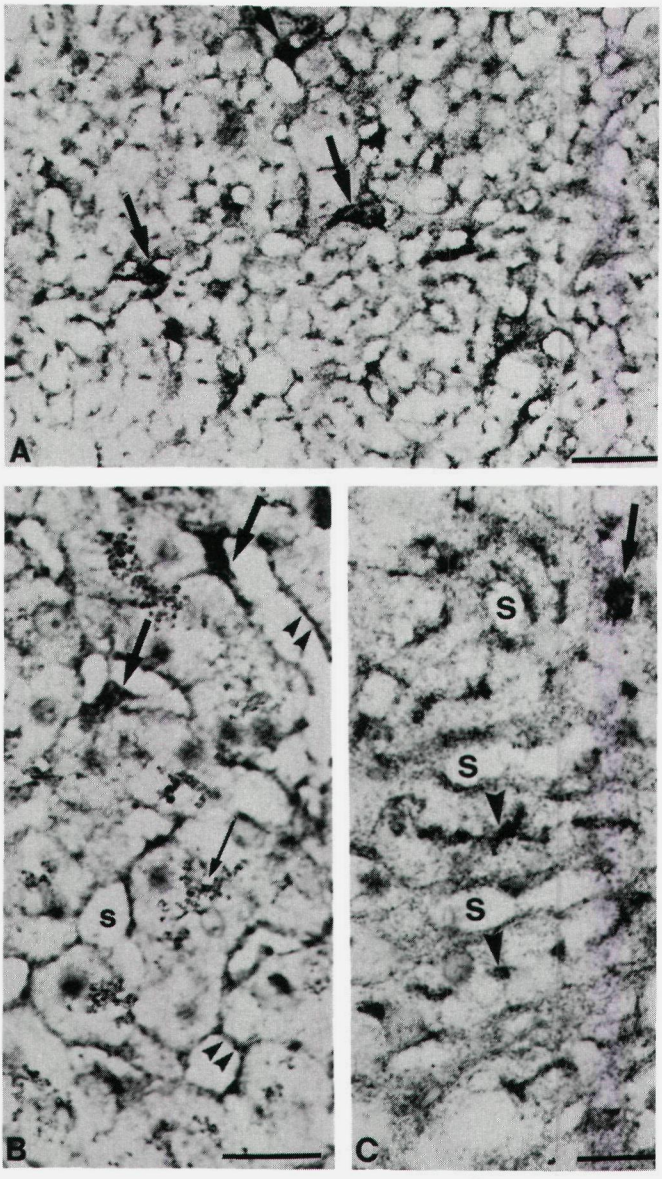


***Figure 4.* Cryostate sections of livers incubated with an antibody against the LDL receptor.**

S = sinusoid. Magnification (A) = 315 x, magnification (B,C) = 600 x, bar (A) indicates 50 μm, bar (B,C) indicates 25 μm.

A: Kupffer cells (thick arrow) are labeled and the parenchymal cells show a slight labeling at the apical side but also at the basolateral side (liver C).

B: Liver H shows a more intense immunostaining of the apical side of the parenchymal cells (double arrowheads). Thin arrow indicates (hemocyderine) pigment.

C: Liver I shows also labeling at the apical side of the parenchymal cells, but also a strong labeling near the bile canaliculi (arrowheads).

Figure 5. Interaction of DiI-LDL with cultured human liver parenchymal cells.

Parenchymal cells (isolation I), 2 days after isolation, incubated with DiI-LDL (10 μg DiI-LDL/ ml) at 37°C or the indicated periods. Magnification = 1360 x, bar indicates 10 μm.

A: Cells not incubated with DiI labeled lipoproteins. **B**: *Same field as in* A. *Autofluorescence* from some small vacuoles and lipid droplets is observed. **C:** Cells were incubated for 60 minutes at 37°C. Punctate fluorescence of DiI-LDL is observed. **D:** Cells were incubated for 180 minutes, the intensity of the punctate fluorescence was increased.

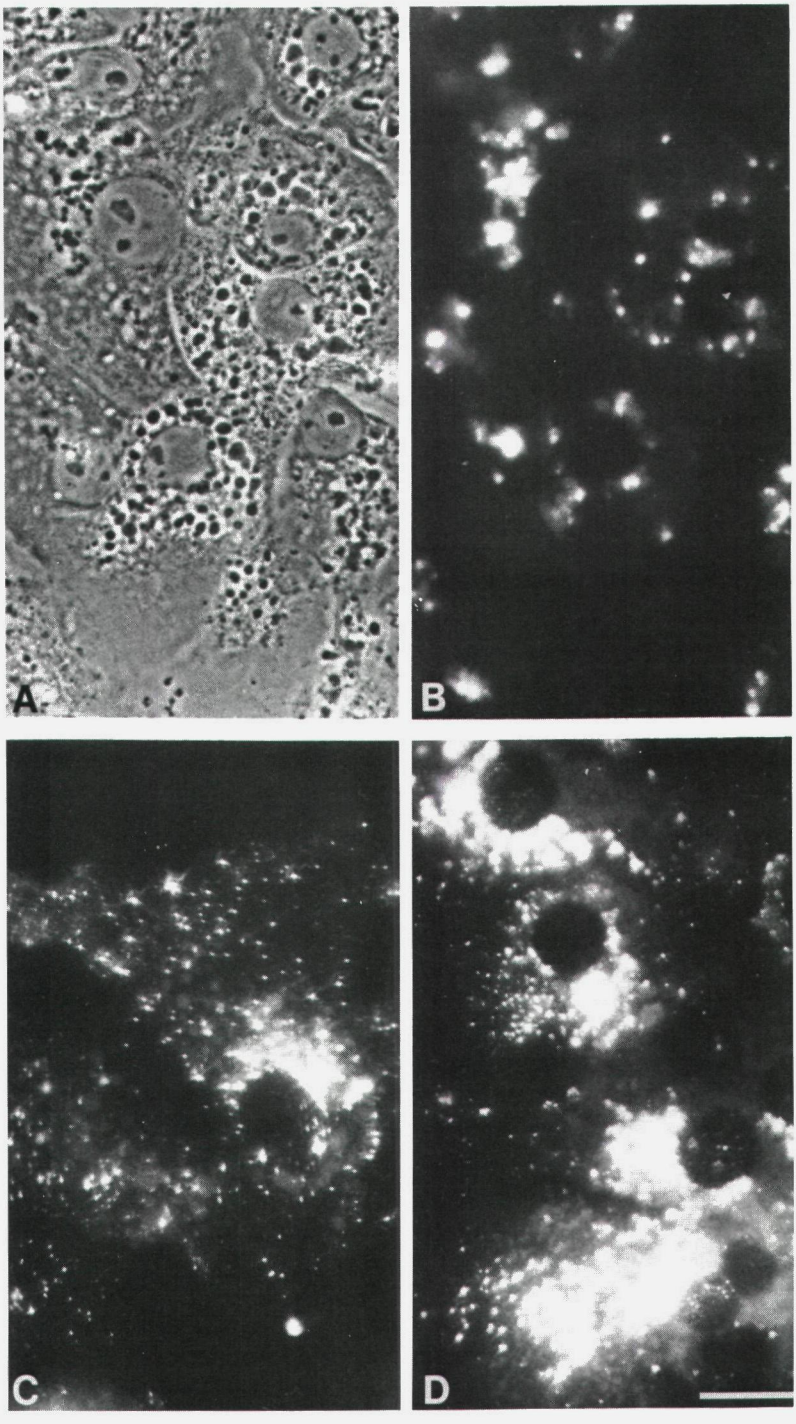

A
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Table I must be completed with:

Perfusion	DiI-AcLDL		DiI-LDL		LDL receptor	
	EC	KC	PC	KC	PC	KC
H	nd	nd	+++	+++	++	+++
I	nd	nd	+++	++++	++	++

Page 134.
Table II must be completed with:

Isolation number	DiI-LDL	DiI-HDL
VI	++	nd

Page 139-141.
Ref. 36 (Oram et al.) up to ref. 62 (Weisgraber et al.) must be numbered 37 up to 63.

Page 123, 2nd sentence.
Reference Hardens-Spengel must be (18) and Nanjee and Miller must be (36)

Isolation number	Dil-LDL	Dil-HDL
I	++++	++
II	+++	+
III	+	±
IV	++	−
V	+	−

Table II. The relative interaction of Dil-LDL and Dil-HDL with parenchymal cells from different isolations.

The relative fluorescence intensity is indicated using + indicating presence of fluorescence or − indicating absence of specific fluorescence.

creased fluorescence of Dil-LDL whereas preincubation with HDL fractions resulted in a slightly increased fluorescence of Dil-LDL (not shown). This suggests a regulation of the LDL receptor activity in cultured human hepatocytes.

The interaction of apo E free Dil-HDL with the hepatocytes varied between the cell preparations (Table II). Incubation of hepatocytes derived from two different donors, with apo E free Dil-HDL for 60 minutes resulted in a punctate association of fluorescent HDL with the cell membrane. A small further increase was observed at incubations for 180 min (fig 7a). No specific intracellular fluorescent labeling was observed. Addition of an excess of unlabeled apo E free HDL abolished the membrane associated labeling (fig 7b). Apo E free Dil-TNM-HDL displayed also an interaction with the membranes of the hepatocytes, but at a lower level than did Dil-HDL (Fig 7c). Also this interaction was diminished by adding an excess of unlabeled nitrosylated HDL. Although we observed a clear interaction of Dil-HDL with these parenchymal cell preparations, in other preparations the interaction of apo E free Dil-HDL with hepatocytes was much lower or absent whereas Dil-LDL showed a clear interaction with the same cell preparations. The donor specific morphological observations concerning the intensity of the interaction with HDL paralleled the simultaneously performed biochemical experiments concerning the amount of high affinity HDL binding (Schouten *et al.*, submitted for publication).

DISCUSSION

We have visualized the interaction of Dil- labeled LDL, HDL AcLDL with various human liver cells. The observed differences in intensity of the interaction of lipoproteins with the livers from different donors can reflect the differences in the donors history e.g. genetic background, diet, hospital treatments hormones etc. It is known that insulin, thyroid hormones and dexamethasone regulate the presence of the LDL receptor on hepatocytes of rats (49, 50) and on Hep G-2 (60). The effects of these hormones on the interaction of HDL with its binding site are largely unknown.

The presence of LDL receptors on adult and fetal human liver parenchymal cells has been reported earlier (2, 9, 15, 21, 24, 28). The presence of an LDL receptor on

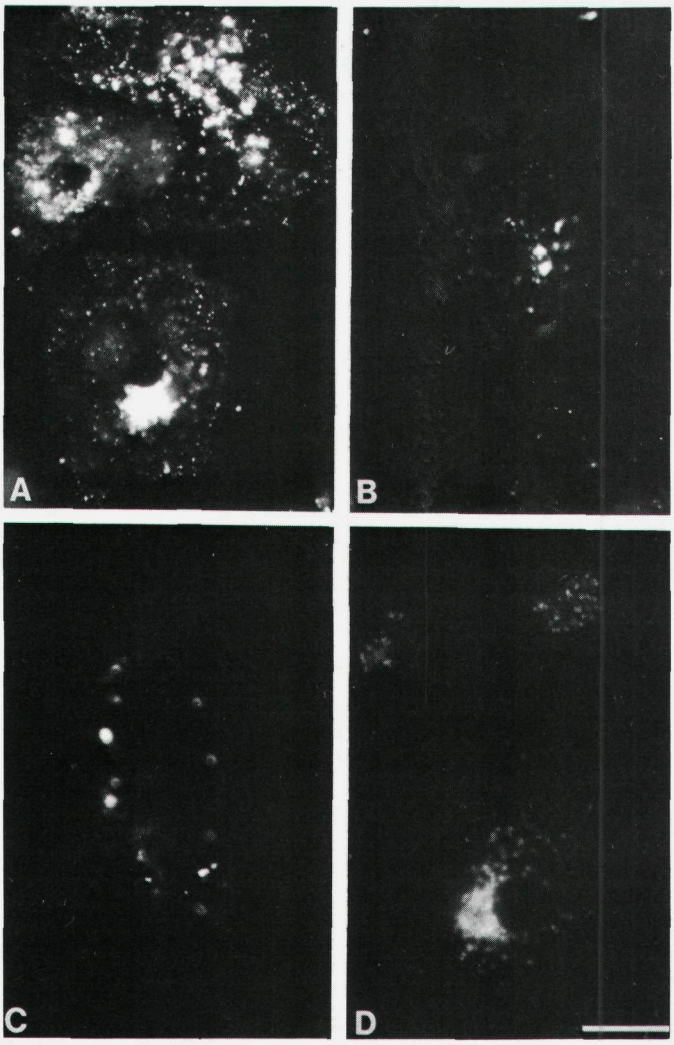

Figure 6. Specificity of DiI-LDL interaction with cultured human liver parenchymal cells.

Parenchymal cells (isolation VII), 2 days after isolation, incubated with DiI labeled lipoproteins (10 μg DiI-lipoprotein/ml) at 37°C, for 3 hours. Magnification = 1134 x, bar indicates 10 μm.

A: Cells incubated with DiI-LDL.

B: Cells incubated in the presence of a 15 fold excess unlabeled LDL.

C: Cells incubated with DiI-HOET-LDL.

D: Cells incubated with DiI-MeLDL.

Kupffer cells was previously reported in rats (20), but to our knowledge no reports were published on human Kupffer cells. There was a specific LDL time and concentration dependent interaction of DiI-LDL with both hepatocytes and Kupffer cells. The uptake was mediated by the LDL receptor, since DiI-LDL uptake was inhibited by addition of an excess of unlabeled LDL and by modification of lysine residues. These amino acid residues are known to be involved in the recognition of LDL by its receptor (63). The uptake of DiI-LDL by parenchymal cells varied strongly between different donors whereas DiI-LDL uptake by Kupffer cells differed only slightly. This suggests that the LDL receptors on Kupffer and parenchymal cells are under a different metabolic control.

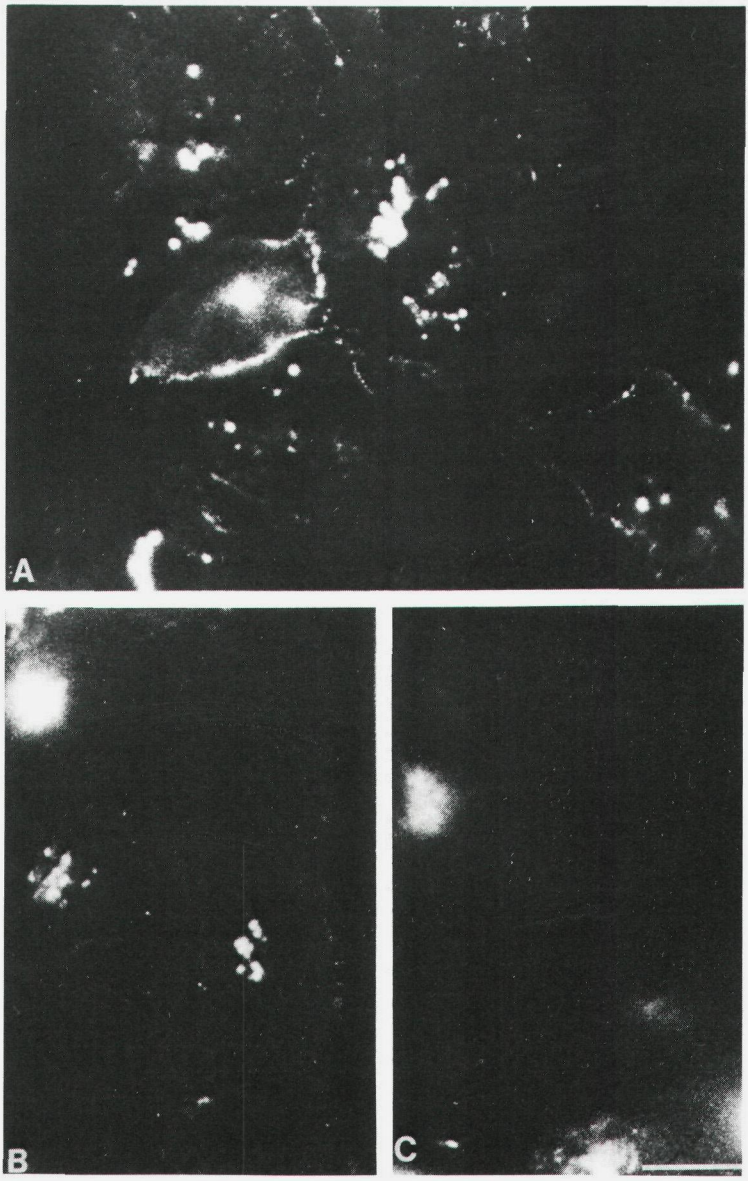

Figure 7. Interaction of DiI-HDL with cultured human liver parenchymal cells.

Parenchymal cells (isolation I), 2 days after isolation, incubated with DiI-HDL or with DiI-TNM-HDL (10 μg DiI-lipoprotein/ml) at 37°C, for 180 minutes. Magnification = 1134 x, bar indicates 10 μm.

A: Punctate fluorescence of DiI-HDL is associated with the membrane of the parenchymal cells.

B: Fluorescence is greatly decreased by addition of an excess unlabeled HDL.

C: The intensity of the fluorescence of DiI-TNM-HDL, associated with the membrane of the parenchymal cells is much lower then after incubation with unmodified HDL.

The interaction of apo E free HDL with the hepatocytes is strikingly different from the interaction of LDL with these cells. Whereas LDL is internalized, apo E free HDL remains at the membrane. Other investigators presented evidence that the interaction of HDL apoprotein with the liver differed from that of the cholesterol moiety (1, 16, 31) and

that cholesterolester uptake by the liver cells exceeded the amount of cell associated apoprotein. That HDL is capable of exchanging cholesterol and cholesterol esters without internalization was demonstrated for steroid producing tissue (47), fibroblasts (37), and hepatocytes (5, 43). A binding site for apo E free HDL is present on human hepatocytes (23, Schouten *et al.* submitted for publication). Kambouris (26) showed that a binding protein of 80 kDa HDL was present on Hep-G2 cells. Since we have also biochemical evidence that the apoprotein moiety of apo E free HDL is not handled via the lysosomal pathway (Schouten *et al.* submitted for publication), we postulate that the HDL binds to its high affinity site but that the HDL shell (apoprotein and phospholipid) is not internalized by human hepatocytes. The pathway of the cholesterol moiety in human hepatocytes remains to be further established.

We have clearly demonstrated the substantial presence of a scavenger receptor on the endothelial cells of the human liver *in situ*. So far the presence of a scavenger receptor in humans was only speculative. Kesäniemi (27) observed a faster clearance of acetaldehyde modified LDL in humans and he suggested a role for the liver. The involvement of endothelial liver cells was extensively demonstrated in rats, dogs and guinea pigs (7, 14, 32, 33, 42, 59) but not in humans. Babaev (2) found that contaminating cells in hepatocyte cultures took up FITC labeled formaldehyde treated serum albumin and only based on studies in rat, he identified these cells as human liver endothelial cells.

We conclude that human hepatocytes and Kupffer cells possess LDL receptors. Liver endothelial cells possess substantial numbers of scavenger receptors. HDL interacts on the membranes of cultured human liver parenchymal cells. The cellular localization of the receptors and the cell types involved in the uptake of DiI labeled lipoproteins are the same as in rats. This may indicate that studies initially performed in rats may be ligitimately extrapolated to the human situation.

Acknowledgements

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Everyone, who was involved in the isolation and culturing of the human hepatocytes, mostly in the middle of the night, is gratefully thanked for their contribution. Dr Knot from the Erasmus University, Dr Bosma and Dr Ligidakis from the Academic Medical centre are kindly thanked for their cooperation concerning the donation of the human livers. Tom Glaudemans is thanked for his photographic work.

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Page 129.
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	EC	KC	PC	KC	PC	KC
H	nd	nd	+++	+++	++	+++
I	nd	nd	+++	++++	++	++

Page 134.
Table II must be completed with:

Isolation number	DiI-LDL	DiI-HDL
VI	++	nd

Page 139-141.
Ref. 36 (Oram et al.) up to ref. 62 (Weisgraber et al.) must be numbered 37 up to 63.

Page 123, 2nd sentence.
Reference Hardens-Spengel must be (18) and Nanjee and Miller must be (36)

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SUMMARY

Lipids in plasma are transported in vehicles, called lipoproteins. These consist of proteins (apoproteins), triglycerides, cholesterol, cholesterol esters, phospholipids and other fat soluble substances. The function of lipoproteins is the transport of dietary fat and of endogenously synthesized lipids to tissues where they are utilized for oxidative metabolism, hormone synthesis or maintenance of membrane integrity. In humans, the majority of cholesterol (60-70%) is carried in humans by low density lipoprotein (LDL). The function of LDL is the delivery of cholesterol to cells. LDL plasma levels are influenced to a great extent, by the amount of LDL receptors in the liver. High density lipoprotein (HDL) also carries an important part (20-30%) of plasma cholesterol. Epidemiological studies on the relationship between plasma cholesterol levels and the prevalence of atherosclerosis has led to the conclusion that LDL-cholesterol levels are positively correlated with coronary heart diseases, whereas HDL-cholesterol levels are negatively correlated. This has been explained by the ability of HDL to mediate in the removal of excess cholesterol deposited in peripheral cells. Glomset (1968) proposed that HDL could deliver this excess cholesterol to the liver, a process called reverse cholesterol transport. The liver is then able to remove cholesterol from the body circulation. Cholesterol is then secreted in the bile wich is discharged into the intestine. In many respects the liver plays a decisive role in the regulation of lipid levels in bloodplasma. It is therefore important to determine the mechanisms by wich the liver recognizes lipoproteins and to analyse the subsequent processing of the different lipoprotein constituents. The liver consists of various cell types, which may all contribute to total liver lipoprotein metabolism. In this thesis, the interaction of LDL and HDL with the liver, including the role of the various liver cell types is determined.

To visualize the interaction of lipoproteins with the liver cells on the light microscopical level the fluorescent phospholipid analog 1'1' dioctadecyl 3 3 3'3'tetramethyl indocarbocyanine (DiI) was used. It was demonstrated that Kupffer cells were actively involved in the uptake of DiI-LDL in both normal rats as well as in rats treated with ethinyl estradiol (EE). The uptake of LDL by parenchymal cells of untreated rats was low, and EE treatment resulted in a large increase in LDL uptake by these cell types. In EE treated rats it was found that the fluorescence of DiI-LDL accumulated in the bile canalicular area. The intracellular pathway of LDL was further investigated at the ultrastructural-microscopical level in EE treated rats. Antibodies against apolipoprotein B, the only protein present in LDL were visualized with electron dense colloidal gold particles to detect the presence of LDL in the liver sections. It was revealed that the initial binding of LDL occured on the microvilli on top of the parenchymal cells. LDL was subsequently taken up in vesicles inside the cells. At later time points the amount of intracellular labeling was increased, mainly in the area around the bile canaliculi. Indications were obtained for the presence of immunoreactive apo B inside bile canaliculi.

The appearance of apo B (fragments) in the bile was quantified in rats which were

permanently equipped with catheters in the bile duct, duodenum and heart. After administration of ^{125}I-tyramine cellobiose labeled LDL in untreated catheterized rats, 5% of the injected radioactivity appeared in the bile during the first 3 hours. EE treatment increased this amount up to 25% of the injected radioactive LDL. The amount of radioactivity in bile was largely decreased by methylation of 125I-Tc-LDL. Because methylation of LDL blocks the recognition of this particle by its receptor, this observation indicated that the excretion of apo B (fragments) in the bile was coupled to recognition by the LDL receptor. Apo B fragments found in bile were still immunoreactive with the antibody and their molecular weight ranged from 120-200 kDa. This is considerably smaller than that of intact apo B (MW 650,000). The function of these fragments of apo B in bile is presently unclear.

The interaction of HDL with liver parenchymal, endothelial and Kupffer cells was characterized *in vivo* and *in vitro*. HDL was radioactively labeled in the apoprotein moiety and injected into rats. At 10 minutes after injection, the liver associated radioactivity was for 75% recovered with parenchymal cells and for 12% and 13% respectively with endothelial and Kupffer cells. To investigate whether this association was mediated by the HDL receptor, the recognition of HDL by its receptor was prevented by modifying HDL with tetranitromethane (TNM-HDL). Instead of the anticipated inhibition of uptake of the modified particle by the liver there was a rapidely increased uptake. It was shown that the liver endothelial cells participated strongly in the uptake of TNM-HDL. Competition experiments indicated that a very active so called scavenger receptor, which is present on liver endothelial cells, mediated the uptake of TNM-HDL.

Studies with freshly isolated liver parenchymal, endothelial and Kupffer cells, indicated that all three cell types expressed a specific high affinity binding site for HDL. It was demonstrated, that for all three cell types, these binding sites possessed similar properties. HDL is expected to function as an cholesterol acceptor from Kupffer and endothelial cells, while it should be capable of delivering cholesterol to parenchymal cells. From these considerations it is concluded that one type of HDL binding site may mediate both cholesterol efflux and influx. The direction of this flux is probably dependent on the cell type studied and also on the relative cholesterol load of the cell. The mechanism wich regulates of the direction of this flux is most likely to be beyond the initial binding of HDL to its binding site.

The characteristics of the HDL binding site were also investigated using human hepatocytes. Evidence was provided for a binding site on human hepatocytes with similar properties to those on rat hepatocytes. Using fluorescent DiI labeled HDL it was visualized that the interaction of HDL with the human liver cells was mainly at the cell membrane. Experiments with radioactive labeled HDL also supported the idea that HDL apoproteins were not actively internalized and degraded by the cells. It is therefore suggested that cholesterol (ester) uptake by hepatocytes occurs while the HDL particle re-

mains in the vicinity of the membrane.

The interaction of LDL with human liver cells was visualized by incorporating fluorescent DiI into LDL. In contrast to HDL, it was found that LDL was taken up in small vacuoles by human liver parenchymal and Kupffer cells. DiI-LDL became intensively associated with human Kupffer cells, while a variable association was noticed with parenchymal cells. This interaction of LDL with both cell types was specific for LDL and appeared to be mediated by the LDL receptor.

The presence of LDL receptors on human parenchymal and Kupffer cells was also indicated by immunostaining with an antibody directed to the LDL receptor. The extent of immunostaining was related to the extent of uptake of DiI-LDL by these cell types.

Modification of LDL by acetylation (AcLDL) resulted in a preferential uptake of AcLDL by human liver endothelial cells. This uptake was mediated by the scavenger receptor, since polyinosinic acid, a known inhibitor of the scavenger receptor, inhibited uptake.

It is concluded that in human liver, Kupffer cells express active LDL receptors, while the parenchymal cell receptor appear to be expressed in a more variable way. This may indicate an independant metabolic regulation of the parenchymal and Kupffer cell receptor. Human endothelial cells express active scavenger receptors, a property also exerted by rat liver endothelial cells.

In general it appears that the properties of lipoprotein interaction with human liver cells are comparable with those of rat liver cells. However, the interaction of LDL with parenchymal cells from normal rat liver is very low as compared with the human situation. The similarity between rat liver and human liver in terms of its interaction with lipoproteins may indicate that lipoprotein studies, initially performed in rats, may be extrapolated to humans.

SAMENVATTING

Lipoproteinen zijn bolvormige deeltjes die in het bloedplasma cholesterol(esters) en vetten transporteren. Lipoproteinen bezitten bovendien één of meerdere eiwitten (apo-proteinen). De vetachtige stoffen die door lipoproteinen getransporteerd kunnen worden zijn triglyceriden, cholesterol, cholesterolesters, fosfolipiden, vitaminen en andere vetoplosbare stoffen. Op grond van de relatieve hoeveelheden vet en eiwit (soortelijke dichtheid) kunnen verschillende typen lipoproteinen worden onderscheiden. De door de lipoproteinen getransporteerde vetachtige substanties worden in verschillende weefsels gebruikt voor de energievoorziening of voor het in stand houden van de integriteit van de celmembranen of voor synthese van hormonen.

Het merendeel (60-70%) van het cholesterol in het plasma wordt bij de mens vervoerd door het lage dichtheids lipoproteine (LDL). Dit deeltje voorziet de cellen dan ook voornamelijk van cholesterol. LDL wordt voor een groot deel door cellen in de lever opgenomen, dit orgaan vervult dan ook een belangrijke rol bij de regulatie van het LDL niveau in bloedplasma. Het hoge dichtheids lipoproteine (HDL) transporteert eveneens een groot deel van het plasma cholesterol (20-30%). Uit bevolkingsonderzoeken is gebleken dat het cholesterolgehalte in de LDL fractie sterk positief gecorreleerd is met het ontstaan van hart- en vaat ziekten, terwijl het cholesterolgehalte in HDL hiermee negatief correleert. Als mogelijke verklaring is geopperd dat LDL kan leiden tot lipidestapeling in de vaatwand, terwijl HDL een rol zou spelen bij het verwijderen van een teveel aan cholesterol uit weefsels. Volgens Glomset (1968) zou HDL dit teveel aan cholesterol aan de lever kunnen afgeven, een proces dat "omgekeerd cholesterol transport" genoemd wordt. De lever kan cholesterol in de gal uitscheiden, waarna het via de faeces het lichaam kan verlaten.

De lever speelt in vele opzichten een beslissende rol in de regulatie van lipoproteine niveau's in het bloed. Het is daarom belangrijk om het mechanisme te bestuderen waarmee de lever de verschillende lipoproteinen herkent en te bepalen welke cellen (leverparenchym-, endotheel- en Kupffercellen) bij de opname betrokken zijn en hoe vervolgens de verschillende lipoproteineonderdelen door de levercellen verwerkt worden. In dit proefschrift worden een aantal aspecten van de interakties van LDL en van HDL met de verschillende typen levercellen zowel biochemisch als morphologisch beschreven.

De opname van lipoproteinen door de verschillende levercellen werd lichtmicroscopisch zichtbaar gemaakt door de lipoproteinen te merken met een fluorescerend fosfolipide analogon: 1',1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchloraat (DiI).
Na injectie van fluorescerend DiI-LDL in ratten werden op verschillende tijdstippen de (betrokken) celtypen geidentificeerd die een interactie waren aangegaan met dit deeltje. Hiebij werden zowel onbehandelde als ethinyl estradiol (EE) behandelde ratten gebruikt. EE behandeling induceert LDL receptoren in de lever op parenchymcellen,

waardoor de intracellulaire afhandeling van het LDL deeltje beter geanalyseerd kan worden. In onbehandelde ratten bleek Dil-LDL zeer sterk door de Kupffer-cellen opgenomen te worden. De leverparenchymcellen van deze ratten namen weinig Dil-LDL op. Na EE behandeling was de opname van Dil-LDL specifiek in de parenchymcellen sterk verhoogd waarbij een intense Dil- fluorescentie in de gebieden rond de galkanaaltjes werd waargenomen.

De intracellulaire route van het LDL in de EE behandelde ratten is verder onderzocht met behulp van het electronenmicroscoop. Antilichamen tegen het apo B, het enige eiwit in het LDL deeltje, werden, in combinatie met electronendichte colloidale goud deeltjes, gebruikt om LDL in levercoupes aan te wijzen. Het bleek dat het LDL in eerste instantie hecht aan de celuitstulpingen aan de sinusoidale zijde van de parenchymcellen en daar door de cel wordt opgenomen in kleine blaasjes. Vervolgens kon het goudlabel (apo B-LDL) in structuren rond de galkanaaltjes nl; in kleine blaasjes en in zogenaamde "multivesicular bodies" worden aangetoond . Tevens werd goudlabel (apo B) in de galkanaaltjes aangetroffen.

Het verschijnen van apo B (fragmenten) in de gal werd gekwantificeerd in ratten waarbij permanente catheters waren aangebracht in de galgang, dunne darm en het hart. LDL werd voor deze toepassing radioactief gemerkt met ^{125}I-tyramine cellobiose (^{125}I-Tc-LDL). In de eerste drie uur na toediening in normale ratten werd slechts 5% van de toegediende radioaktiviteit in de gal aangetroffen. In EE behandelde ratten was dit percentage verhoogd tot 25%. De toename van radioactiviteit in gal werd geblokkeerd door modificering van aminozuren die van belang zijn voor de herkenning van LDL door de LDL receptor. Hieruit kan geconcludeerd worden dat de verschijning van apo B (fragmenten) in de gal gekoppeld is aan de herkenning van LDL door de LDL receptor. Immunoreactieve fragmenten van apo B die in de gal aangetroffen werden varieerden in grootte van 120 kDa to 200 kDA, hetgeen aanzienlijk kleiner is dan het intakte apo B (MG = 650 000). Over de functie van deze apo B fragmenten in de gal kunnen nog geen definitieve cinclusies getrokken worden.

De interactie van HDL met de verschillende cellen van de rattelever is onderzocht zowel *in vivo* als *in vitro*. Het HDL werd met ^{125}I radioaktief gemerkt in het eiwitgedeelte. Nadat 125I-HDL aan de ratten was toegediend, bleek dat binnen de lever, 75% geassocieerd was met de leverparenchymcellen, en respectievelijk 12% en 13% met de endotheel- en Kupffercellen. Teneinde te detecteren of deze associatie van HDL met de levercellen het gevolg was van een interaktie met een HDL receptor werden de aminozuren die betrokken zijn bij deze receptorherkenning gemodificeerd met tetranitromethaan (TNM-HDL). In plaats van de verwachte verlaging van de associatie van radioaktief gelabeld TNM-HDL met de lever werd een sterk verhoogde interaktie gevonden. De leverendotheelcellen bleken voornamelijk betrokken te zijn bij de verhoogde opname van TNM-HDL uit het bloedplasma. Met behulp van competitie experimenten

kon worden aangetoond dat een zogenaamde scavenger receptor, die aanwezig is op deze cellen, verantwoordlijk was voor de aktieve opname van TNM-HDL door de lever.

De herkenning van natief HDL door de verschillende cellen van de rattelever werd verder onderzocht *in vitro* met geisoleerde levercellen. De lever parenchym-, endotheel- en Kupffercellen bleken alle een specifiek bindingsplaats voor HDL te bezitten, waarbij geen verschillen in herkenningskarakteristieken van HDL werd aangetroffen. Omdat HDL, cholesterol (-esters) uit endotheel- en Kupffer cellen moet kunnen opnemen en vervolgens deze cholesterol (-esters) aan parenchymcellen moet kunnen afgeven, lijkt het waarschijnlijk dat één type HDL bindings plaats betrokken is bij zowel de opname als bij de afgifte van cholesterol. In hoeverre interaktie van HDL met de receptor leidt tot opname dan wel afgifte van cholesterol hangt dan af van het celtype en/of van het relatieve cholesterol (-ester) gehalte van de cellen.

De interaktie van HDL met menselijke leverparenchymcellen is eveneens onderzocht, teneinde te bepalen in hoeverre de eerdere bevindingen bij de rat ook gelden voor de mens. De interaktie van HDL met humane leverparenchymcellen vertoonde een specificiteit welke vergelijkbaar was met de eerder aangetroffen interaktie met ratteleverparenchymcellen. Tevens bleek dat de apoproteinen van het HDL niet of nauwelijks door de cellen werden opgenomen en afgebroken. Lichtmicroscopische experimenten met Dil-HDL lieten zien dat HDL zich voornamelijk associeerde met de celmembraan en niet in vacuoles werd aangetroffen. Hieruit kan worden afgeleid dat de afgifte van cholesterol(-esters) aan de leverparenchymcellen kennelijk kan geschieden onder condities waarbij het HDL deeltje aan het celoppervlak blijft.

Evenals met HDL werd ook de interactie van LDL met de humane levercellen werd zichtbaar gemaakt met behulp van Dil. In tegenstelling tot HDL werd Dil-LDL wel opgenomen door humane leverparenchymcellen, waarbij fluorescentie werd aangetroffen in vacuoles. Ook humane Kupffercellen namen Dil-LDL op. Bij alle bestudeerde levers werd een sterke interaktie van LDL met Kupffercellen waargenomen terwijl de interaktie met lever parenchymcellen sterk varieerde. Voor beide celtypen werd de opname gemedieerd door de LDL receptor. De aanwezigheid van LDL receptoren op beide celtypen werd verder bevestigd met behulp van specifieke antilichamen tegen de LDL receptor. De mate van de immuno-(LDL receptor)-kleuring aan de sinusoidale zijde van parenchymcellen in levercoupes van diverse donoren correleerde met de opname van Dil-LDL door levercellen afkomstig van dezelfde donoren.

Naast het bezit van een specifieke bindingsplaats voor HDL en receptoren voor LDL blijkt de humane lever ook geacetyleerd LDL (AcLDL) te kunnen opnemen. Net als in de rattelever werd AcLDL in de humane lever ook door de leverendotheelcellen opgenomen. Omdat de opname geremd werd door polyinosinezuur, een bekende remmer van opname via de scavenger receptor, kon geconcludeerd worden dat ook in humane lever een aktieve scavenger receptor aanwezig is op endotheelcellen.

Concluderend kan gesteld worden dat humane Kupffercellen een aktieve LDL receptor bezitten. De expressie van LDL receptoren op parenchymcellen lijkt variabel te zijn, hetgeen mogelijk samenhangt met individuele verschillen in metabole regulatie. De endotheelcellen van humane lever bezitten evenals rattelever endotheelcellen een *scavenger receptor*.

In het algemeen blijken er grote overeenkomsten te bestaan in de interakties van lipoproteinen met humane levercellen en die met cellen van rattelever. De mate van opname van LDL door de leverparenchymcellen van normale ratten is echter erg laag vergeleken met de humane situatie. De overeenkomsten tussen de ratte en de humane lever wijzen uit dat studies met betrekking tot het lipoproteine metabolisme die in eerste instantie in de rat zijn uitgevoerd waarschijnlijk geextrapoleerd kunnen worden naar de humane situatie.

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Curriculum Vitae

De schrijfster van dit proefschrift werd op 31 oktober 1957 geboren in Den Helder. Het atheneum-b diploma werd in 1978 behaald aan de scholengemeenschap Nieuwediep in Den Helder. In september van datzelfde jaar begon zij haar biologiestudie aan de Rijksuniversiteit Utrecht. In november 1981 werd het kandidaatsexamen afgelegd. De doctoraalfase, opgebouwd uit een hoofdvak electronenmicroscopische structuuranalyse en de bijvakken biochemie, neurobiologie en didaktiek van de biologie, werd afgesloten met het behalen van het diploma in augustus 1985. Op 1 september 1985 werd een aanvang gemaakt met het promotie onderzoek, bij het Instituut voor Experimentele Gerontologie, (IVEG) te Rijswijk in samenwerking met de sectie Biofarmacie (Centrum voor Biofarmaceutische Wetenschappen) van de Rijksuniversiteit Leiden onder leiding van Prof.Dr. D.L.Knook en Prof.Dr. Th.J.C. van Berkel.

LIST OF PUBLICATIONS

1. Nitrosylated high density lipoprotein is recognized by a scavenger receptor of rat liver. M.F. Kleinherenbrink-Stins, D.Schouten, J.vd Boom, A. Brouwer, D.L. Knook and Th.J.C. van Berkel. Journal of Lipid Research 30 , 511-520 (1989).
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10. Interaction *in vivo* and *in vitro* of apolipoprotein E free high density lipoprotein with parenchymal, endothelial and Kupffer cells from rat liver. D. Schouten, M.F. Kleinherenbrink-Stins, A. Brouwer, D.L. Knook and Th.J.C.v. Berkel. Biochemical Journal 256, 615-621 (1988).