Moniek Pieters

The interaction of high-density lipoproteins with the liver:

role in reverse cholesterol transport

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Stellingen

behorende bij het proefschrift van Moniek Pieters, getiteld: 'The interaction of high-density lipoproteins with the liver: role in reverse cholesterol transport'.

1. De selectieve afgifte van cholesterylesters van hoge-dichtheidslipoproteïnen aan de lever wordt niet beïnvloed door de relatieve hoeveelheid apolipoproteïne A-I en A-II.
   Dit proefschrift

2. In tegenstelling tot leverendotheelcellen zijn Kupffercellen in staat om het transport van cholesterol naar de parenchymcellen via een direkte route te laten verlopen.
   Dit proefschrift

3. De afwezigheid van een verhoogde incidentie van hart- en vaatziekten bij patiënten met een HDL-deficiëntie, kan wijzen op óf een efficiënt reverse cholesterol transport, óf de aanwezigheid van actieve, initiële cholesterolacceptoren.
   C.R. Castro & C.J. Fielding, Biochemistry 1988:27;25-30

4. Het gezegde 'hardlopers zijn doodlopers' is niet van toepassing als het gaat om het verhogen van het serum HDL-gehalte

5. Als alle Nederlanders zich chauvinistischer zouden opstellen ten aanzien van de aanschaf van nederlandse produkten, zou dit in het nederlandse bedrijfsleven een ware opleving veroorzaken.

6. Het verstrekken van dozen aan zwervenden in Rotterdam, is als het sturen van kauwgom naar de Sahel.

7. De kosten voor het uitschrijven van een standaard doktersrecept na telefonische aanvraag staan totaal niet in verhouding met de geleverde dienst (circa *f*35,- p. min.).

8. 'Als een samenleving ermee tevreden is, dat van de twee geslachten slechts één van de verworvenheden van de eeuw kan genieten, dan blijft het voor meer dan de helft een zwakke samenleving'
   Kemal Atatürk

9. Termen als afvalverbranding en waterzuivering wekken ten onrechte de suggestie dat afval kan verdwijnen.

The interaction of high-density lipoproteins with the liver:
role in reverse cholesterol transport

Proefschrift

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aan de Rijksuniversiteit te Leiden,
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Monique Nicole Pieters
geboren te Rotterdam in 1964

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CONTENTS

CHAPTER ONE

General introduction

1.1. Introduction

Atherosclerosis is characterized by an excessive deposition of cholesterol in the artery wall. The development of atherosclerotic plaques can finally result in the obstruction of the vascular sytem. Heart attacks and strokes are the clinical consequences of severe atherosclerosis. In the western society, coronary and peripheral vascular diseases are major death causes. An important risk factor for atherosclerosis is an elevated level of LDL cholesterol [1,2]. In contrast, elevated levels of HDL cholesterol are correlated with a decreased risk for coronary artery disease [3,4]. Studies on lipoprotein metabolism have led to an understanding of the mechanism of cholesterol transport and its potential relation with atherosclerosis. This chapter will give a brief overview of lipoprotein metabolism in general. A review on the role of HDL in reverse cholesterol transport will be provided in Chapter 2. Since in the scope of this thesis specifically the interactions of lipoproteins with the liver will be discussed, a short introduction on the liver structure and function will be given.

1.2. The liver

The liver plays a central role in the regulation of the metabolism of lipids, sugars, proteins and xenobiotic compounds. The liver is the major catabolic site for plasma lipoproteins and plays a key role in maintaining whole-body cholesterol homeostasis. Parenchymal cells (also denoted as hepatocytes) form the major class of cell types in the liver. In rats, they comprise 78% of the total liver volume while liver endothelial cells and Kupffer cells contribute 2.8% and 2.1%, respectively [5]. The contribution to total liver protein is 92.5 % for parenchymal cells, 3.3 % for endothelial cells and 2.2 % for Kupffer cells. Parenchymal cells are arranged in one layer cords, separated by the sinusoid and radiating from branches of portal veins to branches of the central veins. Parenchymal cells are separated from the sinusoid by the Space of Disse. The sinusoidal lining is formed by a single layer of endothelial cells. Kupffer cells, the liver macrophages, are located in the sinusoids whereas fat-storing cells (lipocytes) are present in the perisinusoidal spaces. The pronounced fenestration of the sinusoidal lining (diameter of fenestrae approx. 100 nm) and the absence of a basal membrane, facilitates the exchange between liver parenchymal cells and the blood compartment.

An important secretion product of liver parenchymal cells is bile. Bile is secreted into the bile canaliculi which are sealed from sinusoidal blood by tight junctions and run between adjacent parenchymal cells. The bile canaliculi form a continuous network which drain into the bile duct and finally in the duodenum. The main constituents of bile are bile acids, biliary lipids (mainly phospholipids and cholesterol) and various organic anions. In the intestine, bile acids play a role in the emulsification and absorption of lipids. The secretion of cholesterol into the bile either in its unesterified form or converted into bile acids, forms

the only quantitively important route by which the body can dispose of its excess of cholesterol. Bile acids are formed from cholesterol through a cascade of reactions in the cell, of which the 7α-hydroxylation of cholesterol on the endoplasmatic reticulum, is the rate-limiting step (for a review see [6]).

1.3. Lipoprotein metabolism

Cholesterol forms an essential element of cellular membranes and is therefore of vital importance for maintaining the organisms integrity. Cholesterol also functions as a precursor for the biosynthesis of steroid hormones, vitamins and bile acids. Cholesterol enters the body with the diet, or is synthesized de novo. In the blood, cholesterol is transported by plasma lipoproteins, water-soluble, high molecular weight particles consisting of lipids and apolipoproteins. The nonpolar constituents of the lipoprotein, cholesteryl esters and triacylglycerols, are situated in the core of the particle. Free cholesterol, apolipoproteins and a monolayer of phospholipids form the shell of the lipoprotein. Apolipoproteins play an important role in the receptor-mediated binding and endocytosis of lipoproteins, as a cofactor for enzymatic activities and in stabilizing the lipoprotein particle. In human plasma four major lipoprotein classes can be distinguished, according to their density as determined by ultracentrifugation. The four classes are: chylomicrons, very-low density lipoproteins (VLDL), low density lipoproteins (LDL) and high-density lipoproteins (HDL). The lipoprotein classes show differences in size, lipid and apolipoprotein composition (Table 1.).

Table 1. Physical properties and composition of human plasma lipoproteins

	Chylomicron	VLDL	LDL	HDL
density (g/ml)	< 0.96	0,96-1.006	1.019-1.063	1063-1.210
diameter (nm)	75-1200	30-80	19-25	5-12
protein	1-2	6-10	18-22	45-55
triacyglycerol	80-95	45-65	4-8	2-7
phospholipid	3-6	5-20	18-24	26-32
cholesteryl ester	2-4	6-22	45-50	5-20
free cholesterol	1-3	4-8	6-8	3-5
major apolipoproteins	A1,A4,B48,C1-3,E	B100,C1-3,E	B100	A1,A2,E

The values given for protein, triacylglycerol, phospholipid, cholesteryl ester and free cholesterol are expressed as percentage of total weight

Fig. 1 shows a schematic diagram of lipoprotein metabolism. Chylomicrons carry dietary lipids and are the biggest among the lipoproteins. They are synthesized in the intestine and contain primarily triacylglycerol. The majority of triacylglycerol is hydrolyzed to free fatty acids by lipoprotein lipase, an enzyme bound to the luminal surface of capillary endothelium [8,9]. After transferring surface components to nascent HDL particles the resulting particle, the chylomicron remnant, is relatively enriched in cholesterol and apoE [10] and has a mean diameter of 90-200 nm [11]. The chylomicron remnants are highly efficiently endo-

cytosed by the liver parenchymal cells through a receptor which is putatively called "the remnant receptor" (apoE-receptor). The liver assembles and secretes VLDL which contributes to the maintainance of whole-body energy balance (for a review see [12]). In the starved state, VLDL triacylglycerols serve as an important source for muscle-energy [13]. Like chylomicrons, VLDL particles undergo lipolysis. VLDL remnants are removed from the blood by the remnant receptor. VLDL is the precursor for LDL, the major cholesterol carrying lipoprotein class in man. In man, LDL carries two-third of the serum cholesterol and is removed from the plasma through apoB,E (LDL) receptors [1,2]. Modified LDL is removed from the circulation by scavenger-receptors which are mainly present on liver endothelial and Kupffer cells [14-16]. HDL is secreted as a nascent form by the liver and intestine. Maturation of the HDL particles occurs in the circulation in which HDL accepts surface components (phospholipids, cholesterol and apolipoproteins) of chylomicrons and VLDL.

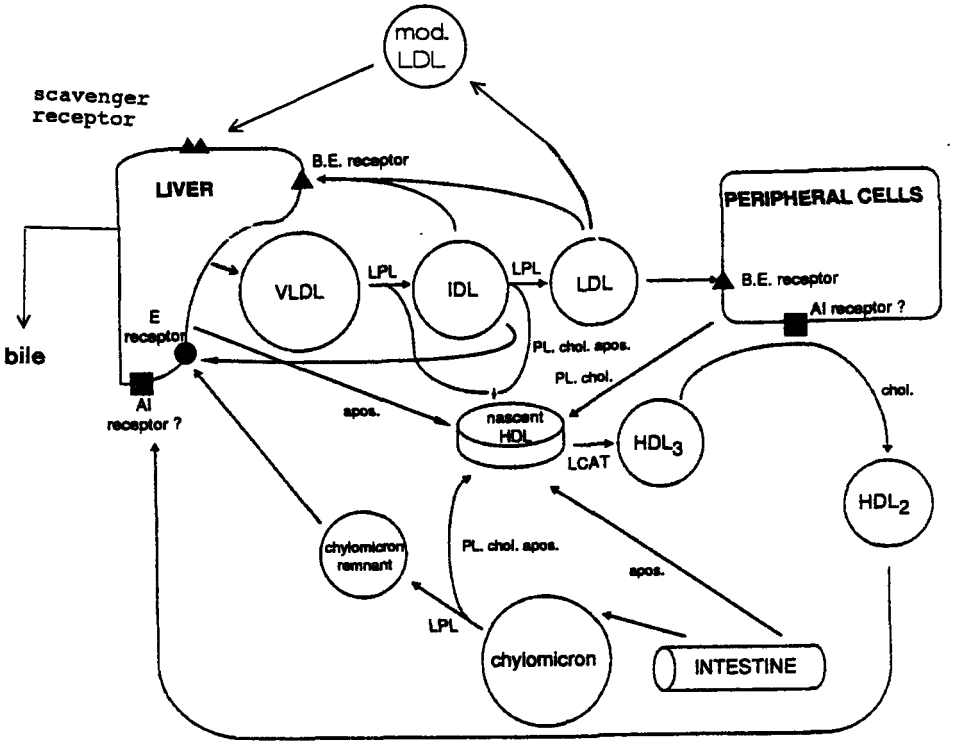

Fig. 1. Schematic representatation of lipoprotein metabolism (modification of fig. presented in [47]). See text for explanation of figure. LDL: low-density lipoproteins; HDL: high-density lipoproteins; VLDL: very-low-density lipoproteins: PL: phospholipids; chol: cholesterol; LPL:lipoprotein lipase; LCAT: lecithin-cholesterol acyltransferase; mod. LDL: modified LDL

The enzyme lecithin:cholesterol acyltransferase (LCAT) catalyzes the esterification of free cholesterol on HDL [see 17]. The function of HDL in transporting excess peripheral cholesterol to the liver is referred to as "reverse cholesterol transport" [18]. In the liver parenchymal cells, excess of cholesterol can be secreted into the bile. Reverse cholesterol transport is considered to be a protective mechanism against the formation of atherosclerotic plaques. In Chapter 2. the role of HDL in reverse cholesterol transport is reviewed.

1.4. Cholesterol metabolism in the cell

Cellular cholesterol homeostasis is a finely regulated balance between cholesterol synthesis, cholesterol uptake via lipoproteins and cholesterol efflux. The rate-limiting enzyme in cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is localized on the endoplasmic reticulum [19]. Cells aquire cholesterol via the receptor-mediated internalization of LDL. Increased levels of cellular cholesterol down-regulate the LDL-receptor and suppress HMG-CoA reductase activity [2]. Furthermore, the activity of acylCoA:cholesterol acyltransferase (ACAT), which esterifies cholesterol, is stimulated [20]. In case of liver parenchymal cells cholesterol can leave the cells via the bile as unesterified cholesterol or as bile acids. Apart from steroid hormone producing tissues, peripheral cells cannot degrade their excess of cholesterol. In order to prevent accumulation of cholesterol (esters) in these cells, HDL accept cholesterol from peripheral tissues. Epidemiological studies have shown that high levels of HDL-cholesterol are inversely correlated with the incidence of atherosclerosis (see Chapter 2).

1.5. Modified LDL and cholesteryl ester accumulation

In the onset of atherosclerosis, macrophage-derived "foam cells" are formed in the artery wall. These foam cells exhibit an extensive accumulation of cholesteryl esters. An elevated level of LDL-cholesterol is a major risk factor for coronary artery disease. However, as mentioned above, peripheral cells effectively down-regulate the LDL-receptor when the cellular cholesterol level rises. Even at very high concentrations of LDL, cholesterol ester accumulation does not occur [20,21]. In 1979, Goldstein and Brown proposed that modification of LDL was a prerequisite for excessive macrophage uptake [20]. Besides the LDL-receptor another receptor is present on macrophages, designated "the scavenger receptor", which is not down-regulated by an increased cholesterol content of the cell [21]. In early studies it was shown that LDL, chemically modified by acetylation, was rapidly taken up by macrophages [20]. Later studies revealed that not only acetylation evoked an enhanced macrophage uptake. Modification by malondialdehyde (MDA) [22], 4-hydroxynonenal [23], heavy metals [24] or lipoxygenase [25], all enhanced the uptake by macrophages. Also exposure to cultured endothelial cells [26, 27] or smooth muscle cells [28] led to the modification of LDL. Cell cultures oxidize LDL by generating lipoperoxides, probably via the action of cellular 15-lipoxygenase [29,30]. Reactive intermediates of lipid degradation such as aldehydes and ketones form complexes with apoB [31]. Due to the derivatization of lysine residues, modified LDL looses its affinity for the LDL-receptor. It has been reported that a cluster of negative charges on the ligand is necessary for recognition by the scavenger receptor [32,33]. However, it has also been suggested that certain epitopes of the apoprotein, exposed by the oxidative modification, are recognized by the scavenger receptor [34]. For a

review on the free radical mediated modification of LDL see [35].

Evidence that Ox-LDL is a pathophysiological, atherogenic lipoprotein, is derived from the reaction of specific antibodies directed to Ox-LDL with the atherosclerotic plaque [36]. The presence of endogenous antioxidants (vitamin E, β-carotene and ubiquinol-10) in LDL protect the lipoprotein against oxidation [37,38]. The capacity of probucol to decrease atherosclerotic plaques in animal models may be related to its antioxidant properties [39-41]. Also the lipophilic anti-oxidant butylated hydroxytoluene has been shown to prevent the oxidation of LDL [42]. Epidemiological studies have shown that high levels of ascorbic acid and α-tocopherol in plasma are related to a lower incidence of coronary artery disease [43]. The fatty acid composition of LDL may also influence the susceptibility of LDL for oxidation. It has been reported that LDL rich in oleic acid is highly resistent to oxidative modification [44]. Though oxidation of LDL probably occurs in the intima, it is likely that some oxidized LDL will leak out of injured tissue. Also mildly (minimally) oxidized LDL may be formed in the plasma. Antibodies against Ox-LDL have been shown to be present in the human and rabbit plasma [35]. Atherosclerotic patients exhibit higher titers of antibodies against oxidized LDL than do "healthy" persons [45,46]. The rapid removal of modified lipoproteins from the plasma [14-16], may form an effective protection system against the presence of atherogenic particles in the blood.

1.6 Scope of this thesis

This thesis focuses on the role of HDL in reverse cholesterol transport. Though the concept of reverse cholesterol transport is generally accepted, in vivo evidence was limited. Mechanisms for cholesterol efflux from cultured peripheral cells to HDL had been proposed (see Chapter 2), but only a few data were available for the transport of HDL cholesterol to the liver in vivo. In this thesis, the efficiency of delivery of HDL cholesterol (esters) to the liver has been studied. Considerable attention has been given to the kinetics of biliary secretion, since this is the only route the liver can use for irreversible disposal of cholesterol either in its unesterified form or as bile acids. Moreover, an in vivo reverse cholesterol transport system has been used in order to study cholesterol transport from Kupffer or endothelial cells to parenchymal cells and bile. The mechanism by which the transport of cholesterol from non-parenchymal cells to parenchymal cells is achieved, has been further studied in an ex vivo perfused rat liver system and by electron microscopical autoradiography.

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CHAPTER TWO

In vitro and in vivo evidence for the role of HDL in reverse cholesterol transport

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Contents

2.1 In vitro evidence for cholesterol transfer from cells to high-density lipoproteins

a. Introduction

High-density lipoproteins (HDL) play an important role in maintaining whole body cholesterol homeostasis. The inverse correlation of HDL cholesterol levels with the incidence and prevalence of coronary artery disease (1) can be explained by the role of HDL in reverse cholesterol transport, a term originally introduced by Glomset in 1968 (2). Reverse cholesterol transport identifies a series of metabolic events resulting in the transport of peripheral cholesterol to the liver (Fig. 1). The removal of peripheral free cholesterol by HDL is followed by the esterification of free cholesterol by the enzyme lecithin:cholesterol acyltransferase (LCAT) which utilizes apoA-I present on HDL as a cofactor. By converting cholesterol to its insoluble esterified form, LCAT forms the driving force for a concentration gradient from the plasmamembrane to HDL. The newly formed cholesteryl esters can be transferred from HDL to other lipoproteins by the cholesteryl ester transfer protein (CETP) and delivered to the liver for secretion in the bile either by HDL itself or via VLDL or LDL.

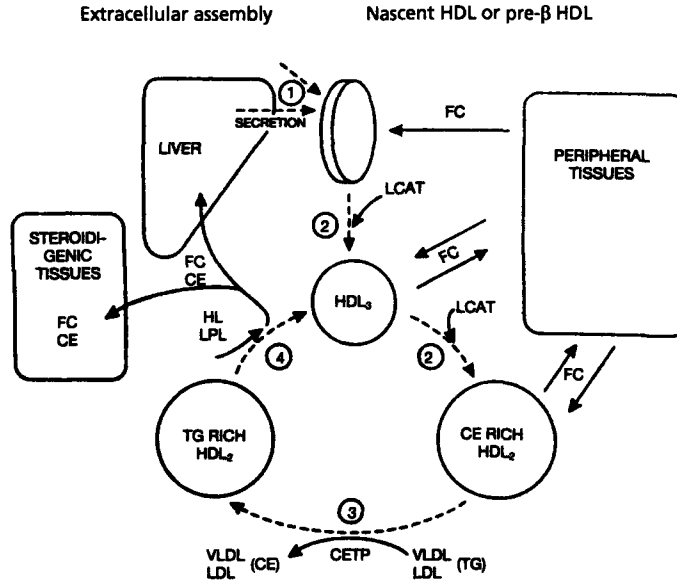

Fig. 1. HDL metabolism and cholesterol transfer. The major steps of HDL metabolism are indicated by the dotted arrows. The transfer of cholesterol by HDL and tissues are indicated by the solid arrows. (1) Nascent HDL is produced by secretion from the liver and intestine or by extracellular assembly in the interstitial fluid. (2) Sequential maturation of nascent HDL into HDL_3 and HDL_2 through the esterification of HDL free cholesterol(FC) by LCAT. (3) Exchange of cholesteryl esters (CE) from HDL with triglyceride (TG) from VLDL and LDL, resulting in the formation of TG-rich HDL_2. (4) TG-rich HDL_2 particles serve as substrate for hepatic lipase and/or lipoprotein lipase (LPL). Derived from Johnson et al. [7].

Though the concept of reverse cholesterol transport is generally accepted, the exact mechanism by which reverse cholesterol transport is regulated, is still not elucidated. Also, in vivo evidence for reverse cholesterol transport remains scarce. In this article, the interactions of HDL with cells and the in vivo evidence for reverse cholesterol transport are reviewed.

b. The mechanism of HDL-mediated cholesterol efflux from cells: current hypothesis

Cell cholesterol homeostasis is a balance between cholesterol synthesis, cholesterol uptake from lipoproteins and cholesterol efflux. The first step in reverse cholesterol transport is the efflux of cholesterol from peripheral cells. Different mechanisms for cellular cholesterol efflux has been proposed. The *first* mechanism considers bidirectional diffusion of cholesterol between the plasmamembrane and the acceptor (3-5). Unesterified cholesterol may be lost from the cell by aqueous diffusion and taken up by HDL (6, for a review see 7). The rate limiting step is the desorption of cholesterol from the plasmamembrane. The diffusion process of cholesterol may be regulated by the size and composition of the accepting HDL particle. The smaller the HDL particle, the higher its diffusion coefficient when compared to the unstirred water layer of the cell surface (3).

A *second* mechanism for cholesterol efflux is proposed by Oram, Bierman and colleagues. Peripheral cell cholesterol may be taken up by HDL via a HDL receptor mediated process. Binding of HDL to its binding protein stimulates translocation of intracellular sterol to the plasmamembrane where it can be removed by HDL (8,9). This stimulation appeared to be initiated by the activation of protein kinase C (8,10).

The *third* mechanism considers internalization and resecretion of HDL (11-15). In this

model, HDL is internalized and extralysosomally processed. During this process HDL may accept cholesterol from the cell (i.e. with cholesterol-loaded macrophages) and is subsequently resecreted.

c. HDL as cholesterol acceptor: role of subfractions

HDL consists of a very heterogenous population of particles which differ in size, density, composition and electrophoretic mobility (16,17). By using ultracentrifugal techniques, HDL can be divided in two major classes: HDL_2 with a density range of 1.063-1.125 g/ml, which is more lipid rich than HDL_3, $1.125 < d < 1.21$, which is consequently more protein rich. Quantitatively, HDL_3 comprises the most important part of the HDL fraction (about 75% (18)). In humans most of the variation in HDL levels is exerted in the HDL_2 fraction. In vitro HDL3 is more efficient in promoting cholesterol efflux from cells than HDL_2 (19). Since both HDL_2 and HDL_3 are still heterogenous in apolipoprotein composition, much attention has been paid to HDL subfractions isolated with immunoaffinity chromatography. The protein components of HDL include apoA-I (70%) apoA-II (20%) and small amounts of apoE and apoC. Based on apolipoprotein composition, the HDL population can be further subdivided into two major fractions: lipoproteins containing apoAI and no Apo-AII (LpA-I) and lipoproteins containing both apoA-I and apoA-II (LpA-I/A-II) (16,20). Clinical studies showed that low levels of HDL in coronary artery disease correlated with low LpA-I levels, while LpA-I/A-II levels were not different (21). Immunopurified subfractions of HDL as well as artificial particles (proteoliposomes) have been used in a number of studies by several investigators in order to study the role of the apolipoprotein composition on cholesterol efflux from peripheral cells. It has been reported that proteoliposomes containing apoA-I are more efficient acceptors of cellular cholesterol than proteoliposomes containing other apolipoproteins (22,23). With immunopurified HDL subfractions, it was shown that LpA-I, LpA-IV and LpA-I/A-IV particles decreased mouse adipocyte cholesterol levels, whereas LpA-I/A-II and LpA-II particles had no effect (20,24,25). These results however, may be restricted to lipid-loaded adipocytes, since no difference in cholesterol efflux could be detected when other cell types were used (26).

Besides apolipoprotein composition, the size and lipid composition of the HDL particles may influence its capacity to accept cellular cholesterol. Only minor subspecies of HDL may initially accept cholesterol from cells (27,28). Castro and Fielding (29) proposed that lipoprotein particles with pre-β mobility, containing only apoA-I as apolipoprotein, were the principle acceptors of free cholesterol from the plasma membrane (see Fig. 2). The conformation of the apolipoprotein in pre-β migrating HDL is different from that in α-HDL (28). Experiments with [^{3}H]cholesterol labelled fibroblasts showed that after 1 min of incubation of cells with normolipaemic serum, pre-β migrating particles with an apparant molecular weight of 70 kD (pre-β_1-particles) had a 30-fold higher specific activity than the bulk (96%) of α-migrating HDL particles (29). At 2 min of incubation, a shift of radioactivity to larger pre-β_2 particles and α-migrating HDL occurred (29). The pre-β_2 particles originated from pre-β_1 particles and resembled the discoidal HDL present in LCAT-deficient patients (30). With LCAT-deficient patients, high concentrations of pre-β HDL could be detected (31). The conversion of small pre-β_1 particles into larger pre-β_2 particles has been proposed to be caused by a transforming factor (30) which has been detected in human plasma (32). Fielding and coworkers also detected a pre-β_3 particle, containing LCAT,

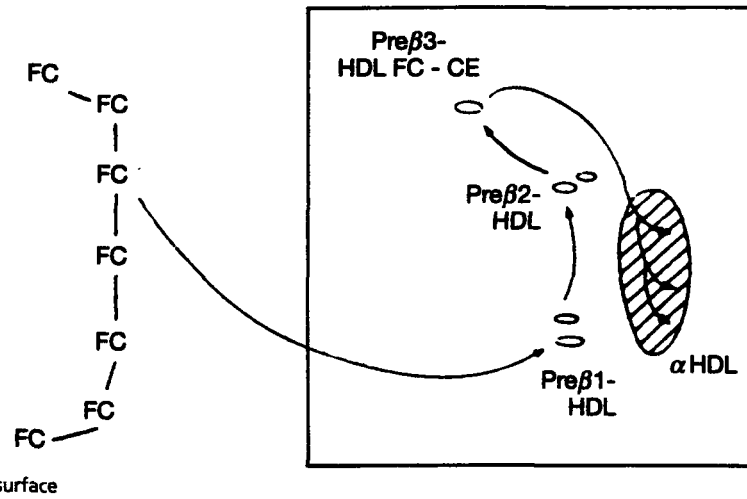

Fig. 2. The formation of a HDL particle with pre-β mobility from cell-derived cholesterol in human plasma. [^{3}H]cholesterol-labelled fibroblasts were incubated with human plasma. After 1 min of incubation, pre-β1 particles could be detected with two-dimensional electrophoresis, which were subsequently converted into pre-β_2 and pre-β_3 particles. Pre-β_3 particles were then transformed to α-HDL. From Francone, Gurakar and Fielding [33].

CETP, apoAI and apoD. It is thus suggested that the small pre-β_1 particles are the principal cholesterol acceptors and the larger pre-β_3 particles are the site of esterification (33). The conversion of pre-β HDL upon incubation with plasma has also been reported by other groups (34-36). The movement of pre-β HDL to α-HDL was shown to be linked to LCAT-activity, since inhibitors of LCAT blocked the disappearance of pre-β migrating particles. Interestingly, Kunitake et al. (36) showed that pre-β HDL can be formed by incubation of α-HDL with CETP and LDL, suggesting that the transfer of cholesteryl esters from HDL to LDL resulted in a smaller pre-β particle. Increased concentrations of apoA-I in the pre-β fraction could be detected in plasma of patients with several classes of hyperlipidemia and familial hypercholesterolemia (27). In plasma of patients with very low HDL levels, hardly no pre-β migrating particles could be detected (28). The concentration of pre-β migrating HDL, rather than total HDL, may be rate-limiting for reverse cholesterol transport.

Studies with peripheral lymph partly supported the role of pre-β migrating particles in reverse cholesterol transport (37,38). Peripheral cells including foam cells in atheromatous lesions are primarily exposed to lipoproteins in the interstitial fluid rather than plasma. The distribution of particle size of apoA-I containing lipoproteins in lymph differs from that in plasma (see 39). Peripheral lymph contains a considerable amount of discoidal HDL particles ("nascent" HDL), which are rich in free cholesterol and phospholipids (40). Also free apolipoproteins (A-I, A-II, A-IV and E) are present in the peripheral lymph (40-42). Hara and Yokoyama (37) showed that free apoA-I, apoA-II and apoE were able to remove unesterified cholesterol from cholesterol-loaded mouse peritoneal macrophages, whereas apoC-III had no effect. ApoA-I, A-II and E can form small and stable disc-like particles with phospholipids (43,44), while apoCIII forms incomplete ellipsoid micellar complexes (45). The products formed with apoA-I and apoA-II migrated slower on agarose electrophoresis

(pre-β to α_2 mobility) (37). The lipids in the formed pre-β HDL-like particles could be transferred to model lipoproteins during incubation at 37°C. It is suggested that pre-β HDL-like particles are generated by the interaction of apolipoproteins with the cell membrane (38). The low LCAT activities present in peripheral lymph suggest that the esterification reaction occurs only after the HDL particles have entered the blood stream (39). In the blood stream, conversion of pre-β particles into α-HDL may occur. Recently, it has been reported that the association of cellular free cholesterol with particles with a pre-β mobility is followed by a rapid redistribution of cholesterol to α-HDL and LDL (46). In this way, LDL was suggested to function as a temporary store of cellular cholesterol. Subsequently, the cholesterol could be either removed from the circulation by the rapid uptake of LDL in the liver, or redelivered to HDL for esterification.

d. HDL as cholesterol donor: role of subfractions

In the concept of reverse cholesterol transport, HDL particles remove cholesterol from peripheral cells and convert free cholesterol into its esterified form by the action of the enzyme lecithin:cholesterolacyl transferase. Subsequently, cholesterol (esters) are delivered to the liver. In the body the liver and steroid hormone producing organs, are quantitatively the only important tissues that can metabolize free cholesterol. In the liver this can lead to the secretion of cholesterol into the bile either as bile acids or as free cholesterol. Three routes are available for the hepatic uptake of HDL cholesterol (esters). First: the particulate uptake of HDL (11-15), second: the selective delivery of HDL cholesteryl esters (47-57) and third: the transfer of HDL cholesteryl esters to apoB,E containing lipoproteins (58,59) which are then taken up by apoB,E recognizing receptors (60). Liver parenchymal cells may also directly take up apoE-rich HDL (61). In humans the transfer of cholesteryl esters from HDL to lipoproteins of lower density is quantitatively the most important route. In mice and rats no active CETP is present (62) and hepatic uptake of HDL cholesteryl esters is determined by the selective uptake pathway. Selective uptake of HDL cholesteryl esters without the parallel uptake of the apoprotein moiety, has been reported in a variety of cell types in vivo and in vitro (47-57). Selective delivery of HDL cholesteryl esters does not require metabolic energy (53). Recently (57), it has been reported that cholesteryl esters which are selectively delivered to $HepG_2$ cells can be partly removed by exposure to unlabelled HDL(cholesteryl esters). The authors suggest that two pools of cholesteryl esters may exist: one which is reversible and one which is inaccessable. The reversible cholesteryl ester pool in the membrane may be an initial uptake site before the irreversible internalization of cholesteryl esters occurs (57).

In vivo the liver was found to be the main site of uptake of HDL-derived cholesteryl esters. However, the most active organs in cholesteryl ester uptake/mg wet weight of tissue were the adrenals and ovary. Cellular depletion of cholesterol increased selective uptake (63-65) while cholesterol loading of fibroblasts led to a down-regulation of the selective uptake of cholesteryl esters (63). We established that in the liver, the selective uptake of cholesteryl esters is restricted to the liver parenchymal cells (56). In the parenchymal cells cholesterol (esters) are converted into bile acids and secreted into the bile. Parenchymal cells utilize HDL derived cholesteryl esters more efficiently for bile acid synthesis than LDL cholesteryl esters (56).

The role of apolipoproteins in the selective delivery of HDL cholesteryl esters is relatively

unexplored. Proteoliposomes containing apoA-I, A-II or C-III are all known to bind to hepatocytes and liver membranes. With immunopurified HDL, the LpA-I fraction showed a higher binding to pig liver membranes than LpA-I/A-II (62). In a study with $HepG_2$ cells LpA-I binding was similar to the binding of LpA-I/A-II. LpA-I did have a higher selective delivery of cholesteryl esters to the cells, though differences were found to be rather small 67). Recently however, it has been reported that selective delivery of cholesteryl esters is independent of HDL-binding to the isolated plasmamembranes of hepatocytes and HepG2 cells (68). Since inhibition of HDL-binding to the membranes did not affect the selective delivery of cholesteryl esters, it was suggested that distinct membrane sites are involved in HDL-binding and cholesteryl ester delivery.

We have shown recently that in vivo in rats no difference existed between the selective delivery of cholesteryl esters from [^{3}H]cholesteryl ester labelled LpA-I and LpA-I/A-II (69). The further processing of LpA-I cholesteryl esters in the liver parenchymal cells to bile acids however, occurred at a higher rate than for LpA-I/A-II cholesteryl esters. This observation indicates that though apolipoprotein composition does not influence selective delivery of cholesteryl esters to the cells, it does influence cholesteryl ester processing and may therefore influence the efficiency of reverse cholesterol transport.

2.2 The role of HDL binding sites in cholesterol transfer

The aqueous diffusion model as proposed by Phillips et al. (3) denies a role of HDL binding in cellular cholesterol efflux. In the signal transduction pathway (Oram and coworkers (8,9)), binding of HDL is the key event in the stimulation of cholesterol transport from intracellular stores to the plasmamembrane. HDL high affinity binding sites have been reported on a number of cell types (70-77). The HDL binding appeared to be specific for HDL, saturable, reversible, Ca^{2+}-independent and pronase insensitive (75-77). Differences exist between the binding of HDL_2 and HDL_3 (78). The binding was thought to be mediated by apoA-I and apoA-II (20,54,73,77,79,80) or apoA-IV (81-82). The binding of HDL is reported to be upregulated after cholesterol loading of the cells (83). However, using cholesterol-loaded fibroblasts, Mendel and Kunitake (84) found that despite a higher HDL binding to the cells, cholesterol efflux was not greater than in presence of other cholesterol acceptors (albumin or phospholipid vesicles).

Though stated here as two distinct mechanisms, it is possible that both mechanisms play an active role in the process of reverse cholesterol transport. In a combined model, the binding of HDL to peripheral cells and the subsequent induction of a second messenger pathway, stimulates the transfer of intracellular cholesterol to the plasmamembrane, possibly in specific domains. The cholesterol-enriched plasmamembrane then looses its unesterified cholesterol through aqueous diffusion to HDL (if available) or any other cholesterol acceptor present. The two proposed mechanisms, HDL binding versus aqueous diffusion, may therefore be additional to each other rather than exclusive.

Since HDL binding sites are reported to be present on a numerous cell types, several attempts have been made to identify and isolate a receptor specific for HDL. Binding proteins mainly identified in blotting experiments and recognizing apoA-I and HDL, have

been detected in various tissues. A HDL-binding protein with an apparent molecular weight of 78-80 kD has been identified by Western blot analysis in sheep adrenocortical membrane fractions (85), rat liver and rat kidney (86), $HepG_2$ cells (87) and human placental membranes (88). It has been reported that on liver two HDL binding proteins are present (89-91). In porcine liver, a 90 kD-binding protein was found to be localized in hepatocytes, whereas a 180 kD-protein could be detected on the lining of sinusoids (89). Tozuka and Fidge identified two different HDL binding proteins on rat liver membranes, designated HB_1 and HB_2 with molecular weights of 100 and 120 kD respectively (91). The binding of iodinated HDL was decreased after treatment of the rats with simvastatin plus cholestyramine (92). In a study with antibodies raised against regions of apoA-I, it was suggested that a specific region of the carboxyl-terminus of apoA-I was responsible for the binding of HDL_3 to cells (93).

In a number of peripheral cell types, Oram and coworkers (94) demonstrated the presence of a HDL binding protein with an apparent molecular weight of 110 kD and a minor binding protein of 130 kD. Incubation of cells with cholesterol led to increased binding of iodinated HDL to a trypsin sensitive binding site. Also inhibition of cell proliferation by adding interferon resulted in an upregulation of the expression of the binding protein (95). Recently, Oram et al. reported the cloning of a HDL-binding protein (HBP) with a molecular weight of 150 kD (96). The HBP was suggested to undergo cell-specific processing which generates several peptides that differ in mass and relative HDL binding properties. The authors suggest that the processing and the instability of HBP may have given rise to the observation that the HDL binding protein has apparent molecular weights ranging from 60 to 130 kD. The membrane-binding sites for HDL with molecular masses of less than 10 kD (73) or approx. 16 kD (98) as detected with radiation inactivation correspond in size with monomers or dimers of the HBP repeat units. The proposed HBP is very different from the structure of a classical receptor. The protein lacks a hydrophobic membrane spanning domain and no defined extracellular and cytoplasmic regions can be indicated. The binding protein may actually represent a signal transducing receptor instead of a receptor mediating the cellular uptake of HDL.

2.3 In vivo evidence for reverse cholesterol transport

Though the concept of reverse cholesterol transport is generally accepted, in vivo data supporting the role of HDL in cholesterol transport are relatively scarce (99). Studies in humans or intact animals are very complex and difficult to interpret. Studies in patients with HDL-metabolism related disorders, show conflicting effects of HDL cholesterol levels on atherosclerosis (see chapter 4). Experimental evidence, supporting the beneficial role of HDL in atherosclerosis has been obtained with cholesterol-fed rabbits. Weekly injections of HDL induced regression of fatty streak lesions (100,101). With transgenic mice, the introduction of a human apoA-I gene in mice resulted in elevated levels of plasma apoA-I, due to the expression of the human gene. The high levels of apoA-I were found to be associated with high levels of HDL (102,103) and led to the presence of HDL_{2b} and HDL_{3a}. The FCR of apoA-I was high (104). The variation in gene loci affecting the apoA-I expression and HDL concentration, have been found to have a major protective effect on diet-induced atherosclerosis in these mice (105). Recently, it has been reported that transgenic mice

expressing both human A-I and A-II are not protected, supporting that apoA-I particles are specifically anti-atherogenic (106).

Since the liver is the major cholesterol processing organ, the secretion of cholesterol in the bile in its unesterified form or processed into bile acids is an important route for the body to dispose of excess cholesterol. In man, it has been shown that free cholesterol from HDL is the major substrate for biliary cholesterol (107). In rats, we have shown that liver parenchymal cells secrete bile acids derived from HDL cholesteryl esters at a higher rate than LDL derived cholesteryl esters (56). These studies all indicate that HDL cholesterol (esters) are a preferential substrate for bile acid synthesis and secretion.

To study the mechanism of reverse cholesterol transport, a perfused rat spleen in conjunction with the perfused liver has been used as a semi in vivo model (108,109). In this model, the spleen is labelled in vivo with ^{3}H-cholesterol by injecting radiolabeled erythrocytes. Perfusion with whole blood showed that 51% of the resecreted radioactivity was associated with lipoproteins, the remainder being associated with erythrocytes. The highest specific radioactivity however, was present in the serum HDL fraction, in which re-esterification of the free cholesterol occurred (108). When the whole blood perfusate of the spleen was used to perfuse an isolated liver, the liver took up radioactivity from both serum lipoproteins and erythrocytes and secreted radiolabelled bile acids (109).

In our group we used an in vivo model for studying reverse cholesterol transport. In this model liver endothelial cells were rapidly labelled with [^{3}H]cholesteryl esters by injecting labelled acetylated LDL. The hydrolysis of cholesteryl esters and subsequent transport of [^{3}H]cholesterol through the serum compartment to the liver parenchymal cells and secretion into the bile were followed (110). By using unrestrained bile-cannulated rats the time-dependent appearance of radiolabelled cholesterol and bile acids can be quantified (see Fig. 3). When liver endothelial cells were loaded with ^{3}H-cholesteryl esters by injecting radiolabelled acetylated LDL, we found that resecreted radiolabelled free cholesterol used HDL as a transport vehicle. The specific activity of radiolabelled cholesterol was found to be the highest in the HDL fraction (Fig. 4). In the serum [^{3}H]cholesterol was converted into cholesteryl esters which is consistent with the action of LCAT. Serum lowering of the HDL levels by pretreatment of the rats with ethinyl estradiol resulted in a less efficient transport of radiolabeled bile acids to the parenchymal cells and bile, thus supporting in vivo the essential role of HDL in mediating reverse cholesterol transport. Recently, we have shown that apolipoprotein composition of the HDL particles influences the efficiency of reverse cholesterol transport (69). Cholesterol esters transported by HDL particles containing apoA-I without apoA-II were more efficiently secreted into the bile as bile acids than cholesterol esters transported by HDL containing both apoA-I and apoA-II (Fig.5).

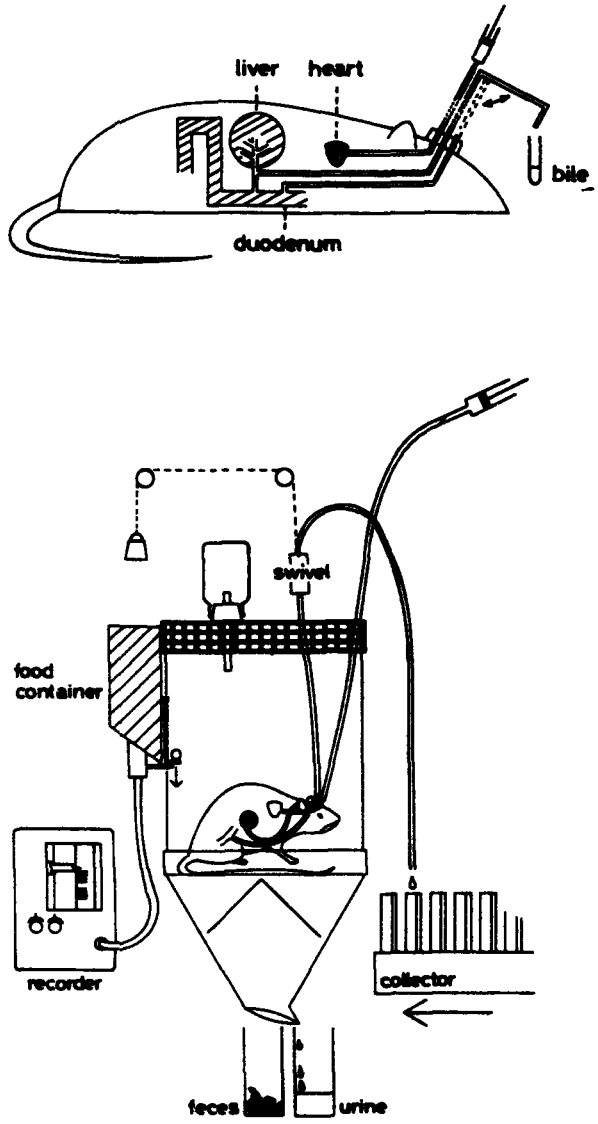

Fig. 3. (a) Schematic diagram of a rat cannulated in the bile duct, duodenum and the heart. (b). The sampling of bile from an unrestrained rat. From Kuipers et al. (127).

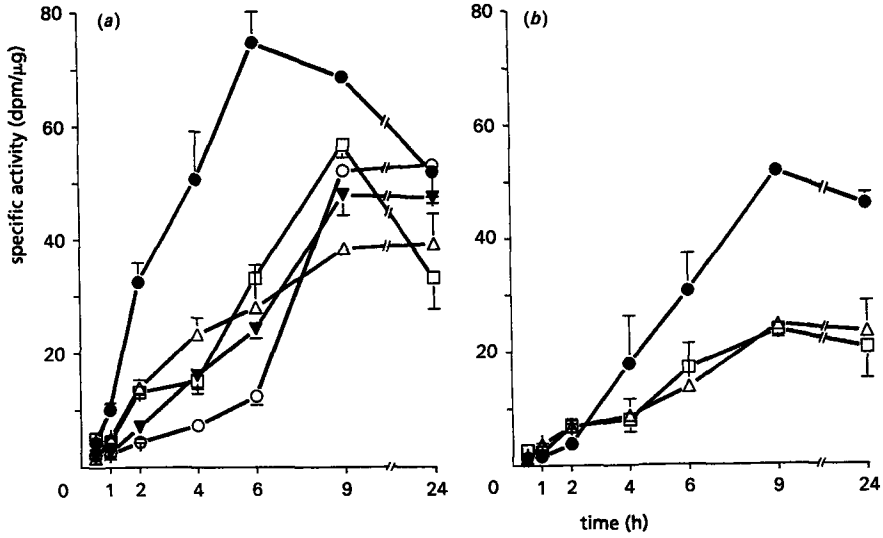

Fig. 4. Specific activities of [^{3}H]cholesterol (a) and [H]cholesteryl ester (b) in the different fractions of the blood after injection of [^{3}H]cholesteryl oleate-labelled LDL in rats. At different time points after injection of [3H]cholesteryl oleate labelled acetyl LDL in rats, the serum was subjected to density ultracentrifugation and the various lipoprotein fractions were isolated. Values are expressed as counts of free or esterified cholesterol in the VHDL (○), HDL (●), LDL (□) and VLDL (△) fractions of the serum, and in the erythrocytes (▼). It can be concluded that the highest specific activities are associated with the HDL fraction. From Bakkeren et al. (110)

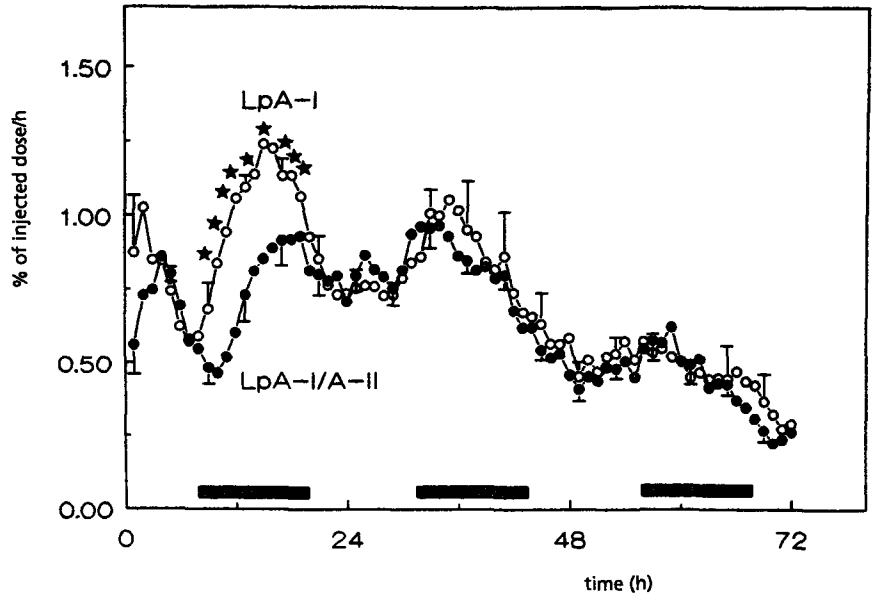

Fig. 5. Biliary secretion of H-radioactivity during 72 h after injection of [^{3}H]cholesteryl oleate labelled LpA-I (○) or LpA-I/A-II (●). Though the selective delivery of ^{3}H-cholesteryl esters to the liver was similar for LpA-I and LpA-I/A-II, the biliary secretion of radiolabelled bile acids derived from [^{3}H]cholesteryl oleate labelled LpA-I was higher. From Pieters et al. (69).

2.4 Clinical relevance of reverse cholesterol transport

Epidemiological studies have shown that HDL cholesterol levels are inversely correlated with the incidence of atherosclerosis (1). The HDL cholesterol level is an important predictor of risk in patients with coronary artery disease (CAD) (111). Lowered HDL levels in patients suffering from CAD were linked to low LpA-I levels, whereas LpA-I/A-II levels were not changed (21,112,113). In humans apoA-I present on LpA-I is faster metabolized than on apoA-I on LpA-I/A-II (114). The increased cardiovascular risk associated with low HDL levels has also been reported in individuals with mutations that cause HDL deficiency syndromes. HDL deficiency due to synthetic defects of apolipoproteins (apoA-I and apoA-I/C-II deficiencies), is associated with premature atherosclerosis (115). Hypercatabolism of HDL however, has a much lower risk for atherosclerosis. Further support that HDL act as a protective agent can be derived from individuals having high levels of HDL, due to a genetic defect in CETP, and a reduced risk for atherosclerosis (116).

On the other hand however, besides for patients with complete absence of apoA-I, none of the many abnormalities in HDL metabolism is associated with severe atherosclerosis. In case of Tangier disease, familial LCAT-deficiency and fish-eye disease, no evidence for premature atherosclerosis has been reported (117,118). In mutant chickens with a HDL deficiency syndrome, no correlation could be observed between low levels of HDL and the susceptibility to either diet-induced or spontaneous atherosclerosis (119). It is difficult to reconcile the low levels of HDL found in Tangier disease and other HDL-related disorders with the low or not apparent increase in susceptability for atherosclerosis. It may therefore be concluded that low HDL cholesterol levels are not atherogenic, per sé. A decrease in HDL cholesterol levels could be caused by an increased turnover of HDL, corresponding with a highly efficient reverse cholesterol transport.

It is however not excluded that the beneficial effects of HDL to prevent atherosclerosis reside in factors which are not related to reverse cholesterol transport. HDL and apoA-I have been reported to prevent the aggregation of LDL (120). LDL readily forms aggregates with itself when subjected to brief vortexing (121) or treatment with Phospholipase C (122). These aggregates are taken up via LDL-receptor mediated phagocytosis by macrophages, resulting in the formation of cholesterol-loaded foam cells. An other possibility is that HDL prevent oxidative modification of LDL (123,124) and inhibit LDL-induced cytotoxicity (125,126). High levels of HDL may also reflect an increased lipoprotein lipase activity. These HDL-linked effects may, in addition to the role of HDL in reverse cholesterol transport, contribute to the beneficial effect of high levels of HDL cholesterol.

2.5 Conclusive remarks

In this article the role of HDL in reverse cholesterol transport is discussed. Many investigators focused on the first step of reverse cholesterol transport: the role of HDL as cholesterol accepting particle. Binding of HDL to membranes does enhance intracellular cholesterol transport (8,9), while also evidence has been provided that peripheral cholesterol leaves the cell by diffusion through the aqueous water layer (3-5). Since the majority of peripheral cells is exposed to the interstitial fluid rather than to the blood compartment, experiments concerning the cholesterol efflux from cells in medium corresponding to the interstitional

fluid are very relevant. The concept that only a small fraction of HDL (pre-β particles) is involved in the initial uptake of cholesterol (29-38), may explain the controversial observations concerning HDL cholesterol levels and atherosclerosis (Chapter 4). The fact that pre-β particles are small enough to pass through the sieving of the interstitial wall, inforces the concept of pre-β particles being the primary cholesterol acceptors. Concerning the role of HDL as cholesterol (ester) donating particle, it is now known that HDL selectively delivers its cholesteryl esters to the liver and other tissues (47-57). Within the liver only parenchymal cells which convert cholesterol into bile acids, exert selective uptake (56). Evidence for in vivo reverse cholesterol transport is still limited. The obtained results however, do sustain the overall view of the ability of HDL in transporting cholesterol in the body. HDL do pick up peripheral cholesterol and deliver cholesterol (esters) to the liver (110). The efficient secretion of bile acids derived from HDL cholesterol esters (56) confirms the supposed essential role of HDL in reverse cholesterol transport.

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CHAPTER THREE

Selective uptake of cholesteryl esters from apolipoprotein-E-free high-density lipoproteins by rat liver parenchymal cells in vivo is efficiently coupled to bile acid synthesis

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Summary

[^{3}H]cholesteryl ester labelled human high density lipoprotein (HDL) was injected into rats and its decay, intrahepatic cellular distribution and the kinetics of biliary secretion were determined. At 10 min after injection the hepatic uptake of cholesteryl esters from HDL was 3-fold higher as compared to the apolipoprotein. Selective uptake was exerted only by parenchymal cells (5-6 more cholesteryl esters than apolipoprotein) and not by liver endothelial or Kupffer cells. The kinetics of biliary secretion of processed cholesteryl esters initially associated with HDL or low density lipoprotein (LDL) were compared in unrestrained rats, equipped with permanent catheters in bile duct, duodenum and heart. At 72 hours after injection of [^{3}H]cholesteryl oleate labelled HDL 51.0 ± 2.5% of the injected dose was recovered as bile acids which is about twice as high as the secretion of biliary radioactivity after injection of [^{3}H]cholesteryl oleate labelled LDL. Estradiol treatment stimulated only liver uptake of LDL cholesteryl esters and resulted in a 2-fold higher liver uptake as with HDL. However, the rate of radioactive bile acid formation from [^{3}H]cholesteryl oleate labelled HDL was still more rapid than for LDL. It is concluded that the selective uptake pathway for cholesteryl esters from HDL in parenchymal cells is more efficiently coupled to the formation of bile acids than the cholesteryl ester uptake from LDL. This efficient coupling may facilitate the role of HDL in reverse cholesterol transport.

Introduction

The inverse correlation between plasma high density lipoprotein (HDL) cholesterol levels and the incidence and prevalence of atherosclerosis has led to the suggestion that HDL plays a protective role against atherosclerosis. According to the generally accepted concept of Glomset [1] HDL takes up cholesterol from peripheral cells and delivers it to the liver. The biliary secretion of cholesterol, either in unchanged form or as bile acids is the major route in the body for eliminating cholesterol from the circulation. Cholesterol and cholesteryl esters are delivered to the liver by various lipoproteins, and the influence of lipoproteins on bile acid biosynthesis and secretion have been studied in vitro as well as in vivo.

In isolated rat hepatocytes very-low density lipoproteins (VLDL) and chylomicron remnants [2,3] but also apolipoprotein (apo) E-rich rat HDL [4,5] have been reported to increase bile acid synthesis. Addition of apo E-free HDL had no effect on bile acid synthesis. The uptake of apo E-rich HDL cholesterol was probably mediated by the remnant or LDL receptor, since methylation of HDL abolished its capacity for stimulating bile acid synthesis [5]. In a recent study with rat hepatocytes Junker & Davis [6] demonstrated that both apo E-free LDL and apo E-rich HDL were able to stimulate bile acid synthesis to the same extent. Binding of apo E-free LDL could be competed for by apo E-rich HDL, and vice versa, thus suggesting a LDL-receptor-mediated pathway. Both LDL and apo E-containing HDL also stimulated bile acid synthesis in cultured rabbit hepatocytes [7]. Cholesteryl esters rather than unesterified cholesterol of apo E-containing HDL were metabolized into bile acids by chicken embryo hepatocytes [8].

Studies in vivo in humans, in which radiolabelled HDL and LDL were administered to subjects with a bile fistula, showed that free cholesterol from HDL is the major substrate for biliary cholesterol [9]. Recently it has been reported that, in rats, free cholesterol from HDL_2 rather than from HDL_3 is preferentially utilized for bile acid synthesis [10]. However, interpretation of these in vivo studies is hampered by the fact that unesterified cholesterol readily exchanges between lipoproteins or membrane surfaces. In the present study we have labelled HDL in the cholesteryl ester moiety and studied the kinetics of liver uptake and biliary secretion in the rat. The rat does not possess an active cholesteryl ester transfer protein [11], and therefore the label will remain associated with the HDL particle in the circulation. Furthermore, we have administered relative low amounts of labelled lipoproteins so that no mass changes in bile acid synthesis were induced. The data are compared with the characteristics of LDL uptake and bile acid formation.

Materials and methods

Isolation and labelling of HDL.

Human HDL was isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave et al. [12]. HDL ($1.063 < d < 1.21$) was dialyzed against 10.1 mM phosphate-buffered saline (PBS)/1 mM EDTA pH 7.4 and labelled with [^{3}H]-cholesteryl oleoyl ether or [^{3}H]cholesteryl oleate (Amersham Corp., Arlington Heights, IL, U.S.A.) by exchange from donor particles. The method, as specified below, forms a modification of the procedure originally described by Pittman et al. [13].

Donor [^{3}H] particles.

Egg yolk phosphatidylcholine (Fluka, Buchs, Switzerland) and cholesteryl oleate (BDH Chemicals, Poole, Dorset, U.K.) were added in chloroform/methanol (1:1, v/v) in a mass ratio of 60:1 to a 25 ml glass scintillation vial and dried under a stream of N_2. Then, 250 μCi of cholesteryl[1α,2α(N)-^{3}H]oleate or cholesteryl[1α,2α(N)-^{3}H]oleoyl ether (Amersham) was then added to the mixture, dried under N_2 and placed overnight in a vacuum desiccator to remove residual solvent. Then 7.5 ml of a 0.1 M KCl/10 mM Tris/1mM EDTA/0.025% NaN_3 buffer, pH 8.0 was added, flushed with N_2 and sonicated with a MSE Soniprep 150 for 40 minutes, amplitude 12 microns, temperature 52 °C under

a constant stream of N_2. The microemulsion was adjusted to a density of 1.21 g/ml and subsequently subjected to density ultracentrifugation (Redgrave et al. [12]) in order to isolate donor particles with a density of approximately 1.03 g/ml. Phospholipid content of the particles was measured by an enzymatic colorimetric assay (Phospholipids kit, Boehringer, Mannheim, Germany).

Labelling of HDL.

2 ml of human lipoprotein deficient serum, extensively dialyzed against 10.1 mM PBS/1 mM EDTA was added to human HDL (1 ml, containing approx. 3.5 mg protein) and donor particles. The mass ratio of HDL protein and donor particle phospholipids was 8:1. The mixture was then incubated for 5 hours at 37 °C in a shaking water bath under N_2. To separate HDL from the donor particles the density of the mixture was raised to 1.21 g/ml with KBr and subjected to differential ultracentrifugation. After reisolation the radiolabelled HDL was dialyzed and passed through a heparin Sepharose affinity column in order to remove apo E [14]. The apo E free fraction was checked on the presence of apo E and albumin by SDS/polyacrylamide gelelectrophoresis with 5-22.5% acrylamide gels, followed by Coomassie Blue staining. Almost all of the radioactivity could be recovered in the cholesteryl ester moiety of HDL as determined by Bligh and Dyer extraction [19] and thin-layer chromatography. $1.0 \pm 0.1\%$ and $2.0 \pm 0.1\%$ were recovered as ^{3}H-unesterified cholesterol in case of [^{3}H]cholesteryl oleoyl ether and [^{3}H]cholesteryl oleate labelling respectively. [^{3}H]cholesteryl ester or -ether labelled HDL was checked for phospholipid, cholesterol and cholesteryl ester content (phospholipids kit and CHOD-PAP kit, Boehringer, Mannheim, Germany), density and electrophoretic α-mobility. Iodinated [^{3}H] cholesteryl ester or -ether HDL had the same plasma decay kinetics and liver association as reported previously [15]. HDL was iodinated by the ICl method of McFarlane [16] as modified by Bilheimer et al. [17]. In negatively stained preparations the labelled HDL had the same appearance as its unlabelled counterpart.

Oestradiol treatment

When indicated, rats received for 3 successive days a subcutaneous injection of 17α-ethinyl oestradiol (Sigma, St.Louis USA) in propyleneglycol at a dose of 5 mg/kg body wt.

Serum decay in vivo and liver association.

Rats were anaesthetized by intraperitoneal injection of Nembutal (1 ml/kg bodywt). After the abdomen was opened, radiolabelled HDL was injected into the vena penis. Blood sampling and liver lobule excising was performed as described previously [18].

Tissue distribution.

Rats were anaesthetized with ether and radiolabelled HDL was administered via the vena penis. After 4 or 24 hours rats were sacrificed, individual tissues weighed and samples combusted in a Packard Sample Oxidizer 306. The samples were counted for radioactivity and corrected for the contribution of the serum to the total counts as described earlier [18].

Cell isolation.

The hepatic cellular distribution was studied by using a low temperature cell isolation technique as described earlier [18]. After injection of radiolabelled HDL in the vena penis, the

vena porta was cannulated at the indicated time and the liver was perfused with oxygenated Hanks' buffer, containing Hepes (1.6 g/l), pH 7.4 at 8 °C. To determine total liver uptake, a lobule was tied off at 8 min after starting the perfusion. The perfusion was continued for 20 min with Hanks'/Hepes buffer containing 0.05% (w/v) collagenase and 1 mM $CaCl_2$. Parenchymal cells were isolated by mincing the liver in Hanks' buffer with 0.3% BSA, filtering it through a nylon gauze and centrifuged four times 30 s at 50 g. The pellet consisted of pure parenchymal cells as judged by light microscopical examination. The non-parenchymal cells present in the supernatants were centrifuged for 10 min at 400 g and stored on ice. The remainder on the nylon gauze was incubated with Hanks'/Hepes buffer containing 0.3% BSA and 0.25% pronase for 10 min at 8 °C. The cell suspension was centrifuged at 400 g and the pellet combined with the stored non-parenchymal cell pellet. By means of centrifugal elutriation the non-parenchymal cells were subdivided into an endothelial cell fraction and a Kupffer cell fraction [19]. The purity of each cell fraction was checked by light microscopy after staining for peroxidase activity and samples were counted for radioactivity.

Bile sampling.

Bile was collected from unrestrained 3 month old male Wistar rats as reported previously [20]. Rats received tap water and standard chow ad libitum. Rats were equipped with permanent catheters in the bile duct, the duodenum and the heart. In order to maintain an intact enterohepatic circulation, the bile duct and duodenum catheters were connected immediately after surgery. Rats were allowed to recover for 1 week. Lipoproteins were introduced via the heart catheter. The bile duct catheter was then connected to a fraction collector and bile was collected for 72 h. Bile samples were counted for radioactivity after combustion in a Packard sample oxidizer 306.

In order to separate bile acids from cholesterol and cholesteryl esters, samples were extracted according to Bligh & Dyer [21]. The aqueous layer containing bile acids was counted for radioactivity. Cholesterol and cholesteryl esters were separated by thin layer chromatography with as developing solvent heptane/diethylether/acetic acid (60:40:1). Spots were visualized by colouring with I_2, scraped off and counted for radioactivity.

Protein determination.

Protein was determined according to Lowry et al. [22] with BSA as standard.

Results

Serum decay and liver association.

Fig. 1 depicts the serum decay of iodinated HDL and [^{3}H]cholesteryl ester or [^{3}H]cholesteryl oleoyl ether labelled HDL. The cholesteryl ester or cholesteryl ether labelled moieties of HDL both follow a similar monoexponential decay and are cleared much faster than the iodinated apolipoproteins. These findings are in accordance with earlier published data [23-25] and are indicative of a similar selective removal of HDL cholesteryl esters or ethers from the circulation.

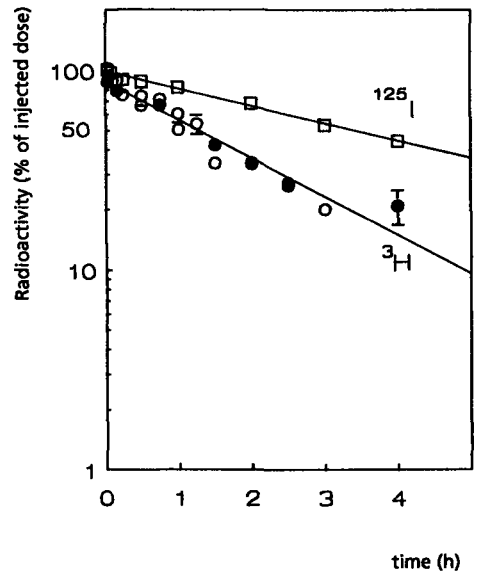

Fig. 1. Serum decay of apoE-free [^{3}H]cholesteryl oleate-labelled HDL and apoE-free ^{125}I-apoprotein labelled HDL. ApoE-free [^{3}H]cholesteryl oleoyl ether (●) or oleate (○) labelled HDL (250 000 dpm, 125 μg), or ^{125}I - HDL (□) (25 x 10^6 cpm, 100 μg) was injected into the vena penis of anaesthetized rats. At the indicated time points serum values were obtained, and the data were expressed as percentages of injected dose.

Tissue distribution

Tissue distribution studies at 24 hours after injection of [^{3}H]cholesteryl ether labelled HDL (Fig. 2a) indicate that the liver is the major site of cholesteryl ether accumulation. At the same time point the liver association with [^{3}H]cholesteryl ester labelled HDL is significantly lower and accompanied by an increase of radioactivity in the small and large intestines (Fig. 2b). The relative importance of the liver as the initial site of uptake is however evident at 4 hours after injection (Fig. 2c). These data are consistent with a primary hydrolysis of cholesteryl esters in the liver which is necessary for the subsequent secretion in the intestine, presumably as radiolabelled bile. Total liver association calculated as the sum of liver, small intestine and large intestine associated radioactivity was identical for [^{3}H]cholesteryl ester (57.8 ± 7.6%) and [^{3}H]cholesteryl ether (62.5 ± 3.5%) labelled HDL.

Intrahepatic cellular distribution of [^{3}H]cholesteryl ester HDL.

To identify the intrahepatic uptake sites for [^{3}H]cholesteryl ester labelled HDL, the various liver cell types, parenchymal, Kupffer and liver endothelial cells were isolated at 10 min after injection. With total liver, the association of the cholesteryl ester moiety of HDL was 3-fold higher than the apolipoprotein moiety (Fig. 3). The uptake of HDL cholesteryl esters in parenchymal cells was 5-6 times higher than the apolipoprotein uptake while no significant difference between HDL cholesteryl esters and apolipoprotein was observed with liver endothelial and Kupffer cells. The relative contribution of each liver cell type to the total uptake of the liver can be calculated by using the specific uptake/mg cell protein and taking into account their relative contribution to total liver protein [26] (Table 1).

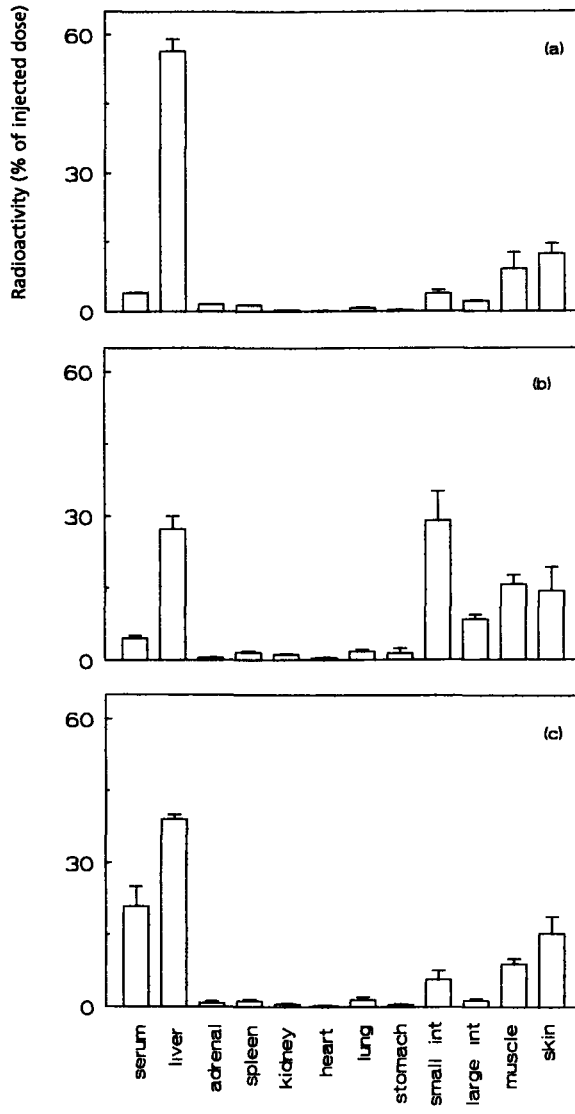

Fig.2 Recovery sites of ^{3}H-radioactivity at 24 h after injection of [^{3}H]cholesteryl oleoyl ether labelled HDL (a) and at 4h (c) and 24 h (b) after injection of [^{3}H]cholesteryl oleate labelled HDL.
Radiolabelled HDL (500 000 dpm, 250 μg) was injected into control rats. At the indicated time, rats were killed and tissues weighed and sampled. Tissue samples were corrected for serum contribtion. Data are expressed as percentage of injected dose (n=3 ± S.E.M.)

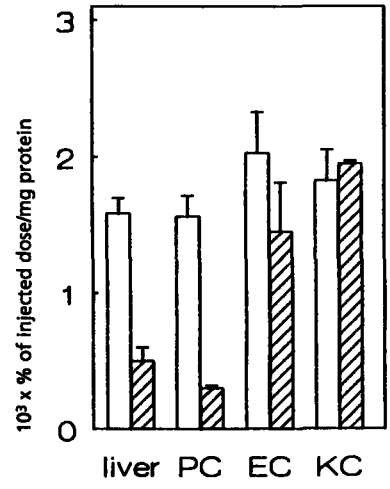

Fig. 3. In vivo distribution of apo E-free [³H]cholesteryl ester labelled HDL and ¹²⁵I-HDL between parenchymal, liver endothelial and Kupffer cells, 10 min after injection. At 10 min after administration of [³H]cholesteryl ester labelled HDL the liver was perfused and parenchymal cells (PC), endothelial cells (EC) and Kupffer cells (KC) were isolated with collagenase and pronase at 8°C. Values are expressed as % of ID x 10^3/mg cell protein for [³H]HDL (open bars) (2 x 10^6 dpm, 1000 μg) or ¹²⁵I-HDL (shaded bars) (10 x 10^6 cpm, 40 μg) [15].

Table 1. Relative contributions of parenchymal, liver endothelial and Kupffer cells to the total uptake of apoE-free [³H]HDL and apoE-free ¹²⁵I-HDL by the rat liver. Relative contributions of parenchymal, liver endothelial and Kupffer cells to the total uptake of [³H]HDL (2x10^6 dpm, 1000 μg) and ¹²⁵I-HDL (10 x10^6 dpm, 40 μg) [15] were calculated by taking into account the contribution of each liver cell type to the total of liver protein, which are 92.5%, 3.3% and 2.5% for parenchymal, liver endothelial ad Kupffer cells respectively. Values are means ± S.E.M of three experiments.

	Percentage of total liver associated radioactivity	
Cell type	[³H]HDL	¹²⁵I-HDL
Parenchymal	90.8±0.7	77.8±2.4
Endothelial	4.4±0.3	10.8±0.8
Kupffer	5.2±0.8	11.3±1.7

The great majority of the cholesteryl esters from HDL appears to be directed to parenchymal cells. The total contribution by liver endothelial cells and Kupffer cells is relatively low and significantly less than for the apoprotein moiety as a consequence of the absence of preferential cholesteryl ester uptake in these cell types. At 10 min after injection no difference in cellular uptake was observed between [^{3}H]cholesteryl oleate labelled or [^{3}H]cholesteryl oleoyl ether labelled HDL (results not shown).

Biliary secretion of [³H] radioactivity in control rats.

In order to investigate the kinetics of the secretion of initial HDL associated cholesteryl esters into bile, rats were equipped with permanent catheters in bile duct, duodenum and

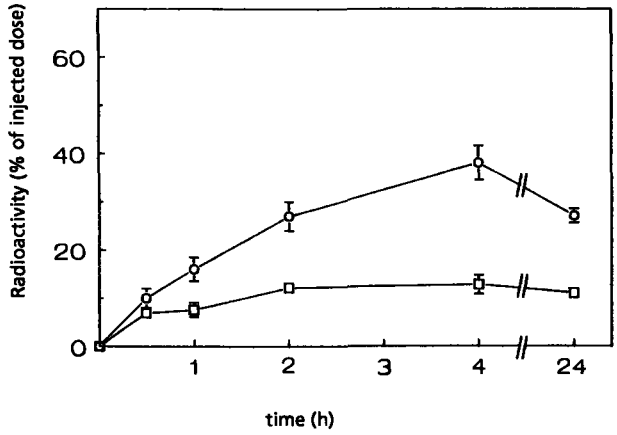

Fig. 4. Liver association of [^{3}H]cholesteryl ester HDL and [^{3}H]cholesteryl ester labelled LDL in control rats. Liver association of [^{3}H]cholesteryl oleate HDL (○) (250,000 dpm, 125 μg) and [^{3}H] cholesteryl oleate LDL (□) (800,000 dpm, 50 μg) [28] was determined in control rats at the indicated time points. Values are corrected for serum contribution and are the mean of 3 experiments ± S.E.M.

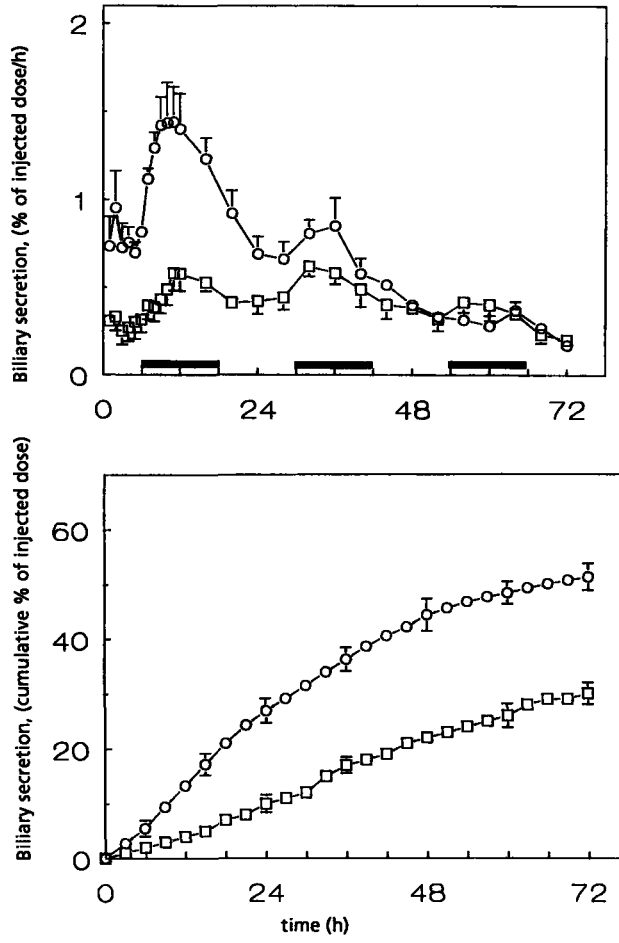

Fig. 5. Biliary secretion of ^{3}H-radioactivity after administration of [^{3}H]cholesteryl oleate HDL (○) or [^{3}H]cholesteryl oleate LDL (□) in control rats. Values are expressed as percentage of injected dose per hour (Fig. 5A) and as cumulative percentage of injected dose (Fig. 5B).
Bile was collected for 72 h in time-intervals of 1 h after injection of [^{3}H]cholesteryl oleate HDL (500,000 dpm, 250 μg) in unrestrained control rats (○) and compared to values of [3H]cholesteryl oleate labelled LDL (8×10^6 dpm, 500 μg) (□) [28,29]. Values represent the mean of 4 experiments ± S.E.M. Closed horizontal bars indicate the dark periods. For the clarity of fig. 5A, data points between 12-72 h after injection are only presented every 4th h.

heart. The liver uptake and kinetics of appearance of biliary radioactivity were compared with data obtained for human LDL. In rats, human LDL has the same metabolic behaviour as homologous LDL with respect to catabolic sites and rate of liver uptake [27] which validates the use the human LDL in the rat. The liver association of [^{3}H]cholesteryl oleate labelled HDL as compared to [^{3}H]cholesteryl oleate labelled LDL in control rats is higher (at 4 h after injection 3-fold higher) (Fig. 4). At 24 hours after injection the liver association of [^{3}H]HDL radioactivity is decreased.

The secretion of biliary radioactivity after the administration of radiolabelled lipoprotein in the heart catheter of the rat expressed as percentage of injected dose/h, is relatively constant during the first 6 hours (Fig. 5a). A marked increase in secretion rate is noticed during the dark period. During the first 24 hours after lipoprotein injection the rate of appearance of radioactivity originating from HDL is about 2-fold higher as with LDL. The day/night variation is also evident with LDL. The higher rate of radioactivity secretion leads to a higher cumulative recovery of radioactivity in bile from [^{3}H]cholesteryl ester labelled HDL when compared to LDL (Fig. 5b). At 72 hours after injection of [^{3}H]HDL, 51% of the injected dose was secreted in the bile, whereas with [^{3}H]LDL 30% of the radioactivity was recovered in the bile [28,29]. Bile analysis revealed that both for LDL and HDL the radioactivity in the bile was mainly present in the form of bile acids (94.2 ± 3.3%).

In agreement with the low amount of radioactivity recovered in the intestine after administration of [^{3}H]cholesteryl ether labelled HDL (Fig. 2a), radioactivity from [^{3}H]cholesteryl oleoyl ether labelled HDL appeared in the bile at a slow rate. At 24 hours only 4.3% of the injected dose was recovered in the bile whereas with [^{3}H]cholesteryl oleate labelled HDL biliary secretion reaches 26.5% of injected dose (Fig. 6). With the rats injected with cholesteryl oleoyl ether-labelled HDL, the radioactivity in bile were mainly be present as unchanged cholesteryl ethers and no hydrolysed products or bile acids were found.

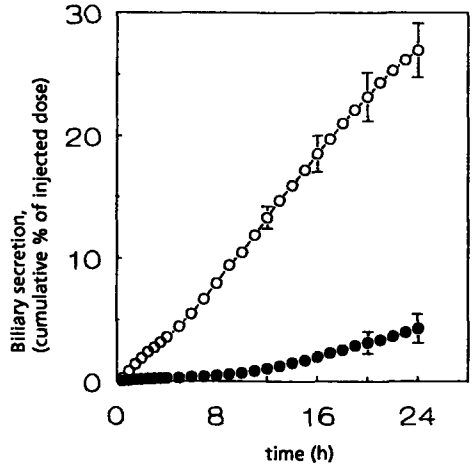

Fig. 6. Comparison of the biliary secretion of [^{3}H]radioactivity after administration of [^{3}H]cholesteryl oleate labelled HDL (○) or [^{3}H]cholesteryl oleyl ether labelled HDL (●). Radiolabelled HDL (500,000 dpm, 250 μg) was injected intracardially to unrestrained catheterized rats. Bile was collected immediately after injection. Secretion is cumulatively expressed as percentage of the injected dose. Values represent the mean of 4 experiments ± S.E.M.

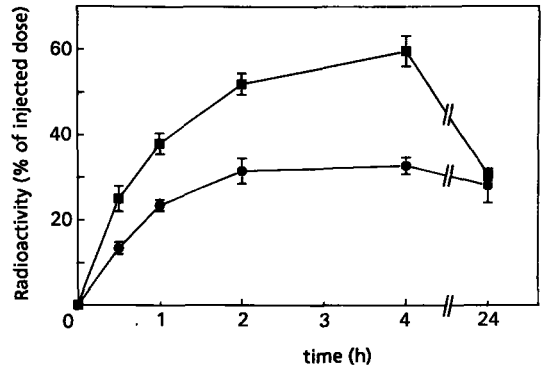

Fig. 7. Liver association of [^{3}H]cholesteryl ester labelled HDL and [^{3}H]cholesteryl ester labelled LDL in estradiol treated rats. Liver association of [^{3}H]cholesteryl oleate HDL (●) (250,000 dpm, 125 μg) or [^{3}H] cholesteryl oleate LDL (■) (800,000 dpm, 125 μg) [28] was determined in estradiol treated rats. Values are corrected for serum contribution and are the mean of 3 experiments ± S.E.M.

Biliary secretion of [^{3}H]radioactivity in oestradiol treated rats.

In earlier studies [28,29] we have shown that initial uptake of LDL in Kupffer cells in the rat is not efficiently coupled to bile acid synthesis and secretion. The major part of [^{3}H]cholesteryl ester labelled HDL is however directed to parenchymal cells (Table 1). In order to allow a comparison of the rate of processing of cholesteryl esters derived from LDL and HDL in parenchymal cells we have treated rats with ethinyl oestradiol. Oestradiol treatment selectively induces apo B,E receptors on parenchymal cells [30]. With [^{3}H]cholesteryl ester labelled LDL we have shown earlier that oestradiol treatment enhances specifically LDL cholesteryl ester uptake in parenchymal cells and that the processing of radiolabelled cholesteryl esters into bile acids is then coupled very efficiently to liver uptake [28]. In Fig. 7 the liver association of [^{3}H]HDL and [^{3}H]LDL in oestradiol treated rats is compared. Oestradiol treatment strongly stimulates the liver association of LDL, whereas liver association of radioactivity from [^{3}H]HDL is hardly affected. The elevated liver association of LDL is accompanied by a higher rate of biliary [^{3}H] secretion (Fig. 8a). When [^{3}H]HDL is injected in oestradiol treated rats an initial high secretion rate is observed. The latter cannot be explained by an increased association of HDL with the liver, since serum decay and liver association of [^{3}H]HDL and ^{125}I-HDL (results not shown) hardly changed in oestradiol treated rats. The cumulative appearance of radioactivity in the bile is greatly enhanced for LDL in estrogen treated rats (55% at 72 hours after injection versus 30% in control rats, Fig. 8b). However, it appears that although the liver association of [^{3}H]HDL is about half of that of [^{3}H]LDL in oestradiol treated rats, biliary secretion of radioactivity from [3H]HDL still occurs at a higher initial rate than with LDL (Fig. 8a).

Discussion

Selective uptake of HDL cholesteryl esters without the parallel uptake of its apoprotein moiety has been reported in a variety of cell types in vivo and in vitro [13,24,31]. Selective uptake of HDL cholesteryl esters has been shown to occur in hepatic [13,23-25,32,33], extrahepatic [34] and steroid producing tissues [23,24,31,35] and could be down-regulated by cholesterol loading of the cell [34,36]. The liver is the major site of cholesteryl ester

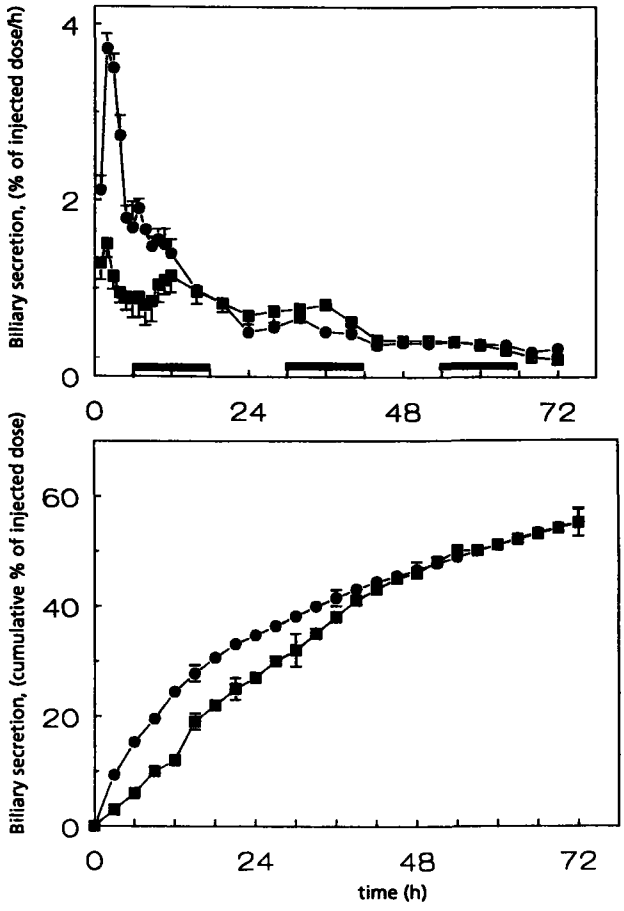

Fig. 8. Biliary secretion of [^{3}H]radioactivity after administration of [^{3}H]cholesteryl oleate labelled HDL (●) or [3H]cholesteryl oleate labelled LDL (■) in estradiol treated rats. Values expressed as percentage of injected dose per hour (Fig. 8A) and as cumulative percentage of injected dose (Fig. 8B).
Bile was collected for 72 h in time-intervals of 1 h, after injection of [^{3}H]cholesteryl oleate HDL (500,000 dpm, 250 μg) in unrestrained estradiol treated rats (●) and compared to values of [^{3}H]cholesteryl oleate labelled LDL (■) (8×10^6 dpm, 500 μg) [28,29]. Values represent the mean of 3 experiments ± S.E.M. Closed horizontal bars indicate the dark periods. For the clarity of Figure 8A, data points between 12-72 h are only presented every 4th h.

uptake and in this study we show in vivo that the selective uptake in the liver is exerted only by the parenchymal cells. At 10 min after injection, the uptake of HDL cholesteryl esters in parenchymal cells is 5-6 times higher than the apoprotein uptake ($p < 0.0001$, Student t-test). In contrast, liver endothelial and Kupffer cells exerted no significant difference between cholesteryl ester and apolipoprotein uptake. It can be calculated that parenchymal cells were responsible for 90.8% of HDL cholesteryl ester uptake in the liver.

High affinity binding sites have been identified on liver cells of various species [37-42]. Recently we have shown that specific high affinity binding sites for HDL are present on rat parenchymal, liver endothelial and Kupffer cells [15] as well as on human parenchymal cells

[43]. The binding properties of parenchymal, liver endothelial and Kupffer cells for apoE-free HDL appeared to be identical for the three cell types [15]. In this study we show that the mechanism by which HDL delivers cholesteryl esters to parenchymal, liver endothelial and Kupffer cells is clearly different. Selective uptake of HDL cholesteryl esters occurs in parenchymal cells whereas liver endothelial cells and Kupffer cells appear to take up HDL cholesteryl esters in parallel with the apoprotein. It can be concluded that although the initial recognition site for HDL on various cells may be identical, the selective uptake of HDL cholesteryl esters must depend on HDL-binding independent factors. It can be anticipated that the high affinity binding of HDL to cells will be a prerequisite for selective uptake but that additional specific cellular properties are needed for selective cholesteryl ester uptake.

According to the generally accepted concept of Glomset [1], HDL is expected to deliver cholesterol (esters) from peripheral cells to the liver in which cholesterol can be converted into bile acids and irreversibly removed from the circulation. Although this sequence of events is generally accepted, evidence for this pathway in vivo is scarce [44]. In literature data are available concerning the processing of HDL labelled in its unesterified cholesterol [9,10]. Free cholesterol however, rapidly exchanges between plasmamembranes and lipoproteins. In our experiments we have used human apoE-free HDL labelled in its cholesteryl ester moiety. Because rat plasma lacks functional cholesteryl ester transfer activity [11] the label remains associated with the particle until it is cleared from the circulation. To quantify the conversion of HDL cholesteryl esters into biliary products we have used unrestrained catheterized rats. It appears that in vivo [^{3}H]cholesteryl ester labelled HDL is actively processed by the liver. At 72 hours after administration 51% of the injected dose was recovered in the bile in the form of bile acids. This is about twice the amount of secreted radioactivity of that obtained with [^{3}H]cholesteryl ester LDL [28]. Furthermore, hydrolysis of cholesteryl esters appeared to be necessary for biliary secretion, since with [^{3}H]cholesteryl oleoyl ether labelled HDL the secretion of radioactivity in the bile appeared to be very slow and at 24 hours after administration only 4.3% of the injected dose was recovered in the bile.

In earlier studies [28,29] we have shown that initial uptake of LDL in Kupffer cells in the rat is not efficiently coupled to bile acid synthesis and secretion. To compare the kinetics of processed cholesteryl esters initially associated with LDL and HDL, we have also used oestradiol treated rats. In oestradiol treated rats more than 90% of both HDL and LDL cholesteryl esters are directed to parenchymal cells. The liver association of HDL cholesteryl esters is hardly affected by oestradiol treatment. It appears that the liver association of cholesteryl esters from LDL versus HDL in oestradiol treated rats is 2-fold higher. However, the secretion of labelled bile acids after injection of [^{3}H]HDL is initially twice as high as for LDL. This indicates that the selective uptake pathway for cholesteryl esters from HDL is apparently more efficiently coupled to the formation of bile acids as with LDL. Studies of Havel et al. have indicated that the LDL-receptor dependent pathway in the liver utilizes the lysosomal route [45] although some apoB may use a more direct route [46]. It remains to be established if the more rapid delivery of cholesteryl esters from HDL into bile is mediated by an extralysosomal route which may be a similar direct "higher speed" pathway. Both LDL and HDL derived cholesteryl esters are secreted in the bile mainly in the form of bile acids. Thus, both have to follow the biosynthetic pathway of bile acids. The difference between LDL and HDL cholesteryl ester processing may therefore not be the conversion in

bile acids itself, but rather the rate of availability of the cholesterol moiety from the lipoprotein for bile synthesizing enzymes in the cell. It is remarkable that although the serum decay and liver association of [^{3}H]cholesteryl esters from HDL are hardly influenced by oestradiol treatment, a marked increase in the bile acid secretion rate occurs during the first 24 hours as compared to control rats. This is in contradiction with the increased acyl-CoA:cholesteryl acyltransferase activity [28,47] and the decreased 7α-hydroxylase activity [48,49] caused by oestradiol treatment. Further investigations on the mechanism of the selective uptake pathway of HDL cholesteryl esters may lead to a better understanding of oestradiol-induced changes.

Recently we showed that, in vivo, HDL can serve as an acceptor for cholesterol from liver endothelial cells and is necessary for an efficient delivery of cholesterol to parenchymal cells [50]. During circulation in the blood [^{3}H]cholesterol was converted into [^{3}H]cholesteryl esters. In the present study we show that uptake of HDL cholesteryl esters in parenchymal cells is coupled to the rapid synthesis of bile acids and thus we provide further evidence in vivo for the properties of HDL as an effective mediator in the reversed cholesterol transport system.

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CHAPTER FOUR

Cholesterol esters selectively delivered in vivo by high-density lipoprotein subclass LpA-I to rat liver are faster processed into bile acids than are LpA-I/A-II derived cholesterol esters

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Biochem J. 1993:292;819-823

Summary

High-density lipoprotein (HDL) subclass LpA-I has been reported to promote cholesterol efflux from mouse adipose cells in vitro whereas LpA-I/A-II has no effect. To investigate whether the apolipoprotein composition of HDL plays a role in the selective delivery of cholesteryl esters to the liver in vivo, we labelled HDL in its cholesterol ester moiety and separated [^{3}H]cholesterol oleate-labelled HDL into subclasses LpA-I and LpA-I/A-II by immuno-affinity chromatography. Serum decay and liver association of LpA-I and LpA-I/A-II were compared for the apoprotein and cholesterol ester moiety. Both LpA-I and LpA-I/A-II selectively delivered cholesteryl esters to the liver in with similar kinetics.

The kinetics of biliary secretion of processed cholesteryl esters, initially associated with LpA-I or LpA-I/A-II, were studied in rats equipped with permanent catheters in bile, duodenum and heart. For both LpA-I as LpA-I/A-II, liver association was coupled to bile acid synthesis with an increase in secretion rate during the night. During the first night period the biliary secretion of LpA-I derived radioactivity was significantly greater than for LpA-I/A-II. The data indicate that both with LpA-I and LpA-I/A-II selective delivery of cholesteryl esters from HDL to the liver occurs, but that cholesteryl esters delivered by LpA-I are more efficiently coupled to bile acid synthesis.

Introduction

Plasma levels of high-density lipoprotein (HDL) cholesterol are inversely correlated with the incidence of atherosclerosis [1,2]. It has been generally accepted that HDL exerts its protective effect by removing cholesterol from peripheral cells and delivering it to the liver, a process called reverse cholesterol transport [3]. The exact mechanism by which HDL can 'extract' cholesterol from peripheral tissues is still in dispute. It has been proposed that the efflux of cholesterol from cholesterol-loaded cells depends on the desorption of cholesterol from the plasma membrane to cholesterol acceptors [4-6]. An alternative mechanism considers the binding of HDL to be necessary for the translocation of intracellular cholesterol to the plasma membrane and eventually to HDL [7-9]. High-affinity binding sites for HDL

have been identified on a variety of cell types including liver cells [10-14]. The major HDL apolipoproteins apoA-I and apoA-II have both been reported to bind to cells or plasma-membranes [15-17].

Within the HDL population, subclasses can be distinguished with different apolipoprotein compositions [19-20]. One subclass contains both apoA-I and apoA-II (LpA-I/A-II particles) whereas apoA-II is absent from the other (LpA-I particles). Levels of LpA-I and LpA-I/A-II have been shown to be affected differently by drug treatment [21]. Clinical studies showed that low HDL levels in coronary artery disease (CAD) were linked to low LpA-I levels, while LpA-I/A-II levels were not different [23,24]. The levels of LpA-I and LpA-I/LpA-II have also been reported to correlate with gender. Females, which are in general more resistent to CAD have higher levels of LpA-I [25].

In vitro studies with cholesterol-loaded mouse adipocytes showed that LpA-I was able to induce cholesterol efflux from the cell, whereas addition of LpA-I/A-II had no effect [20]. However, with other cell types, it has been reported that LpA-I and LpA-I/A-II are nearly equally efficient in promoting cholesterol efflux [26]. In vivo in man, it has been demonstrated that apoA-I on LpA-I has a shorter residence time than apoA-I on LpA-I/A-II, suggesting different catabolic pathways [27]. With $HepG_2$ cells it has been reported that LpA-I and LpA-I/A-II differ in apoprotein binding and uptake and in selective delivery of cholesteryl ethers, though differences were found to be very small [28].

In a previous in vivo study we showed that HDL can serve as a cholesterol acceptor [29]. Subsequent delivery to liver parenchymal cells and secretion of radioactivity in the bile was impaired when low levels of HDL were present, thus supporting the role of HDL in reverse cholesterol transport. Selective delivery of HDL cholesteryl esters to the liver is specific for parenchymal cells and coupled to the formation of bile acids [30]. To investigate whether the apolipoprotein composition of HDL is important for the delivery of cholesteryl esters to the liver and for the kinetics of bile acid formation, in the present study we compared the fate of cholesteryl esters of LpA-I and LpA-I/A-II in the rat.

Materials and methods

Isolation and labelling of HDL

Human HDL was isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave et al. [31]. HDL ($1.063 < d < 1.21$) was dialysed against 10.1 mM phosphate-buffered saline (PBS)/1 mM EDTA, pH 7.4 and labelled with ^{3}H-cholesterol oleate (Amersham Corp, Arlington Heights, IL, U.S.A.) by exchange from donor particles as reported previously [30]. Donor ^{3}H-labelled particles were formed by sonication of egg-yolk phosphatidylcholine (Fluka, Buchs, Switzerland), cholesterol oleate (BHD Chemicals, Poole, Dorset, U.K.) and 250 μCi of [1α,2α(n)-3H]cholesteryl oleate (Amersham). The phosphatidylcholine/cholesteryl oleate mass ratio was 60:1. Sonication was carried out with a MSE Soniprep 150 for 40 min (amplitude 12 μm) at 52°C under a constant stream of N_2 in a 0.1 M-KCl/10 mM Tris/1 mM-EDTA/ 0.025% NaN_3 buffer, pH 8.0. Donor particles with a density approx. 1.03 g/ml were isolated by density-gradient

ultracentrifugation. Phospholipid content of the particles was measured by an enzymic colorimetic assay (Phospholipids Kit, from Boehringer, Mannheim, Germany).

HDL was labelled by incubating HDL with donor particles (mass ratio HDL protein:particle phospholipid = 8:1) in the presence of human lipoprotein deficient serum for 5 h at 37 °C in a shaking water bath under N_2. Radiolabelled HDL was re-isolated by density-gradient ultracentrifugation, dialysed and passed through a heparin-Sepharose affinity column to remove apo E [32]. The apo E-free fraction was checked for the presence of apo E and albumin by SDS/PAGE with 5-22.5%-acrylamide gels, followed by Coomassie Blue staining. Almost all of the radioactivity in HDL could be recovered in the cholesterol ester moieties as determined by Bligh & Dyer extraction [33] and thin layer chromatography, 2.2 ± of the radioactivity was present as unesterified cholesterol.

Isolation of HDL subclasses

The immunosorbents with monoclonal antibodies directed against apo A-I (A05, A17, A30) and apo A-II (G03, G05, G11) [34,35] were prepared by coupling the antibodies to CNBr Sepharose 4B at a concentration of 8 mg ligand/ml in accordance with the procedure of the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden). For isolation of the particles radiolabelled apo E-free HDL was applied to the anti apo A-II immunosorbent at a flow rate of 10 ml/h equilibrated with 0.01 M Tris, 0.15 M NaCl, 0.01 M EDTA. The column was washed with the same buffer containing 0.5 M NaCl at a flow rate of 60 ml/h to eliminate non-specifically bound proteins. The retained fraction (LpA-I/A-II particles) was then eluted with 3M NaSCN at a flow rate of 60 ml/h and immediately filtered through a column packed with Sephadex G25 Coarse to remove most of the thiocynate from the lipoprotein particles. The unbound lipoproteins free of apoA-II were then chromatographed on the anti apoA-I column as described above for the anti apoA-II column. The fraction retained by the anti A-I column corresponded to the LpA-I particles. LpA-I and LpA-I/A-II particles were finally dialysed against PBS/1 mM EDTA and checked on the presence or absence of apolipoproteins by electrophoresis in sodium dodecyl sulphate gels. The chemical composition of the particles was determined by using commercial enzyme kits (Boehringer) for cholesterol, triacylglycerols and phospholipids. Analysis of apolipoprotein composition was carried out by using an e.l.i.s.a with monoclonal antibodies. Radioiodination of ^{3}H-LpA-I and ^{3}H-cholesteryl ester-labelled LpA-I/A-II was carried out according to a modification [36] of the ICl method described by McFarlane [37].

Serum decay and liver association

Male Wistar rats (12 weeks old) were anaesthetized by intraperitoneal injection of 20 mg of Nembutal. The body temperature was maintained at 36.5-37 °C by an i.r. heating lamp monitored as the rectal temperature. After the abdomen was opened, radiolabelled LpA-I and LpA-I/A-II were injected into the vena penis. Single labelled ([^{3}H]-cholesterol oleate) LpA-I or LpA-II/A-II was injected in order to determine the serum decay and liver association of the cholesterol ester moiety, whereas double-labelled (^{125}I/^{3}H) particles were injected to study the clearance of the apoprotein moiety. At the indicated time points, blood sampling and liver lobule excision were performed as described previously [38]. Liver and serum samples of experiments with [^{3}H]cholesterol ester-labelled LpA-I or [^{3}H]cholesterol ester-labelled LpA-I/A-II were combusted in a Packard sample oxidizer and counted for

radioactivity in a Packard liquid scintillation counter. Samples from $^{125}I/^{3}H$-particles were counted in a Packard gamma-counter. To calculate liver associated radioactivity, corrections were made for the contribution of serum to the total liver associated radioactivity.

Bile sampling

Bile was collected from unrestrained 3-month-old male Wistar rats, as reported previously [39]. Rats received tap water and standard chow ad libitum. Rats were equipped with permanent catheters in the bile duct, the duodenum and the heart. Bile duct and duodenum catheters were connected immediately after surgery in order to maintain an intact enterohepatic circulation. Rats were allowed to recover from surgery for one week. Then 100-150 μg LpA-I or LpAI/A-II was introduced via the heart catheter and the bile duct catheter was connected to a fraction collector. Bile samples were collected hourly. A 500 μl portion of bile was de-colourized by adding 100 μl 75% H_2O_2 solution. Hionic fluor scintillation fluid (Packard) was added and the samples were counted for radioactivity in a Packard Liquid Scintillation Analyser.

Protein determination

Protein was determined as described by Lowry et al [40], with BSA (Sigma, U.S.A.) as standard.

Results

The chemical composition of [^{3}H]cholesterol ester-labelled LpA-I and [^{3}H]cholesterol ester-labelled LpA-I/A-II particles is given in Table 1. Almost all of the protein moiety of LpA-I was apoA-I (99.1%) whereas in case of LpA-I/A-II the apoA-I:apoA-II:apoC-III proportions were 53.9:43.0:0.2. The chemical composition (protein, cholesterol, triacylglycerols and phospholipids) was similar for LpA-I and LpA-I/A-II.

In order to determine whether both LpA-I and LpA-I/A-II could selectively deliver cholesteryl esters to the liver, the apoprotein moiety of the particles was labelled by iodination.

Table 1. Chemical composition of LpA-I and LpA-I/A-II. [^{3}H]cholesteryl oleate labelled HDL was separated into LpA-I and LpA-I/A-II subclasses by immunoaffinity chromatography. The chemical composition of the particles from two isolations was determined. ND, not detectable

	Mass (%)			
	Protein	Cholesterol	Triacylglycerols	Phospholipids
LpA	57.2	15.5	ND	27.3
	54.2	16.4	2	27.2
	Apolipoprotein composition (mol%)			
	A-I	A-II	C-III	
LpA	99.1	ND	0.9	
	99.1	ND	0.9	
LpA-I/A-II	42.8	57.0	0.2	
	64.9	34.9	0.2	

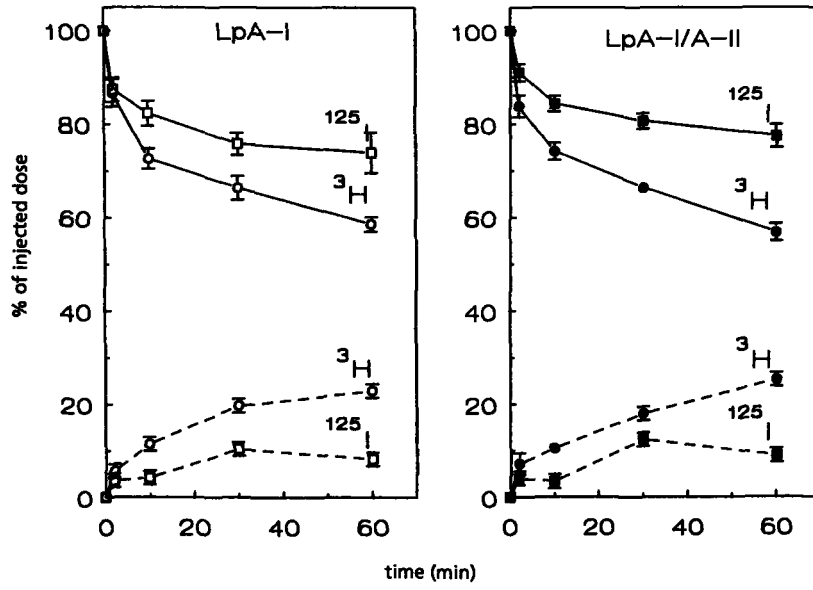

Fig. 1. Serum decay and liver association of LpA-I and LpA-I/A-II in the rat. 100-150 μg (100,000-150,000 dpm) of ^{3}H-cholesteryl ester-labelled LpA-I (a,○ or ^{3}H-cholesteryl ester-labelled LpA-I/A-II (b, ●) were injected into the vena penis of anaesthetized rats. At the indicated time points serum was withdrawn from the vena cava inferior and a liver lobule was tied off, weighed, combusted in a Hewlett Packard sample oxidizer 306 and counted for radioactivity. Serum decay (solid line) and liver association (broken line) were calculated. A correction was made for the contribution of serum to the total liver associated radioactivity [38]. The data for the cholesterol ester moiety are compared with data of the [^{125}I]apolipoprotein moiety of LpA-I and LpA-I/A-II respectively (□ ,■). Data are expressed as percentage of injected dose ± S.E.M. (n = 4).

Figure 1 depicts the serum decay and liver association of LpA-I (1a) and LpA-I/A-II (1b). It appears that the initial serum decay kinetics of LpA-I and LpA-I/A-II are similar, and also liver uptake is identical. At 60 min after injection of LpA-I the liver association of the iodinated apoprotein was 8.2 ± 1.0% of the injected dose, whereas for the ^{3}H-cholesterol ester moiety 22.9 ± 1.3% was recovered in the liver. For LpA-I/A-II these values were 8.9 ± 0.5% and 25.3 ± 1.3% respectively. The data for LpA-I and LpA-I/A-II were not significantly different. Selective delivery of cholesteryl esters to the liver thus occurred with both LpA-I and LpA-I/A-II, indicating that the process of selective delivery is independent of this variation in apolipoprotein composition.

To investigate the kinetics of biliary secretion of initially LpA-I- and LpA-I/A-II associated cholesteryl esters, rats were equipped with permanent catheters in the bile duct, duodenum and the heart. Since only a small amount of LpA-I and LpA-I/A-II (100-150 μg) was injected, no mass effects were induced by administration of the particles. The biliary secretion of radioactivity, after injection of radiolabelled LpA-I and LpA-I/A-II, expressed as percentage of injected dose per hour is shown in Figure 2. In case of LpA-I a clear day/night rhythm can be observed. The marked increase of secretion of radioactivity in the bile during the first dark period in case of LpA-I is less evident for LpA-I/A-II. In case of LpA-I/A-II the increase is delayed and reaches a lower level than for the LpA-I particles. In the first dark period (8-20 h after injection) the secretion of radioactivity derived from LpA-I is thus sig-

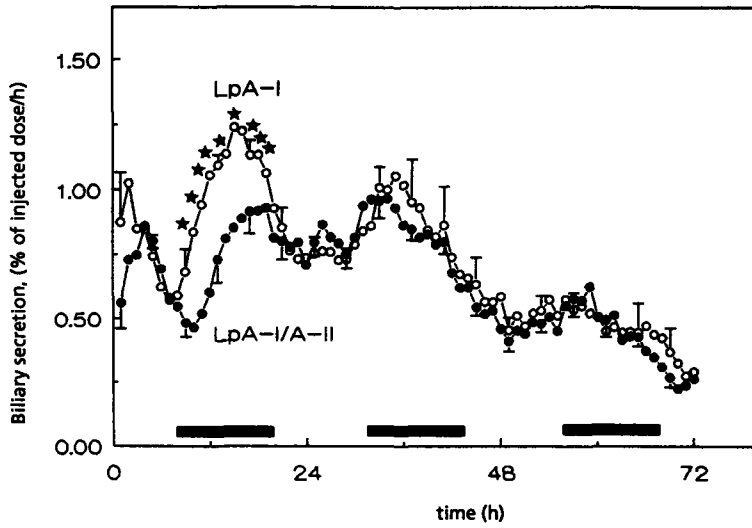

Fig. 2. Biliary secretion of ^{3}H radioactivity after injection of [^{3}H]cholesterol ester-labelled LpA-I or [^{3}H]cholesterol ester-labelled LpA-I/A-II. Bile was collected for 72 hour in 1 h time intervals after injection of 100-150 μg (100,000-150,000 dpm) of LpA-I (○) or LpA-I/A-II (●) in unrestrained cannulated rats. Values are expressed as percentages of injected dose/h and represent the mean of six experiments ± S.E.M. Black horizontal bars indicate the dark periods. Points marked with an asterisk are significantly different ($P<0.05$).

nificantly higher than for LpA-I/A-II (Student t test, $P<0.05$). For the reason that no mass changes occur in bile secretion, it appears that the specific radioactivity is higher for LpA-I than for LpA-I/A-II. The higher biliary secretion rate in the first dark period for LpA-I leads to a higher biliary secretion when expressed cumulatively (Figure 3). For both particles radioactivity was secreted mainly as bile acids (89 ± 2% and 81 ± 6% for LpA-I and LpA-I/A-II respectively).

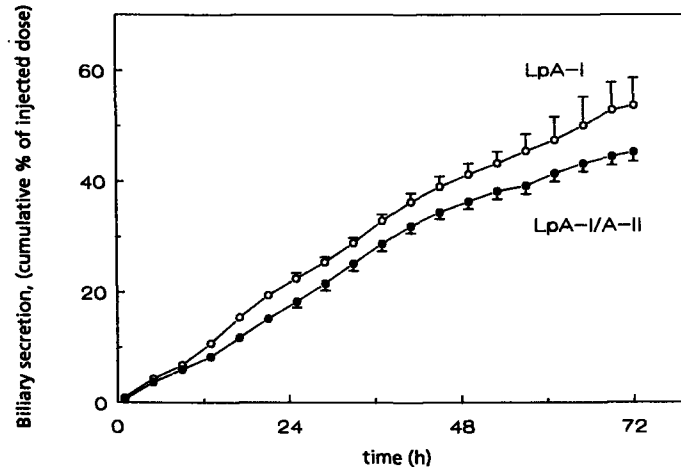

Fig. 3. Biliary secretion of ^{3}H-radioactivity after injection of [^{3}H]cholesterol ester-labelled LpA-I or LpA-I/A-II. Bile was collected for 72 hour in 1 hour time intervals after injection of 100-150 μg (100,000-150,000 dpm) of LpA-I (○) or LpA-I/A-II (●) in unrestrained cannulated rats. Data from Fig. 2 are expressed as percentage of injected dose ± S.E (n=6). For clarity data points are shown every fourth hour.

Discussion

Human HDL is a heterogeneous population of particles that differ in size, density, apoprotein and lipid composition. In this study we isolated HDL by preparative ultracentrifugation, labelled HDL with ^{3}H-cholesterol oleate and re-isolated HDL by a second ultracentrifugation step. To avoid interaction of HDL with apoE binding sites in the liver, the radiolabelled HDL was made apoE-free. In the concept of reverse cholesterol transport, the liver is of major importance as a cholesterol accepting and degrading tissue. We have shown previously [30] that HDL, isolated and labelled as described here, is able to exert selective cholesterol ester delivery specifically to the liver parenchymal cells. We have also shown that in the liver ^{3}H-cholesteryl esters are efficiently metabolized to radiolabelled bile acids and secreted into the bile. In this study we further analysed the particular importance of HDL subclasses LpA-I and LpA-I/A-II for this pathway.

Studies in vitro concerning the binding of HDL subclasses LpA-I and LpA-I/A-II and subsequent cholesterol efflux from the cells have indicated that LpA-I has a higher potency to reduce cellular cholesterol levels [41, 42]. In the present study we isolated the subclasses LpA-I and LpA-I/A-II from cholesterol ester labelled HDL by immunoaffinity chromatography and studied the in vivo behaviour of ^{3}H-cholesteryl esters initially associated with LpA-I and LpA-I/A-II.

It can be concluded that cholesteryl esters initially associated with LpA-I and LpA-I/A-II are both selectively cleared from the circulation as compared to the iodinated apoprotein moiety. At 60 min after injection the liver uptake of the ^{3}H-cholesteryl esters is 2.8 fold higher than that of the [^{125}I]apoprotein moiety. Both subclasses deliver their cholesteryl esters to the liver to the same percentage which indicate that cholesteryl esters, initially associated with LpA-I or LpA-/A-II particles, reach the liver with similar kinetics. Of course we cannot exclude that redistribution of apolipoproteins in vivo occurs. However, in vivo experiments with radiolabelled apolipoproteins in man suggested that a rapid exchange of apolipoproteins is limited and that at least part of the apolipoproteins exchanges at a slower rate than the catabolic rate [27]. Moreover, as will be mentioned later, the difference in kinetics of biliary secretion support that the liver can discriminate between the two different classes of injected particles. Experiments in vitro with pig liver and adrenal membranes show a higher binding of LpA-I than of LpA-I/A-II [43]. With $HepG_2$ cells the higher binding of LpA-I was coupled to a slightly increased uptake of cholesteryl linoleyl ethers [28]. The specific binding of LpA-I and LpA-I/A-II by $HepG_2$ cells however is much lower than the binding of HDL to isolated rat parenchymal cells [13]. Furthermore, bile acid biosynthesis of $HepG_2$ cells is different from rat hepatocytes [44] and does not exhibit clear day/night rhythms.

By using bile catheterized rats we were able to study the kinetics of biliary secretion of radioactivity derived from ^{3}H-cholesterol ester labelled LpA-I and LpA-I/A-II. A marked difference in biliary secretion between LpA-I and LpA-I/A-II was noticed in the first dark period (8-20 h after injection). The day/night rhythm of biliary secretion was much more evident for LpA-I than for LpA-I/A-II particles. The higher secretion rate in the first night leads to a higher percentage of radioactivity secreted in the bile for LpA-I when expressed as cumulative percentage of injected dose. For both ligands, radioactivity was recovered main-

ly as bile acids, indicating that the metabolic pathway for the particles were similar.

Since we did not detect a significant difference between serum decay and liver association of ^{3}H-cholesterol ester-labelled LpA-I and ^{3}H-cholesterol ester-labelled LpA-I/A-II the higher biliary secretion of LpA-I derived radioactivity must be exerted after the initial uptake of cholesteryl esters. It may be anticipated that specifically the binding of LpA-I to the liver parenchymal cell in vivo stimulates an efficient intracellular cholesterol transport to bile acid synthesizing enzymes. Although the presence of apoA-II on the LpA-I/A-II does not have implications for the delivery of cholesteryl esters to the liver, it may hamper the further processing of HDL cholesteryl esters to bile acids. Further work on the mechanism of selective delivery and its coupling to bile acid formation will however be necessary in order to analyse the precise mechanism of the effects of apoA-I versus apoA-I/A-II on this proces.

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CHAPTER FIVE

Cholesteryl esters from oxidized low-density lipoproteins are in vivo rapidly hydrolysed in rat Kupffer cells and transported to liver parenchymal cells and bile

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submitted for publication

Summary

Human low-density lipoprotein (LDL) was labelled in its cholesteryl ester moiety with [^{3}H]cholesteryl oleate or [^{3}H] cholesteryl oleoyl ether and oxidized by exposure to 10 μM of $CuSO_4$. The in vivo metabolism of cholesteryl esters of oxidized LDL (Ox-LDL) was determined after injection into rats. When Ox-LDL was labelled with [^{3}H]cholesteryl oleoyl ether, a non-hydrolyzable analog of cholesteryl oleate, Kupffer cells contributed for 55.1 ± 4.1 % to the total liver uptake at 10 min after injection. When [^{3}H]cholesteryl oleate labelled Ox-LDL was injected, the radiolabelled cholesterol esters were nearly completely hydrolysed within 1 h after injection. Within this time, the Kupffer cell associated radioactivity declined to 32% of the maximal uptake value. In serum the highest specific resecreted [^{3}H]cholesterol (esters) were associated with the serum HDL fraction, thus suggesting a role for HDL as an in vivo cholesterol acceptor. The kinetics of biliary secretion were studied in rats equipped with catheters in the bile, duodenum and heart. At 1 h after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL, 4.15 ± 0.67 % of the injected dose was secreted in the bile, mainly as bile acids. At 6 h after injection this value was 19.2 ± 1.2 %. These values are 3-fold higher than for injected [^{3}H]cholesteryl oleate labelled Ac-LDL, which is initially mainly taken up by liver endothelial cells. The rapid processing of cholesteryl esters derived from Ox-LDL to bile acids, indicate that Kupffer cells form an efficient protection system against the atherogenic action of Ox-LDL in the blood compartment.

Introduction

It has been well established that in vitro modification of low-density lipoproteins (LDL) by acetylation [1], acetoacetylation [2], malondialdehyde treatment [3] or oxidation [4] all lead to the formation of atherogenic particles. In the artery wall, the uptake of modified LDL by macrophages mediated by the scavenger receptor leads to lipid accumulation [5] and eventually to the formation of foam cells, an important event in the developing atherosclerotic plaque. We have shown previously, by using iodinated oxidized LDL (Ox-LDL), that in the rat the liver is highly effective in removing Ox-LDL from the circulation. We provided evi-

dence that on various liver cell types different "scavenger receptors" do exist, recognizing acetylated LDL (Ac-LDL), Ox-LDL or both [6]. By scavenging atherogenic particles from the blood compartment, the liver forms a major protection system of the body. It has been established that oxidized LDL rather than acetylated LDL is the physiological representative for modified LDL [7]. Therefore, in this study, we focused on the in vivo fate of the cholesterol ester moiety from Ox-LDL in the rat. The possible involvement of high-density lipoproteins (HDL) as transport vehicles for intercellulair transport of cholesterol (esters) was also studied. The potential role of HDL in the so called "reverse cholesterol transport", the transport of peripheral cholesterol to the liver as proposed by Glomset [8], is generally accepted. The exact mechanism however, by which HDL removes cholesterol from the cells is still not clear. It has been stated that cholesterol efflux from the cell is based on passive diffusion [9, for review see 10]. It has also been reported that binding of HDL induces the activation of a signal-transduction pathway which results in the translocation of intracellular cholesterol to the plasma membrane [11,12]. Recently, it has been suggested that in cells cholesterol is present in slow and fast kinetic pools. Increased cholesterol efflux from cells upon binding of apoAI was proposed to be linked to a a higher participation of the fast kinetic cholesterol pool in the efflux [13].

In a study with Ac-LDL, we have shown previously [14] that the uptake of radiolabelled cholesterol esters by liver endothelial cells is followed by a resecretion of radiolabelled free cholesterol into the serum. The results indicated that HDL is necessary for an efficient transport of cholesterol from liver endothelial cells to parenchymal cells, thus providing evidence for the role of HDL in reverse cholesterol transport. HDL has been shown to selectively deliver its cholesteryl ester moiety to hepatic [15,16,17], steroidogenic [16,17] and extrahepatic tissues [18]. We have provided evidence that hepatic selective delivery of HDL cholesterol esters is restricted to the liver parenchymal cells and efficiently coupled to bile acid formation and secretion [19]. In the present study we investigated therefore also the role of HDL as an in vivo cholesterol acceptor for Kupffer cell associated cholesterol (esters). Furthermore we followed the kinetics of appearance of radiolabelled bile in order to test the efficiency of biliary secretion of metabolized cholesteryl esters initially associated with Kupffer cells.

Materials and methods

Isolation, labelling and oxidation of LDL

Human LDL was isolated from plasma of healthy volunteers as described by Redgrave et al. [20]. After density ultracentrifugation LDL ($1.019 < d < 1.063$) and lipoprotein deficient serum (LPDS, $d > 1.21$) were collected and dialysed against 10.1 mM-phosphate buffered saline/1 mM EDTA, pH 7.4. [^{3}H]Cholesteryl oleate was incorporated into LDL according to Blomhoff et al. [21]. In short, 25 μCi of [1α,2α(n)-^{3}H]-cholesteryl oleate (Amersham, USA) was dried under N_2 and dissolved in 100 μl acetone. 1 ml of human LPDS was added, placed under a stream of N_2 for 10 min, and incubated for 10 min at 37 °C. Then, 1 ml of LDL was added and incubated at 37 °C during 5 h. [^{3}H]Cholesteryl oleate labelled LDL was reisolated by density ultracentrifugation and dialysed against phosphate buffered saline containing 10 μM EDTA, pH 7.4. LDL (0.2 mg/ml) was then oxidized by exposure

to $CuSO_4$ (10 μM of free copper ions) during 20 h at 37 °C. The oxidation of LDL was stopped by the addition of EDTA (1 mM final concentration). [^{3}H]Cholesteryl oleate labelled LDL was filtrated through a Millipore Millex-GV4 0.45 μm filter to remove possible aggregates and its relative electrophoretic mobility checked on agarose gelelectrophoresis (Ox-LDL: Rf = 0.52 ± 0.01 (n = 6, ± S.E.M.; Control LDL: Rf = 0.21 ± 0.01 (n = 6, ± S.E.M.). Specific activity of [^{3}H]Ox-LDL was 12.5 ± 1.5 dpm/ng (n = 6, ± S.E.M.). 79.4 ± 3.6 % of the radiolabel could be recovered as [^{3}H]cholesteryl esters as determined by Bligh and Dyer extraction [22] and thin layer chromatography [23]. For some experiments [^{3}H]cholesteryl oleoyl ether, a non-hydrolyzable analog of cholesteryl oleate, was incorporated into LDL and subsequently oxidized (specific activity 7.9 dpm/ng, Rf = 0.54). [^{3}H]Ox-LDL was used for experiments within 3 weeks after oxidation. Acetylation of [^{3}H]cholesteryl oleate labelled LDL was carried out as described by Basu et al. [24]. Specific activity of [^{3}H]Ac-LDL was 15.2 ± 1.7 dpm/ng (n = 3, ± S.E.M.). 95.8 ± 0.9 % of the radiolabel could be recovered as cholesteryl esters after lipid extraction and t.l.c.

Serum decay and liver association

Throughout this study 12 week old male Wistar rats were used. Rats were anaesthetized by an intraperitoneal injection of Nembutal (1 ml/kg body wt.). The abdomen was opened and [^{3}H]cholesteryl oleate labelled Ox-LDL was injected in the vena penis. At the indicated time points blood and liver samples were taken. Corrections were made for the contribution of entrapped serum to the liver uptake (90 μl serum/ g wet tissue) as described previously [25]. In order to separate [^{3}H]cholesteryl esters from [^{3}H]free cholesterol, liver lobules were put on fluid nitrogen immediately after excision in order to stop further hydrolysis. Liver samples were homogenized and extracted according to Bligh and Dyer [22]. To separate free cholesterol from cholesterol esters, the lipid fraction of the Bligh and Dyer extraction was subjected to thin layer chromatography with n-heptane/di-ethylether/glacial acetic acid as eluens (60:40:1 by vol.) [23]. To determine serum radioactivity at time points longer than 1 h after injection, rats were anaesthetized with diethylether and injected in the vena penis. Blood samples were taken by capillary puncture of the orbital plexus.

Cell Isolation

For determination of the hepatic cellular distribution in vivo, [^{3}H]cholesteryl oleate or [^{3}H]cholesteryl oleoyl ether labelled Ox-LDL were injected into the vena penis. At the indicated time points the vena porta was cannulated and the liver perfused with oxygenated Hanks' buffer plus Hepes (1.6 g/l), pH 7.4 at 8 °C. In order to determine the total liver uptake a liver lobule was tied off after 8 min perfusion (flow rate 14 ml/min). The various liver cell types were then isolated by a low temperature (8 °C) perfusion method with 0.05% collagenase in Hanks'/Hepes buffer. The separation of parenchymal cells was carried out as described earlier [26]. The non-parenchymal liver endothelial and Kupffer cells were isolated by centrifugal elutriation [27]. The purity of isolated cell fractions (> 90%) were checked light microscopically after staining for peroxidase radioactivity. Calculation of the contribution of the different celtypes to total liver uptake was performed as described previously [27]. In these calculations parenchymal cells contributed for 92.5%, liver endothelial cells for 2.5% and Kupffer cells for 3.3% to the total liver uptake.

Bile sampling

Bile was collected from unrestrained 3-month-old male Wistar rats [28]. Rats received tap water and standard chow ad libitum. Rats were equipped with permanent catheters in the bile duct, the duodenum and the heart. Bile duct and duodenum catheters were connected immediately after surgery in order to maintain an intact enterohepatic circulation. Rats were allowed to recover from surgery for 1 week. 40-50 μg [^{3}H]cholesteryl oleate labelled Ox-LDL (app. 500,000 dpm) was injected via the heart catheter. The bile duct catheter was then connected to a fraction collector and bile samples were collected hourly. 100 μl bile was de-colourized by adding 10 μl 30% H_2O_2 solution. The samples were counted for radioactivity after addition of hionic fluor scintillation fluid (Packard) in a Packard Liquid Scintillation Analyser. To separate bile acids from the lipid fraction, bile samples were extracted according to Bligh and Dyer [22]. The aqueous layer containing the bile acids was counted for radioactivity. Cholesterol and cholesterol esters were separated by thin layer chromatography as described above.

Protein was determined as described by Lowry et al [29], with BSA as standard. Cholesterol and cholesteryl esters were quantified by using a commercial kit (Boehringer, Mannheim).

Results

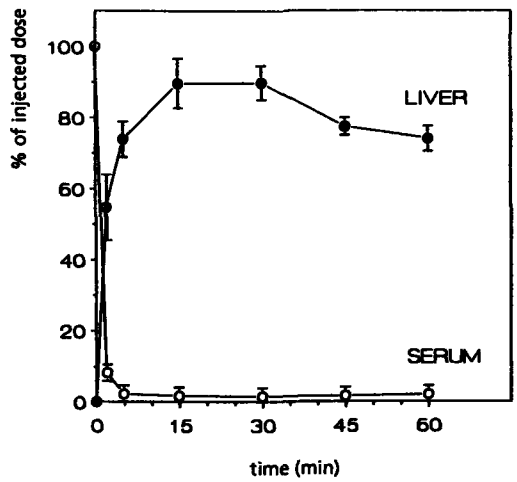

Fig. 1. Serum decay and liver association of [^{3}H]cholesteryl oleate labelled oxidized LDL. 40-50 μg (app. 500,000 dpm) of [^{3}H]cholesteryl oleate Ox-LDL was injected into the vena penis of anaesthetized rats. At the indicated time points serum was drawn from the vena cava inferior and serum decay (○) was calculated. In order to determine liver association (●) a liver lobule was tied off, weighed, combusted in a Hewlett Packard sample oxidizer 306 and counted for radioactivity. A correction was made for the contribution of serum to the total liver associated radioactivity [25]. Data are expressed as percentage of injected dose ± S.E.M. (n=4).

Serum decay and liver association

The serum decay and liver association of [^{3}H]cholesteryl oleate-labelled Ox-LDL is shown in Fig. 1. The rapid decay of [^{3}H]cholesteryl oleate labelled Ox-LDL from the serum resembles the clearance of iodinated Ox-LDL [6]. At two min after injection 91.8 ± 0.2 % of the injected dose has been removed from the serum. Radioactivity of [^{3}H]cholesteryl oleate labelled Ox-LDL was almost quantitively recovered in the liver. At 15 min after injection 89.6 ± 7.0 % of the injected dose was liver associated. At later time points the liver associated radioactivity decreased, indicating processing of the Ox-LDL derived [^{3}H]cholesteryl esters. Fig. 2 shows the percentage of [^{3}H]free cholesterol in the liver at different times after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL. At 5 min after injection already 50% of the liver associated radioactivity was present as free cholesterol. Subsequently, the percentage of free cholesterol in the liver increased upto 80% at 45 min after injection. The data also indicate that the liver is still able to hydrolyze cholesteryl esters of LDL after oxidation.

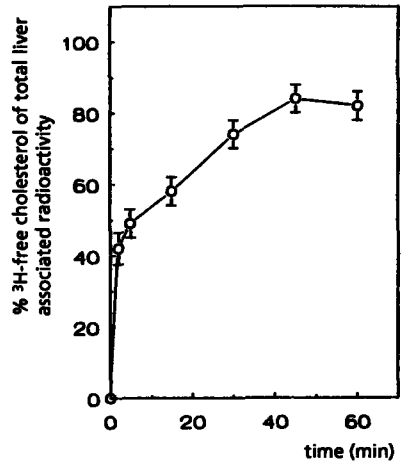

Fig. 2 Percentage of free cholesterol of total liver associated radioactivity. Anaesthetized rats were injected with 40-50 μg [^{3}H]cholesteryl oleate labelled Ox-LDL. At the indicated time points liver lobules were tied off and immediately put in fluid nitrogen in order to stop hydrolysis. Liver samples were extracted according to Bligh and Dyer [22] and subjected to thin layer chromatography. Spots corresponding with free cholesterol and cholesterol esters were scraped off and counted for radioactivity. Data are presented ± S.E.M. (n=4).

Cellular distribution studies of [^{3}H]Ox-LDL in the liver

We have shown previously that Ox-LDL iodinated on its apolipoprotein moiety is mainly taken up by Kupffer cells [6]. When [^{3}H]cholesteryl oleate labelled Ox-LDL is injected into the rat the highest specific activity is also found to be associated with Kupffer cells (Fig. 3). At first sight, a discrepancy between Kupffer cell involvement seems to exist between the apolipoprotein and the cholesteryl ester moiety. At 10 min after injection the specific activity (% of injected dose (ID) x10^3/mg cell protein) of Kupffer cells is 1163 ± 202 and 764 ± 65 for the apolipoprotein and cholesteryl ester part, respectively. Consequently, the calculated relative contribution of the Kupffer cells to the total liver radioactivity is less for [^{3}H]cholesteryl ester labelled Ox-LDL than for ^{125}I-Ox-LDL (Table I). However, when LDL is labelled with a non-hydrolyzable cholesteryl ester analog (cholesteryl oleoyl ether), the involvement of Kupffer cells (966 ± 77) is similar as for the iodinated Ox-LDL. The significant difference ($p < 0.05$, Student's t-test) between the [^{3}H]cholesteryl ester and [^{3}H]cholesteryl oleoyl ether uptake suggests a rapid hydrolysis of cholesteryl esters and a

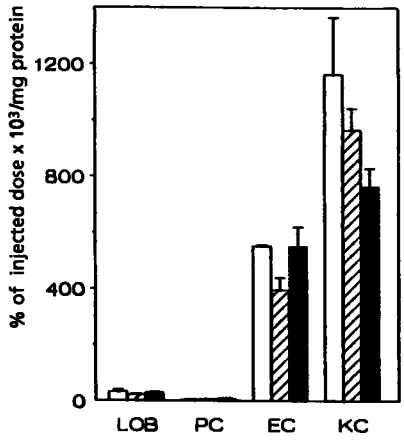

Fig. 3 Cellular distribution of Ox-LDL in the liver. 40-50 ug of Ox-LDL labelled on the apolipoprotein (^{125}I, open bars, from [6]) or cholesteryl ester moiety (cholesterol oleoyl ether, hatched bars or cholesterol oleate, filled bars) was injected into rats and a low temperature perfusion was carried out at 10 min after injection. Cells were separated into parenchymal cells (PC), liver endothelial cells (EC) and Kupffer cells (KC). LOB represents the total liver uptake. Data are expressed as specific activity ± S.E.M. (n=4).

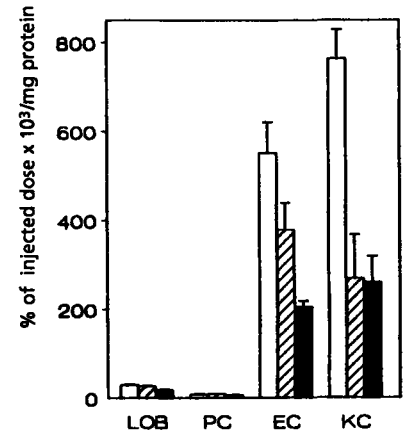

Fig. 4 Cellular distribution of [^{3}H]cholesteryl oleate labelled Ox-LDL at different times after injection. 40-50 μg cholesterol oleate labelled Ox-LDL was injected into rats and parenchymal cells (PC), liver endothelial cells (EC) and Kupffer cells (KC) were isolated at 10 (open bars), 60 (hatched bars) and 120 min (filled bars) after injection. LOB represents the total liver uptake. Data are expressed as specific activity ± S.E.M. (n=4).

rapid secretion of [^{3}H]free cholesterol by the Kupffer cells. Within 1 h after injection a further reduction of Kupffer cell associated radioactivity can be observed (Fig. 4). At 60 min after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL the specific activity of Kupffer cells declined to 32% of the maximal uptake as determined with [^{3}H]cholesteryl oleoyl ether labelled Ox-LDL. After the rapid secretion of [^{3}H]free cholesterol from Kupffer cells in the first hour, the Kupffer cell associated radioactivity remained constant upto 2 h after injection.

Table 1. Relative contribution of different liver cell types to the total liver uptake of Ox-LDL, labelled on its apolipoprotein or cholesteryl ester moiety at 10 min after injection. Parenchymal cells (PC) contribute for 92.5% to the total liver, endothelial cells (EC) 3.3% and Kupffer cells (KC) 2.2%. The amount of radioactivity per mg cell protein was multiplied with the amount that each cell type contributes to the total liver volume, in order to calculate the relative contribution [27]. Values represent the mean of four experiments ±n S.E.M.

	Ox-LDL labelled with		
Cell type	^{125}I	[^{3}H]chol. oleate	[^{3}H]chol. oleoyl ether
PC (%)	6.9 ± 1.2	16.8 ± 3.5	12.7 ± 2.4
EC (%)	36.3 ± 4.2	40.3 ± 3.1	31.9 ± 2.4
KC (%)	56.8 ± 3.0	43.0 ± 2.2	55.1 ± 4.1

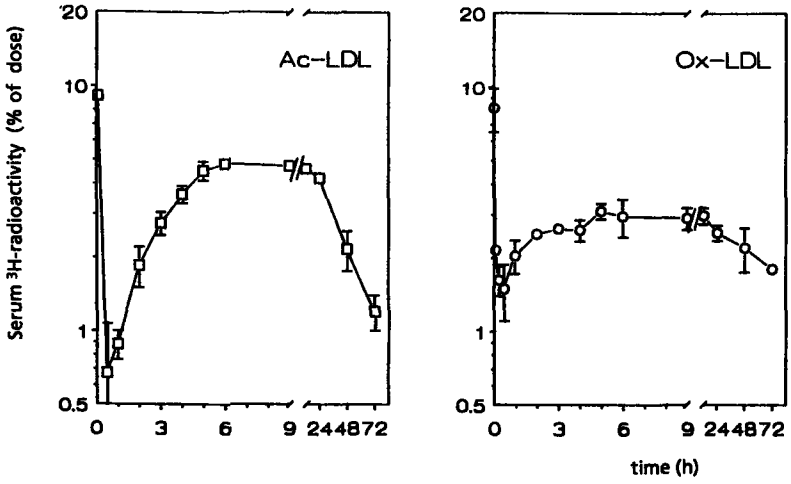

Fig. 5. Serum radioactivity after the injection of [³H]cholesteryl oleate labelled Ox-LDL or Ac-LDL. [³H]Ox-LDL (Fig. 5A) or [³H]Ac-LDL (Fig. 5B) (120-160 μg, 2,000,000 dpm) was injected into rats via the vena penis. At the indicated time points blood was drawn from the orbital plexus. Serum was counted for radioactivity and serum decay was calculated. Results are the mean of 4 experiments ± S.E.M.

Resecretion of ³H-radioactivity into the serum

When the serum radioactivity was determined at different time points after injection of ³H-cholesterol oleate labelled Ox-LDL, the initial rapid decay is followed by a reappearance of radioactivity (Fig. 5). In Fig. 5a and 5b the serum radioactivity upto 72 h after injection of [³H]cholesteryl oleate labelled Ox-LDL or [³H]cholesteryl oleate labelled Ac-LDL can be compared. At 30 min after injection of [³H]cholesteryl oleate labelled Ox-LDL only 1.5 ± 0.4% of the injected dose was present in the serum. Subsequently, serum radioactivity increased up to 3% of the injected dose (significantly different from 1.5% at 30 min after injection, ($p < 0.01$, Student's t-test) and remained constant for upto 12 h after injection. When [³H]cholesteryl oleate labelled Ac-LDL was injected, the serum radioactivity decreased to 0.7% of the injected dose and subsequently increased upto 5%. In the serum the

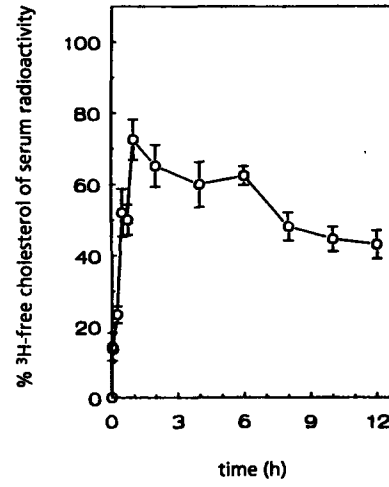

Fig. 6 Percentage of free cholesterol in serum after injection of [³H]cholesteryl oleate labelled Ox-LDL. Serum of rats injected with [³H]cholesteryl oleate labelled Ox-LDL was extracted according Bligh and Dyer [22]. Free cholesterol was separated from cholesterol esters by thin layer chromatography. Spots were scraped off and counted for radioactivity, scraped off and counted for radioiactivity. Data are presented ± S.E.M. (n=4)

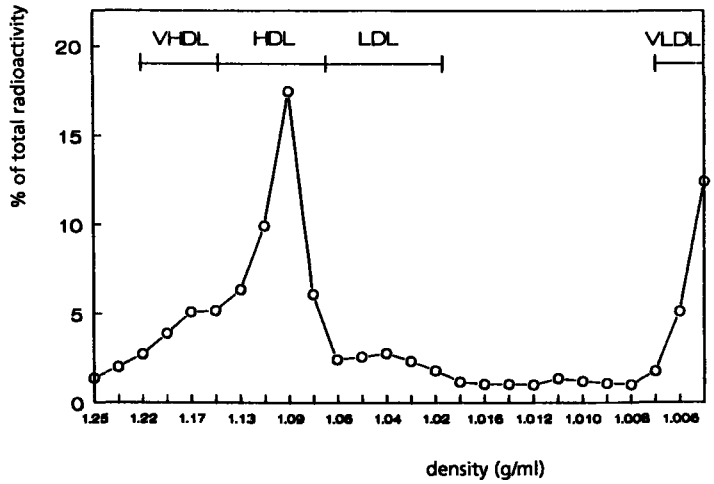

Fig. 7 Density gradient distribution of ^{3}H radioactivity in the serum and specific activities in serum fractions at 3 h after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL. [^{3}H]cholesteryl oleate labelled Ox-LDL (150-200 μg) was injected into the vena penis of a rat. At 3 h after injection, serum was obtained and subjected to KBr density-gradient centrifugation at 4 °C. The gradient was subdivided into 500 ul fractions, starting from the bottom (7A). The profile is a representative for 4 experiments. Specific activity of ^{3}H in the different serum fractions were measured and expressed as dpm/μg free (open bars) or esterified cholesterol (hatched bars) (7B).

reappearance of radiolabel after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL (Fig. 6) was initially mainly in the form of free cholesterol. This indicates that after hydrolysis of cholesteryl esters in the liver, [^{3}H]free cholesterol is secreted into the serum compartment. Serum density ultracentrifugation at 3 h after injection (Fig. 7a) revealed that 55.5 ± 1.1 % of the radioactivity could be recovered in the HDL density range ($1.05 < d < 1.13$). For LDL and VLDL these values were 11.9 ± 2.0 and 11.2 ± 2.8 %, respectively (n=4 ± S.E.M.). Evidence for re-esterification in the serum compartment can be derived from the relative decrease of free cholesterol associated radioactivity at the later time points (Fig. 6). The specific activity of radioactivity of free cholesterol and cholesteryl esters in the different lipoprotein fractions is indicated in Fig b. The specific radioactivity of free cholesterol is similar for HDL, LDL and VLDL. The specific radioactivity of cholesteryl esters however, was in HDL 3.7-fold and 3.5-fold higher than for LDL and VLDL, respectively.

Biliary secretion of ^{3}H radioactivity

In order to study the kinetics of biliary secretion after injection of [^{3}H]cholesteryl ester labelled Ox-LDL, we used rats catheterized in bile duct, duodenum and heart. Immediately after the administration of [^{3}H]Ox-LDL via the heart catheter, bile collection was started. The biliary secretion of ^{3}H-radioactivity is illustrated in Fig. 8. Fig. 8a shows the kinetics of biliary secretion expressed as percentage of injected dose secreted per hour. The immediate rapid secretion of radiolabelled bile resulted in a high initial peak during the first hours after injection of [^{3}H]cholesteryl ester labelled Ox-LDL. At 6 h after injection already 19.2% of the injected dose could be recovered in the bile. For [^{3}H]cholesteryl oleate labelled Ac-LDL the initial biliary secretion rate was much lower. Upto 6 h after injection only 6.3% of the

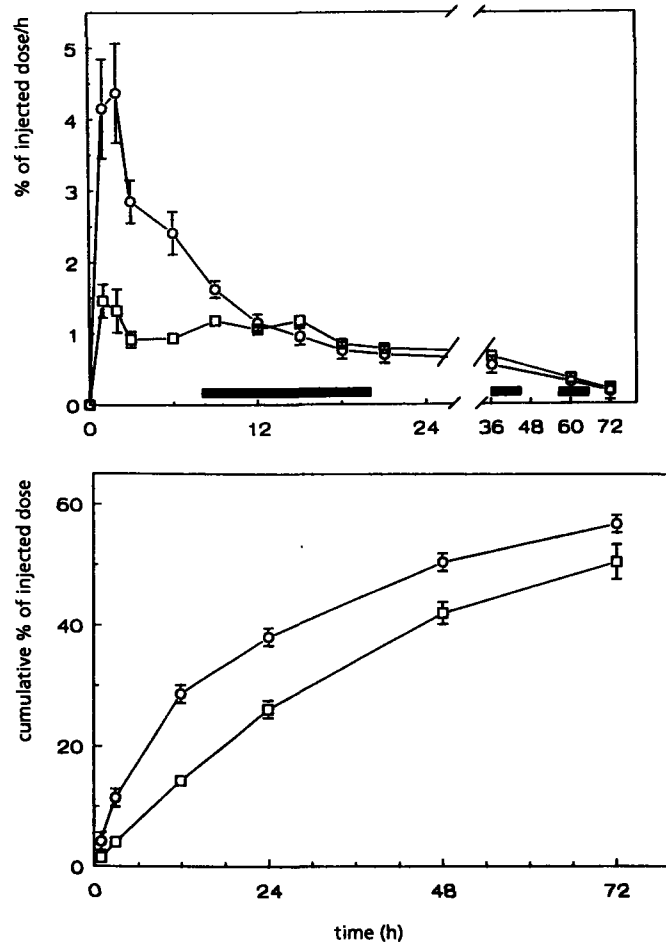

Fig. 8 Biliary secretion of 3H radioactivity after injection of [3H]cholesteryl oleate labelled Ox-LDL (○) or Ac-LDL (□). Bile was collected for 72 hour in 1 hour time intervals after injection of 40-50 μg of [^{3}H]cholesteryl oleate labelled Ox-LDL or Ac-LDL in unrestrained cannulated rats. Values are expressed as percentages of injected dose per hour (8A) or as cumulative percentage of injected dose (8B) and represent the mean of 4 experiments ± S.E.M. Black horizontal bars indicate the dark periods.

injected dose was secreted into the bile [14]. Most of the biliary radioactivity (91.3 ± 2.6%) could be recovered in the aqueous layer after Bligh and Dyer extraction [12], indicating that radioactivity was present as bile acids. The remainder was recovered as labelled free cholesterol. When the biliary secretion is expressed cumulatively (Fig. 8b), it appears that the great initial difference in biliary secretion after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL or Ac-LDL is diminished at later time points. Especially in the first 24 h after injection, the biliary output of [^{3}H]cholesteryl oleate labelled Ox-LDL derived radioactivity is higher than with [^{3}H]cholesteryl oleate labelled Ac-LDL. At 72 h after injection of Ox-LDL 56.8 ± 1.5 % of the injected dose has been secreted in the bile while for [^{3}H]cholesteryl oleate labelled Ac-LDL this value is 50.5 ± 2.2%.

Discussion

In a previous study with Ac-LDL we provided evidence for the role of HDL in reverse cholesterol transport in vivo. It appeared that HDL could act as an acceptor for liver endothelial cell associated cholesterol, with subsequently transport of cholesterol to liver parenchymal cells and bile [14]. Since oxidized LDL rather than acetylated LDL is the pathophysiological representative for atherogenic LDL particles, we investigated in the present study the in vivo fate of [^{3}H]cholesterol oleate labelled Ox-LDL.

[^{3}H]cholesteryl oleate labelled Ox-LDL was rapidly cleared from the serum and almost quantitatively recovered in the liver. The appearance of [^{3}H]free cholesterol in the liver indicated that the liver rapidly hydrolyzed the cholesteryl esters derived from Ox-LDL. Ox-LDL particles are taken up as integral particles from the blood circulation as indicated by the similar cellular distribution of the apolipoprotein and cholesteryl ether moiety. When Ox-LDL labelled with [^{3}H]cholesteryl oleate was injected, it appeared that already in the first 10 min after injection cholesteryl esters were processed and some ^{3}H-free cholesterol was released from the Kupffer cells. The specific radioactivity of Kupffer cells at 10 min after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL was 79% of the specific activity after injection of [^{3}H]cholesteryl oleoyl ether labelled Ox-LDL. At 1 h after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL the Kupffer cell associated radioactivity was declined to 32% of the maximal uptake value. The rapid hydrolysis and rapid secretion of [^{3}H]free cholesterol was reflected by a modest increase in serum radioactivity. The increase in serum radioactivity reached a value of approximately 3% of the injected dose and remained at this level during 12 h after injection. The ratio of radiolabelled free cholesterol to cholesteryl esters in the serum initially rapidly increased, which indicates that free cholesterol is secreted into the serum compartment. By gradient ultracentrifugation most of the radioactivity could be recovered in the HDL range. Subsequently the relative proportion of cholesteryl esters to the total radioactivity increased, a process consistent with serum conversion of cholesterol to cholesteryl esters by the enzyme lecithin cholesteryl acyl transferase (EC 2.3.1.43). The specific radioactivity of HDL was 3.7- and 3.5-fold higher than for other lipoproteins which supports the role of high density lipoproteins as initial cholesterol acceptors and serum site for conversion into cholesterol esters. We have shown previously, that HDL cholesteryl esters are in vivo selectively delivered to liver parenchymal cells and efficiently processed into bile acids [19]. In the present study, the rapid secretion of [3H]free cholesterol from Kupffer cells appeared to be linked to a rapid biliary secretion of radioactivity. Almost all of the radioactivity could be recovered in the aqueous phase after extraction, indicating an almost complete intracellular processing into bile acids in the parenchymal cells. Collection of bile at shorter time intervals showed that the lag-phase of biliary secretion of radioactivity was only 15 min (data not shown). After this time point biliary secretion rapidly increased. Upto 6 h after injection, the biliary secretion of radioactivity from [^{3}H]cholesteryl oleate labelled Ox-LDL was much higher than for [^{3}H]cholesteryl oleate labelled Ac-LDL. It thus appears that the Kupffer cell mediated uptake as compared to liver endothelial cells, is more efficiently coupled to bile acid formation. The molecular mechanism for this more efficient appearance of bile acids radioactivity from [^{3}H]cholesteryl oleate labelled Ox-LDL might be explained in two ways.

In case of [^{3}H]cholesteryl oleate labelled Ac-LDL, the minimal serum value (at 30 min after

injection) is two times lower than after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL (0.7% versus 1.5%). Furthermore, resecretion percentages in the serum are higher for Ac-LDL as for Ox-LDL (5% versus 3%). Comparing the kinetics of biliary secretion, it is clear that radiolabelled bile acids derived from Ox-LDL are secreted at an initially 3-fold higher rate than for Ac-LDL. Anatomically, biliary secretion is linked to liver parenchymal cells. Thus, cholesterol transport from liver endothelial cells and Kupffer cells to parenchymal cells is obligatory. It is possible that Kupffer cells secrete vesicles containing cholesterol and other lipids, which are rapidly taken up by liver parenchymal cells. It has been reported that mouse macrophages and human monocyte-macrophages secrete apolipoprotein E (apoE), which can be incorporated in lipoproteins [30]. Increases in cellular cholesterol content markedly stimulate the apoE production [31]. Immunogoldlabelling of rat liver showed that also Kupffer cells do stain for apoE [32]. ApoE, secreted by Kupffer cells, may be associated with a lipid vesicle and evoke an apoE-mediated uptake by parenchymal cells. A high turnover of these vesicles would explain the pattern of serum radioactivity for Ox-LDL. We tried to test this hypothesis by blocking apo-E mediated parenchymal cell uptake by lactoferrin [33]. However, no effect of lactoferrin on the biliary secretion rate was noticed (M.N. Pieters et al., unpublished). The second explanation is related to the difference in localisation of liver endothelial cells and Kupffer cells. Liver endothelial cells are separated from parenchymal cells by the space of Disse. Therefore, cholesterol from liver endothelial cells has to be transported through the serum compartment in order to reach the parenchymal cells. Kupffer cells are located in the sinusoids, but pseudopodia of Kupffer cells are able to penetrate through the endothelial fenestrae and can be in direct contact with the microvilli of parenchymal cells. This would enable the Kupffer cell to directly transport cholesterol from the Kupffer cell to the parenchymal cell. To discriminate between these possiblities and to explain the molecular mechanisms of the rapid cholesterol transport from Kupffer cells to parenchymal cells further experiments will be necessary.

In conclusion, cholesteryl esters from oxidized LDL are in vivo mainly taken up by Kupffer cells and rapidly hydrolyzed to free cholesterol. Resecretion of cholesterol to serum HDL occurs, supporting a role of HDL in reverse cholesterol transport. A relative rapid secretion of radiolabelled bile acids is noticed after injection of [^{3}H]Ox-LDL which was 3-fold higher than for [^{3}H]Ac-LDL. The rapid processing of cholesteryl esters from Ox-LDL by Kupffer cells and the efficient conversion to bile acids and secretion into bile indicate that Kupffer cells form an efficient major protection system against the atherogenic action of Ox-LDL in the blood compartment.

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CHAPTER SIX

The biliary secretion rate of processed cholesteryl esters from modified LDL depends on the contribution of liver endothelial or Kupffer cells to the hepatic uptake.

Evidence that Kupffer cells rapidly transfer cholesterol to parenchymal cells

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submitted for publication

Summary

Human low-density lipoproteins (LDL) were labelled with [^{3}H]cholesteryl oleate and modified by acetylation (Ac-LDL) or oxidation (Ox-LDL). In vivo Ac-LDL was mainly directed to the liver endothelial cells, whereas Ox-LDL was mainly taken up by the Kupffer cells. In bile cannulated rats, the secretion of bile acids derived from Ox-LDL was 10-13 fold higher than the secretion of bile acids derived from Ac-LDL. This indicates that the transport of cholesterol from Kupffer cells to parenchymal cells and bile is highly efficient. To investigate whether the presence of blood components and extrahepatic factors were obligatory for these differential secretion pathways, we used isolated rat livers perfused with artificial medium. After in vivo uptake of either [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL by the liver, a recirculating perfusion was performed during 2 hours. The biliary secretion of bile acids derived from Ox-LDL in the perfused livers was similar efficient as observed in vivo and was 40 times higher than the secretion of bile acids derived from Ac-LDL. In both cases, more than 90% of the biliary radioactivity was secreted in the form of bile acids. The [^{3}H]cholesterol efflux from the Ox-LDL-loaded liver to the perfusate was 2-3 fold higher than for Ac-LDL. The addition of HDL did enhance the cholesterol efflux to the perfusate, but exerted no effect on the biliary secretion rate. The results indicate that the high biliary secretion rate after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL in vivo, does not depend on cholesterol transport via serum HDL but may utilize a more direct route from Kupffer cells to parenchymal cells.

Introduction

It is well established that the uptake of modified low-density lipoproteins (LDL) by macrophages can lead to the formation of foam cells [1], an early event in the atherosclero-

tic plaque [2]. The uptake of modified LDL is mediated via the scavenger receptor, which is not subjected to down-regulation by increasing amounts of cellular cholesterol (esters) [3]. Previously applied modifications of LDL include acetylation [3] and oxidation [4], of which oxidation is considered to reflect a pathophysiological relevant process [5]. We have shown previously [6] that in rats iodinated acetylated LDL (Ac-LDL) is mainly taken up by the liver endothelial cells, whereas iodinated oxidized LDL (Ox-LDL) is directed to the Kupffer cells. The cholesteryl ester moiety of Ac-LDL has been shown to be hydrolyzed in the liver endothelial cells and secreted in its unesterified form to serum high-density lipoproteins (HDL) [7]. HDL can subsequently act as a transport vehicle for transfer of cholesterol to the parenchymal cells, thus supporting the supposed role of HDL in reverse cholesterol transport [8]. In this process, cholesteryl esters from HDL utilize the so-called "selective uptake route" exerted by the liver [9,10,11]. In the parenchymal cell, cholesterol is converted into bile acids and secreted into the bile. In a recent study we investigated in vivo the metabolism of cholesteryl esters derived from Ox-LDL. It was shown that the initial secretion of radiolabelled bile acids derived from cholesteryl oleate labelled Ox-LDL was high in spite of low concentrations of radiolabelled unesterified cholesterol in the serum (Pieters, M.N., Esbach, S., Schouten, D., Brouwer, A., Knook, D.L. & Van Berkel, Th.J.C., submitted). Based on these results, we speculated that besides transfer via HDL an additional pathway may exist which enables the Kupffer cell to transfer the cholesterol to the parenchymal cells rapidly.

In the present study we investigated the hypothesis that the anatomic structure of the liver, i.e. liver endothelial cells (separated from the parenchymal cells by the Space of Disse) versus Kupffer cells (pseudopodia may have direct contact with parenchymal microvillii), may be responsible for the different rate of transport of cholesterol to the parenchymal cells. The use of a perfused rat liver enabled us to exclude the role of blood components and extrahepatic tissues while the liver architecture remained similarly organized as in vivo.

Materials and methods

Isolation of LDL and HDL; labelling and modification of LDL

Human LDL and HDL were isolated from plasma of normolipidemic volunteers as described by Redgrave et al. [12]. LDL ($1.024 < d < 1.063$ g/ml), HDL ($1.063 < d < 1.21$ g/ml) and lipoprotein deficient serum (LPDS, $d > 1.21$ g/ml) were collected and dialysed against 10.1 mM-phosphate buffered saline/1 mM EDTA, pH 7.4. Incorporation of [^{3}H]cholesteryl oleate or [^{3}H]cholesteryl oleoyl ether in LDL was carried out according to Blomhoff et al. [13]. [^{3}H]Cholesteryl oleate or oleoyl ether labelled LDL was reisolated by density ultracentrifugation and dialysed against phosphate buffered saline containing 10 μM EDTA, pH 7.4. Subsequently, oxidation of the labelled LDL was then carried out by exposure to 10 μM of free copper ions during 20 h at 37 °C as previously described [6]. [^{3}H]Cholesteryl oleate or oleoyl ether labelled LDL was acetylated according to Basu et al. [14]. Oxidized [^{3}H]Cholesteryl oleate labelled LDL was filtrated through a Millipore Millex-GV4 0.45 μm filter in order to remove any aggregates. The relative electrophoretic mobility of labelled oxidized or acetylated LDL was checked on agarose gelelectrophoresis (Ox-LDL: $Rf = 0.51 \pm 0.01$ ($n = 3$, ± S.E.M.); Ac-LDL: $Rf = 0.52 \pm 0.01$ ($n = 3$, ± S.E.M).

Control LDL: Rf = 0.21 ± 0.01 (n = 3, ± S.E.M.). Specific activity of [^{3}H]cholesteryl oleate labelled ligands were 14.9 ± 4.4 dpm/ng (n = 3, ± S.E.M.).

Serum decay and liver association

Throughout the study 12-week old rats (225-275 g) have been used. To determine the hepatic uptake of [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL, rats were anaesthesized by an intraperitoneal injection of Nembutal (1 ml/kg bodywt). After the abdomen was opened, 30-40 μg [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL (app. 500,000 dpm) were injected into the vena penis. Blood samples were drawn from the vena cava inferior. To determine liver association, liver lobules were tied off. Serum decay and liver associated were calculated as described previously [15].

Cell Isolation

For determination of the hepatic cellular distribution in vivo, [^{3}H]cholesteryl oleoyl ether labelled Ox-LDL or Ac-LDL were injected into the vena penis of rats. At 10 min after injection, the vena porta was cannulated and the liver perfused with oxygenated Hanks' buffer plus Hepes (1.6 g/l), pH 7.4 at 8 °C. The various liver cell types were isolated by a low temperature (8 °C) perfusion method with 0.05% collagenase in Hanks'/Hepes buffer. After isolation of the parenchymal cells, non-parenchymal liver endothelial and Kupffer cells were separated by centrifugal elutriation [16]. The purity of isolated cell fractions were checked light microscopically after staining for peroxidase. Calculation of the contribution of the different cell types to total liver uptake was performed as described previously [16].

In vivo bile sampling

Bile was collected from unrestrained 3-month-old male Wistar rats equipped with permanent catheters in the bile duct, the duodenum and the heart [17]. Rats were allowed to recover from surgery for 1 week and received tap water and standard chow ad libitum. Approx. 100 μg [^{3}H]cholesteryl oleate labelled Ox-LDL or Ac-LDL (1.2-1.5x10^6 dpm) was injected via the heart catheter. Bile samples were collected in 10 min time intervals upto 2 h after injection. Samples of 50 μl bile were decolourized by adding 10 μl 30% H_2O_2 solution and counted for radioactivity after addition of hionic fluor scintillation fluid (Packard) in a Packard Liquid Scintillation Analyser. Bile samples were extracted according to Bligh and Dyer [18]. The aqueous layer containing the bile acids was counted for radioactivity. The lipid fraction containing cholesterol and cholesteryl esters was subjected to thin layer chromatography with heptane:diethylether:acetic acid (60:40:1 by vol.) as solvent as described earlier [19]. Spots were coloured with I_2-vapor and scraped off. Hionic scintillation fluid was added and the samples counted.

Isolated rat liver perfusion experiments

Detailed description of the methods for the surgical isolation and the apparatus for perfusion has been published earlier [20]. Rats of 220-250 g were anaesthetized by an intraperitoneal injection of Nembutal (1 ml/kg body wt.). The abdomen was opened and 300-350 μg [^{3}H]cholesteryl oleate labelled Ox-LDL or Ac-LDL was injected in the vena penis. In order to allow initial uptake by the liver, ligands were circulated for 2 min. At 2 min after injection, the vena porta, the inferior vena cava in the thorax and the bile duct were cannulated. At approx. 3 min after injection of the ligand the liver was flushed free of blood with

oxygenated Krebs-bicarbonate buffer, supplemented with 1% bovine serum albumin (Boseral, Organon, Oss, The Netherlands) and glucose (1 g/l). The liver was placed in a thermostatically controlled cabinet of 37 °C. Once prepared, recirculating perfusion with or without the addition of HDL (22.5 mg/100 ml) was started at 9-11 min after injection and maintained for 2 hours. The volume of the perfusate was approx. 100 ml (90-110 ml). During the perfusion a taurocholate infusion (15μmol/h) was used in order to replace the secreted bile acids. Immediately after switching to recirculating perfusion, bile collection was started. A bile sample and an 1 ml sample of the perfusate were taken every 10 min and counted for radioactivity. After 2 h of recirculation, the liver was weighed. Liver samples were combusted in a Packard sample oxidizer and counted for radioactivity. The perfusate was collected, the exact volume measured and subjected to density ultracentrifugation and Bligh and Dyer extraction [18]. Free cholesterol and cholesteryl esters were separated by t.l.c. (see above) and counted for radioactivity.

Protein determination

Protein was determined as described by Lowry et al [21], with BSA as standard.

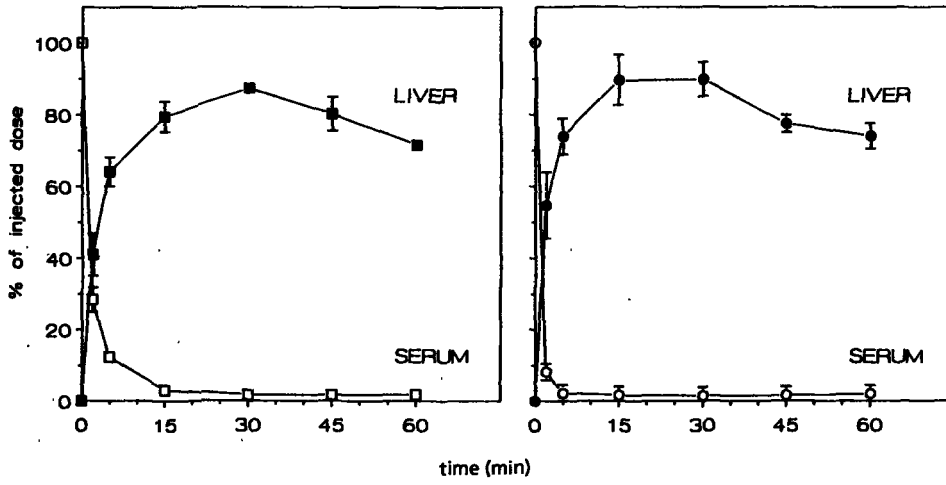

Fig.1. Serum decay and liver association of [^{3}H]cholesteryl oleate labelled Ac-LDL and Ox-LDL in the rat. 30-40 μg of [^{3}H]cholesteryl oleate labelled Ac-LDL (Fig. 1A) or Ox-LDL (Fig. 1B) as injected in the vena penis of anaesthetized rats. At the indicated time points blood was withdrawn from the vena cava inferior and serum decay (open symbols) was calculated. Liver association (closed symbols) was determined by taking out liver lobules which were weighed, combusted in a sample oxidizer and counted for radioactivity. A correction was made for the contribution of serum to the total radioactivity [16]. Data are expressed as % of injected dose ± S.E.M. (n = 3)

Results

Fig. 1 shows the in vivo serum decay and liver association of [^{3}H]cholesteryl oleate labelled Ac-LDL (Fig. 1A) and [^{3}H]cholesteryl oleate labelled Ox-LDL (fig. 1B). Both ligands are cleared very fast from the serum and radioactivity is quantitatively recovered in the liver. The hepatic cellular distribution of Ac-LDL and Ox-LDL however, is different.

Table 1. Relative contribution of different cell types to the total liver uptake of Ox-LDL and Ac-LDL labelled with [^{3}H]cholesteryloleoyl ether at 10 min after injection. Parenchymal cells (PC) contribute for 92.5% to the total liver, endothelial cells (EC) 3.3% and Kupffer cells (KC) 2.2%. The amount of radioactivity per mg cell protein was multiplied with the amount that each cell type contributes to the total liver volume, in order to calculate the relative contribution [27]. Values represent the mean of four experiments ±n S.E.M.

Cell type	Ox-LDL	Ac-LDL
PC (%)	12.7±2.4	28.7±2.7
EC (%)	31.9±2.4	63.4±1.6
KC (%)	55.1±4.1	7.8±1.8

Table 1 shows the relative percentual contribution of the different cell types in the liver for Ac-LDL and Ox-LDL uptake, which were labelled with [^{3}H]cholesteryl oleoyl ether, a non-hydrolyzable analog of cholesteryl oleate. At 10 min after injection Ac-LDL is mainly recovered in the liver endothelial cells, whereas for Ox-LDL the Kupffer cells are the main cellular site of uptake.

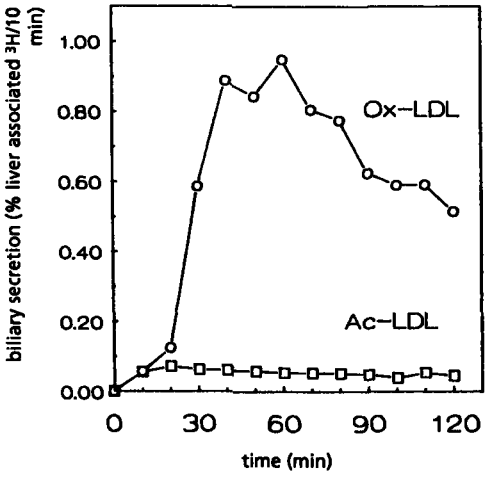

Fig. 2. In vivo biliary secretion of 3H-radioactivity after injection of [^{3}H]cholesterol oleate labelled Ac-LDL or Ox-LDL. Bile was collected for 2 hours in 10 min time intervals after injection of 50-100 μg of [^{3}H]cholesterol oleate labelled Ac-LDL (□) or Ox-LDL (○) in unrestrained bile cannulated rats. Values are expressed as percentage of the liver associated radioactivity per 10 min and are the mean of 2 experiments.

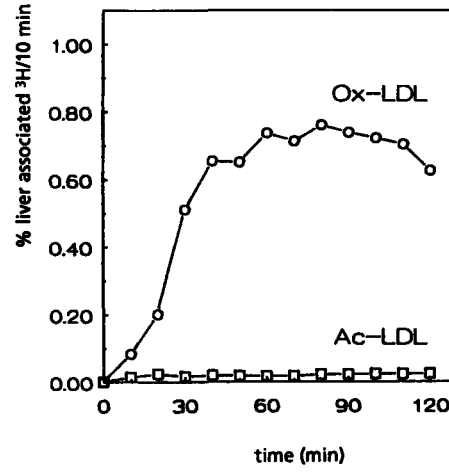

Fig. 3. Biliary secretion of 3H-radioactivity by the perfused rat liver after previous in vivo uptake of [3H]cholesterol oleate labelled Ac-LDL or Ox-LDL. 300 μg of [^{3}H]cholesteryl oleate labelled Ac-LDL (□) or Ox-LDL (○) was injected into rats. After 2 min of circulation, the liver was cannulated and perfused in order to remove the blood from the liver. At 9-11 min after injection, recirculating perfusion was started (zero time) at 37 °C in a thermostatically controlled cabinet. Bile was collected every 10 min during 2 h of perfusion, weighed, de-colourized and counted for radioactivity. Values are expressed as % of liver associated radioactivity per 10 min and represent the mean of two experiments.

In order to study the in vivo kinetics of biliary secretion after injection of [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL, we used rats, cannulated in the bile duct, duodenum and the heart. Fig. 2 shows the biliary secretion rates at 10 min time intervals. After a lag-phase of app. 20 min, the biliary secretion rate of Ox-LDL derived radioactivity increases to 0.9% of the liver associated dose/10 min at 40 min after injection. This value is 13 times higher than the secretion rate of Ac-LDL derived radioactivity. At 70 min after injection, the biliary secretion rate of Ox-LDL derived radioactivity decreases. At 120 min after injection however, Ox-LDL biliary secretion is still 11-fold higher than the secretion of Ac-LDL radioactivity. Bligh and Dyer extraction [18] revealed that for both ligands radioactivity is mainly (>90%) recovered as bile acids. At 120 min after injection, 0.7% and 7.4% of the liver associated radioactivity for [^{3}H]cholesteryl oleate labelled Ac-LDL and Ox-LDL respectively, has been secreted in the bile. The secretion of radiolabelled bile acids in the bile implies that [^{3}H]free cholesterol must have been transported to parenchymal cells, in which cholesterol can be converted into bile acids. The mechanism of transport from endothelial and Kupffer cells to parenchymal cells and bile was subsequently investigated in an ex vivo perfusion technique. In this model the secretion of radioactivity from non-parenchymal cells to the "blood compartment" (perfusate buffer) can be controlled without interference of extrahepatic sites or metabolic conversion in the blood compartment. [^{3}H]Cholesteryl oleate labelled Ac-LDL or Ox-LDL were in vivo injected into the vena penis of rats and allowed to circulate for 2 min. The liver associated radioactivity was calculated by summation of the liver associated radioactivity after 2 h of recirculating perfusion and the total radioactivity secreted in the perfusate and the bile. For Ox-LDL 39.2 ± 1.1 % of the injected dose (n = 9 ± S.E.M.) and for Ac-LDL 27.9 ± 1.6 % of the injected dose (n = 8 ± S.E.M.) was liver associated at 2 min after injection. The relatively short circulation time was chosen in order to prevent early catabolism of the modified LDL. The liver was cannulated in the bile duct, flushed free of blood and connected to a recirculating perfusion system. The subsequent secretion of radioactivity in the bile is shown in Fig. 3. During the ex vivo perfusion biliary secretion of Ox-LDL derived radioactivity also showed a lag-phase but remained fairly constant after reaching its highest value at 60 min after injection. At this time point, biliary secretion of Ox-LDL derived radioactivity was more than 40 times higher than that of Ac-LDL derived radioactivity. At 120 min after injection, 7.1% of the liver associated radioactivity has been secreted into the bile. For Ac-LDL this

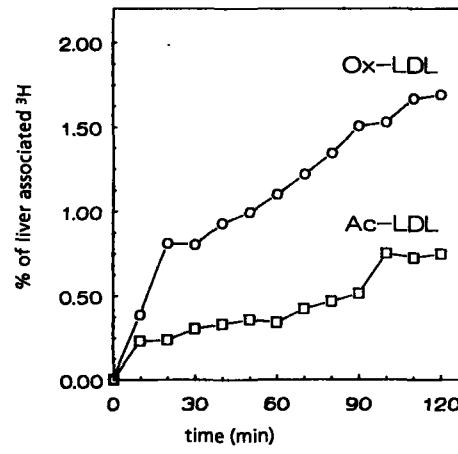

Fig. 4. Release of radioactivity in the perfusate. 300 μg of [^{3}H]cholesteryl oleate labelled Ac-LDL (□) or Ox-LDL (○) was injected into rats. After 2 min of circulation, the liver was cannulated and perfused in order to remove the blood from the liver. At 9-11 min after injection, recirculating perfusion was started (zero time) at 37 °C in a thermostatically controlled cabinet. 1 ml samples of the perfusate were taken every 10 min and counted for radioactivity. Values are expressed as % of liver associated radioactivity and represent the mean of two experiments.

value was 0.26% of the liver associated radioactivity. For both ligands biliary radioactivity was mainly in the form of bile acids (>95%), indicating adequate metabolic conversion in parenchymal cells.

To study the possible secretion of radiolabel in the perfusate, 1 ml-samples of the perfusate were taken every 10 min. Since the perfusate buffer was recirculated, the efflux to the perfusate was measured as a cumulative efflux. The efflux of ^{3}H-radioactivity to the perfusate for Ac-LDL and Ox-LDL "loaded" livers is presented in Fig. 4. The efflux of radioactivity from the liver to the perfusate after Ox-LDL injection is 2-3-fold higher than for Ac-LDL. Resecreted radioactivity was mainly recovered (>90%) as unesterified free cholesterol, indicating that only hydrolyzed cholesteryl esters were secreted.

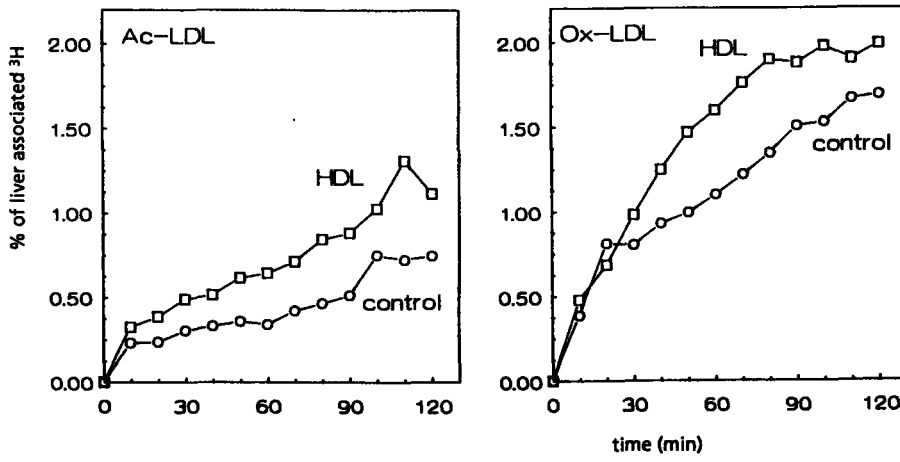

Fig. 5. The effect of HDL on the cholesterol efflux from the liver to the perfusate. 300 μg of [^{3}H]cholesteryl oleate labelled Ac-LDL (Fig.5A) or Ox-LDL (Fig.5B) was injected into rats. After 2 min of circulation, the liver was cannulated and perfused in order to remove the blood from the liver. At 9-11 min after injection, recirculating perfusion was started (zero time). The perfusate contained no HDL (○) or 22.5 mg HDL/100 ml (□). 1 ml samples of the perfusate were taken every 10 min and counted for radioactivity. Values are expressed as cumulative % of liver associated radioactivity and represent the mean of two experiments.

The possible role of HDL in promoting cholesterol efflux from the cells and as transport vehicle to the parenchymal cells was studied by adding HDL to the perfusate buffer. As is shown in Fig. 5A and 5B, the presence of HDL in the perfusate does stimulate the cholesterol efflux to the perfusate though differences are relatively small. Density ultracentrifugation revealed that the radioactivity was associated with the very dense and very light fractions of the gradient when no HDL was added to the perfusate (Fig. 6). When HDL was present, radiolabelled unesterified cholesterol was found to be mainly associated with the HDL-density range. The addition of HDL to the perfusate did not enhance radiolabelled bile acid secretion (data not shown).

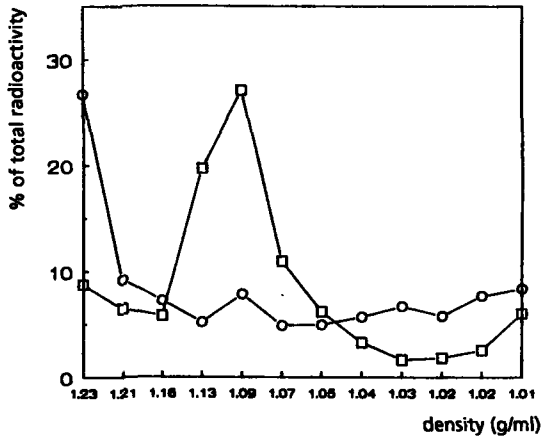

Fig. 6 Density gradient distribution of ^{3}H-radioactivity in the perfusate without or in the presence of HDL after previous in vivo uptake of [^{3}H]cholesteryl oleate labelled Ox-LDL. At 2 h after starting recirculating perfusion, the perfusate with (□) or without (○) HDL (22.5 mg/100 ml) was collected and kept on ice. The perfusate was then subjected to KBr density-gradient centrifugation at 4°C. The gradient was subdivided into 1 ml fractions, starting from the bottom. After measuring the density of each fraction, the fractions were counted for radioactivity.

Discussion

We have shown previously that the liver forms a major protective system for atherogenic particles in the blood compartment. Non-parenchymal cells rapidly take up and degrade modified LDL [6]. However, to estimate the relevancy of non-parenchymal cells as a definitive protection system, it is necessary to establish to which extent and by which mechanism cholesteryl esters from modified LDL can reach the bile. Secretion in the bile allows irreversible secretion from the body. In the liver different types of scavenger receptors exist on the various liver cell types, recognizing Ac-LDL, Ox-LDL or both [6]. When Ac-LDL and Ox-LDL are labelled with [^{3}H]cholesteryl oleoyl ether, a non-hydrolyzable analog of cholesteryl oleate, the hepatic cellular distribution is generally the same as found when the ligands are labelled on the apolipoprotein (^{125}I, [6]). This indicates that both Ac-LDL and Ox-LDL are taken up as integral particles. We have established earlier that cholesteryl esters derived from Ac-LDL [7] or Ox-LDL are in vivo converted into bile acids and secreted into the bile. In the present study we show that in the first 2 hours after injection, the rate of biliary secretion for Ox-LDL derived bile acids is 10-13 fold higher than for Ac-LDL derived bile acids. This observation is very surprising, since in vivo both ligands are similarly rapid cleared from the serum and taken up by the liver with similar kinetics. Also, in vivo hydrolysis of cholesteryl esters derived from Ox-LDL and Ac-LDL occur both at a high rate. The rapid secretion of radiolabelled bile acids after injection of [^{3}H]cholesteryl oleate labelled Ox-LDL may be the result of a more rapid transport of unesterified cholesterol from the Kupffer cells to the parenchymal cells as compared to transport from endothelial cells. Such a rapid transport may be mediated by serum components, for example HDL which mediates reverse cholesterol transport as proposed by Glomset [8]. A second mechanism may be related to the liver architecture. Liver endothelial cells are separated from the parenchymal cells by the Space of Disse, whereas Kupffer cells can have direct access to the microvilli of

parenchymal cells. We tried to discriminate between these two mechanisms by using an ex vivo perfused rat liver, which had been in vivo "loaded" with either [^{3}H]cholesteryl oleate labelled Ac-LDL (liver endothelial cells) or [^{3}H]cholesteryl oleate labelled Ox-LDL (Kupffer cells). Bile was collected during two hours of recirculating perfusion and the secreted radiolabelled bile acids, expressed as % of liver associated radioactivity, were compared for Ac-LDL and Ox-LDL. Bile acids derived from Ox-LDL were secreted at a 40-fold higher rate than bile acids derived from Ac-LDL. The secretion of ^{3}H-bile acids after injection of Ox-LDL was comparable with the values as found in vivo. The lag-phase in the biliary secretion which can be observed in unrestrained, cannulated rats was also apparant in the ex vivo perfused liver. With the Ac-LDL loaded liver, the biliary secretion in the perfused liver was 2-3 times lower than in vivo which may indicate a role for the blood compartment and extrahepatic tissues in the further processing of Ac-LDL cholesteryl esters. To investigate the secretion of radiolabel from the cells to the perfusate buffer, we collected perfusate samples every 10 min. The efflux of radiolabelled cholesterol was 2-3 times higher for an Ox-LDL-loaded liver than for an Ac-LDL-loaded liver. The fact that almost all of the radioactivity (>90%) in the perfusate for both ligands was present as free cholesterol indicates that the cholesteryl esters had been hydrolysed prior to secretion into the perfusate. The absence of [^{3}H]cholesteryl esters in the perfusate eliminates the possibilities that resecretion of radioactivity in the perfusate was caused by washing off liver associated but not internalized ligand or retroendocytosis of the ligand.

In vivo studies have shown earlier that HDL can act as cholesterol accepting and transporting vehicles [7]. Cholesteryl esters from HDL can be selectively delivered to the parenchymal cells [10]. In order to study the role of HDL as acceptor and transport vehicle for cholesterol to the parenchymal cells and bile, we added HDL to the perfusate. When HDL was absent from the perfusate buffer, radiolabelled cholesterol was found to be mainly associated with the very dense fraction containing albumin (BSA). The remainder of the radioactivity was diffusely distributed among different density fractions. However when HDL was present, the radiolabelled cholesterol was associated with the HDL particles. Some [^{3}H]cholesterol was also present in the very light top fraction, suggesting that part of the free cholesterol may be secreted together with newly synthesized VLDL [22]. Though the addition of HDL did enhance the cholesterol efflux to the perfusate, no effect was observed on the biliary secretion. Increasing the amount of HDL from 225 to 675 μg/ml further enhanced the secretion of [^{3}H]free cholesterol to the perfusate, but still had no effect on the secretion rate of ^{3}H-labelled bile acids. In vivo, lowering HDL levels by ethinyl estradiol treatment resulted in a decrease of biliary secretion between 6-24 h after injection of [^{3}H]cholesteryl oleate labelled Ac-LDL [7] while no effect on biliary secretion rate was noticed in the first hours after injection. So, the time course of the ex vivo perfusion experiments (two hours) was too short for showing an effect of HDL in stimulating biliary secretion and it seems likely that metabolic conversion (i.e. LCAT-activity leading to cholesteryl ester formation) is needed for HDL's mediating role in net cholesteryl (ester) transport. Both the rapid transport of cholesterol from Kupffer cells (Ox-LDL derived cholesteryl esters) as evidenced by the rapid biliary output and the limited release of radioactive cholesterol into the perfusate suggest that direct transport of cholesterol from Kupffer cells to parenchymal cells can occur. Alternatively, Kupffer cells may secrete lipid vesicles [24] that are rapidly taken up by neighbouring parenchymal cells before the perfusate leaves the liver. Anyway, the intact

architecture of the liver is of vital importance for the transport of cholesterol or other secretory products from Kupffer cells to parenchymal cells.

In conclusion, we show that in vivo in the rat, as well in an ex vivo liver perfusion system, cholesteryl esters of Ox-LDL initially present in Kupffer cells are faster converted into bile acids and secreted into the bile than cholesteryl esters from Ac-LDL which are initially mainly taken up by the liver endothelial cells. This indicates that the rate of biliary secretion of ^{3}H-bile acids depends primarily on the cell type responsible for the hepatic uptake. The presence of HDL in the perfusate did stimulate cholesterol efflux to the perfusate, but did not affect the biliary secretion rate. It thus appears that the high biliary secretion rate after [^{3}H]cholesteryl ester uptake by the Kupffer cells does not depend on HDL-mediated cholesterol transport via the serum compartment. Our results strongly suggest that the intact liver architecture is of a major importance for efficient cholesterol transport from Kupffer cells to parenchymal cells.

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CHAPTER SEVEN

Visualization of the uptake and processing of oxidized low-density lipoproteins in human and rat liver

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Summary

The interaction of oxidized human low density lipoproteins (Ox-LDL) with human and rat liver was analyzed by light and electron microscopy. At the light microscopic level Ox-LDL was visualized by the fluorescent dye 1,1' dioctadecyl 3,3,3',3' tetramethyl indocarbocyanine perchlorate (DiI), while at the electron microscopic level, an indirect immunolabeling procedure was used, detecting the apoprotein B of the Ox-LDL. In rats, Ox-LDL was administered intravenously, while uptake by human liver was studied by perfusion of tissue blocks. Both in human and in rat liver, DiI-Ox-LDL was mainly found to become concentrated in Kupffer cells, and, to a lesser extent, in endothelial cells. In both species the cell association of DiI-Ox-LDL could be inhibited by pre-administration of polyinosinic acid, indicating a scavenger receptor-mediated process.

At the electron microscopic level, Ox-LDL was found to bind mainly to areas of the plasma membrane of the Kupffer cells without clathrin coating, although binding to coated regions was also noticed. Internalization of the ligand occurred via coated vesicle formation, and via membrane folding of interacting lamellipodia and worm-like structures. No indication for phagocytosis of aggregated Ox-LDL particles was noticed. Following internalization, the immunoreactive Ox-LDL was detected in relatively electron lucent endosomes, and, subsequently, in lysosomes. Endothelial cells internalized Ox-LDL solely via coated pits, after which the particles were transferred via endosomes into lysosomes. The endosomes often contained tubular extensions, which were devoid of immunolabel. In human Kupffer and endothelial liver cells essentially the same organelles were demonstrated to be involved in the internalization and processing of Ox-LDL as in the rat.

Our morphological results confirm earlier biochemical data on the relative involvement of the various liver cell types in the uptake of Ox-LDL in rats, while the relevance of these data for the human situation is indicated. The uptake process, coupled to Ox-LDL recognition by Kupffer cells, as presently analyzed, indicates that both rat and human Kupffer cells are equipped with a similar removal system to protect the body against the occurrence of the atherogenic Ox-LDL particles in the blood.

Introduction

Macrophage derived foam cell formation is characteristic in the early atherosclerotic lesion (1). Although the level of serum LDL cholesterol is positively correlated with the occurrence of atherosclerotic lesions, native LDL does not provoke excessive accumulation of cholesterylesters in tissue macrophages, even when exposed to high concentrations of LDL for prolonged periods (2, 3). Modified forms of LDL, however, are taken up with high efficiency by macrophages via the interaction with the so called scavenger receptor, resulting in massive cholesterol accumulation within the cells (4). Various types of modification of LDL have been described. These modifications include acetylation (4), malondialdehyde treatment (5) and oxidation (6).

In rats, it was previously demonstrated that acetylated LDL (Ac-LDL) is rapidly cleared from the circulation by the liver via the scavenger receptor on liver endothelial cells (7). More recently evidence is obtained which suggests that oxidized LDL is the real patho-physiological form of modified LDL. Ox-LDL was detectable in, and even extractable from rabbit and human atherosclerotic lesions (8). The possible mechanisms responsible for LDL oxidation and the atherogenic characteristics of Ox-LDL have been recently reviewed by Witztum and Steinberg (9).

Upon injection into rats Ox-LDL is rapidly cleared from the circulation by the liver similarly as Ac-LDL (10). However, biochemical data indicate that Kupffer cells, rather than endothelial cells, are responsible for the liver uptake of Ox-LDL (10). Studies with isolated endothelial and Kupffer cells showed evidence for the presence of an additional scavenger receptor, which is specific for Ox-LDL and highly concentrated on Kupffer cells (10). In the present study, we used a morphological approach with light and electronmicroscopical techniques to determine to what extent recognition by scavenger receptors utilizes a phagocytotic or another endocytotic mechanism. Furthermore, the morphological approach also allowed the visualization of the uptake of Ox-LDL in human liver tissue which is hitherto not described.

Materials and methods

Human serum albumin (HSA) and polyinosinic acid were purchased from Sigma (St. Louis, MO, USA). Gelatin and glycine were obtained from Merck (Darmstadt, West Germany). Tylose (MH 300) was purchased from Fluka (Buchs, Switzerland) and gold-conjugated antibodies from Aurion (Wageningen, Netherlands). Dulbecco's DMEM was obtained from Flow Laboratories (Irvine, Scotland, UK) and DiI was obtained from Molecular Probes (Eugene, OR).

Lipoprotein isolation and modification

LDL ($1.024 < d < 1.063$) was isolated from human plasma plus 1mM EDTA by density gradient centrifugation according to Redgrave et al. (11). LDL was dialysed against phosphate-buffered saline (PBS) containing 10 μM EDTA before being oxidized (200 μg of protein/ml) by exposure to $CuSO_4$ (5 μM free Cu^{2+} concentration) as described by Van Berkel et al. (10). Oxidation was arrested by cooling and addition of 200 μM EDTA. Oxidation of LDL was tested by assessing the electrophoretic mobility on agarose gel. In comparison

to native LDL, the Rf value of Ox-LDL was increased from 0.21±0.01 to 0.54±0.01, in accordance to the data of Van Berkel et al. (10).

Oxidized LDL was fluorescently labeled with DiI according to Pitas et al. (12). The density of the DiI and lipoprotein mixture was subsequently raised to 1.21, and the lipoproteins were reisolated according to the above described procedure. During the isolation of the labeled lipoproteins by density gradient centrifugation Ox-LDL was seen to be of a higher density in comparison to native LDL in accordance to previous data (13).

Animals and perfusion studies

For the visualisation studies on in vivo endocytosed Ox-LDL, 3-month-old male Wag/Rij rats were used, weighing about 200 grams. Following centrifugation (1 minute, 1200 g), oxidized LDL (50 μg/ml plasma) was injected into the vena cava inferior of overnight fasted rats under halothane anaesthesia and was allowed to circulate 2 or 10 minutes for light microscopical examination and 0.5, 2, 6 and 30 minutes for electron microscopic studies. For practical reasons Ox-LDL was injected into the portal vein to allow localization studies after 30 seconds of circulation. To study the involvement of the scavenger receptors in the uptake of Ox-LDL, polyinosinic acid, when indicated, was injected 1 minute prior to the lipoproteins in a concentration of 4 mg/kg body weight. Subsequently, rat livers were rinsed shortly with PBS up to 1 minute, or directly fixed with 4% paraformaldehyde (PF) and 0.1% glutaraldehyde (GA) in PBS by in situ perfusion via the portal vein for 10 minutes. Livers were stored in 2% PF in PBS. Rat livers that were only used for ultrastructural examination were fixed and stored according to the above described procedure using 2% GA in PBS.

Light- and electronmicroscopical studies

Fixed liver tissue was dissected and 200 μm vibratome slices were prepared. Specimens that were used for immunohistochemical study were embedded in 5% gelatin, and immersed in 2.3 M sucrose in PBS, overnight. Specimens that were used for fluorescence microscopy were directly immersed in 2.3 M sucrose. To prepare cryo-sections, small pieces were placed on a specimen holder and frozen in liquid nitrogen. Semithin or ultrathin cryosections were cut using a Reichert FC-4D Ultracut cryomicrotome at a temperature of -100 °C. To localize DiI fluorescence, semithin sections were placed on a glass cover slip and after being mounted with glycerol viewed with a Leitz ortholux microscope with standard rhodamine excitation and emission filters. Ultrathin sections were placed on carbon coated nickel grids for immunolabeling.

To differentiate between intracellular and extracellular compartments in order to study the internalisation mechanisms, 200 μm vibratome sections were stained en-bloc with the membrane mordant stain ruthenium red according to Handley et al. (14) and, subsequently, dehydrated in a graded series of ethanol and embedded in epon.

Livers that were used only for ultrastructural examination were postfixed in 1% OsO_4 in 0.15 M sodium cacodylate buffer for 45 minutes, dehydrated, and, embedded in epon. Ultrathin sections were examined in a Philips EM 410 electron microscope.
Antibodies and immunolabeling procedure

Antibodies against human apolipoprotein B (apoB), raised in rabbits, were kindly donated by Dr. L. Havekes (IVVO-TNO, Leiden, the Netherlands). The antibody was tested for reactivity with native and modified LDL using the double radial immunodiffusion method as described by Crowle (15). Results showed that Ox-LDL had sufficiently retained antigenicity to allow use of the antibody against native LDL for immunocytochemical studies. Cross-reactivity with the rat lipoproteins LDL, VLDL and HDL, and lipoprotein-deficient rat serum was absent (16). In some cases, mouse antibodies against ED_2 were used in double-labeling experiments to detect Kupffer cells. Antibodies against ED_2 were kindly donated by Dr. C. Dijkstra (VU, Amsterdam, the Netherlands). The specificity has been described previously (17).

For the immunolabeling procedure, antisera and gold conjugates were diluted in PBS containing 0.1% gelatin, 0.5% BSA and 0.1% Tween 20. The dilutions used were: anti-apoB, 600 fold, anti ED_2, 100 fold, goat anti-rabbit IgG-gold (6nm), 30 fold, and protein A-gold (10 nm), 50 fold. For the washing steps the same medium was used, unless otherwise indicated.

Cryosections were incubated with 0.05 M glycine in PBS, washed, incubated with 10% non-immune rat serum followed by incubation with the primary antibody, washed, incubated with the secondary antibody coupled to colloidal gold, washed, washed again with aqua dest, stained with uranylacetate and covered with tylose according to Tokuyasu (18). Double-labeling experiments were done according to the procedure described by Slot et al. (19). In control sections, the primary antibodies were omitted from the procedure and, non-immune rabbit serum was used instead.

Processing and labeling of human liver tissue

Human liver tissue (n=6, 4 female, 2 male, age between 44 and 74) was obtained from patients undergoing partial hepatic resection for liver tumors (Academic Medical Centre and Anthonie van Leeuwenhoek Hospital, Amsterdam, The Netherlands) under protocol of the Medical Ethical Commissions. Tissue blocks with apparently normal liver morphology were used for perfusion experiments within four hours after resection, while during this period liver tissue was preserved at 4°C. Fluorescently labeled Ox-LDL (20 μg/ml DMEM containing 1% HSA) was perfused (5 ml/min) through the pieces of liver via a portal vein for 2 or 10 minutes at 37°C, as previously described (20). To study the involvement of the scavenger receptor polyinosinic acid was perfused simultaneously, in some cases. The liver pieces were shortly rinsed by perfusion with PBS and fixed with 4% PF and 0,1% GA in PBS for 12 minutes. Control tissue was only rinsed with PBS and subsequently fixed according to the above procedure. After fixation, liver tissue was processed for light and electron microscopic examination as described for rat tissue to allow respectively detection of fluorescence and immunodetection of apoB.

Results and dicussions

Light microscopy

At 2 minutes after intravenous injection of DiI-Ox-LDL into rats, DiI fluorescence is found

to be mainly concentrated with Kupffer cells, while, to a lower extent, fluorescence is noticed with liver endothelial cells (Fig. 1a). At 10 minutes, the fluorescence intensity has increased with both cell types (Fig. 1b). Neither parenchymal cells, nor fat-storing cells contain any fluorescent signal at the indicated time points. The intensity of fluorescence concentrated with Kupffer cells is in accordance to previous biochemical data (10), which showed Kupffer cells to be the major cell type in the rat liver involved in the uptake of Ox-LDL. The association of fluorescence is greatly reduced for both Kupffer and endothelial cells, when, 1 minute prior to Ox-LDL polyinosinic acid was pre-injected (Fig. 1c). This demonstrates that the association of fluorescence with both cell types is subject to inhibition by polyinosinic acid and can be defined as scavenger receptor mediated (3, 10).

With human liver, a very similar visualization pattern is observed, showing DiI-Ox-LDL to be highly concentrated with Kupffer cells, while a less intensive fluorescent signal is found with endothelial cells (Fig. 1d, 1e). In contrast to rat liver, auto-fluorescence with human liver is found to be associated with lipid vacuoles of parenchymal cells (indicated by thick arrowhead). Perfusion of polyinosinic acid together with DiI-Ox-LDL strongly reduces fluorescent signal with both human liver Kupffer and endothelial cells (Fig. 1f), demonstrating the involvement of scavenger receptors. In addition to figure 1 liver tissue blocks from 5 other donors were analyzed. Although donors differed in gender, age and health status, the labeling pattern is very reproducible between the different donors. Earlier studies with

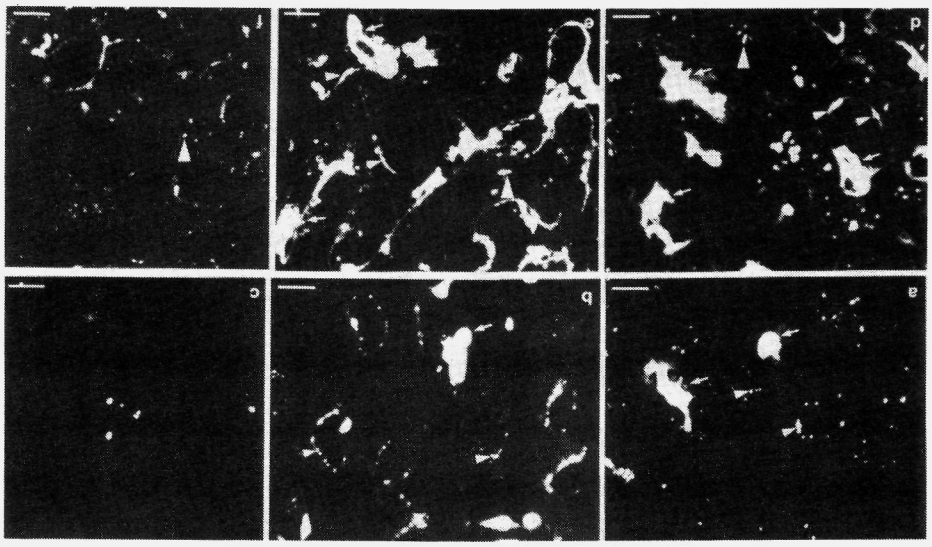

Fig. 1. Fluorescence microscopy of rat (a, b) and human (d,e) liver respectively 2 and 10 minutes after intravenous injection or after ex situ perfusion of DiI-Ox-LDL. Fluorescence is mainly concentrated with Kupffer (arrow), and to a smaller extend with endothelial cells (arrowhead). Addition of polyinosinic acid clearly diminishes the amount of fluorescence present both in rat (c), and human liver (f). Some autofluorescence is present in human parenchymal cells in fat vacuoles (thick arrowhead). Bars 6 μm.

human liver demonstrated considerable differences between donors in the relative uptake of the various liver cell types for native LDL (20). The amount of uptake of native LDL by human hepatocytes by a similar approach was found to correlate with the amount of LDL receptors (20). The receptors involved in the binding and uptake of Ox-LDL are, therefore, suggested to be consistently present in sufficient amounts on liver Kupffer and endothelial cells so allowing an adequate removal of Ox-LDL from the blood circulation. Our results with ex situ perfusion of human liver tissue blocks are sustained by recent biochemical data with isolated human Kupffer cells, which demonstrated that various recognition sites for modified LDL, including a specific Ox-LDL receptor, are present at a relatively high concentration (21).

Kupffer cells do not constitute a homogeneous population. Differences in size and endocytotic capacity of these cells in dependence of their position along the sinusoid have been reported (22, 23). In our experiments, Kupffer cells in portal as well as in central areas of the liver in both rat and human appear to participate in the uptake of Ox-LDL, suggesting that all liver macrophages do contain the receptor needed for the processing of Ox-LDL.

Electron microscopy

In rat liver, 30 seconds after portal vein injection, Ox-LDL, as demonstrated by immunolabeling of apoB, is mainly bound to the exterior of the plasma membrane of Kupffer cells. Immunolabel is localized in small clusters at regions without clathrin coating (Fig. 2a), occasionally in coated membrane invaginations (Fig. 2b), and bound to lamellipodia (2c). At this early time point, immunolabel is also found to be present inside structures localized in the cell periphery which are surrounded by membranes in cross-sections (Fig. 2d). These apparent vacuoles in reality represent cross-sections of 'sponge-like' structures, in which channels of extracellular fluid are surrounded by complex associations of organelle-free lamellipodia, as becomes evident on staining with the membrane mordant stain ruthenium red, which only stains the plasma membrane which is in direct contact with the extracellular medium (Fig. 2e). Although more than one type of scavenger receptor is present on Kupffer cells, as also demonstrated for other cell types (24-26), the majority of Ox-LDL binds to the Ox-LDL specific receptor (10). The low frequency of label in clathrin coated structures suggests that the Ox-LDL specific binding site on the Kupffer cells is predominantly localized outside coated pits.

Figure 3a shows that, besides the apparent vacuoles in the areas extended from the cell body, also tubular structures and apparent vesicles in the peripheral part of the cell body are surrounded by plasma membrane. The tubular structures are often demonstrated to be connected to the apparent vesicles. Similar structures are demonstrated to contain immunolabel at 6 minutes after the administration of Ox-LDL (Fig. 3b). It appears that, without actual internalization, bound ligand is transported into the cell body by movements of lamellipodia. In routinely plastic embedded liver tissue lamellipodia are demonstrated in continuity with worm-like structures (Fig. 4), suggesting that the lamellipodia arise from worm-like structures. The function of worm-like structures and their role in endocytosis of colloids have been debated previously (27). Our observations suggest that worm-like structures are involved in the formation of lamellipodia, and sponge-like structures by widening its enclosed space, and thereby allow binding and subsequent internalization of ligand.

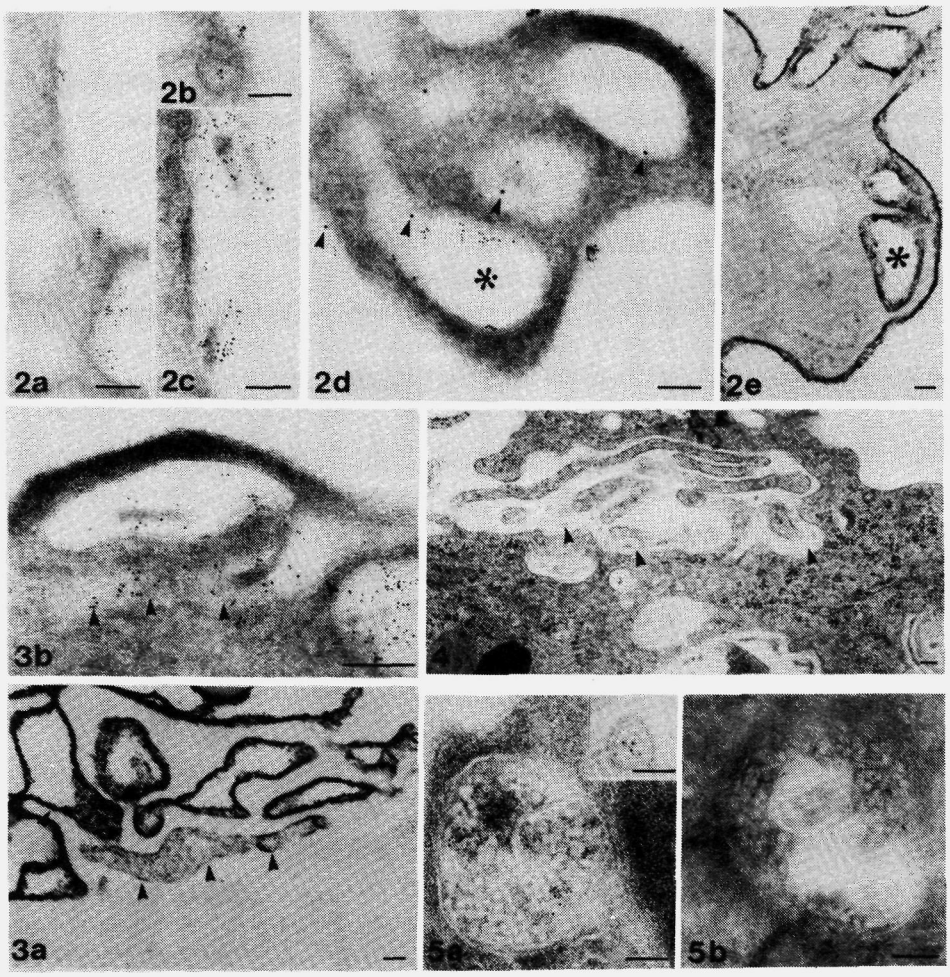

Fig. 2. Immunolabelling with anti-apoB of an ultrathin cryo-section 30 seconds after injection of Ox-LDL (a-d). Immunogold is localized with Kupffer cells at regions of the plasma membrane without clathrin coating (a), in coated pits (b), bound to lamellipodia (c), and d-in close proximity to the membrane of apparent vacuoles (asterisk). The latter structures are demonstrated in figure 4e to be in contact with the extracellular space by ruthenium red stain of subsequently epon embedded specimens (asterisk). Large gold particles in Figure 2e represent the Kupffer cell marker ED_2 (arrowheads). Bars 0.1 μm.

Fig. 3. Electron micrograph of an ultrathin epon section of specimen contrasted with ruthenium red. Plasma membrane and membrane invaginations (arrowheads) of the Kupffer cell are stained (a). These structures (arrowheads) can be demonstrated in Kupffer cells on ultrathin cryosections to contain immunolabel 6 minutes after administration of Ox-LDL (b). Bars 0.1 μm.

Fig. 4. Electron micrograph of rat liver, after fixation with 2% GA. Worm-like structures characterized by the electron dense central line are demonstrated in continuity with lamellipodia (arrowheads). Bars 0.1 μm.

Fig. 5. Appearance of immunoreactive apoB in Kupffer cells on ultrathin cryosections associated with lipoprotein-like particles in coated vesicles (a, inset), endosomes (a) and lysosomes, which also contain lipid inclusions (b), 6 minutes after injection of Ox-LDL. Bars 0.1 μm.

Internalization of Ox-LDL occurs partly through coated vesicle formation, as demonstrated by the occasional presence of labeled coated vesicles at 2 and 6 minutes after administration of Ox-LDL (Fig. 5a, inset). Coated vesicle formation is also involved in the uptake of other modified forms of LDL (24, 28-31). However, internalization of Ox-LDL predominantly seems to occur through membrane folding involving lamellipodia and worm-like structures, based on the amount of immunolabel associated with these structures. The ultrastructure of Kupffer cells has been described in detail already two decades ago with particular emphasis on endocytosis and phagocytosis (27, 32-35). Although involvement of lamellipodia in endocytosis has not yet been described, similar membrane folding and plasma membrane extensions have been described in peritoneal macrophages to be involved in the internalization of β-VLDL conjugated to colloidal gold (36, 37). The interaction of the lamellipodia with Ox-LDL is clearly different from pseudopodia engulfing particular matter during phagocytosis. Previous studies showed pre-aggregated modified LDL to be internalized by macrophages via phagocytosis (38, 39, 40). In these studies aggregates ranging in size up to several μm were demonstrated in different stages of engulfment. Phagocytosis of aggregates was never seen to occur in vivo in our studies.

At 6 minutes after injection of Ox-LDL, label is mainly demonstrated dispersed over vesicles ranging in size up to 0.6-0.7 μm. The label is associated with spherical particles of about 23 nm in diameter, probably representing still intact Ox-LDL (Fig. 5a). These particles are only observed in liver of animals that were injected with Ox-LDL, and not in untreated animals. Vesicles with this size are not stained by ruthenium red (not shown), suggesting these structures to represent the first intracellular compartment. The apparent detachment of particles and immunolabel from the vesicular membrane, may represent uncoupling of Ox-LDL from its receptor, a process which is known to take place in endosomes. Together with the ruthenium red exclusion, and timing we define this compartment as endosomal. Transport of ligand through endosomes to lysosomes is apparent from the presence of immunoreactive apoB in vesicles which contained not only label and ligand detached from the vesicle membrane, but also membranous material and lipid inclusions (Fig. 5b). Lysosomes as defined by their capacity to degrade biological material, are the final compartment of the Ox-LDL particles as demonstrated by biochemical experiments (10). The appearance of labeled lysosomal structures already at 6 minutes after injection of Ox-LDL is in agreement with the rapid degradation of Ox-LDL by Kupffer cells (10). The appearance of lipid in the lysosomes is previously demonstrated in peritoneal macrophages by Fukuda et al. (29). At 30 minutes after administration, immunolabel is exclusively localized in endosomes and lysosomes and no longer bound to the plasma membrane or in apparent vesicular or tubular structures.

Ox-LDL is also observed in the endothelial cells of the rat liver. Thirty seconds after portal vein injection of Ox-LDL, some label is detectable bound to uncoated regions of the plasma membrane, but most label is present in coated pits (Fig. 6a). The scavenger receptors involved in binding of Ox-LDL by endothelial cells are apparently mainly localized in coated pits, comparable to the Ac-LDL-scavenger receptor (31). At 2 minutes after injection less label is detectable at the plasma membrane than at 30 seconds, most of the immunogold is present dispersed over large electron lucent vesicles ranging in size up to 0.7 μm (Fig. 6b). These structures are intracellular, as similar structures are excluded from the ruthenium red

staining. In analogy to the Kupffer cells, these observations support the idea of the endosomal nature of these early label containing vacuoles. These structures often show tubular membrane extensions, which are not labeled (Fig 6b). This compartment might be functionally comparable to the Compartment of Uncoupling Receptor and Ligand (CURL), as described by Geuze et al. for liver parenchymal cells (41). Detachment of Ox-LDL particles from its receptor is indicated by the appearance of ligand in the vesicular lumen. The absence of immunolabel in the tubular extensions may indicate that the extensions are more likely to be involved in the recycling of the (scavenger) receptors to the plasma membrane than in ligand delivery. At 6 and 30 minutes after injection of Ox-LDL, the number of the vesicles that contain immunolabel increased. Besides in large electron lucent vesicles, immunolabel is present in more electron dense vesicles (Fig 6c). The gradual increase in electron density of the endosomal structures is in accordance to the maturation of endosomes into lysosomes (27, 31, 42, 43), consistent with biochemical data that demonstrated lysosomal degradation of Ox-LDL (10) in liver endothelial cells.

Besides Kupffer and endothelial cells, no other cell types in the rat liver could be demonstrated to contain significant amounts of immunolabel.
In human liver, due to the fact that anti-apoB antiserum recognizes also apoB from endoge-

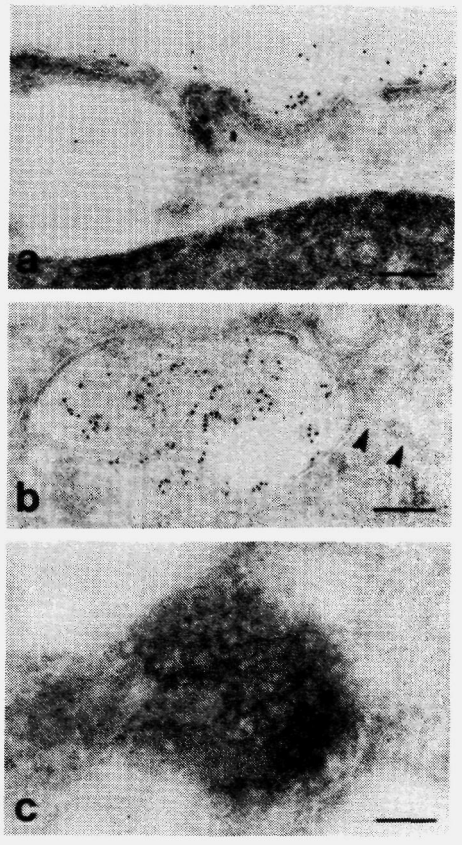

Fig. 6. Ultrathin cryosections of rat liver endothelial cells after injection of Ox-LDL respectively for 30 seconds (a), 2 (b) and 6 minutes (c). Immunolabel is localized, 30 seconds after administration bound to uncoated and coated areas of the plasma membrane. After 2 minutes immunolabel is localized dispersed over vesicles, which often contained membrane extensions which were devoid of immunolabel (arrowheads). Increasing with time immunolabel is noticed dispersed over vesicles of a higher electron density (c). Bars 0.1 μm.

nous origin, human parenchymal cells contained substantial amounts of immunolabel, also when the liver was not perfused with Ox-LDL. This endogenous apoB was mainly localized in the bile canalicular zone in multi-vesicular structures and associated with the Golgi-apparatus. Some label was also seen close to the plasma membrane in multi-vesicular structures and extracellularly (not shown). These observations are in accordance with synthesis of apoB and VLDL excretion as morphologically illustrated by Alexander et al. (44). Endothelial and Kupffer cells were never found to contain any immunolabel with control livers.

At 2 minutes after beginning of Ox-LDL perfusion, immunoreactive apoB was localized in Kupffer cells in small clusters bound to the plasma membrane, to lamellipodia (Fig. 7a), and in electron lucent roundish structures (Fig 7b). The electron lucent roundish structures were often seen to continue into more electron dense, tubular shaped structures, showing great similarity to the earlier described tubulo-vesicular structures from rat liver Kupffer cells (Fig. 2d). After 10 minutes, immunolabel is still localized in the above mentioned structures, but is now also found dispersed over vesicles of a higher electron density (Fig. 7c). Lysosomes were not seen to contain immunoreactive apoB at any of the time points studied. Coated vesicles nor coated vesicle formation was noticed.

After 2 and 10 minutes of circulation human liver endothelial cells showed immunostaining, mainly dispersed over relatively electron lucent vesicles, while part of the label is also bound to the plasma membrane. After 10 minutes, low amounts of label are detectable at the plasma membrane, but immunolabel was mostly restricted to the relatively electron lucent vesicles and to some vesicles of a higher electron density (Fig. 7d), comparable to structures labeled in the rat endothelial cells (Fig. 6b-c).
Although not all described structures, that were found to be involved in the processing of Ox-LDL in rat liver Kupffer and endothelial cells, could be demonstrated in the human liver endothelial and Kupffer cells, all Ox-LDL containing structures found in human liver, were also seen in rat liver. Coated pits and coated vesicles could not be demonstrated in human tissue, probably because there is some delay in actual fixation, which may allow some continuation of internalization after Ox-LDL has been removed from the perfusate. Immunolabel was not observed in lysosomal structures of human Kupffer cells, probably because of a slower processing of the Ox-LDL particles in this ex situ perfusion system. A more rapid degradation in human liver versus rat liver is not likely, since one would then expect lipid or membranous material to accumulate in the lysosomes as noticed with rat liver (Fig. 5b).

In conclusion, our results show that Ox-LDL, intravenously injected into rats, or perfused through human liver tissue becomes rapidly concentrated in Kupffer cells and to a lesser extent in endothelial liver cells. Characterization of the in vivo interaction of Ox-LDL in rats by immunoelectron microscopy indicates that binding of Ox-LDL to Kupffer cells occurs primarily to regions of the plasma membrane without clathrin coating. For internalization different mechanisms occurred simultaneously a) by coated vesicles, and b) by lamellipodia and worm-like structures. Subsequently immunoreactive Ox-LDL was detected in relatively electron lucent endosomes, whereafter finally the lysosomal compartment was reached. For rat endothelial cells the mechanism of uptake exhibited a mechanism clearly

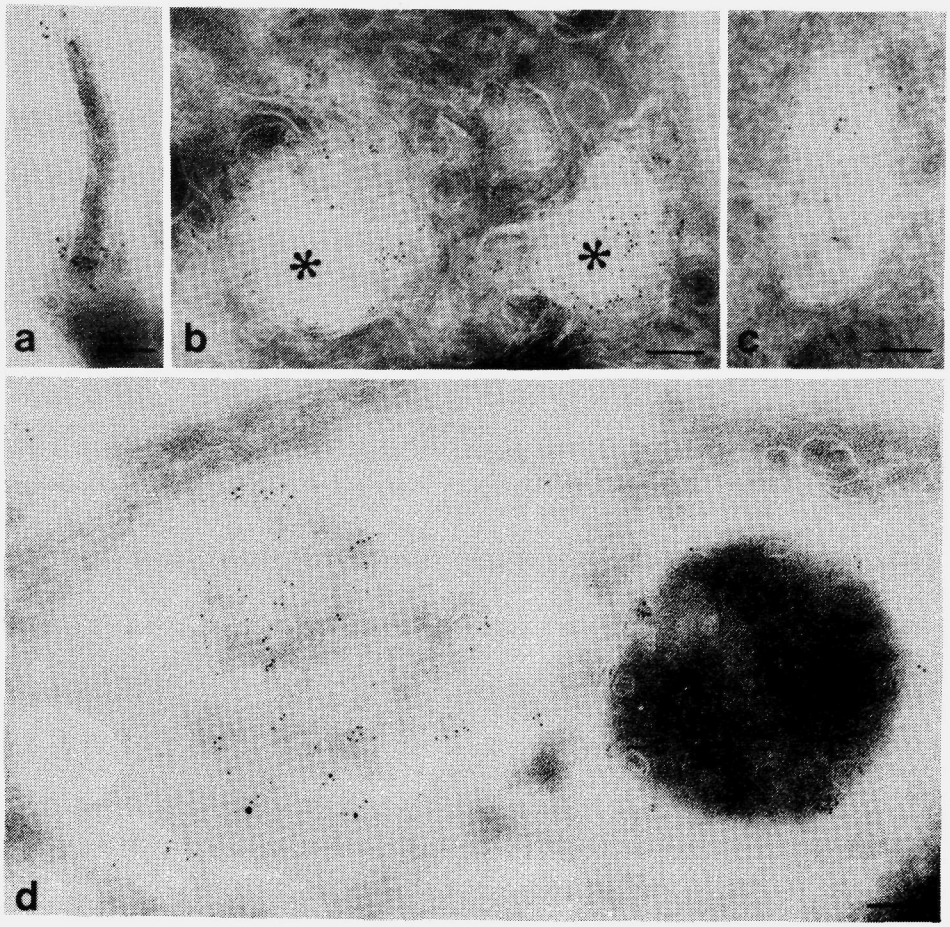

Fig. 7. Electron micrograph of ultrathin cryosections of human liver cells 2 (a, b) and 10 minutes (c, d) after the beginning of perfusion. a - Immunogold is localized bound to the exterior of the plasma membrane of Kupffer cells to lamellipodia (a), and b - in close proximity to the membrane of apparent vacuoles, which are connected to more electron dense tubular structures (asterisk). c - At 10 minutes after perfusion immunolabel representing apoB is also localized in electron lucent vesicles in Kupffer cells. Endothelial cells contained immunoreactive apoB, localized in vesicles of different electron densities (d). Bars 0.1 μm.

different from that of Kupffer cells. Binding of Ox-LDL to liver endothelial cells is mainly observed in coated pits, while cellular processing involves large electron lucent endosomes (size up to 0.7 μm) before the lysosomal compartment is reached. The mechanism of uptake and processing of Ox-LDL by human liver utilize similar ultrastructural features as observed with rat liver cells. These observations allow an extension of the animal studies to the human situation establishing that liver cells, particularly Kupffer cells form a highly effective protective system against the occurrence of atherogenic Ox-LDL particles in the blood.

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CHAPTER EIGHT

An electron microscopic autoradiography study on the uptake and processing of [^{3}H]cholesteryl oleate labelled acetylated or oxidized low density lipoproteins in rat liver.

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submitted for publication

Summary

The role of rat liver cell organelles in the uptake and transport of acetylated or copper-oxidized human low-density lipoproteins (Ac-LDL and Ox-LDL, respectively), labelled with [^{3}H]cholesteryl oleate, was visualized with electron microscopic autoradiography. At 2, 15 and 30 min after injection of Ac-LDL or Ox-LDL into rats, livers were fixed by perfusion fixation. In liver endothelial cells, large endosomes and irregularly formed lysosomes were observed, containing lipoprotein particles. In Kupffer cells, lipoprotein particles were associated with worm-like structures and extensive plasma membrane foldings. Kupffer cells showed long plasma membrane extensions in the space of Disse, which made contact with the microvilli of parenchymal cells. In parenchymal cells, the appearance of electron dense dots, associated with the periphery of mitochondria and rough endoplasmic reticulum proximate to mitochondria, was noticed. Their appearance was time-dependent which suggested a relation with the processing of cholesterol and lipids derived from Ac-LDL or Ox-LDL. In liver endothelial cells, autoradiographic grains, representing the cholesterol moiety derived from Ac-LDL or Ox-LDL, were initially associated with the endosomal compartment, whereas in Kupffer cells grains were initially localized on worm-like structures. In both cell types, grains shifted to the lysosomal compartment at later time points. A small number of grains was also localized on the rough endoplasmic reticulum. The association of grains on the microvilli of parenchymal cells after injection of Ac-LDL, was only apparent at 2 min after injection, indicating direct interaction of Ac-LDL with this cell type. However, when Ox-LDL was injected, association of grains with the microvilli was also prominent at 15 and 30 min after injection. The latter observation is indicative for the transport of (free) cholesterol from Kupffer cells to parenchymal cells. Most of the grains in parenchymal cells, were localized on the periphery of mitochondria and on cisternae of the rough endoplasmic reticulum associated with mitochondria. No grains were observed on lysosomes or peroxisomes. These results indicate, that the cholesteryl ester moiety of Ac-LDL and Ox-LDL is processed by endothelial and Kupffer cells, prior to the transport of free cholesterol to the parenchymal cells. In the parenchymal cells, mitochondria and endoplasmic reticulum are of major importance for the processing of cholesterol derived from Ac-LDL or Ox-LDL.

Introduction

The uptake of modified low-density lipoproteins (LDL) by macrophages leads to an accumulation of cholesteryl esters in the cell and finally to the formation of foam cells [1]. The different forms of modification of LDL include acetylation [2] and oxidation [3], of which oxidation is pathophysiologically relevant [4]. In the blood, acetylated LDL (Ac-LDL) and oxidized LDL (Ox-LDL) are rapidly removed by the liver sinusoidal cells [5]. In this way the liver forms a protective system against atherogenic particles in the blood compartment. With radioactive iodine tracer studies [5] we have shown earlier, that Ac-LDL is rapidly taken up by the liver endothelial cells, whereas Ox-LDL is mainly directed to the Kupffer cells. In recent studies we showed that a major part of the cholesteryl ester moiety of Ac-LDL [6] and Ox-LDL [M.N. Pieters et al., submitted] is metabolized to bile acids and secreted into the bile. The biliary secretion rate of bile acids derived from Ox-LDL cholesteryl esters was 10-13 fold higher than of bile acids derived from Ac-LDL. With isolated livers perfused with medium, we provided evidence that the rapid secretion of bile acids derived from Ox-LDL, was not serum-dependent and, therefore, did not depend on HDL as a cholesterol transport vehicle. We suggested that Kupffer cells may utilize a direct route to transport cholesterol to the parenchymal cells.

In other recent studies we visualized the processing of Ac-LDL [S. Esbach, et al., submitted] and Ox-LDL [7] on the light and electron microscopical level. We showed that the uptake of modified LDL by endothelial cells was mediated by coated vesicles. Kupffer cells mediated the uptake of modified LDL by membrane foldings and worm-like structures, while also uptake through coated vesicles could be observed. In the present study we focused on the processing of cholesteryl esters from Ac-LDL and Ox-LDL and visualized the fate of the cholesteryl ester moiety of these ligands by electron microscopic autoradiography.

Materials and methods

Isolation and labelling of human LDL

LDL and lipoprotein deficient serum (LPDS) were isolated from normolipidemic serum by density ultracentrifugation as described by Redgrave et al. [8]. After dialysis against 10.1 mM phosphate-buffered saline (PBS) pH 7.4/ 1 mM EDTA, [1α,2α(n)-^{3}H]cholesteryl oleate (Amersham, USA) was incorporated according to Blomhoff et al. [9]. [^{3}H]cholesteryl oleate labelled LDL was reisolated by density ultracentrifugation [8], dialysed against PBS/10μM EDTA and modified by acetylation or oxidation. Acetylation of ^{3}H-LDL was carried out by the repetitive addition of acetic anhydride [10]. ^{3}H-LDL (200 μg/ml) was oxidized by exposure to 10 μM free copper ions ($CuSO_4$) at 37 °C during 20 h [5]. The relative oxidative mobility (Rf) on agarose gel were 0.51 for Ac-LDL and 0.52 for Ox-LDL.

Uptake of Ac-LDL or Ox-LDL and perfusion of rat liver

Male Wistar rats (12 weeks old) were fasted overnight and anaesthesized by an intraperitoneal injection of Nembutal (1 ml/kg bodywt). Approx. $40x10^6$ dpm (2 mg of protein) of [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL was injected into the vena penis of the rat. At 2, 15 or 30 min after injection the vena porta was cannulated and the liver was preperfused at 5 ml/min with 0.15 M sodium cacodylatebuffer pH 7.2 at room temperature

during 1-2 min. Subsequently the liver was fixated by perfusion with 2% glutaraldehyde in 0.15 M sodium cacodylate buffer during 5 min. The liver was excised and stored in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer at 4°C.

Electron microscopic autoradiography

Liver tissue was dissected and 150 μm-thick tissue blocks were prepared using a vibratome (Oxford, Oxford, England). These blocks were postfixed with 1% OsO_4 in 0.15 mM sodium cacodylate buffer pH 7.2, containing 1.5% potassium ferrocyanide during 1 h at 4 °C. The blocks were washed 5-7 times with 0.15 M sodium cacodylate buffer (pH 5.5) for 30 min and stained en bloc with Waltons lead aspartate solution at 60 °C for 30 min [11,12]. Subsequently, the tissue blocks were washed 4 times in 0.15 M sodium cacodylate buffer (pH 5.5) during 5-7 minutes, dehydrated in graded series of ethanol at 4 °C and the tissue was embedded in Epon (LX 122, Ladd Research Industry Inc., Burlington, Vt).
The embedded tissue blocks were trimmed and ultrathin sections (± 90 nm) were cut using an ultramicrotome (Reichert OmU2, Vienna, Austria). The sections were transferred to microscope slides which had been previously coated with formvar. After drying overnight, the sections were carbon coated (4-6 nm layer). The slides were then dipped in L4 emulsion (Ilford TTD, Basildon, England) diluted 1:2 (v/v) in twice-distilled water and dried at room temperature for 15-30 min. Slides were kept in closed plastic boxes in the presence of silica.

After an exposure time of 120 days at 4 °C, the ultrathin sections were developed according to Kopriwa [13] using Elon developing agent (Eastman Kodak Company, Rochester, NY) and preceded by gold latensification to improve the sensitivity of the emulsion [14]. The formvar film carrying the section and developed emulsion was separated from the slide. Grids were placed on the film and dried overnight. The electron microscopic autoradiograms were examined using a Philips-410 electron microscope (Philips, Eindhoven, The Netherlands).

Distribution of grains among the non-parenchymal liver cells

To estimate the distribution of radiolabel among the liver endothelial and Kupffer cells, 20-50 endothelial cells and 10-20 Kupffer cells were scored on the presence of grains at each time point. For Ac-LDL most of the endothelial cells were positive (i.e. cells contained grains), whereas only one out of six Kupffer cells showed grains. When Ox-LDL was injected, the majority of both cell types was positive. The distribution pattern of grains between liver endothelial and Kupffer cells reconciled with the biochemical data [5]. Since the total amount of radiolabel in the cells was fairly low, quantification of the results with statistical evaluation was not possible. Therefore, the results presented are descriptive rather than absolute.

RESULTS

Electron microscopical structure of liver endothelial, Kupffer and parenchymal cells after injection of modified LDL.

In liver endothelial cells, Ac-LDL and Ox-LDL were taken up by a process of coated pits and coated vesicles. Already at 2 min after injection, particles with a size of approx. 18 nm

were present in endosomes (Fig 1a), which were interpreted as being modified LDL particles. At later time points, large irregular lysosomal structures were formed (Fig. 1b), in which these particles could no longer be recognized.

In Kupffer cells, the initial uptake of Ac-LDL or Ox-LDL was mediated through worm-like structures and extensive membrane foldings (Fig 1c,e). At 2 min after injection, lipoprotein particles could be recognized in these structures. At 15 and 30 min after injection of modified LDL, the number of lysosomes in Kupffer cells was increased. Long plasma membrane extensions of Kupffer cells were observed, insinuating the endothelial lining and making contact with the microvilli of the parenchymal cells (Fig 1c,d). Very often Kupffer cells directly lined to parenchymal cells (Fig 1d)

In parenchymal cells, electron dense dots could be observed after injection of modified LDL. These dots (60-100 nm) were localized on cisternea of the rough endoplasmic reticulum proximate to mitochondria and also on the periphery of mitochondria (Fig 1f). Since these dots were hardly observed in control livers, or in livers at 2 min after injection, we suggest that they may contain lipid derived from modified LDL.

Autoradiographic analysis of the uptake and processing of cholesteryl esters from Ac-LDL and Ox-LDL

The uptake and processing of [^{3}H]cholesteryl oleate labelled Ac-LDL and Ox-LDL were visualized by means of autoradiography. The routing of grains in the cells, representing the routing of the cholesterol (ester) moiety of modified LDL in the cells, appeared to be the same for Ac-LDL as for Ox-LDL. Therefore, the presented pictures are representative for both forms of modified LDL. Table 1 shows the percentual distribution of grains over the liver cell organelles at different time points after injection. In liver endothelial cells, at 2 min after injection of modified LDL, most of the grains were localized on coated vesicles or on endosomal structures (Fig. 2a,b). At 15 and 30 minutes, grains were mainly observed on the lysosomal compartment (Fig. 2c) and sometimes on tubular stuctures (2b) and membranes of the rough endoplasmic reticulum (fig 2h). In Kupffer cells, at 2 and 15 min after injection of modified LDL, radiolabel was present on the plasmamembrane and in worm-like structures (Fig. 2d). Localization of grains on lysosomes and RER was evident at 15 and 30 min after injection (Fig. 2e).

The association of grains with the microvilli of parenchymal cells (2h) after injection of Ac-LDL was only evident at 2 min after injection (Table 1), indicating initial association of

Fig. 1. Ultrastructure of liver endothelial cells (a,b), Kupffer cells (c-e) and cytoplasm of parenchymal cells (f) at 2 min (a,c,d) and 15 min (b,d-f) after i.v. injection of [^{3}H]cholesteryl oleate labelled acetylated (a, b) or oxidized (c-h) low density proteins.
a and b: detail of an endothelial cell showing pinocytotic vesicles (pv), endosomes (end) along the sinusoidal side (sin) (a) and lysosomes (lys) along the Space of Disse (sd). c - e: overview (c,d) and detail (e, from c) of a Kupffer cell showing worm-like structures (WL) and long cellular extensions from the Kupffer cells running to and intermingled with the microvilli of parenchymal cells (PC). f: detail of the cytoplasm of parenchymal cell showing electron dense dots (arrows) with a diameter of 80 n. These dots are associated with cisternae of rough endoplasmic reticulum and with the outermembrane of mitochondria. Note the presence of the - particles with a diameter of about 18 nm (small arrowheads) in pinocytotic vesicles and endosomes (a) of endothelial cells and in the worm-like structures (e) in Kupffer cells suggesting the modified LDL-particles injected. Original magnification:a and b 22,000x; c 5,300 x; d 8,800x; e 27,500x and f 47,500 x.

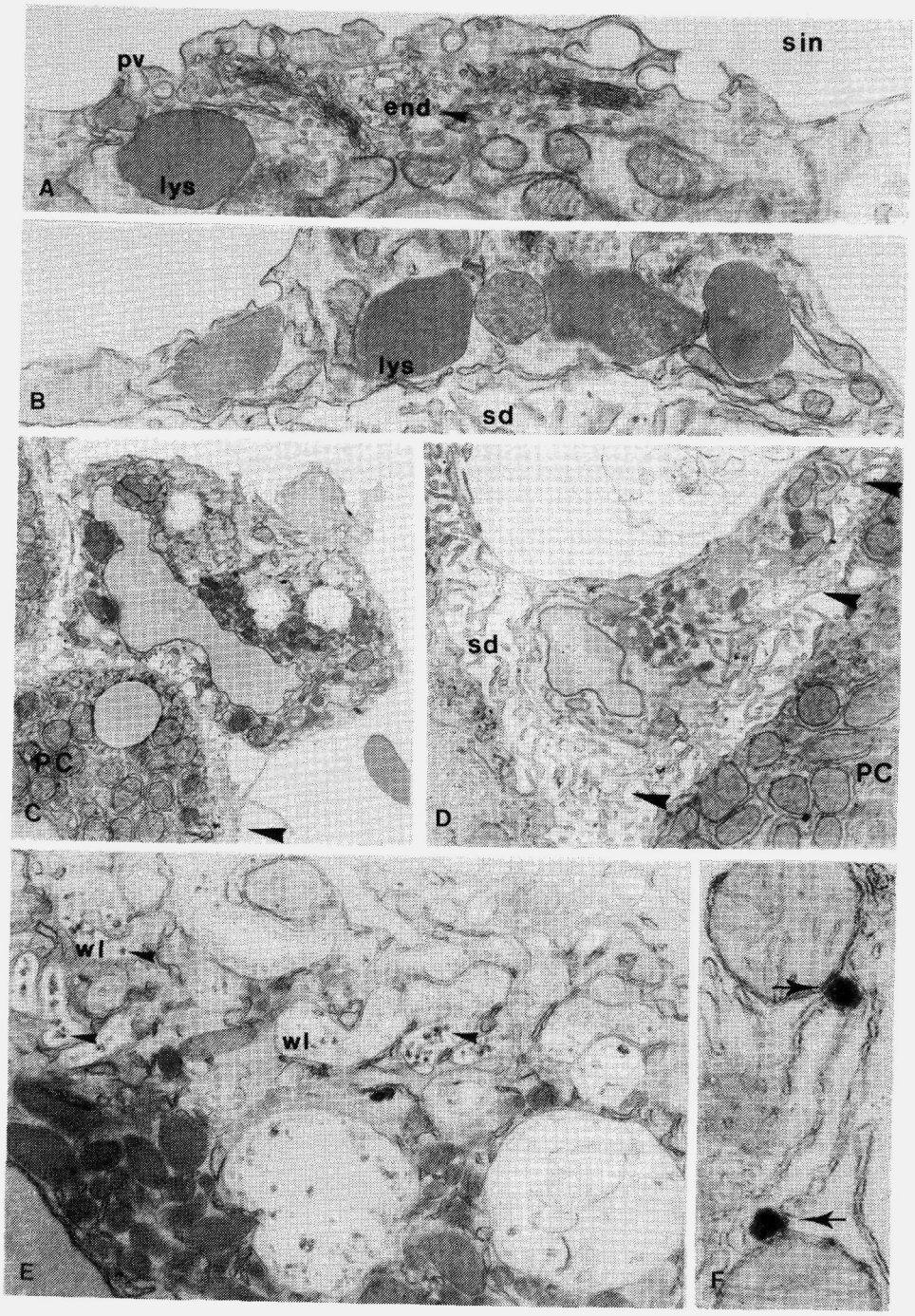
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Table 1. Percentual distribution of autoradiographic grains over liver cells at different time points after i.v. injection of [^{3}H]cholesteryl labelled Ac-LDL or Ox-LDL expressed as a percentage of the total number of grains counted.

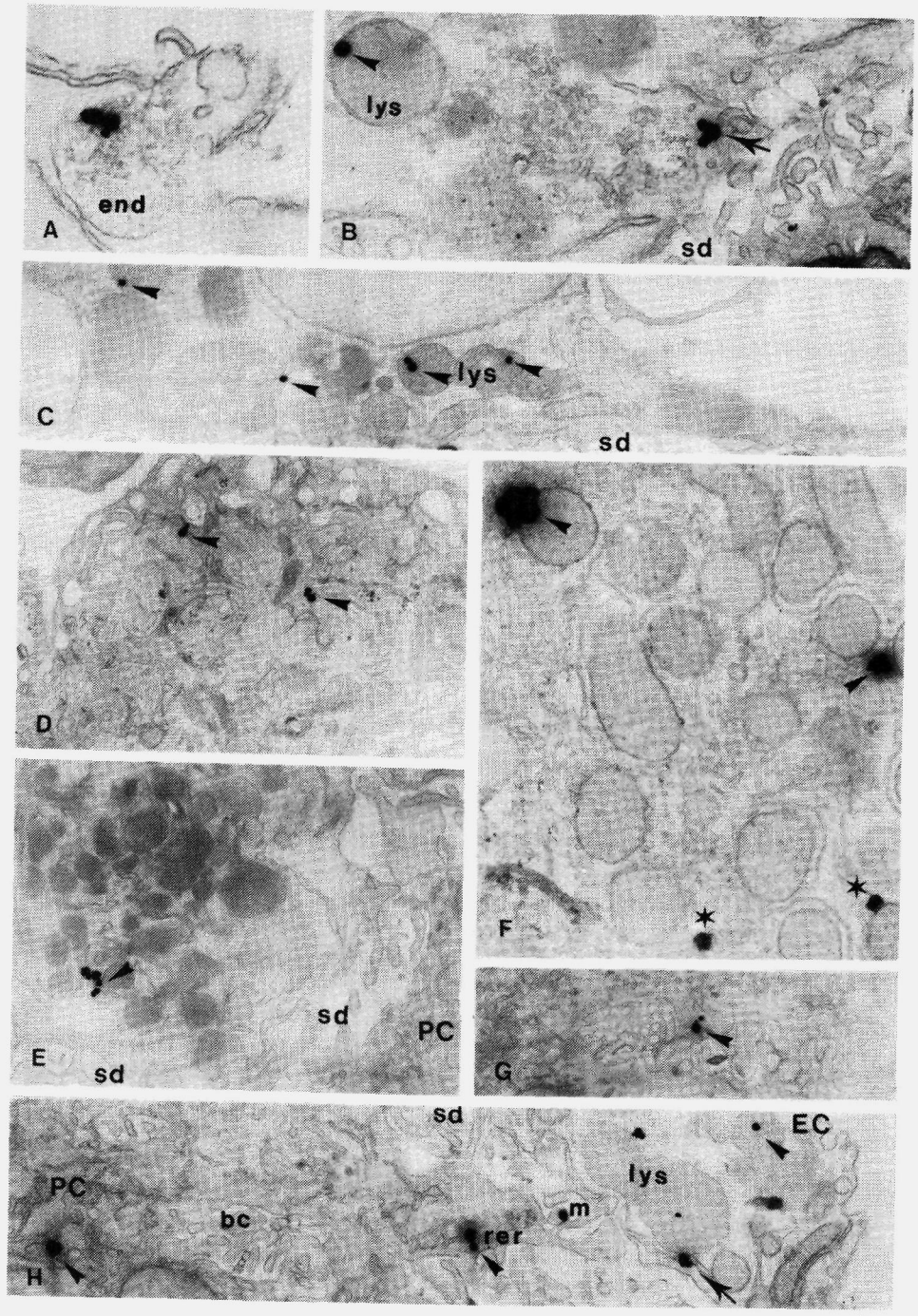
	Endothelial cells						Kupffer cells						Parenchymal cells					
	Ac-LDL			Ox-LDL			Ac-LDL			Ox-LDL			Ac-LDL			Ox-LDL		
	2'	15'	30'	2'	15'	30'	2'	15'	30'	2'	15'	30'	2'	15'	30'	2'	15'	30'
Plasma membrane/endosomes	81	93	29	60	28		33	40	50									
Worm-like structures							33	20		100	14							
Microvilli													26		3	17	22	33
Vesicles at plasma membrane															3	9	11	
Lysosomes		7	24	40	63	100	33	40	50		43	83						
Golgi apparatus			6										17	8	13	17	13	
RER	13		35		2						43	17	22	20	42	17	24	
SER													13				11	
Mitochondrial membranes													29	56	10	50	26	44
Lipid droplets														8	6		12	
Nucleus	6		6		7								1		13	9		
Bile canalicus													1	12	3		5	22
Number of grains	16	14	17	5	46	10	6	5	2	3	7	6	77	25	31	12	76	9
Percentage of positive cells	31	29	54	18	52	26	14	10	20	50	35	60	nd	nd	nd	nd	nd	nd

Ac-LDL with parenchymal cells. However, when Ox-LDL was injected, grains associated with the microvilli could be observed at all time points. A substantial part of the grains in parenchymal cells was observed on membranes of the rough endoplasmic reticulum associated with mitochondria and on the outermembrane of mitochondria (Fig 2f). No label was observed in lysosomes, peroxisomes or multivesicular bodies (Table 1). Sometimes label was found in vesicles near bile canaliculi (Fig 2h) or close to membranes of bile canaliculi (Fig 2g).

Discussion

In previous biochemical studies we have established the contribution of liver endothelial and Kupffer cells to the total hepatic uptake of Ac-LDL and Ox-LDL [5]. Labelling Ac-LDL and Ox-LDL in its cholesteryl ester moiety with [^{3}H]cholesteryl oleate resulted in the processing of cholesteryl esters and the secretion into the bile as bile acids [7, and M.N. Pieters et al., submitted]. In this electron microscopical autoradiography study, we visualize the uptake and processing of cholesteryl ester-labelled Ac-LDL and Ox-LDL in the rat liver. The contribution of liver endothelial cells and Kupffer cells to the total hepatic uptake of Ac-LDL and Ox-LDL, is reflected by the distribution of grains among these cell types.

Fig. 2. Electron microscopic autoradiograms of liver endothelial cells (a,b,c,h), Kupffer cells (d,e) and parenchymal cells (f,g,h) with grains at 2 min (a,b,d) and 15 min (c,e-h) after i.v. injection of [^{3}H] cholesteryl oleate labelled acetylated (a-c) or oxidized (d-h) low-density lipoproteins.
a - c,h: detail of endothelial cells, showing grains on endosomes (a), lysosomes (arrow heads) (b,c), tubular structures (arrow) (b) and a short strand of rough endoplasmic reticulum (arrow) (h). d and e: Kupffer cell showing grains (arrow heads) on worm-like structures and a short strand of endoplasmic reticulum (e).
f - g: detail of parenchymal cell showing grains (arrow heads) on microvilli (m) (h), on vesicles in the neighbourhood of bile canaliculus (bc) (h) or close to the bile canaliculus (g), and on strands of rough endoplasmic reticulum in close association with mitochondria (f).
Please, note the presence of electron dense dots (asterix) associated with strands of the rough endoplasmic reticulum in the endothelial (b,h) and the Kupffer cells (e). These strands are close to the sinusoidal sites of these cells. Original magnification: a 47,500x; b 27,500x; c,d and g 17,000x; e,f and h 22,000x.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It is clear that both liver endothelial cells and Kupffer cells direct Ac-LDL and Ox-LDL to the lysosomal compartment. In endothelial cells, grains marking Ac-LDL or Ox-LDL gradually shift from the pinocytotic vesicles on the plasmamembrane to endosomes and lysosomes, during the time course of the experiment. In Kupffer cells, grains end up rapidly in lysosomes after initially being internalized through worm-like structures. Also the mitochondria and the rough endoplasmic reticulum seem to be involved in the processing of the cholesteryl ester moiety from modified LDL. This indicates that within 15 min after injection, the lipid moiety of modified LDL is separated from the apolipoprotein moiety which is degraded in the lysosomes. Hydrolysed cholesterol is then rapidly transported from the lysosomes through membrane structures. In contrast with liver endothelial and Kupffer cells, parenchymal cells showed no grains on lysosomal structures when Ac-LDL or Ox-LDL was injected, and also peroxisomes were always unlabelled.

The relatively strong association of grains with microvilli at 2 min after injection of Ac-LDL is indicative for the initial uptake of part of the Ac-LDL particles by parenchymal cells. With Ox-LDL however, grains on microvilli were also present at the later time points, indicating the transport of cholesterol derived from Ox-LDL from Kupffer to parenchymal cells. From biochemical data it is clear that the fate of this cholesterol moiety is secretion into the bile. For both types of modified LDL we found no evidence for a lysosomal routing in parenchymal cells nor for the involvement of peroxisomes. The presence of grains on the mitochondria and the rough endoplasmatic reticulum associated with mitochondria, strongly suggest that these cell organelles are associated with the transport of cholesterol in parenchymal cells. Moreover, at 15 and 30 min after injection of Ac-LDL or Ox-LDL, electron dense dots could be observed. These electron dense dots may contain cholesterol and other lipids, which are processed along the rough endoplasmic reticulum for secretion into the bile.

In a previous biochemical study in rats, we showed that the biliary secretion of radio-labelled bile acids derived from [^{3}H]cholesteryl oleate labelled Ox-LDL was higher than with Ac-LDL, though resecretion of radiolabelled cholesterol in the serum was lower [M.N. Pieters et al., submitted]. We suggested that Kupffer cells utilize a direct route for transporting cholesterol to parenchymal cells. Under in vivo conditions, this may be mediated through membrane extensions of Kupffer cells and microvilli of parenchymal cells. It can be anticipated that such a direct transfer of cholesterol between Kupffer and parenchymal cells will be rapid and therefore difficult to visualize, since the detection of the grains depends on the residence time of the label in a particular compartment. The results obtained in this study, however, do support that such a rapid cholesterol transfer may exist.

It is clear that also liver endothelial cells do have contact with the microvilli of parenchymal cells. However, this contact seems to be due only to the membrane activity of parenchymal cells, rather than to the membrane activity of endothelial cells. In contrast, Kupffer cells are highly membrane-active cells, as is shown by the occurrence of worm-like structures and membrane-foldings, which appear to be induced after the injection of modified LDL particles. They also diplay numerous cellular extensions which are intermingled with the microvilli of the parenchymal cells. Membrane activity may therefore be a prerequisite for the rapid transport of cholesterol from non-parenchymal to parenchymal cells.

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CHAPTER NINE

Results and general discussion

9.1 Selective delivery of HDL cholesteryl esters to the liver

Epidemiological studies have shown that high levels of HDL are correlated with an decrased risk for atherosclerosis [1]. The beneficial effect of HDL in the pathogenesis of atherosclerosis has been related to the potential role of HDL in transporting cholesterol from peripheral tissues to the liver [2]. In order to perform its role in reverse cholesterol transport HDL should be (i) an efficient acceptor for peripheral cholesterol and (ii) an efficient donor of HDL cholesterol to the liver. The role of HDL as cholesterol acceptor has mainly been studied in vitro (see chapter 2), and different theories concerning the mechanism of cholesterol efflux from cells have been proposed [3-9]. The role of HDL as cholesterol donating particle has been studied in vitro as well as in vivo. It has been shown, that HDL selectively delivers its cholesterol (esters) to a variety of cell types, including liver and steroid hormone producing cells [10-19]. In chapter 3, in vivo evidence is provided that HDL selectively delivers its cholesteryl esters to the liver and that this selective delivery is exerted within the liver only by the parenchymal cells. At 10 min after injection of HDL labelled with [^{3}H]cholesteryl oleate in the rat, the uptake of HDL cholesteryl esters was 5-6 fold higher than the apolipoprotein. In contrast, liver endothelial and Kupffer cells exerted no significant difference between between cholesteryl ester and apolipoprotein uptake. Parenchymal cells appear to be responsible for 90.8% of the HDL cholesteryl ester uptake by the rat liver. It was shown earlier [20] that high affinity binding sites with similar binding characteristics exist on liver parenchymal, endothelial and Kupffer cells. It can, therefore, be concluded that although the recognition sites of HDL on the various cell types may be identical, the selective delivery of cholesteryl esters to parenchymal cells in vivo depends on additional specific cellular properties.

Liver parenchymal cells can secrete cholesterol into the bile, either as unmodified cholesterol or after metabolic conversion into bile acids. In order to study the kinetics of biliary secretion, we used rats cannulated in the bile duct, the duodenum and the heart [21, 22]. It appeared that HDL cholesteryl esters were more efficiently processed to bile acids when compared with LDL cholesteryl esters, under conditions that liver uptake of HDL and LDL were similar. Hydrolysis of cholesteryl esters appeared to be necessary for biliary secretion, since with [^{3}H]cholesteryl oleoyl ether labelled HDL, the secretion in the bile was very slow. With both [^{3}H]cholesteryl oleate labelled HDL and LDL, secretion in the bile was mainly in the form of bile acids. The difference between HDL and LDL cholesteryl ester processing may therefore not be the conversion into bile acids itself, but rather the rate of availability of the cholesterol moiety of the lipoprotein for bile acid synthesis. The efficient coupling of the selective uptake of HDL cholesteryl esters by the parenchymal cells to bile acid synthesis supports the role of HDL in reverse cholesterol transport.

9.2 Role of apolipoprotein composition

Clinical studies have shown that low HDL levels in coronary artery disease are linked to low levels of HDL containing only apoA-I (denoted as LpA-I), whereas levels of HDL containing both apoA-I and apoA-II (LpA-I/A-II) were not different [23]. The role of apolipoproteins in the HDL-mediated cholesterol efflux has been discussed by various groups. Studies in vitro have indicated that the HDL subclass LpA-I has a greater potency to decrease cellular cholesterol levels [24-26]. However, in vivo data concerning the role of apolipoproteins in the selective cholesteryl ester delivery to the liver were not available. In chapter 4 immunopurified HDL is utilized and it appears that cholesteryl esters initially associated with LpA-I and LpA-I/A-II can both be selectively cleared from the circulation when compared to the apolipoprotein moiety. The kinetics of serum decay and liver association of cholesteryl esters from LpA-I and LpA-I/A-II were similar. Thus, the selective delivery of cholesteryl esters from HDL to the liver does not depend on the apolipoprotein binding to the cell. However, a significant difference in biliary secretion between LpA-I and LpA-I/A-II was apparent in the first dark period (8-20 h after injection). A clear day/night rhythm was much more evident for LpA-I and resulted in a higher biliary secretion of radiolabelled bile acids when expressed cumulatively. It can be concluded that though the selective delivery of cholesteryl esters is independent of the apolipoprotein composition, apolipoproteins may influence the subsequent intracellular cholesterol transport to bile acid synthesizing enzymes.

9.3 HDL as an in vivo acceptor of cholesterol from non-parenchymal liver cells

The in vivo injection of [^{3}H]cholesteryl oleate labelled Ac-LDL or [^{3}H]cholesteryl oleate labelled Ox-LDL in rats, leads to a major uptake in liver endothelial and Kupffer cells, respectively (chapter 5, 6). The transport of radiolabelled cholesterol from liver endothelial cells (injection of Ac-LDL) to parenchymal cells was shown to be mediated by serum HDL [27], supporting in vivo the role of HDL in reverse cholesterol transport. When [^{3}H]cholesteryl ester labelled Ox-LDL was injected, cholesteryl esters were rapidly hydrolyzed and transported from the Kupffer cells to the parenchymal cells. The biliary secretion in the first 2 h after injection was found to be 3 fold higher than with Ac-LDL (chapter 5) and showed a lag-phase of only approx. 15 min. When expressed in 10 min time intervals, the biliary secretion was approx. 10-13 fold higher than with Ac-LDL (chapter 6). The percentage of radioactivity associated with HDL however, was 2-fold less than with Ac-LDL. This suggested that cholesterol was transferred to the parenchymal cells through a more direct route. The rapid transport of cholesterol between Kupffer cells and parenchymal cells may be mediated by direct cell-cell contact. It has been established [28] that pseudopodia of Kupffer cells can penetrate through the endothelial fenestrae and make contact with the microvilli of the parenchymal cells. To investigate this hypothesis we utilized isolated perfused rat livers (chapter 6). In this system the influence of extrahepatic tissues is excluded, while the perfusion medium can be modulated. The secretion of radiolabelled bile acids after injection of Ox-LDL was 40-fold higher than with Ac-LDL and comparable with the in vivo situation. It thus appears that an intact liver architecture is essential for the rapid cholesterol transport between Kupffer and parenchymal cells. The efflux of radiolabelled cholesterol from Kupffer cells to the perfusion medium, could be promoted by the addition of HDL, supporting the potential role of HDL as cholesterol accepting particle. However,

the increased cholesterol efflux to the perfusate did not influence the secretion of radiolabelled bile acids. It can thus be concluded that the serum compartment does not play a role in the rapid transport of cholesterol from Kupffer cells to parenchymal cells.

9.4. The visualization of the processing of Ac-LDL and Ox-LDL

Chapter 7 describes the visualization of Ox-LDL by light and electron microscopy. The distribution of Ox-LDL between the different liver cell types was similar as found biochemically [29]. At the electron microscopical level, differences do exist between the mechanism of uptake of Ox-LDL by Kupffer cells and liver endothelial cells. Ox-LDL bound to Kupffer cells was internalized by membrane-folding and worm-like structures or via coated vesicle formation. Liver endothelial cells took up Ox-LDL only via coated pits. The results in rat liver and human liver were essentially the same, indicating that the processing of Ox-LDL in rat liver is representative for the human situation. In chapter 8 the processing of cholesteryl esters derived from [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL is studied by means of electron microscopic autoradiography. The association of grains to the microvilli of parenchymal cells after injection of Ac-LDL was only evident at 2 min after injection. This indicates the initial interaction of Ac-LDL with parenchymal cells and subsequent internalization. In contrast, when Ox-LDL was injected, grains associated with the microvilli of parenchymal cells were also prominent at 15 and 30 min after injection. This suggest that cholesteryl esters, initially taken up and hydrolyzed by the Kupffer cells, had been transported to the parenchymal cells. The association of grains with the endoplasmic reticulum in liver endothelial and Kupffer cells, suggest that a stabilizing protein may be added to the lipid particle (for example: sterol carrier protein$_2$). In Kupffer cells, worm-like structures and rapid membrane-movements appear to be induced after injection of Ac-LDL or Ox-LDL. These worm-like structures and rapid membrane movements may provide the Kupffer cells with an unique system to rapidly transport cholesterol to the parenchymal cells.

9.5 HDL and cholesterol transport between liver cells: a conclusive model

This thesis describes the role of HDL in reverse cholesterol transport and the interaction of HDL with liver cells in particular. The HDL-mediated cholesterol efflux from non-parenchymal liver cells, the selective delivery of HDL cholesteryl esters to parenchymal cells and the suggested rapid direct route for the transport of cholesterol from Kupffer cells to parenchymal cells and bile are summarized in Fig. 1.

Ac-LDL is mainly internalized by the liver endothelial cells through binding of Ac-LDL to the scavenger receptor. Within the endothelial cells, hydrolysis of cholesteryl esters occurs in the lysosomes. Free cholesterol can be released by the endothelial cells by either i) diffusion through the aqueous layer present between the plasmamembrane and HDL or ii) released to HDL after binding of HDL to the cell. In HDL free cholesterol is converted into cholesteryl esters by the enzyme LCAT and transported to the core of the HDL particle. HDL then delivers its cholesteryl esters selectively to the parenchymal cells. Within the parenchymal cells, cholesteryl esters of HDL are hydrolysed and converted into bile acids which are then secreted in the bile.

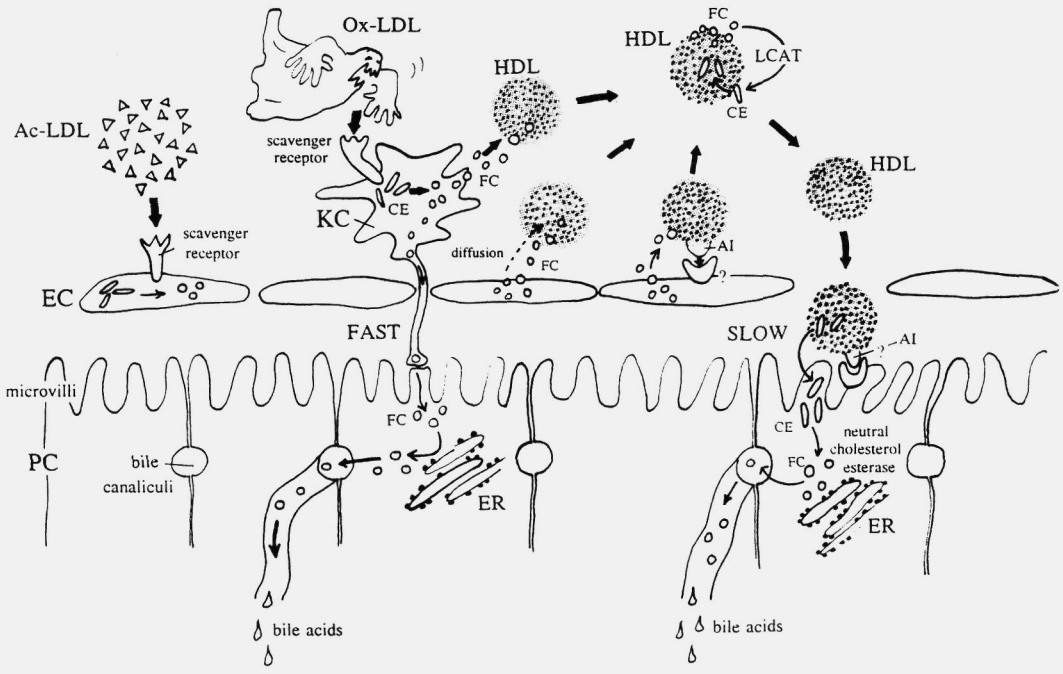

Fig. 1 Schematic representation of cholesterol transport from liver endothelial and Kupffer cells to parenchymal cells and bile. O = free cholesterol; ⬭ = cholesteryl ester.

Ox-LDL is mainly taken up by the Kupffer cells through binding to the scavenger receptor (Ox-LDL receptor). Internalization of Ox-LDL is predominantly accomplished by membrane-foldings and worm-like structures. Hydrolysis of cholesteryl esters in the lysosomes occurs. Free cholesterol is then transported from the Kupffer cells to the parenchymal cells by two different routes. The major route which occurs very fast, depends on direct cell-cell contact between Kupffer and parenchymal cells. Free cholesterol entering the parenchymal cell is directly converted into bile acids and secreted into the bile. Additionally, Kupffer cells may release free cholesterol to HDL on which cholesterol is esterified and transported to the parenchymal cells as described above. The latter route occurs at a slower rate.

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PERSPECTIVES

Though the role of HDL in reverse cholesterol transport is generally accepted, the exact mechanism by which HDL accepts cholesterol from peripheral cells and subsequently delivers to the liver is still not elucidated. It has been shown before, that cholesteryl esters of HDL are selectively delivered to the liver and other tissues. In this thesis is shown that within the liver, only parenchymal cells exert selective uptake of HDL cholesteryl esters. The mechanism by which cholesteryl esters are specifically taken up by the liver remains unclear, although it has been suggested that specific domains in the plasmamembrane may be more susceptible for the selective delivery of cholesteryl esters to the cell [1]. If this is the case, parenchymal cells should exhibit more cholesteryl ester delivery plasma membrane sites than liver endothelial cells and Kupffer cells. When HDL is labelled with cholesteryl oleoyl ether instead of cholesteryl oleate, selective delivery is quantitatively identical. This implies that hydrolysis of cholesteryl esters does not contribute to the selective delivery route. Future experiments may be carried out by labelling HDL with fluorescent analogs for the cholesteryl ester moiety, and analyse the uptake site by means of low light video microscopy. Additional information, obtained by electron microscopy, might contribute to the elucidation of the involvement of specific membrane structures in the uptake of HDL cholesteryl esters.

In this thesis the processing of cholesteryl esters derived from Ac-LDL and Ox-LDL is described. It is clear by labelling Ac-LDL and Ox-LDL in the apolipoprotein and the cholesteryl ester moiety, that modified LDL is take up as an integral particle. It has been established earlier [2], that Ac-LDL is in vivo mainly directed to the liver endothelial cells, whereas the majority of Ox-LDL is taken up by the Kupffer cells. Differences also exist in the further processing of cholesteryl esters derived from Ac-LDL and Ox-LDL with respect to the transport of secreted cholesterol and the velocity of secretion in the bile. It appears that when cells do not directly interact with liver parenchymal cells (i.e. liver endothelial cells and peripheral cells), HDL is important as a transport vehicle for cholesterol. On the contrary, when cells do have a direct interaction with parenchymal cells (i.e. Kupffer cells), transport by serum HDL is of minor importance. In this thesis we suggest that cholesterol can be delivered to parenchymal cells much faster by membrane-membrane interactions. This phenomenon would indicate a very efficient intercellular communication system between parenchymal and Kupffer cells. Upon injection of modified lipoproteins, worm-like structures and rapid membrane movements are induced in the Kupffer cell. The function of the worm-like structures is still not known. The results of our studies suggest that worm-like structures may be involved in the processing and transport of cholesteryl esters from modified lipoproteins. If this is the case, worm-like structures may provide the Kupffer cells with an unique transport system, not only for cholesterol but possibly for numerous other compounds (i.e. endotoxin compounds). Future research may provide further evidence for a potential role of worm-like structures and membrane-foldings in intercellular communication in the liver. The importance of cellular contact between liver parenchymal cells and Kupffer cells, may be visualized by coculturing parenchymal and Kupffer cells (i.e. at opposing sites of a filter). Cells may be able to make contact through the pores of the filter and exchange metabolites in this way. Such studies may provide additional information on the quantitative aspects and the mechanism of direct cell-cell interactions in the liver.

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SUMMARY

High levels of high-density lipoprotein (HDL) cholesterol in the blood are correlated with a decreased risk for coronary artery disease. The protective effect of HDL against atherosclerosis has been related to the role of HDL in "reverse cholesterol transport", the transport of peripheral cholesterol to the liver. In the liver parenchymal cells, cholesterol is eliminated by secretion into the bile, either unmodified as free cholesterol or after metabolic conversion into bile acids. In this thesis the role of HDL as transporting vehicle for cholesterol has been studied. HDL was labelled with [^{3}H]cholesteryl oleate and its in vivo processing was investigated in the rat. Cholesteryl esters of HDL were selectively delivered to the liver at a 3-fold higher rate than the apolipoprotein moiety. The selective uptake of HDL cholesteryl esters was only exerted by the liver parenchymal cells. Parenchymal cells, which took up 5-6 fold more cholesteryl esters than apolipoprotein, contributed for 90.8 % to the total liver uptake. In contrast, liver endothelial cells and Kupffer cells took up cholesteryl esters in parallel with the apolipoprotein. When compared to cholesteryl esters from low-density lipoproteins (LDL), cholesteryl esters of HDL were more efficiently processed to bile acids and secreted into the bile. In rats, cannulated in the bile, duodenum and heart, the secretion of radiolabelled bile acids derived from HDL was 2-fold higher than with LDL, under conditions that the kinetics of liver uptake of HDL and LDL cholesteryl esters were similar. The efficient processing of HDL cholesteryl esters by the liver does support the role of HDL in reverse cholesterol transport.

In order to study the involvement of apolipoprotein composition in the processing of HDL cholesteryl esters, [^{3}H]cholesteryl oleate labelled HDL was subjected to immunoaffinity chromatography. The HDL subclass containing only apolipoprotein A-I (LpA-I) was separated from the HDL subclass containing both apolipoproteins A-I and A-II (LpA-I/A-II). In rats, both LpA-I and LpA-I/A-II showed selective delivery of cholesteryl esters to the liver with similar kinetics. However, a significant difference in biliary secretion was apparent at 8-20 h after injection (first dark period). The secretion of bile acids derived from cholesteryl esters initially associated with LpA-I, was significantly higher than the secretion of bile acids derived from LpA-I/A-II. It thus appears that, though selective delivery does not depend on apolipoprotein composition, apolipoproteins influence the further processing of HDL cholesteryl esters.

In vivo, the role of HDL in reverse cholesterol transport was investigated by "loading" liver endothelial and Kupffer cells with [^{3}H]cholesteryl oleate and studying the transport of cholesterol to the parenchymal cells. "Loading" of liver endothelial or Kupffer cells with cholesteryl esters, was accomplished by the injection of [^{3}H]cholesteryl oleate labelled Ac-LDL or Ox-LDL, respectively. These modified lipoproteins are rapidly removed from the circulation by the scavenger receptor present on liver sinusoidal cells. When liver endothelial cells were "loaded" with cholesteryl esters derived from Ac-LDL, cholesterol was transported by HDL from endothelial cells to the parenchymal cells which subsequently secreted cholesterol as bile acids into the bile. When Kupffer cells were "loaded" with cholesteryl esters from Ox-LDL however, the role of HDL in cholesterol transport to parenchymal cells was less evident, though secretion of radiolabelled bile acids occurred at a higher rate. This suggested that Kupffer cells utilize a more direct route for the transport of cholesterol to parenchy-

mal cells. Cholesterol may be rapidly transferred by the membrane interaction of pseudopodia of Kupffer cells and microvilli of parenchymal cells. This was further investigated by using isolated rat livers, perfused with medium. In this system the influence of extrahepatic tissues is excluded. It appearred that also in this system, cholesteryl esters from Ox-LDL were more rapidly transported from Kupffer cells to parenchymal cells and bile than cholesteryl esters from Ac-LDL. HDL stimulated the efflux of cholesterol to the perfusate. However, the increased cholesterol efflux to HDL did not influence the secretion of radiolabelled bile acids. It is concluded that the intact liver architecture is essential for the rapid cholesterol transport between Kupffer and parenchymal cells.

In order to visualize the uptake and processing of cholesteryl esters of Ac-LDL and Ox-LDL, an electron microscopic autoradiography study was carried out. Liver endothelial cells internalized Ac-LDL and Ox-LDL only via coated vesicles, whereas Kupffer cells internalized these modified LDL-particles mainly through membrane-foldings and worm-like structures, though some uptake through coated vesicles did occur. In both cell types Ac-LDL and Ox-LDL were directed to the lysosomes. The association of grains with the microvilli of parenchymal cells, after injection of Ac-LDL was only evident at 2 min after injection. This indicated initial association of Ac-LDL to parenchymal cells. When Ox-LDL was injected, however, the association of grains with the microvilli of parenchymal cells was evident at all time points, which suggested that cholesterol derived from Ox-LDL was rapidly transported to the parenchymal cells. It is concluded that the high-membrane activiy of Kupffer cells (worm-like structures and membrane-foldings) provide the Kupffer cell with a mechanism for rapidly transporting cholesterol to the microvilli of parenchymal cells.

SAMENVATTING

In het bloed worden cholesterol en andere lipiden getransporteerd in de vorm van kleine bolletjes, de lipoproteïnen. Bij de mens kunnen vier verschillende klassen van lipoproteïnen onderscheiden worden, waarvan het lage-dichtheids lipoproteïne (LDL) kwantitatief de belangrijkste is. Een hoog LDL-cholesterol gehalte is een belangrijke risicofactor voor het ontstaan van hart- en vaatziekten. In tegenstelling hiermee is een hoog gehalte van het hoge-dichtheids lipoproteïne (HDL) gerelateerd aan een verlaagd risico voor atherosclerose. Aangenomen wordt, dat dit veroorzaakt wordt door de rol van HDL in het zogenaamde "omgekeerde cholesterol transport", het transport van cholesterol van de bloedvaten naar de lever. In de lever kan cholesterol irreversibel uit het lichaam worden verwijderd door secretie in de gal. Hierbij wordt cholesterol ofwel direct, ofwel na omzetting van cholesterol tot galzuren, uitgescheiden. In dit proefschrift is de rol van HDL in het transport van cholesterol naar de lever bestudeerd. Hiertoe werd HDL gemerkt met radioactieve cholesterolesters en ingespoten in de rat. In de lever werden relatief meer cholesterolesters dan eiwit (apolipoproteïne) vanuit HDL opgenomen. Deze selectieve opname van cholesterolesters werd geheel veroorzaakt door de parenchymcellen van de lever. De parenchymcellen zijn de belangrijkste leverceltypen en verantwoordelijk voor de secretie van gal in de darm. De leverendotheelcellen en de Kupffercellen, namen de cholesterol-esters en de apolipoproteïnen van HDL in gelijke mate op. Teneinde de secretie van cholesterol en galzuren in de gal te bestuderen, is gebruikt gemaakt van een proefdiermodel, waarbij ratten werden gecannuleerd in het galkanaaltje, de dunne darm en de halsader. HDL, gemerkt met radioactieve cholesterolesters, werd via de halsader ingespoten. Door vervolgens de gal af te tappen, werd de secretie van radioactiviteit in de gal gevolgd. Het bleek dat cholesterolesters afkomstig van HDL efficiënt door de parenchymcellen werden omgezet tot galzuren en uitgescheiden in de gal. Vergeleken met cholesterolesters van LDL werden, onder omstandigheden van gelijke leverassociatie, tweemaal zoveel radioactieve galzuren gesecreteerd. Deze efficiënte verwerking van cholesterolesters van HDL, vormt een ondersteuning van de rol van HDL als transportmiddel voor cholesterol van de bloedvaten naar de lever.

Om te onderzoeken of het eiwitgedeelte van het HDL een rol speelt bij de afgifte van cholesterolesters aan de lever, werd het HDL met behulp van immunoaffiniteits-kolomchromatografie in twee klassen gescheiden. Eén subklasse bevatte het apolipoproteïne A-I, terwijl de andere subklasse zowel

apolipoproteïne A-I als A-II bevatte. De selectieve afgifte van cholesterolesters van HDL aan de lever was onafhankelijk van de apolipoproteïnesamenstelling. Wel was een verschil waarneembaar in de snelheid van galsecretie. HDL met alleen apolipoproteïne A-I, vertoonde een hogere galsecretie dan HDL met zowel apolipoproteïne A-I als A-II. Hoewel de eiwitsamenstelling van HDL dus niet direct de snelheid van afgifte van cholesterolesters bepaalt, blijkt de eiwitsamenstelling wel de verdere verwerking tot galzuren te beïnvloeden.

Teneinde het omgekeerde cholesteroltransport optimaal te laten verlopen, dient HDL niet alleen zijn cholesterolesters efficiënt aan de leverparenchymcellen af te geven, maar moet HDL ook in staat zijn om cholesterol uit cellen buiten de lever op te nemen. Het transport van cholesterol tussen leverendotheel- en Kupffercellen is bestudeerd in intacte ratten, waarbij gebruik gemaakt is van, met radioactieve cholesterolesters gemerkt, geacetyleerd (Ac-LDL) of geoxideerd LDL (Ox-LDL). Door acetylering of oxydatie wordt de eiwitstructuur van LDL zodanig veranderd, dat LDL herkend wordt door de zogenaamde "scavenger-receptor". Opname via deze receptor gaat zeer snel en blootstelling van leverendotheel- en Kupffercellen aan gemodificeerd LDL, leidt dan ook tot een snelle "oplading" van de cellen met cholesterolesters. Na intraveneuze toediening wordt Ac-LDL voornamelijk opgenomen door de leverendotheelcellen, terwijl Ox-LDL juist voornamelijk wordt opgenomen door de Kupffercellen. De opgenomen cholesterolesters worden door beide leverceltypen snel gehydrolyseerd tot vrij cholesterol. Bij leverendotheelcellen (opgeladen met Ac-LDL) bleek, dat het transport van cholesterol van de leverendotheelcel naar de parenchymcel en uiteindelijk naar de gal afhankelijk was van HDL. Daarentegen bleek het cholesteroltransport van Kupffercellen naar parenchymcellen (Kupffercellen opgeladen met Ox-LDL) sneller te verlopen. Hierbij was de secretie van gemerkt cholesterol naar HDL in het serum twee keer zo laag als wanneer leverendotheelcellen waren opgeladen met Ac-LDL, terwijl de galsecretie drie keer zo hoog lag. Mogelijk kunnen de Kupffercellen door een direct membraancontact met de parenchymcellen dit snelle cholesteroltransport bewerkstelligen. Morfologisch zijn er aanwijzingen dat de pseudopodia ("pootjes") van de Kupffercellen zich door de fenestrae ("kleine gaatjes") van de leverendotheelcellaag kunnen dringen, waardoor contact met de, onder de endotheelcellen liggende, parenchymcellen mogelijk is. Om deze hypothese te onderzoeken is gebruik gemaakt van een geïsoleerde rattelever, doorstroomd met een fysiologische oplossing. Door de rat eerst te injecteren met Ac-LDL of Ox-LDL, werden respectievelijk de leverendotheelcellen of Kupffercellen "opgeladen" met

gemerkte cholesterolesters. Tijdens de doorstroming van de lever met de fysiologische oplossing werden zowel de efflux van cholesterol naar de doorstoomde oplossing, alsook de secretie in de gal gemeten. Ook onder deze condities werd na Kupffercelbelading een snel cholesteroltransport naar de gal (en dus de parenchymcellen) gevonden. De uitscheiding van radioactief gemerkte galzuren na het opladen van Kupffercellen, was 40 keer hoger dan na het opladen van leverendotheelcellen. Toevoegen van HDL aan de door de lever stromende oplossing, leidde tot een verhoogde cholesterolefflux van de lever naar de fysiologische oplossing, maar beïnvloedde niet de snelheid van secretie in de gal. Dit bevestigt de hypothese dat Kupffercellen beschikken over een directe, snelle route voor het transport van cholesterol naar de parenchymcellen.

De verwerking van Ox-LDL in de rattelever en humane lever werd lichtmicroscopisch bestudeerd met behulp van fluorescerend Ox-LDL. Tevens werden electronenmicroscopische technieken gebruikt. Kupffercellen nemen Ox-LDL voornamelijk op via membraanstructuren, die zich snel de cel in verplaatsen (membraanvouwingen en "wormachtige" structuren). Het mechanisme van opname was identiek voor rattelever en humane lever en geeft aan, dat in de mens Ox-LDL op dezelfde manier verwerkt wordt. De worm-achtige structuren in Kupffercellen dragen mogelijk bij tot het snelle cholesteroltransport in de Kupffercel. Met behulp van een autoradiografische techniek werden radioactief gemerkte cholesterol(esters) in de cel zichtbaar gemaakt. Hiertoe werd een fotografische emulsie (film) op de cellen aangebracht. Na ontwikkeling van deze film bleek dat cholesterol, afkomstig van Ox-LDL en initieel voornamelijk opgenomen door Kupffercellen, op alle onderzochte tijdstippen geassocieerd was met het plasmamembraan van parenchymcellen. Dit was in tegenstelling tot cholesterol, afkomstig van Ac-LDL en initieel voornamelijk opgenomen door endotheelcellen, dat alleen vlak na inspuiting van Ac-LDL met het plasmamembraan van de parenchymcel was geassocieerd. Deze resultaten ondersteunden de hypothese dat direct cholesteroltransport wel optreedt van Kupffercellen naar parenchymcellen, maar niet van endotheelcellen naar parenchymcellen. Er werd geconcludeerd dat het directe, snelle cholesteroltransport tussen Kupffer- en parenchymcellen waarschijnlijk wordt bepaald door de hoge membraanactiviteit van de Kupffercellen.

LIST OF ABBREVIATIONS

Abbreviations

ACAT	Acyl-CoA:cholesterol acyltransferase
Ac-LDL	Acetylated low-density lipoprotein
Apo	Apolipoprotein
BSA	Bovine serum albumin
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
DiI	Dioctadecyl-tetramethyl-indocarbocyanine perchlorate
EDTA	Ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid
EC	Endothelial cell
ER	Endoplasmatic reticulum
FC	Free cholesterol
HDL	High-density lipoprotein
KC	Kupffer cell
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
Ox-LDL	Oxidized low-density lipoprotein
PAAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Parenchymal cell
SDS	Sodium dodecyl sulphate
VLDL	Very-low density lipoprotein

NAWOORD

Dit proefschrift is het produkt van vier jaar onderzoek. Een tijd van vallen, opstaan en hard werken. Het is echter ook een tijd geweest van veel gezelligheid (borrels!) en leuke congressen. Graag zou ik langs deze weg enkele mensen willen noemen, die mij gedurende deze tijd op verschillende wijzen tot steun zijn geweest.

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CURRICULUM VITAE

Monique Nicole Pieters werd geboren op 10 juli 1964 te Rotterdam. In 1982 behaalde zij het Atheneum B diploma aan het St. Laurenscollege te Rotterdam.

Van 1982 tot 1988 studeerde zij Levensmiddelentechnologie aan de Landbouw Universiteit te Wageningen met als afstudeervakken: toxicologie (hoofdvak), levensmiddelenchemie en celbiologie en immunulogie. In het kader van de toxicologie werd een stage doorlopen aan het Unilever Research Laboratory, Sharnbrook, Engeland. In september 1988 werd het doctoraalexamen behaald.

Van december 1988 tot en met december 1992 was zij werkzaam als onderzoeker in opleiding (o.i.o.) voor de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) bij de afdeling Biofarmacie van het "Leiden-Amsterdam Center for Drug Research". Vanaf 1 april 1993 is zij werkzaam als toxicoloog aan het Rijksinstituut voor volksgezondheid en milieuhygiëne (RIVM) te Bilthoven.

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submitted for publication