High density lipoproteins and reverse cholesterol transport

Hoge dichtheids lipoproteïnen en omgekeerd cholesterol transport

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Voor mijn ouders

Contents

Abbreviations

acLDL	acetylated LDL
apo	apolipoprotein
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
catLDL	cationized LDL
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
DMEM	Dulbecco's modified Eagle's medium
dma	dimethyl acetal
DMS	dimethyl suberimidate
EC	esterified cholesterol
FC	free (unesterified) cholesterol
FCS	fetal calf serum
GLC	gas-liquid chromatography
HAT	hypoxanthin/aminopterin/thymidin
HSA	human serum albumin
HDL	high density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
MUFA	monounsaturated fatty acids
PAGE	polyacrylamide gel electrophoresis
PL	phospholipid
PUFA	polyunsaturated fatty acids
SDS	sodium dodecyl sulphate
SFA	saturated fatty acids
SPM	sphingomyelin
TC	total cholesterol
TG	triglyceride
TNM	tetranitrometane
UC	unesterified (free) cholesterol
VHDL	very high density lipoprotein
VLDL	very low density lipoprotein

16:0	palmitic acid
18:1	oleic acid
18:2	linoleic acid
20:4	arachidonic acid
20:5	eicosapentaenoic acid

Chapter 1

General Introduction

1.1 Cholesterol homeostasis and high density lipoprotein-mediated reverse cholesterol transport

In Western societies coronary heart disease is responsible for about 25% and 15% of the total mortality of men and women, respectively, in the age group of 45 to 64 years [1]. Atherosclerosis plays a crucial role in the development of coronary heart disease because this process causes the narrowing of blood vessels. The structure of the inner layer of the vascular wall, the intima, changes during atherosclerosis. The intima consists of the endothelial layer, a subendothelial space and the lamina interna elastica. The endothelium is an important barrier between the blood and the underlying tissues of the vessel wall. Early in the process of atherogenesis, when fatty streaks are formed, the morphology of the endothelial layer changes and the endothelium accumulates lipid droplets [2,3]. These changes may be associated with a changed endothelial barrier function, for instance increased permeability towards lipoproteins or decreased removal of lipoproteins from the intima [3]. Smooth muscle cells from the media migrate into the intima during the formation of atherosclerotic plaques, and foam cells are formed by lipid accumulation in macrophages and smooth muscle cells in the intima. In advanced lesions, cholesterol is also deposited extracellularly, together with necrotic and calcified materials and collagen. The lipids in the lesions, of which cholesterol is most abundant, are derived mainly from plasma lipoproteins. Epidemiological studies have shown that a high level of plasma cholesterol is an important risk factor for the future development of coronary heart disease [4]. Cholesterol is transported in plasma in different types of lipoproteins. The major part of plasma cholesterol is present in lipoproteins called LDL. The various lipoprotein classes in human plasma, characterized by their buoyant densities are: very low density lipoprotein (VLDL) with a density < 1.006 g/ml, low density lipoprotein (LDL) with a density ranging from 1.006-1.063 g/ml and high density lipoprotein (HDL) with a density ranging from 1.063-1.21 g/ml. After the consumption of fat the intestine secretes an additional type of lipoprotein, the chylomicrons, which contain large amounts of triglycerides (density < 0.95 g/ml). The chemical composition

Table I
Chemical composition of human plasma lipoproteins[a]

lipoprotein	TG	PL	UC	CE	protein	apolipoproteins
chylomicron (remnants)	86-94	3-8	≤1	1-3	1-2	B-48, A-I, A-IV, C's and E
VLDL	55-65	12-18	6-8	12-14	5-10	B-100, C's and E
LDL	8-12	20-25	5-10	35-40	20-24	B-100
HDL	3-6	20-30	3-5	14-18	45-55	A-I, A-II, C's and E

[a] The composition is given in weight percentages.

of human plasma lipoproteins is given in table I.

Although a high intracellular concentration of cholesterol is toxic, cholesterol is an important natural component of all cells. For example, it is essential for the maintenance of cellular membrane structures. Apart from the endogenous synthesis of cholesterol, which is present in almost all cells of the body, cells can also take up cholesterol from the plasma. LDL can be taken up via binding to the LDL receptor and subsequent adsorptive endocytosis. This system is present on a wide variety of cells grown in tissue culture or taken directly from the body [5], including hepatocytes, smooth muscle cells, endothelial cells, fibroblasts and adipocytes. In addition, when the LDL-receptor activity is down-regulated because of the high concentration of LDL in plasma, cells can take up LDL by way of nonspecific pinocytosis or adsorptive endocytosis of LDL bound to low affinity sites [6,7]. This latter, receptor-independent, uptake of LDL accounts for about one-half of LDL catabolism in normal man [8]. Scavenger receptors, by which acetylated or malondialdehyde-modified LDL can be taken up, are present on macrophages and in smaller quantities on endothelial cells [5]. LDL, which is oxidized in vitro, can also be taken up by similar receptors. Oxidized LDL particles will hardly be detectable in the circulation because the formation is prevented by the antioxidants in the blood stream, but in subendothelial spaces oxidized LDL may be formed and taken up via the scavenger receptor pathway.

Because cells can accumulate cholesterol via various mechanisms, which were mentioned above, they need an efficient system to remove any possible overload of intracellular cholesterol. Non-hepatic cells, apart from steroid hormone producing cells, cannot degrade or excrete cholesterol and can only eliminate cholesterol by transporting

it back to the plasma. Cell-derived cholesterol can be transferred to the liver via the circulation. Hepatocytes can convert cholesterol into bile acids, which are excreted in the bile, together with unconverted cholesterol. A mechanism for the transport of cholesterol from peripheral cells to the liver was first postulated by Glomset in 1968, and called reverse cholesterol transport [9]. In this process the activity of the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) plays a crucial role. LCAT preferentially acts on HDL particles since apo A-I is the most potent activator of the enzyme [10]. In vivo LCAT maintains a low concentration of unesterified cholesterol in HDL, and therefore HDL can take up cellular cholesterol. Also in interstitial fluid, where the HDL concentration exceeds the concentration of other lipoproteins, this transport of cholesterol from cells to HDL can be imagined to be very efficient. Miller and Miller [11] again focused the attention on the role of HDL in reverse cholesterol transport by combining all the evidence on the inverse relation between high HDL cholesterol levels and a low risk of coronary heart disease. Since then the hypothesis of reverse cholesterol transport is supported by numerous different studies. Data from epidemiological studies, e.g. the Framingham study, reinforced the role of HDL [12]. In vitro, HDL decreases cellular cholesterol levels: this was first demonstrated for Landschütz ascites cells [13] and later for numerous other cell types. In vivo, there was a reduction of the amount of atherosclerotic lesions and the amount of lipid deposits per lesion when a mixture of rabbit HDL and VHDL (density range 1.063-1.25 g/ml) was injected into cholesterol-fed atherosclerotic rabbits [14]. Another example of the possible role of HDL in reverse cholesterol transport in vivo is the almost complete inhibition of the formation of fatty streaks by atherogenic diets in transgenic mice expressing high amounts of plasma human apo A-I and with about 2-fold increased plasma HDL cholesterol levels, if compared with control mice [15]. All these data support the role of HDL in reverse cholesterol transport, but the exact mechanism of the process is still unknown. There are several different hypotheses about the mechanism of uptake of cellular cholesterol by HDL particles. The data are controversial on this point. The main difference between the different ideas is whether binding of HDL to specific high affinity binding sites for HDL is essential for efflux of cholesterol from cells or not [16,17]. It is generally assumed that HDL takes up unesterified cellular cholesterol by diffusion [16,17], but there are also reports describing efflux as a retroendocytosis process [18]. The role of HDL binding in HDL-mediated efflux of cellular cholesterol and the different hypotheses of the mechanism of HDL-mediated efflux are discussed in sections 1.2 and 1.3, respectively.

The processes occurring after the uptake of cell-derived cholesterol by HDL will be discussed only briefly here. Unesterified cholesterol diffuses between HDL particles and liver cells. The depletion of HDL phospholipids by hepatic lipase may enhance a net

flux of cholesterol from HDL to these cells [19,20]. The major site for uptake of HDL cholesteryl esters is also the liver [21-23]. The LCAT-derived HDL cholesteryl esters are delivered to the liver by several pathways. Firstly, intact HDL particles can be taken up by liver cells [21,22], which express high affinity binding sites for HDL, and binding may result in uptake and subsequent degradation of HDL. Apo E-containing HDL can also be cleared from the circulation via the apo B/E (LDL) receptor on hepatocytes. Secondly, cholesteryl esters from HDL can be taken up in a HDL-receptor independent pathway by liver cells, a process known as selective uptake [24]. The mechanism of this process is not yet fully understood, but it is assumed that only the cholesteryl esters are transferred to the hepatocytes without concomitant uptake of the whole HDL particle. A third, indirect, pathway involves the transfer of HDL-cholesteryl esters to other lipoproteins by cholesteryl ester transfer protein (CETP) in plasma. Cholesteryl esters are transferred to the VLDL and LDL fraction and will be cleared from the circulation via the apo B/E (LDL) receptor on hepatocytes. Species lacking active CETP, e.g. rat and pig, cannot clear HDL cholesteryl esters via this pathway. The importance of this pathway was demonstrated by the increased clearance of HDL cholesteryl esters from plasma and higher accumulation of radioactive label in the liver after the simultaneous injection of CETP and [^{3}H]cholesteryl ether-labelled HDL in rats, if compared with the clearance of HDL cholesteryl esters without injection of CETP [23]. In rabbits, the uptake of HDL particles by the liver was responsible for 10% of the clearance of HDL cholesteryl esters, 20% was cleared via the selective uptake of HDL cholesteryl esters and about 70% of the esters were taken up by the liver via transfer by CETP and subsequent uptake of LDL [25].

Although the idea of reverse cholesterol transport is generally accepted, the enormous amount of research on the anti-atherogenic properties of HDL since 1975 still does not prove that coronary heart disease is prevented by a high HDL level. There is a higher incidence of coronary heart disease in people with a moderately reduced HDL level (< 0.9 mM HDL cholesterol), if compared with people with higher HDL levels. But there are also reports on people with very low HDL levels (HDL cholesterol < 10th percentile), due to genetic defects like fish-eye disease or Tangier disease, who only have a moderate increased risk of coronary heart disease [26]. Probably certain HDL subfractions are responsible for the anti-atherogenic properties of HDL, or other factors are also important for the prevention of atherogenesis. It is known that the concentration of HDL cholesterol in plasma is inversely correlated with the plasma triglyceride concentration, implying the possibility that not high HDL is preventing atherogenesis, but that low triglyceride levels are the protective factor. A high level of plasma triglycerides suggests an impaired clearance of triglyceride-rich particles. In the circulation the

triglycerides of VLDL and chylomicrons are hydrolysed by lipoprotein lipase, which is present on the vascular endothelial cells. During lipolysis, chylomicron- and VLDL-derived surface fragments are taken up by HDL. Possibly, low levels of HDL indicate that triglyceride-rich particles are present in the circulation for a long time, exerting atherogenic effects. Another reason for the inverse correlation between HDL-cholesterol and plasma triglyceride levels is the activity of CETP in plasma. CETP catalyses the exchange of HDL cholesteryl esters for triglycerides derived from VLDL or chylomicron (remnants). So, a high CETP activity, together with high plasma triglycerides, results in low concentrations of HDL cholesterol. Experiments of Rubin *et al.* [15], using transgenic mice expressing high levels of human apo A-I, do not implicate an important role of triglycerides because HDL influenced atherogenesis without affecting plasma triglycerides, indicating that HDL itself is responsible for the inhibition of the fatty streak formation. Although HDL-dependent reverse cholesterol transport is important for body cholesterol homeostasis, this process may not be the only factor responsible for the protection against atherogenesis and more research must further clarify the role of HDL in atherosclerosis.

1.2 The role of HDL binding in HDL-mediated efflux of cellular cholesterol.

As mentioned in section 1.1 it is controversial whether binding of HDL to specific membrane receptors and HDL-mediated efflux of cellular cholesterol are directly linked. A saturable high affinity binding of HDL to numerous types of cells has been measured (see table II) indicating the presence of specific high affinity receptors for HDL on plasma membranes. Some investigators could not measure a saturable binding of HDL to endothelial cells [32,33], but after preincubation of the cells with 25-hydroxy cholesterol the HDL binding was saturable [31]. Table II shows a large variation in the affinity of high affinity HDL binding sites, even for similar cells or membrane preparations: the K_d ranged from 2-95 μg HDL protein/ml. This may have been caused by the different incubation temperatures during the incubations, the use of different HDL preparations or the methods used to calculate the K_d (nonspecific binding measured by addition of excess unlabelled HDL or calculated from the linear component of the binding plot at high HDL concentrations). The loading of cells with cholesterol did not change the affinity for HDL [40,41], but an increase of the amount of HDL binding after cellular cholesterol enrichment is reported for various cell types, e.g., fibroblasts [30], endothelial cells [31,40,41], macrophages [38] and hepatocytes [36]. The high affinity binding of HDL to whole cells or membrane fractions is generally characterized as calcium-independent [28,42,45] and relatively insensitive to pronase treatment [29,42,45].

Table II
High affinity binding of HDL to whole cells or plasma membranes

	K_d	ref.
human fibroblasts	n.d.[a]	[27,28]
idem	9 μg/ml[b]	[29]
idem	2 μg/ml[c]	[30]
bovine vascular endothelial cells	5-10 μg/ml[bc]	[31]
idem	non-saturable[b]	[32]
porcine vascular endothelial cells	non-saturable[b]	[33]
smooth muscle cells	n.d.	[28,30]
rat hepatocytes	11 μg/ml	[34]
rat adrenal cortical cells	n.d.	[35]
HepG2 cells	n.d.	[36,37]
mouse peritoneal macrophages	95 μg/ml	[38]
mouse adipocytes	18-24 μg/ml	[39]
human EA.hy 926 endothelial cells	20 μg/ml[b]	[40]
idem	37 μg/ml	[41]
idem	31 μg/ml[c]	[41]
rat liver membranes	75 μg/ml[d]	[42]
idem	12 μg/ml[c]	[43]
rat testis membranes	70 μg/ml	[44]
idem	13 μg/ml[c]	[43]
rat kidney membranes	40 μg/ml	[45]
human liver plasma membranes	60 μg/ml	[36]
human adipocyte membranes	10-15 μg/ml	[46]
porcine liver, adrenal and skeletal muscle membranes	11-18 μg/ml	[47]

[a] not determined.
[b] Binding of HDL was measured at 37 °C instead of 4 °C.
[c] Binding of HDL was up-regulated by preincubation of the cells with FC, 25-OH-FC, catLDL or acLDL.
[d] Liver membranes derived from estradiol-treated rats were used.
[e] Binding of HDL was measured at 22 °C instead of 4 °C.

These characteristics also distinguish between binding of high and low density lipoproteins, indicating the existence of two separate binding sites. The normal binding of HDL to LDL-receptor negative fibroblasts [28,29] or to hepatic membranes from a LDL receptor-deficient patient [36] also indicates the existence of separate binding sites for HDL and LDL. Other arguments for the existence of different binding sites for HDL and LDL on plasma membranes are 1) the different effect of cholesterol loading of

cultured cells on binding of HDL and LDL [30] and 2) the different effect of oestradiol treatment on binding of HDL and LDL to isolated rat liver membranes [42]. Many competition experiments also indicate that the high affinity HDL binding site is specific for HDL, because unlabelled LDL was not able to inhibit the binding of iodinated HDL (human fibroblasts [29], membranes of rat liver, testis and kidney [42-45] and rat and human hepatocytes [34,36]). However, there are also studies in which a partial competition of the HDL binding by LDL was measured (endothelial cells [40,48] and human fibroblasts [28,30]). In addition, several investigators reported a relaxed specificity of the HDL binding sites: in these experiments HDL and LDL inhibited the binding of iodinated HDL similarly, indicating that both HDL and LDL bind to the same sites (endothelial cells [31,32] and human adipocyte membranes [46]). These different specificities of HDL binding sites may be explained by the existence of more than one type of HDL binding site on different cells or the regulation of the specificity of the HDL binding sites by the cholesterol status of the cells (in the studies mentioned above both normal and cholesterol-loaded cells were used).

The nature of the HDL binding sites was further investigated by comparison of the apolipoprotein involvement in the binding of HDL. Apo A-I, the major protein of HDL, is generally considered to be a ligand for the HDL binding proteins. Leblond and Marcel [49] investigated which epitopes of apo A-I were responsible for the binding of apo A-I to HepG2 cells. Using monoclonal antibodies against epitopes in the regions of the amphipatic α-helical repeats of apo A-I, they reported that not one of the antibodies was capable of inhibiting more than 15% of the binding of ^{125}I-HDL, although a mixture of these monoclonals or polyclonal anti-A-I inhibited 43% and 95% of the binding, respectively. This indicates that not one of the exclusive epitopes of apo A-I was responsible for the binding, but that the accessibility of the entire apo A-I molecule and cooperative binding of the amphipatic α-helix repeats are needed for maximal interaction of apo A-I with the HepG2 cells. These results do not fully agree with the study of Morrison *et al.* [50]. Using cyanogen bromide fragments of apo A-I, complexed to phosphatidylcholine, Morrison *et al.* [50] reported that only the apo A-I fragment containing the carboxyl-terminal region of apo A-I (amino acid residues 147 to 243) showed similar binding characteristics to rat liver plasma membranes, if compared with native apo A-I/phosphatidylcholine complexes. The amphipatic helix-containing epitopes, who were detected by the monoclonals of Leblond and Marcel [49], were situated along the whole apo A-I molecule, and not just in the residues 147 to 243. Not only apo A-I binds to high affinity HDL binding sites, but other apolipoproteins are also able to interact with high affinity binding sites. For various cell types or membrane preparations a specific saturable binding of iodinated proteoliposomes of apo A-I, apo A-II or apo A-IV was shown [51-53]. On ligand blots, A-I or A-II containing proteoliposomes bound to

the same cellular (110 kDa) HDL binding protein as native HDL_3 [54]. Proteoliposomes, containing A-I, A-II or A-IV, displaced each other or HDL_3, also indicating binding to one type of receptor site [51-53]. This was confirmed in our own experiments (see chapter 8) and by de Crom *et al.* [47] using the immunopurified HDL subfractions LP A-I and LP A-I/A-II. It is not clear yet whether HDL only binds via apo A-I or also via other HDL apolipoproteins (e.g. apo A-II). Vadiveloo and Fidge [55] measured the binding of apo A-II-enriched HDL_3 particles to bovine aortic endothelial cells. The apo A-II enrichment did not change the binding characteristics and the A-II-HDL_3 displaced the normal HDL_3 particles. So both apo A-I and apo A-II seemed equally important in the recognition of HDL by its binding site. Recently, these authors reported that the maximal binding of immuno-purified LP A-I to endothelial cell membranes was 3- to 4-fold higher than the binding of HDL_3 or apo A-II-enriched HDL_3 [56]. They now suggest that apo A-I binds to only one receptor and that apo A-II interacts with four receptors. In contrast, Fong *et al.* [57] and Schmitz *et al.* [58] reported that only apo A-I is important for the binding of HDL. In studies from Fong *et al.* [57] enrichment of HDL_3 particles with apo A-II lowered the total binding to human adipocytes compared to the binding of normal HDL_3 particles. The binding to the adipocytes correlated with the amount of apo A-I in the HDL_3 particles. Schmitz *et al.* [58] concluded that only apo A-I is the ligand for the HDL receptor, since only anti-apo A-I inhibited the binding of HDL to peritoneal macrophages. Antibodies against apo A-II, apo C or apo E did not inhibit the HDL binding. Although apo A-II seems to be able to bind to HDL receptors, it is possible that HDL only binds via apo A-I when both apo A-I and A-II are present on the same particle. This could explain the lack of effect of anti-apo A-II on HDL binding in the experiments described in [58]. The minor HDL apolipoproteins, apo E and apo C's, are not considered to be very important for the binding to the HDL receptor, as was demonstrated in [34,36,45,51,54,58]. In general, only apo E-free HDL is used in HDL binding experiments, in order to eliminate binding of HDL to the LDL (B/E) receptor.

Specific HDL binding proteins have been visualized in membrane preparations of various cell types (from fresh tissue as well as from cultured cells, see table III). In most of these studies HDL binding proteins were visualized using ligand blotting. However, the results obtained from different laboratories are sometimes conflicting, and the molecular weights for the HDL binding proteins vary from 58 to 210 kDa. The smaller differences in molecular weights may be caused by variation in the degree of glycosylation of the proteins [64,65], but also the existence of more than one HDL binding protein is not unlikely because of the different metabolic routes of HDL in different organs. E.g., different HDL binding proteins may be involved in hepatic uptake of HDL and in extrahepatic reverse cholesterol transport. Another explanation for the

Table III
HDL binding proteins visualized using SDS-PAGE

source	MW (in kDa)	ref.
sheep adrenal cortex	78	[59]
rat liver	78	[60]
rat kidneys	78	[60]
human skin fibroblasts	110	[54]
Swiss 3T3 fibroblasts	110	[54]
bovine aortic endothelial cells	110	[54]
J774 macrophages	110	[54]
HepG2 cells	110	[54]
rat hepatocytes	110	[54]
human arterial smooth muscle cells	110	[54]
human placenta	80	[54]
J774 macrophages	110	[61]
Fao hepatocytes	110	[61]
human placenta	120 (complex of 30 and 50 kDa monomers)	[62]
diff. Ob1771 mouse adipocytes	70/100 (proteins identified with chemical cross-linkers)	[52]
rat and human liver	100/120	[63,64]
mouse adipocytes (Ob17MT18 line)	80/92	[65]
rat ovaries	58	[66]
rat liver	95	[67]
Hep3B human hepatoma cells	60/100/210	[68]
porcine liver	90/180(110/130 minor bands)	[69]

conflicting results may be the method used for isolation of the plasma membranes. It is difficult to isolate pure plasma membranes, and some bands in ligand blotting experiments probably originate from HDL binding proteins in intracellular membranes. In hepatocytes these intracellular binding proteins may be necessary for the secretion of nascent HDL, which is produced in these cells. Recently, McKnight *et al.* [70] visualized 110 and 130 kDa HDL binding proteins in bovine aortic endothelial cell membranes using ligand blotting, although the signal of the 130 kDa-band was only weak. However, the endothelial cells membranes contained similar amounts of the 110 and 130 kDa binding proteins when an anti-110 kDa was used in immunoblotting. This implies that the HDL binding activity of the 130 kDa protein is very weak, if compared with the HDL binding of the 110 kDa membrane protein. Similar results were found in cells transfected with the gene for the 110 kDa HDL binding protein (HBP), in which the 130 and 150

kDa immunoreactive bands showed virtually no HDL binding activity. The extra HDL binding activity after overexpression of the HBP gene in BHK cells was localized intracellular (lysosomes, Golgi and endoplasmatic reticulum), and had only little effect on the binding of iodinated HDL to these cells. From the amino acid sequence it was predicted that this HBP gene product lacked a typical membrane spanning domain and that it may contain many amphipatic helixes, resembling the structure of human apolipoprotein A-IV) [70]. Previously, Graham and Oram [54] reported that, after trypsinization and subsequent isolation of the membranes of endothelial cells, proteolytic digestion abolished the HDL binding activity of the membrane proteins on ligand blots. Recently, Hidaka and Fidge [64] further characterized 100 and 120 kDa HDL binding glycoproteins from rat liver. They also reported that the HDL binding activity on ligand blots disappeared after proteolysis of the membranes with trypsine or pronase, but that the removal of sialic acid residues from the glycoproteins did not destroy HDL binding activity. However, there are also reports denying the existence of saturable high affinity HDL receptors on cells. Tabas and Tall [33] demonstrated that specific binding of iodinated HDL_3 to porcine endothelial cells was not saturable and that the binding of HDL to these cells was not susceptible to proteolytic treatment of either HDL (trypsinization) or the cells (pronase or trypsine treatment). Both proteolytic enzymes, pronase and trypsine, cleave proteins at specific peptide bonds and it is possible that these enzymes cannot splice off the HDL binding epitopes. Tabas and Tall [33] hypothesize that binding of HDL to cellular binding sites is based on lipid interactions and that the upregulation of HDL binding after cholesterol loading was determined mainly by the cell surface cholesterol content. Using radiation inactivation of membrane binding sites (a technique which reconfirmed the molecular weight of the LDL receptor), Mendel *et al.* [71,72] also reported that the HDL binding sites on membranes from cultured fibroblasts and human liver cannot be classical high molecular weight membrane proteins: they showed that binding of HDL disappeared after irradiation of plasma membrane sites of 10 and 16 kDa for liver and fibroblasts, respectively.

The observation that cholesterol loading results in up-regulation of HDL binding to fibroblasts [30] and in an increase of the amount of plasma membrane HDL binding protein on ligand blots [54], suggests a functional role for these proteins in the removal of an excess of cellular cholesterol. Since the mechanism of cellular cholesterol efflux is still unclear, the role of the HDL binding proteins in this process remains uncertain. Recently, Oram and co-workers suggested the role of high affinity binding of HDL in intracellular cholesterol trafficking [73]. This hypothesis is discussed in detail in section 1.3. However, there is a substantial amount of literature in which the importance of HDL binding to membrane proteins in efflux of cellular cholesterol is denied. The relation

between high affinity binding of HDL and HDL-mediated cholesterol efflux was studied by comparison of the dependencies on HDL concentration of high affinity binding and HDL-mediated efflux. The shape of the curves was different, indicating that there was no direct linkage between binding and efflux [74-76]. Mendel and Kunitake [75] demonstrated that albumin or phosphatidylcholine alone were able to induce cholesterol efflux, only higher concentrations were needed than with HDL_3. Cholesterol loading of human fibroblasts resulted in up-regulation of specific HDL binding, but efflux was enhanced similarly when HDL_3, albumin or phospholipid vesicles were used as cholesterol acceptors. So, the increase of specific binding of HDL_3 was not correlated with a specific HDL_3-mediated efflux, but also with efflux induced by "nonspecific cholesterol acceptors" [75]. This indicates that efflux is not mediated by binding of HDL to specific HDL binding sites. Another method to study the effect of HDL binding on cellular cholesterol efflux is the use of chemically modified HDL particles, which do not bind to specific high affinity receptors for HDL. In this way the involvement of specific amino acid residues in binding and efflux can be investigated. Acetylation or methylation of lysine residues [43,77], reductive alkylation of cysteine residues [77] and cyclohexadione treatment of arginine residues [43,77] did not alter binding of HDL. Treatment of HDL with tetranitromethane effectively inhibited HDL binding to membranes of rat liver [78], to Fu5AH cells [74] and to human fibroblasts [74,77]. It is not known whether the nitration of tyrosine groups eliminated the HDL binding, since tetranitromethane also caused extensive cross-linking of HDL apolipoproteins (see chapter 5 and [78]). The chemical cross-linker dimethyl suberimidate also eliminated binding of native HDL to high affinity binding sites (see chapter 5 and [78]), so cross-linking of HDL apolipoproteins probably causes the inhibition of binding of both tetranitromethane- and dimethyl suberimidate-treated HDL to specific high affinity binding sites for native HDL. Neither tetranitromethane [74] nor dimethyl suberimidate treatment of HDL [76] altered cholesterol efflux if compared with native HDL. Tetranitromethane-modified HDL_3 also induced a substantial amount of cholesterol efflux in experiments of Brinton *et al.* [77], but these authors interpreted their results differently than Karlin *et al.* [74] and Johnson *et al.* [76]. Brinton *et al.* [77] concluded, that the high affinity component of cholesterol efflux was abolished when tetranitromethane-modified HDL_3 was used. It is not known which fraction of total efflux corresponds with the proposed "high affinity component" of cholesterol efflux, so the importance of this component of the cholesterol efflux is unclear.

Another approach to study the involvement of specific apolipoproteins in HDL-mediated efflux is the use of phospholipid vesicles with different apolipoproteins or immunopurified HDL subfractions with distinct apolipoprotein compositions for efflux

experiments. These cholesterol acceptors can also give information about the importance of binding of specific HDL apolipoproteins in the efflux of cellular cholesterol. For more information see section 1.4.

1.3 The different hypotheses of the mechanism of cellular cholesterol efflux

The aqueous diffusion model and the role of membrane kinetic pools in efflux of cellular cholesterol

Serum proteins (e.g. BSA or apo A-I) [13,75,80] or phospholipids alone [13,75,80,81] are able to induce efflux of labelled cellular cholesterol, but apolipoprotein/phospholipid complexes [13,80], HDL [14,75] or whole calf serum [80] proved to be far more efficient in removing cholesterol from cells. The fact that phospholipids alone also remove cholesterol from cells, indicates that binding of specific apolipoproteins to cell membrane receptors is not absolutely necessary for efflux. Cholesterol can exchange between different liposomes, between liposomes and lipoproteins and between different lipoproteins by diffusion, and Phillips *et al.* [81] reported that the diffusion of cholesterol out of cells to liposomes was similar to the diffusion of cholesterol between different liposomes. This mechanism for diffusion of cellular cholesterol is described in the aqueous diffusion model [81]. The aqueous diffusion model is in agreement with the literature denying the role of high affinity HDL binding in cholesterol efflux (for reviews see Johnson *et al.* [16] and Phillips *et al.* [82]). According to this model, the diffusion of cholesterol is only dependent of the cholesterol concentration of the donor particle. The rate limiting step for efflux of cellular cholesterol is the desorption of cholesterol from the plasma membrane into the water phase. The cholesterol acceptor concentration is the limiting factor for efflux only at low concentrations, when the distance for diffusion of cholesterol in the aqueous phase determines the diffusion rate. Similar kinetics for diffusion of cholesterol out of whole cells and out of plasma membrane vesicles from these cells demonstrated the key function of the plasma membrane in determining the rate of cellular cholesterol efflux [83]. For Fu5AH and WIRL rat liver cells different rate constants for efflux of cholesterol from plasma membrane vesicles were measured, but these differences between the two cell types were eliminated when efflux was measured from vesicles of only the plasma membrane lipids [83]. This indicates that the efflux rate was determined by other factors than the plasma membrane lipid composition alone, e.g. the membrane proteins in certain membrane domains, which may determine local diffusion rates for cholesterol.

Efflux of cellular cholesterol can be described by first order kinetics with respect to cellular cholesterol content, when the acceptor is present in excess. This means that on each time point a fixed percentage of the cellular cholesterol content (characterized by a rate constant) desorbs from the plasma membrane. Cholesterol is released from cholesterol-enriched cells by exactly the same mechanism: the rate constant for efflux of cellular cholesterol, k_e, was identical for normal and cholesterol-loaded fibroblasts [76]. Net mass flux of cholesterol from cells to cholesterol acceptors (liposomes or HDL) is determined by efflux of cholesterol from cells to the acceptors minus influx of cholesterol from acceptors to cells. For both influx and efflux the cholesterol fluxes are determined by the same mechanism, and dependent on the cholesterol concentration of cells and acceptors, respectively, net efflux or net influx occurs.

For J774 macrophages, human GM 3468 and mouse L-cell fibroblasts it was reported that complexes of apo A-I and egg phosphatidylcholine were more efficient in removal of cellular cholesterol than complexes of other apolipoproteins (apo C's or apo A-II) [84,85]. However, for Fu5AH [84,85], HepG2 [84] and smooth muscle cells [85] all apolipoprotein/phosphatidylcholine complexes induced similar efflux, indicating that the more efficient removal of cellular cholesterol by apo A-I complexes was specific for certain cell types. It was recently suggested that this stimulating effect of apo A-I on cholesterol efflux is correlated with an increased percentage of cholesterol in fast kinetic pools in the plasma membrane [85,86]. This hypothesis is based on recent knowledge of cholesterol distributions in different microdomains of the plasma membrane, as reviewed by Schroeder *et al.* [87]. The packing of cholesterol in cholesterol-poor and cholesterol-rich microdomains in the plasma membrane can be influenced by several factors, for instance membrane protein [88], cholesterol content [88], phospholipid unsaturation [89], or by the interaction of the plasma membrane with cholesterol acceptors [90]. Mahlberg and Rothblat [85] reported that cholesterol efflux from certain cell types, e.g., J774 and Fu5AH cells, was best fitted to a curve describing the release of cellular cholesterol from two kinetic compartments. According to these observations the cholesterol efflux can be divided in efflux from a fast pool (with a short half-time for efflux) and from a slow pool, which has a long half-time. The more efficient efflux with apo A-I/phosphatidylcholine complexes, as measured in J774 macrophages [85], was correlated with a higher contribution of the fast kinetic cholesterol pool to total efflux of cholesterol. For smooth muscle cells, where efflux was described by the release of cholesterol from only one kinetic pool, apo A-I did not enhance the contribution of a fast kinetic pool. The nature of the interaction between apo A-I and the cells is not known yet. Apo A-I does not necessarily have to interact with membrane proteins to influence the membrane microdomains. Interactions of apo A-I with membrane lipids can also induce very specific

changes in the cells, like the induction of a signal transduction pathway as proposed in [91].

The signal-transduction pathway in HDL-mediated reverse cholesterol transport.

Oram, Bierman and coworkers [17] state that HDL-mediated efflux of cellular cholesterol consists of two components: 1) binding of HDL to specific HDL binding proteins in the plasma membrane and subsequent translocation of cholesterol from intracellular pools to the plasma membrane and 2) the diffusion of membrane sterols to extracellular cholesterol acceptors. The experiments, leading to these two conclusions will be discussed briefly here.

According to Oram *et al.* [92] HDL binds reversibly to specific high affinity receptors and is released into the medium without cellular uptake of the particle. The transfer of [^{3}H]cholesterol from the plasma membrane to native HDL_3 or TNM-HDL_3 was identical, indicating no involvement of binding of apolipoproteins in this process [93]. This was confirmed in experiments using other modifications of HDL_3 (pronase- or trypsine-treated HDL_3, which also do not bind to high affinity HDL receptors) [73]. So, although binding was not necessary for efflux of membrane cholesterol, these investigators reported that binding of HDL was essential for translocation and efflux of cholesterol from intracellular pools. Translocation of labelled cellular cholesterol from internal pools to the plasma membrane by HDL is measured using the cholesterol oxidase method [93]. It is assumed that cholesterol oxidase can only convert cholesterol in the plasma membrane, without oxidizing intracellular cholesterol. Cholesterol-loaded fibroblasts were incubated with [^{3}H]mevalonic acid in order to label only intracellular cholesterol pools with [^{3}H]cholesterol. HDL_3 was able to induce the translocation of this labelled intracellular cholesterol to the plasma membrane. In my opinion, this translocation does not necessarily has to be caused by specific signal transduction pathways: the desorption of plasma membrane cholesterol by HDL may result in the diffusion of cholesterol from intracellular pools to the plasma membrane in order to maintain the plasma membrane cholesterol content. The HDL-mediated translocation is observed using microsomal [^{3}H]cholesterol (synthesized from [^{3}H]mevalonolactone) as well as lysosomal [^{3}H]cholesterol ([^{3}H]cholesterol from degradation of LDL cholesteryl ester) as intracellular cholesterol pools [94]. Virtually no translocation of intracellular [^{3}H]cholesterol from cholesterol-loaded cells was measured when modified HDL_3 (TNM-, pronase- or trypsine-treated HDL_3) was used instead of native HDL_3 [73,93,94]. Although the modified HDL_3 particles did not induce translocation of cholesterol from intracellular pools to the membrane, these particles were able to induce efflux of microsomal, [^{3}H]mevalonolactone-derived, cholesterol to the medium, to an

extent varying from 10 to 50% of the efflux induced by native HDL_3 [73,93,94]. This efflux was called the low affinity component of cholesterol efflux. It is not known what the contribution of this low affinity efflux is when higher HDL concentrations are used (e.g., in vivo) and how important the high affinity efflux is in reverse cholesterol transport. Johnson *et al.* [95] did not report differences in efflux of cholesterol from lysosomal pools mediated by HDL_3 or DMS-HDL_3, another type of HDL modification which abolishes HDL high affinity binding.

According to the results of Oram and coworkers the mechanism of cholesterol efflux from loaded cells is substantially different from the mechanism involved in efflux from unloaded cells. The translocation of intracellular cholesterol to the plasma membrane was identical for native HDL_3 and TNM-HDL_3 using normocholesterolemic fibroblasts and macrophages [94], an observation clearly opposite to the results in cholesterol-loaded cells. This is in contrast with the aqueous diffusion model, in which the mechanism of efflux of cellular cholesterol is the same for control and cholesterol-loaded cells.

It is proposed that the induction of a signal-transduction pathway after the binding of HDL to plasma membrane receptors is the link between binding of HDL and translocation of intracellular cholesterol. Not only binding of HDL or apo A-I, but also apo A-II, apo A-IV and apo C's can induce translocation of intracellular cholesterol (J.F. Oram, personal communication). Mendez *et al.* [96] reported the involvement of protein kinase C (PKC) in this pathway. Activators of PKC, e.g., the phorbol myristate acetate, 1,2-dioctanoyl-glycerol and the calcium ionophore A23187, mimic the HDL_3- or apo A-I-induced translocation of intracellular cholesterol. The inhibition of PKC, by sphingosine or by long term treatment with phorbol esters, reduced the translocation and efflux of intracellular cholesterol [96]. The activation of PKC after addition of HDL_3 to mouse adipocytes was also reported by Theret *et al.* [97]. Earlier, these investigators have postulated that binding of apo A-I to plasma membrane HDL receptors induced cholesterol efflux from mouse adipocytes, and that apo A-II was an antagonist (both apo A-I and apo A-II bind to the plasma membrane high affinity HDL receptors) [65]. Theret *et al.* [97] showed that degradation of phosphatidylcholine (but not the degradation of phosphatidylinositol) and formation of diacylglycerol by mouse adipocytes were induced by HDL_3 and by apo A-I/phosphatidylcholine complexes, but not by TNM-HDL_3 or apo A-II/phosphatidylcholine complexes. The role of PKC in this process was demonstrated by the induction of efflux of cellular cholesterol mass by phorbol esters plus phospholipid vesicles, whereas phospholipid vesicles alone did not decrease cell cholesterol. Although there is no evidence that HDL_3 activates PKC via the breakdown of phosphatidylinositol there is prove that in some way HDL_3 interacts with the phosphoinositide cycle, because

HDL_3 was able to inhibit the LDL- or bradykinin-induced inositoltriphosphate production in endothelial cells [98]. It is not yet clear whether the release of calcium from intracellular stores, induced by the addition of HDL_3 to human fibroblasts, as reported by Pörn *et al.* [99], also takes part in the same signal transduction pathway because for calcium release specific high affinity binding of HDL was not necessary: modification of HDL_3 with tetranitromethane or dimethyl suberimidate did not inhibit the mobilization of calcium. Another possibility for the influence of HDL on signal transduction pathways is the stimulation of cellular prostaglandin synthesis. HDL increases the prostacyclin synthesis in arterial smooth muscle and endothelial cells [100,101]. Prostaglandins are modulators of adenylate cyclase activity, which activate cAMP-dependent protein kinase (also called protein kinase A). No effects of prostaglandins on intracellular cholesterol translocation are reported, but it is known that prostacyclin can regulate the activity of cellular cholesteryl ester hydrolases [100].

The retroendocytosis pathway for HDL-mediated cholesterol efflux

An alternative pathway for cellular cholesterol efflux is retroendocytosis. Schmitz *et al.* [38] reported that internalized acetylated LDL and HDL were found in different cellular compartments (in cholesterol-loaded mouse peritoneal macrophages): gold-labelled acetylated LDL was seen in acidic, lysosomal, compartments and gold-labelled HDL was seen in endosomes. These endosomes sometimes were in close contact with, or fused with, cytoplasmatic lipid droplets. Vesicles containing acetylated LDL or HDL containing vesicles were found at separate surface regions of the macrophages after longer incubations, and secretion of the gold-labelled content of the vesicles into the medium was seen. Recently, it was reported that when HDL was visualized using specific antibodies, instead of using gold-labelled HDL, only very little uptake of HDL was seen (B. Harrach, personal communication). Probably gold-labelled HDL is processed differently from native HDL in macrophages, and therefore data obtained with gold-labelled HDL may be artefacts. Schmitz *et al.* [58] measured little degradation of HDL after incubation of iodinated HDL with macrophages for 4 h at 37 °C, but it is not known whether this degradation is absent after longer incubations. Inhibition of cellular cholesterol esterification (ACAT activity) in cholesterol-loaded macrophages increased HDL binding and HDL-mediated cholesterol efflux [58]. Schmitz and coworkers [18,102] reported that efflux of cellular cholesterol existed of an HDL-dependent and an HDL-independent pathway. The HDL-dependent pathway can be stimulated by inhibition of cellular ACAT activity, which results in the formation of lamellar bodies from cytoplasmatic lipid droplets. These lamellar bodies fuse with the HDL containing endosomes, and HDL, enriched with unesterified cholesterol, is secreted into the

medium. The HDL-independent pathway is regulated by protein kinase A and can be stimulated by calcium antagonists. Complexes of cholesterol, phospholipids and apo E are secreted after the increased formation of lamellar bodies, which originate from the lysosomes. The increased secretion of apo E-rich phospholipid discs by cholesterol-loaded macrophages was reported before by Brown and Goldstein [5], but they stated that the discs did not contain cholesterol, that synthesis and secretion of apo E were not necessary for efflux of cellular cholesterol, and that cholesterol was removed from the cells only in the presence of HDL. Alam *et al.* [103] also reported the resecretion of apo E-enriched HDL, but on careful reading of this paper it becomes clear that in these experiments the major part of "resecreted" HDL is extracellular surface-bound HDL, which is released from the membranes at 37 °C.

The resecretion of HDL was also reported for hepatocytes [104,105]. Kambouris *et al.* [104] reported that the HDL resecretion increased after cholesterol loading of the HepG2 cells, indicating a role for retroendocytosis in cellular cholesterol homeostasis. For cells with normal cholesterol levels they showed that there was no change in size of the HDL particles after retroendocytosis. DeLamatre *et al.* [105] reported that the size of the HDL particles was changed and that they contained less cholesteryl esters after retroendocytosis by Fu5AH cells. The decreased endogenous sterol synthesis after preincubation with HDL also indicated the intracellular accumulation of cholesterol. These results do not relate to the HDL-mediated efflux of cholesterol from cells, but can probably explain the selective uptake of HDL cholesteryl esters by liver cells.

1.4 The effect of acceptor composition on cholesterol efflux

The effects of the cholesterol acceptor composition on the efflux of cellular cholesterol can be divided into the effects of the lipid and of the apolipoprotein component of the cholesterol acceptor in the medium. These aspects will be discussed separately in this section.

Influence of the lipid composition of the cholesterol acceptor

The unesterified cholesterol molecules in HDL are dissolved in the HDL phospholipids. Therefore changes in HDL cholesterol and phospholipid concentrations may influence the net mass uptake of cellular cholesterol by HDL. The unesterified cholesterol content of HDL can be decreased by incubation of HDL with LCAT, which converts HDL cholesterol into HDL cholesteryl esters. The effect of LCAT on HDL-

mediated efflux was examined for human fibroblasts [106,107], rat aortic smooth muscle cells [106] and rat hepatoma Fu5AH cells [108]. The effect of LCAT on both efflux of labelled cellular cholesterol and on the net mass transfer of cellular cholesterol to HDL were determined. All these experiments showed that efflux of cellular [^{3}H]cholesterol to HDL/serum was not changed by LCAT [106-108], but that cellular cholesterol mass only decreased when LCAT activity or LCAT-treated HDL was present in the medium during the incubations [107,108]. These results indicate that LCAT decreased the influx of cholesterol from HDL, because LCAT decreased cell cholesterol mass without changing the efflux of cellular cholesterol. In later experiments this was confirmed using reconstituted HDL with varying UC/PL ratios: only influx of cholesterol was dependent on the UC/PL ratio of the reconstituted particles [76]. For cells with normal cholesterol levels, where cholesterol efflux equals cholesterol influx, these changes in influx caused by the UC/PL ratio of the particles in the medium, can change cellular cholesterol levels. But in cells with increased cholesterol levels efflux normally exceeds cholesterol influx several times, and only very small or no effects of the composition of the cholesterol acceptors in the medium may be expected (see also chapter 4 and ref. [106]). Stein *et al.* [106] reported that in LDL/chloroquine-loaded human fibroblasts the inhibition of LCAT in VLDL/LDL-free serum did not affect the net mass transfer of cellular cholesterol to the medium. It is important to note that the cholesterol status of the cells is very important when changes of the acceptor properties are studied: in cholesterol-loaded cells changes in influx will probably not be detected and will not affect net mass flux of cholesterol, because influx is relatively small, if compared with efflux. For all the experiments described in the rest of this section normocholesterolemic cells were used.

The effect of the HDL phospholipid content can be studied using phospholipase- or hepatic lipase-treated HDL, which increases the UC/PL ratio of the HDL particles without affecting the cholesterol/protein ratio. Bamberger *et al.* [111] and Johnson *et al.* [112] reported that Fu5AH cell cholesterol mass was higher after incubation with hepatic lipase- or phospholipase A_2-treated HDL, if compared with control HDL. Later, these investigators showed that this was largely caused by the increased uptake of HDL cholesterol from lipase-treated HDL, which was approximately 4-fold the uptake of HDL cholesteryl esters [20]. The increased cholesterol mass transfer from phospholipid-depleted HDL to liver cells may be the way by which hepatic lipase can stimulate reverse cholesterol transport [19,20]. In erythrocytes Chollet *et al.* [109] reported no effect of phospholipase A_2 treatment of HDL on cholesterol mass and efflux of erythrocyte [^{3}H]cholesterol after 9 h of incubation with control or phosphatidylcholine-depleted HDL (30 to 90% degradation of HDL phosphatidylcholine). This is not in

agreement with the results from Johnson *et al.* [112], who reported that phospholipid depletion decreased the rate constant for efflux, k_e, without affecting the constant for influx, k_i. Collet *et al.* [110] also reported that the influx of [^{3}H]cholesterol from HDL into endothelial cells was not influenced by previous phospholipase treatment of the HDL particles (0 to 5 h of incubation of the cells with labelled HDL).

Not only the total HDL phospholipid concentration, but also the composition of the phospholipids may influence the capacity of the acceptor to take up cellular cholesterol. Esteva *et al.* [113] investigated the effect of HDL lipid composition on efflux of labelled cholesterol from human fibroblasts. The different HDL preparations, used in the efflux experiments, were isolated from persons on diets containing different types of fats. This not only changed the fatty acyl composition of the HDL phospholipids, but also the concentration of HDL triglyceride, unesterified and esterified cholesterol. The overall changes in HDL composition and the unsaturated character of the HDL phospholipids were not correlated with the efflux of [^{3}H]cholesterol from human fibroblasts, but the phospholipid chain length was correlated with cellular cholesterol efflux. However, these data are hard to interpret, because of the many changes in the HDL composition after the various diets. Surprisingly, the highest efflux was measured when HDL isolated after a diet high in milk-fat was used. This HDL had a very high free cholesterol content (molar UC/PL ratio 1.1-1.6), and theoretically it would be a bad acceptor for cell cholesterol. Especially because of the large differences of the UC/PL ratios in the HDL particles after the various diets, changes in cholesterol influx can be expected in these experiments, and only measurement of net mass flux or both influx and efflux can give reliable information in this kind of experiments. This was also demonstrated by Brinton *et al.* [114], who reported that the cholesterol content of HDL did not influence the initial efflux of labelled cellular cholesterol, but after longer incubations, when an equilibrium was reached between cells and HDL, the HDL cholesterol content determined the net flux of cellular cholesterol to HDL.

The influence of HDL subfraction type and apolipoprotein composition of the cholesterol acceptor

HDL is a very heterogeneous population of lipoprotein particles. They differ in size, lipid and apolipoprotein composition and the most widely used subfractionation of HDL is based on particle density: HDL_2 with a density range of 1.063-1.125 g/ml, HDL_3 with a density range of 1.125-1.21 g/ml and VHDL, isolated from the density range 1.21-1.25 g/ml. The composition of the HDL subfractions is given in table IV. Quantitatively, HDL_3 comprises the most important part of the HDL fraction (about 75%) [115]. In vitro, HDL_3 and VHDL are more efficient in removing cell cholesterol than HDL_2

[119,120]. It is not possible to detect a specific role for certain apolipoproteins in reverse cholesterol transport using subfractions like HDL_2 and HDL_3, because these HDL subfractions still consist of a very heterogeneous population of lipoprotein particles, as shown by gradient gel electrophoresis [121,122]. Immunopurified HDL subfractions or apolipoprotein/phospholipid complexes are more useful for the detection of specific functions of subfractions with different apolipoprotein compositions in efflux of cellular cholesterol. Based on apolipoprotein composition, HDL consists of at least two types of

Table IV
Human high density lipoprotein subfractions

A. Composition

subfraction	chemical composition[a]					apolipoprotein composition[a]		
	PL	CE	UC	TG	protein	A-I	A-II	others
HDL_2[b]	30	20	5	3	42	73	23	4
HDL_3[b]	25	16	3	1	55	64	31	5
VHDL[c]	29	3	0.3	5	62	nd.[d]	nd.	nd.
LP A-I[e]	24	18	5	3	50	>96	--	<4
LP A-I/A-II[e]	22	16	4	2	56	56	40	<4

B. Plasma concentration

subfraction	A-I (mg/l)	A-II (mg/l)	TC (mM)
HDL_2[b]	300	95	0.45
HDL_3[b]	877	427	0.78
LP A-I[f]	507	--	0.62
LP A-I/A-II[f]	842	325	1.00

[a] Values given are weight %.
[b] Data are calculated with the results from ref. [115] for males. HDL_2 and HDL_3 were isolated by ultracentrifugation (density range 1.063-1.125 and 1.0125-1.21, respectively).
[c] VHDL was isolated by ultracentrifugation (density range 1.21-1.25 g/ml) [116].
[d] not determined.
[e] This thesis.
[f] Mean values for males [118].

particles: particles containing apo A-I, but no apo A-II (LP A-I, also called LP A-I/wo A-II by some authors), and the second type of lipoprotein particles containing both apo A-I and apo A-II on the same particle (LP A-I/A-II). These two HDL subfractions can be isolated using immuno-affinity chromatography (see chapter 8 of this thesis and [123]). To eliminate possible interactions with apo B/E receptors all apo B and apo E containing lipoproteins can be removed by chromatography on a heparin Sepharose column. These subfractions also contain small amounts of apo C's (ranging from 0 to 5% of total HDL apolipoproteins) and apo D [117]. For detailed information about the chemical composition of immunopurified HDL subfractions see chapter 8. Recently, it was reported that there are also particles containing apo A-IV without apo A-I (LP A-IV) and that the LP A-I fraction can be further separated in a HDL subfraction containing A-IV (LP A-I/A-IV) and particles containing no apo A-IV (LP A-I/wo A-IV) [53]. The greater part of apo A-IV is present in the free form in human plasma, i.e. not bound to lipoprotein particles, so quantitatively A-IV containing particles are not a very important HDL subfraction. Additionally, a minor fraction of plasma apo A-II (about 10%) is present on LP A-II particles, which contain apo A-II but no apo A-I [117,124].

Immunopurified HDL subfractions or proteoliposomes can be used for the study of the effect of apolipoprotein type on cholesterol efflux. Proteoliposomes are not physiological acceptors, but the advantage is that these particles have identical lipid compositions, which is not the case for immunopurified HDL subfractions. Studies using both kinds of acceptors will be discussed in this section. Several investigators demonstrated that apo A-I/phosphatidylcholine complexes are more efficient acceptors of cellular cholesterol than complexes with other apolipoproteins [84,85,125]. Whether this more efficient efflux by apo A-I was related with binding to receptors and signal-transduction pathways or with interactions of apo A-I with cellular lipids, as described in section 1.3, remains unclear. Barbaras *et al.* [125] and Steinmetz *et al.* [53] found rather drastic effects of apolipoprotein type of the acceptor on efflux: Apo A-II containing vesicles did not induce cholesterol efflux and apo A-I or apo A-IV containing vesicles did induce efflux. Similar results were obtained with immunopurified HDL subfractions: LP A-I, LP A-IV and LP A-I/A-IV particles decreased mouse adipocyte cholesterol levels, in contrast with LP A-I/A-II en LP A-II particles [53,123,124]. However, these results are in clear contrast with our own observations of the efflux of cellular cholesterol mediated by immunopurified LP A-I and LP A-I/A-II, as described in chapter 7 of this thesis. In these experiments, in which human fibroblasts, smooth muscle cells and rat hepatoma cells were used, we showed that both LP A-I and LP A-I/A-II had the same capacity to take up cell cholesterol. This holds for labelled cholesterol as well as for unlabelled cholesterol from sterol-enriched cells. The

discrepancy between our experiments and the studies using mouse adipose cells could be due to the method used for cholesterol enrichment of the cells or to the cell type. A specific role of certain LP A-I particles in efflux of cholesterol from fibroblasts has also been proposed by Castro and Fielding [126]. Fibroblasts were labelled with [^{3}H]cholesterol and after short incubations with plasma the lipoprotein particles were separated by two-dimensional agarose-polyacrylamide gel electrophoresis. After 1 minute of incubation ^{3}H-cholesterol was only present in apo A-I-containing HDL particles with pre-ß mobility on agarose gel electrophoresis. These pre-ß1 LP A-I particles of 70 kDA (not containing apo A-II) were postulated to take up cell-derived cholesterol preferentially. After another 2 minutes of incubation of the plasma with unlabelled fibroblasts, the label also occurred in 325 kDa pre-ß2 LP A-I particles and in the bulk of α-migrating HDL particles, which comprise about 96% of total HDL. In later experiments a pre-ß3 LP A-I subspecies was detected [127]. This particle contains LCAT and CETP and could be responsible for the further transfer of cholesterol from the pre-ß2 fraction to the bulk of HDL particles. This specific function of certain LP A-I particles cannot fully explain the specific role of LP A-I in the mouse adipocyte experiments. In these experiments the efflux was similar for LP A-I particles isolated from plasma, HDL_2 or HDL_3 [124]. In HDL isolated by ultracentrifugation no LCAT or CETP activity is present and the pre-ß3 particles obviously are not intact anymore. Numerous in vitro experiments by various investigators have shown that HDL, isolated by ultracentrifugation, can take up cell cholesterol, and this implies that the preferential transfer of cell-derived cholesterol via the pre-ß LP A-I particles to the bulk of α-migrating HDL is not the only pathway by which HDL can take up cellular cholesterol.

1.5 The effect of cellular composition on cellular cholesterol efflux

Many studies showed that the efflux of cellular cholesterol was already maximal at low concentrations of HDL, i.e. far below the physiological HDL concentration. (see chapter 3 and ref. [128,129]). So other factors, like cellular esterified and unesterified cholesterol content, cholesteryl ester hydrolase activity, intracellular cholesterol transport or desorption of cholesterol out of the plasma membrane, may be rate limiting factors for cholesterol efflux.

The influence of the cellular cholesterol content on efflux

Johnson *et al.* [76] reported that the efflux of cellular cholesterol is linear with respect to the cellular unesterified cholesterol content, i.e., that the efflux is always the

same percentage of the cellular cholesterol content. The transfer of cholesterol from intracellular stores is not considered to be the rate limiting factor for efflux of cellular cholesterol. Johnson *et al.* [95] showed that lysosomal cholesterol was transported to the medium in maximally 40 minutes, and that after this initial lag time the cholesterol efflux rate was similar for efflux of lysosomal and plasma membrane derived cholesterol. Also it is reported that the half-time for transport of newly synthesized cholesterol to the plasma membrane varies from 10 minutes to 2 hours (dependent on cell type) and that the mean half-time for efflux of cellular cholesterol for different cell types is about 10 hours, which is far more than the half-time known for intracellular cholesterol transport [82].

When cells store a large percentage of cell cholesterol in the esterified form, the hydrolysis of esterified cholesterol may be a rate limiting factor for cholesterol efflux. Macrophages, which have a very active cholesteryl ester cycle, i.e., a high rate of cholesterol esterification and cholesteryl ester hydrolysis, store the major part of intracellular cholesterol as esters. Therefore the inhibition of ACAT activity in macrophages, which leads to the increase of cellular unesterified cholesterol, results in more HDL-mediated net mass efflux of cellular cholesterol [58,130]. Stimulation of cholesteryl ester hydrolysis in J774 macrophages by cAMP analogues also increased cholesteryl ester clearance and the HDL-mediated net mass efflux of cholesterol [131]. The pathway for the activation of cholesteryl ester hydrolase via cAMP and protein kinase A is still unclear. Probably, HDL can indirectly influence cellular hydrolase activity: it is reported that HDL induces the endogenous synthesis of prostaglandins [100,101], which are mediators of adenylate cyclase activity, the enzyme responsible for the formation of intracellular cAMP.

The type of fatty acyl chain of the esterified cholesterol molecules may also be important for the rate of hydrolysis and subsequent efflux of cholesterol: cholesteryl ester clearance is higher from cells which contain high amounts of unsaturated cholesteryl esters, if compared with saturated esters [132]. There are reports suggesting a substrate specificity of neutral cholesteryl ester hydrolase for unsaturated cholesteryl esters [133,134], but there are also articles denying any preference [132,135]. When the cholesteryl ester fatty acyl composition of Fu5AH cells was compared before and after incubation with HDL, there was no difference in the fatty acid composition of cellular cholesteryl esters (HDL induced a 50% reduction in cell cholesteryl ester content) [135]. Later, it was reported that these differences in cholesteryl ester clearance may be explained by the physical state of the cholesteryl ester droplets [132]. When the cytoplasmatic lipid droplets contain triglycerides, or when the cholesteryl esters contain many unsaturated fatty acids, the droplets are in a isotropic, liquid, state. Saturated

cholesteryl esters lead to the formation of anisotropic, more crystalline, droplets in which the cholesteryl esters are less accessible to the hydrolases, decreasing the rate of cholesteryl ester hydrolysis.

Influence of membrane lipid composition on cholesterol efflux

Not always the increase of the unesterified cellular cholesterol concentration leads to more efflux, because other factors may limit efflux, e.g. the desorption of cholesterol from the plasma membrane. This desorption rate may be influenced by the membrane lipid composition. The unsaturated character of phospholipids influences the exchange of cholesterol. Bloj and Zilversmit [136] and Lund-Katz *et al.* [137] reported that the efflux (exchange) of labelled cholesterol from UC/PC vesicles was faster in the presence of unsaturated phosphatidylcholine. Using cholesterol-labelled UC/PL vesicles or erythrocytes it was reported that a higher sphingomyelin content of the vesicle or plasma membrane decreased the exchange of cholesterol to the acceptor vesicles [137,138]. This was explained by the stronger van der Waals interactions between cholesterol and sphingomyelin, if compared with the interactions between cholesterol and phosphatidylcholine. However, when the membrane of human fibroblasts was depleted of sphingomyelin by treatment with sphingomyelinase, there was no effect on the efflux of cellular [^{3}H]cholesterol [139]. The sphingomyelin depletion caused a mobilization of plasma membrane cholesterol, resulting in an increase of the intracellular cholesterol esterification. In steroid producing cells the mobilized cholesterol from the membrane was transferred to the mitochondria for hormone synthesis when cells were treated with sphingomyelinase [140].

1.6 The measurement of cellular cholesterol efflux

Net mass flux of cholesterol from cells to HDL is composed of two components: influx of HDL cholesterol into cells, and efflux of cholesterol from cells to the HDL particles in the medium. The critical step of both influx and efflux is considered to be based on the same mechanism, i.e. desorption of cholesterol, followed by diffusion through the aqueous phase [82]. Net mass transfer of cholesterol from cells to the medium only occurs when efflux of cholesterol exceeds influx. The best method to measure net fluxes of cholesterol is to determine cholesterol mass in cells and/or medium before and after the incubations. Because this method is rather time-consuming, many investigators measure only the release of labelled cellular cholesterol. Data obtained with this limited approach must be interpreted with caution, because efflux of

labelled cholesterol does not necessarily correspond with net mass transfer of cholesterol! This was demonstrated by Ranganathan *et al.* [141] and Savion and Kotev-Emeth [142]. Ranganathan *et al.* [141] reported that incubation of human skin fibroblasts with normal cholesterol levels with LDL, HDL_2 or HDL_3 induced efflux of 32 to 38% of cellular [^{3}H] cholesterol to the medium, but only HDL_3 caused a net removal of cholesterol from the cells of 27%. HDL_2 did not change cellular cholesterol levels and incubation with LDL even increased the cellular cholesterol content by 20%. Another example of the discrepancy between efflux of labelled cholesterol and net mass transfer is given by Savion and Kotev-Emeth [142]: efflux of about 39% of cellular [^{3}H]cholesterol from bovine vascular endothelial cells by HDL corresponded with a 15% decrease of cellular cholesterol mass. Only when cholesterol-free acceptor particles are used, e.g. cholesterol-free proteoliposomes [143] or delipidated HDL [128], the influx of cholesterol is eliminated (at least initially) and unidirectional flow of cholesterol from cells to acceptors can be measured using labelled cholesterol. With physiological cholesterol acceptors, e.g. HDL, the measurement of bidirectional cholesterol fluxes is necessary to solve the problem. This technique implies that cells as well as acceptor particles are labelled with different radioactive cholesterol isotopes, resulting in the measurement of the movement of cholesterol between cells and medium in both directions. Johnson *et al.* [112] studied the bidirectional flux of cholesterol between HDL and Fu5AH cells with normal cholesterol levels. They labelled the cells with [^{14}C]cholesterol and HDL with [^{3}H]cholesterol. The distribution of cellular and HDL-derived isotopically labelled cholesterol was in equilibrium after 8 hours of incubation, without changes of the cholesterol content of the cells, indicating that only exchange of cholesterol between cells and HDL had occurred. But not for all cell types incubation of normocholesterolemic cells with HDL results in only exchange: Fielding *et al.* [129] reported that there was a net flux of sterols from endothelial cells to whole serum during short-term experiments (up to 60 minutes of incubation). It may be expected that the serum depletes the cells of cholesterol and that the efflux will stop after longer incubations because an equilibrium is reached. Another example of differences in cholesterol efflux from different cell types is given by Savion and Kotev-Emeth [142]. They reported that the efflux of labelled cholesterol from cells to medium was higher for bovine aortic endothelial cells than for smooth muscle cells, and that the influx of HDL cholesterol into both cell types was identical. After 24 h of incubation with 300 μg HDL/ml, the ratio of efflux:influx was 3.9 and 3.0 for endothelial and smooth muscle cells, respectively. For both cell types the actual decrease in cholesterol content was smaller than expected, based on the calculated transfer of labelled cholesterol (efflux minus influx). It could be calculated that , based upon the substraction of cholesterol influx from efflux, 28% of endothelial total cell cholesterol was taken up by the HDL particles in the medium after

24 hours of incubation (200 μg HDL/ml). However, the actual decrease of cellular cholesterol after the incubation was only 15%, which may have been explained by the endogenous synthesis of cholesterol. These experiments show that, even when bidirectional fluxes are measured, the calculated net flux cannot always predict the actual cellular cholesterol content after incubation. These experiments show that the specific activity of labelled cholesterol may change during the incubations, either by endogenous cholesterol synthesis or by the distribution of labelled cholesterol in different cellular pools, and that it is dangerous to extrapolate radioactive counts to net mass equivalents, even when bidirectional fluxes are measured.

Net mass fluxes of cholesterol can be measured more easily from cholesterol-loaded cells than from cells with normal cholesterol levels, because the net mass transfer of cholesterol from cells to medium is larger for cholesterol-enriched cells. Johnson *et al.* [76] reported that, in the presence of an excess of HDL, the efflux of cellular cholesterol was linear with the cellular content of unesterified cholesterol. In cells with a 2-fold increased cholesterol content, the efflux was about 2 times higher, if compared with control cells, and the influx of cholesterol was identical to the influx into control cells. This resulted in net mass transfer of cholesterol out of the cholesterol-loaded cells. It is generally accepted that in cholesterol-loaded cells the efflux of labelled cholesterol will be linked to net transport of cholesterol from cells to medium, but because of influx of cholesterol the efflux of labelled cholesterol overestimates the net mass transfer of cholesterol out of the cells. The experiments of Johnson *et al.* [76] imply that the actual release of cholesterol from loaded Fu5AH cells is only half of the measured cellular [^{3}H]cholesterol efflux. The chance of a change of the specific activity of the cellular cholesterol label is smaller, because it can be expected that the endogenous cholesterol synthesis is virtually zero in loaded cells, but the existence of different cellular cholesterol pools with different specific radioactivities is still possible and may influence the resulting data.

1.7 Scope of this thesis

The experiments described in this thesis were performed in order to clarify the mechanism of efflux of cellular cholesterol, a key step in reverse cholesterol transport. It is not known which factors influence the rate of transfer of cholesterol from cells to HDL. Rate limiting steps are important steps in the efflux process and may give more insight into the mechanism of the efflux of cellular cholesterol. Possible rate limiting steps are the cholesteryl ester hydrolysis and subsequent transport of cholesterol to the

plasma membrane, the desorption of cholesterol from the membrane, the binding of HDL to the membrane or the composition of the HDL particles.

The major part of the experiments described in this thesis were performed with human endothelial cells (EA.hy 926 cell line). These cells are hybrids of human umbilical vein endothelial cells with lung carcinoma cells (A549 line) and show many characteristics of differentiated endothelial cells, e.g., the expression of von Willebrand factor, tissue plasminogen activator, plasminogen activtor inhibitor-type 1, and the production of prostacyclin. In addition, the cells formed confluent monolayers with contact-inhibition of cell growth. For obvious reasons (see section 1.6) we choose to measure only net mass transfer of cholesterol from the cells and first developed a method to enrich the cells with cholesterol using cationized LDL (see chapter 2). Subsequently, we studied the efflux of cholesterol from loaded EA.hy 926 cells by different plasma lipoproteins, and the effects of the plasma enzyme lecithin:cholesterol acyltransferase, as described in chapters 3 and 4. In chapter 5 the effect of HDL binding on HDL-mediated cholesterol efflux was studied using modifications of HDL with tetranitromethane and dimethyl suberimidate. The effect of cell membrane phospholipid composition on HDL binding and cholesterol efflux is described in chapter 6. All these experiments were carried out with ultracentrifugally isolated HDL or HDL_3. HDL is a heterogeneous population of lipoprotein particles and HDL subfractions with distinct apolipoprotein compositions can be isolated from plasma with immuno-affinity columns. The different apolipoprotein composition of these HDL subfractions are expected to be important for the interaction with possible plasma membrane HDL binding proteins and may therefore clarify the role of HDL binding in the efflux of cellular cholesterol. Chapter 7 and 8 contain a description of our investigations of the role of different immunopurified HDL subfractions in the efflux of cellular cholesterol from various cell types as well as in the uptake of HDL by hepatocytes.

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chapter 2

Endothelial EA.hy 926 cells can be loaded with cationized LDL, but not with acetylated LDL

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Abstract EA.hy 926 cells, a human endothelial cell line, show characteristics of differentiated endothelial cells. Endothelial cells normally express membrane scavenger receptors. Therefore modified LDL, e.g., acetylated LDL, can be taken up, causing accumulation of mass amounts of cholesterol. We have shown that EA.hy 926 cells cannot be loaded with cholesterol using acetylated LDL, but can be efficiently enriched with cholesterol by incubation with cationized LDL. The loaded cells may serve as models for studies on reverse cholesterol transport.

The EA.hy 926 cell line shows characteristics of differentiated human endothelial cells, e.g., expression of von Willebrand factor [1], tissue plasminogen activator [2], plasminogen activator inhibitor-type 1 [2] and production of prostacyclin [3]. The advantages of the use of a cell line, instead of primary cultures, are the constant availability of cells and the fact that there is no risk of loss of differentiated functions, like in senescent primary cultures. Cells of the permanent EA.hy 926 line still express typical differentiated characteristics after more than 100 cumulative population doublings [1,2].

In vivo, endothelial cells are constantly in contact with high concentrations of cholesterol-carrying plasma lipoproteins, and serve as an important barrier between plasma and underlying cells of the vessel wall. Plasma cholesterol is constantly taken up by endothelial cells by way of fluid and/or adsorptive endocytosis, even in the presence of down-regulated LDL receptor-mediated endocytosis [4], or by uptake of chylomicron-derived cholesterol [5]. Therefore an efficient system of reverse cholesterol transport is necessary [6]. Efflux of cellular cholesterol can be studied in cultured endothelial cells. Substantial amounts of net mass efflux of cholesterol can only be measured from cholesterol-enriched cells. It was reported that endothelial cells, derived from bovine aorta or human umbilical vein, can be loaded with modified LDL [7-9]. This uptake is mediated via plasma membrane scavenger receptors. Cellular cholesteryl ester levels increased at least 3-fold after incubation of bovine aortic endothelial cells with 25 μg acetylated LDL/ml [7,8]. Stein and Stein [7] reported similar results for endothelial cells derived from human umbilical vein. Malondialdehyde-modified LDL, which is also taken up by scavenger receptors, increased total cell cholesterol by 77% after 72 h of incubation with 100 μg LDL-protein/ml [9]. Loading of endothelial cells never resulted in more than 2-fold increase of total cell cholesterol [8,9]. We studied the loading of EA.hy 926 cells with different types of modified LDL in order to use these cells for cholesterol efflux experiments.

The endothelial cell line EA.hy 926 was generously provided by Dr. C.-J.S. Edgell [1]. EA.hy 926 cells were cultured in DMEM (Flow Laboratories), supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and HAT (100 μM hypoxanthin, Merck No. 4517; 0.4 μM aminopterin, Serva No. 13170; 16 μM thymidin, Merck No. 8206) at 37 °C in 5% CO_2 and 95% air. J774 macrophages were cultured in DMEM, supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. EA.hy 926 and J774 cells were plated in multiwell dishes (35 mm, $1 \cdot 10^5$ cells/well). Confluent EA.hy 926 monolayers were used for loading with various cholesterol-containing media. Subconfluent cultures of macrophages were used 2-3 days after plating. Cholesterol-loading media consisted of DMEM, supplemented with 10% FCS, 0.2 or 2% BSA (Sigma No. A-4378), L-glutamine, penicillin, streptomycin and the indicated amounts of LDL or cholesterol suspension (cholesterol was added to FCS in ethanol, final concentration of ethanol in medium was 1%). The cells were incubated for 24 to 48 h at 37 °C. After the incubations the wells were rinsed three times with 0.9% NaCl, containing 0.2% BSA and 50 mM Tris-HCl (pH 7.4) and three times with 0.9% NaCl/50 mM Tris-HCl (pH 7.4). Subsequently the cells were scraped into 1 ml of 0.9% NaCl/50 mM Tris-HCl (pH 7.4). Cellular cholesterol was measured as in ref. [10]. HDL_3 (density range 1.125-1.21 g/ml) and LDL (density range 1.006-1.063) were isolated by

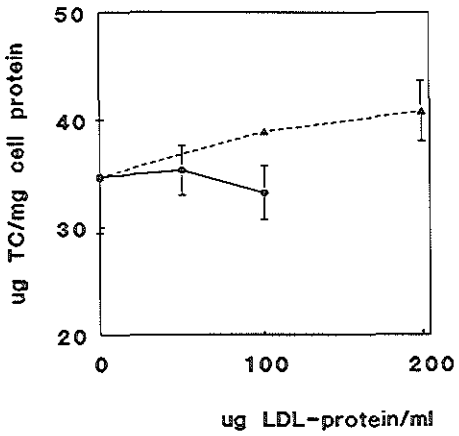

Fig. 1 EA.hy 926 cells were incubated with native LDL (▲) or acetylated LDL (●) in 10% FCS-containing medium for 48 h at 37 °C. Total cell cholesterol was determined as described and expressed as μg per mg cell protein (means ± SEM, n=2-3).

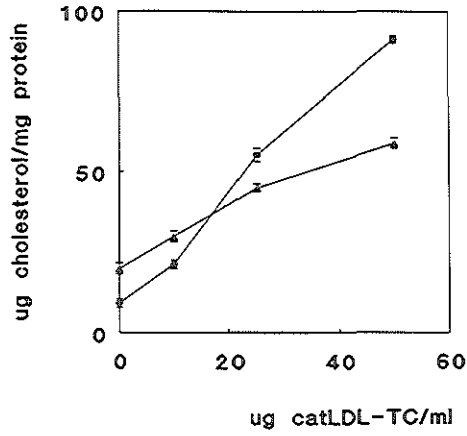

Fig. 2 EA.hy 926 cells were incubated with cationized LDL in 10% FCS-containing medium for 24 h at 37 °C. Total and unesterified cell cholesterol were determined as described. Esterified cholesterol was calculated as the difference of total and unesterified cholesterol.
Unesterified cholesterol (▲); esterified cholesterol (●). Values are means ± SEM (n=6).

Table I
Incubation of EA.hy 926 cells with various cholesterol-containing media

medium[a]	*n*	μg TC/mg	μg UC/mg	μg EC/mg
experiment 1				
FCS	13	30.9 ± 5.3	27.0 ± 4.6	3.9 ± 5.5
BSA	11	25.4 ± 10.3	23.4 ± 5.9	2.0 ± 5.6
BSA + acLDL	12	31.4 ± 9.2	28.9 ± 7.8	2.5 ± 4.9
BSA + native LDL	5	29.0 ± 4.2	28.1 ± 5.5	0.9 ± 1.4
experiment 2				
FCS	3	40.3 ± 2.4	40.4 ± 2.0	-0.1 ± 1.3
FCS + catLDL	3	119.1 ± 7.4	67.6 ± 3.3	51.5 ± 4.1
FCS + UC	3	122.1 ± 15.2	103.5 ± 11.8	18.6 ± 4.1

[a] Confluent monolayers were incubated with 10% FCS or 2% BSA at 37 °C for 48 h in experiment 1 and for 24 h in experiment 2. LDL and acLDL concentrations used were 50 μg LDL protein/ml. 25 μg catLDL protein/ml or 25 μg UC/ml was used in experiment 2. After incubation the cells were washed, scraped into buffer, extracted and total and unesterified cell cholesterol were measured. Esterified cholesterol was calculated from total and unesterified cholesterol. Values are means ± SD.

sequential ultracentrifugation of human plasma [11]. LDL was acetylated or cationized according to Basu *et al.* [12]. All lipoprotein preparations were dialyzed against 0.9% NaCl, containing 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, and filter sterilized (0.45 μm for HDL_3, LDL and acetylated LDL; 0.8 μm for cationized LDL). LDL-cholesterol was measured enzymatically using a commercially available kit (cholesterol kit No. 310328 and cholesterol esterase No. 161772, Boehringer Mannheim). Protein was measured according to ref. [13]. The protein content of cationized LDL was calculated from the cholesterol concentration of cationized LDL and the protein:cholesterol ratio of native LDL, because of disturbances of protein measurements after cationization. LDL, acetylated LDL and cationized LDL were stored at 4 °C.

In a number of experiments we tried to load EA.hy 926 cells with different preparations and concentrations of acetylated LDL. Figure 1 shows cellular total cholesterol levels after incubation of EA.hy 926 cells with different concentrations of acetylated LDL or native LDL in medium containing 10% FCS. Native LDL was used as a control, since no large increase in cholesterol after incubation with LDL was reported for endothelial cells [9]. EA.hy 926 cell cholesterol increased 18% after 48 h of incubation with 200 μg native LDL/ml. Surprisingly, incubation with acetylated LDL

had no effect on EA.hy 926 cell cholesterol. We used FCS- as well as BSA-containing media for incubation of EA.hy 926 cells (see table I). Native LDL as well as acetylated LDL only slightly increased total cell cholesterol if compared with incubation with BSA alone. Again, acetylated LDL was not taken up more than native LDL. Cells grown with 10% FCS and cells incubated with LDL or acetylated LDL had identical cholesterol levels. Prolonged incubation (72 h) with acetylated LDL or native LDL gave similar results (not shown). These results suggest that active scavenger receptors are not present on EA.hy 926 cells. Table I also shows that EA.hy 926 cells can be loaded very effectively with cationized LDL. Total cell cholesterol increased 3-fold, and 43% of this cholesterol was esterified. Loading with a suspension of cholesterol in FCS also resulted in a 3-fold increase of total cell cholesterol, but only 15% of cell cholesterol was in the esterified form. The amount of cell protein per well was decreased after incubation with a suspension of cholesterol (217 ± 16 μg, mean ± SD), if compared with incubation with FCS alone (243 ± 10 μg). This suggests that the cholesterol suspension was toxic to the cells at the concentration used. Since esterified cholesterol is the more physiological storage form, loading with cationized LDL resulted in a more relevant model. Cholesterol loading with cationized LDL was concentration-dependent, as can be seen in figure 2. Figure 2 also shows that both esterified and unesterified cholesterol increased after incubation with cationized LDL. The cellular esterified:unesterified cholesterol ratio increased after loading the cells with higher concentrations of cationized LDL. This may be caused by a lysosomal accumulation of undegraded cationized LDL. It was reported that the binding of cationized LDL is very high, if compared with native LDL [14,15]. This high binding of cationized LDL (about 100 times more than for native LDL [16]) may result in relatively high rates of uptake of cationized LDL, while rates of hydrolysis of [^{3}H]cholesteryl linoleate from cationized or native LDL are comparable [12].

Table II shows the cell cholesterol levels of J774 macrophages after incubation with native LDL, acetylated LDL or cationized LDL. Incubation of macrophages with native LDL did not increase cellular cholesterol levels. Total cellular cholesterol of J774 macrophages increased up to 5-fold after incubation with 100 μg acetylated LDL-protein/ml. About 50-60% of cell cholesterol was esterified. As expected, incubation with acetylated LDL resulted in the formation of foam cells within 24 h (not shown). These data indicate that LDL was acetylated effectively and that the lack of uptake of acetylated LDL by EA.hy 926 cells was not caused by improperly acetylated LDL. Like EA.hy 926 cells, J774 macrophages can be cholesterol-loaded with cationized LDL. Incubation with 35 μg cationized LDL/ml increased total cell cholesterol nearly 3-fold, if compared with incubation with 10% FCS only. About 60% of cell cholesterol was esterified. Incubation with FCS alone slightly increased cholesteryl esters (by 10 μg/mg cell protein), if compared with BSA. Human skin fibroblasts also become enriched with

cholesterol after incubation with cationized LDL [14,15]. Cationized LDL is neither taken up by the LDL receptor, nor by the scavenger receptor. It is endocytosed after nonspecific binding of the cationized LDL particles to anionic sites on plasma membranes [14]. This implies that cationized LDL may be used for cholesterol loading of many different cell types. After endocytosis the cationized LDL particles are directed to the lysosomes and subsequently degraded, suggesting that the processing of cationized LDL is comparable to that of native LDL [14]. Dependent on the relative rates of lysosomal cholesteryl ester hydrolysis and cellular cholesterol esterification in a specific cell type, the cholesterol derived from cationized LDL will accumulate either as lysosomal or cytoplasmatic cholesteryl esters.

Table III shows HDL_3-dependent efflux of unesterified cholesterol from loaded EA.hy 926 cells and J774 macrophages. It can be expected that some cationized LDL is present at the external cell surface during these experiments [14,15]. There was no increase of cholesteryl esters in the culture media after 24 h of incubation with BSA or HDL_3 (not shown). This indicates that possible extracellular cationized LDL is very

Table II
Loading of J774 macrophages with acetylated LDL and cationized LDL

treatment (*n*)	LDL (μg protein/ml)	μg TC/mg	μg UC/mg	μg EC/mg
experiment 1[a]				
control (6)	--	25.9 ± 1.5[c]	23.5 ± 1.5	2.4 ± 2.2
native LDL (5)	50	27.7 ± 2.6	25.9 ± 2.7	1.8 ± 0.6
acLDL (6)	50	91.4 ± 18.0	42.6 ± 7.3	48.8 ± 11.9
acLDL (2)	100	146.1 ± 6.4	65.8 ± 8.5	80.3 ± 2.1
experiment 2[b]				
BSA (3)	--	27.7 ± 3.1	28.1 ± 3.3	-0.4 ± 1.1
BSA + acLDL (3)	100	153.0 ± 0.8	56.0 ± 0.8	97.0 ± 0.4
FCS (3)	--	39.0 ± 1.3	28.9 ± 2.4	10.1 ± 1.2
FCS + catLDL (3)	35	108.8 ± 5.0	45.2 ± 1.6	63.6 ± 3.5

[a] J774 macrophages were incubated in DMEM, supplemented with L-glutamine, penicillin, streptomycin and lipoproteins as indicated for 48 h at 37 °C. After the incubations cells were rinsed, scraped into buffer, extracted and total and unesterified cell cholesterol were measured. Esterified cholesterol was calculated as the difference of total and unesterified cholesterol.
[b] J774 macrophages were incubated for 24 at 37 °C in DMEM with L-glutamine, penicillin, streptomycin, 0.2% BSA or 10% FCS and lipoproteins as indicated.
[c] Values are means ± SD.

Table III
HDL_3-mediated cholesterol efflux from EA.hy 926 and J774 cells

HDL_3 conc. (mg protein/ml)	nmol cholesterol efflux/well[a]		
	EA.hy 926 loaded with catLDL	J774 loaded with catLDL	J774 loaded with acLDL
0	1.8 ± 1.0	1.7 ± 0.7	3.3 ± 0.3
0.1	-	8.7 ± 1.0	10.6 ± 1.2
0.2	10.7 ± 0.4	10.2 ± 1.5	12.3 ± 1.0
0.5	10.2 ± 1.6	8.5 ± 1.0	14.2 ± 0.8
1.0	-	12.6 ± 4.7	12.9 ± 2.4

[a] Cells were loaded with 50 μg catLDL TC/ml or 100 μg acLDL protein/ml for 24 h at 37 °C (1 ml/well). Subsequently the cells were incubated with 1 ml of DMEM, supplemented with 0.2% BSA, L-glutamine, penicillin, streptomycin and HDL_3 as indicated. After 24 h of incubation at 37 °C the plates were cooled on ice, media were collected and a small amount of dislodged cells was spun down. Efflux was measured as the increase of unesterified cholesterol in the medium. Cholesterol was assayed enzymatically using a commercially available kit ($n=6$ for EA.hy 926 cells and $n=3$ for J774 cells, values are means ± SD).

tightly bound to the plasma membrane and is not released from the cells during the efflux period. Stein et al. [15] reported that extracellular cationized LDL was bound very tightly to fibroblasts. Desorption of plastic-bound cationized LDL cannot interfere with the efflux described in this report. The cholesterol concentrations in culture media of empty wells after 24 h of incubation were identical in untreated wells and in wells preincubated with cationized LDL (not shown). Efflux reached a maximum at about 0.2 mg HDL_3 protein/ml for both EA.hy 926 and J774 cells. Table III also shows that efflux from J774 cells was similar for acetylated LDL- and cationized LDL-loaded cells. These data indicate that loading of cells with cationized LDL is a suitable model to study subsequent efflux of intracellular cholesterol from both cell types.

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chapter 3

High density lipoprotein-mediated efflux of cholesterol from human endothelial cells

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Abstract EA.hy 926 cells, a human endothelial cell line, show characteristics of differentiated endothelial cells. The cells express saturable binding of apo E-free ^{125}I-high density lipoprotein$_3$ (HDL_3). B_{max} increased from 71 to 226 ng HDL_3 bound/mg cell protein after cholesterol loading of the confluent endothelial cells with cationized low density lipoprotein (LDL). The affinity did not change after cholesterol enrichment (K_d was 37 μg HDL_3 protein/ml for control cells and 31 μg/ml for loaded cells).
Incubation of cholesterol-loaded EA.hy 926 cells with native HDL and LDL had different effects on cellular cholesterol levels. Incubation with HDL decreased both esterified and unesterified cellular cholesterol, but LDL did not change total cellular cholesterol. However, LDL tended to increase cellular cholesteryl esters, with a concomitant decrease of unesterified cellular cholesterol. Incubation of endothelial cells with both HDL and LDL also resulted in decreased total cellular cholesterol levels. These data show that cationized LDL-loaded human endothelial EA.hy 926 cells can be used to study HDL-mediated efflux of cellular cholesterol, the first step in reverse cholesterol transport.

Introduction

High density lipoprotein (HDL) is believed to play a role in the transfer of peripheral cholesterol back to the liver, a process called reverse cholesterol transport. HDL binding membrane proteins were visualized in different cell types [1-5], but the physiological role of these HDL binding proteins is still unclear. For various cell types, including bovine vascular endothelial cells, up-regulation of HDL binding activity after enrichment of the cells with cholesterol is reported [6-10]. This suggests a functional role for HDL binding proteins in the removal of excess cellular cholesterol. However, there are also investigators denying the binding of HDL to membrane proteins [11,12] or the role of HDL binding in cholesterol efflux [13-15].

In vivo, vascular endothelial cells are in contact with high concentrations of plasma lipoproteins. The endothelial cell monolayer is the primary site of interaction between the vessel wall, blood cells and plasma lipoproteins. It exerts a protective role against thrombosis and atherogenesis. Lipoprotein uptake by vascular endothelial cells has been implicated in the initiation and development of atherosclerosis [16,17]. In vivo, cholesterol uptake by endothelial cells can take place via several mechanisms. As it can be expected that LDL receptors are down-regulated in vivo, receptor-mediated endocytosis of apo B or apo E containing lipoproteins is unlikely. It was reported however that endothelial cells can take up LDL via nonspecific endocytosis or pinocytosis [18]. A second possibility is the uptake of cholesterol from chylomicrons, as suggested

by Fielding *et al.* [19].

The first step of reverse cholesterol transport, egress of cholesterol from endothelial cells, can be studied in vitro. EA.hy 926 cells, a human endothelial cell line, show characteristics of differentiated endothelial cells, e.g., expression of von Willebrand factor [20], tissue plasminogen activator [21], plasminogen activator inhibitor-type 1 [21] and production of prostacyclin [22]. These cells still express typical differentiated characteristics after more than one hundred cumulative population doublings [20,21]. We studied net mass efflux of cholesterol from these endothelial cells after enrichment of the cells with intracellular cholesterol derived from cationized LDL. Although the effects of cholesterol enrichment on binding of HDL_3 to endothelial cells are studied before [6,7], very little is known about the efflux of cholesterol from endothelial cells. In this study we examined 1) the effect of cell cholesterol enrichment on binding of apo E-free HDL_3 to EA.hy 926 cells; 2) whether HDL-mediated cholesterol efflux from EA.hy 926 cells is dependent on the cellular concentration of cholesterol; and 3) whether incubation with LDL or LDL plus HDL is also able to induce efflux of cholesterol from loaded EA.hy 926 cells. Our results indicate that EA.hy 926 cells are suitable for further studies on the mechanism of HDL-mediated efflux of cellular cholesterol.

Materials and methods

Cell culture

The endothelial cell line EA.hy 926 was generously provided by Dr. C.-J.S. Edgell [20]. EA.hy 926 cells were cultured in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and HAT (100 μM hypoxanthin, Merck No. 4517; 0.4 μM aminopterin, Serva No. 13170; 16 μM thymidin, Merck No. 8206) at 37 °C in 5% CO_2 and 95%. Cells were plated in multiwell dishes (22 mm: $0.5 \cdot 10^4$ cells/well, 35 mm: $1 \cdot 10^5$ cells/well) 4 to 5 days prior to each experiment. Medium was changed every 2 or 3 days. The confluent cells were loaded with cholesterol during a 24 h incubation of the cells with cationized LDL in growth medium as described earlier [23]. Control cells were preincubated similarly with growth medium (containing 10% FCS) only.

Lipoproteins

HDL (density range 1.063-1.21 g/ml), HDL_3 (density range 1.125-1.21 g/ml) and LDL (density range 1.006-1.063) were isolated by sequential ultracentrifugation of human plasma according to Havel *et al.* [24]. Apo E-free HDL_3 was isolated by chromatography of HDL_3 on a heparin-Sepharose column [25]. Apo E-free HDL_3 was iodinated using the iodine monochloride method (using $Na^{125}I$ obtained from Amersham International) as described earlier [26]. The molar iodine to protein ratio of the ^{125}I-HDL_3 preparations ranged from 0.22 to 0.49; 95.4 ± 0.8% of the label was protein-bound, 1.7 ± 0.6% was in the HDL-lipids and 2.9 ± 0.5% was free. All lipoproteins were dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Before cell experiments, the lipoproteins were dialyzed for a second time against DMEM and filter sterilized (0.45 μm).

LDL was cationized according to Basu *et.al.* [27]. An equal volume of LDL (20 to 30 mg of protein) was added to a 2 M solution of 3-dimethylaminopropylamine (Aldrich No. 24.005-2) in H_2O at room temperature. The pH was adjusted to 6.5 with HCl. After stirring this mixture, 100 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (Sigma No. E-7750) was added. The pH was maintained at 6.5 with 0.1 M HCl during the reaction. After 3 hours, the pH of the solution did not change anymore; the reaction mixture was left at 4 °C overnight. The cationized LDL was dialyzed against 0.9 % NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, filter sterilized (0.8 µm) and stored. Storage of cationized LDL at +4 or -20 °C did not influence the uptake by EA.hy 926 cells.

Binding of ^{125}I-HDL$_3$ to EA.hy 926 cells

Confluent EA.hy 926 cells (in 22 mm wells) were preincubated with 50 µg catLDL-TC/ml in growth medium for 24 h (0.5 ml/well). Control cells were preincubated similarly with growth medium only. After cholesterol enrichment, the wells were rinsed three times with DMEM containing 2 mg BSA/ml (Sigma No. A-4378) and penicillin/streptomycin at room temperature, followed by three rinses with cold DMEM containing only penicillin/streptomycin. Subsequently, media were added (DMEM, supplemented with 2 mg BSA/ml, L-glutamine, penicillin/streptomycin and lipoproteins as indicated, 0.5 ml/well) and the cells were incubated 4 h at 4 °C in an atmosphere with 5% CO_2. After the incubations, the cells were rinsed three times with 2 mg BSA/ml in 0.9% NaCl/50 mM Tris-HCl (pH 7.4) and three times with 0.9% NaCl/50 mM Tris-HCl (pH 7.4). Cell protein was dissolved in 300 µl 1 M NaOH. The radioactivity in this solution was counted using a Packard Minaxi 5000 gamma counter and protein was measured [28] in the same aliquot.

Saturable binding of apo E-free ^{125}I-HDL$_3$ to control cells (after preincubation with growth medium only) and cholesterol-loaded cells (after preincubation with growth medium plus cationized LDL) was measured at 5-100 µg ^{125}I-HDL$_3$ protein/ml (specific activity 200 cpm/ng). Nonspecific binding was measured in the presence of 1 mg unlabelled HDL$_3$/ml. Specific binding was calculated by difference of total and nonspecific binding.

Efflux experiments

After loading with cationized LDL the cells (in 35 mm wells) were rinsed twice with DMEM, containing 2 mg BSA/ml and penicillin/streptomycin, and twice with DMEM, plus penicillin/streptomycin only, at room temperature. The cells were subsequently incubated for 24 h at 37 °C with 1 ml efflux medium in an atmosphere with 5% CO_2 and 95% air. Efflux media consisted of DMEM, supplemented with L-glutamine, penicillin/streptomycin and 2 mg BSA/ml (Sigma A-4378) and lipoproteins as indicated. Efflux experiments were ended by cooling the culture dishes on ice. The culture media were collected and small amounts of dislodged cells were spun down (500 x g_{max}, 4 °C). Cell media were stored at -20 °C until cholesterol determinations. The wells were rinsed three times in 0.9% NaCl with 2 mg BSA/ml and 50 mM Tris-HCl (pH 7.4), followed by three rinses with 0.9% NaCl/Tris-HCl (50 mM, pH 7.4). The cells were scraped into 1 ml 0.9% NaCl/Tris-HCl (50 mM, pH 7.4) with a rubber policeman and lipids were extracted using the method of Bligh and Dyer [29]. The resulting protein pellet was dissolved in 500 µl 0.1 M NaOH containing 10% SDS (w/v). Cell protein was measured according to Lowry *et al.* [28]. Cellular lipids were dried down under nitrogen and dissolved in 300 µl 2-propanol. The lipid extracts were stored up to 1 day at -20 °C and unesterified and total cholesterol were determined separately using a slight modification of the method described by Heider and Boyett [30]. For determination of unesterified cholesterol the reaction mixture consisted of 0.15 mg *p*-OH-phenylacetic acid/ml (Sigma No. H-4377), 5 U peroxidase/ml (Boehringer Mannheim No. 108081) and 3.2 µg cholesterol oxidase/ml (Boehringer Mannheim No. 396818) in sodium phosphate buffer (50 mM, pH 7.4). The reaction mixture of the total cholesterol determination was as for unesterified cholesterol, plus 6.4 µg cholesteryl esterase/ml (Boehringer Mannheim No. 161772), 5 mM taurocholate (Calbiochem No. 580217) and 0.17 mM PEG-6000 (Serva No. 33137). 0.75 ml reaction mixture was added to 40 µl cell extract in 2-propanol. A 100 µg/ml solution of cholesterol (Sigma No. C-

8253) in 2-propanol was used as a standard. After the incubation (37 °C, 30 min for unesterified cholesterol and 120 min for total cholesterol assays) the reaction was ended by addition of 1.5 ml 0.5 M NaOH and fluorescence was measured with a Perkin-Elmer LS-3B fluorimeter (excitation 325 nm, emission 415 nm). Total and unesterified cholesterol in the incubation media were measured using a commercially available kit (cholesterol kit No. 310328, cholesterol esterase No. 161772, Boehringer Mannheim).

Statistical analyses

The nonparametric Mann-Whitney test was used for comparison between treatment groups with unpaired observations. For paired observations the nonparametric paired Wilcoxon test was used. The differences were considered statistically significant if $P<0.05$.

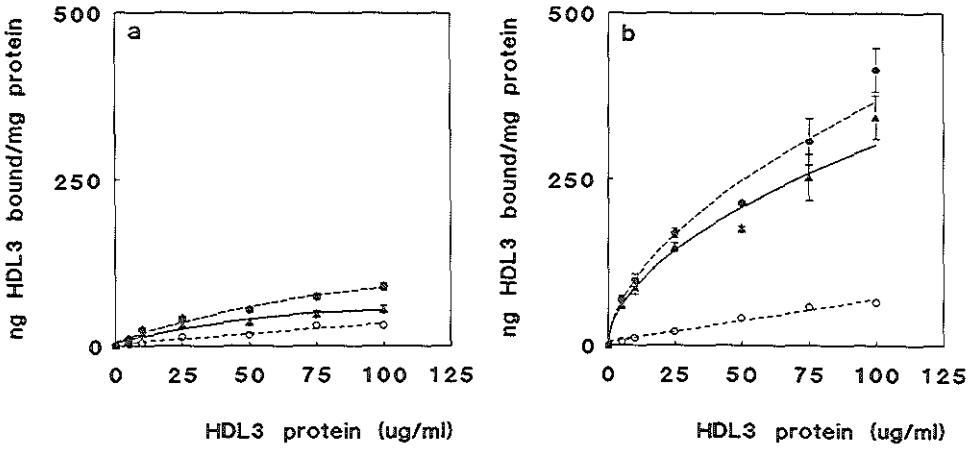

Fig. 1 Binding of apo E-free ^{125}I-HDL_3 to control (preincubated in medium with 10% FCS, fig. a) and cholesterol-loaded EA.hy 926 cells (preincubated in medium with 10% FCS plus 50 μg catLDL-TC/ml, fig. b). Total (●), specific (▲) and nonspecific (○) binding per mg cell protein were measured after 4 h incubation at 4 °C. Nonspecific binding was determined after addition of 1 mg unlabelled HDL_3/ml, specific binding was calculated by difference of total and nonspecific binding. Values are means ± SEM ($n=3$).

Table I

Binding characteristics of apo E-free HDL_3 to control and cholesterol-loaded EA.hy 926 cells.

	K_d[a]	B_{max}[b]
control cells		
experiment 1	31	62
experiment 2	42	79
catLDL-loaded cells[c]	31 ± 4	226 ± 64

[a] Values are in μg HDL_3 protein/ml.
[b] Values are in ng HDL_3 protein bound/mg cell protein.
[c] Confluent EA.hy 926 cells were incubated with 50 μg catLDL-TC/ml for 24 h at 37 °C. Values are means ± SEM of 4 separate experiments.

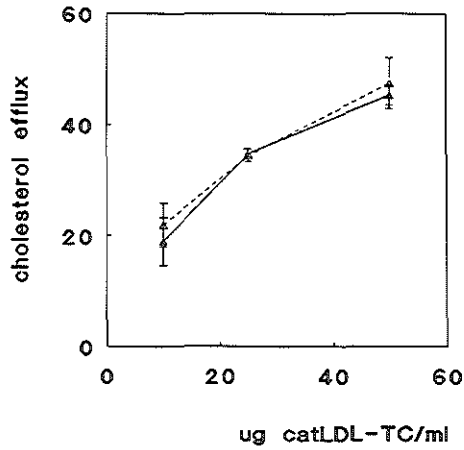

Fig. 2 EA.hy 926 cells were loaded with increasing concentrations of catLDL for 24 h at 37 °C. Subsequently, the HDL-mediated cholesterol efflux was measured as the increase of cholesterol in the medium after 24 h of incubation with 0.2 mg HDL_3/ml at 37 °C. Medium UC (▲) and TC (△) were both determined. Efflux was expressed in nmol cholesterol efflux per mg cell protein (means ± SEM, $n=6$).

Results

Figure 1 shows the binding of apo E-free ^{125}I-HDL_3 to control and cholesterol-loaded EA.hy 926 cells. Total as well as specific binding was higher for the cholesterol-enriched cells. Table I shows the K_d and B_{max} of binding of apo E-free HDL_3 to control and cholesterol-loaded EA.hy 926 cells. Cholesterol enrichment did not change the affinity for HDL_3 (K_d was about 37 and 31 μg/ml, respectively), but the maximal binding increased from about 71 to 226 ng HDL_3/mg cell protein for control and cholesterol-loaded cells.

Table II shows that after 24 h of incubation of EA.hy 926 cells with 50 μg of catLDL-TC/ml total cellular cholesterol increased about 4 fold. Both unesterified and esterified cholesterol increased after loading with catLDL. Subsequent incubation of cholesterol-enriched cells with HDL decreased cell cholesterol significantly, in comparison to incubation with BSA alone. After incubation with 2 mg of HDL/ml, both cell UC and EC levels decreased.

After loading EA.hy 926 cells with different concentrations of cationized LDL, efflux was measured after 24 h of incubation with 0.2 mg HDL_3 protein/ml (see fig. 2). After the preincubation with 0, 10, 25 and 50 μg catLDL-TC/ml, cellular cholesterol was

34±1, 56±7, 105±6 and 155±3 μg/mg cell protein, respectively (means ± SD, $n=6$). The efflux of cholesterol, measured as the increase of cholesterol in the medium, was dependent on the loading of the cells (see fig. 2). Under all conditions tested, the increase in medium total cholesterol quantitatively consisted of unesterified cholesterol.

Figure 3 shows that efflux was specific for HDL, since LDL did not lower cellular cholesterol content. HDL-mediated efflux was already maximal at 0.15 mg of HDL protein/ml. All HDL concentrations, ranging from 0.15 to 2.0 mg of HDL protein/ml, reduced total cell cholesterol significantly ($P<0.01$). At all LDL concentrations used, no significant reduction of total cellular cholesterol by LDL could be detected. Comparable results were obtained if cell cholesterol was expressed as μg cholesterol per mg cell protein (not shown). HDL-mediated efflux resulted in a decrease of both cell UC and EC (see fig. 4a). At low concentrations of HDL, the reduction of cell TC was caused mainly by efflux of unesterified cholesterol. Cell UC was significantly

Table II
Loading and HDL-mediated efflux of cholesterol from EA.hy 926 cells

	μg cholesterol/mg cell protein[a]		
	TC	UC	EC
control[b]	46.3 ± 5.0	39.7 ± 5.8	6.6 ± 1.5
loaded	166.4 ± 12.0	96.0 ± 6.2	70.5 ± 11.5
after 24 h efflux			
BSA	165.6 ± 0.4	101.5 ± 3.8	64.1 ± 3.9
+ HDL	121.7 ± 9.3[c]	76.6 ± 7.8[c]	45.1 ± 3.1[c]

[a] Values are means ± SD ($n=3$).
[b] Cells were preincubated for 24 h, control cells in medium with 10% FCS and loaded cells in the same medium plus 50 μg cationized LDL-TC/ml. After the preincubation the loaded cells were rinsed, and incubated for 24 h in efflux medium with BSA (0.2% w/v) only or with BSA plus 2 mg HDL-protein/ml.
[c] Significantly different from BSA alone (Mann-Whitney, $P<0.05$).

Table III
Efflux of cell cholesterol after incubation of EA.hy 926 cells with HDL and LDL

treatment (n)[a]	μg TC/mg cell protein
BSA (2)	145.7 ± 2.5
BSA + HDL (3)	129.1 ± 6.3[b]
BSA + LDL (3)	143.7 ± 2.0
BSA + HDL + LDL (3)	129.2 ± 6.8[b]

[a] After loading the EA.hy 926 cells with cationized LDL the cells were washed and incubated with DMEM, containing L-glutamine, penicillin/streptomycin, 2 mg BSA/ml and lipoproteins as indicated (concentrations used were 2 mg of HDL protein/ml and 1 mg of LDL protein/ml). After 24 h of incubation at 37 °C the cells were washed, scraped into buffer, extracted and cell TC and protein were determined as described in *Materials and methods*. Values are means ± SD ($n=2$-3).
[b] Significantly different from incubation with LDL (nonparametric Mann-Whitney test, $P<0.05$).

decreased at all HDL concentrations used, if compared with incubation with BSA only (nonparametric Mann-Whitney test, $P < 0.01$). Also cell EC was significantly lowered after incubation with 0.15-2.0 mg HDL/ml ($P < 0.05$). Concentrations of about 0.2 mg HDL/ml and higher resulted in maximal efflux of cell cholesterol: cell UC decreased 20-25% and cell EC decreased 10-15%. After incubation with LDL cell EC tended to increase, with a concomitant decrease in UC (see fig. 4b). The decrease of cell UC reached statistical significance, if compared with the UC concentration after incubation with BSA only (nonparametric Mann-Whitney test, $P < 0.01$), at concentrations of 1.0-2.4 mg LDL protein/ml. Table III shows the cell TC levels after incubation of loaded EA.hy 926 cells with BSA, or physiological concentrations of HDL, LDL and HDL plus LDL. As expected, HDL decreased cell TC, and LDL did not. Cell cholesterol also decreased when the cells were incubated with a combination of HDL and LDL. Incubation with HDL alone or HDL plus LDL resulted in significantly lower cell TC levels than incubation with LDL, indicating that LDL did not inhibit HDL-mediated efflux.

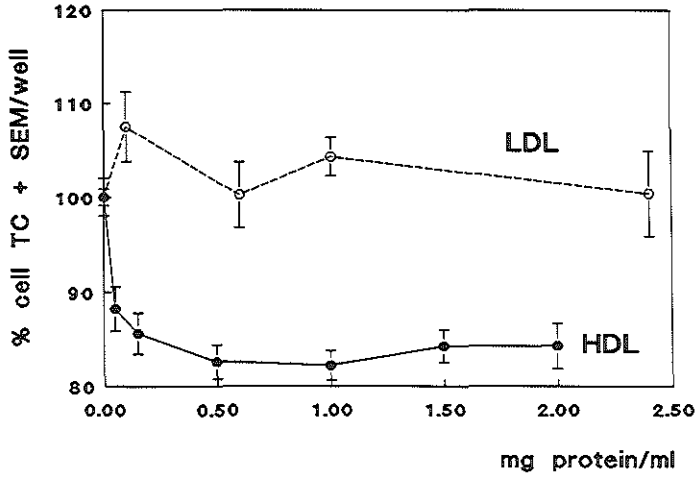

Fig. 3 Efflux of cell TC from cholesterol-loaded EA.hy 926 cells by HDL (—●—) and LDL (--o--). After 24 h of incubation with DMEM, supplemented with L-glutamine, penicillin/streptomycin, 2 mg BSA/ml and lipoproteins as indicated, cells were washed, scraped into buffer, extracted and cell TC was measured fluorimetrically as described in *Materials and methods*. Values are expressed as % of cell TC after incubation with BSA alone (means ± SEM).

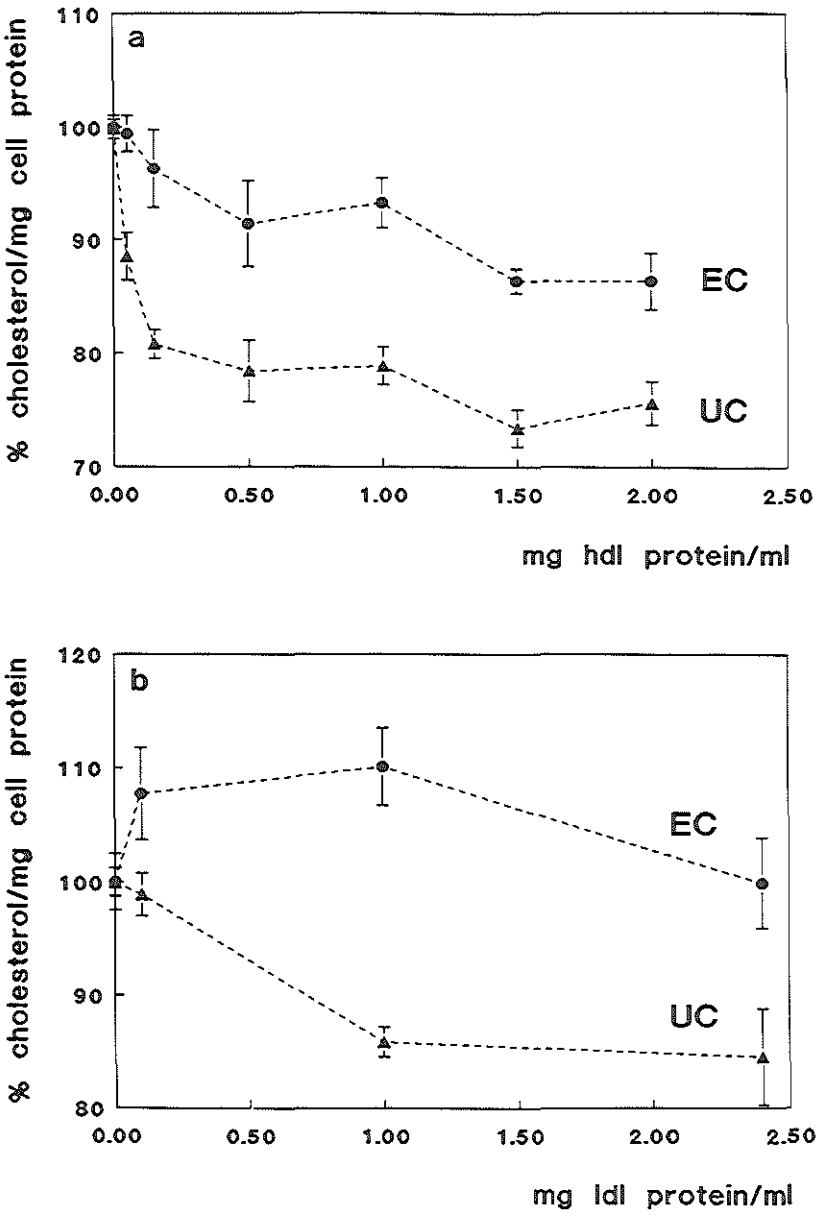

Fig. 4 Efflux of cell UC (▲) and EC (●) from cholesterol-loaded EA.hy 926 cells by HDL (fig. a) and LDL (fig. b). Cells were incubated and analyzed as in *Fig. 4*. Both TC and UC were measured in the cellular lipid extracts, EC was calculated by difference of TC and UC. Values are expressed as % of cell UC and EC after incubation with BSA alone (means ± SEM).

Discussion

Up-regulation of HDL binding after cholesterol enrichment of endothelial cells, e.g., by incubation of the cells with acetylated LDL [6], with 25-hydroxy cholesterol [7] or with unesterified cholesterol [31], was reported before. The present paper reports that, after cholesterol enrichment with cationized LDL, EA.hy 926 cells also show increased binding (B_{max}) of apo E-free ^{125}I-HDL_3, without having a significant effect on the dissociation constant (K_d). The K_d for HDL_3 binding to EA.hy 926 cells (31-37 μg/ml) was higher than for bovine vascular endothelial cells (3-10 μg/ml,-see ref. [6,7]). A large variation in the K_d values for HDL binding is found in the literature: values ranging from 5 to 95 μg/ml [6-10].

Efflux of cholesterol was measured in two ways, 1) by measurement of the increased cholesterol concentration in the incubation medium and 2) by assay of the decrease in cell cholesterol after extraction of the cells. The first method showed similar increases of unesterified and total cholesterol in the medium after 24 h of incubation with HDL_3 (see fig. 2). As cholesteryl esters did not appear in the medium, these data indicate that cholesterol leaves the cells in the unesterified form and that esterification does not occur in the medium during the incubation of the cells with HDL. In separate experiments we could not detect any lecithin:cholesterol acyltransferase activity in ultracentrifugally isolated HDL, which was used in the experiments (unpublished observation). The lack of increase of medium cholesteryl esters also indicates that no (eventually) extracellularly bound cationized LDL was released from the cells during incubation. Cholesterol efflux data, obtained with the second method (i.e., assay of the decrease of cellular cholesterol, see fig. 3), indicate that efflux was almost maximal with HDL concentrations as low as 0.15 mg/ml. Apparently, the efflux was limited by other factors than the HDL concentration, e.g., the desorption of cholesterol out of the plasma membrane, the transfer of intracellular cholesterol to the plasma membrane or the hydrolysis of intracellular cholesteryl esters. The decrease of cellular cholesterol consisted of a decrease of both esterified and unesterified cholesterol (after incubation with HDL, see fig. 4a). LDL also decreased cellular unesterified cholesterol, but cholesteryl esters increased, resulting in virtually unchanged cell TC levels (see fig. 4b). Probably this can be explained by a stimulation of the intracellular cholesterol esterification by LDL. If both HDL and LDL were present together during the efflux experiment, cell TC decreased similarly to incubation with HDL alone (see table III). So, HDL-mediated efflux of cell cholesterol is not inhibited by LDL.

Summarizing, the EA.hy 926 cells respond normally to cholesterol loading with respect to HDL binding and HDL-mediated cellular cholesterol efflux. Therefore, the cationized LDL-loaded EAhy 926 cell model can be used to study the relation between

HDL binding to specific high affinity binding sites and the HDL-mediated efflux of cellular cholesterol.

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chapter 4

Effect of LCAT on HDL-mediated cholesterol efflux from loaded EA.hy 926 cells

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Abstract

1. Human endothelial cells (EA.hy 926 line) were loaded with cholesterol using cationized LDL, and the effect of lecithin:cholesterol acyltransferase (LCAT) on cellular cholesterol efflux, mediated by high density lipoproteins (HDL), was measured subsequently.
2. In plasma, lecithin:cholesterol acyltransferase (LCAT) converts unesterified HDL cholesterol into cholesteryl esters, thereby maintaining the low UC/PL ratio of HDL. It was tested if further decrease in UC/PL ratio of HDL by LCAT influences cellular cholesterol efflux in vitro.
3. Efflux was measured as the decrease of cellular cholesterol after 24 h of incubation with various concentrations of HDL in the presence and absence of LCAT. LCAT from human plasma (about 3000-fold purified) was added to the cell culture, resulting in activity levels in the culture media of 60-70% of human serum.
4. Although LCAT had a profound effect on HDL structure (UC/TC and UC/PL ratio's decreased), the enzyme did not enhance efflux of cellular cholesterol, using a wide range of HDL concentrations (0.05-2.00 mg HDL protein/ml).
4. The data indicate that the extremely low unesterified cholesterol content of HDL, induced by LCAT, does not enhance efflux of cholesterol from loaded EA.hy 926 cells. It is concluded that the HDL composition (as isolated from plasma by ultracentrifugation) is optimal for uptake of cellular cholesterol.

Introduction

LCAT plays a crucial role in the concept of reverse cholesterol transport as proposed by Glomset (1968), the process in which cholesterol from peripheral cells is transported back to the liver. In vivo, LCAT indeed plays an important role in the postulated reverse cholesterol transport system, illustrated by the accumulation of cholesterol in kidneys, spleen and bone marrow of patients with LCAT deficiency (Norum *et al.*, 1989). Plasma LCAT esterifies cholesterol in HDL and the resulting cholesteryl esters are transferred to the core of the HDL particles. Cellular cholesterol leaves cells in the unesterified form (Johnson *et al.*, 1991; Oram *et al.*, 1991). The lower the UC/PL ratio of the acceptor particles, the larger the cholesterol gradient between the plasma membrane and acceptor particles, possibly resulting in a faster diffusion of cholesterol from cells to acceptor lipoproteins. HDL has the lowest ratio of UC/PL, if compared with other plasma lipoproteins (LDL and VLDL), and

this low UC/PL ratio of HDL makes these particles good acceptors of cellular cholesterol. It was hypothesized that in vitro LCAT may enhance transfer of cellular cholesterol to HDL by a further decrease of the UC/PL ratio of HDL particles. However, the effect of LCAT in vitro is not very clear and the data from literature are conflicting. Ray *et al.* (1980) and Fielding and Fielding (1981) stated that active LCAT in the culture media increased net mass efflux of cellular cholesterol. But earlier Stein *et al.* (1978) had reported that eggress of cholesterol from cholesterol-enriched fibroblasts was not increased by LCAT. These contradicting results may be explaned by differences in the cholesterol status of the cells or by the methods used to modify the HDL particles. In the present study we investigated whether LCAT influences the HDL-mediated efflux of cholesterol from loaded EA.hy 926 cells, a human endothelial cell line.

Materials and methods

EA.hy 926 cells were cultured in DMEM, supplemented with 10% FCS, L-glutamine, penicillin/streptomycin and HAT (100 μM hypoxanthin, Merck No. 4517; 0.4 μM aminopterin, Serva No. 13170; 16 μM thymidin, Merck No. 8206) at 37 °C in 5% CO_2 and 95% air. At confluency, the cells were loaded with 50 μg cationized LDL-cholesterol/ml in growth medium for 24 h. LDL was cationized as described previously (Kilsdonk *et al.*, 1992). Subsequently the cells were rinsed three times with DMEM containing 2 mg BSA/ml (Sigma No. A-4378) and penicillin/streptomycin and three times with DMEM with penicillin/streptomycin only. Cells were incubated with efflux medium for 24 h at 37 °C in an atmosphere with 5% CO_2 and 95% air. Efflux media consisted of DMEM, containing 2 mg BSA/ml, L-glutamine, penicillin/streptomycin, HDL at the concentrations indicated and either 10% (v/v) of a partially purified LCAT preparation or PBS (0.9% NaCl, 10 mM sodium phosphate and 1 mM EDTA, pH 7.4). HDL (density range 1.063-1.21 g/ml) was isolated by sequential ultracentrifugation of human plasma according to Havel *et al.* (1955). The LCAT preparation was dialyzed against PBS. LCAT was partially purified from the $d>1.18$ g/ml fraction of human plasma using phenyl-Sepharose and CM-cellulose chromatography, as described by Morton and Zilversmit (1982) for the isolation of CETP. The column fractions were tested for both LCAT and CETP activity using excess exogenous substrate as described by Glomset and Wright (1964) and Groener *et al.* (1986), respectively. The fractions with maximal LCAT activity did not contain any CETP activity and were pooled, concentrated by membrane pressure dialysis (Amicon YM10 membranes), and dialyzed against PBS. The LCAT preparation contained 130-165 μg protein/ml and the specific activity was increased about 3000 times, if compared with human plasma. The activity of this preparation was 650%/ml (LCAT activity was expressed relative to the LCAT activity of a human plasma pool). The LCAT activity level in the incubation media was 65% before and 60% after the 24 h incubation with the cells (not shown), indicating that the enzyme was not inactivated during the efflux experiments. After 24 h of incubation of cholesterol-loaded cells with the efflux media, the cells were cooled on ice and rinsed three times with 2 mg BSA/ml in 0.9% NaCl/Tris-HCl (50 mM, pH 7.4), followed by three rinses with NaCl/Tris-HCl buffer without BSA. Cellular lipids were extracted with the method of Bligh and Dyer (1959) and the resulting protein pellet was dissolved in 500 μl 0.1 M NaOH with 10% SDS. Cell protein was measured according to Lowry *et al.* (1951). Cellular total cholesterol and unesterified cholesterol were measured fluorimetrically in the lipid extracts as described previously (Kilsdonk *et al.*, 1992). Total and unesterified cholesterol in the incubation media were determined enzymatically (Boehringer Mannheim, cholesterol kit No. 310328 and cholesterol esterase No. 161772).

Results and Discussion

Table I gives the UC/TC ratios in HDL before and after incubations with endothelial cells. The ratio varied from 0.18 to 0.23. The HDL UC/TC ratio increased up to 0.31 after 24 hours of incubation of the cholesterol-enriched cells with HDL, due to the uptake of unesterified cholesterol from the cells (see table I). This increase is caused by the rise in medium unesterified cholesterol, indicating that cholesterol leaves the cells in the unesterified form (not shown). Other investigators also reported that cholesterol leaves the cells in the unesterified form (Johnson *et al.*, 1991; Oram *et al.*,1991). Addition of LCAT to the incubation media had a profound effect on HDL composition. The UC/TC ratio decreased markedly, from about 0.20 before to 0.04-0.11 after the incubation. It can be calculated that the UC/PL ratio of these particles must have decreased from about 0.25 to about 0.04-0.20 (depending on the HDL concentration). Table II shows the effect of LCAT on HDL-mediated efflux of cellular cholesterol. HDL significantly reduced total and unesterified cell cholesterol, if compared with cells incubated with BSA alone (nonparametric Mann-Whitney test, $P<0.05$). Cellular cholesteryl esters were significantly decreased after incubation with 0.5 mg HDL/ml or more ($P<0.05$). At all HDL concentrations used, there were no differences in total cell cholesterol between cells incubated plus or minus LCAT ($P>0.05$). Johnson *et al.* (1988) reported that recombined HDL particles with decreaments in the UC/PL ratio, similar to the ones induced by LCAT,

Table I

The composition of HDL before and after incubation with cholesterol-loaded EA.hy 926 cells: effect of LCAT

HDL (mg protein/ml)	molar UC/TC ratio of HDL		
	t=0 h	t=24 h minus LCAT	t=24 h plus LCAT
0.05	0.20 ± 0.01	0.32 ± 0.02	0.04 ± 0.01
0.15	0.23 ± 0.02	0.31 ± 0.04	0.05 ± 0.01
1.10	0.18 ± 0.01	0.21 ± 0.01	0.04 ± 0.01
1.50	0.22 ± 0.02	0.24 ± 0.02	0.12 ± 0.01

Data are from 3 experiments with n=3-6 for each separate experiment. HDL-UC and -TC were measured in the incubation media before and after incubation with cells. LCAT activity was 65% of the activity of human plasma.

Table II
The effect of LCAT on HDL-mediated efflux from cholesterol-loaded EA.hy 926 cells

HDL (mg/ml)	*n*	LCAT[a]	cellular cholesterol[b]		
			TC	UC	EC
0	(12)	–	100.0 ± 3.0	100.0 ± 2.9	100.0 ± 6.2
0	(8)	+	101.1 ± 7.1	100.1 ± 6.7	101.2 ± 7.8
0.05	(6)	–	94.3 ± 3.4[c]	88.6 ± 5.2[c]	99.4 ± 3.9
0.05	(5)	+	91.2 ± 4.5[c]	86.1 ± 4.8[c]	95.7 ± 5.4
0.50	(3)	–	80.8 ± 3.0[c]	72.2 ± 1.4[c]	91.1 ± 5.0
0.50	(3)	+	83.2 ± 6.3[c]	75.1 ± 5.2[c]	93.2 ± 8.7
1.10	(3)	–	84.8 ± 4.0[c]	79.2 ± 2.7[c]	90.7 ± 5.4[c]
1.10	(3)	+	83.7 ± 2.2[c]	74.9 ± 2.5[c]	92.8 ± 4.4[c]
2.00	(3)	–	74.4 ± 1.6[c]	63.6 ± 4.8[c]	87.8 ± 5.2[c]
2.00	(3)	+	68.9 ± 4.8[c]	61.4 ± 3.8[c]	78.2 ± 6.1[c]

[a] LCAT activity, if present, was 60-65% of human plasma. There were no significant differences in cellular cholesterol between + and - LCAT incubations.
[b] Values are expressed as percentages of cellular cholesterol (μg/mg cell protein) after incubation with BSA alone (means ± SD).
[c] Significantly different from cells incubated with BSA alone (nonparametric Mann-Whitney test, $P<0.05$).

significantly enhanced net cholesterol flux from fibroblasts, so the changes in UC/PL ratio's induced by LCAT were large enough to influence cholesterol flux in other model systems. The apparent discrepancy between our data and the results of Johnson *et al.* (1988) may be a different cholesterol status of the cells.

Net mass flux of cholesterol between cells and acceptor particles is determined by two processes: efflux and influx of cholesterol. The difference of efflux of cellular cholesterol and the influx of cholesterol from the acceptor particles into the cells is the net mass transfer of cholesterol. Influx and efflux are approximately equal for the incubation of normocholesterolemic cells with normal HDL (Johnson *et al.*, 1986). Various investigators (Stein *et al.*, 1978; Ray *et al.*, 1980; Fielding and Fielding, 1981) reported that net mass transfer of cholesterol out of cells was increased by LCAT, but that efflux of labelled cellular cholesterol was not affected by LCAT. These results indicate that LCAT only decreases influx of cholesterol from HDL into cells, without changing the efflux of cellular cholesterol. This idea was confirmed by Johnson *et al.* (1988), who reported a decreased influx of cholesterol

from reconstituted HDL with a lowered UC/PL ratio into fibroblasts. In all these experiments cells with normal cholesterol levels were used. Using LDL/chloroquine-loaded fibroblasts Stein *et al.* (1978) found that inhibition of LCAT in the culture media did not change net mass transfer of cellular cholesterol, which is in agreement with our own results. Like for normal cells, LCAT also did not change the efflux of labelled cholesterol from loaded cells (Stein *et al.*, 1978). It can be expected that LCAT also somewhat decreased influx of cholesterol from HDL into these cells. Johnson *et al.* (1988) reported that after cholesterol loading only efflux of cellular cholesterol increased, while influx remained unchanged. Because LCAT only decreases influx of HDL cholesterol into cells, it can be concluded that LCAT does not change the net mass flux of cholesterol from cholesterol-loaded cells since for these cells efflux of cholesterol exceeds the influx several times.

The present experiments show that HDL-mediated net mass flux of cholesterol from loaded EA.hy 926 cells is not affected by addition of LCAT activity to the incubation media. These results also indicate that the composition of the HDL used in our experiments was optimal for uptake of cellular cholesterol, because in vitro LCAT did not enhance HDL-mediated efflux. The results implicate that HDL composition is not rate limiting for efflux of cholesterol from loaded EA.hy 926 cells.

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chapter 5

Binding of modified high density lipoproteins to endothelial cells: relation with cellular cholesterol efflux?

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Abstract Human endothelial cells (EA.hy 926 line) were enriched with cholesterol using cationized LDL. Cholesterol-loaded cells interacted with native apo E-free HDL_3 as well as with dimethyl suberimidate-modified HDL_3 (DMS-HDL_3). At 4 °C both HDL preparations showed a saturable high affinity binding with a K_d of 31 and 50 μg of protein/ml and a B_{max} of 226 and 436 ng/mg cell protein for native HDL_3 and DMS-HDL_3 particles, respectively. Competition of binding of 5 μg apo E-free ^{125}I-labelled HDL_3/ml by unlabelled DMS-HDL_3 and tetranitromethane-treated HDL_3 (TNM-HDL_3) was very poor, whereas unlabelled native HDL_3 competed very effectively with ^{125}I-labelled HDL_3 binding. Thus, both types of modified HDL did not compete for the high affinity binding sites for native HDL. Unlabelled native HDL_3 and unlabelled DMS-HDL_3 both competed for the binding of ^{125}I-labelled DMS-HDL_3 very effectively. These experiments indicate that there are two distinct high affinity binding sites for HDL on cationized LDL-loaded EA.hy 926 cells: one specific HDL binding site, which only binds native HDL, and a second binding site for both native HDL and DMS-HDL. The modified HDL fractions were used to study the relation between HDL binding and HDL-mediated efflux. Efflux of cell cholesterol was measured as the increase of cholesterol mass in the medium after 24 h of incubation with 0.2 mg native HDL_3/ml, or the same amount of modified HDL_3. DMS-HDL_3-mediated efflux was identical to efflux mediated by native HDL_3. TNM-HDL_3 also induced efflux of cell cholesterol, however; efflux induced by TNM-HDL_3 was only 45-50% of the amount obtained with native HDL_3. So both DMS- and TNM-modified HDL_3 induced efflux of cholesterol, although these particles do not bind to the specific high affinity binding sites for native HDL. These results do not indicate a link between binding of HDL to specific receptors for native HDL and HDL-mediated efflux of cholesterol from loaded endothelial cells.

Introduction

High density lipoprotein (HDL) is believed to play a role in reverse cholesterol transport, i.e., the transfer of peripheral cellular cholesterol back to the liver. In different cell types HDL binding membrane proteins were visualized [1-5], but the physiological role of these HDL binding proteins is still unclear. Oram *et al.* [6] showed up-regulation of HDL binding activity in fibroblasts and smooth muscle cells when cells were loaded with cholesterol, suggesting a functional role for these proteins in the removal of an excess of cell cholesterol. Modified HDL particles which do not bind to high affinity HDL receptors, can be very useful for research on the function of HDL binding in the efflux process. Brinton *et al.* [7] concluded, using tetranitromethane-modified HDL (TNM-HDL), that the high affinity component of cholesterol efflux was abolished when

high affinity binding to cellular HDL binding sites was inhibited. However, the literature is not consistent on this point and (by using the same kind of modified HDL) Karlin *et al.* [8] denied inhibition of cholesterol efflux after TNM treatment of HDL particles. There are more reports that disagree with the notion of a direct linkage of HDL binding and HDL-mediated cholesterol efflux, using a different HDL modification [9].

In this paper we describe the interaction of native HDL and two types of chemically modified HDL particles with endothelial cells (the human EA.hy 926 cell line). The following questions were asked: 1) do modified lipoproteins bind to cells, and 2) do modified HDL and native HDL bind to the same binding site? We used DMS-HDL for these experiments, because this modification of HDL produces no changes in chemical composition of HDL [10]. In addition, TNM-HDL was used as a second type of modified HDL, although it is known that TNM modification also affects the lipid structure of HDL particles by cross-linking of phospholipids and cholesteryl esters to apolipoproteins [11]. We also describe the effects of cross-linking of HDL apolipoproteins on net mass efflux of cholesterol from cholesterol-loaded endothelial cells. Studies involving assays of efflux of radiolabelled cholesterol from cells are more difficult to interpret, unless bidirectional fluxes are measured [9,12]. It is obvious that exchange processes can occur using native as well as modified HDL particles. In order to overcome possible problems of interpretation of results we measured net mass transfer of cholesterol from loaded cells to HDL. This is the first report on efflux of cholesterol from endothelial cells, in which two types of modified HDL_3 (DMS-HDL_3 and TNM-HDL_3) are compared. The experiments were carried out to clarify the putative link between binding of HDL to high affinity receptors and HDL-mediated cholesterol efflux.

Materials and methods

Cell culture

The endothelial cell line EA.hy 926 was generously provided by Dr. C.-J.S. Edgell [13]. The EA.hy 926 cells were cultured in DMEM (Flow Laboratories), supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml and HAT (100 µM hypoxanthin, Merck No. 4517; 0.4 µM aminopterin, Serva No. 13170; 16 µM thymidin, Merck No. 8206) at 37 °C in 5% CO_2 and 95% air. Cells were plated in multiwell dishes (35 mm: $1 \cdot 10^5$ cells/well, 22 mm: $5 \cdot 10^4$ cells/well) 4 to 5 days prior to each experiment. Medium was changed every 2 or 3 days.

Only confluent monolayers were used for binding and efflux experiments. For cholesterol loading, the confluent cells were preincubated with cationized LDL in growth medium for 24 h (50 µg catLDL-cholesterol/ml medium). Control cells were preincubated similarly with growth medium (containing 10% FCS) only.

Lipoproteins

LDL (*d* 1.006-1.063 g/ml), HDL (*d* 1.063-1.21 g/ml) and HDL_3 (*d* 1.125-1.21 g/ml) were isolated by sequential ultracentrifugation of human plasma according to Havel *et al.* [14]. Apo E-free HDL_3 was isolated by chromatography of HDL_3 on a heparin-Sepharose column [15]. All lipoproteins were dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Before cell experiments lipoproteins were dialyzed for a second time against DMEM and filter sterilized (0.45 μm). Total cholesterol, unesterified cholesterol, phospholipids and triacylglycerols were measured by enzymatic methods. Cholesteryl esters were calculated by difference of total and unesterified cholesterol. The following kits were used: cholesterol (Boehringer Mannheim No. 310328), phospholipids (Bio-Mérieux No. 61491) and triacylglycerols (Boehringer Mannheim No. 877557). Total cholesterol was measured using the cholesterol kit after addition of cholesterol esterase (Boehringer Mannheim No. 161772).

LDL was cationized according to Basu *et al.* [16]. An equal volume of LDL (20 to 30 mg of protein) was added to a 2 M solution of 3-dimethylaminopropyl-amine (Aldrich No. 24.005-2) in H_2O at room temperature. The pH was adjusted to 6.5 with HCl. After stirring this mixture, 100 mg of 1-ethyl-3(3-

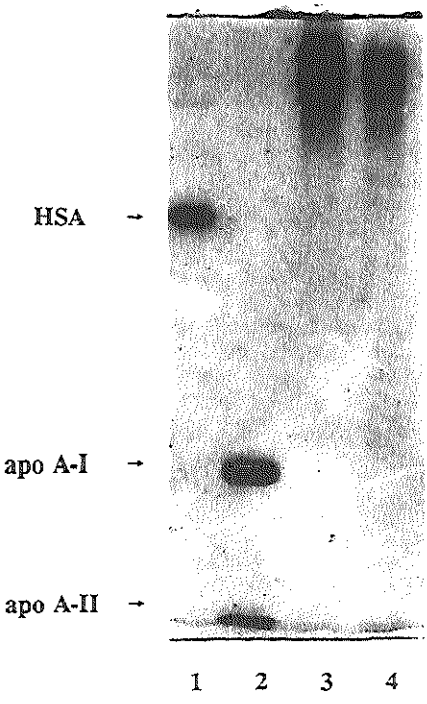

Fig. 1 12 % SDS-PAGE of native and modified HDL_3 preparations. Lane 1, human serum albumin (HSA); lane 2, native HDL_3; lane 3, DMS-HDL_3; lane 4, TNM-HDL_3. The gel was stained with Coomassie Brilliant Blue; 5 μg of protein was applied on each lane.

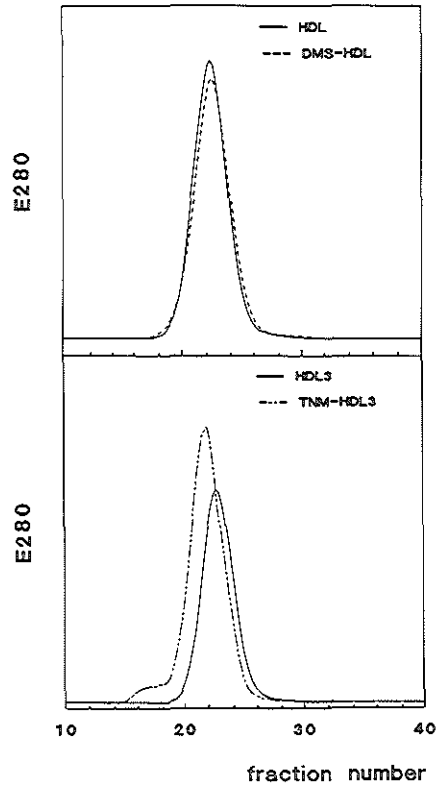

Fig. 2 Gel filtration profiles of native HDL and DMS-modified HDL (upper panel) and of native HDL_3 and TNM-modified HDL_3 (lower panel).

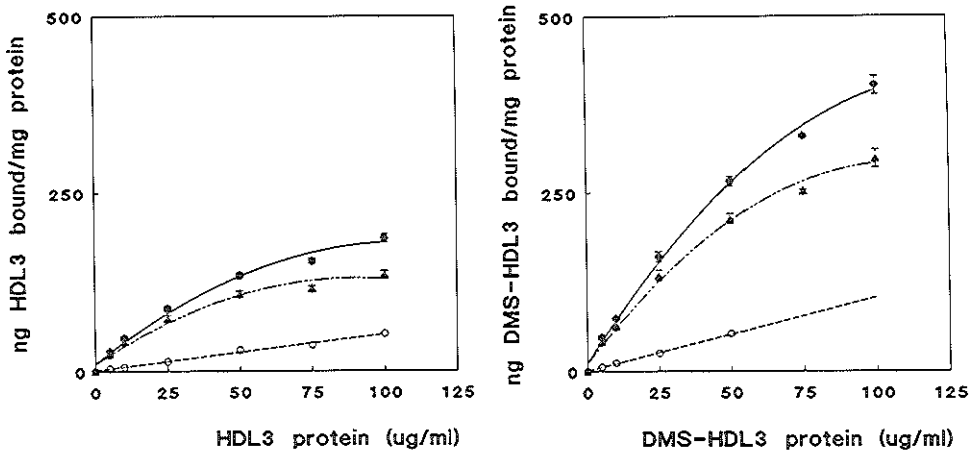

Fig. 3 Binding of ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3 to cholesterol loaded EA.hy 926 cells. Total (●), specific (▲) and nonspecific (○) binding per mg cell protein were measured after 4 h incubation at 4 °C. Nonspecific binding was determined after addition of 1 mg unlabelled lipoprotein/ml. Values are means ± SEM (n=3).

dimethylaminopropyl)-carbodiimide (Sigma No. E-7750) was added. The pH was maintained at 6.5 with 0.1 M HCl during the reaction. After 3 h (the pH of the solution did not change further), the reaction mixture was left at 4 °C overnight. Cationized LDL was dialyzed against 0.9 % NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, filter sterilized (0.8 µm) and stored (-20 °C). Storage at 4 or -20 °C did not influence the loading of the cells.

HDL and HDL_3 were treated with dimethyl suberimidate (Pierce No. 20668) according to Chacko *et al.* [10]. HDL and HDL_3 (5 mg protein/ml) were incubated with 5 mg DMS/ml in a 0.09 M triethanolamine-HCl buffer (pH 9.5) for 2 h at room temperature. After the reaction, DMS-HDL was reisolated on a Sephadex column (G25 Fine, 1 x 30 cm, Pharmacia). The column was eluted with 0.9% NaCl containing 2 mM EDTA (pH 7.4). HDL_3 was modified with tetranitromethane (Aldrich No. T2500-3) as described in [17]. In short, a 10-fold molar excess of reagent was incubated with HDL_3 for 1 h at room temperature in a 50 mM Tris-HCl buffer (pH 8.0) with 0.1 M NaCl. TNM-HDL_3 was reisolated as described above for DMS-HDL. For both DMS- and TNM-HDL_3 the cross-linking of the apolipoproteins was checked by electrophoresis on 12% polyacrylamide gels containing 0.1% SDS [18]. The sizes of native HDL and HDL_3, DMS-HDL and TNM-HDL_3 were measured by gel filtration according to [19], using a Superose 12 column (Prep grade, 1 x 30 cm, Pharmacia).

Apo E-free HDL_3 and DMS-HDL_3 were iodinated using the iodine monochloride method (using Na^{125}I from Amersham International) as described earlier [20]. The mean molar iodine to protein ratio of iodinated HDL_3 and DMS-HDL_3 was 0.35 and 0.37, respectively. For HDL_3 and DMS-HDL_3, respectively, 95.4 ± 0.8% and 96.2 ± 1.1% of the label was protein-bound, 2.3 ± 0.9% and 2.2 ± 0.8% was in the HDL lipids and 2.3 ± 0.9% and 1.6 ± 0.3% was free.

Measurement of binding and total cell association

After cholesterol loading of the cells (in 22-mm wells), the saturable binding of apo E-free ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3 was measured. The wells were rinsed three times with DMEM containing 2 mg BSA/ml (Sigma No. A-4378) and penicillin /streptomycin, followed by three rinses at 0 °C with DMEM and penicillin/ streptomycin to cool the cells. Subsequently, the cells were incubated with medium (DMEM with 2 mg BSA/ml, L-glutamine, penicillin/streptomycin and iodinated HDL_3 or DMS-

HDL_3 as indicated, 0.5 ml/well) for 4 h at 4 °C in an atmosphere with 5% CO_2. After the incubations the cells were rinsed three times with 0.9% NaCl containing 2 mg BSA/ml and 50 mM Tris-HCl (pH 7.4), followed by three rinses with 0.9% NaCl/50 mM Tris-HCl (pH 7.4) at 0 °C. Cell protein was dissolved in 300 µl 1 M NaOH. The radioactivity of this cell suspension was determined using a Packard Minaxi 5000 gamma counter and protein was measured [21] in the same aliquot. Nonspecific binding was measured in the presence of 1 mg unlabelled lipoprotein/ml. Specific binding was calculated by the difference of total and nonspecific binding.

The specificity of the HDL and DMS-HDL binding sites was examined by competition experiments. The binding of 5 µg iodinated lipoprotein/ml was competed for by native HDL_3, DMS-HDL_3 and TNM-HDL_3 (0 to 500 µg unlabelled protein/ml). The experiments were carried out as described above.

Total cell association of ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3 was measured using 20 µg protein/ml medium (specific activity 150 cpm/ng protein). The composition of the media and preincubation, rinsing and analyses of the cells were the same as for the binding experiments described above. The cells were incubated with the lipoproteins for 24 h at 37 °C in 5% CO_2 and 95% air.

Efflux experiments

After cholesterol loading of the cells (in 35-mm wells), the monolayers were rinsed twice with 2 mg BSA/ml in DMEM with penicillin/streptomycin and twice with DMEM (plus penicillin/streptomycin) at room temperature. Subsequently, the cells were incubated with efflux medium for 24 h at 37 °C in 5% CO_2 and 95% air. Efflux medium consisted of DMEM, supplemented with L-glutamine, penicillin/streptomycin, 2 mg BSA/ml (Sigma No. A-4378) and lipoproteins as indicated. The efflux was ended by cooling the culture dishes on ice, culture media were collected and a small amount of dislodged cells was spun down (500 x g_{max}, 4 °C) and discarded. The supernatant was used for cholesterol determinations. Cholesterol in the media was measured enzymatically using a commercially available kit (No. 310328, Boehringer Mannheim). The cells were rinsed three times with 2 mg BSA/ml in 0.9% NaCl/Tris-HCl (50 mM, pH 7.4), followed by three rinses with NaCl/Tris-HCl buffer alone. Cell protein was dissolved in 500 µl 0.1 M NaOH with 10% SDS and was measured according to Lowry *et al.* [21].

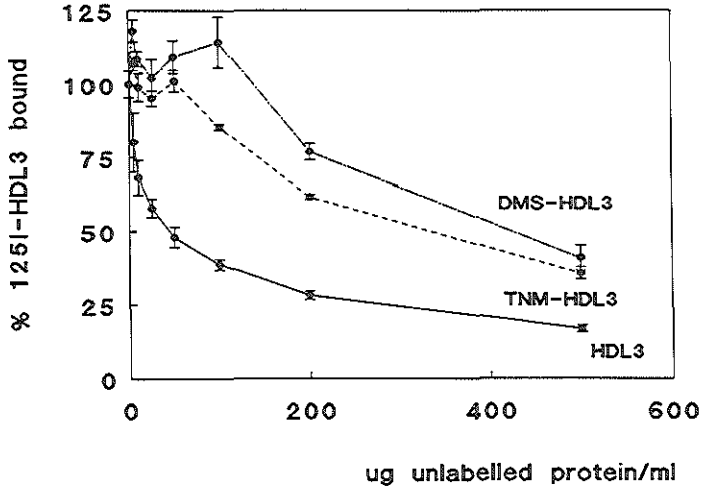

Fig. 4 Competition of native (iodinated) apo E-free HDL_3 binding (5 µg protein/ml) to cholesterol-loaded EA.hy 926 cells by unlabelled native HDL_3, DMS-HDL_3 and TNM-HDL_3. Values are means ± SEM (n=6 for native HDL_3 and DMS-HDL_3 and n=3 for TNM-HDL_3).

Statistical analysis

The nonparametric Mann-Whitney test was used for comparison between treatment groups. For paired observations the nonparametric paired Wilcoxon test was used. The differences were considered statistically significant when $P<0.05$.

Results

The composition and size of native and modified HDL particles.

The apolipoprotein structure was changed extensively after modification of the HDL and HDL_3 particles. Figure 1 shows that DMS-HDL_3 did not contain any normal apo A-I (MW 28 kDa) anymore, and that only traces of intact apo A-II (MW 17 kDa) were present. The cross-linked apolipoproteins were seen as diffuse high molecular weight bands. SDS-PAGE of iodinated native HDL_3 and DMS-HDL_3 gave exactly the same results (not shown). The SDS-PAGE apolipoprotein pattern of TNM-HDL_3 was similar to that of DMS-HDL_3 (see fig. 1). All HDL apolipoproteins were cross-linked, resulting in molecular weights of 100-200 kDa.

The size of the different HDL preparations was measured by gel filtration chromatography on Superose 12 columns. The column was operated and calibrated as described earlier [19]. As shown in figure 2 the size of native HDL was identical to the size of DMS-HDL. Mean particle weights were 202 kDa and 194 kDa for native HDL and DMS-HDL, respectively. Modification with TNM resulted in a change in size distribution of the HDL_3 particles: the largest native HDL_3 particles were about 400 kDa, but TNM-HDL_3 started eluting from the column at about 1000 kDa, indicating that cross-linking between different HDL_3 particles had occurred to some extent. However, the mean particle weight of the major part of TNM-HDL_3 was increased by only 19%, if compared to native HDL_3.

Table I summarizes the lipid compositions of native HDL_3, DMS-HDL_3 and TNM-HDL_3. The lipid compositions of native HDL_3 and DMS-HDL_3 were identical. TNM-HDL_3 had a slightly decreased percentage of cholesteryl esters in experiment 1 as well as in experiment 2. This resulted in an increase of the ratio of unesterified/total cholesterol.

Saturable binding of ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3 to EA.hy 926 cells.

Figure 3 shows that both native HDL_3 and DMS-HDL_3 bind to cholesterol-loaded endothelial cells. Specific binding of modified DMS-HDL_3 appeared to be higher

Table I

The lipid composition of native HDL_3, DMS-HDL_3 and TNM-HDL_3

	HDL_3	DMS-HDL_3	TNM-HDL_3
experiment 1			
PL[a]	49	49	52
FC	5	4	5
CE	40	40	35
TG	7	7	8
FC:TC[b]	0.17	0.16	0.20
FC:PL	0.19	0.18	0.20
experiment 2			
PL	60	61	61
FC	5	5	6
CE	30	29	27
TG	5	5	6
FC:TC	0.22	0.22	0.27
FC:PL	0.16	0.16	0.20

[a] Values are given as weight % of total lipid. A mean molecular mass of 651 was used for esterified cholesterol.

[b] Data are expressed as molar ratios.

Table II

Binding characteristics of HDL_3 and DMS-HDL_3 obtained with cholesterol-loaded EA.hy 926 cells

	K_d	B_{max}
HDL_3 ($n=4$)	31 ± 4[a]	226 ± 64[b]
DMS-HDL_3		
experiment 1	47	432
experiment 2	53	440

EA.hy 926 cells were loaded with cholesterol by incubation of the confluent monolayers with 50 μg catLDL-TC/ml growth medium for 24 h. Binding of ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3 was measured for 4 h at 4 °C as described in *Materials and methods*.

[a] Values are in μg HDL_3 protein/ml (mean ± SEM).

[b] Values are in ng HDL_3 protein bound/mg cell protein (mean ± SEM).

than the specific binding of native HDL_3 particles. Scatchard analysis (see table II) gave an average K_d of 31 μg protein/ml for native HDL_3 and 50 μg/ml for DMS-HDL_3. B_{max} was also somewhat higher for DMS-HDL_3 compared to native HDL_3: 436 ng/mg cell protein versus 226 ng/mg. These differences were of borderline statistical significance ($P<0.064$). The data indicate that, although the apolipoproteins are extensively changed after cross-linking with DMS, modified particles can still bind to endothelial cells. The affinities of cell membrane binding sites for DMS-HDL_3 and native HDL_3 are comparable. Table III shows the total cell association, i.e., binding plus cellular uptake, of native HDL_3 and DMS-HDL_3 at 20 μg HDL_3-protein/ml. Cell association of native HDL_3 and DMS-HDL_3 increased significantly after enrichment of EA.hy 926 cells with cholesterol. For both control and loaded cells the cell association of DMS-HDL_3 was significantly higher, if compared with native HDL_3.

However, figure 4 shows that the interaction of DMS-HDL_3 with high affinity

Table III

The cell association of HDL_3 and DMS-HDL_3 in normal and cholesterol-enriched EA.hy 926 cells at 37 °C

preincubation	cell association[a]	
	native HDL_3	DMS-HDL_3
control cells	86.8 ± 0.8	157.7 ± 12.5[b]
cells loaded with cationized LDL	200.2 ± 12.1[c]	308.7 ± 9.8[b,c]

[a] The confluent EA.hy 926 cells were preincubated with 50 μg catLDL-TC/ml growth medium for 24 h. Control cells were preincubated with growth medium alone. The cell association was measured with 20 μg of ^{125}I-labelled HDL_3 or ^{125}I-labelled DMS-HDL_3/ml for 24 h at 37 °C, as described in *Materials and methods*. Values are ng ^{125}I-labelled lipoprotein/mg cell protein (means ± SEM ($n=3$).

[b] Significantly different from native HDL_3 (nonparametric Mann-Whitney test, $P<0.05$).

[c] Significantly different from value for control cells (nonparametric Mann-Whitney test, $P<0.05$).

Table IV

Efflux of cholesterol from cationized LDL-loaded EA.hy 926 cells by HDL

μg catLDL-TC/ml	μg cell TC/mg protein (t=0)	increase of cholesterol in medium after 24 h, measured as: μg FC/mg	μg TC/mg
0	33.4 ± 1.4	nd.[a]	nd.[a]
10	55.8 ± 6.6	19.0 ± 10.5	21.9 ± 9.7[b]
25	105.1 ± 5.9	34.8 ± 2.1	34.5 ± 2.7[b]
50	154.9 ± 2.8	45.3 ± 4.4	47.4 ± 11.2[b]

The confluent EA.hy 926 cells were preincubated with various concentrations of catLDL ($n=12$ wells per concentration) and rinsed as described in *Materials and methods*. For each concentration 6 wells were scraped and initial cellular cholesterol was determined, and 6 wells were incubated with DMEM containing 0.2 mg HDL_3 protein/ml, 0.2% BSA, L-glutamine, penicillin and streptomycin. After 24 h efflux was measured as the increase of unesterified and total cholesterol in the medium.

[a] not determined.

[b] Equal if compared with the increase in μg FC/mg (nonparametric paired Wilcoxon test, $P>0.75$).

binding sites was distinct from the interaction of native HDL_3 with its receptor. Competition of the binding of ^{125}I-labelled HDL_3 by unlabelled HDL_3 is very effective: a 10-fold excess of unlabelled HDL_3 inhibits the binding of iodinated HDL_3 by 50%, whereas the same concentration of unlabelled DMS-HDL_3 does not inhibit at all. So, although the affinity of binding of DMS-HDL particles to endothelial cells was only slightly lower than for native HDL_3, these competition experiments showed that it is not likely that modified HDL_3 binds to the same high affinity receptor. Competition of native ^{125}I-labelled HDL_3 binding with another type of modified HDL_3 (TNM-HDL_3), gave similar results (see fig. 4).

Competition of binding of native ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3 by unlabelled DMS-HDL_3 (fig. 5, lower panel) showed that DMS-HDL_3 inhibited the binding of ^{125}I-labelled DMS-HDL_3 very effectively. Unlabelled DMS-HDL_3 competed poorly with ^{125}I-labelled HDL_3 binding. The capacity of unlabelled HDL_3 to inhibit the binding of iodinated HDL_3 was very similar to the inhibition of the binding of iodinated DMS-HDL_3 by unlabelled HDL_3 (see fig. 5, upper panel). So unlabelled native HDL_3 inhibited both the binding of native HDL_3 and of DMS-HDL_3, in contrast with unlabelled DMS-HDL_3, which competed only for the binding of DMS-HDL_3.

Table V
Efflux of cholesterol from loaded EA.hy 926 cells by modified HDL particles

	cholesterol efflux (nmol/mg cell protein[a])	in % of the efflux by native HDL
experiment 1		
HDL	55.4 ± 0.9	100
DMS-HDL	53.6 ± 5.2	97
experiment 2		
HDL_3	52.0 ± 1.6	100
DMS-HDL_3	51.0 ± 1.9	98
TNM-HDL_3	23.3 ± 1.2	45
experiment 3		
HDL_3	63.7 ± 1.1	100
TNM-HDL_3	31.2 ± 1.1	49

[a] Confluent EA.hy 926 cells were loaded with 50 μg catLDL-TC/ml and subsequently, efflux of cellular cholesterol was measured as described in *Materials and methods*. Values are expressed as means ± SEM ($n=6$ for exp. 1 and 2, $n=3$ for exp. 3).

Efflux of cholesterol from endothelial cells by modified HDL_3 particles.

Control cells contained 30-40 μg cholesterol/mg cell protein, of which about 90% was unesterified. Preincubation of EA.hy 926 cells with cationized LDL resulted in a dose-dependent increase of cellular cholesterol, which was about 4- to 5-fold after incubation with 50 μg cationized LDL-TC/ml. After loading 50 to 60% of cellular cholesterol was unesterified. Table IV shows the HDL-mediated efflux of cellular cholesterol after loading the cells with 10, 25 or 50 μg cationized LDL-TC/ml. Efflux of cellular cholesterol was determined as the increase of cholesterol in the medium after 24 h of incubation of the cells with 0.2 mg HDL_3 protein/ml. The cholesterol increase was measured as the increase of unesterified medium cholesterol as well as of total medium cholesterol (see table IV). The increase in total cholesterol was identical to the increase of unesterified cholesterol, indicating that there was no increase of cholesteryl esters in the medium during the efflux period.

Efflux of cholesterol from loaded EA.hy 926 cells by different HDL or HDL_3 preparations is given in table V. Efflux was measured as the increase of unesterified cholesterol in the cell media after 24 h of incubation with 0.2 mg protein/ml of native or modified HDL. Native HDL or HDL_3 caused a release of 15 to 25% of cell cholesterol. Efflux in control medium with 2 mg BSA/ml was only 15% of the HDL-mediated efflux (not shown). In different experiments HDL-mediated efflux of cholesterol (55.4 ± 0.9 for HDL and 52.0 ± 1.6 nmol/mg for HDL_3 in experiments 1 and 2, respectively) and DMS-HDL-mediated efflux (53.6 ± 5.2 for DMS-HDL and 51.0 ± 1.9 nmol/mg for DMS-HDL_3 in experiments 1 and 2, respectively) were identical. TNM-HDL_3 was also capable of inducing cholesterol efflux, although this efflux was only about half of the efflux induced by native HDL_3. In experiments 2 and 3, TNM-HDL_3 induced a cholesterol efflux of 23.3 ± 1.2 and 31.2 ± 1.1 nmol/mg respectively.

Discussion

The implication of our experiments is that the interaction of HDL with specific high affinity binding sites for native HDL is not necessary for efflux of cellular cholesterol. DMS-HDL_3 completely mimicked the effects of native HDL_3, and TNM-HDL_3 also induced efflux, although impaired if compared to native HDL_3. Using comparison of the concentration dependencies of HDL binding and HDL-mediated cholesterol efflux, or using modified HDL particles, both Johnson *et al.* [9] and Mendel and Kunitake [22] also denied a direct relation between specific HDL binding and HDL-mediated cholesterol efflux. On the contrary, Brinton *et al.* [7] and Slotte *et al.* [23]

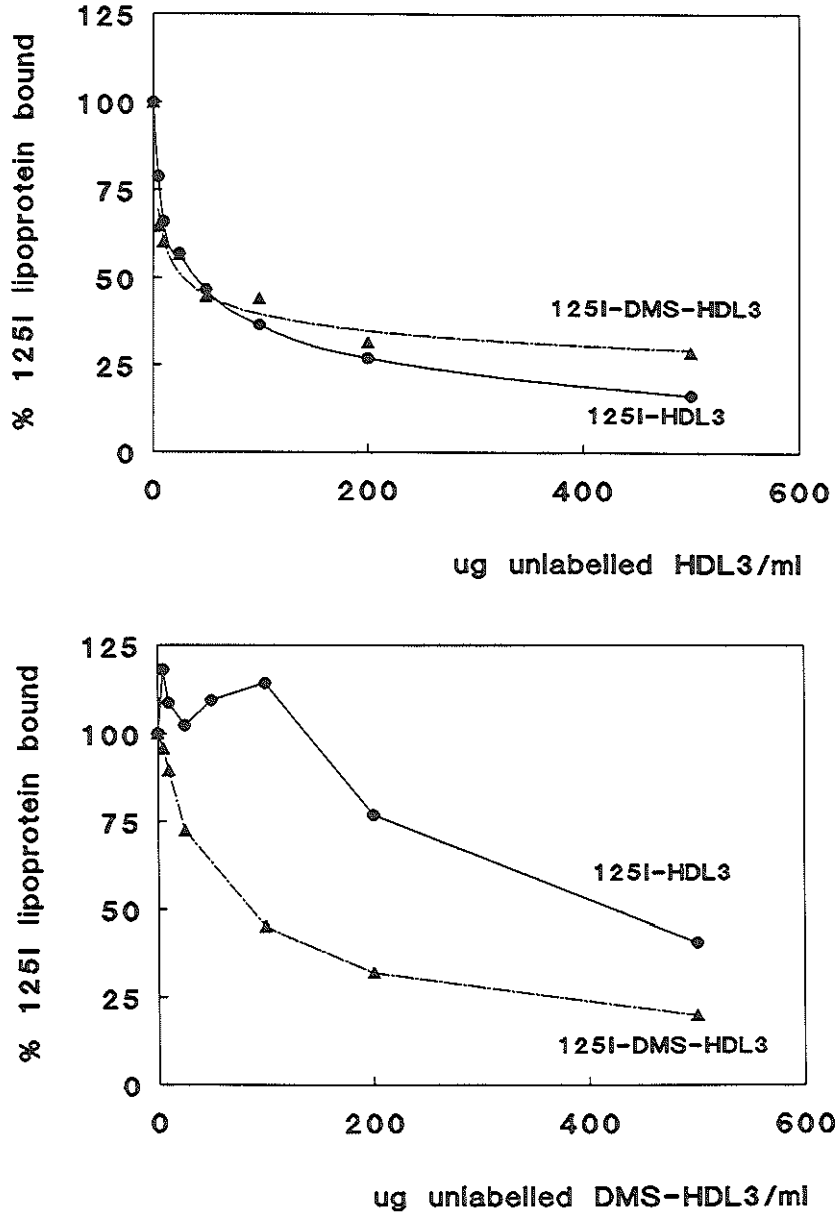

Fig. 5 Competition of iodinated native HDL_3 (●) or iodinated DMS-HDL_3 (▲) binding to cholesterol-loaded EA.hy 926 cells by unlabelled HDL_3 (upper panel) or by unlabelled DMS-HDL_3 (lower panel). Part of these data are also shown in *Fig. 4*.

concluded that HDL binding to high affinity HDL receptors is necessary to induce efflux of cholesterol from loaded cells.

Modification of HDL particles with DMS or TNM resulted in highly cross-linked HDL apolipoproteins without large effects on particle size. The size distribution for DMS-HDL was even identical to that of native HDL as shown by Superose 12 gel filtration (see fig. 2). TNM-HDL_3 showed a small increase in mean particle size and a small amount of aggregated HDL_3 particles. Using the same modification procedure other investigators [17,24] did not report an increased particle size of TNM-HDL. It is known that, apart from cross-linking between apolipoproteins, TNM also gives rise to cross-linking between apolipoproteins and cholesteryl esters [11]. This could explain the decreased amount of esterified cholesterol measured in the TNM-HDL_3 fraction in our experiments (see table I): the esterase used in the cholesterol determinations may not be able to attack cross-linked cholesteryl esters properly.

We showed that, apart from native HDL_3 particles, DMS-modified HDL_3 particles can also interact with human endothelial cells (see fig. 3). Binding at 4 °C is even higher for iodinated DMS-HDL_3 than for iodinated native HDL_3. Cholesterol enriched EA.hy 926 cells have a higher B_{max} and a slightly decreased affinity for DMS-HDL_3 compared to native HDL_3. Senault *et al.* [25] also measured a higher B_{max} for DMS-HDL_3 (although not significantly different), but found similar K_d values for native HDL_3 and DMS-HDL_3 binding to rat liver membranes. Probably, differences in K_d for native HDL_3 and DMS-HDL_3 occur only in cholesterol-enriched cells. Also human GM3468 fibroblasts gave similar bindings of iodinated HDL_3 and DMS-HDL_3, if measured at 5 μg lipoprotein/ml [10].

In vivo experiments have shown that the hepatic uptake of DMS-HDL_3 was more rapid than that of native HDL_3 [25]. This is in agreement with the higher cell association of ^{125}I-labelled DMS-HDL_3 at 37 °C, compared with native HDL_3, reported in the present paper. We did not study the interaction of TNM-HDL_3 with EA.hy 926 cells. It was shown before that TNM-HDL can be bound and taken up by ovarian cells [26] and liver endothelial cells [24]. The binding plus uptake of TNM-HDL, like for DMS-HDL in our experiments, was faster than for native HDL. Binding of iodinated DSP-HDL (DSP is an analog of DMS) to rat peritoneal macrophages was inhibited by acetylated LDL, a known ligand for the scavenger receptor [27]. So, the mechanism responsible for the cellular uptake of DMS-HDL and TNM-HDL could be the scavenger receptor pathway. The involvement of the scavenger receptor on liver endothelial cells in uptake of TNM-HDL was also proposed [24]. It is unlikely, however, that the uptake of modified HDL particles in our experiments is mediated by scavenger receptors. This type of receptor is not active in our cell system because incubation of EA.hy 926 cells with acetylated LDL did not result in storage of intracellular cholesterol, whereas the same

acetylated LDL preparation caused a large increase of intracellular cholesterol in J774 macrophages [28].

Up to 100 μg/ml of unlabelled DMS- and TNM-HDL_3 did not inhibit the binding of native HDL_3 at 4 °C, whereas unlabelled HDL_3 itself was very effective at these low concentrations (see fig. 4). This lack of competition of native HDL_3 binding by DMS-HDL_3 and TNM-HDL_3 was also shown for rat liver membranes [10,17], human fibroblasts [7,10] and rat ovarian cells [26]. We conclude from these experiments that the modified HDL_3 particles (DMS-HDL_3 and TNM-HDL_3) did not bind to specific high affinity receptor sites for native HDL_3. However, when a very high excess of unlabelled modified HDL_3 particles was used (40- to 100-fold), ^{125}I-labelled HDL_3 binding was inhibited up to 60%. There are several explanations for this observation. 1) Native HDL_3 may interact with two different types of binding sites (one specific for native HDL and a second type binding both native HDL and modified HDL). 2) The competition might be explained by a very reduced affinity of modified HDL_3 for specific receptor sites for native HDL_3, resulting in competition for HDL_3 binding only at very high concentrations of unlabelled ligands. This would mean that HDL modification decreased the affinity for HDL binding sites about 50-fold. 3) Theoretically, there may still be another explanation for the inhibition of HDL_3 binding by high concentrations of unlabelled DMS-HDL_3 or TNM-HDL_3. It is possible that there is a small percentage of unmodified HDL_3 particles present in the DMS- and TNM-HDL_3 preparations. With 2% of unmodified HDL_3 this would mean that 10 μg of unmodified HDL_3 particles/ml could be present in the incubation media at the highest concentration of modified HDL_3. As shown in fig. 4, 10 μg native HDL_3/ml may inhibit ^{125}I-labelled HDL_3 binding effectively. However, no indication of the presence of native apo A-I and A-II in modified HDL_3 is seen in fig. 1.

The nature of the native HDL_3 and DMS-HDL_3 binding sites was examined further using competition experiments (see fig. 5). Unlabelled DMS-HDL_3 strongly inhibited ^{125}I-labelled DMS-HDL_3 binding, but failed to compete effectively for the binding of native ^{125}I-labelled HDL_3. Fig. 5 also shows that unlabelled HDL_3 can inhibit the binding of both ^{125}I-labelled HDL_3 and ^{125}I-labelled DMS-HDL_3. Therefore we conclude that cationized LDL-loaded EA.hy 926 cells express one binding site which is specific for native HDL_3 and a second binding site, that interacts with both native HDL_3 and DMS-HDL_3. Both binding sites appear to bind native HDL_3 with high affinity, because Scatchard analysis does not reveal two distinct sites (not shown). It is unexplained at present why the maximal binding of native HDL, if binding at both sites, is lower than the maximal binding of DMS-HDL, which interacts with one binding site only. Our conclusion is different from that of Senault *et al.* [25], who proposed the existence of two distinct binding sites for HDL_3 and DMS-HDL_3 on rat liver membranes.

In the experiments of Senault *et al.* [25] there was also competition of the binding of iodinated DMS-HDL_3 by unlabelled HDL_3, although this competition was weaker than in our experiments. In our opinion, the results of Senault *et al.* [25] do not exclude the possibility that native HDL and DMS-HDL bind to the same binding site. Chacko *et al.* [17] measured the competition of the binding of ^{125}I-labelled TNM-HDL_3 by unlabelled native HDL_3 and by unlabelled TNM-HDL_3, and showed that both lipoproteins gave an identical inhibition of the binding of iodinated TNM-HDL_3. These data also suggest that TNM-HDL may bind to a binding site that also interacts with native HDL (and possibly also with DMS-HDL).

Table IV shows that the cells released cholesterol only in the unesterified form, which is consistent with the literature [8,9,29]. This also indicates that, eventually extracellularly-bound, cationized LDL is not released from the cells during the incubation with HDL. In that case medium cholesteryl ester content would have increased also, which was not observed. So, the measured increase of cholesterol in the medium is consistent with real efflux of cellular cholesterol.

DMS modification of HDL did not inhibit HDL-induced cholesterol efflux in our experiments. DMS-HDL-mediated efflux was also identical with efflux induced by native HDL_3 in experiments of Johnson *et al.* [8]. Although DMS-HDL particles do not bind to the specific binding sites for native HDL (see fig. 5) efflux is still maximal. This implies the possibility that the membrane binding sites, recognizing both native and DMS-HDL, are involved in cholesterol efflux. However, these conclusions are only based on cross-competition experiments. Additional experiments (e.g. using specific antibodies against the two HDL binding sites) are necessary to give further information about the importance of these binding sites in efflux of cellular cholesterol. In our experiments TNM-HDL_3 also induced efflux of cell cholesterol, but its capacity was reduced by about 50%. The reason for this decreased efflux could be the structure of the lipids in TNM-HDL_3 particles. Because of cross-linking of phospholipids and cholesteryl esters to HDL apolipoproteins [11], the structure of TNM-HDL_3 particles may have become more rigid. This may inhibit diffusion of cholesterol between cells and HDL particles, thereby decreasing efflux rate. Brinton *et al.* [7] also measured an impaired efflux of cell cholesterol if TNM-HDL_3 was used instead of native HDL_3. These data appear to be consistent with our findings. Brinton *et al.* [7] label the TNM-HDL-mediated efflux as "low affinity cholesterol efflux", and conclude that TNM-HDL reduces the "high affinity component of cholesterol efflux". The latter operates up to a concentration of about 6 μg of HDL protein/ml. This HDL concentration seems to be too low to induce a substantial amount of mass cholesterol efflux in vitro.

In conclusion our experiments show that modified HDL binds differently to cationized LDL-loaded EA.hy 926 cells, if compared with native HDL. Cholesterol-

loaded EA.hy 926 cells may express two high affinity binding sites for HDL: one site is specific for native HDL, and the second site may bind both native HDL and DMS-HDL (and probably also TNM-HDL). Native HDL and DMS-HDL induced identical efflux of cholesterol from cholesterol-enriched EA.hy 926 cells. The interaction of the cholesterol-loaded cells with DMS-HDL is sufficient to facilitate cholesterol efflux, probably by shortening the distance for diffusion of cholesterol through the aqueous phase.

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chapter 6

Effect of phospholipid fatty acid composition of endothelial cells on cholesterol efflux rates

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Abstract Human endothelial cells (EA.hy 926 line) were loaded with cationized low density lipoprotein (LDL) and subsequently incubated with fatty acid/bovine serum albumin complexes. The fatty acids were palmitic, oleic, linoleic, arachidonic and eicosapentaenoic acids. The preincubations resulted in extensively modified fatty acid profiles in cell membrane phospholipids and in cellular cholesteryl esters. The cholesterol efflux from these fatty acid-modified cells was measured using 0.2 mg high density lipoprotein$_3$ (HDL_3)/ml medium. The efflux was significantly higher for the palmitic acid-treated cells, compared to all other fatty acid treatments. These differences in efflux rates were not caused by changes in the binding of HDL_3 to high affinity receptors on the EA.hy 926 cells. Efflux mediated by dimethyl suberimidate-treated HDL_3, which does not interact with high affinity HDL receptors, was similar to efflux induced by native HDL_3 after all fatty acid treatments. Our results indicate that high affinity HDL receptors are not important for HDL-mediated efflux of cell cholesterol. The fatty acid composition of the cell membrane phospholipids may be an important determinant.

Introduction

High density lipoproteins (HDL) are believed to play a role in reverse cholesterol transport, i.e., the transfer of peripheral cell cholesterol back to the liver. HDL binding membrane proteins were visualized in different cell types [1-5], but the physiological role of these HDL binding proteins is still unclear. Oram *et al.* [6] showed up-regulation of HDL binding activity in fibroblasts and smooth muscle cells after loading the cells with cholesterol, suggesting a functional role for HDL binding proteins in the removal of excess cell cholesterol. When HDL_3 was unable to bind to the high affinity receptors, the high affinity component of the efflux of cholesterol was also abolished [7]. However, other investigators showed that there was no relation between binding of HDL and HDL-mediated efflux of cell cholesterol [8-10]. The desorption of cholesterol out of the cell membrane is considered the rate limiting factor for efflux [11]. In the present study we determined the effect of membrane composition on cholesterol efflux. Fatty acids added to the media of cells in culture, even at confluency, are readily incorporated in the cell membranes [12], resulting in extensively modified fatty acid profiles of the membrane phospholipids. The physical properties of the membrane, e.g., the fluidity, may change by modification of this fatty acid profile, leading to modifications of membrane-dependent cellular functions (for reviews see ref. [13,14]). When the desorption of cholesterol out of the cell membrane is an important factor, these modifications in membrane composition will affect the cholesterol efflux. The importance of the high affinity binding of HDL for cholesterol efflux was examined in

two ways. First, by determining possible differences in binding after fatty acid modification of the membrane phospholipids, and second, by measuring cholesterol efflux using modified HDL particles, which do not bind to the high affinity HDL binding sites. The conclusions of this study are based on net mass efflux of cholesterol, measured by increases of cholesterol in the medium. No isotopically labeled cholesterol was used. This was done in order to avoid the complications of isotope effects caused by exchange of labeled cell cholesterol for unlabeled lipoprotein cholesterol.

Materials and methods

Cell culture

The endothelial cell line EA.hy 926 was generously provided by Dr. C.-J.S. Edgell [15]. The EA.hy 926 cells were cultured in DMEM (Flow Laboratories), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml and HAT (100 µM hypoxanthin, Merck No. 4517; 0.4 µM aminopterin, Serva No. 13170; 16 µM thymidin, Merck No. 8206) at 37 °C in 95% air and 5% CO_2. Cells were plated in multiwell dishes (35 mm: $1 \cdot 10^5$ cells/well, 22 mm: $5 \cdot 10^4$ cells/well) 4 to 5 days prior to each experiment. Medium was changed every 2 or 3 days.

The confluent monolayers were loaded with cholesterol during a 24-h incubation of the cells with cationized LDL in growth medium (50 µg LDL-cholesterol/ml medium). After enrichment with cholesterol, the cells were incubated with fatty acid/BSA complexes for 48 h. The medium was changed once during this incubation. The fatty acids used were palmitic acid (Merck No. 509), oleic acid (sodium salt, Sigma No. O-7501), linoleic acid (sodium salt, Sigma No. L-8134), arachidonic acid (Sigma No. A-9798) and eicosapentaenoic acid (Sigma No. E-2011). Stock solutions (50 mM) were prepared by dissolving the fatty acids in methanol with butylated hydroxytoluene (12.5 µg/ml). The media used for fatty acid modification contained 0.1 mM fatty acid, 2 mg BSA/ml (Sigma No. A-4378) and 0.005% n-octyl ß-D-glucopyranoside (Sigma No. O-8001). An equimolar amount of NaOH was added when the acid form was used instead of the sodium salt.

Lipoproteins

LDL (density range 1.006-1.063 g/ml) and HDL_3 (density range 1.125-1.21 g/ml) were isolated by sequential ultracentrifugation of human plasma according to Havel *et al.* [16]. All lipoproteins were dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Before the cell experiments the lipoproteins were dialyzed for a second time against DMEM and filter sterilized (0.45 µm).

LDL was cationized according to Basu *et al.* [17]. A volume of LDL (20 to 30 mg of protein) was added to an equal volume of 2 M solution of 3-dimethyl-aminopropylamine (Aldrich No. 24.005-2) in H_2O at room temperature. The pH was adjusted to 6.5 with HCl. After stirring this mixture, 100 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (Sigma No. E-7750) was added. The pH was maintained at 6.5 with 0.1 M HCl during the reaction. After 3 hours, the pH of the solution did not change anymore; the reaction mixture was left at 4 °C overnight. Cationized LDL was dialyzed against 0.9 % NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, filter sterilized (0.8 µm), and stored. Storage at 4 or -20 °C did not influence the loading of the cells.

Apo E-free HDL_3 was isolated by chromatography of HDL_3 on a heparin-Sepharose column [18]. Modification of HDL_3 with DMS was performed as described previously [19]. The apo E-free HDL_3 (5 mg protein/ml) was incubated with 5 mg DMS/ml in a 0.09 M triethanolamine-HCl buffer (pH 9.5) for 2 h at room temperature. After the reaction, DMS-HDL_3 was reisolated on a Sephadex column (G25 Fine, 1 x 30 cm, Pharmacia). The column was eluted with 0.9% NaCl with 2 mM EDTA (pH 7.4). The cross-linking of

the DMS-HDL_3 apolipoproteins was checked by electrophoresis on 12% polyacrylamide gels containing 0.1% SDS. No apo A-I monomers were visible on the cross-linked HDL_3 particles. The sizes of native HDL_3 and DMS-HDL_3 particles were measured by gel filtration as described [20], using a Superose 12 column (Prep grade, 1.0 x 30 cm, Pharmacia). The size of both HDL_3 preparations was exactly the same, indicating that cross-linking only occured between apolipoproteins on the same HDL_3 particle. HDL_3 was iodinated using the iodine monochloride method (using $Na^{125}I$ obtained from Amersham International) as described earlier [21]. The molar iodine to protein ratio of the ^{125}I-labeled HDL_3 preparations ranged from 0.22 to 0.49; 95.4 ± 0.8 % of the label was protein-bound, 1.7 ± 0.6% was in the HDL lipids and 2.9 ± 0.5% was free.

Efflux experiments

Efflux was virtually negligible in the absence of HDL. Both BSA alone and BSA plus variable amounts of LDL did not result in significant cholesterol efflux from loaded cells (not shown). After cholesterol loading and fatty acid modification, cells were rinsed twice with 2 mg BSA/ml in DMEM with penicillin/streptomycin (1 ml/well) and twice with DMEM only, plus penicillin/streptomycin, at room temperature. The assay of cholesterol efflux was started by addition of efflux medium and incubation at 37 °C in 5% CO_2 and 95% air. The efflux medium consisted of DMEM, supplemented with L-glutamine, penicillin, streptomycin, 2 mg BSA/ml (Sigma No. A-4378), and lipoproteins as indicated. The efflux was ended after 24 h by cooling the culture dishes on ice. The culture media were collected and dislodged cells were spun down (500 x g_{max}, 4 °C). The cell media were stored at -20 °C untill cholesterol determinations. The wells were rinsed three times with 2 mg BSA/ml in 0.9% NaCl with 50 mM Tris-HCl (pH 7.4), followed by three rinses with 0.9% NaCl/50 mM Tris-HCl (pH 7.4). The cells were scraped into 1 ml 0.9% NaCl/50 mM Tris-HCl (pH 7.4) with a rubber policeman and lipids were extracted using the method of Bligh and Dyer [22]. The resulting protein pellet was dissolved in 500 µl 0.1 M NaOH with 10% SDS (w/v). Cell protein was measured according to Lowry *et al.* [23]. Cell lipids were dried down under nitrogen and dissolved in 300 µl 2-propanol. The lipid extracts were stored up to 1 day at -20 °C and unesterified and total cholesterol were determined separately using a slight modification of the method described by Heider and Boyett [24]. For determination of unesterified cholesterol the reaction mixture consisted of 0.15 mg *p*-OH-phenylacetic acid/ml (Sigma No. H-4377), 5 U peroxidase/ml (Boehringer Mannheim No. 108081) and 3.2 µg cholesterol oxidase/ml (Boehringer Mannheim No. 396818) in sodium phosphate buffer (50 mM, pH 7.4). The reaction mixture of the total cholesterol determination was as for unesterified cholesterol, plus 6.4 µg/ml cholesteryl esterase (Boehringer Mannheim No. 161772), 5 mM taurocholate (Calbiochem No. 580217) and 0.17 mM PEG-6000 (Serva No. 33137). Reaction mixture (0.75 ml) was added to 40 µl cell extract in 2-propanol. A 100 µg/ml solution of cholesterol (Sigma No. C-8253) in 2-propanol was used as a standard. After the incubation (37 °C, 30 min for unesterified cholesterol and 120 min for total cholesterol assays) the reaction was ended by addition of 1.5 ml 0.5 M NaOH and fluorescence was measured with a Perkin-Elmer LS-3B fluorimeter (excitation 325 nm, emission 415 nm).

Total and unesterified cholesterol in the media were measured enzymatically using a commercially available kit (cholesterol kit No. 310328, cholesterol esterase No. 161772, Boehringer Mannheim). Cholesteryl esters were calculated by difference between total and unesterified cholesterol.

Lipoprotein binding experiments

After the preincubations with cationized LDL and fatty acids, the cells (in 22 mm wells) were rinsed three times with 2 mg BSA/ml and penicillin/streptomycin in DMEM at room temperature, followed by three rinses at 0 °C with DMEM and penicillin/streptomycin to cool the cells. Subsequently, media (DMEM with 2 mg BSA/ml, L-glutamine, penicillin/streptomycin and lipoproteins as indicated) were added (0.5 ml/well) and the cells were incubated 4 h at 4 °C in an atmosphere with 5% CO_2. After the incubations the cells were rinsed three times with 0.9% NaCl containing 2 mg BSA/ml and 50 mM Tris-HCL (pH 7.4) and three times with 0.9% NaCl containing only 50 mM Tris-HCL (pH 7.4). The cells were dissolved in 300 µl 1 M NaOH, the radioactivity was counted using a Packard Minaxi 5000 gamma counter, and protein was measured according to Lowry *et al.* [23]. The incubation media contained 15 or 100 µg apo E-free ^{125}I-labeled HDL_3/ml (specific activity 200 cpm/ng). Nonspecific binding was measured in the presence of 1 mg

Table I

Cholesterol levels in EA.hy 926 cells after cholesterol enrichment and fatty acid treatment

treatment	μg TC/mg protein[a]	μg UC/mg protein	% unesterified cholesterol
control cells	45.3 ± 4.0	39.8 ± 3.6	87.9 ± 4.6
cholesterol-rich cells[b]:			
palmitic acid	169.8 ± 7.5[c,d]	99.0 ± 4.7[d]	57.4 ± 2.6[d,e]
oleic acid	173.8 ± 10.7[c]	96.8 ± 5.5	55.8 ± 2.7[c,d]
linoleic acid	166.4 ± 15.6[d]	91.5 ± 10.3	54.9 ± 2.5[c,f]
arachidonic acid	157.0 ± 10.0[d,f,g]	96.7 ± 6.7	61.7 ± 3.0[d,e,g]
eicosapentaenoic acid	178.0 ± 3.0[c,e,f]	90.4 ± 4.4[f]	50.8 ± 2.5[c,e,f,g]

[a] Cholesterol mass (mean ± SD).

[b] Confluent EA.hy 926 cells were preincubated for 24 h with 50 μg cationized LDL-TC/ml, followed by 48 h of incubation with different free faty acid/BSA complexes (0.1 mM fatty acid). The cells were then rinsed, scraped, and cell cholesterol was determined as described in *Materials and methods*. Data are from one typical experiment (6 wells per treatment).

[c] Significantly different from arachidonic acid treatment (Mann-Whitney, $P<0.05$).

[d] Significantly different from eicosapentaenoic acid treatment (idem).

[e] Significantly different from linoleic acid treatment (idem).

[f] Significantly different from palmitic acid treatment (idem).

[g] Significantly different from oleic acid treatment (idem).

Table II

Phospholipid level and molar unesterified cholesterol:phospholipid ratio after cholesterol enrichment and fatty acid treatment of the EA.hy 926 cells.

treatment	μg PL/mg protein[a]	FC:PL (mol:mol)
control cells	612 ± 18	0.098 ± 0.007
cholesterol rich cells[b]:		
palmitic acid	693 ± 17	0.282 ± 0.008
oleic acid	616 ± 11	0.269 ± 0.011
linoleic acid	594 ± 52	0.285 ± 0.032
arachidonic acid	590 ± 13	0.305 ± 0.011
eicosapentaenoic acid	616 ± 23	0.268 ± 0.006

[a] Values are means ± SD, $n=2$.

[b] For preincubation conditions, see *Table I*. After the preincubation, the cells were trypsinized and sonicated. The resulting cell suspensions were used for the total phosphorus, cholesterol and protein determinations.

unlabeled HDL_3/ml. Specific binding was calculated by difference between total and nonspecific binding. Binding was expressed per mg cell protein. In competition experiments 5 μg apo E-free ^{125}I-labeled HDL_3/ml was used with 0-200 μg/ml unlabeled apo E-free HDL_3 or DMS-HDL_3.

Determination of fatty acid composition of cell phospholipids and cholesteryl esters

Cells from a 75-cm^2 flask (Costar No. 3375) were preincubated with 7.5 ml of cholesterol-enriched medium (growth medium plus cationized LDL) followed by fatty acid/BSA containing medium (15 ml). After the preincubation period the cells were trypsinized and resuspended in 0.9% NaCl with 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA. EDTA was added to inhibit phospholipase C activity. Fatty acid profiles were determined both in phospholipids and cholesteryl esters according to Lamers *et al.* [25]. In short, the lipids were extracted by the method of Bligh and Dyer [22] and dissolved in a small amount of chloroform-methanol 1:2. The lipid classes were separated by thin-layer chromatography on activated silica gel 60 plates (Merck No. 5721) in hexane-diethylether-acetic acid 60:40:1 with 0.02% butylated hydroxytoluene. Phospholipid and cholesteryl ester spots were scraped off and the lipids were eluted from the silica with methanol (phospholipids) or chloroform-methanol 2:1 (cholesteryl esters). The solvent was evaporated and boron trifluoride-methanol (Sigma No. B-1127) was added for transmethylation (14% BF_3 for phospholipids and 5% for cholesteryl esters). After methylation at 100 °C under N_2, the methyl esters were extracted with pentane-H_2O 2:1, dried with sodium sulphate, dissolved in iso-octane and the methyl esters were separated by GLC (split injector, 270 °C; N_2 carrier gas, inlet pressure 95 kPa; CP9000 capillary column (Chrompack) equipped with a coated, fused silica caplillary CP-Sil 88 column; column temperature 190 °C during first 15 min, increasing to 220 °C (15 °C/min), total run time 31 min; flame ionization detection, 300 °C).

Total phospholipid-derived phosphorus was determined using the method of Bartlett [26] in the membrane pellets of 5% trichloroacetic acid-treated cell suspensions. Inorganic potassium phosphate was used as a standard.

Statistical analysis

Analysis of variance was used for comparison of the different fatty acid treatments when possible ($n>9$, variances identical in all treatment groups), Bonferroni t-tests were used for comparison among treatment groups when results of the analysis of variance were significant. The nonparametric Mann-Whitney test was used to compare two treatment groups of $n<9$. The differences were considered statistically significant when $P<0.05$.

Results

Cell cholesterol and phospholipid composition after preincubation with cationized LDL and free fatty acids

Table I shows the cholesterol levels after preincubation of the cells. Incubation with cationized LDL increased cell cholesterol levels 3- to 4-fold. In normal EA.hy 926 cells almost 90% of this cholesterol was unesterified. The esterification level was much higher in the loaded cells: 40-50% was esterified. Total cell cholesterol, expressed per mg cell protein, was lowest for arachidonic acid-treated cells (significantly lower than after 16:0, 18:1, and 20:5 treatment). Preincubation with eicosapentaenoic acid resulted in the highest level of total cell cholesterol, which was significantly higher than after

Table III

Fatty acid composition of EA.hy 926 cell phospholipids

	treatment[a]					
	control	palmitic acid	oleic acid	linoleic acid	arachidonic acid	eicosapentaeonic acid
16:0	26.5[b]	27.0	11.1	18.7	17.7	17.3
18:0	14.2	14.8	9.1	13.3	11.9	10.7
18.0dma[c]	nd.[d]	4.0	1.2	2.3	2.6	2.1
22.0	0.9	1.1	2.5	1.2	0.9	2.9
24.0	1.0	1.2	0.8	1.0	0.9	0.5
16:1 n-7	2.9	3.3	1.7	3.2	1.8	2.1
18:1 n-9/n-7	24.6	21.4	48.2	29.0	18.3	18.2
20:1 n-9	0.4	0.5	1.3	0.6	0.3	0.2
18:2 n-6	12.1	11.2	6.7	14.2	7.7	6.9
18:3 n-3	0.1	0.1	2.0	0.5	0.3	2.8
20:3 n-6	2.1	2.1	1.4	2.2	1.2	1.1
20:4 n-6	5.6	6.4	8.1	6.7	24.3	5.2
20:5 n-3	0.8	0.9	0.6	1.0	0.3	16.1
22:4 n-6	0.6	0.5	0.4	0.5	9.1	1.0
22:5 n-3	1.9	1.9	1.6	1.8	1.0	11.1
22:6 n-3	3.0	2.8	1.8	2.4	0.8	0.8
Others	3.4	1.1	1.5	1.4	0.9	1.0
% SFA	42.9	48.2	24.9	36.7	34.1	33.8
% MUFA	30.4	25.8	52.4	33.7	21.0	21.2
% PUFA	26.7	26.1	22.7	29.6	44.8	45.1
unsaturation index[e]	119	114	132	128	186	212

[a] Data are from one typical experiment, 1 flask (75 cm^2) of cells was used for each fatty acid treatment (preincubations as in *Table I*). At the end of the preincubation the cells were trypsinized, lipids were extracted, phospholipids were separated and methylated for GLC analysis. The variation between replicate GLC injections was <2%. Even the relatively small differences between 16:0 and 18:2 treatments were reproduced in 2 separate experiments.

[b] Values are % of total fatty acids in the phospholipid fraction, fatty acids were listed when in one of the treatments the percentage was > 1%.

[c] 18:0 dimethyl acetal.

[d] Not detectable.

[e] Unsaturation index = Σ(% of each fatty acid · amount of double bonds per FA).

Table IV

Fatty acid composition of EA.hy 926 cell cholesteryl esters.

	treatment[a]					
	control	palmitic acid	oleic acid	linoleic acid	arachidonic acid	eicosapenta-enoic acid
16:0	17.5[b]	23.1	15.2	19.4	14.2	15.7
18:0	2.6	2.7	2.5	2.8	1.8	2.5
22:0	2.5	0.4	0.4	0.4	0.4	1.0
16:1 n-7	4.0	3.6	3.3	4.3	3.8	3.6
18:1 n-9/n-7	23.3	24.5	40.8	27.9	20.1	19.9
20:1 n-9	0.1	3.6	1.8	0.4	nd.	nd.
22:1 n-9	1.0	3.4	0.9	0.4	0.4	0.5
24:1 n-9	nd.[c]	3.7	1.2	0.4	nd.	0.2
18:2 n-6	40.0	29.6	26.7	37.9	33.0	34.4
18:3 n-3	4.0	0.6	0.7	0.7	0.6	1.0
20:4 n-6	2.3	1.3	1.4	1.9	17.1	2.5
20:5 n-3	0.3	0.3	0.2	0.2	nd.	11.0
22:4 n-6	0.5	nd.	0.5	0.4	6.5	nd.
22:5 n-3	nd.	0.2	0.5	nd.	nd.	5.2
others	3.1	3.0	3.9	2.9	2.1	2.5
% SFA	22.6	30.2	22.5	26.2	19.6	23.1
% MUFA	28.4	37.6	46.5	32.5	23.7	23.5
% PUFA	49.0	32.2	31.0	41.3	56.8	53.4
unsaturation index[d]	141	108	119	124	185	185

[a] The same cells were used for fatty acid analysis of cholesteryl esters and phospholipids (see *Table III*).
[b] Values are % of total fatty acids in the cholesteryl ester fraction, fatty acids were listed when in one of the treatments the percentage was > 1%.
[c] Not detectable.
[d] Unsaturation index = Σ(% of each fatty acid · amount of double bonds per FA).

treatment with 16:0, 18:2, and 20:4. The percentage of cell cholesterol that was unesterified was highest for arachidonic acid-treated cells (62±3%, which was significantly higher than for 18:1-, 18:2-, and 20:5-treated cells). The lowest percentage unesterified cholesterol was found after preincubation with eicosapentaenoic acid (51±3%). All other fatty acid treatments gave significantly higher percentages unesterified cholesterol than eicosapentaenoic acid treatment. The absolute level of unesterified cholesterol per mg cell protein was highest in palmitic acid-treated cells (significantly different only from 20:5-treated cells).

Palmitic acid treatment also tended to increase the cell phospholipid level (13% increase, see table II). Treatments with all the other fatty acids did not alter cellular phospholipid levels compared to normal confluent EA.hy 926 cells. Table II shows an almost 3-fold increase in the molar ratio of FC:PL in cells after enrichment with cholesterol. The FC:PL ratio ranged between 0.268 and 0.305 for the different fatty acid treatments.

Table III shows that the fatty acid profile of the cell phospholipids differed greatly after the various fatty acid treatments. The control incubation, in which the cholesterol-loaded cells were incubated without fatty acids (but with the solvents), differed only slightly when compared with palmitic acid treatment: the total of saturated fatty acids in the phospholipid fraction increased from 43 to 48%. Preincubation with oleic acid resulted in 48% oleic acid in the cell phospholipids, and the total of monounsaturated fatty acids in phospholipids increased to 52%. Linoleic acid treatment gave the highest percentage of linoleic acid in cell phospholipids (compared to the other fatty acid treatments) although the increase was small. Arachidonic and eicosapentaenoic acid treatment both lead to very high amounts of the respective polyunsaturated fatty acids in the cell phospholipid fraction (24 and 16% respectively). Not only the percentages of these added fatty acids increased, but there was also a marked increase of the elongation products of arachidonic and eicosapentaenoic acid, resulting in 9% of 22:4 n-6 and 11% of 22:6 n-3, after the respective treatments. Palmitic acid treatment resulted in a 4% increase in dimethylacetal esters from stearic acid, which is also an elongation product. For the other fatty acid treatments there was no clear increase of elongation products. In summary, the preincubations of the EA.hy 926 cells with the various fatty acids resulted in five types of endothelial cells with clearly different fatty acid profiles of the cell phospholipid fraction.

There was also an effect on fatty acid profiles of the cell cholesteryl esters (see table IV). Independent of the fatty acid type used for modification, the cell cholesteryl esters contained 27 to 38% linoleic acid, which was mostly derived from the cationized LDL used for cholesterol enrichment. Although the differences caused by the fatty acid treatments were less than in the phospholipid fraction, the effect of the added fatty acids

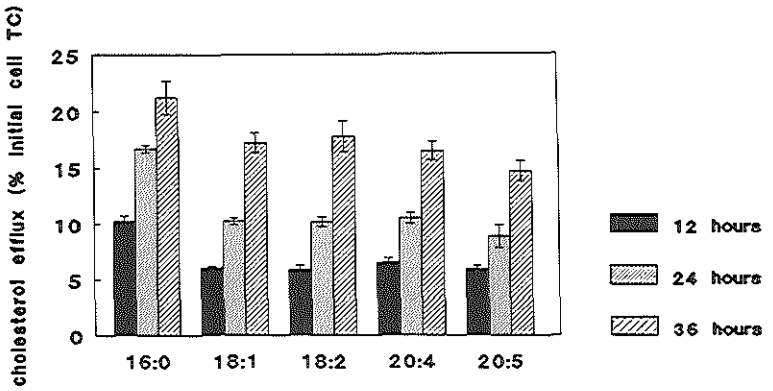

Fig. 1 Efflux of cell cholesterol after 12, 24 and 36 h of incubation with 0.2 mg HDL_3/ml, expressed as the percentage of the initial cell cholesterol per well appearing in the medium (data are from one typical experiment, means ± SEM, $n=6$ wells). The cholesterol-enriched cells were preincubated with palmitic acid, oleic acid, linoleic acid, aracidonic acid or eicosapentaenoic acid. For each fatty acid treatment efflux was significantly different between the three time points (Mann-Whitney, $P<0.02$). For each fatty acid treatment, cell protein (μg per well) after 0, 12, 24 and 36 h of incubation was, respectively: palmitic acid 257±11, 250±4, 251±6 and 258±5; oleic acid 226±12, 234±11, 219±7 and 226±8; linoleic acid 219±21, 229±12, 239±7 and 236±6; arachidonic acid 225±20, 211±5, 216±10 and 233±9; eicosapentaenoic acid 217±6, 234±6, 263±13 and 248±8 (means ± SD). For initial cell cholesterol levels, see *Table I*.

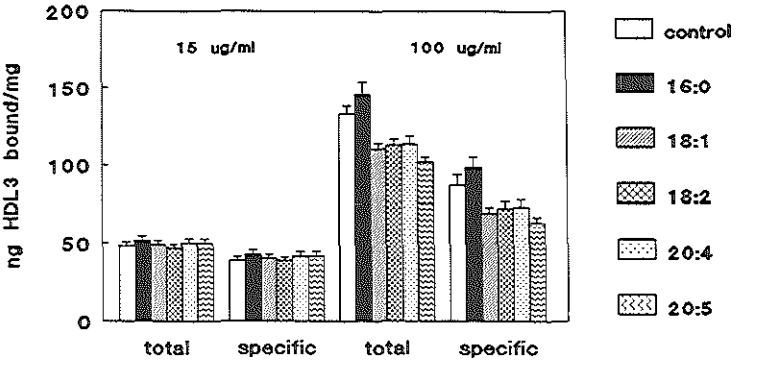

Fig. 2 Binding of apo E-free HDL_3 to EA.hy 926 cells after fatty acid treatment of cholesterol-loaded cells. The binding was measured at 15 and 100 μg ^{125}I-labeled HDL_3/ml for 4 h at 4 °C. Specific binding is the difference of total and nonspecific binding (measured with an excess of 1 mg unlabeled HDL_3/ml). The data are means ± SEM from 4 and 3 separate experiments at 15 and 100 μg/ml, respectively ($n=3$ wells for each fatty acid treatment in each experiment). Analysis of variance showed no significant differences between the fatty acid treatments for total and specific binding at 15 μg HDL_3/ml. At 100 μg/ml of HDL_3 total as well as specific binding to palmitic acid-treated cells was higher than to 18:1-, 18:2-, 20:4-, and 20:5-treated cells ($P<0.02$). Total binding to control cells is higher in comparison with 18:1 and 20:5 treatment ($P<0.05$). Specific binding to control cells is higher than to 20:5-treated cells ($P<0.05$).

Table V

HDL_3-mediated efflux of cholesterol from cholesterol-enriched fatty acid-modified EA.hy 926 cells

treatment (n)	cholesterol increase in medium (nmol/mg cell protein/24 h)[a]
palmitic acid (30)	90.6 ± 1.9[b]
oleic acid (11)	66.3 ± 1.8
linoleic acid (18)	63.9 ± 3.9
arachidonic acid (14)	60.7 ± 2.5
eicosapentaenoic acid (10)	52.8 ± 2.6

[a]Values are means ± SEM from 4 separate experiments (n = total number of wells).
[b]Significantly different from other fatty acid treatments (Bonferroni t-test, $P < 0.001$).

Table VI

Competition of ^{125}I- labeled HDL_3 binding by unlabeled native HDL_3 or DMS-HDL_3

competitor (μg protein/ml)	% ^{125}I-labeled HDL_3 bound[a]: native HDL_3	DMS-HDL_3
0	100 ± 5	100 ± 5
5	80 ± 10	118 ± 4
10	68 ± 6	109 ± 3
25	58 ± 3	102 ± 6
50	47 ± 4	109 ± 6
100	39 ± 2	114 ± 9
200	28 ± 1	77 ± 3

[a] Binding was measured with 5 μg apo E-free ^{125}I-labeled HDL_3/ml and 0-200 μg of unlabeled native HDL_3 or DMS-HDL_3/ml. The data are expressed as % of the amount of binding without competitors (means ± SEM from 2 separate expe-riments, $n = 3$ wells for each experiment).

was visible in the cholesteryl ester moieties. Palmitic acid treatment gave 23% 16:0 in the cell cholesteryl esters. The added oleic acid was incorporated very well in the cholesteryl esters: 41% cholesteryl oleate after oleic acid addition. Linoleic acid resulted in 38% cholesteryl linoleate in the cell cholesteryl ester fraction. Preincubation with arachidonic acid gave 17% 20:4 n-6 in the cholesteryl esters and 7% of its elongation product 22:4 n-6. Eicosapentaenoic acid treatment resulted in a cholesteryl ester fraction with 11% 20:5 n-3 and 5% 22:5 n-3.

HDL_3-mediated efflux and HDL_3 binding of fatty acid-modified cholesterol-enriched EA.hy 926 cells

Efflux of cell cholesterol to HDL_3 was measured after 12, 24 and 36 h of incubation (fig. 1). The increase of unesterified and total cholesterol in the incubation media was identical (not shown). The amount of cholesterol leaving the cells increased in time for all the treatment groups. The efflux of cholesterol had different rates for the various fatty acid treatments. After 12 and 24 h of incubation, the efflux was highest for

palmitic acid-treated cells; respectively 10.3±0.5% and 16.7±0.3% of initial cell cholesterol was released from the cells (means ± SEM, Mann-Whitney, $P<0.01$). After 36 h of incubation with HDL_3, 21.2±1.5% of the cell cholesterol was transferred to the medium. The differences in efflux between the palmitic acid-treated cells and the other fatty acid-treated cells decreased at this time point, but efflux after palmitic acid treatment remained significantly higher than after treatment with 18:1, 20:4, and 20:5 (Mann-Whitney, $P<0.05$). No significant correlations were observed between the efflux and initial cell cholesterol levels or grade of esterification.

Table V shows the average efflux (mean nmol cholesterol ± SEM/mg cell protein) after fatty acid treatment of the cholesterol-enriched cells after 4 separate experiments. Initial cell cholesterol was measured in only 2 of the 4 experiments (not shown) and was comparable with the data shown in table I. Efflux was 90.6±1.9 for palmitic acid-treated cells, 66.3±1.8 for oleic acid-treated cells, 63.9±3.9 for linoleic acid-treated cells, 60.7±2.5, for arachidonic acid-treated cells, and 52.8±2.6 after eicosapentaenoic acid treatment. Palmitic acid treatment resulted in significantly more efflux than the other four fatty acid treatments (Bonferroni *t*-test, $P<0.001$).

The binding of apo E-free HDL_3 to the fatty acid-treated cells was measured at 15 and 100 μg HDL_3 protein/ml (see fig. 2). Both total binding and nonspecific binding were determined (nonspecific binding was measured with an excess of 1 mg unlabeled HDL_3/ml). Specific binding is defined as the difference between total binding and non-specific binding. At 15 μg/ml no differences between the fatty acid treatments were found for total HDL_3 binding or specific HDL_3 binding. At higher HDL_3 concentrations (i.e., 100 μg/ml, above the K_d, where most of the high affinity HDL_3 sites are occupied) the palmitic acid-treated cells showed a significantly higher total and "specific" binding than all the other treatments (Bonferroni *t*-test, $P<0.05$).

To test whether binding to the high affinity HDL receptor was important for cholesterol efflux, we used DMS-HDL_3. This modified HDL does not compete with binding of native HDL_3 to high affinity binding sites, as is shown in table VI (see also ref. 19). Fig. 3 shows the HDL_3-or DMS-HDL_3-mediated cholesterol efflux for three different fatty acid modifications. The efflux was similar for both HDL_3 preparations after all treatments. The efflux was significantly higher for palmitic acid-modified cells, if compared to oleic or eicosapentaenoic acid treatment using DMS-HDL_3 as well as native HDL_3 (Mann-Whitney, $P<0.025$).

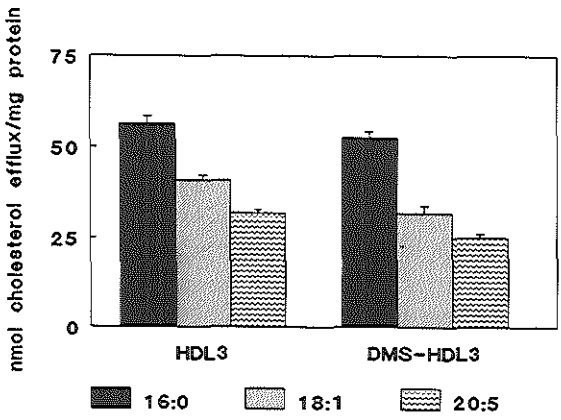

Fig. 3 Efflux of cell cholesterol after 24 h of incubation with 0.2 mg native HDL_3/ml (left) or 0.2 mg DMS-HDL_3/ml (right). Cholesterol-enriched cells were preincubated with palmitic acid, oleic acid or eicosapentaenoic acid. The efflux was measured as the increase of cholesterol in the medium, and expressed per mg cell protein (data are from one experiment, means ± SEM, $n=6$ wells). Efflux was significantly different between the three fatty acid treatments for both native HDL_3 and DMS-HDL_3 (Mann-Whitney, $P<0.025$).

Discussion

These results demonstrate that HDL-mediated efflux of cell cholesterol is dependent on the type of fatty acid used to modify the fatty acid profiles of cell phospholipids and cholesteryl esters. Binding of apo E-free HDL_3 to high affinity receptors on the cell membranes does not seem to be involved in the efflux process. This conclusion is based on two observations: 1) the specific binding of HDL_3 at 15 μg/ml was not changed after fatty acid treatment and did not correlate with cholesterol efflux; and 2) DMS-HDL_3, which does not bind to high affinity HDL receptors on cell membranes, gave efflux similar to that of native HDL_3 after each fatty acid treatment. The lack of relation between HDL binding and HDL-mediated cholesterol efflux was also reported by Karlin *et al.* [8], Johnson *et al.* [9], and Mendel and Kunitake [10], using a variety of cell types. These conclusions were based on studies involving modified HDL [8,9] and comparison of HDL-concentration dependencies of HDL binding and HDL-mediated efflux [8,10]. However, Brinton *et al.* [7] concluded from their experiments with fibroblasts, in which tetranitromethane-modified HDL particles were used, that HDL binding to high affinity receptors is necessary to induce efflux from cholesterol-loaded cells.

The enrichment of the confluent endothelial cells with cationized LDL caused an increase in cellular unesterified cholesterol and cholesteryl esters as well as in the FC:PL ratio. It was possible to measure a net mass cholesterol efflux from cells to HDL_3 particles. This efflux was measured as the increase of cholesterol in the medium which is far more accurate than the measurement of the decrease of cellular cholesterol. Measurement of total as well as unesterified cholesterol increase gave similar results,

indicating that cholesterol leaves the cells in the unesterified form and that no esterification of cholesterol in the medium took place during the incubation. The measurement of LCAT activity confirmed this conclusion: LCAT activity was absent in the HDL preparation used, which was isolated by ultracentrifugation. The fact that no increase in cholesteryl esters in the culture medium was measured after 24 h of efflux also indicated that no extracellularly bound cationized LDL was released from the cells during the incubation [27,28]. Stein *et al.* [28] reported that extracellular cationized LDL is very tightly bound to the plasma membrane.

The various types of fatty acids used resulted in specific changes in fatty acid profile of cell phospholipids. Addition of unsaturated fatty acids to the incubation media had more effect on fatty acid profiles than addition of saturated fatty acids (see table III). This may have been due to endogenous phospholipase A_2 activity of the cells, resulting in a preferential incorporation of unsaturated fatty acyl chains on the sn_2 position. It is likely that unsaturated fatty acids present at the sn_2 position of phospholipids have a faster turnover than saturated fatty acids, mostly present at the sn_1 position. The data presented in fig. 1 show that the different fatty acid modifications resulted in changes in rates of cholesterol efflux. After 36 h of incubation the differences among the various treatments were smaller than after 12 - 24 h of incubation. This may be explained by a reversal of the cell phospholipid fatty acid profiles, resulting from substantial exchange of phospholipids between cells and HDL particles after long-term incubations [13].

Cell protein per well was virtually constant during the 36 h incubation with HDL_3 (see legend fig. 1). These data indicate that palmitic acid had no toxic effects on the cells. Therefore, cell death can not have caused apparent cholesterol efflux after palmitic acid treatment. The small differences in initial cholesterol levels observed after the various fatty acid treatments (see table I) are too small to explain the differences in efflux as shown in table V, assuming that the efflux is linear with initial cholesterol levels. The differences in efflux rates among the fatty acid treatments may have been caused by efflux of cholesterol derived from different kinetic pools of membrane cholesterol, as proposed recently by Mahlberg and Rothblat [29]: different plasma membrane microdomains [30] may represent different pools of cholesterol from which efflux occurs with different rates. Modification of membrane acyl composition may affect the packing of cholesterol in different microdomains of the plasma membrane, thereby facilitating efflux from certain microdomains with fast kinetic cholesterol pools.

The fatty acid composition of cell cholesteryl esters also changed during the preincubation with the various fatty acids. It is unlikely that this will influence the rate of cholesterol efflux in our experiments. It is controversial whether the rate of cholesteryl ester hydrolysis varies for the different types of cholesteryl esters. There are reports

suggesting a substrate specificity of neutral cholesterol esterase for unsaturated cholesteryl esters [31,32], but there are also articles denying any preference [33,34]. It was reported that the clearance of cholesteryl esters from oleic acid- or linoleic acid-treated cells was higher than the clearance from palmitic acid-modified cells [34]. The authors concluded that this was due to a more isotropic condition of cytoplasmic lipid droplets, resulting from a higher triacylglycerol content and more unsaturated fatty acyl chains in the cholesteryl esters, facilitating the action of neutral cholesteryl ester hydrolases. So it is unlikely that the higher efflux rate from palmitic acid-treated cells, observed in our experiments, can be explained by increased hydrolysis of cholesteryl palmitate.

Although there was no significant correlation between the free cholesterol levels at t=0 and cholesterol efflux during 24 h, the palmitic acid-treated EA.hy 926 cells had a relatively high free cholesterol content (see table I). This could be due to a lower ACAT activity after this treatment, like for palmitic acid-treated J774 cells [35]. A lower ACAT activity renders more cholesterol directly available for efflux. McCloskey *et al.* [35] did not report whether the lower ACAT activity of palmitic acid-treated macrophages was due to a lower enzyme activity, or to changes in the availability of the cholesterol substrate in the membranes of the endoplasmatic reticulum. In contrast, Murthy *et al.* [36] measured a higher ACAT activity in CaCo-2 cells after palmitic acid treatment.

Other investigators, measuring exchange of cholesterol, observed a relatively low cholesterol exchange between dipalmitoylcholine vesicles and vesicles of egg phosphatidylcholine [37]. This indicates that net mass efflux of cholesterol from cholesterol-enriched cells involves mechanisms different from cholesterol exchange processes. We did not measure whether the various fatty acid treatments lead to different amounts of phospholipid subclasses. It is conceivable that the amount of sphingomyelin, containing relatively high amounts of saturated fatty acids, may change. Although a high sphingomyelin content of membranes or vesicles decreases the cholesterol exchange [37,38], the [^{3}H]cholesterol efflux from sphingomyelinase-treated fibroblasts remained unchanged [39].

The physical properties of the cell membrane can be expected to influence membrane-dependent cellular functions, like cholesterol desorption and receptor activities. Platelet aggregation [40], the phagocytotic capacities of peritoneal macrophages [41], binding properties of the insulin receptor [42], and the affinity as well as maximal binding of LDL to the LDL-receptor on peripheral blood mononuclear cells [43], U937 monocytes [44], and HepG2 cells [45] are influenced by membrane fatty acid composition. We could not detect any differences in the binding of apo E-free HDL_3 to fatty acid-modified cells at low HDL_3 concentrations (15 μg/ml). At this concentration,

binding is mostly to high affinity binding sites. At higher HDL_3 concentrations (100 μg/ml), the low affinity binding becomes more important. This concentration is 3-5 times the K_d. We measured a K_d of 37 μg/ml for untreated EA.hy 926 cells (not shown) and others reported a value of about 20 μg/ml [46]. We found small (statistically significant) differences at HDL_3 concentrations of 100 μg/ml. These results suggest that there may be differences in the low affinity binding sites after fatty acid treatment of cholesterol-enriched cells.

The results of the DMS-HDL_3-mediated efflux experiments also show that binding of HDL_3 to specific high affinity receptors is not important for efflux of cell cholesterol because for the three fatty acid treatments, efflux with HDL_3 and DMS-HDL_3 was similar (fig. 3). This is in agreement with results obtained by other investigators [9].

Our experiments demonstrate that efflux of cholesterol from cholesterol-loaded EA.hy 926 cells is possible without binding of HDL_3 to specific high affinity binding sites. The composition of the cell membranes is likely to be important in determining the rate of efflux. The degree of saturation of fatty acids in membrane phospholipids could influence the distribution of cholesterol-poor and cholesterol-rich domains in cell membranes, which may play a role in cholesterol efflux [29,30].

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chapter 7

Cholesterol efflux from cells to immunopurified subfractions of human high density lipoprotein: LP A-I and LP A-I/A-II

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Abstract Using immunoaffinity chromatography, we separated human high density lipoprotein (HDL) into two subfractions: LP-AI, in which all particles contain apolipoprotein AI (apo-AI) but no apo-AII, and LP-AI/AII, in which all particles contain both apo-AI and apo-AII. To compare LP-AI and LP-AI/AII as acceptors of cell cholesterol, the isolated subfractions were diluted to 50 μg phospholipid/ml, and then incubated with monolayer cultures of cells in which whole-cell and lysosomal cholesterol has been labeled with ^{14}C and ^{3}H, respectively. We used three cell types (Fu5AH rat hepatoma cells, normal human skin fibroblasts, and rabbit aortic smooth muscle cells). When these cells were prepared to contain normal physiological quantities of cholesterol (20-35 μg/mg protein), LP-AI and LP-AI/AII were nearly equally efficient in promoting efflux of both whole-cell and lysosomal cholesterol. For whole-cell cholesterol, the rate constants for efflux to LP-AI and LP-AI/AII were: 0.050/h and 0.053/h, respectively, with Fu5AH cells; 0.0063/h and 0.0074/h with GM3468 human skin fibroblasts; and 0.0076/h and 0.0079/h with rabbit aortic smooth muscle cells. When cholesterol in hepatoma cells or fibroblasts was elevated 2- to 3-fold above normal, there was still no difference in efflux of whole-cell cholesterol to LP-AI and LP-AI/AII. In long term incubations, the net depletion of cholesterol mass from cholesterol-enriched cells was either identical with the two HDL subfractions, or somewhat greater with LP-AI/AII. The present results suggest that with several types of mammalian cells: 1) LP-AI and LP-AI/AII function equally well in removing cholesterol that originates in either the plasma membrane or lysosomes; and 2) efflux to both fractions is likely to be governed primarily by the unmediated diffusion of cholesterol between the plasma membrane and the lipoprotein particles.

Introduction

The major portion of cholesterol in nucleated cells of mammals is found in the plasma membrane, although significant amounts also are found in intracellular membranes and lipid storage droplets [1]. In most extrahepatic cells, there is little or no oxidative degradation of cholesterol, and sterol homeostasis requires that excess cholesterol be removed and transported to the liver. These removal and transport processes collectively are termed reverse cholesterol transport, and are mediated by plasma lipoproteins (reviewed in ref. [2]). The initial removal of sterol from cells involves the transfer of cholesterol from the plasma membrane and possibly from intracellular membranes to particles within the high density lipoprotein (HDL) fraction of blood plasma and interstitial fluid [3-5].

Using immobilized antibodies to the major apolipoproteins of HDL (apo-AI and

apo-AII), plasma HDL can be separated by immunoaffinity chromatography into two major subfractions: LP-AI, in which all particles contain apo-AI but no apo-AII, and LP-AI/AII, in which all particles contain both apo-AI and apo-AII [6]. By nondenaturing two-dimensional gel electrophoresis, it has been shown that plasma HDL contains minor components that migrate to the pre-beta position on agarose gel electrophoresis and that contain apo-AI and apo-D, but no apo-AII [7]. These pre-beta particles should contribute to the LP-AI fraction of HDL.

In studies on the efflux of cell cholesterol to immunopurified subfractions of HDL, Barbaras *et al.* [8] found that LP-AI can efficiently remove excess cholesterol from cholesterol-enriched OB1771 mouse adipocytes, whereas LP-AI/AII has little or no effect on cell sterol levels. Similarly, Castro and Fielding [9] have reported that when fibroblasts are incubated with human plasma, the primary acceptors of cell cholesterol are pre-beta HDL particles, which contain apo-AI, but no apo-AII. These observations suggest that particles within the HDL fraction that contain apo-AI, but no apo-AII, may have critical functions in the removal of sterol from cells and in the initiation of reverse cholesterol transport. It has been proposed that LP-AI induces the mobilization and removal of cell cholesterol through interaction with HDL-specific binding sites on the adipocyte surface, and that LP-AI/AII, which also binds to HDL-specific sites on cells, antagonizes this process [8,10].

In the present studies, we prepared LP-AI and LP-AI/AII by accepted procedures and compared the two subfractions as acceptors of cholesterol derived from several types of cells. We chose cell types that are established tissue-culture models for studies on sterol movement between HDL and cells. The cell labeling procedures permitted examination of the efflux of both plasma membrane and LDL-derived lysosomal cholesterol. In addition, we investigated the ability of the subfractions to produce net depletion of cell cholesterol mass, and possible changes in efflux induced by the enrichment of cells with cholesterol.

Materials and methods

Materials

Commercial sources of chemicals, supplies, solvents, and isotopic compounds were as described previously [11]. Human low density lipoprotein (LDL, d = 1.019-1.063 g/ml), HDL_3 (d = 1.125-1.21 g/ml), and lipoprotein deficient serum (density > 1.21 g/ml), as well as bovine delipidized serum protein and human low density lipoprotein reconstituted with [1,2-^{3}H]cholesteryl oleate (r[^{3}H-CO]LDL) were prepared as described previously [11]. Compound 58-035 (an inhibitor of acyl-coenzyme A:cholesterol acyltransferase, ACAT) was a gift of Dr. John Heider, Sandoz Incorporated (Hanover, NJ).

Immunopurified subfractions of HDL (LP-AI and LP-AI/AII) were prepared from human plasma as described by Kilsdonk *et al.* [12]. All preparation procedures and storage were done at 4°C or on ice. The

major proteins of LP-AI/AII, as determined by denaturing polyacrylamide gel electrophoresis, were apo-AI and apo-AII, whereas the major protein of LP-AI was apo-AI, with no detectable apo-AII. Neither fraction contained detectable apo E. The phospholipid-to-protein ratios in LP-AI/AII and LP-AI were 0.39 and 0.46 (w/w), respectively. The corresponding free cholesterol-to-phospholipid ratios were 0.30 and 0.47 (mole/mole), respectively. Complete compositional data on LP-AI and LP-AI/AII are provided by Kilsdonk *et al.* [12]. Prior to experiments, the fractions were dialyzed exhaustively against phosphate buffered saline and then against 30-50 volumes of MEM tissue culture medium (containing 14 mM HEPES buffer, pH 7.4, and 50 μg/ml of gentamicin). After dialysis, the fractions were sterilized by filtration (0.45 μm pore size), and then assayed for phospholipid content [13]. To prepare efflux media, the fractions were diluted to a concentration of 50 μg phospholipid/ml (approx. 120 μg protein/ml). This concentration approximates the level of HDL in mammalian interstitial fluid [14], and is within a range used previously to demonstrate dramatic differences between LP-AI and LP-AI/AII in the ability to remove cholesterol from OB1771 mouse adipocytes [8]. Typically, 1-2 weeks elapsed between the drawing of blood and the use of immunopurified fractions in experiments.

Plasma from female donors was found to contain high concentrations of both LP-AI and LP-AI/AII, whereas male donor plasma typically contained very little LP-AI. To ensure well-controlled comparisons with adequate amounts of both subfractions, all experiments were conducted with subfractions obtained from female donors. In a given experiment, LP-AI and LP-AI/AII were from a single collection of plasma obtained from one donor. For both LP-AI and LP-AI/AII, there appear to be only minor compositional differences between male- and female-derived materials [15,16]. Thus, it seems unlikely that the results of the present studies were biased significantly by using only subfractions from female donors.

The cell lines used in these experiments were Fu5AH rat hepatoma cells, GM3468A human skin fibroblasts, GM0637 human fibroblasts, and rabbit aortic smooth muscle cells (grown from an explant in this laboratory). All cells were grown in monolayer culture. The growth media for routine propagation of cells were 5% bovine calf serum/95% MEM (for Fu5AH cells) and 10% fetal bovine serum/90% MEM (for fibroblasts and smooth muscle cells). The Fu5AH cell was chosen for initial comparisons between subfractions because of this cell's ability to release cholesterol rapidly from its plasma membrane [2]. This property allows incubation times to be kept relatively short. In addition, it increases the likelihood that the efflux of internal sterol will be rate-limited by intracellular transport rather than desorption from the cell surface, thus improving the reliability of using efflux of internal sterol as a measure for delivery of the sterol to the plasma membrane.

Methods

Unless otherwise indicated, all work with cells was performed at 37°C. In a given experiment, cells were prepared in one of two ways.

I) To examine the efflux of both whole-cell cholesterol and LDL-derived lysosomal cholesterol, cells were prepared in 22-mm wells of 12-well tissue culture plates as described in Johnson *et al.* [11]. Briefly, this involved incubation of cell monolayers for 2 days in MEM-based medium that contained 10 mg protein/ml of human lipoprotein deficient serum, and 0.025-0.05 μCi/ml [4-^{14}C]cholesterol (dispersed with 5 μg/ml egg phosphatidylcholine and 0.1% ethanol), followed by a 5-h, 15°C pulse with r[^{3}H-CO]LDL (10 μg protein/ml), and then incubation at 37°C in efflux medium, which consisted of HEPES-buffered MEM containing HDL, an HDL subfraction, or bovine serum albumin (BSA). During the 2-day incubation with [^{14}C]cholesterol, this tracer should label all cellular pools to the same specific activity. Thus, it labels whole-cell cholesterol and is expected to be present mostly in the plasma membrane at the beginning of the efflux period [17]. Previously, we have found that the efflux of whole-cell cholesterol from intact cells is representative of cholesterol desorption from the plasma membrane [18]. During the 5-h incubation at 15°C, reconstituted LDL is delivered to endosomes by LDL receptor-mediated endocytosis [19]. Subsequently, when cells are warmed to 37°C, the LDL is delivered rapidly to lysosomes, where it is degraded, resulting in the generation of free [^{3}H]cholesterol in lysosomes. This label becomes available for efflux only after transport to the plasma membrane.

II) To examine the efflux of whole-cell cholesterol from cells that were enriched with free cholesterol, cells were prepared as described by Johnson *et al.* [20]. Briefly, this involved incubation of cell

monolayers with human lipoprotein-deficient serum, followed by a 1-day incubation with cholesterol enrichment medium, which contained 50 μg protein/ml of native human LDL, 100 μg cholesterol/ml of cholesterol-enriched liposomes (cholesterol/egg phosphatidylcholine ≥ 2 mole/mole), 1 μg/ml of the ACAT inhibitor Sandoz compound 58-035 (dispersed with 0.5% dimethylsulfoxide), and 0.05-0.1 μCi/ml [^{14}C]cholesterol (dispersed with 0.1% ethanol and 1% fetal bovine serum). Cells then were incubated with efflux medium that was supplemented with compound 58-035 (dispersed with 0.5% dimethylsulfoxide). Control (unenriched) cells were treated identically, except that the enrichment medium was replaced with medium that contained cholesterol-free liposomes and did not contain LDL. The enrichment conditions raised the content of cellular cholesterol 2- to 3-fold above normal control levels.

At the initiation of efflux, cultures were 80-100% confluent. One-half ml of efflux medium was added to each well. HDL subfractions were used at a concentration of 50 μg phospholipid/ml. Maximum incubation times were 8 h for Fu5AH cells and 24 h for fibroblasts and smooth muscle cells. Incubations were ended, and the cell and medium samples were analyzed as described previously [11,21].

All incubations were performed at least in triplicate. Each value is the mean ± 1 SD of replicate determinations, unless otherwise indicated. The significance of differences was assessed by the two-tailed Student *t*-test for unpaired observations.

Results

Cholesterol efflux from Fu5AH rat hepatoma cells to LP-AI and LP-AI/AII

The efflux of whole-cell and lysosomal cholesterol from Fu5AH cells to LP-AI and LP-AI/AII is shown in figure 1. Three important parameters were monitored over an 8-h efflux period: the release of whole-cell [^{14}C]cholesterol (fig. 1a), the lysosomal hydrolysis of LDL [^{3}H]cholesteryl oleate (fig. 1b), and the release of the lysosomally generated free [^{3}H]cholesterol from the cells (fig. 1c). As the data indicate, the time course of each of these processes was essentially the same with either LP-AI or LP-AI/AII in the incubation medium. The cumulative release of cholesterol tracers during the 8 h incubations was 14% for whole-cell cholesterol and 15% for lysosomal cholesterol, suggesting rapid equilibration of lysosomal cholesterol into the various whole-cell pools. In several other experiments under the same conditions, the release of whole-cell and lysosomal cholesterol to media containing BSA (2 mg/ml), but no lipoproteins, was less than 1% (data not shown). Thus, the spontaneous release of cholesterol was expected to be quite low in this experiment and should not have obscured any significant differences in efflux to the two types of HDL. In the present experiment, esterification of [^{14}C]cholesterol in media was less than 1% after 8 h (data not shown), indicating that the activity of lecithin:cholesterol acyltransferase (LCAT) in the two subfractions was minimal. Approximately 10% of the ^{3}H label in both media was esterified after 8 h. The kinetics of appearance of this tracer in the media suggested that it was due to a small amount of desorption or retroendocytosis of undegraded LDL from the cells early in the incubations (data not shown), rather than any preferential esterification of lysosomally derived cholesterol.

To determine whether the enrichment of cells with cholesterol induces a difference in the responsiveness of efflux to LP-AI and LP-AI/AII, we prepared Fu5AH cells to contain normal (control) and elevated amounts of free cholesterol. In both cases, whole-cell cholesterol was labeled with ^{14}C, and the labeling of cells and the efflux of cholesterol were examined in the presence of compound 58-035 to prevent cellular esterification of cholesterol. The levels of cholesterol in control and enriched cells were 28 and 68 μg/mg protein, respectively. Thus, the enrichment procedure elevated cell cholesterol 2.4-fold above the control level. As shown in figure 2, there was very striking similarity in efflux to the two forms of HDL, in both control cells (fig. 2a) and

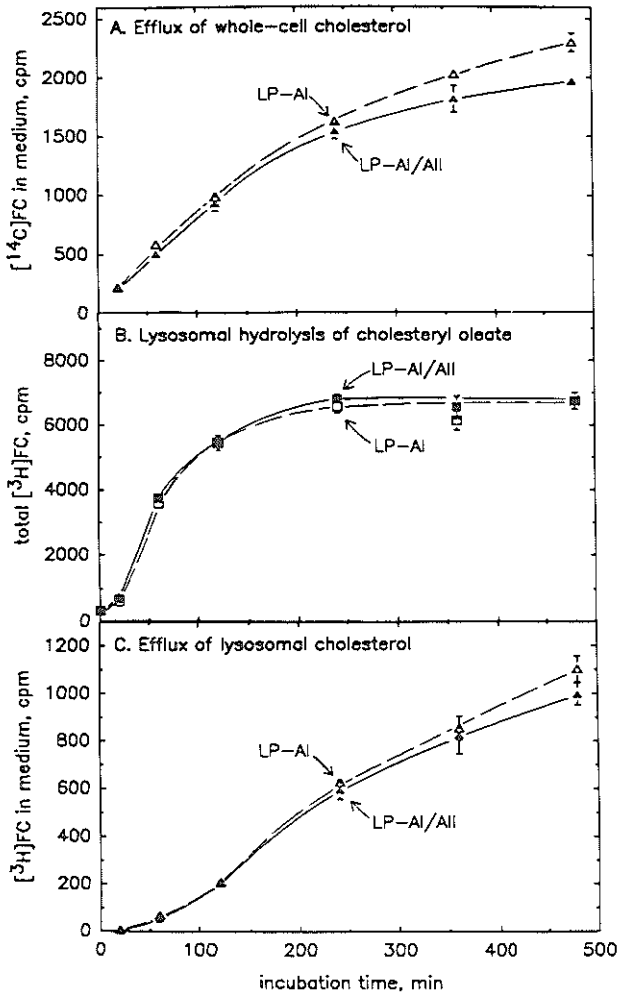

Fig. 1 Efflux of whole-cell and lysosomal cholesterol from Fu5AH rat hepatoma cells to human LP-AI and LP-AI/AII. The preparation of cells and lipoproteins was as described in *Materials and Methods*. The concentration of each lipoprotein was 50 μg phospholipid/ml. Initial (t=0) cell parameters were as follows: cell protein=0.204 ± 0.021 mg/well; total [^{14}C]cholesterol= 16001 ± 635 cpm/well (free/total= 0.958 ± 0.005); total [^{3}H]cholesterol=9528 ± 966 cpm/well (free/total=0.036 ± 0.005); uptake of reconstituted LDL=684 ± 71 ng LDL protein/mg cell protein. Cell cholesterol mass was not measured in this experiment, but typically was 20-25 μg/mg protein.

Panel A. Efflux of unesterified [^{14}C]cholesterol ([^{14}C]FC) to LP-AI (△) and LP-AI/AII (▲) versus time in min.

Panel B. Total recovery of [^{3}H]FC from cells and medium during incubation with LP-AI (□) and LP-AI/AII (■).

Panel C. Efflux of lysosomally generated [^{3}H]FC to LP-AI (△) and LP-AI/AII (▲). Each value is the mean ± 1 SD of at least three determinations. When not visible, bars indicating SD are covered by symbols.

cholesterol-enriched cells (fig. 2b). After 8 h incubation, the cumulative release of the whole-cell tracer was approximately 17% from control cells, and 12% from enriched cells. Thus, efflux from the enriched hepatoma cells was somewhat sluggish, although it was equally efficient with the two forms of HDL. The enrichment did not induce any difference in efflux to LP-AI and LP-AI/AII.

As shown in table I, the content of cholesterol mass in sterol-enriched Fu5AH cells was changed very little by incubation for 8 h with LP-AI, LP-AI/AII, HDL_3, or BSA. There was clearly no tendency for LP-AI to produce greater depletion of cell sterol mass than either LP-AI/AII or HDL_3.

Table I

Free cholesterol content of cholesterol-enriched Fu5AH cells before and after 8-h incubation with LP-AI, LP-AI/AII, HDL_3, or bovine serum albumin (BSA)

incubation time	medium	cell cholesterol content
h		*μg FC/mg protein*
0		68 ± 8
8	LP-AI	60 ± 1
8	LP-AI/AII	59 ± 2
8	HDL_3	57 ± 0.3
8	BSA	64 ± 2

Enrichment conditions were as described in *Fig. 2*. During the 8-h efflux period, lipoproteins were used at a concentration of 50 μg phospholipid/ml, and BSA at a concentration of 100 μg/ml. BSA was not added to efflux media that contained lipoproteins. For t = 0, $n = 6$. For each value at t = h, $n = 3$. At t = 0, cell protein was 0.304 ± 0.028 mg/well; after 8 h, cell protein averaged 0.303 mg/well and varied by no more than 4% between treatment groups. Among the 8-h cell cholesterol content values, two differences were significant ($P < 0.05$): HDL_3 versus LP-AI and BSA versus HDL_3.

Table II

Free cholesterol content of cholesterol-enriched human skin fibroblasts before and after 24-h incubation with LP-AI, LP-AI/AII, or BSA

incubation time	medium	cell cholesterol content
h		*μg FC/mg protein*
0		85 ± 11
24	LP-AI	66 ± 10
24	LP-AI/AII	46 ± 3
24	BSA	58 ± 3

The preparation of cells and efflux conditions were as described in *Fig. 4*. For t = 0, $n = 11$. For each value at t = 24 h, $n = 3$. At t = 0, cell protein was 35.9 ± 2.1 μg/well; after 24 h, cell protein averaged 44.2 μg/well and varied by no more than 6% between treatment groups. Among the 24-h cell cholesterol content data, the following differences were significant: LP-AI versus LP-AI/AII ($P = 0.03$) and BSA versus LP-AI/ AII ($P = 0.004$).

Cholesterol efflux from GM3468 human skin fibroblasts to LP-AI and LP-AI/AII

To examine the efflux of whole-cell and lysosomal cholesterol from fibroblasts, cells were prepared for experiments and incubated with lipoproteins as described above for the Fu5AH cells. Since efflux from the plasma membrane of fibroblasts is 5- to 10-fold slower than from the Fu5AH cell, the maximum incubation time was extended to 24 h. As shown in figure 3, the efflux of whole-cell and lysosomal cholesterol as well as the hydrolysis of LDL cholesteryl oleate in fibroblasts was the same with either LP-AI or LP-AI/AII in the incubation medium. After 24 h, the cumulative release of the whole-

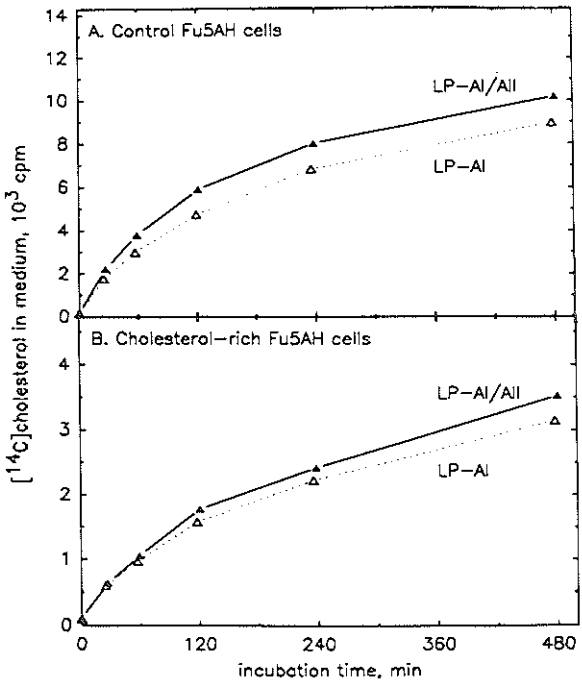

Fig. 2 Effect of enriching cells with cholesterol on the efflux of whole-cell cholesterol from Fu5AH cells to LP-AI and LP-AI/AII. Cells were prepared and labeled with [^{14}C]cholesterol as described in *Materials and methods*. Efflux media contained 1 μg/ml of the ACAT inhibitor compound 58-035 (dispersed with 0.5% dimethylsulfoxide) and 50 μg phospholipid/ml of either LP-AI or LP-AI/AII. Initial cell parameters were as follows. 1) For control cells: cell protein = 0.327 ± 0.026 mg/well; free cholesterol/protein = 28.2 ± 2.1 μg/mg; total [^{14}C]cholesterol = 58098 ± 507 cpm/well (free/total = 0.984 ± 0.001). 2) For cholesterol-enriched cells: cell protein = 0.304 ± 0.028 mg/well; free cholesterol/protein = 68.4 ± 8.1 μg/mg; total [^{14}C]cholesterol = 27979 ± 814 cpm/well (free/total = 0.985 ± 0.001). **Panel A.** Efflux of [^{14}C]FC from control cells in the presence of LP-AI (△) and LP-AI/AII (▲) versus incubation time in min. **Panel B.** Efflux of [^{14}C]FC from cholesterol-enriched cells to LP-AI and LP-AI/AII. Each value is the mean of at least three determinations. All SD were quite small (maximum = 189 and 77 cpm for control and enriched cells, respectively), and therefore were not plotted.

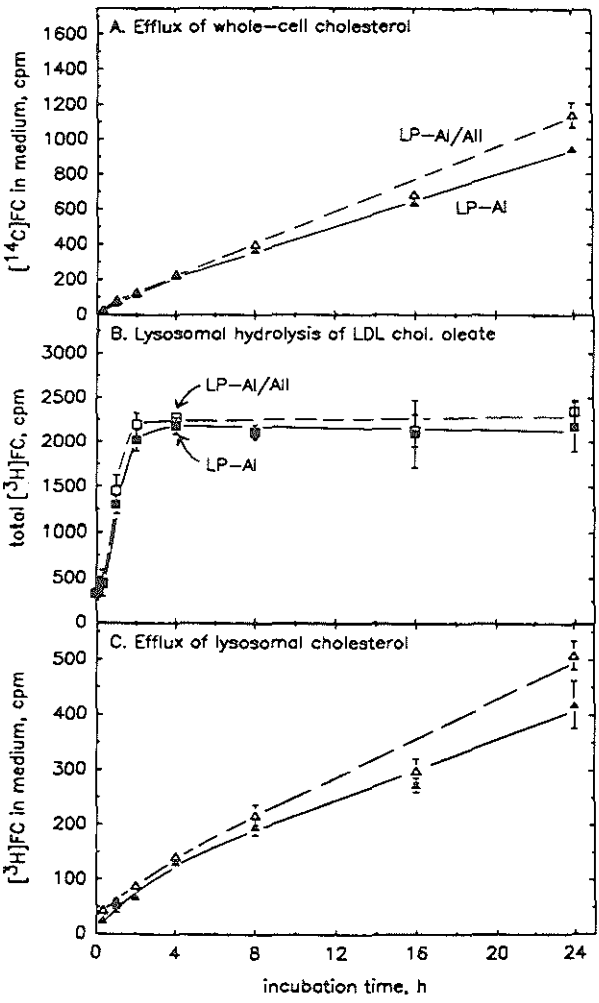

Fig 3. Efflux of whole-cell and lysosomal cholesterol from human skin fibroblasts to LP-AI and LP-AI/AII. Conditions were as described in *Fig. 1*, except the maximum incubation time for efflux was 24 h. Initial cell parameters were as follows: cell protein = 40.4 ± 1.5 μg/well; free cholesterol/protein = 33.5 ± 5.8 μg/mg; total [^{14}C]cholesterol = 7012 ± 337 cpm/well (free/total = 0.996 ± 0.001); total [^{3}H]cholesterol = 3424 ± 312 cpm/well (free/total = 0.095 ± 0.003).
Panel A. Efflux of whole-cell [^{14}C] free cholesterol versus incubation time with LP-AI (▲) and LP-AI/AII (△).
Panel B. Total [^{3}H]free cholesterol recovered from cells and medium versus incubation time during incubation with LP-AI (■) and LP-AI/AII (□).
Panel C. Efflux of lysosomally generated [^{3}H]free cholesterol to LP-AI (▲) and LP-AI/AII (△).

cell ^{14}C-labeled tracer was approximately 19%, whereas the release of the lysosomally generated ^{3}H-labeled tracer was approximately 20%.

The cholesterol enrichment procedure raised the content of cholesterol in fibroblasts to 85 μg/mg protein, approximately 2.5-fold above the normal control level in these cells. Efflux of whole-cell cholesterol from the enriched fibroblasts was virtually identical during incubations with LP-AI and LP-AI/AII (fig. 4). The net depletion of cell cholesterol mass during 24 h incubation (table II) was greatest in the presence of LP-AI/AII (46%), whereas the depletion produced by LP-AI (22%) was not significantly different from that produced by BSA (32%). In this experiment, cell protein/well after

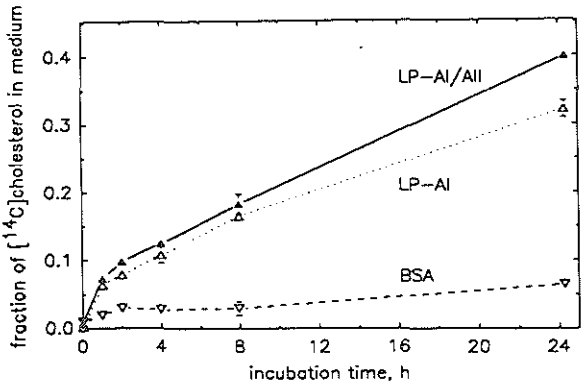

Fig. 4 Efflux of whole-cell cholesterol from cholesterol-enriched fibroblasts to LP-AI, LP-AI/AII, and BSA. The preparation of cholesterol-enriched cells and the efflux conditions were as described in *Fig.* 2. During incubation with BSA, its concentration was 100 μg/ml. BSA was not added to media containing LP-AI or LP-AI/AII. Initial cell parameters were as follows: protein=36 ± 2 μg/well; free cholesterol=85 ± 11 μg/mg protein; total [^{14}C]cholesterol=927 ± 45 cpm/well (free/total=0.996 ± 0.003). Control fibroblasts (not prepared in this experiment) typically contain 30 μg free cholesterol/mg protein. Data are plotted as the fractional release of [^{14}C]free cholesterol versus incubation time in h. Symbols: △ LP-AI; ▲LP-AI/AII; ▽ BSA.

24 h was increased by approximately 23% under all three incubation conditions (legend table II). Thus, most of the apparent depletion of cellular cholesterol (reduced cholesterol-to-protein ratio) observed with LP-AI or BSA could be attributed to increased cell protein, rather than loss of cholesterol to the extracellular medium.

Cholesterol efflux from other cell lines to LP-AI and LP-AI/AII

The efflux of whole-cell and lysosomal cholesterol to LP-AI and LP-AI/AII was also examined using rabbit aortic smooth muscle cells and a second human skin fibroblast line (GM0637). With both cells, efflux of whole-cell and lysosomal cholesterol to the two forms of HDL was similar to that observed with GM3468 fibroblasts. There was no indication of preferential release of cholesterol to LP-AI (data not shown).

Rate constants for efflux of whole-cell cholesterol to LP-AI and LP-AI/AII

Estimates of k_e (the rate constant for efflux of cell cholesterol) can be obtained by analyzing time-course data on the efflux of whole-cell cholesterol in terms of a kinetic model for tracer equilibration between two pools [20,21]. Table III summarizes estimates of k_e obtained in the above-described comparisons between LP-AI and LP-AI/AII. The kinetic analyses suggest that with both control and cholesterol-enriched cells, LP-AI is

slightly less efficient (by a factor of 4-15%, depending on experiment and cell type) than LP-AI/AII at promoting efflux of whole-cell cholesterol.

Discussion

During incubation of cells with HDL, cholesterol is known to diffuse bidirectionally between the plasma membrane and the HDL particles [21]. Net efflux is thought to occur when the plasma membrane is relatively enriched with cholesterol, or the lipoprotein is relatively depleted of cholesterol [2]. In the present studies, the free cholesterol-to-phospholipid ratios of LP-AI and LP-AI/AII were 0.47 and 0.30 (mole/mole), respectively [12]. Thus, LP-AI was enriched with cholesterol relative to LP-AI/AII. The above considerations predict that when LP-AI and LP-AI/AII are diluted to the same phospholipid concentration, efflux of plasma membrane cholesterol to the two acceptors ought to be similar, whereas there may be greater influx of cholesterol

Table III
Rate constants for efflux of whole-cell cholesterol to LP-AI and LP-AI/AII

type of cell	treatment	cell cholesterol	efflux k_e, h^{-1}		$\frac{\text{LP AI}}{\text{LP-AI/AII}}$ x 100%
			LP-AI/AII	LP-AI	
		μg/mg			
Fu5AH rat hepatoma	control	23	0.048	0.058	96 ± 23%
		n.a.	0.041	0.038	
		28	0.070	0.053	
	FC-rich	68	0.034	0.032	94%
GM3468 human fibroblasts	control	34	0.0074	0.0063	85%
	FC-rich	85	0.018	0.017	94%
rabbit SMC	control	23	0.0079	0.0076	96%

Control and cholesterol-enriched cells were prepared as described in *Materials and methods*. LP-AI and LP-AI/AII were used at a concentration of 50 μg phospholipid/ml. Each pair of values for k_e was obtained in a single experiment by fitting time-course data on the efflux of ^{14}C-labeled whole-cell cholesterol to a kinetic model for tracer equilibration between two pools [20,21]. Each time course contained data from at least six time points, with triplicate incubations performed at each time point (e.g., fig. 1A). The percentage value (in the far right column) with an SD assigned is an average based on data from the three indicated experiments. Each of the other percentages was calculated from k_e values obtained in a single experiment. Values for k_e can be converted to half times ($t_{1/2}$) for efflux with the formula: $t_{1/2} = (\ln 2)/(k_e)$. Abbreviations: FC, free cholesterol; SMC, smooth muscle cells; k_e, rate constant for efflux of cell cholesterol; n.a., not assayed.

from LP-AI (due to its greater content of cholesterol). This would result in reduced net depletion of cell cholesterol mass during incubation with LP-AI. In the present studies, we found that when LP-AI and LP-AI/AII were diluted to the same phospholipid concentration, the two lipoproteins promoted similar efflux of whole-cell cholesterol from rat hepatoma cells, fibroblasts, and smooth muscle cells (figs. 1-4, table III), and that LP-AI produced net depletions of cell cholesterol mass that were either the same as or somewhat less than the depletions produced by LP-AI/AII (tables I and II). Thus, the observed features of cholesterol movement between cells and the HDL subfractions were essentially those predicted by a model assuming unmediated diffusion of sterol between the plasma membrane and extracellular sterol carriers. This relatively simple outcome suggests that the unmediated bidirectional flux of cholesterol at the plasma membrane is likely to be the predominant factor governing sterol movement between the immunopurified HDL subfractions and the cells that were used in the present studies.

In these studies, we also found that LDL-derived lysosomal cholesterol was readily available for efflux, and that LP-AI and LP-AI/AII were equally efficient at removing the lysosomal sterol from cells (figs. 1 and 3). These results are consistent with previous data showing that the transport of cholesterol from lysosomes to the plasma membrane is rapid and not regulated by the type of sterol acceptor in the extracellular medium [11,22-24]. In most cells lysosome-to-plasma membrane cholesterol transport is fast in comparison to efflux from the plasma membrane. Thus, it seldom is rate-limiting for the removal of lysosomal cholesterol from cells.

In previous comparisons between LP-AI and LP-AI/AII, Barbaras *et al.* [8] found that LP-AI is able to remove excess cholesterol from cholesterol-enriched OB1771 mouse adipocytes, whereas LP-AI/AII promotes little or no net efflux from these cells. Additional data suggest that the movement of cholesterol from these cells to LP-AI requires the presence of HDL-specific binding sites on the adipocyte surface [10]. Since both LP-AI and LP-AI/AII bind with high affinity to HDL binding sites on adipocytes, it has been proposed that the specific binding of apo-AI (in the absence of apo-AII) stimulates the mobilization of excess cholesterol in these cells, and that the specific binding of apo-AII antagonizes this mobilization [8,10]. Two of the cells examined in the present studies (Fu5AH hepatoma cells and GM3468 fibroblasts) are known to express HDL-specific binding sites [20,25]. Thus, the lack of preferential efflux to LP-AI observed with these cells cannot be explained by the lack of HDL receptors. We speculate that the contrast between the present results and those obtained with adipocytes may be related to the unique ability of adipocytes to store very large quantities of lipid. This specialization may be accompanied by some fundamentally unique mechanism for sterol mobilization.

Recent data of Castro and Fielding [9] and Francone *et al.* [26] suggest that

when fibroblasts are incubated with human plasma, the initial acceptor of cholesterol that desorbs from the cells is an apo AI-containing pre-beta lipoprotein. This lipoprotein should be a component of the LP-AI fraction of plasma. Thus, isolated LP-AI might be expected to promote greater efflux than isolated LP-AI/AII. The fact that our results are not consistent with this prediction may have a number of explanations. The simplest possibility is that pre-beta HDL, although an efficient sterol acceptor, may not be required for the rapid efflux of sterol from cells. In the absence of pre-beta HDL, other lipoproteins (such as those in LP-AI/AII) may be able to serve as efficient acceptors of the cholesterol that is continually desorbing from cell plasma membranes. This suggestion is consistent with the fact that a variety of sterol acceptors (including nonlipoprotein phospholipid vesicles and phospholipid/bile acid micelles) can support rapid efflux of cholesterol from cells [27].

Pertinent to the issue of pre-beta HDL, the procedures used for isolation of HDL subfractions in the present studies involved a preliminary gel filtration step that eliminated plasma proteins outside the particle weight range of approximately 100-700 kDa [12]. This step may have resulted in low recoveries of the 71-kDa form of pre-beta HDL [7]. Moreover, it is thought that pre-beta HDL are short-lived and transformed rapidly to alpha-HDL particles [7]. If not regenerated in the absence of whole plasma, pre-beta HDL may disappear quickly from purified HDL subfractions. Thus, the LP-AI used in the present studies may have lacked a fully functional pre-beta HDL system. Consistent with this suggestion, we could detect very little esterification of cell-derived cholesterol in either LP-AI or LP-AI/AII (cf. Results and ref. [26]). In the present studies, we did not examine the immunopurified fractions for the presence of pre-beta HDL. The postulated importance of pre-beta HDL might be addressed least equivocally by studying efflux of cell cholesterol to pure stable forms of this lipoprotein in comparison to the efflux obtained with other types of acceptors at similar particle and phospholipid concentrations. Because of the low abundance and apparent lability of naturally occurring pre-beta HDL, such experiments probably will require the reconstitution of artificial pre-beta-like particles.

The present data show that purified human LP-AI and LP-AI/AII are equally capable of removing both plasma membrane and lysosomal cholesterol from several types of mammalian cells. These results support the hypothesis that efflux of cellular cholesterol is governed largely by the unmediated diffusion of cholesterol between the plasma membrane and HDL particles. The results also are consistent with previous data that suggest that the movement of cholesterol from lysosomes to the plasma membrane is rapid and not regulated by specific extracellular acceptors. There is a striking contrast between the present data, showing similar efflux to LP-AI and LP-AI/AII, and data reported by others, showing that LP-AI specifically stimulates efflux from sterol-rich

mouse adipocytes. This contrast suggests that any unique ability of LP-AI to initiate reverse cholesterol transport is confined to a limited range of cell types. Thus far, only variants of the OB17 mouse adipocyte have demonstrated any unique responsiveness to LP-AI. Why these cells respond to LP-AI and others do not, remains to be explained.

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chapter 8

Characterization of human high-density lipoprotein subclasses LP A-I and LP A-I/A-II and binding to HepG2 cells

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Abstract Plasma HDL can be classified according to their apolipoprotein content into at least two types of lipoprotein particles: lipoproteins containing both apo A-I and apo A-II (LP A-I/A-II) and lipoproteins with apo A-I but without apo A-II (LP A-I). LP A-I and LP A-I/A-II were isolated by immuno-affinity chromatography. LP A-I has a higher cholesterol content and less protein compared to LP A-I/A-II. The average particle weight of LP A-I is higher (379 kDa) than the average particle weight of LP A-I/A-II (269 kDa). The binding of ^{125}I-LP A-I to HepG2 cells at 4 °C, as well as the uptake of [^{3}H]cholesteryl ether-labelled LP A-I by HepG2 cells at 37 °C, was significantly higher than the binding and uptake of LP A-I/A-II. It is likely that both binding and uptake are mediated by apo A-I. Our results do not provide evidence in favor of a specific role for apo A-II in the binding and uptake of HDL by HepG2 cells.

Introduction

High density lipoproteins (HDL) play an important role in the transport of cholesterol from extrahepatic tissues to the liver, a process called reverse cholesterol transport. This role of HDL has been reinforced by studies in cultured cells, showing that HDL can accept cholesterol from cells in a process regulated by their cholesterol status [1,2]. The exact mechanism of this process is still unknown. Specific lipoprotein receptors [2-4], desorption of unesterified cholesterol from the plasma membrane followed by diffusion through the water phase [5-7] and enzyme activities like lecithin: cholesterol acyltransferase (LCAT, EC 2.3.1.43) and cholesteryl ester transfer protein (CETP) may play a role [8-10]. Although several investigators have visualized a specific binding protein for HDL [11-15], the question remains whether binding and net mass cholesterol transport are directly related [6,7]. Mendel [16-17] measured a molecular mass of approximately 10 to 16 kDa for the functional unit of the putative HDL binding protein using radiation inactivation, while ligand blotting experiments point to a binding protein molecular mass ranging from 78 to 110 kDa [11-15].

It has been shown that liver and steroidogenic tissues are especially active in metabolizing HDL particles. Cholesteryl esters from HDL are taken up at a greater fractional rate than apo A-I, i.e., cholesteryl esters are taken up without parallel uptake of HDL-apo A-I [18-20] in a process called selective uptake.

It is known that plasma HDL consists of a heterogeneous population of lipoprotein particles. The various HDL subclasses have different densities, which are used for the isolation of HDL_2 and HDL_3 by ultracentrifugation. Within these subfractions, particles with diameters ranging from 8 to 11 nm can be detected using

gradient gel electrophoresis [21,22]. After subfractionation of HDL in seven or nine subfractions by density gradient ultracentrifugation apo A-I and apo A-II were abundant in all subfractions [23].

Chromatography on specific antibody columns is the method of choice for the isolation of HDL particles with different apolipoprotein composition [23,24]. HDL consists of at least two types of particles: lipoproteins containing both apo A-I and apo A-II (LP A-I/A-II), and lipoprotein particles with apo A-I but without apo A-II (LP A-I). In the present study we characterized the chemical compositions of LP A-I and LP A-I/A-II and measured their size by gel filtration. This is the first report on the binding and uptake of LP A-I and LP A-I/A-II by the human hepatoma cell line HepG2.

Materials and methods

Anti-apo A-I and anti-apo A-II immuno affinity chromatography columns, as well as the antibodies used for ELISA of apolipoproteins, were generously provided by Prof. J.C. Fruchart and Dr. P. Puchois from the Institut Pasteur in Lille (France).

Blood was collected from healthy, normolipidemic volunteers in tubes containing EDTA (final concentration 1.5 mg/ml) and cooled on ice immediately. The subjects were starved overnight. Plasma was collected by centrifugation for 15 min at 1500 x g_{max} and 4 °C. All following isolation procedures were performed at 4 °C. Plasma HDL was obtained by gel filtration of 2 ml plasma using a Superose 6 column (prep. grade, 1.6 x 50 cm, Pharmacia, Uppsala, Sweden) as described in [25]. The elution buffer consisted of 0.9% NaCl, containing 2 mM sodium phosphate (pH 7.4), 5 mM EDTA and 0.02% NaN_3. Plasma cholesteryl (esters), phospholipids and triacylglycerols were eluted with high recoveries [25]. The HDL fractions obtained after two runs (2 ml of plasma each) were pooled and applied on a combination of a Sephadex column (G25 Fine, 1.6 x 35 cm, Pharmacia, Uppsala, Sweden) directly connected with a heparin-Sepharose column (1.3 x 29 cm), and eluted with 2 mM sodium phosphate buffer (pH 7.4), containing 0.1% NaN_3 [26]. No apo E was detectable in the HDL fraction obtained after this procedure using ELISA as described in [27].

The next isolation steps consisted of immuno-affinity column chromatography, as described by Barbaras *et al.* [24]. For isolation of the LP A-I/A-II particles, the apo E-free HDL fraction was passed through the immuno-affinity column with anti-apo A-II (flow 10 ml/h). The eluate was controlled for remaining apo A-II using a highly sensitive ELISA technique [27]. The resulting apo A-II-free HDL was subsequently applied on an anti-apo A-I column, which bound all LP A-I. Both antibody columns were washed with 150 ml of 0.9% NaCl containing 0.1 M sodium phosphate (pH 7.4), 0.1% EDTA and 0.1% NaN_3, followed by 30 ml 0.5 M NaCl with 10 mM sodium phosphate (pH 7.4) to elute nonspecifically bound material. The HDL-subfractions were eluted with 30 ml 3 M sodium thiocyanate, immediately followed by gel filtration on a Sephadex column (G25 Coarse, 2.6 x 60 cm, Pharmacia, Uppsala, Sweden). Both subfractions were concentrated by pressure dialysis using YM10 membranes (Amicon, Danvers, MA) and dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA.

Labelling of LP A-I and LP A-I/A-II with ^{125}I or [^{3}H]cholesteryl linoleyl ether

The HDL subfractions were iodinated using the ICl method (with $Na^{125}I$ from Amersham International, U.K.), as described previously [28]. Labelling efficiency was 52 to 68% and the iodine/protein ratio ranged from 0.58 to 0.73. Less than 1% of the radioactivity of the preparations was

free (non-precipitable with trichloroacetic acid), 2.2-4.6% was lipid-associated and 94 to 97% was protein-bound.

LP A-I and LP A-I/A-II were labelled with [1α,2α(n)-^{3}H]cholesteryl linoleyl ether (obtained from Amersham International, U.K.) by incubation of the subfractions with cholesteryl ether containing vesicles in the presence of CETP. The assay method and partial purification of CETP were described before [29]. LP A-I or LP A-I/A-II were incubated for 4 h at 37 °C with phosphatidylcholine/cholesteryl ether vesicles (ratio of 5:1, formed by sonication, L-α-phosphatidylcholine P-5388 from Sigma, St. Louis, MO, U.S.A.) in the presence of 0.01% EDTA and enough purified CETP to give the CETP activity level present in human plasma. 1 mM IAA was added to inhibit LCAT. The amount of phosphatidylcholine used during the incubations was kept as low as 1% of the total HDL phospholipids, in order to prevent changes in the phospholipid content of the particles. Lipoproteins and CETP were separated after incubation by density gradient ultracentrifugation and the HDL subfractions were reisolated from the density range 1.063-1.21 g/ml. The specific radioactivity ranged from 50 to 80 dpm/ng protein.

After labelling, the subfractions were dialyzed against 0.9% NaCl containing 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. If necessary, the fractions were dialyzed against the cell culture medium prior to the binding experiments.

Cell culture

HepG2 cells were cultured in DMEM/F10$^+$ (1:1) medium (DMEM was purchased from Flow Laboratories and F10$^+$ from Gibco), supplemented with L-glutamine, penicillin/streptomycin and 10% FCS, at 37 °C in 95% air and 5% CO_2. Cells were plated in multiwell dishes (35 mm, 0.5-1.0 · 10^6 cells/well) 3 days prior to each experiment. After 2 days the medium was removed, cells were rinsed three times with 0.9% NaCl containing 10 mM sodium phosphate (pH 7.4) and new medium, containing either 10% FCS or 2% BSA (A-4378, Sigma, St. Louis, MO), was added to the wells. After a preincubation period of 24 h the cells were ready for use in the binding experiments. At that time cells were near confluency.

Lipoprotein binding studies

After three washes with cold 0.9% NaCl, containing 10 mM sodium phosphate (pH 7.4), the cells were placed on ice and 1 ml test medium was added. The test medium used was culture medium (see above) in which the FCS was replaced by 2% BSA. The amount of iodinated lipoproteins in the test media ranged from 0 to 20 μg protein/ml. Nonspecific binding was measured after addition of 100 μg protein/ml of unlabelled LP A-I or LP A-I/A-II. This low amount of HDL-protein was used for practical reasons. Addition of high concentrations (e.g., 1 mg protein/ml) is impractical, due to the limited capacity of the antibody columns. Therefore we measured an apparent "specific binding", which is somewhat lower than the real specific binding. Competition was measured using 5 μg iodinated lipoprotein LP A-I or LP A-I/A-II per ml medium with 0-175 μg/ml unlabelled protein of the competing lipoprotein particle. For all experiments the cells were incubated for 4 h at 4 °C, under a H_2O-saturated atmosphere with 5% CO_2 and 95% air. After incubation the dishes were placed on ice, the medium was removed and the cells were washed three times with 0.2% BSA in 0.9% NaCl with 50 mM Tris-HCl (pH 7.4) to remove nonspecifically bound material. Then the cells were rinsed three times with 0.9% NaCl containing 50 mM Tris-HCl (pH 7.4). The cell protein was dissolved in 2 ml 1 M NaOH. The cell-bound radioactivity was measured using a gamma counter, and protein was measured according to Lowry *et al.* [30].

Binding plus uptake of iodinated or [^{3}H]cholesteryl ether-labelled LP A-I and LP A-I/A-II were measured at 37 °C at a concentration of 20 μg/ml of protein. This binding plus uptake will be referred to as total cell association. The overall procedure for these experiments at 37 °C is identical with that described above for the experiments at 4 °C.

Lipoprotein analyses

The protein content of the HDL subfractions was measured according to [30]. Total cholesterol, unesterified cholesterol, triacylglycerols and phospholipids were measured by enzymatic methods. The following kits were used: cholesterol, No. 310328 and cholesterol esterase (Boehringer Mannheim, F.R.G.); triacylglycerols, No. 877557 (Boehringer Mannheim, F.R.G.); phospholipids, No. 61491 (Bio-Mérieux, Craponne, France). Cholesteryl esters were calculated by difference of total and unesterified cholesterol.

The purity of the isolated HDL subfractions was determined by two different methods: apolipoprotein measurements with ELISA techniques [27] and 15% SDS-polyacrylamide gel electrophoresis [31]. Autoradiograms were made after electrophoresis of iodinated HDL subfractions. The dried gel was exposed to Hyperfilm-MP (Amersham International, U.K.) for 3 days at -80 °C. The apo A-I and apo A-II concentrations were determined by immuno-electrophoresis [32]. The molar ratio of apo A-I to apo A-II was calculated using a M_r of $2.8 \cdot 10^4$ and $1.7 \cdot 10^4$ for apo A-I and apo A-II respectively.

Particle weights of LP A-I and LP A-I/A-II were determined by gel filtration on a Aca 34 column (Ultrogel, Reactifs IBF, Villeneuve-la Garenne, France). The column was calibrated with reference proteins with known molecular masses (gel filtration calibration kits No. 17-0441-01 and 17-0445-01, Pharmacia, Uppsala, Sweden). The reference proteins were: thyroglobulin (M_r = $6.69 \cdot 10^5$), ferritine (M_r = $4.4 \cdot 10^5$), catalase (M_r = $2.32 \cdot 10^5$) and lactate dehydrogenase (M_r = $1.40 \cdot 10^5$). Dextran Blue (M_r = $2.0 \cdot 10^6$) was used as a marker of the void volume. The resulting K_a values were plotted against the log M_r resulting in a regression line with a correlation coefficient of 0.999.

For statistical analysis the nonparametric Wilcoxon test was used [33]. Results were regarded as significantly different if $P<0.05$.

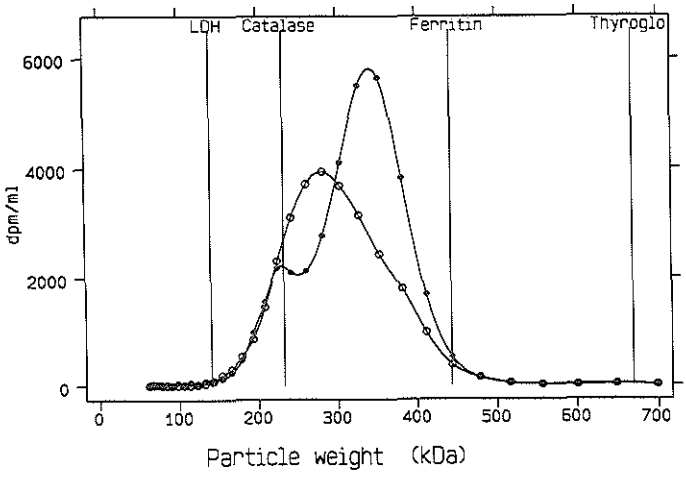

Fig. 1 A typical example of separate gel filtrations of [^{3}H]cholesteryl ether labelled LP A-I (●) and LP A-I/A-II (○) on a ACA 34 column at 4 °C. The column was calibrated with the following proteins: lactate dehydrogenase (LDH), catalase, ferritin and thyroglobulin (Thyroglo).

Table I
The chemical composition of the human HDL subclasses LP A-I and LP A-I/A-II

	LP A-I	LP A-I/A-II
protein	55.2 ± 3.0[a]	60.3 ± 2.4[b]
phospholipids	25.6 ± 2.2	23.7 ± 2.0
total cholesterol	17.0 ± 1.2	13.9 ± 0.7[c]
unesterified cholesterol	6.0 ± 0.8	3.5 ± 0.1
esterified cholesterol	11.4 ± 2.1	10.2 ± 0.6
triacylglycerols	2.9 ± 0.4	2.1 ± 0.1

[a] Values given are weight % (mean ± SD, $n=6$).
[b] Significantly different from LP A-I, $P=0.025$.
[c] Idem, $P=0.005$.

Results

Table I shows that the two HDL subfractions, LP A-I and LP A-I/A-II, differ in chemical composition. LP A-I contains less protein and more cholesterol than LP A-I/A-II. The difference in cholesterol content is caused mainly by a higher level of unesterified cholesterol in LP A-I. Consequently, LP A-I/A-II has a higher proportion of esterified cholesterol than LP A-I. The molar ratio of esterified cholesterol to unesterified cholesterol is 3.0 for LP A-I/A-II and only 1.9 for LP A-I. Not only do the chemical compositions of LP A-I and LP A-I/A-II differ, also the average particle weights are significantly different. The value for LP A-I is 379 ± 62 kDa, (mean ± SD, $n=3$) and the value for LP A-I/A-II is 269 ± 17 kDa (mean ± SD, $n=3$) (see also fig. 1). Figure 1 demonstrates that LP A-I as well as LP A-I/A-II consists of particles with a wide range of diameters. The mean size of LP A-I and LP A-I/A-II varies between different individuals, but for each individual LP A-I is always bigger than LP A-I/A-II. The cholesteryl ether label and the phospholipids have similar size distributions for LP A-I as well as LP A-I/A-II (not shown). The gel filtration profile of LP A-I shows two peaks (fig. 1): a main peak that corresponds with a mean spherical particle radius of 6.2 nm and a smaller peak with a radius of approximately 5.2 nm. The profile of LP A-I/A-II shows only one peak (fig. 1), corresponding with a mean particle radius of 5.5 nm.

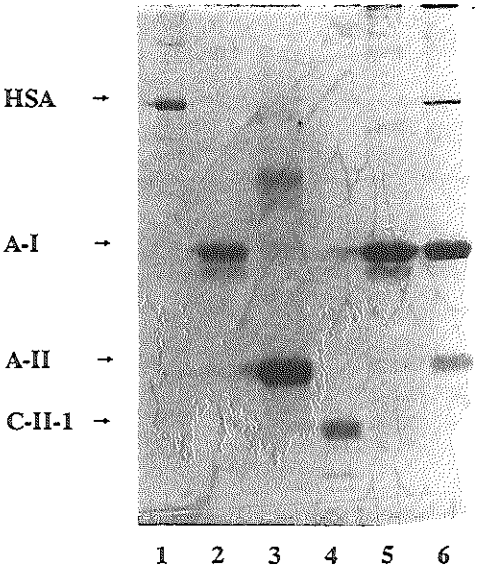

Fig. 2 Non-reduced 15% SDS-polyacrylamide gel electrophoresis of the HDL-subfractions. Lane 1 HSA marker, lane 2 apo A-I marker, lane 3 apo A-II marker, lane 4 apo C-III-1 marker, lane 5 LP A-I and lane 6 LP A-I/A-II. The gel was stained with Coomassie Brilliant Blue.

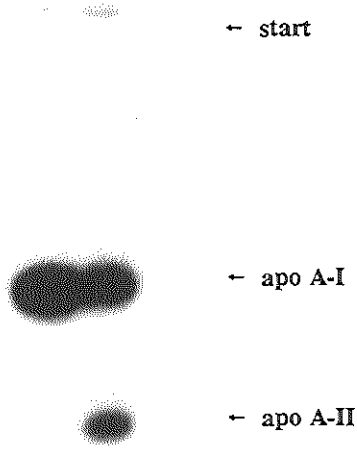

Fig. 3 Autoradiogram of ^{125}I-LP A-I (left lane) and LP A-I/A-II (right lane) after non-reduced 15% SDS-polyacrylamide gel electrophoresis.

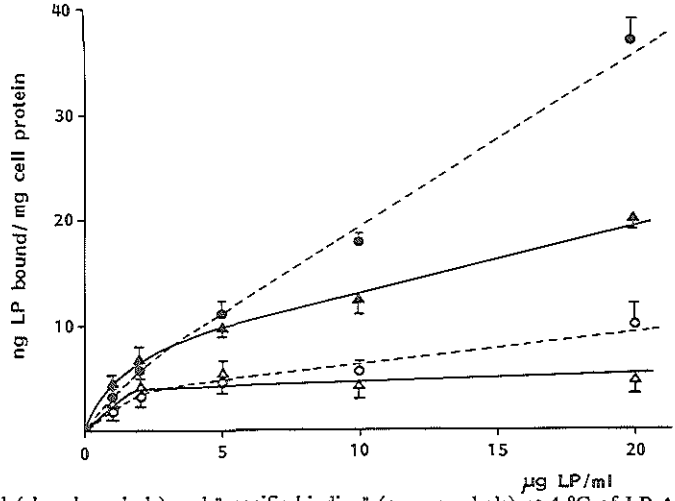

Fig. 4 The total (closed symbols) and "specific binding" (open symbols) at 4 °C of LP A-I (● , ○) and LP A-I/A-II (▲ , △) to HepG2 cells (ng HDL protein/mg cell protein, values are given as means ± SEM, $n=3$).

No apo A-II could be detected in the LP A-I fraction on SDS-polyacrylamide gel electrophoresis (fig. 2). There was only a very small amount of contaminating protein (co-migrating with HSA) visible in the LP A-I/A-II fraction. A minor protein band, migrating slightly faster than the main band of apo A-I, was present in LP A-I as well as in the pure apo A-I marker. The nature of this minor band, which is not present in LP A-I/A-II, is presently unknown. It is likely to be a degradation product of apo A-I. Evidence in favor of a high sensitivity for proteolytic degradation in specific HDL subfractions has been published recently [34]. ELISA assays showed that the percentage of apo A-II in the LP A-I subfraction is less than 0.1%, apo C-III (quantitatively the most important apo C in HDL) ranged from 1-3%, and apo E was less than 0.5% of total apolipoprotein. The molar ratio of apo A-I/apo A-II in the LP A-I/A-II subfraction, measured in delipidated samples, was 1.4 ± 0.2 (mean ± SEM, $n=5$).

Figure 3 shows an autoradiogram obtained after SDS-polyacrylamide gel electrophoresis of iodinated LP A-I and LP A-I/A-II. Only very little radioactivity is

Table II

Competition of the binding of iodinated LP A-I and iodinated LP A-I/A-II by unlabelled LP A-I/A-II or LP A-I in HepG2 cells at 4 °C

Ligand (5 μg/ml)	Competitor	(μg/ml)	% Labelled ligand bound	
^{125}I-LP A-I	LP A-I/A-II	0	100[a]	(n=3)
		35	92 ± 16[b]	(n=3)
		75	65 ± 1	(n=2)
		135	60 ± 4	(n=2)
		175	59 ± 1	(n=2)
^{125}I-LP A-I/A-II	LP A-I	0	100	(n=3)
		35	62 ± 6[b]	(n=3)
		75	51 ± 1	(n=2)
		135	49 ± 5	(n=2)
		175	43 ± 1	(n=2)

[a] Values are means (± SEM).

[b] Significant difference between the % found for LP A-I and LP A-I/A-II competition (Wilcoxon's nonparametric test, $P<0.05$).

Table III

The binding and uptake of the protein and cholesteryl ester moieties of LP A-I and LP A-I/A-II by HepG2 cells at 37 °C

	^{125}I-protein	^{3}H-lipid	ratio ^{3}H/^{125}I
Preincubation of cells in 10% FCS:			
LP A-I	0.36 ± 0.02[a]	1.24 ± 0.05[a]	3.4
LP A-I/A-II	0.29 ± 0.02[b]	0.74 ± 0.06[b]	2.5
Preincubation of cells in 2% BSA:			
LP A-I	0.34 ± 0.01	0.77 ± 0.01	2.3
LP A-I/A-II	0.29 ± 0.01[b]	0.54 ± 0.03[b]	1.9

[a] All values are cell-associated radioactivity expressed in % of added radioactivity (mean ± SEM, $n=3$).

[b] Significantly different from the value for LP A-I, $P < 0.05$.

present in proteins other than apo A-I or apo A-II.

Figure 4 shows the binding of iodinated LP A-I and LP A-I/A-II to HepG2 cells, measured at 4 °C. The total binding of LP A-I was high compared to the binding of LP A-I/A-II. Both HDL subfractions showed a saturable "specific binding" of about 50% of the total binding, if measured at a lipoprotein concentration of 5 μg/ml. The construction of accurate Scatchard plots for the calculation of binding affinities and maximal binding was not possible, due to the limited number of measurements. Table II shows the competition of LP A-I/A-II for ^{125}I-LP A-I binding and of LP A-I for ^{125}I-LP A-I/A-II binding. Both subfractions are able to displace each other, although LP A-I can displace more ^{125}I-LP A-I/A-II, especially at low concentrations. Table III gives the data on total HDL association to HepG2 cells at 37 °C. The cells were incubated with 20 μg HDL protein/ml, using different preincubation conditions. Two differently labelled preparations were used: ^{125}I-apolipoprotein label and [^{3}H]cholesteryl ether label. The results show that the binding of LP A-I is significantly higher than that of LP A-I/A-II. This finding is not dependent on the preincubation conditions. The cholesteryl ether-derived cell-associated radioactivity was higher than that derived from iodinated lipoproteins (expressed as % of the added radioactivity), indicating a preferential uptake of cholesteryl ether over apolipoproteins. This selective uptake occured for both LP A-I and LP A-I/A-II. The ratio cholesteryl ether uptake/apolipoprotein uptake ranged from 1.9 to 3.4.

Discussion

The differences between LP A-I and LP A-I/A-II resemble the known differences between HDL_2 and HDL_3. Both LP A-I and HDL_2 have a relatively low protein content and a relatively high total cholesterol content. The esterification level is highest in LP A-I/A-II and HDL_3. The observed protein contents of both LP A-I and LP A-I/A-II (55 and 60%, respectively) are higher than those reported for HDL isolated by ultracentrifugation [35]. HDL_2 and HDL_3 contain about 40% and 53% protein, respectively. The most likely explanation for these differences in protein content between the two isolation methods is "stripping" of HDL-apolipoproteins during ultracentrifugation [36,37]. Kunitake *et al.* [36] showed that 20% of the total apo A-I of human HDL was lost after one 24 h ultracentrifugation at high salt concentration. Cheung and Wolf [37] found that 14% of the apo A-I of LP A-I was found in the $d > 1.21$ g/ml fraction after ultracentrifugation. About 9% of rat HDL-apo A-I is lost during one ultracentrifugal step [38,39].

The high protein content of LP A-I and LP A-I/A-II is a common finding after isolation of HDL subfractions by immuno-affinity column chromatography. McVicar *et al.* [40] isolated apo A-I containing particles with 62-70% protein. Cheung and Wolf [37] isolated LP A-I particles with 43-56% and LP A-I/A-II particles with 55-60% protein. Lower values (34-46% of protein) were calculated by summation of the specific apolipoprotein concentrations [41]. However, absolute standardization of these apolipoprotein assays is difficult and total protein determination will probably result in more reliable data.

The isolated HDL subfractions had average particle weights of about 380 and 270 kDa for LP A-I and LP A-I/A-II, respectively. The particle weight for LP A-I/A-II measured by us was higher than the values reported for HDL_3 [42,43]. This again may be caused by the avoidance of ultracentrifugation (see above). Changes in apo A-I containing particles due to ultracentrifugation were described before [40,44]. Vezina *et al.* [44] showed that apo A-I containing lipoproteins may have particle weights up to 815 kDa, if separated by gradient gel electrophoresis. After prior ultracentrifugation, no apo A-I containing lipoproteins with average particle weights above 390 kDa could be detected anymore. In another study [40], apo A-I containing particles, isolated by immuno-affinity chromatography, also had higher mean particle radii than HDL isolated by ultracentrifugation.

The subdivision of LP A-I in two subfractions (see fig. 1) is also evident on gradient gel electrophoresis [45,46]. In [45] the main band consisted of lipoproteins of approximately 300 kDa and the smaller band was about 164 kDa. The LP A-I isolated in our study was bigger: the major component has a particle weight of approximately 380 kDa. The mean particle size of LP A-I and LP A-I/A-II reported in part of the existing literature is somewhat smaller than the values obtained in the present study. This may be partly due to the method of analysis: measurements by gel filtration give slightly higher values for the radii of HDL particles, compared to gradient gel electrophoresis [47]. From our data we calculated mean particle radii of 6.2 nm for LP A-I and of 5.5 nm for LP A-I/A-II. Using the same method, i.e., gel filtration, Cheung *et al.* [47] found similar results for LP A-I (5.7 to 6.5 nm), and higher values for LP A-I/A-II (6.0 to 6.3 nm). Size measurements using the electron microscope showed apo A-I containing particles with a radius ranging from 5 to 6 nm [40].

We find a molar ratio of apo A-I/apo A-II in LP A-I/A-II particles of 1.4 ± 0.2. From a study of Ohta *et al.* [41] a ratio of 1.5 for women and 1.6 for men can be calculated. James *et al.* [48] measured a ratio of 1.5 ± 0.1. Cheung and Albers [45] isolated particles with a ratio of 2 and showed that ultracentrifugation had no effect on the apo A-I/apo A-II molar ratio. However, the major fraction of the LP A-I/A-II particles they isolate contains 2 molecules of apo A-I and 1-2 molecules of apo A-II

[49]. In our study at least 3 molecules of apo A-I and 2 molecules of apo A-II appear to be present on each LP A-I/A-II particle. For LP A-I, 2-4 apo A-I molecules per particle were proposed before [50,51]. From the data obtained in this study we calculate that the average LP A-I particle may contain 7-8 molecules of apo A-I. This high number follows from the combination of a relatively high protein content as well as particle weight of the LP A-I subfraction. Separate gel filtration of the two subfractions (Fig. 1) showed that both subfractions consisted of particles with greatly variable size. This is not caused by an overloading of the column. Comparable amounts of protein of the calibration standards gave narrow peaks. This implies that affinity chromatography is necessary to isolate pure LP A-I and LP A-I/A-II, and that gel filtration alone or ultracentrifugation alone cannot provide pure subfractions.

In all our experiments the binding and uptake of LP A-I was higher compared to LP A-I/A-II. For both subfractions the specific binding was about 50% of the total amount of bound HDL. Other investigators also measured a relatively high nonspecific binding of HDL to HepG2 cells [52]. When the binding of LP A-I/A-II is related to the concentration of apo A-I the binding curves of LP A-I and LP A-I/A-II do not differ anymore (not shown), suggesting that only apo A-I mediates the binding. The fact that LP A-I was able to displace bound ^{125}I-LP A-I/A-II also supports the role of apo A-I in the binding of HDL to its binding site. These competition experiments are not interfered with by exchange of labelled apo A-I (on bound iodinated lipoprotein) with unlabelled competing apo A-I. This type of exchange could cause apparent competition [53]. Exchange of apolipoproteins between different particles does not occur at 4 °C however [54].

Whether both apo A-I and apo A-II are ligands for the binding of HDL remains controversial. There are only two other studies in which LP A-I and LP A-I/A-II particles were used in binding experiments. Pig liver, adrenal and skeletal muscle membranes [55] also show a higher binding of LP A-I compared to LP A-I/A-II. Differentiated Ob1771 mouse adipocytes show binding of apo A-I- as well as apo A-II-containing liposomes [56]. In this study, a striking difference in the function of the two HDL-subfractions was found when cholesterol efflux from adipocytes was measured [24,56,57]. Apo A-II-containing liposomes and LP A-I/A-II failed to promote cholesterol efflux, resulting in the hypothesis that apo A-II has an antagonistic effect on cholesterol efflux. Another study shows that apo A-II on HDL inhibits HDL binding to adipocyte cell membranes [58]. HDL_3 binding to human adipocyte membranes decreased 92% after enrichment of HDL_3 with apo A-II. In our study with HepG2 cells LP A-I and LP A-I/A-II were both bound to about the same extent and cholesteryl ether uptake from HDL was comparable. In several studies using ligand blotting, apo A-I as well as apo A-II are able to bind to a receptor

protein in membranes of various cell types, e.g., liver, adrenal cortex, kidney, fibroblasts, smooth muscle cells, macrophages and adipocytes [13,57,59]. In contrast, Schmitz *et al.* [60] state that only apo A-I is the ligand for the HDL receptor, since only anti-apo A-I was able to inhibit binding of HDL to peritoneal macrophages. Anti-apo A-II, anti-apo E and anti-apo C's did not inhibit HDL binding.

A selective uptake of HDL-cholesteryl ether, compared to HDL-apolipoprotein, was observed in HepG2 cells, measured at 37 °C, confirming earlier reports [19,61]. In our experiments [^{3}H]cholesteryl ether was used as a non-degradable label. Theoretically the ^{125}I label will be released from the cells after degradation of the apolipoproteins. However, no degradation could be measured during the short incubation periods used in our experiments (not shown). The selective uptake was higher for LP A-I than it was for LP A-I/A-II, but the differences were small. Our results do not support a specific role for apo A-II in the uptake of HDL-cholesteryl esters.

The effects of CETP, secreted by HepG2 cells [62], could influence our results. However, no CETP activity is detectable in the medium after 4 h of incubation (not shown). Recently Rinninger and Pittman [19] showed that secreted CETP activity does not influence HDL-cholesteryl ether uptake during short incubation periods.

This study describes for the first time the binding and uptake of HDL subfractions, isolated by immuno-affinity chromatography, to HepG2 cells. No big differences in the binding or uptake of LP A-I and LP A-I/A-II were observed and no specific role of apo A-II in HDL binding or uptake of cholesteryl esters was detected.

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chapter 9

General Discussion

The experiments described in this thesis were performed to gain more insight into the mechanism of reverse cholesterol transport. The efflux of cellular cholesterol, the first step in reverse cholesterol transport, was studied in several ways. Factors which may limit the rate of HDL-mediated efflux of cellular cholesterol are the concentration and/or composition of the cholesterol acceptor in the medium, and the composition of the plasma membrane. Therefore, immunopurified HDL subfractions, LCAT activity, binding of HDL to putative receptors and the fatty acid profile of cellular phospholipids received ample attention. In addition, we examined the last step of reverse cholesterol transport (uptake of HDL cholesterol by the liver) and measured whether there were differences in cholesterol delivery to HepG2 cells by the various immunopurified HDL subfractions.

The use of cationized LDL-loaded EA.hy 926 cells for measurement of cellular cholesterol efflux

The model, used for the major part of the experiments in this thesis, is described in chapters 2 and 3. Human endothelial EA.hy 926 cells, a cell line derived from fusion of human umbilical vein endothelial cells with lung carcinoma cells, were used [1]. This line has many characteristics of differentiated human endothelial cells, e.g., expression of von Willebrand factor [1], tissue plasminogen activator [2], plasminogen activator inhibitor-type 1 [2] and production of prostacyclin [3]. Cells of this permanent EA.hy 926 line still express typical differentiated characteristics after more than one hundred cumulative population doublings [1,2]. It was reported that endothelial cells, derived from bovine aorta or human umbilical vein, can be loaded with LDL which was modified with acetic anhydride or malondialdehyde [4-6]. These modified LDL particles are taken up via plasma membrane scavenger receptors. However, EA.hy 926 endothelial cells did not have active scavenger receptors, but these cells could be loaded with cholesterol via incubation with cationized LDL (see chapter 2). Cationized LDL is taken up by cells via

a LDL receptor-independent pathway [7]. LDL is more positively charged after cationization and may interact with unspecified anionic sites on plasma membranes [8]. The bound particles are probably internalized during the course of membrane turnover, in analogy with the receptor-independent internalisation of native LDL bound to low affinity binding sites [9]. Basu *et al.* [7] reported that internalized cationized LDL is degraded in the same way as native LDL, i.e., the internalized particles are directed to the lysosomes. The binding of cationized LDL to cells is very high. In our studies 30 to 40% of the added cationized LDL is cell-associated after 24 h of incubation. Basu *et al.* [10] reported that the binding of cationized LDL to fibroblasts is about 100 times higher than the binding of native LDL. Using pulse-labelling experiments, it was shown that 60% of initially cell-bound cationized LDL was internalized after 24 hours of incubation, and that about half of this internalized material is degraded [10]. This indicates that a substantial part of the internalized cationized LDL particles accumulates before lysosomal degradation can take place. So, the degradation of cationized LDL by the cells is slow, if compared with the high binding. Basu *et al.* [7] reported that the actual degradation of cationized LDL cholesteryl esters is equal to or even somewhat higher than for cholesteryl esters from native LDL. This was shown by the rates of [^{3}H]cholesterol formation from [^{3}H]cholesteryl ester-labelled cationized or native LDL by human fibroblasts [7]. Although it is known that a substantial amount of cationized LDL can still be present extracellularly after 24 hours of incubation [10], this extracellularly-bound cationized LDL did not disturb our efflux experiments. We never measured an increase of the medium cholesteryl ester content during our assays. Because of the absence of cholesterol esterification in the medium, an increase of medium cholesteryl esters can only be caused by a release of extracellularly-bound cationized LDL. It can be concluded therefore that extracellularly-bound cationized LDL was not released from the cells during the incubation of the EA.hy 926 cells with BSA or HDL. Stein *et al.* [11] also showed that extracellularly-bound cationized LDL is bound to the plasma membrane very tightly and cannot be removed easily from the cells. Combining all these data we conclude that cationized LDL-loaded EA.hy 926 cells can be used to study efflux of intracellular cholesterol.

Incubation of confluent EA.hy 926 cells with 50 μg cationized LDL cholesterol/ml increased cellular cholesterol about 4-fold (see chapter 3), and about 40% of this cholesterol was esterified. After loading the cells with varying concentrations of cationized LDL, the cholesterol efflux was dependent on the initial cellular cholesterol concentration (see chapter 3). The best correlation between cell cholesterol content and cellular cholesterol efflux was found for the cellular concentration of unesterified cholesterol ($r = 0.998$, $P < 0.002$, not shown). These observations are in agreement with the

results of Johnson *et al.* [12], who reported that the rate of cellular cholesterol efflux was linear with the cellular concentration of unesterified cholesterol.

Effect of the acceptor on efflux of cholesterol from cationized LDL-loaded EA.hy 926 cells

In chapter 3 we showed that HDL is most efficient in decreasing cellular cholesterol levels. Although LDL decreased the cellular unesterified cholesterol concentration, there was no decrease of total cellular cholesterol. We tested whether LCAT, which decreases the unesterified cholesterol content of HDL, enhanced the cellular cholesterol efflux (see chapter 4). This modification of HDL may enhance the diffusion of cholesterol into the HDL particles, because LCAT increases the concentration gradient of unesterified cholesterol between cells and HDL particles. It was found however, that LCAT treatment did not influence the release of cellular cholesterol mass at HDL concentrations varying from 50 to 2000 μg/ml. These results indicate that LCAT-treated HDL, with a very low UC/PL ratio, does not extra stimulate efflux and that HDL, isolated by ultracentrifugation, has an optimal composition for uptake of cell cholesterol. The lack of effect of LCAT treatment on efflux from cholesterol-loaded cells was also reported by Stein *et al.* [13], but LCAT can enhance the net mass transfer of cellular cholesterol when cells with normal cholesterol levels are used [13-15]. Influx and efflux of cholesterol are about equal during the incubation of normocholesterolemic cells with HDL [16]. It is postulated that LCAT decreases the influx of cholesterol into cells, without affecting efflux, and that LCAT treatment therefore results in net mass efflux of cholesterol from the cells [12-14].

In our model system, HDL-mediated efflux was already maximal at about 150 μg HDL protein/ ml (see chapter 3). This implies that, at higher HDL concentrations, the rate of cholesterol efflux was not determined by the acceptor in the medium, but that efflux was limited by other factors. Possible factors which may influence the cholesterol efflux rate are the hydrolysis of intracellular cholesteryl esters and the subsequent transport to the plasma membrane, and the desorption of cholesterol from the membrane into the aqueous phase. The desorption of cholesterol from the plasma membrane is likely to be influenced by the composition of this membrane. Therefore we studied the effect of plasma membrane composition on cholesterol efflux rate.

Effect of plasma membrane composition on efflux of cellular cholesterol

Bellini *et al.* [17] showed that membrane proteins influence the desorption of cholesterol from the plasma membrane. In our studies we examined the effects of differences in plasma membrane phospholipid composition on HDL-mediated efflux of

cellular cholesterol (see chapter 6). Both membrane cholesterol content and phospholipid fatty acyl composition determine the fluidity of the membrane, i.e., the motion of membrane lipids [18,19]. When the desorption of cholesterol from the plasma membrane is a rate limiting step in the efflux of cellular cholesterol, changes in the fatty acyl composition of cellular phospholipids may influence efflux of cellular cholesterol. To study the role of cellular phospholipid composition on cholesterol efflux, confluent cationized LDL-loaded EA.hy 926 cells were incubated with complexes of fatty acids and albumin. The following fatty acids were used: palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4) and eicosapentaenoic acid (20:5). The incubations resulted in endothelial cells with clearly different fatty acid profiles of cellular phospholipids. Incubation with the various fatty acids also resulted in changes of the cellular cholesteryl ester composition, but the differences between the various fatty acid treatments were less pronounced than for the phospholipids. Subsequently, the efflux of cellular cholesterol was measured as the increase of cholesterol in the medium after 24 hours of incubation of the cells with HDL_3. Cholesterol efflux was significantly higher after palmitic acid treatment, if compared with all other fatty acid treatments. We investigated whether the differences in efflux in our experiments, observed after the various fatty acid treatments, were related with specific high affinity binding of HDL to the EA.hy 926 cells. Earlier, it was reported that modification of cellular phospholipids with fatty acids changes the LDL-receptor activity [20,21]. The specific high affinity binding of HDL was virtually identical after treatment with the various fatty acids and the binding did not correlate with cholesterol efflux. Another reason to conclude that specific high affinity binding of HDL is not involved in HDL-mediated cholesterol efflux was that, using DMS-HDL_3 instead of native HDL_3 to induce efflux of cellular cholesterol, the differences in efflux between the various fatty acid treatments were still present (DMS-HDL_3 does not bind to specific high affinity binding sites for native HDL). These experiments support the hypothesis that the plasma membrane plays an important role in determining the efflux of cellular cholesterol. Possibly, the efflux of cholesterol was higher after palmitic acid treatment in order to maintain the fluidity of the cellular membranes. It was reported before that it is hard to change the fluidity of biological membranes by incubation with fatty acids, because cells tend to maintain a constant membrane fluidity (for reviews see [18,19]). Both a high cholesterol content and a high percentage of saturated fatty acids in phospholipids may decrease membrane fluidity and the rapid release of cellular cholesterol after palmitic acid treatment may have helped to maintain membrane fluidity. The distribution of cholesterol in different microdomains in the plasma membrane, corresponding with different kinetic pools of cholesterol efflux, could provide another explanation for the higher efflux of cholesterol after palmitic acid treatment. Effects of phospholipid unsaturation on cholesterol microdomains were

reported earlier [22], and Rothblat *et al.* [23] hypothesized that efflux of cellular cholesterol consists of cholesterol derived from two kinetic compartments. These two compartments can be represented by membrane microdomains with "fast" and "slow" cholesterol pools. Palmitic acid treatment may have shifted more cholesterol into the "fast" kinetic pools, due to the changes in membrane phospholipid composition, and resulting in a higher efflux of cellular cholesterol. The experiments described in this thesis are the first report of the effects of cellular fatty acid modification on net mass efflux of cellular cholesterol, but there are earlier studies in which the exchange of labelled cholesterol from vesicles with saturated or unsaturated phospholipids to acceptor vesicles was measured. Bloj and Zilverschmit [24] and Lund-Katz *et al.* [25] reported that exchange of labelled cholesterol between phosphatidylcholine vesicles was enhanced when phosphatidylcholine with unsaturated fatty acid chains, instead of "saturated" phosphatidylcholine, was used for the donor vesicles. This was explained by the stronger van der Waals interactions between cholesterol and saturated phosphatidylcholine, resulting in a decreased exchange of labelled cholesterol. Pal and Davis [26] reported that treatment of normocholesterolemic fibroblasts with eicosapentaenoic acid increased the efflux of labelled cellular cholesterol, if compared with linoleic acid treatment [26]. The results from the exchange experiments are in apparent contradiction with our studies, which showed that efflux was highest after palmitic acid treatment of the cholesterol-loaded EA.hy 926 cells. However, we measured net mass flux of cholesterol from cells to HDL and therefore our results cannot have been influenced by exchange phenomena. We did not measure whether the fatty acid treatments changed the distribution of phospholipid subclasses in the cellular membrane. It is obvious that differences in phospholipid subclass distribution could also affect the desorption of cholesterol from the plasma membrane, resulting in differences in efflux of cellular cholesterol. Indeed, it was reported that the sphingomyelin content of [^{3}H]cholesterol labelled liposomes or erythrocytes influenced the exchange of labelled cholesterol [25,27].

The relation between binding of HDL and HDL-mediated efflux of cellular cholesterol

The main difference between the various hypotheses about the mechanism of efflux of cellular cholesterol is the possible involvement of the binding of HDL to specific, saturable, high affinity HDL binding sites [28,29]. The effect of membrane phospholipid composition on efflux of cellular cholesterol, as described in chapter 6, indicates that the plasma membrane composition plays an important role in determining cholesterol efflux rate, and that specific binding sites for native HDL are not necessarily involved in cellular cholesterol efflux. We also used chemically modified HDL to study the relation between HDL binding and HDL-mediated efflux of cellular cholesterol.

Various authors used dimethyl suberimidate (DMS)- or tetranitromethane (TNM)-treatment of HDL to prevent the high affinity binding of HDL to cells or membranes [30-32]. These studies indicate that the ability to compete for the binding of iodinated native HDL disappeared after modification of HDL. Also in our experiments, using EA.hy 926 cells loaded with cationized LDL, unlabelled DMS-HDL_3 and TNM-HDL_3 were poor competitors for the binding of iodinated native HDL_3. Recently, it was reported that rat liver plasma membranes contain different high affinity binding sites for native HDL_3 and for DMS-HDL_3 [33]. We also measured the saturable, high affinity binding of iodinated DMS-HDL_3, using cholesterol-loaded EA.hy 926 cells. However, our experiments do not indicate the existence of one specific binding site for native HDL_3 and a second site which only binds DMS-HDL_3. The experiments, described in chapter 5, show that cationized LDL-loaded EA.hy 926 cells express one binding site, specific for native HDL_3 only, and a second site which binds both native HDL_3 and DMS-HDL_3. This explains the inhibition of ^{125}I-DMS-HDL_3 binding both by unlabelled native HDL_3 and DMS-HDL_3 (see chapter 5). Possibly, TNM-HDL_3 also binds to the native HDL/DMS-HDL site because Chacko *et al.* [30] reported that binding of iodinated TNM-HDL_3 was also inhibited both by unlabelled TNM-HDL_3 and native HDL_3 . This hypothesis can be tested in future experiments, measuring the competition of the binding of iodinated DMS-HDL_3 and TNM-HDL_3 with unlabelled TNM-HDL_3 and DMS-HDL_3, respectively.

Efflux mediated by DMS-HDL and TNM-HDL (see chapter 5) was identical (DMS-HDL_3) or about half of the efflux mediated by native HDL (TNM-HDL_3). We conclude that binding of HDL to the specific high affinity sites for native HDL is not necessary for efflux of cellular cholesterol. The half maximal efflux, induced by TNM-HDL_3, can probably be explained by the limited diffusion of cell-derived cholesterol into the TNM-HDL_3 particles because of the rigid structure of the modified particles: TNM not only causes cross-linking of HDL apolipoproteins, but also cross-links HDL phospholipids and cholesteryl esters to apolipoproteins [34]. Based on the decreased capacity of TNM-HDL to take up cellular cholesterol, it can be expected that cell cholesterol levels will be higher after incubation with TNM-HDL, if compared with native HDL. Brinton *et al.* [31] also reported less mass efflux of cholesterol by TNM-HDL_3. This might explain the different effects of HDL and TNM-HDL in the translocation experiments of Oram and coworkers [29]: if the desorption of cellular cholesterol is lower for TNM-HDL, this may result in a decreased flow, i.e., translocation, of intracellular cholesterol to the plasma membrane. In this way the "specific" intracellular translocation of cholesterol by native HDL may be explained by nonspecific diffusion processes. However, lipid-free apo A-I or activators of protein kinase C (in the absence of HDL) also induced translocation of intracellular cholesterol

in loaded fibroblasts [35], indicating the role of a signal transduction pathway in intracellular cholesterol fluxes.

From our data we cannot conclude whether the interaction of HDL or modified HDL with the second class of binding sites (which binds both native and modified HDL) is involved in the efflux of cell cholesterol. It is possible that this interaction enhances the efflux of cholesterol by diminishing the distance for diffusion of cholesterol between the cells and lipoprotein particles. More experiments are necessary to clarify the role of these different cellular binding sites in efflux of cellular cholesterol. Assuming that these binding sites are membrane proteins, isolation and subsequent production of specific antibodies may be used as an approach to study the role of the different receptor proteins in the efflux process. It is generally accepted that binding to saturable high affinity sites involves binding to receptor molecules on the plasma membrane (protein-protein interactions). Because of the cross-linking of the apolipoproteins in the chemically modified HDL particles it is unlikely that modified HDL is bound via protein-protein interactions, although it remains possible that binding via the apolipoproteins is unchanged after modification. Another possibility is that modified HDL binds to certain membrane lipid domains via lipid-lipid interactions, as proposed for native HDL in [36,37]. When the binding is restricted to certain specific membrane microdomains, this will also result in saturable binding. Rothblat *et al.* [23] hypothesized that, although efflux occurs without binding of apolipoproteins to cells, the efflux rate of cholesterol from certain cell types is enhanced by the interaction of apo A-I with specific microdomains in the membrane (protein-lipid interactions). It is not known which part of the apo A-I molecule is involved in this interaction and whether it is prevented by chemical modification of apo A-I. It may be speculated that this protein-lipid interaction is impaired when HDL is treated with TNM. This could explain the decreased capacity of TNM-HDL_3 to take up cellular cholesterol in our experiments (see chapter 5).

The role of LP A-I and LP A-I/A-II in reverse cholesterol transport

The role of immunopurified HDL subfractions in different processes of reverse cholesterol transport is described in chapters 7 and 8. Both the capacity of different HDL subfractions to take up cellular cholesterol as well as the uptake of these subfractions by HepG2 cells were studied. HDL mainly consists of two subfractions: LP A-I (HDL particles with apo A-I but without apo A-II) and LP A-I/A-II (particles containing apo A-I as well as apo A-II). The efflux of cellular cholesterol, using a variety of cell types, was virtually the same when LP A-I or LP A-I/A-II was used as cholesterol acceptor (see chapter 7). In these experiments we did not detect a clear antagonistic role of apo A-II. Hodenberg *et al.* [38] also reported that HDL subfractions with different apo A-I/A-II

ratios induced similar efflux of cholesterol from loaded macrophages. However, Barkia *et al.* [39] reported that only LP A-I is capable of removing cholesterol from mouse adipocytes, and that LP A-I/A-II is unable to change cellular cholesterol levels. A specific function of LP A-I particles was also found by Castro and Fielding [40] and by Fielding *et al.* [41]. They showed that a specific LP A-I population, with pre-ß migrating mobility on agarose gels, was more efficient in taking up labelled cellular cholesterol than the bulk of α-migrating particles. So, although we did not detect a clear specific function of apo A-II, it is possible that the two major HDL subfractions have different effects on certain cell types.

The specific binding of iodinated HDL to HepG2 cells was highest for LP A-I, and is probably mediated by apo A-I (see chapter 8). The total cell association (binding plus uptake at 37 °C) of ^{125}I-labelled LP A-I was about 20 % higher than for LP A-I/A-II. Although these differences were significant, the difference was small if related to the pronounced difference in apolipoprotein composition between the subfractions. The uptake of cholesteryl esters from LP A-I was about 80% higher than the uptake from LP A-I/A-II. This could indicate an antagonistic role of apo A-II in the selective uptake of cholesteryl esters from HDL, but may also have been caused by the differences in lipid composition between the two HDL subfractions (for example, the cholesteryl ester content of LP A-I is higher than of LP A-I/A-II at equal protein concentrations). Recently, Rader *et al.* [42] reported that in healthy humans apo A-I on LP A-I particles is catabolized more rapidly than apo A-I on LP A-I/A-II particles, indicating divergent metabolic pathways for LP A-I and LP A-I/A-II. These differences may have been caused by a faster hepatic uptake of LP A-I as revealed by our experiments showing its higher binding and uptake by HepG2 cells.

Summarizing we conclude that 1) HDL-mediated efflux of cellular cholesterol is independent of its interaction with specific high affinity binding sites for native HDL. 2) HDL and HDL_3 (the main HDL subfraction), isolated by ultracentrifugation, are efficient cholesterol acceptors: a decrease of the unesterified cholesterol content of HDL by LCAT does not further stimulate the HDL-mediated efflux. 3) the chemical composition of the plasma membrane (fatty acid profile of the phospholipids) is likely to be an important determinant of the efflux process. 4) Both LP A-I and LP A-I/A-II can release cholesterol from cells, but LP A-I has a higher capacity for cholesteryl ester delivery to hepatocytes.

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Summary

High density lipoproteins are postulated to play an important role in reverse cholesterol transport, the process in which peripheral cell cholesterol is transported back to the liver. The experiments described in this thesis were performed in order to clarify the mechanism of efflux of cellular cholesterol, the initial step in reverse cholesterol transport. In addition, the uptake of HDL by liver cells (the final step in the process) was studied.

EA.hy 926 cells, a human endothelial cell line which expresses characteristics of differentiated endothelial cells, were used for the major part of the experiments described in this thesis. These cells can be loaded with mass amounts of cholesterol after incubation of the confluent monolayers with cationized LDL. Total cellular cholesterol increases about 4-fold after 24 h of incubation with cationized LDL (50 μg/ml), and about 40% of this cholesterol is esterified. The high affinity binding of apo E-free HDL_3 to the cells increased after cholesterol loading, without affecting the affinity. The cholesterol-enriched cells can be used to study efflux of cellular cholesterol. HDL was capable of inducing efflux of cholesterol from the cells, but LDL did not change cellular cholesterol levels. The HDL-mediated efflux was dependent on the initial cell cholesterol level and efflux was maximal at concentrations of 0.2 mg HDL protein/ml. The relation between binding of HDL and HDL-mediated efflux was studied using two types of chemically modified HDL: dimethyl suberimidate (DMS)- and tetranitromethane (TNM)-treated HDL. Both iodinated native HDL_3 and DMS-HDL_3 showed a saturable, high affinity binding to loaded EA.hy 926 cells. Competition experiments revealed that these cells express two different high affinity binding sites: one site which is specific for native HDL_3 and another site, which binds both native HDL_3 and DMS-HDL_3. TNM-HDL_3, like DMS-HDL_3, did not interact with the specific site for native HDL_3, but both modified HDL particles were able to induce efflux of cellular cholesterol: DMS-HDL_3-mediated cholesterol efflux and native HDL_3-mediated efflux were identical, and TNM-HDL_3 induced about 50% of the efflux, if compared with native HDL_3. We conclude that interaction of HDL with specific high affinity membrane binding sites for native HDL is not necessary for the efflux of cholesterol. In order to investigate the role of the fatty acid composition of plasma membrane phospholipids in determining the cholesterol efflux rate, we measured the HDL-mediated efflux of cell cholesterol after fatty acid modification of cationized LDL-loaded EA.hy 926 cells. The incubation with fatty acid/BSA complexes extensively changed the fatty acid profile of cellular phospholipid (which reflects the plasma membrane profile), and had less pronounced effects on cellular cholesteryl ester fatty acid composition. HDL_3-mediated efflux of cellular

cholesterol was highest after palmitic acid treatment, if compared with the other fatty acid treatments (oleic, linoleic, arachidonic or eicosapentaenoic acid treatment). The differences in efflux after the various fatty acid treatments were not mediated by the high affinity binding of HDL_3, because firstly, this binding did not correlate with the cholesterol efflux and secondly, using DMS-HDL_3, which does not bind to specific high affinity sites for native HDL, the differences in cholesterol efflux among the various fatty acid treatments were still present. These experiments demonstrate the important role of the membrane phospholipid fatty acid composition in determining the rate of cellular cholesterol efflux. It is known that HDL is a very heterogenous population of lipoprotein particles with different compositions of lipids as well as of apolipoproteins. Using immuno-affinity chromatography, HDL subfractions with clearly distinct apolipoprotein compositions can be isolated. Human HDL consists of two major subfractions: LP A-I particles containing apo A-I, but no apo A-II and LP A-I/A-II particles containing both apo A-I and apo A-II. The capacity of LP A-I and LP A-I/A-II to induce efflux of cellular cholesterol from human fibroblasts, Fu5AH hepatoma cells and aorta smooth muscle cells was compared. Both subfractions showed similar efflux of lysosomal- as well as membrane-derived cholesterol. These data indicate that LP A-I and LP A-I/A-II are both capable to remove cellular cholesterol. No clear antagonsitic role of apo A-II could be detected in our experiments. In another study it was found that the binding of iodinated LP A-I to HepG2 cells was higher than for LP A-I/A-II. The binding to liver cells is probably mediated by apo A-I. The total cell association (i.e., binding plus uptake) and selective uptake of cholesteryl esters was also highest for LP A-I. So, both HDL subfractions can deliver cholesterol to liver cells, but that LP A-I is most active in this respect.

It is concluded that both LP A-I and LP A-I/A-II can release cholesterol from cells, that the composition of the plasma membrane (fatty acid profile of the phospholipids) is likely to be an important factor in the efflux of cellular cholesterol, and that binding to specific high affinity sites for native HDL is not involved in this process.

Samenvatting

Er wordt aangenomen dat hoge dichtheids lipoproteïnen (HDL) een belangrijke rol spelen in het omgekeerde cholesterol transport, het proces waarin cholesterol uit perifere cellen naar de lever wordt getransporteerd. De experimenten, die in dit proefschrift beschreven worden, werden uitgevoerd om het mechanisme van de efflux van cellulair cholesterol, de eerste stap in het omgekeerde cholesterol transport, op te helderen. Tevens werd de opname van HDL door levercellen (de laatste stap in het proces) bestudeerd.

EA.hy 926 cellen, een humane endotheliale cellijn met karakteristieke kenmerken van gedifferentiëerde endotheel cellen, werden gebruikt voor het grootste gedeelte van de experimenten die in dit proefschrift beschreven zijn. EA.hy 926 cellen kunnen worden verrijkt met cholesterol door incubatie van de confluente cellen met gecationiseerd LDL. Dit leidt tot een circa viervoudige stijging van het celcholesterol na 24 uur incubatie (50 μg gecationiseerd LDL/ml), waarvan ongeveer 40% veresterd is. De hoge affiniteits binding van apo E-vrij HDL_3 nam toe na het opladen van de cellen met cholesterol, terwijl de affiniteit gelijk bleef. De cholesterol-verrijkte cellen kunnen worden gebruikt voor de bestudering van de efflux van cholesterol uit cellen. HDL was in staat de concentratie van celcholesterol te verlagen, terwijl LDL geen effect op het cholesterol niveau had. De efflux onder invloed van HDL was afhankelijk van de initiële cholesterol concentratie in de cellen en de efflux was maximaal bij een HDL concentratie van 0.2 mg HDL eiwit/ml en hoger. De relatie tussen de binding van HDL en de door HDL geïnduceerde cholesterol efflux werd bestudeerd met behulp van twee soorten chemisch gemodificeerd HDL, dimethyl suberimidaat (DMS)- en tetranitromethaan (TNM)-behandeld HDL. Zowel gejodeerd natief HDL_3 als DMS-HDL_3 gaven een verzadigbare hoge affiniteits binding te zien aan de cholesterol-verrijkte EA.hy 926 cellen. Competitie experimenten toonden aan dat deze cellen twee verschillende high affinity bindingsplaatsen hebben: één plaats, specifiek voor natief HDL_3 en een andere plaats, die zowel natief HDL_3 als DMS-HDL_3 bindt. TNM-HDL_3, evenals DMS-HDL_3, bindt niet aan de specifieke bindingsplaatsen voor natief HDL_3, maar beide soorten gemodificeerd HDL waren in staat de celcholesterol concentratie te verlagen: de efflux, gemediëerd door natief HDL_3 en door DMS-HDL_3 was gelijk, en TNM-HDL_3 gaf circa 50% cholesterol efflux in vergelijking met natief HDL_3. Wij concluderen dat de interactie van HDL met specifieke bindingsplaatsen voor natief HDL op de plasmamembraan niet noodzakelijk is voor de efflux van cholesterol uit EA.hy 926 cellen. Het effect van de vetzuursamenstelling van de fosfolipiden in de plasma membraan op de effluxsnelheid werd onderzocht door het meten van de efflux onder

invloed van HDL na modificatie van de cholesterolrijke cellen met diverse vetzuren. De incubatie met complexen van vetzuren en albumine leidde tot een aanzienlijke verandering van het vetzuurspectrum van de celfosfolipiden (die de verandering van de plasmamembraan fosfolipiden weerspiegelen). In mindere mate werd ook de vetzuursamenstelling van de intracellulaire cholesterolesters veranderd. De efflux van celcholesterol was het hoogste na behandeling met palmitinezuur, in vergelijking met cellen behandeld met oliezuur, linolzuur, arachidonzuur of eicosapenteenzuur. De verschillen in efflux tussen de diverse vetzuurbehandelingen werden niet veroorzaakt door veranderingen in HDL binding, omdat ten eerste, deze binding niet gecorreleerd was met de efflux, en ten tweede, omdat de verschillen in efflux tussen de diverse vetzuurbehandelingen hetzelfde waren wanneer DMS-HDL (dat niet aan de specifieke bindingsplaatsen voor natief HDL bindt) als acceptor gebruikt werd. Deze experimenten tonen aan dat de fosfolipidsamenstelling van de plasmamembraan waarschijnlijk een belangrijke rol speelt bij het bepalen van de effluxsnelheid. Het is bekend dat HDL een heterogene samenstelling heeft: er zijn verschillen in zowel lipid- als in apolipoproteïnensamenstelling tussen HDL deeltjes. Door het gebruik van immuno-affiniteits chromatografie kan de rol van HDL subfracties (met zeer uiteenlopende apolipoproteïnensamenstelling) in het omgekeerde cholesterol transport bestudeerd worden. Humaan HDL bestaat voor het grootste gedeelte uit twee subfracties: een subfractie waarin de HDL deeltjes wel apo A-I maar geen apo A-II bevatten (LP A-I), en een subfractie met zowel apo A-I als apo A-II op elk HDL deeltje (LP A-I/A-II). In humane fibroblasten, FuSAH hepatoma cellen en aorta gladde spier cellen werd vergeleken of LP A-I en LP A-I/A-II beiden de efflux van celcholesterol konden induceren. Beide subfracties gaven eenzelfde efflux van zowel cholesterol afkomstig uit de membraan als van lysozomaal cholesterol. Deze resultaten geven aan dat beide HDL subfracties in staat zijn om celcholesterol op te nemen. Het apo A-II had geen duidelijke antagonistische werking in onze experimenten. In een andere studie werd aangetoond dat de binding van LP A-I aan HepG2 cellen hoger is dan van LP A-I/A-II. De binding van HDL verloopt waarschijnlijk via apo A-I. De totale celassociatie en de selectieve opname van cholesterolesters is ook het hoogste voor LP A-I. Dus beide subfracties kunnen cholesterol afgeven aan levercellen. LP A-I is hierin het meest actief.

Wij concluderen dat zowel LP A-I als LP A-I/A-II cholesterol uit cellen kan opnemen, dat de samenstelling van de plasmamembraan (vetzuurprofiel van de fosfolipiden) waarschijnlijk een belangrijke rol speelt in de efflux van celcholesterol en dat binding aan specifieke high affinity bindingsplaatsen voor natief HDL niet betrokken is in dit proces.

Nawoord

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En als laatste genoemd, maar misschien wel het allerbelangrijkste, was de steun van mijn familie: pa, ma, Tiny en Ad. Op de achtergrond waren jullie er altijd, en menigmaal heeft jullie hulp mij ergens overheen geholpen. Bedankt!

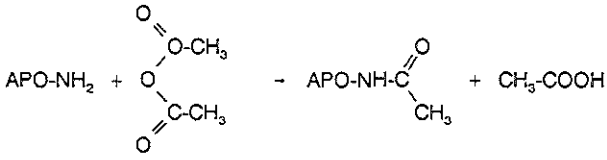
Curriculum Vitae

Liesbeth Kilsdonk werd op 19 juni 1963 geboren te Breda. In 1981 behaalde zij cum laude het VWO diploma aan het Onze Lieve Vrouwe Lyceum te Breda. Aansluitend begon zij de studie Humane Voeding aan de toenmalige Landbouw Hogeschool te Wageningen en behaalde in 1985 cum laude het kandidaats examen. Gedurende de doctoraal fase werd van mei tot december 1986 stage gelopen op de afdeling Biologische Toxicologie van het CIVO-TNO te Zeist. Het ingenieurs diploma werd in september 1987 behaald met als hoofdvakken Toxicologie en Humane Voeding, en als bijvak Organische Chemie. In samenwerking met Ir. G. Smulders won zij de scriptieprijs 1988 van de Nederlandse Vereniging voor Voeding en Levensmiddelen Technologie. Van november 1987 tot en met januari 1992 werd het onderzoek verricht, waarvan de resultaten beschreven zijn in dit proefschrift. Dit onderzoek werd uitgevoerd op de afdeling Biochemie van de Erasmus Universiteit Rotterdam en op de afdeling Physiology and Biochemistry van het Medical College of Pennsylvania (november-december 1989). Zij was gedurende deze tijd in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (november 1987-oktober 1991) en de Erasmus Universiteit Rotterdam (november 1991-januari 1992). Gedurende deze periode behaalde zij in het kader van het AIO-onderwijs het Cambridge Proficiency Certificate en volgde zij de cursus Biostatistiek. Vanaf januari 1993 zal zij werkzaam zijn als post-doctoral fellow op de afdeling Physiology and Biochemistry van het Medical College of Pennsylvania te Philadelphia (stipendium van de Niels Stensen Stichting te Amsterdam).

APPENDIX I Chemical modification of lipoproteins

Acetylation of LDL

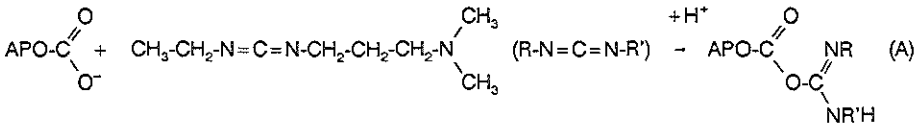
LDL (density range 1.006-1.063 g/ml) and HDL_3 (density range 1.125-1.21 g/ml) were isolated by sequential ultracentrifugation of human plasma [1] and dialyzed against 0.9% NaCl, containing 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. LDL was acetylated with acetic anhydride as described by Basu *et al.* [2]. This reaction is specific for amino groups in a solution of half-saturated sodium acetate [3]. In short, 1 ml of LDL (1-10 mg protein/ml) was added slowly to 1 ml of a saturated solution of sodium acetate (Merck No. 6267, MW 136.08) with continous stirring at 0 °C. After 5 minutes, acetic anhydride (BDH No. 27017, MW 102.1) was added in multiple portions of 2 μl over a period of 1 hour. The total amount of acetic anydride was 1.5-fold the amount of LDL protein (1 μl corresponds with 1.08 mg acetic anhydride). The mixture was stirred for another 30 minutes. Subsequently, the acetylated LDL was dialyzed against 0.9% NaCl, 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, and filter sterilized (0.45 μm).

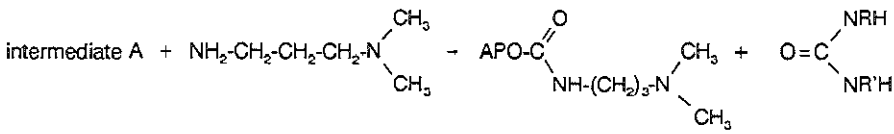


Cationization of LDL

LDL was cationized according to Basu *et al.* [2]. This reaction converts carboxyl groups into amides via a reaction with a water-soluble carbodiimide, followed by a reaction with an amine [3]. A volume of LDL (20-30 mg of protein) was added to an equal volume of a 2 M solution of 3-dimethyl aminopropyl-amine (Aldrich No. 24.005-2, MW 102) at room temperature. The pH was adjusted to 6.5 with HCl and subsequently 100 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma No. E-7750, MW 191.7) was added. The pH was maintained at 6.5 with 0.1 M HCl during the reaction. After 3 h, the pH of the solution did not change anymore, and the reaction mixture was left overnight at 4 °C. The cationized LDL was dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA and filter sterilized (0.8 μm). Total LDL cholesterol was measured enzymatically using a commercially available kit (Boehringer

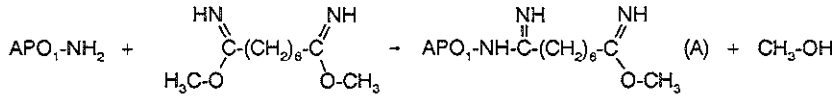
Mannheim, cholesterol kit No. 310328 and cholesterol esterase No. 161772). Protein was measured according to [4]. The protein content of cationized LDL was calculated from the cholesterol concentration of cationized LDL and the protein/cholesterol ratio of native LDL, because of disturbances of protein measurements after cationization of LDL, as was also described by [5]. LDL, acetylated LDL and cationized LDL were stored at 4 °C. Storage of cationized LDL at -20 °C did not change the loading of the EA.hy 926 cells, if compared with storage at 4 °C.

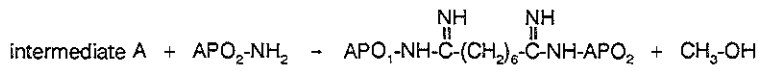




Cross-linking of HDL with dimethyl suberimidate

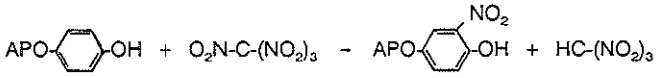
Modification of HDL_3 with dimethyl suberimidate (DMS, Pierce No. 20668, MW 273.2) was performed as described in [6]. Dimethyl suberimidate cross-links proteins via reactions with free amino groups [3]. HDL (5 mg protein/ml) was incubated with 5 mg DMS/ml in a 0.09 M triethanolamine-HCl buffer (pH 9.5) for 2 h at room temperature. After the reaction, DMS-HDL was reisolated on a Sephadex column (G25 Fine, 1 x 30 cm, Pharmacia). The column was eluted with 0.9% NaCl containing 2 mM EDTA (pH 7.4). The lipoprotein fraction was dialyzed against 0.9% NaCl, containing 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA.





Modification of HDL with tetranitromethane

HDL was modified with tetranitromethane (TNM, Aldrich No. T2500-3, MW 196) as described in [7]. Tetranitromethane converts tyrosine functions into 3-nitrotyrosine residues. In addition, tetranitromethane also cross-links apolipoproteins (probably also via the tyrosine residues) [3]. Chacko has reported that cross-links are also formed between apolipoproteins and phopsholipids or cholesteryl esters, which may be due to reactions of tetranitromethane with the double bonds in fatty acyl chains [8]. In short, a 10-fold molar excess of reagent was incubated with HDL for 1 h at room temperature in a 50 mM Tris-HCl buffer (pH 8.0) with 0.1 M NaCl. TNM-HDL was reisolated as described above for DMS-HDL. For both DMS- and TNM-HDL the cross-linking of the apolipoproteins was checked by electrophoresis on 12% polyacrylamide gels containing 0.1% SDS [9]. The sizes of native HDL, DMS-HDL and TNM-HDL were measured by gel filtration according to [10], using a Superose 12 column (Prep grade, 1 x 30 cm, Pharmacia).



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Appendix II Fatty acid composition of EA.hy 926 cells

Fatty acid composition of control and cholesterol-enriched cells

	control	FC-loaded	cationized LDL-loaded	
	phospholipids	phospholipids	phospholipids	cholesteryl esters
16:0 dma	1.2 ± 1.5	3.2	2.4	0.5
16:0	19.6 ± 0.6	23.4	24.1	17.0
18:0 dma	2.4 ± 0.4	2.6	1.7	0.6
18:0	16.4 ± 1.4	16.7	12.5	2.1
22:0	0.7 ± 0.4	0.2	0.9	2.5
24:0	0.6 ± 0.1	1.0	1.0	--
16:1 *n*-7	4.7 ± 0.5	1.9	2.9	4.0
18:1 *n*-9/*n*-7	28.0 ± 1.4	29.0	24.6	23.2
22:1 *n*-9	1.5 ± 0.1	0.2	2.0	1.0
18:2 *n*-6	4.9 ± 1.4	4.0	12.1	40.0
18:3 *n*-3	0.8 ± 1.1	--	0.1	4.0
20:3 *n*-6	--	1.4	2.2	0.5
20:4 *n*-6	7.8 ± 0.2	7.0	5.6	2.3
22:4 *n*-6	1.2 ± 0.2	1.1	0.6	0.5
20:5 *n*-3	1.3 ± 0.2	0.9	0.8	0.3
22:5 *n*-3	2.9 ± 0.5	2.5	1.9	--
22:6 *n*-3	4.3 ± 0.4	4.0	3.0	0.6
others	1.3 ± 1.3	1.0	1.7	1.1
% SFA	41.9 ± 4.2	47.3	42.9	22.9
% MUFA	34.6 ± 1.5	31.9	30.4	28.3
% PUFA	23.5 ± 0.4	20.9	26.7	48.8

Confluent EA.hy 926 cells were incubated 24 h with DMEM containing 10% FCS, HAT, L-glutamine, penicilline, streptomycine and 50 µg cholesterol/ml or 50 µg catLDL-TC/ml for the cholesterol-loaded and cationized LDL-loaded cells, respectively. The cellular phospholipid and cholesteryl ester fatty acid composition was determined as described in *Chapter 6*. Control and cholesterol-loaded cells contained not enough cholesteryl esters to analyse their fatty acid composition. Fatty acids were tabulated if in one of the treatment groups the percentage was >1%.

Fatty acid composition of cellular phospholipids after incubation with fatty acid/albumin complexes

	control EA.hy 926 cells				FC-loaded EA.hy 926 cells			
	control	palmitic acid	oleic acid	linoleic acid	control (+FC)	palmitic acid (+FC)	oleic acid (+FC)	linoleic acid (+FC)
16:0 dma	3.1	2.7	1.5	3.2	3.2	3.0	1.9	3.1
16:0	19.2	28.5	15.4	21.5	23.4	26.0	15.7	22.7
18:0 dma	2.7	1.8	1.3	--	2.6	1.8	1.7	2.2
18:0	17.4	15.0	9.3	14.9	16.7	18.6	10.2	14.7
22:0	0.5	0.4	0.3	1.5	0.2	0.4	0.1	0.2
24:0	0.7	0.9	0.3	0.4	1.0	1.2	0.4	0.6
16:1 *n*-7	5.1	3.6	2.1	3.1	1.9	1.5	0.5	0.8
18:1 *n*-9/*n*-7	27.0	19.9	52.8	20.4	29.0	20.4	53.7	20.0
20:1 *n*-9	--	--	--	--	0.5	0.5	1.8	0.5
22:1 *n*-9	1.4	1.9	0.9	1.2	0.2	0.3	0.5	0.3
24:1 *n*-9	--	--	0.8	0.2	0.3	0.2	1.3	0.4
18:2 *n*-6	3.9	4.3	2.0	18.4	4.0	4.2	2.5	21.3
18:3 *n*-6	0.7	1.2	1.8	0.4	--	0.1	--	--
20:3 *n*-6	--	--	--	--	1.4	2.0	0.6	1.1
20:4 *n*-6	8.0	7.6	5.3	7.5	7.0	8.8	4.8	6.2
20:5 *n*-3	1.4	1.1	0.6	0.6	0.9	1.1	0.3	0.3
22:4 *n*-6	1.0	1.3	1.2	1.3	1.1	1.2	1.2	1.4
22:5 *n*-3	2.6	3.9	1.6	2.1	2.5	3.9	1.2	1.8
22:6 *n*-3	4.1	4.9	1.6	2.1	4.0	4.2	1.5	2.2
others	1.4	0.9	1.4	1.3	0.2	0.6	0.1	0.3
% SFA	39.0	50.3	29.6	42.7	47.3	51.4	30.0	43.7
% MUFA	35.6	25.5	56.5	24.8	31.9	22.9	57.8	22.0
% PUFA	25.4	24.3	13.9	32.4	20.9	25.7	12.1	34.3

The cells were cultured in DMEM, supplemented with 10% FCS, 50 μM fatty acid, HAT, L-glutamine, penicilline and streptomycine for 2 passages (approximately 10 days). At this time point the confluent control cells were trypsinized, and the cholesterol-loaded cells were incubated in medium with 50 μg cholesterol/ml for another 24 h before trypsinization. The fatty acid composition of cellular phospholipids was determined as in *Chapter 6*. Fatty acids were tabulated if in one of the treatment groups the percentage was >1%.

Appendix III Composition of cationized LDL

Fatty acid composition of cationized LDL

fatty acid	total lipids	phospholipids	cholesteryl esters
16:0	23.5	34.7	18.0
18:0	6.3	18.2	2.5
20:0	0.4	1.7	0.2
22:0	0.9	2.5	0.3
24:0	0.7	2.1	0.2
16:1 *n*-7	2.4	0.6	4.2
18:1 *n*-9	17.2	7.1	21.6
18:1 *n*-7	1.8	1.3	--
24:1 *n*-9	0.4	1.0	1.2
18:2 *n*-6	38.1	19.1	46.1
20:3 *n*-6	1.5	3.6	0.5
20:4 *n*-6	3.6	5.0	1.7
20:5 *n*-3	1.0	0.3	0.5
22:6 *n*-3	--	1.2	--
others	2.3	1.7	3.1
% SFA	32.7	60.5	22.2
% MUFA	22.2	10.0	27.0
% PUFA	45.2	29.6	50.8

The fatty acid composition of the total lipid fraction, of the phospholipids and of the cholesteryl esters from cationized LDL was determined as described in *Chapter 6*. Fatty acids were tabulated if in one of the fractions the percentage was >1%.