LIVER CELLS AND LIPOPROTEINS

LEVERCELLEN EN LIPOPROTEINEN

PROEFSCHRIFT

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CONTENTS

SAMENVATTING

De lever speelt een centrale regulerende rol in het metabolisme van suikers, eiwitten en lipiden. In tegenstelling tot suikers dienen lipiden met eiwiten tot zgn. lipoproteinen gecomplexeerd te worden voordat het bloed ze kan transporteren. Afhankelijk van de dichtheid, die bepaald wordt door de verhouding eiwit-lipide kunnen er vier typen lipoproteinen worden onderscheiden; chylomicronen, very-low density lipoproteins (VLDL), low-density lipoproteins (LDL) en high-density lipoproteins (HDL).
Onderzoek naar de relatie tussen het optreden van harten vaatziekten en het niveau van de verschillende lipoproteinen in het bloed hebben aangetoond dat een verhoogd LDL-niveau in het bloed gekoppeld is aan een verhoogd risico. In het bloed wordt LDL gevormd uit VLDL wat afkomstig is uit de lever. De lever is ook het orgaan waar een belangrijk deel van de afbraak van LDL plaatsvindt. De opname van LDL vanuit het bloed door de lever vormt het onderwerp van dit proefschrift. De vier belangrijkste celtypen waaruit de lever is opgebouwd zijn; de parenchymale, de endotheel, de Kupffer en de "fat-storing" cel. De parenchymale cellen vormen 92.5% van de levermassa. Hoewel de niet-parenchymale cellen met elkaar dus slechts 7,5% bijdragen waren er aanwijzingen dat deze cellen een belangrijke rol spelen bij de afbraak van LDL. Echter voordat het mogelijk was de interactie tussen LDL en de verschillende lever celtypen te bestuderen diende er een celisolatieprocedure ontwikkeld te worden welke zuivere en intacte cellen opleverde (appendixartikel II). Een bijkomend resultaat van het zoeken naar een geschikte celisolatieprocedure was de detectie van de blebs in de niet-parenchymale celfracties. Deze deeltjes worden gevormd uit parenchymcellen maar bezitten dezelfde grootte als niet-parenchymale cellen. Zonder speciale voorzorgsmaatregelen worden de blebs dan ook samen met de niet-parenchymale cellen geïsoleerd (appendix artikel I).
De interactie tussen LDL en de levercellen voltrekt zich via een eiwit op het plasmamembraan dat het apoproteine gedeelte van het lipoproteine herkent (de LDL-receptor). In de appendixartikelen III en IV worden de experimenten beschreven waaruit blijkt dat de niet-parenchymale cellen een hogere concentratie LDL (apo B,E) receptoren bezitten dan de parenchymale cellen (uitgedrukt per mg cel eiwit).
Lipoproteinen met eenzelfde dichtheid als LDL kunnen behalve met de apo B,E receptor ook een interactie aangaan met een receptor die LDL herkent met een relatief negatieve lading. Een dergelijke ladingsverandering zou kunnen optreden door een langere circulatietijd van het LDL. Het is aangetoond dat dit gemodificeerde LDL potentieel atherogene eigenschappen bezit omdat opname van dit deeltje door macrofagen kan leiden tot de omzetting van deze cellen tot een soort schuimcellen die in de atherosclerotische plaque zijn aangetoond. Wanneer echter gemodificeerd LDL intraveneus bij ratten wordt ingespoten wordt het niet door de macrofagen in het bloed maar zeer snel door de lever opgenomen. Appendixartikelen III en IV laten zien dat vooral de niet-parenchymale cellen, in vergelijking met parenchymale cellen, sterk verrijkt zijn aan receptoren voor gemodificeerd LDL. Een verdere onderverdeling van het niet-parenchymale celpreparaat gaf aan dat de receptor voor gemodificeerd LDL voornamelijk gelocaliseerd is op de endotheelcel. De receptor voor natief LDL bevindt zich voornamelijk op de Kupffercel (appendixartikelen II en VI).
De eigenschappen van de receptor voor gemodificeerd LDL werden aanvankelijk bestudeerd met LDL dat met behulp van azijnzuur anhydride was geacetyleerd. Het is echter onwaarschijnlijk dat dergelijke chemische reacties plaatsvinden in het bloed. Omdat het was beschreven dat LDL ook biologisch gemodificeerd kon worden, nl. door het te incuberen met navelstrengendotheelcellen, is de interactie tussen dit deeltje en de lever onderzocht. In appendixartikel V wordt aangetoond dat biologisch gemodificeerd LDL wordt herkend door dezelfde receptor op

leverendotheelcellen welke acetyl-LDL herkent. Daar biologisch gemodificeerd LDL atherogene eigenschappen bezit kan de opname door de lever en speciaal de leverendotheelcel een beschermingsmechanisme van het lichaam vormen tegen het optreden van zulke deeltjes in het bloed.
In alle bovenstaande experimenten is, omdat het apoproteine gedeelte van de lipoproteinen het herkenningsteken is voor de receptors, de interactie tussen het apoproteine en de cellen bestudeerd. De verwerking van het lipidegedeelte is echter belangrijker voor het metabolisme. De apoproteinen worden, na opname door de cel, direct afgebroken in het lysosoom en de afbraakproducten verlaten de cel. De lipiden worden echter, voordat verdere verwerking plaats kan vinden, getransporteerd vanuit het lysosoom waardoor beïnvloeding van het cellulaire metabolisme mogelijk is. In appendixartikel VII wordt de *in vivo* opname en verwerking van de cholesterylesters uit acetyl-LDL door de verschillende levercellen beschreven. Het blijkt dat de cellen de cholesterylesters aanvankelijk op dezelfde wijze verwerken als het apoproteinegedeelte. In de parenchymale cellen wordt het vetzuur, vrijgekomen na hydrolyse van de cholesterylesters, gebruikt voor metabole reacties. In de endotheelcellen echter wordt het vetzuur uitgescheiden, opgenomen door de parenchymale cel en daar vervolgens voor metabole reacties gebruikt. In beide celtypen blijft het cholesterol in de vrije vorm. Deze resultaten duiden erop dat de lever in staat is om zowel het eiwit- als het lipidegedeelte van gemodificeerde lipoproteinen te metaboliseren zonder verdere locale pathologische consequenties.

SUMMARY

The liver plays a central regulatory role in the metabolism of sugars, proteins and lipids. In contrast with sugars, lipids have to be complexed with proteins to socalled lipoproteins before they can be transported in the blood. According to their density, which is determined by the lipid-protein ratio, the lipoproteins can be divided into 4 subclasses; chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL).
Studies on the relation between the incidence of coronary artery disease (CAD) and the level of the various lipoproteins in the blood have indicated that an increased LDL-level in the blood leads to a higher risk. LDL is formed in the blood from VLDL which is synthesized in the liver. The liver is also an important organ for the degradation of LDL. The uptake of LDL from the blood by the liver, forms the subject of this thesis.
The four major cell types present in the liver are; the parenchymal, the endothelial, the Kupffer and the fat-storing cells. The parenchymal cells contribute 92.5% to total livermass. Although the non-parenchymal cells contribute only 7,5% it was suggested that these cell types could play an important role in the degradation of LDL. Before the interaction between LDL and the different liver cell types could be studied it was necessary to develop a cell isolation procedure that results in pure intact cells (appendixpaper II). An additional result of the search for a suitable isolation procedure was the discovery of particles (blebs) derived from parenchymal cells with a size similar as non-parenchymal cells. By standard cell isolation procedures these blebs are co-isolated with the non-parenchymal cells unless appropriate precautions are taken (appendixpaper I).
The interaction between LDL and liver cells is mediated by a protein located on the plasma membrane which recognizes the apoprotein of the lipoprotein (the LDL-receptor). In appendixpapers III and IV the experiments are described which indicate that the non-parenchymal cells contain a higher concentration of LDL-(apo B,E) receptors than parenchymal cells (when expressed per mg cell protein).
In addition to the (apo B,E)receptor, lipoprotein particles in the LDL-density range may interact with a socalled scavenger receptor which recognizes LDL with a more negative charge. Such an altered charge could be generated by prolonged LDL circulation in the blood. It has been shown that this modified LDL could be atherogenic because uptake of this particle by macrophages can convert these cells to foam cells as present in the atherosclerotic plaque. However, when modified LDL is injected into rats it is not recovered in the blood macrophages but instead rapidly cleared by the liver. Appendixpapers III and IV describe that the non-parenchymal cells are especially rich in receptors for modified LDL when compared with parenchymal cells. A further subdivision of the non-parenchymal cellfraction indicated that the receptor for modified LDL is predominantly located on endothelial cells. The receptor for native LDL was found to be predominantly present on Kupffer cells (appendixpapers II and VI).
The characteristics of the receptor for modified LDL were initially studied with LDL that was acetylated by acetic anhydrid. It is however unlikely that such a chemical reaction occurs in the bloodstream. It has been reported that a biological modification of LDL could be induced by incubating the LDL with umbilical vein endothelial cells. In appendixpaper V it is shown that such biologically modified LDL is recognized by liver endothelial cells through the same receptor as acetyl-LDL. Because the biologically modified LDL is considered as atherogenic, the uptake of this particle by the liver, and especially the endothelial cell, might form a protective system of the body against the occurrence of potential atherogenic particles in the blood.
In all aforementioned experiments the interaction between the apoproteinmoiety of the lipoproteins and the cells was stud-

ied, because the protein contains the recognition mark for the receptors. The fate of the lipid moiety is however metabolically more important as these components are effective in changing the intracellular metabolism. The apoproteins are directly after uptake degraded in the lysosome and the degradation products are secreted from the cell. The lipids however have to be transported from the lysosome before further processing is possible, in this way they can influence intracellular metabolism. In appendixpaper VII the *in vivo* uptake and processing of the cholesterylesters from acetyl-LDL by the different liver cells is described. It appeared that initially the cholesterylesters were processed in the same way by the various liver cells as the apoprotein of acetyl-LDL. However, after hydrolysis of the cholesterylester in parenchymal cells the fatty acid was used for metabolic reactions. The fatty acid liberated in the endothelial cells is however secreted and transferred to the parenchymal cell where it is then used for biosynthetic reactions as well. In both cell types the cholesterol remained in the free form. These findings indicate that the liver is capable of metabolizing both the protein as the cholesterylester moiety of modified lipoproteins, without concomitant pathological consequences.

INTRODUCTION

1.1. the liver

1.1.1. FUNCTIONS

In the liver many metabolic processes take place which are of vital importance for the functioning of the whole organism. Examples are: glycogenolysis and gluconeogenesis which serve to maintain a rather constant glucose level in the blood, the urea cycle which is important for the elimination of protein degradation products, the cytochrome P 450 system involved in the degradation of xenobiotic substances and lipoprotein processing which functions in the uptake and transfer of cholesterol to the bile. The liver also synthesizes and secretes a variety of macromolecules e.g. albumine, liverlipase and apolipoproteins. In addition to the supporting properties for the whole body, reactions take place that serve the functioning of the liver itself. Many of these reactions proceed at the same time, some share common intermediates and are therefore regulated by a complex network. One of the factors that facilitates the regulation of metabolism is the intra- and intercellular compartmentation of the liver. Specific processes take place at distinguished compartments of the cell. For instance, fatty acids are mainly degraded in the mitochondrion but *de novo* synthesized in the cytoplasm. Characterization of the intercellular compartmentation forms the subject of this thesis.

1.1.2. LIVER ARCHITECTURE

The four major cell types from which the liver is composed are: the parenchymal cells, the endothelial cells, the Kupffer cells and the fat-storing cells. Their contribution to total livervolume is respectively 78%, 2,8%, 2,1% and 1,4%. The remaining volume is formed by the lumina of the sinusoids (10,6%), the spaces of Disse (4,9%) and the bile canaliculi (0,4%) (1). As the protein/volume ratio of the liver cells (except perhaps the fat-storing cells) is equal (2), it can be calculated that

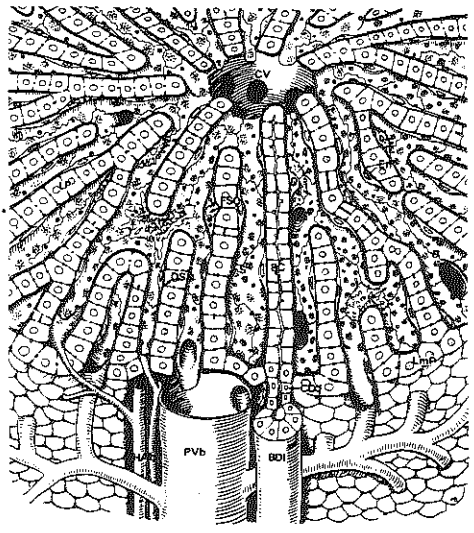

FIGURE 1

Diagram of a lobule

Schematic figure summarizing the three-dimensional structure of the hepatic lobule. CV: central vein; K: Kupffer cell; FSC: fat-storing cell; BC: bile canaliculus; En: endothelial cell; S: sinusoid with fenestrations; DS: Disse's space; HAb: hepatic artery branch; PVb: portal vein branch; CDJ: caniculo-ductular junction; BDl: bile ductule; LmP: limiting plate; LP: liver plates. Modified from Muto, M.: Arch. Histol. Jap. **37**: 369-386 (1975).

the parenchymal cells contribute 92,5%, the endothelial cells 3,3% and the Kupffer cells 2,5% to total liver protein mass. Figure 1 shows the arrangement of the different liver cells. The parenchymal cells form plates or laminae that are interconnected to a continuous three-dimensional lattice. Bile canaliculi and blood vessels are arranged in such a way that they are strictly separated. The endothelial cells form the wall of the blood vessels, while the Kupffer cells are often located at intersections. The fat-storing cell is located in the space of Disse between the endothelial and the parenchymal cell. All cell types have direct contact with the blood, but before substances can reach the parenchymal or fat-storing cell, they first have to pass the fenestrae (average diameter 100 nm) (3) of the endothelial cell.

1.1.3. PROPERTIES OF NON-PARENCHYMAL CELLS.

The parenchymal cells contribute more than 90% to total liver protein and therefore the attention has been focussed mostly on this cell type. Much less is known about the other liver cell types, the Kupffer cells, the endothelial cells and the fat-storing cells, although Kupffer described his Sternzellen as early as 1876 (4). From the observation that Kupffer cells took up dyes, Aschoff included them in 1924 in his Reticulo-Endothelial-System (RES) of phagocytosing cells (5). Later, histochemical and morphological studies indicated that both Kupffer and endothelial cells were involved in the uptake of a variety of substances (6). The opportunity to study these cells biochemically came after the development of cell isolation procedures. Especially the introduction of the collagenase perfusion technique by Berry & Friend (7) and Seglen (8) and of the pronase perfusion technique by Mills and Zucker-Franklin (9) were important landmarks in this respect. After perfusion of the liver with collagenase the resulting cell suspension can be separated into a parenchymal and non-parenchymal cell fraction (10, 11). By pronase perfusion of the liver the parenchymal cells are destroyed leaving the non-parenchymal cells apparently intact. The availability of these techniques made it possible to indicate that non-parenchymal cells contain relatively large amounts of lysosomal enzymes when compared with parenchymal cells (12, 13, 14). Studies on the localization of isoenzymes established that the non-parenchymal cells contain a specific type of pyruvatekinase, the M-type which is kinetically different from the L-type present in parenchymal cells (15). These isoenzymes can be routinely used as markers for the presence of parenchymal and non-parenchymal cells, because their relative amount can be determined in an easy assay by measuring the effect of fructose 1-6-diphosphate on enzyme activity.The aforementioned relatively high concentration of lysosomal enzymes in non-parenchymal cells again suggests the possible involvement of these cells in the uptake and degradation of circulating compounds. This suggestion is sustained by a large number of studies which demonstrated the uptake by non-parenchymal cells of a wide diversity of substances e.g.: lipid emulsions, erythrocytes, modified albumin and lipoproteins (for review see 16). The specific celltypes within the non-parenchymal cell preparations, involved in these processes, have however not always been indicated. The assignment of a certain process to Kupffer or endothelial cells or both might have important implications for the mechanism of uptake.

1.1.4.ISOLATION OF KUPFFER AND ENDOTHELIAL LIVER CELLS.

As mentioned in the previous section the availability of non-parenchymal liver cell preparations enabled the study of the contribution of these cell types to total liver functioning. However a further separation into Kupffer and endothelial cells was only attempted for occasionally. One of the first attempts to obtain pure Kupffer cell preparations was performed by Wattiaux et al. (17). The capacity of the Kupffer cells to phagocytose colloidal iron was applied to purify them, from a suspension of liver cells, by using a magnet (18). However the viability of cells obtained in this way is doubtfull (17).

The property of Kupffer cells to adhere to plastic dishes, while endothelial cells need a more complex adherence matrix, was used by Munthe-Kaas in order to obtain pure Kupffer cell cultures (19). The first direct separation of freshly isolated non-parenchymal cells into endothelial and Kupffer cells was reported by Knook and Sleyster (14). Initially the parenchymal cells were destroyed by pronase and the non-parenchymal cell fraction purified by centrifugation through a density cushion of Metrizamide and separated into an endothelial and Kupffer cell fraction by centrifugal elutriation. (This technique employs the elutriation rotor, cells are washed into the spinning rotor where they are subjected to the centrifugal force and a counterforce generated by pumping liquid from the peripheral tip of the rotor-chamber towards the center. By increasing the

pump speed, cells of increasing size are washed out of the rotor and can be collected).
Cells obtained in this way are suitable for biochemical and morphological studies. However the interaction of exogenously added substances with receptors on the plasma membrane of the cells cannot be directly studied because the pronase treatment will destroy ectoproteins including receptors. The receptor concentration might be restored by culturing the Kupffer cells (18) but it is not certain that the properties of the cultured cells will reflect those of the cells *in vivo*.
Therefore a technique was necessary providing pure endothelial and Kupffer cells suited for binding and uptake studies directly after isolation. Furthermore it was felt necessary to determine the relative contribution of the various liver cells to the uptake of various substances *in vivo*. An isolation procedure was therefore adapted by which the level of *in vivo* cell associated substances was not affected during isolation.

1.2. lipoproteins

In plasma, lipids are transported as integral components of several macromolecular lipidprotein complexes (lipoproteins), which possess characteristic sizes, densities and compositions. All lipoproteins contain protein components (apoproteins), and polar lipids on the surface, which surround a neutral lipid core. Major apoproteins are apo A-I, apo B, apo C and apo E (20).
The apoproteins are distributed differently among the various lipoproteins which, according to their density, based on the lipid/protein ratio, are subdivided into chylomicrons, very low density lipoprotein (VLDL; $d < 1.006$), low density lipoprotein (LDL; $1.019 < d < 1.063$) and high density lipoprotein (HDL; $1.063 < d < 1.13$). (d is expressed as g/ml).
The intestine synthesizes the chylomicrons which carry dietary triglyceride from the intestine to non-hepatic tissues, where they are utilized or stored. Lipoprotein lipase, localized at the surface of extrahepatic endothelial cells, hydrolyses the triglycerides from the chylomicrons. During this extrahepatic metabolism of chylomicrons to so-called chylomicron remnants the apoprotein composition changes and the resulting chylomicron remnant is enriched in apo E and has lost most of its C apolipoproteins. This remnant is subsequently taken up by the liver (21).
VLDL is primarily synthesized by the liver and its extrahepatic metabolism is in general similar to that of chylomicrons. After extrahepatic catabolism by lipoprotein lipase, the resulting VLDL-remnants are, in rats, removed by the liver (22). In humans, the VLDL-remnant is catabolized further to LDL which is the major cholesterol-carrying lipoprotein. LDL isolated from human plasma contains solely apo B while rat LDL preparations contain in addition apo E and apo C. Both in rats and pigs, the liver is the major organ for LDL uptake with a relative percentage of the total catabolism of about 70% (23, 24).
In rats, the majority of the cholesterol in plasma is carried in HDL. The major apoproteins of HDL are apo A-I and A-II. As minor apoproteins apo E and C are present and a further subfractionation of the HDL particles is possible with different relative amounts of these apoproteins. The tissues responsible for HDL catabolism are not clearly defined although recent studies of Munford et al. (25) and Glass et al. (26), indicate that the liver and the kidneys are the most important sites for uptake of the apoproteins of HDL.

1.3. the liver and lipoproteins.

1.3.1. LIPOPROTEIN RECEPTORS

The interaction between liver cells and lipoproteins is mediated by protein molecules, termed receptors. The receptors are located at the plasma membrane where they can interact with the apoprotein part of the circulating lipoproteins. In the liver four types of receptors are now identified; I the remnant (apo E receptor), II the HDL (apo A-I) receptor, III the LDL (apo B,E) receptor and IV the scavengerreceptor. The cellular localization of these

receptors is however largely unknown. When rat-LDL or rat-HDL is injected *in vivo* it was found that 7 times more of the lipoproteins was associated with the non-parenchymal cells than with the parenchymal cells, expressed per mg protein (10). This relative uptake in the various cell types suggested already an important role of the non-parenchymal cells in lipoprotein metabolism.
In this thesis experiments are described which were performed to determine the cellular localization of the LDL (apo B,E) receptor and the "scavenger-receptor" in the liver. The characteristic properties of LDL are already discussed in the section lipoproteins. The possible relevance of the "scavenger-receptor" will be discussed below.

1.3.2. THE SCAVENGER-RECEPTOR

The existence of this receptor was proposed and demonstrated by Brown and Goldstein (27). This proposition was based upon two types of observations.
I: When LDL accumulates to high levels in human plasma, LDL cholesterol is deposited at widespread sites in the body. Especially in people suffering from homozygous familial hypercholesterolemia these depositions are found. Due to a genetic defect these people cannot synthesize functional LDL-receptors. The cholesterol accumulates primarily in macrophages. Because in these patients the LDL could not have been introduced into the macrophages by the LDL receptor pathway, Brown & Goldstein (27) suggested the presence of another receptor on these cells, which could be responsible for the cholesterol deposition. It was suggested that this receptor could recognize LDL that was modified during the prolonged circulation of LDL in the blood of these patients.
II: After in vitro incubation of control-macrophages with LDL no cholesterylester deposition is found. This restriction of cholesterol accumulation in vitro is not only a consequence of the fact that macrophages possess little LDL receptors but also by the finding that these receptors are downregulated at a high cholesterol content of the cell. Cholesterol deposition in these cells could only be provoked by incubation with a chemically modified form of LDL. The LDL was modified by acetylation with acetic anhydrid resulting in a more negative charge of the lipoprotein. Subsequent experiments indicated that macrophages express a specific receptor for acetylated LDL the so-called "scavenger-receptor". For reason that this receptor is not or poorly down-regulated (28) high concentrations of cholesterol can be deposited in the macrophage.In the cell, the cholesterol is esterified by acyl CoA: cholesterolacyltransferase (ACAT) to cholesterylesters which are stored in droplets in the cytoplasm. In this way, macrophages can transform into "foam cells" seen in tissues of patients with high plasma LDL levels. The lysosomal hydrolysis of the LDL cholesterylester, followed by re-esterification in the cytosol, is described as the so-called two-compartment model (Fig. 2). The initial hydrolysis of the cholesterylester by acid lipase is followed by re-esterification of aprox. 50% of the liberated cholesterol, at least under these experimental conditions, whereby exogeneously added fatty acids are used as acceptor.

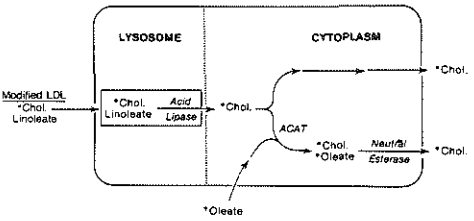

FIGURE 2.

Two-compartment model for cholesteryl ester metabolism in macrophages.
The hydrolysis and re-esterification of cholesteryl esters of modified LDL was demonstrated experimentally by incubating macrophages with ^{14}C-oleate and acetyl-LDL radiolabeld with ^{3}H-cholesteryl linoleate and showing that the cholesterylesters that accumulate within the cytoplasm contain ^{14}C-oleate. From ref. 27.

When the acetylated LDL is injected into rats it is cleared from the circulation by the liver within three minutes (29). This implicates the presence of an active scavenger mechanism in liver which could possibly prevent the interaction of acetyl-LDL with macrophages at other sites of the body. The regulation of the scavenger activity inside the liver may therefore have an important consequence for the formation of foam cells and therefore for the development of atherosclerotic lesions. Part of this thesis is therefore focussed on the protective properties of the liver against the presence of potentially atherogenic lipoproteins in the blood.

RESULTS AND DISCUSSION

2.1. cell isolation procedures for *in vivo* and *in vitro* studies

Freshly isolated non-parenchymal cells obtained by collagenase perfusion and purified by differential centrifugation possess intact lipoprotein receptors (10). In order to obtain pure Kupffer and endothelial cells for receptor studies, it was decided to use this fraction as starting material for further cell purification, applying centrifugal elutriation. Initially this cell fraction was subjected to Metrizamide density gradient centrifugation to remove debris and erythrocytes and subsequently the cell suspension was subjected to centrifugal elutriation to separate endothelial and Kupffer cells according to their difference in size. However biochemical characterization of the obtained cell fractions, done by determination of the type of pyruvatekinase isoenzyme present, indicated the presence of parenchymal cell protein in the isolated endothelial and Kupffer cell fraction. Furthermore the fraction collected at a flowrate of 12 ml/min and rotorspeed of 2500 rpm contained almost exclusively the L-type isoenzyme wich is characteristic for parenchymal cells. These results were in contrast with those from light microscopical examination of this fraction which indicated the total absence of cells with a similar size as parenchymal cells. To explain these apparently contradictional results it was supposed that during the cell isolation parenchymal cells may form particles (blebs) which are tied off and subsequently arrive in the non-parenchymal cell preparations.

Electron microscopical examination (by M. Prado-Figueroa from U.C.L. Brussel and A.M. de Leeuw from REPGO-TNO, Rijswijk) indeed demonstrated the presence of particles in the non-parenchymal cell preparation with a similar size as endothelial and Kupffer cells. Further electronmicroscopical characterization of the purified particles (Appendixpaper I) showed the absence of nuclear material in all particles, some contained a mitochondrion or other cell organelles whereas others were completely translucent. Biochemical examination of the purified blebs confirmed the presence of cytoplasmatic enzymes, varying amounts of marker enzymes for the different cell organelles and a relative enrichment of plasma membrane enzymes as compared to parenchymal cells.

In order to prevent the contamination of the endothelial and Kupffercells with blebs the cell isolation procedure was modified by introducing a second elutriation step in an early stage of the isolation procedure. In this step all parenchymal cells are removed and therefore no blebs with a similar size as endothelial and Kuppfer cells are formed during the further cell purification. A scheme of the isolationprocedure is given in appendixpaper II.

Studies on the *in vivo* interaction between components in the blood and liver cells were hampered by the cell isolation procedure which was performed at 37°C after the injection of radiolabelled compounds. Consequently during cell isolation, part of the cell-associated radioactivity was either degraded or released, leading to a non- quantitative recovery in the purified cells of the cell-associated material originally present. By the development of a so-called low temperature cell isolation procedure by Praaning-Van Dalen (30), this problem is circumvented. Initially the

liver is perfused with a pronase containing buffer solution of 8°C, followed by a further incubation at 8°C. Also the other steps of the isolation procedure are performed at a low temperature. One of the consequences of the low temperature is that all metabolic processes in the cell, including lysosomal degradation proceed at a very low rate. Therefore, the amount of in vivo endocytosed material present in the cells at the start of the perfusion will not change during the subsequent isolation to pure cell fractions. In addition to this procedure for the isolation of endothelial and Kupffercells, we introduced a low temperature isolation technique for the isolation of parenchymal cells. To check whether there was indeed no degradation during the cell isolations a small lobule was tied off just prior to the perfusion with the proteolytic enzymes whereafter the different cell types were isolated with the "cold techniques". After determination of the radioactivity in the isolated cells the amount of radioactivity that should have been present in the liver can be calculated, by taking into account the contribution of each cell type to total liver protein. Comparison of this value with the actually determined amount in the lobule indicated that the recovery of radioactivity in the isolated cells was quantitative.
Moreover, this isolation technique enables to follow *in vitro* the metabolism of *in vivo* endocytosed material. The blockade of metabolism during the isolation procedure can be abolished by warming the cells to 37°C. Incubation of the cells at 37°C for different time-intervals followed by quantification and identification of cell-associated and secreted radioactive material allows determination of the intracellular processing of endocytosed substances.

2.2. in vivo and *in vitro* interaction of acetyl- and native LDL with the different livercell types.

In appendixpaper III the *in vitro* binding and degradation to, resp. in, non-parenchymal cells of acetyl and native LDL, are compared. It appeares that the association of acetyl-LDL to the non-parenchymal cell fraction is twice as high as the binding of native LDL but that acetyl-LDL is degraded at a 30-50 fold higher rate. The high efficiency of acetyl-LDL degradation by the non-parenchymal cells suggests an active role of these cell types in the catabolism of modified lipoprotein.
Therefore, the relative importance of the parenchymal and non-parenchymal cells for the *in vivo* uptake of acetylated LDL was determined. Ten minutes after injection of iodine labeled acetyl-LDL into rats, the isolated non-parenchymal cells contained approximately 30 times more iodine-label than the parenchymal cells (per mg protein). Also a comparison of the interaction of acetyl-LDL with isolated parenchymal and non-parenchymal cells showed that the non-parenchymal cells degraded acetyl-LDL at a fifty times higher rate than parenchymal cells (appendixpaper IV).
However, the question remained which cell type in the non-parenchymal cell fraction was responsible for this high activity. To specify the cell type the *in vivo* uptake of acetyl- and native LDL was determined by isolating the Kupffer and endothelial cells by a low temperature procedure as described in section 2.1. ^{125}I-labelled acetyl-LDL was injected into rats and ten minutes thereafter a cell isolation was started. In appendixpaper II it is shown that the liver endothelial cells are by far the most active in the *in vivo* uptake of acetyl-LDL. Five times, resp. thirty times more accumulates in the endothelial cells than in the Kupffer resp. parenchymal cells.
The distribution of the ^{131}I-labelled native LDL between the different liver cell types ten minutes after injection is shown in appendixpaper VI.It appears that the Kupffer cells are the most active cell types in the *in vivo* uptake of native LDL. They contain five times more radioactivity than the endothelial cells and thirty times more than the parenchymal cells. The same relative importance of the different cell types in the *in vivo* uptake of acetyl- and native LDL is observed thirty minutes after the injection of the liproproteins (not shown). However, for reason that endothelial and Kupffer cells represent only

3,3% and 2,5% resp. of total liver protein, it can be calculated that from the total amount of acetyl-LDL associated with the liver approximately 50% is associated with endothelial cells and 50% with parenchymal cells. A similar relative distribution is found for the LDL association, 50% is associated with the Kupffer cells and 50% with the parenchymal cells.

2.3. nature of interaction sites

Section 2.2. described the interaction of acetyl-LDL and native LDL with the livercells. In the present section the nature of these interaction sites is discussed. A number of observations indicate that the interaction of both lipoproteins with the cells is mediated by a receptor. In the case of acetyl-LDL these observations are: Isolation of endothelial cells with pronase and subsequent incubation with acetyl-LDL results in decreased binding as compared with cells isolated with collagenase. Incubation of endothelial cells with increasing amounts of acetyl-LDL shows that the cell-association is saturable and possesses high-affinity characteristics. Similarly incubation of Kupffer cells with increasing amounts of LDL shows that cell-association proceeds in a high-affinity saturable fashion.

The specificity of the receptors was determined by incubating the cells with the radiolabelled lipoproteins and various amounts of different unlabelled lipoproteins. Incubation of endothelial cells with radiolabelled acetyl-LDL and increasing amounts of unlabelled acetyl-LDL, showed that an excess amount of unlabelled acetyl-LDL could reduce the binding to 20% while an excess of native LDL was ineffective (appendixpaper II). Incubation of Kupffer cells with radiolabelled native LDL and increasing amounts of unlabelled native LDL reduced the binding of the labelled lipoprotein to 50% while unlabelled acetylated LDL was ineffective (appendixpaper V). Moreover the ability of rat-LDL and rat-HDL to compete with acetyl-LDL for binding to non-parenchymal cells was tested. It appeared that both were ineffective (appendixpaper IV). Therefore these data indicate that the interaction of both acetyl-LDL and native LDL proceeds by specific high-affinity receptors.

2.4. substrates for the scavenger-receptor

Initially the presence of the scavenger-receptor was tested by the ability of cells to interact with acetyl-LDL, acetoacetylated LDL or malondialdehyde treated LDL. However the occurence of such chemically modified particles *in vivo* is unlikely. A more relevant modificaton of LDL leading to recognition by the scavenger-receptor was reported by Henriksen et al. (31). After *in vitro* incubation of LDL with umbilical vein endothelial cells (EC-modified LDL) the particle is recognized by macrophages. Acetyl-LDL competes with EC-modified LDL for binding, indicating that EC-modified LDL is recognized by the scavengerreceptor on macrophages. In appendixpaper VI the experiments are described which served to test whether this biologically modified LDL also interacted with the liver scavenger-receptor. As described by Henriksen et al.(31), the incubation of the LDL with umbilical vein endothelial cells led to a time-dependent shift to a higher density and a more negative charge of the particle. We found (appendixpaper V) that this change in physical properties is accompanied by an enhanced serum decay and liver uptake of the lipoprotein after injection into rats. Determination of the liver cell types responsible for the increased liver interaction revealed that EC-modified LDL is recovered at the same hepatic sites as acetyl-LDL, i.e. the endothelial and the parenchymal cell. Also the relative distribution between these cell types was the same, approx. thirty times more EC-modified LDL was associated with the endothelial cell than with the parenchymal cell (per mg protein).

To test whether the EC-modified LDL was recognized by the same receptor as acetyl-LDL endothelial cells were incubated with radiolabelled EC-modified LDL and increasing amounts of unlabelled acetyl-LDL or EC-modified LDL. It was found that acetyl-LDL was even a

better competitor for the binding of EC-modified LDL to endothelial cells than EC-modified LDL itself. These experiments prove that it is possible to modify LDL in a biological system to such an extent that it is recognized by the liver scavenger-receptor. Whether it is the change in density or in charge, or both, or some other yet unknown changed property of the LDL that determines the recognition by the scavenger-receptor remains to be established.

2.5. degradation of acetyl-LDL

The experiments of Brown & Goldstein (27) indicated that in macrophages degradation of endocytosed acetyl-LDL takes place in the lysosomes. This proceeds at a low pH and therefore agents that increase the lysosomal pH are well suited for blocking degradation. Such agents are chloroquine and NH_4Cl. By addition of chloroquine or NH_4Cl to endothelial cells *in vitro* the degradation of the apoprotein of acetyl-LDL was blocked for 90%, indicating that acetyl-LDL is degraded by the endothelial cells within the lysosomes (appendixpaper II). Also the hydrolysis of cholesterylesters from acetyl-LDL, takes place in the lysosomes of macrophages (29). In the section 1.3.2 a two-compartmentmodel is discussed. This model predicts that in the absence of exogeneous acceptors for the free cholesterol a re-esterification will occur leading to storage of cholesterylester droplets in the cytoplasm. In order to determine the mechanism by which livercells metabolize *in vivo* the cholesterylesters from acetyl-LDL radiolabeled cholesterylesters were incorporated into acetyl-LDL, injected *in vivo* into rats and at different time-intervals after injection cell isolations were started. Initially the technique for the introduction of cholesterylester directly into LDL as described by Krieger (32) was used. However in our hands the resulting LDL underwent some modification resulting in a much faster serum decay after injection into rats than native LDL (T 1/2 = 10 min vs. 4h). Therefore, we developed an alternative technique for the incorporation of cholesterylesters into LDL (appendixpaper VII).This technique includes, the extraction of neutral lipids from HDL which are then replaced by radiolabelled cholesterylesters (radiolabelled both in the cholesterol and the fatty acid moiety), subsequently the reconstituted HDL is incubated with LDL in the presence of cholesterylester-transfer protein. After re-isolation of the LDL the radiolabelled cholesterylesters now have the same half-life in serum as the apoprotein moiety of LDL. The LDL was acetylated and injected into rats. It was found that at short time intervals after injection (10 min) both the cholesterol and the fatty acid were recovered at the same hepatic sites and with the same relative distribution as the apoprotein of acetyl-LDL i.e. the endothelial and the parenchymal cell (appendixpaper VII). However at longer time intervals after injection the fatty acid that originates from cholesterylester directly taken up by the parenchymal cell is used for biosynthetic reactions. The fatty acid liberated in the endothelial cell is however secreted and transferred to the parenchymal cell were it is than also used for biosynthetic reactions. In both cell types the cholesterol remained in the free form, probably because it is diluted into a pre-existing pool of free cholesterol.

In studies with macrophages, which possess an active scavenger receptor, it has been shown that approximately 50% of the cholesterol introduced into the cell by acetyl-LDL is converted to deposited cholesterylester. The absence of cholesterylester deposition in the endothelial cells might be caused by the cellular environment (*in vivo*) or the consequence of intracellular processing different from that in macrophages. To discriminate between these possibilities isolated endothelial cells were incubated under similiar conditions as described for macrophages (27). Endothelial cells were incubated with acetyl-LDL in the presence of exogeneous [^{14}C] oleate (which is preferentially used for esterification of the deposited cholesterylester). However only 0.3% of the intracellularly liberated cholesterol was converted to cholesterylester suggesting that, in contrast with macrophages, the

uptake of acetyl-LDL is not coupled to cholesterylester deposition in the cell. The active uptake mechanism for modified LDL inside the liver is supposed to serve as protection system against the action of these atherogenic particles at other sites of the body (27). The absence of deposition of cholesterylesters inside the endothelial or parenchymal cells after uptake of modified LDL fulfills the requirements of a protection system because the cells are fully capable to metabolize the internalized particle.

REFERENCES

1. Blouin, A., Bolender, R.P. and Weibel, E.R. (1977) J. Cell. Biol. **72**: 441-455
2. Knook, D.L. (1984) in the Handbook Series in Aging, Section D, Biological Sciences, Cell Biology (Christofalo, V.J., ed.) Vol. **2**, CRC Press, Boca Raton, Fl. in press
3. Wisse, E., De Zanger, R. and Jacobs, R. (1982) inSinusoidal Liver Cells pp 61-67 (Knook, D.L. and Wisse,E. ed) Elsevier Biomedical Press Amsterdam
4. Kupffer, C. (1876) Arch. Mikr.-Anat. Entwecklgesch.**12**: 353-358
5. Aschoff, L. (1924) Ergeb. Inn. Med. und Kinderheiltz. **26**: 1-118
6. Wisse, E. (1977) in Kupffer cells and other liver sinusoidal cells pp 33-60 (Wisse, E. and Knook, D.L. ed) Elsevier/North-Holland Biomedical Press Amsterdam
7. Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. **59**: 722-734
8. Seglen, P.O. (1979) in Methodological Surveys (B): Biochemistry vol **8**: 25-46 (Reid, E. ed) Ellis Horwood Ltd. Chichester
9. Mills, D.M. and Zucker-Franklin, D. (1969) Amer. J. Pathol. **54**: 147-166
10. Van Berkel, Th.J.C. and Van Tol, A. (1979) Biochim. Biophys. Acta **530**: 299-304
11. Ose, L., Ose, T., Reinertsen, R. and Berg, T. (1980) Exp. Cell Res. **126**: 109-119
12. Van Berkel, Th.J.C., Kruijt, J.K. and Koster, J.F. (1975) Eur. J. Biochem. **58**: 145-152
13. Berg, T. and Bomar, D. (1973) Biochim. Biophys Acta **321**: 585-596
14. Knook, D.L. and Sleyster, E.Ch. (1976) Exp. Cell Res. **99**: 444-448
15. Van Berkel, Th.J.C., Koster, J.F. and Hülsmann, W.C. (1972) Biochim. Biophys. Acta **276**: 425-429
16. Van Berkel, Th.J.C. (1982) in Metabolic Compartmentation pp 437-482 (H. Sies ed.) Academic Press Inc. London
17. Wattiaux, R., Bauhuin, P., Berleur, A. and de Duve, C. (1956) Bioch. J. **63**: 608-612
18. Rous, P. and Beard, J.W. (1934) J. Exp. Med. **59**: 577-591
19. Munthe-Kaas, A.C., Berg, T., Seglen, P.O. and Seljelid, R. (1975) J. Exp. Med. **141**: 1-10
20. Smith, L.C., Pownall, H.J. and Gotto, A.M. (1978) Ann. Rev. Biochem. **47**: 751-777
21. Groot, P.H.E., Scheek, L.M., Dallinga-Thie, G.M., Van Tol, A. and Van Berkel, Th.J.C. (1981) Adv. Physiol. Sci. **12**: 505-514
22. Van Tol, A. and Van Berkel, Th.J.C. (1980) Biochim. Biophys. Acta **619**: 156-166
23. Pittman, R.C., Attie, A.D., Carew, T.E. and Steinberg, D. (1979) Proc. Natl. Acad Sci. U.S.A. **76**: 5345-5349
24. Pittman, R.C., Attie, A.D., Carew, T.E. and Steinberg,D. (1982) Biochim. Biophys. Acta **710** : 7-14
25. Munford, R.G,. Andersen, J.M. and Dietschy, J.M. (1981) J. Clin. Invest. **68**: 1503-1513
26. Glass, C.K., Pittman, R.C., Keller, G.A. and Steinberg, D. (1983) J. Biol. Chem. **258**: 7161-7167
27. Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K. and Goldstein, J.L. (1980) J. Supramol. Struct. **13**: 67-81
28. Mazzone, T. and Chait, A. (1982) Arteriosclerosis **2**: 487-492
29. Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**: 333-337

30. Praaning-Van Dalen, D.P. and Knook, D.L. (1982) FEBS Lett. **141**: 229-232
31. Henriksen, T., Mahoney, E.M. and Steinberg, D. (1983) Arteriosclerosis **3**: 149-159
32. Krieger, M., Brown, M.S., Faust, J.R. and Goldstein, J.L. (1978) J. Biol. Chem. **253**: 4093-4101

APPENDIX PAPER I

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ISOLATION OF PARENCHYMAL CELL-DERIVED PARTICLES FROM NON-PARENCHYMAL RAT LIVER CELL PREPARATIONS

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SUMMARY

Rat liver was perfused with collagenase and the non-parenchymal cells were isolated by means of differential centrifugation. Low magnification microscopical examination indicated that in this non-parenchymal cell fraction less than 1% are parenchymal cells, whereas the observed pyruvate kinase kinetics indicated that 50% of the total amount of pyruvate kinase in this fraction is of parenchymal cell origin. The non-parenchymal cell fraction was further purified by metrizamide density cushion centrifugation followed by centrifugal elutriation. A fraction that consisted of small particles, diameter <5 μm, was collected. The pyruvate kinase activity in this fraction showed characteristics of absolute L-type kinetics and further examination of these particles, called blebs, indicated that they were of parenchymal cell origin. Determination of enzyme markers with regard to the different subcellular structures indicated that the blebs, as compared with parenchymal cells, contained lower specific activities of enzyme markers for the endoplasmic reticulum, mitochondria and especially peroxisomes. Electron micrographs indicated the complete absence of nuclei. It is suggested that the pure isolated blebs form a unique test material to study the involvement of the nucleus and/or peroxisomes in metabolic processes. The identification of these blebs in the non-parenchymal cell preparations might also explain some discrepancies in the literature about the presence of certain metabolic processes in non-parenchymal cells.

For the isolation of non-parenchymal liver cells two methods are most widely used. One method initially involves perfusion and incubation of the liver with collagenase, whereas the second makes use of the hepatocyte-destroying capacity of pronase. By using collagenase a mixture of parenchymal and non-parenchymal cells is obtained, from which the non-parenchymal cells can be purified by differential centrifugation [1–3] or on a metrizamide gradient [4]. The pronase method was originally described by Mills & Zucker-Franklin [5] and is based on the selective destruction of the parenchymal cells by pronase. The method of choice is merely determined on the kind of research one wants to perform (for review see [6]). A disadvantage of the pronase treatment is that an overnight cell culture is necessary before uptake studies can be performed, presumably because pronase destroys receptors and/or ectoenzymes [3, 7]. This makes this method less favourable to perform in vitro receptor-mediated endocytosis studies.

The collagenase treatment is considered to be relatively mild, leaving receptor-mediated uptake processes intact [1, 3]. Using this latter method, we had already noted that there was a discrepancy between the purity of the non-parenchymal cell preparation as indicated by light microscopy and the purity based upon a biochemical assay. Further subdivision of this cell preparation by centrifugal elutriation resulted in the isolation of one fraction of particles with bio-

Table 1. *Activities of cytosolic enzymes in parenchymal cells and blebs*
PEP, phosphoenolpyruvate; FDP, fructose-1,6-diphosphate

Enzyme	Parenchymal cells	*n*	Blebs	*n*
Pyruvate kinase at 1 mM PEP	17.8±2.1	4	22.7±4.1	7
Pyruvate kinase at 1 mM PEP+0.5 mM FDP	200±13.2	4	188.1±17.7	7
Fructose-1,6-diphosphatase	42.0±7.7	4	46.3±7.2	3
Lactate dehydrogenase	4 520±130	4	5 200±110	2

Enzyme activities are expressed as nmoles/min/mg protein ± SEM. Determinations were done in 10000 g supernatant of parenchymal cell and bleb homogenates.

chemical characteristics similar to parenchymal cells and a size similar to non-parenchymal cells. The characterization of these particles, which we called blebs, and which are normally present in collagenase-prepared non-parenchymal cell preparations, forms the subject of this paper.

MATERIALS AND METHODS

Chemicals

Collagenase type I and DNAse grade II were obtained from Sigma, St Louis, Mo. Metrizamide (analytical grade) was obtained from Nyegaard & Co., A/S, Oslo; glucagon from NOVO Industri, A/S, Copenhagen; and [8-^{14}C]ATP (40–60 mCi/mmol) from the Radiochemical Centre, Amersham. All other chemicals used were of the purest grade commercially available.

Cell and bleb isolation

Livers were from male Wistar rats. Throughout this study animals were used who had free access to ordinary laboratory chow and water. Cell isolation was done as described earlier [2], with some modifications. Where indicated EGTA was included in the preperfusion buffer. This had no effect on the recovery of the non-parenchymal cell fraction but led to a better yield of parenchymal cells as described by Seglen [8]. With EGTA the yield was about 70%, without 40–50%. Purity was in most cases complete, although occasionally a number of entangled endothelial cells were present.

During the collagenase incubation of the initial cell suspension 0.5 mg DNAse was included to prevent clotting and in the Hanks' buffer 10 mM Hepes was present. The non-parenchymal cell fraction obtained by differential centrifugation was suspended in 5 ml of Hanks' buffer and mixed with 7 ml 30% metrizamide. The cell suspension was divided over two Sorvall tubes. Carefully a layer of buffer was brought on top of the mixture and the tubes were centrifuged for 15 min at 600 g. The cells that had floated into the top layer plus those present at the interphase were collected and washed once. The cells were resuspended in 5 ml Hanks' buffer, aspirated in a syringe and injected into the mixing chamber of the elutriator rotor. The cells were washed into the rotor and distinct fractions were collected at different pump speeds. The fraction characterized in this paper left the elutriator rotor at a rotor speed of 2500 rpm and a flow of 13.5 ml/min. At this flow speed 150 ml was collected and the blebs were concentrated with a 10 min 400 g centrifugation. Normally the yield was between 5–10 mg protein per liver. Purity was always larger than 95%; occasionally there was a contamination with blood cells.

Glycogenolysis

Freshly isolated cells or blebs were incubated in 3 ml Hanks' buffer without glucose but with 1 mM Ca^{2+}. The final protein concentration was 1 mg/ml. During incubation, cells or blebs were kept under an atmosphere of 95% O_2 and 5% CO_2. At intervals, a 500 μl sample was taken and centrifuged for 5 min at 400 g. Glucose was determined in the supernatant fraction with ABTS according to Werner et al. [9]. Where indicated 10^{-6} M glucagon was present.

Enzyme activities

For determination of enzyme activities the final suspensions were sonicated twice for 30 sec at 21 kHz in a MSE 100 W ultrasonic disintegrator at 0°C. 1.4 mM β-mercaptoethanol was included as routine. Cytosolic enzymes as pyruvate kinase, fructose-1,6-diphosphatase and lactate dehydrogenase were tested in the supernatant of the homogenate that was centrifuged at 10000 g for 10 min at 4°C. All other enzymes were tested in the total homogenate. Pyruvate kinase, fructose-1,6-diphosphatase, adenylcyclase and adenosine formation were tested immediately after isolation. For the other enzyme determinations the final suspension was stored at −80°C. The relative amount of L-

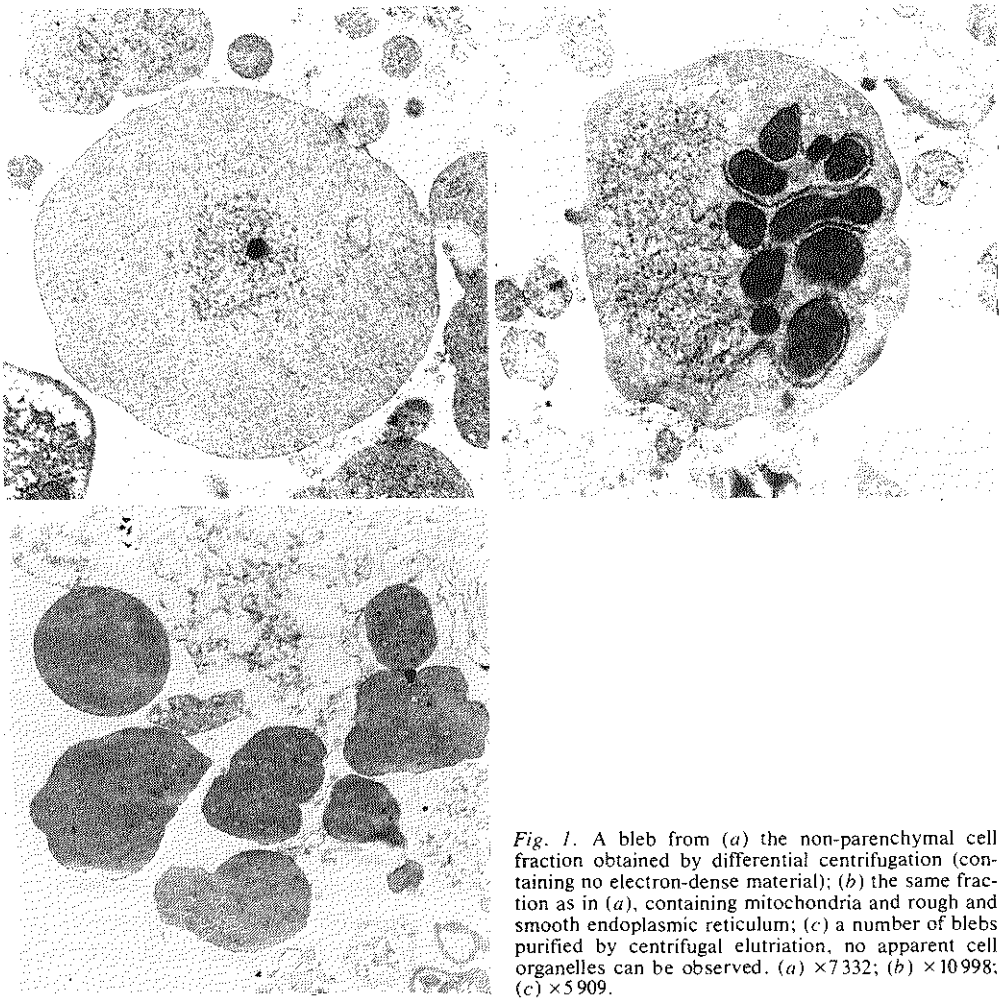

Fig. 1. A bleb from (*a*) the non-parenchymal cell fraction obtained by differential centrifugation (containing no electron-dense material); (*b*) the same fraction as in (*a*), containing mitochondria and rough and smooth endoplasmic reticulum; (*c*) a number of blebs purified by centrifugal elutriation, no apparent cell organelles can be observed. (*a*) ×7332; (*b*) ×10998; (*c*) ×5909.

or M_2-type pyruvate kinase (EC 2.7.1.40) was determined as described by Van Berkel & Kruijt [10] and fructose-1,6-diphosphatase (EC 3.1.3.11) with the method of Black et al. [11], including an AMP-trapping system. Lactate dehydrogenase (EC 1.1.1.27) was tested spectrophotometrically; final concentrations in the cuvet were: Na-K-phosphate buffer, 94 mM; Na-pyruvate, 7.6 mM; and NADH, 0.2 mM. Cyt. *c*-oxidase (EC 1.9.3.1) was determined according to Smith [12] and catalase (EC 1.11.1.6) according to Aebi [13]. The Golgi marker enzyme glucose-6-phosphatase, D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) was tested according to Harper [14]. In order to check if the phosphatase activity was specific towards glucose-6-phosphate a second test was done with β-glycerolphosphate as substrate [15]. A correction was made for the aspecific phosphatase activity. Acid phosphatase (EC 3.1.3.2) was measured as described by Robinson & Willcox [16] with 0.05 mM 4-methylumbelliferylphosphate as substrate. Cathepsin D was tested as described by Gianetto & De Duve [17]. The formation of cAMP by adenylcyclase (EC 4.6.1.1) from ATP, and the formation of adenosine from ATP was measured according to Drummond & Duncan with [^{14}C]ATP as substrate [18]. Alkaline phosphatase activity was measured with the substrate *p*-nitrophenolphosphate according to Lansing et al. [19], measurements were done on a double-beam spectrophotometer with a blank without enzyme to correct for the autohydrolysis of *p*-nitrophenolphosphate. γ-Glutamyltransferase (EC 2.3.2.2) was measured according to Seymour & Peters [39].

Table 2. *Activities of organelle-bound enzymes in parenchymal cells and blebs*

Enzyme	Parenchymal cells	*n*	Blebs	*n*
Glucose-6-phosphatase	53.9±9.8	4	44.3±15.3	4
Cytochrome *c* oxidase	228.4±9.2	3	56.8±8.5	2
Catalase	13.0±2.3	4	0.9±0.1	4
Acid phosphatase	18.5±0.8	7	9.5±0.6	10
Cathepsin D	3.3±0.4	4	3.3±0.2	4

Enzyme activities are expressed as nmoles/min/mg protein ± SEM, except for catalase which is expressed as K/mg protein ± SEM. Determinations were done in total parenchymal cell- and bleb homogenates.

Electron microscopy

Electron micrographs were prepared by Miss A. M. de Leeuw at TNO/REPGO, Rijswijk, The Netherlands. The final suspensions were fixed in 0.15 M cacodylate buffer containing 2% glutardialdehyde and post-fixed in OsO_4. Dehydrated and embedded in Epon 812, contrasted with uranyl acetate and lead citrate.

RESULTS

Morphology

Low magnification phase contrast microscopy of the total non-parenchymal cell preparation, prepared by the collagenase method, gives the impression that no cells of a size similar to parenchymal cells are present in such preparations. By centrifugal elutriation, cells or particles can be separated mainly on the basis of size differences [20]. At a centrifugation speed of 2500 rpm and a flow of 13.5 ml/min, particles with a diameter smaller than 5 μm are washed out of the rotor. High magnification phase-contrast microscopy of this fraction, derived from the total non-parenchymal cell preparation, results in the recognition of particles of various sizes and shapes without nucleus. Some particles are translucent, whereas others have a distinct granulate-like appearance. In contrast with the Kupffer cells they are not coloured by DAB-peroxidase staining and appear light blue after Papanicolou counterstaining.

The electron micrographs in fig. 1*a*, *b* show the ultrastructural appearance of the particles (further called blebs). Fig. 1*a* shows a large bleb with a diameter of 7 μm, which does not contain any cell organelles and is surrounded by a number of smaller blebs which also appear to contain no electron-dense material. The bleb on fig. 2*b* contains several mitochondria with a lining of rough endoplasmic reticulum and a region containing endoplasmic reticulum remnants. It has a diameter of 5 μm and is also surrounded by a number of smaller blebs. Fig. 1*c* displays a number of blebs from the fraction obtained with centrifugal elutriation. They all appear to lack electron-dense material, like that in fig. 1*a*. The residual material visible around the blebs, is probably caused by the instability of the blebs towards fixation, as such material is not expected to leave the elutriator rotor at this speed.

Enzyme activities

With non-parenchymal cell preparations, isolated with the aid of pronase, it has been shown that the non-parenchymal cells contain specific isoenzymes by which they can be distinguished from parenchymal cells [10]. Characteristic enzymatic activities for the different liver cell types and their intracellular organelles were determined in order to learn more about the origin and composition of the blebs. Table 1 shows a number

Table 3. *Activities of plasma membrane-bound enzymes in parenchymal cells and blebs*

Enzyme	Parenchymal cells	*n*	Blebs	*n*
γ-Glutamyltranspeptidase	4.23±0.61	3	11.12±2.16	3
Alkaline phosphatase	2.95±0.16	6	6.82±0.73	4
Adenylcyclase				
−glucagon	0.09±0.01	3	0.27±0.04	3
+glucagon	0.13±0.02	3	0.28±0.01	3
Adenosine formation	nd		6.5±0.2	3

Enzyme activities are expressed as nmoles/min/mg protein ± SEM. Determinations were done in total parenchymal cell- and bleb homogenates.
nd, not detectable.

of activities of enzymes located in the cytoplasm. They were measured in the supernatant of homogenized parenchymal cells and in the supernatant of the bleb fraction. These data show that there is no significant difference in cytoplasmic enzyme activities per mg protein between the two preparations. It is clear that the pyruvate kinase activity in the supernatant of the blebs displays specific L-type behaviour, i.e. low activity without fructose-1,6-diphosphate and a 10-fold increase when this is added [10]. Beside the glycolytic enzyme pyruvate kinase the activity of the gluconeogenic enzyme fructose-1,6-diphosphatase was measured. There is some controversy about the presence of FDPase in non-parenchymal cells [21, 22]. We detected the same amount of FDPase activity in the parenchymal cell and in the bleb preparations. To check if there was any FDPase activity in the non-parenchymal cells we measured pyruvate kinase and FDPase in other cell fractions that were collected from the elutriation rotor. We invariably found that if the pyruvate kinase activity showed explicit M_2-type kinetics, i.e. no stimulation by fructose-1,6-diphosphate, no FDPase activity could be measured. Thus, it might just be possible that FDPase activity [22] and gluconeogenesis [23] that were reported earlier in non-parenchymal cells are due to contamination of the non-parenchymal cell fraction with blebs. The lactate dehydrogenase activities in hepatocytes and in blebs are also comparable. This enzyme is frequently used as a measure for leakage of macromolecules from cells during isolation or incubation. From our data we can conclude that on basis of this criterion the isolated blebs behave as intact cells during the isolation procedure.

Table 2 shows a number of enzyme activities which are used as marker enzymes for the various cell organelles. As marker for the mitochondria cytochrome *c* oxidase was chosen. This enzyme is firmly bound to the mitochondrial inner membrane, and it has been suggested that the enzyme activity reflects the amount of mitochondria present in a certain cell type [24]. When this assumption is applied to the parenchymal and bleb preparations, it can be concluded that blebs possess only one-fourth of mitochondria per mg protein when compared with the parenchymal cells. On the other hand, the activity of glucose-6-phosphatase, a marker for the endoplasmic reticulum, is only slightly less in blebs than in parenchymal cell preparations. Also, when non-specific phosphatase activity is subtracted from the glucose-6-phosphatase activity, the blebs contain 83% of the activity found in parenchymal cells. A dramatic decrease

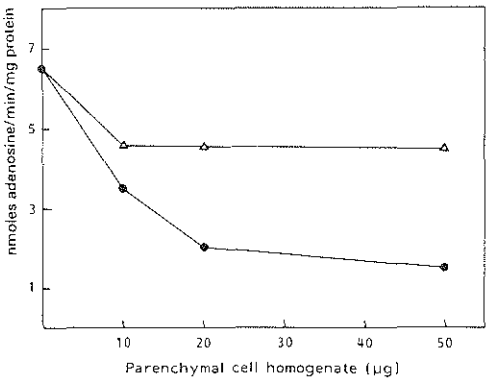

Fig. 2. Effect of addition of parenchymal cell homogenate on adenosine formation by blebs, ●, Parenchymal cell homogenate; △, homogenate heated for 5 min at 100°C.

is seen in the catalase activity, the marker enzyme for peroxisomes [25]. The blebs appear to contain only 7% of the activity found in parenchymal cells.

The activity of two lysosomal enzymes is also shown in table 2. As can be seen from this table, no unambiguous conclusion can be drawn about the lysosomal content of the blebs. The cathepsin D activity in parenchymal cell- and in bleb homogenates is of the similar magnitude, whereas the acid phosphatase activity in the bleb homogenate is half of that of parenchymal cells. Sleyster & Knook [26] showed that parenchymal cells contain multiple forms of acid phosphatase. One explanation for the decreased activity of acid phosphatase in the bleb fraction could be that the blebs do not contain all of the multiple acid phosphatase forms normally present in parenchymal cells. Cathepsin D activity is restricted to the lysosomes, thus it appears that blebs and parenchymal cells contain the same amount of lysosomal activity per mg protein.

Table 3 shows the activities of enzymes representing the plasma membranes. It can be expected that, due to their smaller size, blebs contain plasma membrane activities 3–4 times higher than parenchymal cells. However, another complication is that the enzymes of the plasma membrane are not homogeneously distributed over the entire membrane. In situ there are three topographically distinct areas on the hepatocyte plasma membrane, i.e. blood-sinusoidal, lateral and bile-canalicular [27]. Wisher & Evans [28] prepared plasma membranes from isolated parenchymal cells and found that enzyme activities belonging to the different membrane areas were recovered at the same density in a sucrose gradient as those prepared from whole rat liver. From this they concluded that in isolated cells the distinct domains are also preserved. Bearing this in mind, we tested a number of enzyme activities representing the distinct plasma membrane domains: i.e. alkaline phosphatase and γ-glutamyl-transpeptidase for the bile-canalicular region and glucagon-activated adenylcyclase for the blood–sinusoidal region. We intended to investigate the possibility that when these domains are preserved in isolated cells, one of these would preferentially form blebs which would give unique test material to study processes happening at that particular membrane region. This expectation was not borne out, as can be seen from the data shown in table 3. All tested enzyme activities are about equally enriched in the bleb fraction. There is one exception—the formation of adenosine from ATP, which is under our test conditions probably exerted by the combined action of nucleotide pyrophosphatase and 5'-nucleotidase. This process occurs quickly in the blebs, whereas it is hardly detectable in parenchymal cell homogenates. Moreover, it also proceeds at the same high rate in 'intact' blebs. When the adenosine formation is as-

Table 4. *Hormone-sensitive glucose production in parenchymal cells and blebs*

Substrate	Parenchymal cells	*n*	Blebs	*n*
None				
−glucagon	50± 6	2	7.3±3.9	3
+glucagon	110±13	2	37.5±4.5	2

Glucose production is expressed in nmoles/h/mg proyein ± SEM.

sayed at equal protein concentration in bleb- and parenchymal cell homogenate, a 300 times higher activity is found in the bleb homogenate. However, when protein dependency was tested, it appeared that the low activity in the parenchymal cell homogenates was caused by the presence of an inhibitor. When small amounts of parenchymal cell homogenate are added to blebs, it can be seen (fig. 2) that the adenosine formation of the blebs also decreases severely. This inhibitory activity is temperature-sensitive. When the parenchymal cell homogenate is boiled for 5 min at 100°C the inhibiting ability is greatly diminished.

Glycogenolysis and stability of the blebs upon incubation

Frequently it has been reported that isolated parenchymal liver cells indeed do form cellularly bound blebs when they are in a bad metabolic condition. So it can be questioned if a modification of the parenchymal cell isolation procedure could lead to a change in the amount of blebs [6]. It appears that such modifications as temperature of collagenase perfusion, MgEGTA preperfusion, exclusion of Ca^{2+} from collagenase perfusion does not significantly influence the amount of blebs formed. To verify further the intactness and viability of both parenchymal cells and the blebs we have, next to the trypan blue exclusion test, determined the ability of the cells and blebs to respond to glucagon and to perform glycogenolysis (table 4). In both parenchymal cells and blebs, glucose formation from glycogen occurs which is greatly activated by glucagon. This indicates that the complex cascade to form glucose from glycogen is still present and active in the isolated blebs.

The glucose production in parenchymal cells and in blebs was linear in time up to 1 h of incubation. The stability of the blebs was further tested by incubating them up to 3 h. Adenylcyclase activity was stable for the first 2 h but was slightly less during the third hour. Alkaline phosphatase activity was stable during the whole incubation period and adenosine formation even increased slightly (data not shown).

DISCUSSION

This paper describes a vesicle which, due to its size, is isolated together with the non-parenchymal cells, i.e. the Kupffer and endothelial cells, when isolated by the collagenase procedure [2]. This vesicle contains equal amounts of cytoplasmic and lysosomal enzyme activities and less endoplasmic reticulum, mitochondrial and, especially, peroxisomal enzyme activities when compared with the parenchymal cell. The enzymes from the different plasma membrane regions were all enriched by about the same factor. It can be calculated that particles with the size of blebs will have a 4-fold higher membrane/volume ratio than parenchymal cells. The tested membrane-bound enzymes are indeed enriched by this factor. This indicates that the blebs can originate randomly from every hepatocyte membrane region. A consequence of this is that the bleb population in itself is heterogeneous. Some will have originated from

the bile-canalicular region of the hepatocyte, whereas others are formed at the blood-sinusoidal or lateral sites. Lateral movement of the domains during isolation is another possibility, i.e. loss of preservation of domains, but this is argued against by the results of Wisher & Evans [28], who observed a preservation of the domains in isolated hepatocytes. Heterogeneity of the bleb population was also observed by Cossel [29], who examined electron micrographs of 33 human liver needle biopsies. He found that bleb formation was occurring both on blood-sinusoidal as well as on the lateral and bile-canalicular sides of the hepatocyte. The formation always occurred at hepatocytes located at the periphery of the liver lobule.

Next to this study in whole liver, there are also some studies on bleb formation in vitro [30–33]. In these cases bleb formation is induced mostly by cytochalasin B, but phalloidin is also used. Reith & Seglen [30] induced this bleb formation in vitro with hepatocytes and collected the blebs. They showed that in these particles protein synthesis occurred at about the same rate as in intact liver cells. They also reported that the blebs contained more mitochondria per mg protein and that these showed a normal endoplasmic reticulum display.

From our studies we must conclude that the 'spontaneously' formed blebs contain fewer cell organelles, except for lysosomes. An explanation for this might be that the isolated parenchymal cell when it is on the edge of 'dying' attracts cell organelles to the nuclear region, creating an organelle-poor zone at the periphery of the cell. With the light-microscope this appears as a translucent ring. Electron micrographs of this phenomenon are presented in the thesis of Van Bezooyen [34].

Our present characterization of blebs formed during liver cell isolation—which was made possible by the availability of the elutriation method, enabling us to obtain them in a pure state (>95%)—offers the possibility to study the relative importance of some subcellular organelles for liver cell functioning in general and the metabolism of some compounds in particular. The absence of the nucleus makes it possible to study its importance without the application of protein synthesis inhibitors, acting (specifically?) at the nuclear level. Furthermore, the almost complete absence of peroxisomes makes it also possible to investigate their role in hydrogen peroxide metabolism and/or fatty acid oxidation [35].

It might be questioned to what extent the presence of until now unrecognized parenchymal cell-derived blebs in the non-parenchymal cell preparation might have influenced earlier studies [1, 3, 36–38]. In most studies the purity of the non-parenchymal cell preparation was only checked by low magnification (phase-contrast) microscopy. By this method the contamination of the non-parenchymal cell preparation with intact parenchymal cells can be followed. However, in such a system blebs are overlooked and a high magnification or a biochemical criterium is necessary to quantitate their presence. In fact our present findings are based upon the divergence between low magnification microscopy and the differential assay of L-type and M_2-type pyruvate kinase. It can now be emphasized that such a biochemical criterium must be met before conclusions about the purity of the non-parenchymal cell preparations can be drawn. Beside pyruvate kinase also other parenchymal cell-specific enzymatic activities as, for example, fructose-1,6-diphosphatase can be used to meet such criteria. It seems probable that discrepancies in the literature, like the absence or pres-

ence of gluconeogenesis in non-parenchymal cells can be resolved when such biochemical criteria are more frequently used.

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REFERENCES

1. Nilsson, M & Berg, T, Biochim biophys acta 497 (1977) 171.
2. Van Berkel, Th J C & Van Tol, A, Biochim biophys acta 530 (1978) 299.
3. Steer, C J & Ciarenburg, R, J biol chem 254 (1979) 4457.
4. Munthe-Kaas, A C & Seglen, P O, FEBS lett 43 (1974) 252.
5. Mills, D M & Zucker-Franklin, D, Am j pathol 54 (1969) 147.
6. Seglen, P O, Methods in cell biology (ed D M Prescott) vol. 13, p. 29. Academic Press, New York, London, San Francisco (1976).
7. Munthe-Kaas, A C, Berg, T, Seglen, P O & Seljelid, R, J exp med 141 (1975) 1.
8. Seglen, P O, Exp cell res 82 (1973) 391.
9. Werner, W, Rey, H G & Wielinger, H, Z anal chem 252 (1970) 224.
10. Van Berkel, Th J C & Kruijt, J K, Eur j biochem 73 (1977) 223.
11. Black, W J, Van Tol, A, Fernandes, J & Horecker, B L, Arch biochem biophys 151 (1972) 576.
12. Smith, L, Methods in enzymology (ed S P Colowick & N O Kaplan) vol. 2, p. 732. Academic Press, New York (1955).
13. Aebi, H, Methods of enzymatic analysis (ed H U Bergmeyer) p. 674. Verlag Chemie, Weinheim, New York (1974).
14. Harper, A E, Methoden der enzymatischen Analyse (ed H U Bergmeyer) p. 788. Verlag Chemie, Weinheim, New York (1962).
15. Schrijver, J, Koster, J F & Hülsmann, W C, Eur j clin invest 5 (1975) 7.
16. Robinson, O & Willcox, P, Biochim, biophys acta 191 (1969) 183.
17. Gianetto, R & De Duve, C, Biochem j 59 (1955) 433.
18. Drummond, G J & Duncan, L, J biol chem 245 (1970) 976.
19. Lansing, A I, Belkhode, M L, Lynch, W E & Lieberman, I, J biol chem 242 (1967) 1772.
20. Knook, D L & Sleyster, E Ch, Exp cell res 99 (1976) 444.
21. De Pijper, A, Histochemie 37 (1973) 197.
22. Crisp, D M & Pogson, C I, Biochem j 126 (1972) 1009.
23. Wagle, S R, Hofmann, F & Decker, K, Biochem biophys res commun 71 (1976) 857.
24. Klingenberg, M, Funktion und Morphologie der Zelle (ed P Karlson) p. 69. Springer-Verlag, Berlin (1963).
25. De Duve, C & Baudhuin, P, Physiol rev 46 (1966) 323.
26. Sleyster, E Ch & Knook, D L, Arch biochem biophys 190 (1978) 756.
27. Evans, W H, Biochim biophys acta 604 (1980) 27.
28. Wisher, M H & Evans, W H, Biochem j 164 (1977) 415.
29. Cossel, L, Path res pract 170 (1980) 298.
30. Reith, A & Seglen, P O, J ultrastruct res 50 (1975) 370.
31. Henius, G V, Laris, P C & Woodburn, J D, Exp cell res 121 (1979) 337.
32. Freikopf-Cassel, A & Kulka, R G, FEBS lett 124 (1981) 27.
33. Seglen, P O, Biological separations in iodinated density gradient media (ed D Richwood) p. 107. Information Retrieval Ltd, London and Washington DC (1976).
34. Van Bezooijen, C F A, Thesis, University of Utrecht (1978).
35. Lazarow, P B, Proc natl acad sci US 73 (1976) 2043.
36. Mørland, J & Olsen, H, Drug metabolism and disposition 5 (1977) 511.
37. Kreusch, J, Hofmann, F, Maier, K P & Decker, K, Hoppe Seyler's z physiol chem 258 (1977) 1513.
38. Morrone, S, Dentchev, P G, Baynes, J & Thorpe, S, Biochem j 194 (1981) 733.
39. Seymour, C A & Peters, T J, Clin sci mol med 52 (1977) 229.

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APPENDIX PAPER II

In Vivo and *in Vitro* Uptake and Degradation of Acetylated Low Density Lipoprotein by Rat Liver Endothelial, Kupffer, and Parenchymal Cells*

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Isolation and separation of rat liver cells into endothelial, Kupffer, and parenchymal cell fractions were performed at different times after injection of human ^{125}I-acetyl low density lipoproteins (LDL). In order to minimize degradation and redistribution of the injected lipoprotein during cell isolation, a low temperature (8 °C) procedure was applied. Ten min after injection, isolated endothelial cells contained 5 times more acetyl-LDL apoprotein per mg of cell protein than the Kupffer cells and 31 times more than the hepatocytes. A similar relative importance of the different cell types in the uptake of acetyl-LDL was observed 30 min after injection.

For studies on the *in vitro* interaction of endothelial and Kupffer cells with acetyl-LDL, the cells were isolated with a collagenase perfusion at 37 °C. Pure endothelial (>95%) and purified Kupffer cells (>70%) were obtained by a two-step elutriation method. It is demonstrated that the rat liver endothelial cell possesses a high affinity receptor specific for the acetyl-LDL because a 35-fold excess of unlabeled acetyl-LDL inhibits association of the labeled compound for 70%, whereas unlabeled native human LDL is ineffective. Binding to the acetyl-LDL receptor is coupled to rapid uptake and degradation of the apolipoprotein. Addition of the lysosomotropic agents chloroquine (50 μM) or NH_4Cl (10 mM) resulted in more than 90% inhibition of the high affinity degradation, indicating that this occurs in the lysosomes. With the purified Kupffer cell fraction, the cell association and degradation of acetyl-LDL was at least 4 times less per mg of cell protein than with the pure endothelial cells.

Although cells isolated with the cold pronase technique are also still able to bind and degrade acetyl-LDL, it appeared that 40–60% of the receptors are destroyed or inactivated during the isolation procedure.

It is concluded that the rat liver endothelial cell is the main cell type responsible for acetyl-LDL uptake.

In vitro binding, uptake, and degradation of modified low density lipoproteins has been reported to occur in a number of cell types. Acetylated human low density lipoprotein is taken up and degraded in large amounts by resident mouse peritoneal macrophages, rat peritoneal macrophages, guinea pig Kupffer cells, human monocytes, and, in much smaller amounts, by some other cell types (1). Also, bovine aortic endothelial and smooth muscle cells can degrade acetyl-LDL[1] (2). Malondialdehyde-altered low density lipoprotein is degraded by human monocytes-macrophages (3). The catabolism of modified lipoprotein is suggested to represent the so-called scavenger pathway (4). *In vivo*, 33–66% of LDL is degraded by receptor-mediated endocytosis, in which the receptor is specific for native LDL (5), whereas the remaining part is degraded by the scavenger pathway or other unspecified systems (6). The assumption that uptake of acetyl-LDL reflects a physiological process was recently strengthened because native LDL, incubated with cultured aortic endothelial cells, is converted into a form which is recognized by the acetyl-LDL receptor on macrophages (7). The *in vivo* cellular sites for acetyl-LDL degradation are not known although it was observed that ^{125}I-labeled acetyl-LDL injected intravenously into mice was mainly cleared by the liver (1).

The liver, however, consists of different cell types: hepatocytes, endothelial cells, Kupffer cells, and fat-storing cells. Determinations of the cell type(s) in an organ that is (are) responsible for uptake of a certain substance can be done qualitatively by electron microscopy or autoradiography. From such studies (8), it was concluded that hepatic nonparenchymal cells are responsible for the uptake of acetoacetylated LDL. However, no distinction was made between liver endothelial and Kupffer cells. Furthermore, in autoradiographic studies, the role of the parenchymal cells in uptake is easily under-estimated. The observed concentration of ingested substance in the parenchymal cells may be considerably lower when compared with nonparenchymal cells, but as the parenchymal cell contribution to total liver protein is 92.5%, their actual role in uptake may be considerable.

We performed cell isolation after *in vivo* injection of the radiolabeled substrate and determined quantitatively the contribution of the different cell type(s) to the total liver uptake. Redistribution and degradation during isolation was avoided by using a low temperature isolation procedure with pronase or collagenase.

The availability of pure cells also makes it possible to study the mechanism of uptake and degradation in a defined homogeneous system. We have therefore developed a cell isolation procedure with collagenase by which pure endothelial and Kupffer cell fractions are obtained. The isolated and purified cells can be used directly for uptake and degradation studies of acetyl-LDL.

MATERIALS AND METHODS

Cell Isolations—Throughout the study, 3-month-old male Wistar

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[1] The abbreviations used are: acetyl-LDL, acetylated low density lipoprotein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

rats, who had free access to food and water, are used. Rats are anesthetized with 18 mg of Nembutal given intraperitoneally. After cannulation of the vena porta, perfusion is started with Hanks' buffer, pH 7.4 at 8 °C or 37 °C. For *in vitro* uptake studies, cells are isolated with collagenase at 37 °C according to Fig. 1. Preperfusion, 10 min, flow rate, 14 ml/min, is done with Hanks' buffer at 37 °C. It has been advised to include EGTA in the preperfusions for a better cleaving of the desmosome junction in which Ca^{2+} plays a major role (9). However, it has been reported that the use of EGTA during cell isolation leads to an irreversible depletion of intracellular Ca^{2+} (10). We have observed that cells isolated in the presence of EGTA are less active in binding and degradation; moreover, the inclusion of 1 mM Ca^{2+} during collagenase perfusion was necessary to obtain optimally active cells. After the initial 10 min, perfusion is continued for 20 min with Hanks' buffer, containing collagenase (0.05% w/v) and 1 mM Ca^{2+}. Thereafter, the liver is excised, cut into pieces with a pair of scissors in ice-cold medium, transferred to a plastic beaker, and slowly stirred with a magnetic stirring bar in Hanks' buffer supplemented with 0.4% bovine serum albumin (without collagenase) at 0 °C. This temperature is maintained during the further isolation procedure so that the uptake in nonparenchymal cells of cell debris (from parenchymal cells) or other substances during the isolation procedure is minimal. After 5 min, the suspension is filtered through nylon gauze (mesh width, 95 μm). The remainder on the filter is resuspended in fresh buffer and the procedure is repeated. After this, almost no residue is left on the filter. The filtrate is divided over 8 Sorvall tubes and centrifuged for 15 s at 50 × *g*, after which the supernatants are transferred to 50-ml Falcon tubes. The pellets are resuspended and the whole procedure is repeated. The combined supernatants are then centrifuged for 10 min at 400 × *g*. The pellets of the 400 × *g* centrifugation (mainly nonparenchymal cells) are collected and transferred to the mixing chamber of the Beckman elutriation rotor spinning at 1800 rpm. Cells are washed into the rotor at a flow speed of 18 ml/min. At this rotor speed/flow speed ratio, all particles smaller than 13 μm in diameter are immediately washed out of the rotor. This step is necessary for removal of all the parenchymal cells from the cell mixture. As we have shown earlier (11), collagenase-prepared nonparenchymal cell preparations contain parenchymal cell-derived vesicles with a similar size as nonparenchymal cells. Complete removal of the parenchymal cells at this stage of the isolation procedure prevents the formation of vesicles which otherwise are isolated together with the endothelial and Kupffer cells. After collection of 250 ml of buffer, containing the particles smaller than 13 μm in diameter, they are concentrated by centrifugation (10 min, 400 × *g*). The pellets are resuspended in 5 ml of buffer, mixed with 7 ml of 30% Metrizamide, and divided over two Sorvall tubes. One ml of buffer is layered on top of the mixture and the tubes are spun for 15 min at 1400 × *g*. The cells which have floated into the top phase are aspirated in a syringe, injected into the mixing chamber of the elutriator rotor—this time spinning at 2500 rpm—, and different cell fractions are collected at the flow speeds indicated in Fig. 1, collected by centrifugation (10 min, 400 × *g*), and immediately used for *in vitro* experiments. With this method, about 1–2 mg of protein of pure endothelial and 1 mg of purified Kupffer cells are obtained, representing a yield of 1.5–3% and 2%, respectively. For determination of the distribution of *in vivo* injected substances between Kupffer and endothelial cells, a low temperature pronase method is used (12). The low temperature and the pronase treatment minimize degradation and redistribution, respectively. After an initial 10-min period of perfusion at 8 °C with Hanks' buffer, flow rate, 14 ml/min, a lobule is tied off for the determination of the total liver uptake. Subsequently, the liver is perfused with Hanks' buffer plus 0.25% (w/v) pronase at 8 °C for 20 min. After this step, the isolation procedure follows the same protocol as the collagenase method except that the first elutriation step is omitted because all the hepatocytes are already destroyed by the pronase treatment. With this method, 2–3 mg of protein of endothelial cells and 1–2 mg of Kupffer cells are obtained, which is a yield of 3–4.5% and 2–4%, respectively.

Determination of the *in vivo* uptake by hepatocytes is done by following initially the same protocol as with the cold pronase method but, instead of pronase, collagenase (0.05% w/v) is used. After cutting the liver, the suspension is immediately filtered and the filtrate is spun for 15 s at 50 × *g*. The pellet is washed with Hanks' buffer plus 0.4% bovine serum albumin and centrifuged again. This is repeated three times after which the final preparation was 100% pure. The yield of parenchymal cells is about 200 mg of protein representing a yield of 10%.

Data Used for Calculations on the Contribution of the Different Cell Types to Total Liver Uptake—With the strain of Wistar rats used, liver wet weight is 3.75% of the body weight (13). The wet weight and the protein content of the tied off lobule is determined and from these values total liver uptake can be calculated. The hepatocytes contribute 92.5% of total liver cell volume, the endothelial cells 3.3%, and the Kupffer cells 2.5% (13). The remaining 1.7% is contributed by the fat-storing cells; however, these are not recovered in our preparations. As the protein concentration (in milligrams per ml of cell volume) in both parenchymal and nonparenchymal cells is identical (14, 15), these relative values can be directly transferred to protein. The validity of this assumption is indicated earlier (16) by enzyme distribution studies with peroxidase (a specific marker for Kupffer cells), lysosomal enzymes, and pyruvate kinase (16) (see also Table I).

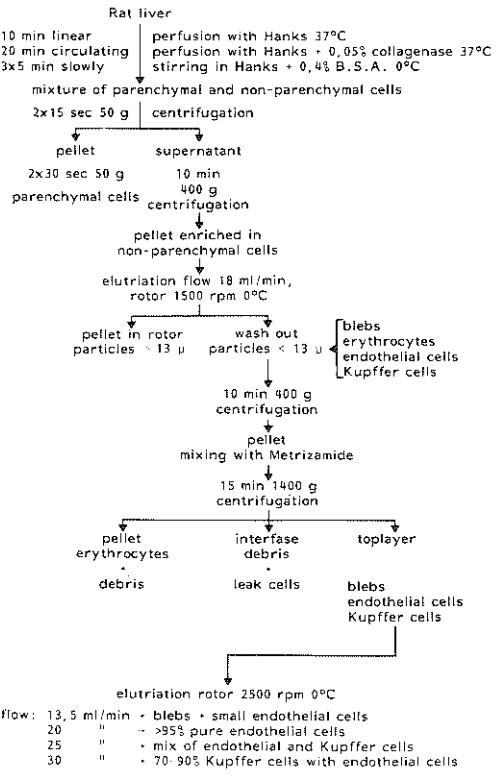

FIG. 1. **Isolation procedure for endothelial and Kupffer cells from rat liver with collagenase at 37 °C.**

Isolation, Modification, and Labeling of LDL—Human LDL is obtained from the blood of healthy volunteers, who had fasted overnight. Isolation, iodination, modification, and characterization of LDL is done exactly as described elsewhere (17). LDL was also acetylated with [^{14}C]acetic anhydride and, from the amount of ^{14}C radioactivity incorporated, it was calculated that at least 10% of the lysine residues of LDL were modified, assuming that 8% of the amino acids in the apoprotein are lysine (18).

In Vivo Uptake of Acetyl-LDL in the Different Liver Cell Types—After anesthesia of the rat, the abdomen is opened and radiolabeled lipoprotein (100–300 μg of apolipoprotein) is injected into the inferior vena cava at the level of the renal veins. After 3 min and just before the start of the perfusion, blood samples are drawn from the inferior vena cava at least 2 cm distal of the injection point. The radioactivity present in the lobule, serum, and isolated cells obtained by the 8 °C methods is counted in a LKB Wallace ultrogamma counter. Counting after injection of ^{3}H- and ^{14}C-labeled compounds was performed in a

Packard Tri-Carb liquid scintillation spectrometer after digestion of the samples with Soluene.

In Vitro Lipoprotein Binding, Uptake, and Degradation—Determination of binding, uptake, and degradation of lipoprotein by the isolated cells and control of viability of the cells during and after incubation was done essentially the same way as in Ref. 17.

Determination of Pyruvate Kinase Activity—This was done spectrophotometrically with 10,000 × *g* supernatants of cell or tissue homogenates according to Ref. 19.

Protein Determination—Protein was determined according to Ref. 20 with bovine serum albumin as a standard.

Electron Microscopy—The final cell preparations were fixed in 0.15 M cacodylate buffer containing 2% glutaraldehyde and postfixed in OsO_4, dehydrated and embedded in Epon 812, and contrasted with uranyl acetate and lead citrate.

Chemicals—Collagenase (type I) was obtained from Sigma and pronase B-grade from Calbiochem-Behring Corp. Metrizamide was purchased from Neygaard & Co A/S, Oslo, Norway; Ham's F-10 medium from Gibco-Europe, Hoofddorp, The Netherlands; [^{3}H]heparin (sodium salt), [^{14}C]acetic anhydride, and ^{125}I (carrier-free) in NaOH were obtained from New England Nuclear, Dreieich, West Germany. All other reagents were analytical grade, obtained commerically.

RESULTS

Characterization of the Isolated Cells—Purity of the different cell fractions was checked by the distribution of the isoenzymes of pyruvate kinase, the distribution of intravenously injected [^{3}H]heparin, light microscopy after 3,3′-diaminobenzedine peroxidase staining (21) followed by Papanicolaou counterstaining (22), and electron microscopy. The liver contains two forms of pyruvate kinase of which the L type is found in the parenchymal cells and M_2 type in the nonparenchymal cells (19). Distinction can be made by measuring the stimulating effect of fructose 1,6-diphosphate on the enzyme activity. Earlier, we have shown that nonparenchymal cell preparations obtained by 37 °C collagenase perfusion and differential centrifugation contain both L type and M_2 type pyruvate kinase (about 50% of both) and we demonstrated that this is caused by the presence of parenchymal cell-derived particles in the nonparenchymal cell fraction (11). By modification of the cell isolation procedure (see "Materials and Methods"), it is now possible to isolate endothelial and Kupffer cell fractions that contain solely the M_2 type pyruvate kinase activity (Table I). Determination of the endothelial cell content in the final fractions was done by isolation of cells from rats who had received [^{3}H]heparin 1 h before cell isolation. Heparin is known to interact specifically with the endothelial cells (23).

Based upon the distribution of [^{3}H]heparin radioactivity between the different fractions, leaving the elutriation rotor at different flow speeds, a discrimination was made in an endothelial cell fraction (EC), a mixed cell fraction (MF), and a Kupffer cell fraction (KC) (Fig. 2). For the light microscopical examination, the cells were stained for 3,3′-diaminobenzedine peroxidase (21), for which the Kupffer cells are positive and endothelial cells negative. To increase the gradual differences between the two cell types, the peroxidase staining was followed by a Papanicolaou counterstaining (22). Kupffer cells are colored gold-brown after this procedure while endothelial cells are deep blue. Also, the halftone plate (Fig. 3, *A* and *B*) allows an easy distinction between both cell types. Both with the cold pronase and the 37 °C collagenase method, the EC fraction contained more than 95% of endothelial cells (Fig. 3), occasionally there was a small contamination (<5%) of white blood cells. The KC fraction contained never less than 70% peroxidase-positive cells (Fig. 3), while the mean percentage of Kupffer cells was 85 ± 5% (n = 10, ±S.E.), the remainder being large or entangled endothelial cells. The MF fraction was of a varying composition of endothelial and Kupffer cells. Parenchymal cells isolated by the 8 °C collagenase method were, as tested by the pyruvate kinase assay (Table I) and light microscopical examination, completely free from nonparenchymal cells. Electron microscopical examination confirmed the intactness and relative composition of the different cell fractions (Fig. 3, *C* and *D*). It can be noticed that after isolation the sieve plates of the endothelial cells, which *in vivo* form the lining of the sinusoid, are encircled around the nuclei.

In Vivo Distribution of the Acetyl-LDL Apolipoprotein—The *in vivo* uptake of acetyl-LDL by the different liver cell types was determined by counting apolipoprotein radioactivity in cell fractions after isolation and purification at low temperature (8 °C). Fig. 4*A* shows the distribution of ^{125}I-acetyl-LDL between the different cell types isolated 10 min after injection. Expressed per mg of cell protein, the endothelial cell fraction contains 5 times more radioactivity than the Kupffer cell fraction and 31 times more than the hepatocytes. Fig. 4*B* shows the distribution of acetyl-LDL between the different cell types 30 min after injection. The endothelial cells contain 6 times more acetyl-LDL per mg of protein than the Kupffer cells and 93 times more than the parenchymal cells.

TABLE I

Pyruvate kinase isoenzyme activities in the different cell preparations

M_2-pyruvate kinase, not being stimulated by fructose-1,6-P_2, is the only isoenzyme present in the EC, MF, and KC preparations. The PC fraction contains only the L-type isoenzyme (18). The number of experiments is given in parentheses. The S.E. is indicated. Measurements were done as described in Ref. 18. EC, endothelial cell; MF, mixed fraction; KC, Kupffer cell; WRL, whole rat liver; PC, parenchymal cell.

Source of homogenate	Activity at	
	1 mM P-enolpyruvate	1 mM P-enolpyruvate + fructose-1,6-P_2
	nmol/min/mg protein	
EC	105.0 ± 1.5 (3)	102.6 ± 1.5 (3)
MF	67.3 ± 2.9 (3)	65.0 ± 5.1 (3)
KC	66.3 ± 1.2 (3)	62.0 ± 2.1 (3)
WRL	20.0 ± 1.5 (6)	117.1 ± 8.3 (6)
PC	13.1 ± 1.3 (4)	133.4 ± 7.5 (4)

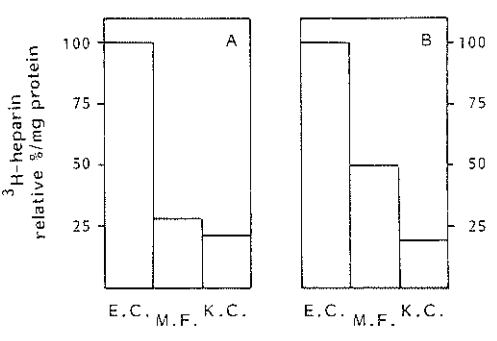

FIG. 2. **Determination of the distribution of intravenously injected [^{3}H]heparin between endothelial and Kupffer cell fractions after cell isolation with pronase at 8 °C (*A*) or collagenase at 37 °C (*B*).** Each value represents the average of two independent determinations, which were within 10% of each other. Cell isolation was started 60 min after heparin injection. *E.C.*, endothelial cell; *M.F.*, mixed fraction; *K.C.*, Kupffer cell. The parenchymal cell value is 0.8%, and of whole rat liver 3.3%. Heparin content per mg of cell protein in the isolated endothelial cells was taken as 100% value.

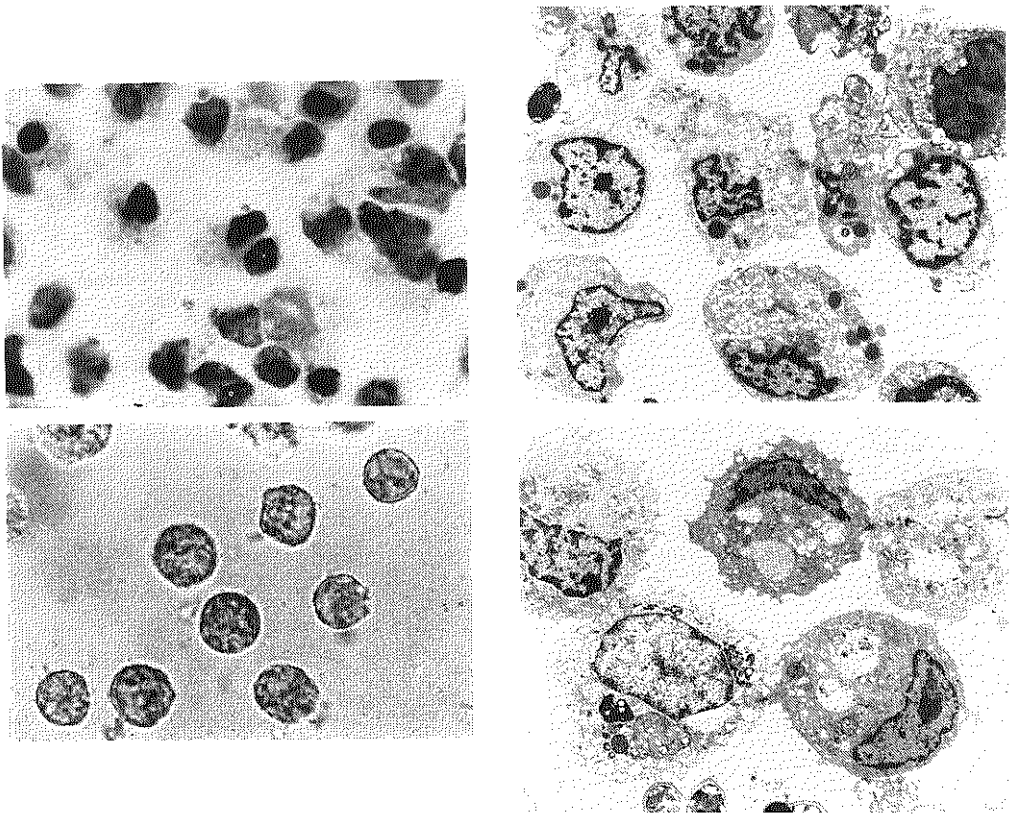

FIG. 3. **Light and electron photomicrographs of the isolated cell fractions.** *A* and *B*, light photomicrographs of the isolated endothelial cell fraction (*A*) and Kupffer cell fraction (*B*) after 3,3'-diaminobenzedine peroxidase staining and Papanicolaou counterstaining. The cells were obtained with the 8 °C pronase method. *C* and *D* electron photomicrographs of the endothelial cell fraction (*C*) and of the Kupffer cell fraction (*D*). Cells were obtained with the 37 °C collagenase method.

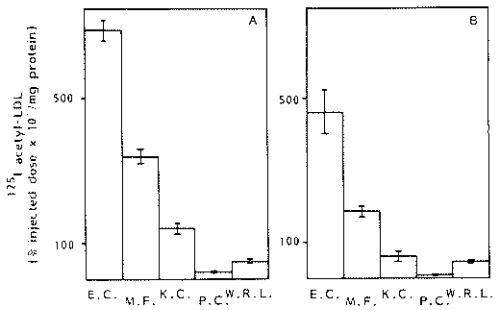

FIG. 4. **Distribution of ^{125}I-acetyl-LDL between endothelial and Kupffer cell fractions after isolation with pronase at 8 °C and parenchymal cell after isolation with collagenase at 8 °C.** Acetyl-LDL was injected intravenously as one single bolus and allowed to circulate for 10 min (*A*) or 30 min (*B*). Each value represents the average of three cell isolations ± S.E. Values are expressed as % injected dose × 10^5 per mg of cell protein. *E.C.*, endothelial cell; *M.F.*, mixed fraction; *K.C.*, Kupffer cell; *P.C.*, parenchymal cell; and *W.R.L.*, whole rat liver.

TABLE II

Relative contribution of the different liver cell types to the total uptake of acetyl-LDL by rat liver

For each individual animal, the amount of apoprotein per mg of cell protein in the isolated cell fractions was multiplied with the amount of mg of protein that each cell type contributes to total liver protein. The values are expressed as percentage of total injected dose and are the means ± S.E. of 3 determinations.

	Acetyl-LDL	
	10-min circulation time	30-min circulation time
Whole liver	78.6 ± 4.5	46.0 ± 6.6
Parenchymal cells	36.5 ± 5.5	9.9 ± 1.6
Endothelial cells	50.6 ± 5.4	34.9 ± 6.0
Kupffer cells	7.0 ± 1.6	3.0 ± 0.8

Table II shows the contribution of the whole population of the various cell types to total liver uptake, taking into account the relative protein contribution of the different cell types to total liver *in vivo* (see also "Materials and Methods"). It demonstrates that both endothelial and parenchymal cells are responsible for the active uptake of acetyl-LDL by the liver. As the Kupffer cell fraction is contaminated with endothelial

cells (5–30%), the actual contribution of the Kupffer cells to the total liver uptake, as indicated in Table II, may be overestimated.

Furthermore, Table II shows that the recovery of acetyl-LDL in the isolated cell fractions is quantitative as compared with the whole liver. This quantitative recovery was ascertained by the strict maintenance of a low temperature during the cell isolation procedure.

For the study of catabolism of *in vivo* endocytosed acetyl-LDL and to compare our present results with data upon *in vivo* distribution, obtained with a 37 °C isolation technique, the initial level and catabolism of acetyl-LDL by endothelial cells isolated by both methods 10 min after *in vivo* injection of ^{125}I-acetyl-LDL was compared (Fig. 5). Endothelial cells isolated at 8 °C contain 4 times more radioactivity than cells isolated at 37 °C. Furthermore, the cell-associated radioactivity of cells isolated at 8 °C is rapidly released in the acid-soluble water phase (non-iodide) by subsequent incubation at 37 °C. This indicates that the intracellular degradation system remains intact upon isolation. (Homogenization of the cells and subsequent incubation at 37 °C did not lead to the detection of acid-soluble radioactivity).

To exclude the possibility that iodination might induce recognition by nonparenchymal cells, we have also investigated the fate of LDL that had been acetylated with [^{14}C] acetic anhydride ([^{14}C]acetyl-LDL). After intravenous injection, a similar cellular distribution was obtained as with ^{125}I-acetyl-LDL (not shown). This indicates that solely the acetylation procedure determines the fate of the modified lipoprotein.

In Vitro Interaction and Degradation of Acetyl-LDL with Isolated Endothelial and Kupffer Cells—In these experiments, nonparenchymal cells isolated both with the 37 °C collagenase method or with the 8 °C pronase method were applied. For the isolation with collagenase, the temperature during the liver perfusion is kept as exactly 37 °C in order to assure an optimal action of the collagenase. However, immediately after perfusion, the temperature is brought to 0 °C in order to prevent uptake of cell debris from the total cell suspension, because such an uptake can influence the final separation in endothelial and Kupffer cells. Furthermore, we observed that cells, obtained by a method that includes incubation with collagenase at 37 °C after perfusion, were less active in the degradation of acetyl-LDL.

Fig. 6 shows the time course of association and degradation of the ^{125}I-labeled apolipoprotein of acetyl-LDL with the freshly isolated endothelial cells. Cell association is rapid and slows down gradually at longer time intervals. Degradation shows a lag phase of about 10 min after which acid-soluble radioactive degradation products (non-iodide) are released from the cells at a constant rate.

After 2 h of incubation, approximately 7,000 ng of apolipoprotein is associated with the cells and 12,000 ng degraded (both expressed per mg of cell protein). Before, during, and after incubation, the viability of the cells was checked by the trypan blue exclusion test which always indicated a more than 95% viability. In order to investigate the involvement of the lysosomes in the degradation process, incubations were done in the presence of the lysosomotropic agents NH_4Cl (10 mM) or chloroquine (50 μM). In the presence of NH_4Cl or chloroquine, the rate of cell association at short time intervals is slower but after 2 h a similar level is reached as in the absence of these agents (see also the legend to Fig. 6). In the presence of NH_4Cl or chloroquine, the degradation of the apolipoprotein is inhibited by more than 90%. Endothelial cells isolated

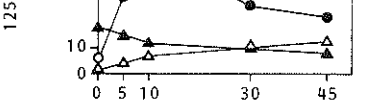

FIG. 5. **Cell association and degradation of ^{125}I-acetyl-LDL as a function of the incubation time.** The endothelial cells were obtained 10 min after intravenous injection of ^{125}I-acetyl-LDL by the 8 °C pronase method (●) or by the 37 °C collagenase method (△). After isolation, cells were suspended in Ham's F-10 at 37 °C and at the indicated times a sample was drawn in which cell-associated and acid-soluble (non-iodide) radioactivity was determined. Values are expressed per mg of protein and are the average of two experiments. *Open symbols* represent acid-soluble, *closed symbols* represent cell-bound radioactivity. As 100% value was taken cell-bound + acid-soluble radioactivity per mg of protein from the pronase-prepared cells.

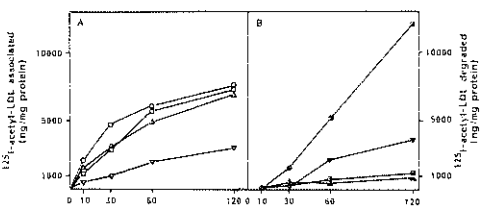

FIG. 6. **Time course of *in vitro* association (*A*) and degradation (*B*) of ^{125}I-acetyl-LDL by the endothelial cells.** Cells isolated with the 37 °C collagenase method were incubated in the absence (○) or presence of NH_4Cl (10 mM) (□) or chloroquine (50 μM) (△). Also, cells obtained with the 8 °C pronase method were tested (▽). Values are expressed as nanograms of apolipoprotein cell-associated (*A*) or degraded as acid-soluble radioactivity (non-iodide) (*B*) per mg of cell protein. Values reached after 120 min of incubation ± S.E. were: 7,700 ± 2,070 (n = 11) (○), 12,000 ± 2,000 (n = 9) (●), 7,300 ± 4,150 (n = 4) (□), 1,270 ± 240 (n = 4) (■), 7,100 ± 2,470 (n = 4) (△), 960 ± 130 (n = 4) (▲), 3,050 ± 550 (n = 5) (▽), and 3,650 ± 400 (n = 5) (▼). The concentration of ^{125}I-acetyl-LDL was 10 μg/ml.

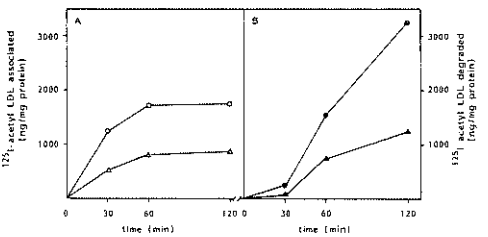

FIG. 7. **Time course of *in vitro* association (*A*) and degradation (*B*) of ^{125}I-acetyl-LDL by the Kupffer cell fraction obtained with the 37 °C collagenase method (○) or the 8 °C pronase method** (△). Values reached after 120 min of incubation ± S.E. were: 1,900 ± 550 (n = 5) (○), 3,200 ± 1000 (n = 5) (●), 900 ± 400 (n = 3) (△), and 1,250 ± 490 (n = 3) (▲). Conditions and presentation of the results are the same as in Fig. 6.

unique possibility to study *in vitro* the processing of *in vivo*-internalized substances, bridging the gap between *in vivo* and *in vitro* studies.

The endothelial cell, although contributing only 3.3% of total liver protein mass, contains more than 50% of total liver cell radioactivity 10 min after intravenous injection of ^{125}I-labeled acetyl-LDL. This calculation on the relative contribution of endothelial cells to the total liver uptake is based on the assumption that the radioactivity associated with the pure, isolated cells reflects the radioactivity of the total population. We can, however, not exclude the possibility that we have isolated a specific subclass of endothelial cells because the recovery of cells as compared to those originally present in liver is rather low. However, up until now, no evidence was available upon the existence of specific subclasses of rat liver endothelial cells and also the calculations on the recovery of radioactivity in the isolated cells as compared to whole rat liver do not give any evidence of such a subclass. The *in vivo* uptake can be explained with the results of *in vitro* studies which indicated the presence of a specific receptor on the endothelial cell that recognizes acetyl-LDL. The cell association and degradation is saturable and the apparent affinity of the cells for acetyl-LDL is high with a half-maximal cell association and degradation at 10–20 μg/ml of apolipoprotein. Binding of acetyl-LDL to this receptor is effectively coupled to degradation and degradation products are detectable after a 10-min lag phase. The involvement of the lysosomes in the degradation process is indicated by the nearly complete inhibition of the degradation *in vitro* in the presence of 10 mM NH_4Cl or 50 μM chloroquine.

We can only speculate about the physiological implication of the specialization of the liver endothelial cell in acetyl-LDL uptake. It has been suggested that the acetyl-LDL receptor is involved in the massive accumulation of cholesterol (ester) in the atherosclerotic plaques and skin macrophages (4). Recently, the existence of such a receptor was demonstrated in the foam cell present in atherosclerotic lesions (25). It might be possible that the active acetyl-LDL receptor on liver endothelial cells combined with their strategic anatomical localization forms an important protection against such an accumulation under normal conditions. Furthermore, the active lysosomal degradation capacity (26)—including acid cholesterol esterase, demonstrated in the whole nonparenchymal cell fraction (27) and by us in pure endothelial and Kupffer cells[2]—enables these cells to hydrolyze the internalized cholesterol esters. Studies on macrophages have led to the concept of a two-compartment model for the handling of cholesterol esters by hydrolysis in the lysosomes and subsequent re-esterification and storage in the cytoplasmic compartment (4). The stored cholesterol can then be released from the cells by, for instance, high density lipoproteins or erythrocytes. However, the anatomical localization of the liver endothelial cells gives them close or even direct contact with the parenchymal cells which might enable a direct transfer of the endocytosed or processed cholesterol from inside the endothelial cells to the parenchymal cells and then to the bile canaliculi. Further studies are planned to test the possibility of this pathway and to determine whether inadequate operation under pathological conditions causes accumulation of cholesterol (esters) in other sites of the body.

[2] J. F. Nagelkerke, K. P. Barto, and T. J. C. van Berkel, unpublished observations.

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REFERENCES

1. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 333–337
2. Stein, O., and Stein, Y. (1980) *Biochim. Biophys. Acta* **620,** 631–635
3. Shechter, I., Fogelman, A. M., Haberland, M. E., Seager, J., Hokom, M., and Edwards, P. A. (1981) *J. Lipid Res.* **22,** 63–71
4. Brown, M. S., Basu, S. K., Falck, J. R., Ho, Y. K., and Goldstein, J. L. (1980) *J. Supramol. Struct.* **13,** 67–81
5. Brown, M. S., and Goldstein, J. L. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3330–3337
6. Brown, M. S., Kovanen, P. T., and Goldstein, J. L. (1981) *Science* **212,** 628–635
7. Henriksen, T., Mahoney, E. M., and Steinberg, D. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6499–6503
8. Mahley, R. W., Weisgraber, K. H., Innerarity, T. L., and Windmueller, H. G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1746–1750
9. Seglen, P. O. (1976) *Methods Cell Biol.* **13,** 29–83
10. Edmondson, J. W., and Bang, N. U. (1981) *Am. J. Physiol.* **241,** C3–C8
11. Nagelkerke, J. F., Barto, K. P., and van Berkel, Th. J. C. (1982) *Exp. Cell Res.* **138,** 183–191
12. Praaning-van Dalen, D. P., and Knook, D. L. (1982) *FEBS Lett.* **141,** 229–232
13. Blouin, A., Bolender, R. P., and Weibel, E. R. (1977) *J. Cell Biol.* **72,** 441–455
14. Munthe-Kaas, A. C., Berg, T., and Sjeljelid, R. (1976) *Exp. Cell Res.* **99,** 146–154
15. Knook, D. L. (1983) in *The Handbook Series in Aging, Section D. Biological Sciences, Cell Biology* (Christofalo, V. J., ed) Vol. 2, CRC Press, Boca Raton, FL, in press
16. van Berkel, Th. J. C. (1982) in *Metabolic Compartmentation* (Sies, H., ed) pp. 437–482, Academic Press, New York
17. van Berkel, Th. J. C., Nagelkerke, J. F., and Kruijt, J. K. (1981) *FEBS Lett.* **132,** 61–66
18. Weisgraber, K. H., Innerarity, T. L., and Mahley, R. W. (1978) *J. Biol. Chem.* **253,** 9053–9062
19. van Berkel, Th. J. C., and Kruijt, J. K. (1977) *Eur. J. Biochem.* **73,** 223–229
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
21. Fahimi, H. D. (1970) *J. Cell Biol.* **47,** 247–261
22. Koss, L. G. (1979) *Diagnostic Cytology*, 3rd Ed. pp. 1211–1222. J. B. Lippincott Co., Philadelphia
23. Praaning-van Dalen, D. P., Brouwer, A., and Knook, D. L. (1981) *Gastroenterology* **81,** 1036–1044
24. van Berkel, Th. J. C., Nagelkerke, J. F., Harkes, L., and Kruijt, J. K. (1982) *Biochem. J.* **208,** 493–503
25. Pitas, R. E., Innerarity, T. L., and Mahley, R. W. (1983) *Arteriosclerosis* **3,** 2–12
26. van Berkel, T. J. C., Kruijt, J. K., and Koster, J. F. (1975) *Eur. J. Biochem.* **58,** 145–152
27. van Berkel, T. J. C., Vaandrager, H., Kruijt, J. K., and Koster, J. F. (1980) *Biochim. Biophys. Acta* **617,** 446–457

with the 8 °C pronase method are significantly less active in binding and degradation of the apolipoprotein of acetyl-LDL than cells prepared with the 37 °C collagenase method (Fig. 6); however, the ratio cell degradation/association is equal.

Fig. 7 shows the time course of cell association and degradation of the apolipoprotein of acetyl-LDL by the freshly isolated Kupffer cell fraction. As already mentioned under "Characterization of the Isolated Cells," the Kupffer cell content of this fraction is 85 ± 5%. The amount of acetyl-LDL that becomes cell-associated and degraded in this Kupffer cell fraction is about 25% of that obtained with the pure endothelial cells. From these data it will be clear that Kupffer cells are much less active or possibly inactive in the catabolism of acetyl-LDL.

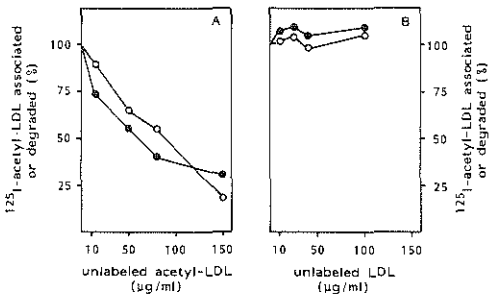

FIG. 8. **Comparison of the ability of unlabeled acetyl-LDL (*A*) or native LDL (*B*) to compete with the cell association and degradation of ^{125}I-acetyl-LDL by endothelial cells.** ●, association; ○, degradation. Values are given as percentage of control incubated in the absence of the unlabeled lipoproteins. Concentration of ^{125}I-acetyl-LDL was 4 µg/ml (*A*) and 2.1 µg/ml (*B*) (expressed as apolipoprotein content). The 100% value for the cell association was 3850 (*A*) and 1850 (*B*) ng/mg of cell protein, respectively and for the degradation 6200 (*A*) and 3300 (*B*) ng/mg of cell protein, respectively. The incubation time was 2 h. Each value represents the average of three experiments.

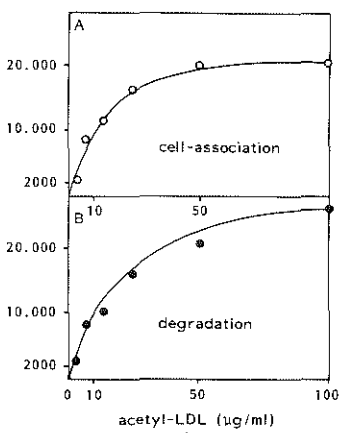

FIG. 9. **Relation of the concentration of acetyl-LDL to the extent of cell association (*upper part*) or degradation (*lower part*) by rat liver endothelial cells obtained with the 37 °C collagenase method.** Values are expressed as nanograms of apolipoprotein per mg of protein. Incubation time was 2 h. Values are from one experiment. In two other experiments, similar results were obtained.

It has been reported that the uptake of acetyl-LDL in macrophages is initiated by recognition of acetyl-LDL by a specific receptor (1). The specificity of acetyl-LDL interaction with the endothelial cells was determined by incubating cells with ^{125}I-labeled apolipoprotein in the absence or presence of increasing concentrations of unlabeled acetyl-LDL. It was found that both the cell association and degradation of the ^{125}I-apolipoprotein are decreased to 30% of the control value with a 35-fold excess of unlabeled acetyl-LDL (Fig. 8), whereas addition of a 50-fold excess of unlabeled native LDL does not affect the cell association and degradation of ^{125}I-acetyl-LDL.

The dependency of the cell association and degradation on the concentration of acetyl-LDL (Fig. 9) indicates that the endothelial cells possess a high affinity and saturable binding site for acetyl-LDL with a half-maximal association or degradation at about 10–20 µg of apolipoprotein/ml.

DISCUSSION

This study shows the specific interaction of acetyl-LDL with the different liver cell types. For the isolation of liver endothelial and Kupffer cells, two methods were applied. The main difference between these isolation techniques is that with the first method the liver is perfused at 8 °C with 0.25% pronase and with the second method perfusion is performed at 37 °C with 0.05% collagenase. With both methods, pure endothelial and purified Kupffer cell fractions were obtained. For the determination of the cellular uptake of intravenously injected acetyl-LDL in endothelial and Kupffer cells, the cold pronase technique with, in addition, a cold collagenase method for the parenchymal cells appears to be superior to the traditional 37 °C isolation methods (24). Degradation of *in vivo* injected substances during cell isolation is minimal and a quantitative recovery of the liver-associated label in the isolated cells is obtained. However, for direct *in vitro* binding and uptake studies, the described 37 °C collagenase technique leads to the isolation of more active cells. Up until now, no methods have been available to isolate pure liver endothelial cells that were directly applicable for *in vitro* uptake studies. Although Kupffer cells have been used for numerous uptake studies, this was only possible after culturing the Kupffer cells at least overnight. A time in culture was necessary because, with the employed isolation technique—pronase was used at 37 °C—all the cellular receptors had been destroyed during isolation. Also, endothelial cells obtained by the 8 °C pronase technique are less active in the *in vitro* binding and degradation than those obtained by the 37 °C collagenase method. This is probably because also at 8 °C pronase destroys receptors present on the cell membrane. However, endothelial cells isolated by the 8 °C pronase method are equally efficient in the degradation of cell-associated acetyl-LDL as collagenase-isolated cells (the degradation/cell-associated ratio is similar at 2 h of incubation for cells isolated by both methods). Furthermore, *in vivo* endocytosed acetyl-LDL is further processed by the pronase-prepared cells upon warming after isolation. So it can be concluded that with both methods liver endothelial cells are obtained with an active intracellular processing system for internalized acetyl-LDL. However, with the 37 °C collagenase method, the total amount of acetyl-LDL processed by the cells is significantly higher, probably because receptors present on the cell membrane are not destroyed by collagenase. Therefore, for *in vitro* studies, the prevalence must be given to this method. In contrast, as mentioned above, for studies on the *in vivo* cellular uptake of rapidly degradable substances, the 8 °C pronase method appears to be superior. Furthermore, this method opens the

Correction:
Throughout the text Mg-EGTA was printed as Mg-EDTA

THE EFFECT OF Ca^{2+} AND TRIFLUOPERAZINE ON THE PROCESSING OF HUMAN ACETYLATED LOW DENSITY LIPOPROTEIN BY NON-PARENCHYMAL LIVER CELLS

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1. Introduction

Studies with human fibroblasts have defined a receptor-mediated pathway by which cells take up and degrade low density lipoprotein (LDL), the major cholesterol(ester)-transport protein in human plasma [1]. Internalization of the particle is followed after recognition by a specific receptor. These receptors are clustered in coated regions of the cell membrane, so-called coated pits. Upon LDL binding the coated pits invaginate and form coated endocytotic vesicles which fuse subsequently with lysosomes. The protein moiety and the cholesterol esters are then degraded by the action of cathepsins and acid cholesterol esterase, respectively. Although the different steps involved in receptor-mediated endocytosis have been described, the molecular mechanisms involved in the process are largely unknown. Peritoneal macrophages possess a binding site for acetylated LDL which is distinct from the native LDL receptor [2]. On macrophages this binding site is coupled to a very active internalization and degradation process. This property facilitates the study of the molecular mechanism of the uptake and degradation pathway. This is also illustrated by the finding that after injection of ^{125}I-labeled acetyl-LDL into rats, these particles are rapidly cleared from the circulation (<3 min). The radioactivity is subsequently merely recovered in the non-parenchymal liver cells (unpublished). This study describes the in vitro binding of both native and acetylated LDL to freshly isolated non-parenchymal liver cells. Binding of acetylated LDL to these cells is only twice as high as the binding of native LDL but the degradation is increased 30–50-fold after acetylation of the LDL. The degradation of acetylated LDL is inhibited by chloroquine and NH_4Cl, indicating a lysosomal process. Mg-EDTA at 2 mM inhibits the degradation of acetyl-LDL by 50% and trifluoperazine (50 μM), an inhibitor of calmodulin [3,4], blocks the degradation completely. The rate of association of acetyl-LDL with non-parenchymal cells is only slightly inhibited by trifluoperazine. It is concluded that the main action of trifluoperazine is exerted on the route of acetyl-LDL to the lysosomes after the initial binding process. The data are consistent with a role of calmodulin in the receptor-mediated endocytotic process although it cannot be excluded that trifluoperazine interacts with another still unknown target.

2. Materials and methods

2.1. *Isolation, modification and labeling of LDL*

Human LDL was isolated exactly as in [5]. The density range was 1.019–1.063. LDL was acetylated with repeated additions of acetic anhydride as in [6]. In short, 1 ml LDL (at 2–10 mg protein/ml) in 0.15 M NaCl, 1 mM EDTA and 8 mM phosphate buffer (pH 7.5) was added to 1 ml of a saturated solution of sodium acetate with continuous stirring in an ice-water bath. Next, acetic anhydride was added in multiple small aliquots (2 μl) over 1 h. After the addition of a total mass of acetic anhydride equal to 1.5-times the mass of protein used, the mixture was stirred for an additional 30 min. The solution was then dialyzed overnight at 6°C against buffer containing 0.15 M NaCl, 1 mM EDTA and 8 mM phosphate buffer (pH 7.5). Both LDL and acetyl-LDL were iodinated at pH 10 by the ICl method [7], as modified for lipoproteins [8]. Free I^- was removed by Sephadex G-50 filtration. The iodine:protein ratio was between 0.6–0.8 atoms/mol for both LDL and acetyl-LDL. Of the radioactivity in the preparation, 3–4% was free, 3–5% was present in phospholipids

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and 92–94% was protein-bound. The only apoprotein present in LDL is apolipoprotein B as determined on SDS–polyacrylamide electrophoresis [10].

2.2. *Isolation of liver cells*

Isolation of rat non-parenchymal liver cells was performed by differential centrifugation (method 2 in [9]).

2.3. *Lipoprotein binding, uptake and degradation*

Incubations of freshly isolated liver cells with the indicated amounts of lipoproteins and cells were performed in Ham's F-10 medium (modified), containing 5% (v/v) human lipoprotein-deficient serum (LPDS; final protein 2.5 mg/ml). The incubations were carried out either in plastic Eppendorf tubes in 1 ml total vol. or in 25 ml Erlenmeyer flasks (siliconized) stoppered with rubber caps with a total incubation volume and time as indicated in the figure legends. At the indicated times 1 ml samples were withdrawn and the cells were centrifuged in an Eppendorf centrifuge for 2 min at 3000 rev./min. The pellets were suspended in 1 ml medium containing 50 mM Tris–HCl (pH 7.4), 0.15 M NaCl and 2 mg bovine serum albumin, incubated for 5 min at 4°C and centrifuged again. This washing procedure was repeated twice. The last washing was performed with 0.15 M NaCl only to enable a reliable protein determination. The cell-associated radioactivity and 0.5 ml of the different supernatants were counted in a LKB-Wallace ultrogamma counter. The radioactivity in the last supernatant was <5% of the cell-associated radioactivity. Degradation of the lipoproteins was measured as in [11]. To 0.5 ml of the first supernatant, 0.2 ml 35% trichloroacetic acid was added followed by incubation for 15 min at 37°C; subsequently the mixture was centrifuged for 2 min at 15 000 rev./min. To 0.5 ml of the supernatants 5 μl 40% KI and 25 μl 30% H_2O_2 were added. After 5 min at room temperature 0.8 ml $CHCl_3$ was added and the mixture was shaken for another 5 min. After centrifugation for 2 min at 15 000 rev./min, 0.4 ml of the aqueous phase (containing iodinated amino acids and small peptides) and 0.5 ml of the $CHCl_3$ phase (containing I_2 formed from I^- by oxidation with H_2O_2) was counted. This sample was corrected for quenching by $CHCl_3$. In the corresponding blanks the lipoproteins were incubated in the absence of cells. Further additions are indicated in the figure legends.

The viability of the cells was >95%. The viability was also checked after addition of chloroquine or trifluoperazine. It was found that high chloroquine concentrations (>100 μM) lead to a lower viability of the cells especial at the longer incubation times. With the concentrations used in this paper no decrease in cell viability was observed.

2.4. *Reagents*

Collagenase (type I) was obtained from Sigma. Trifluoperazine was supplied by Smith, Kline and French Labs.

3. Results

Fig.1 shows the time course of the interaction of ^{125}I-labeled LDL and ^{125}I-labeled acetylated LDL with the freshly isolated non-parenchymal cells. The amount of cell-associated lipoprotein rapidly increases and levels off at the longer incubation times. With rat lipoproteins, the amount of cell-associated radioactivity remained constant from 0.5–3 h incubation [10]. Such a steady-state level is not readily observed with human LDL or acetyl-LDL, although in some experiments the increase in cell-associated radioactivity from 2–3 h incubation was found to be low. The appearance of acid-soluble radioactivity in the water phase follows a completely different time dependency than the amount of cell-associated radioactivity. With acetyl-LDL a lag phase is evident (10–30 min) before the degradation reaches a constant rate (from 1–3 h). This degradation is completely blocked by chloroquine (100 μM, fig.2) or NH_4Cl (10 mM, not shown). Both unrelated compounds inhibit the lyosomal pathway of protein degradation [12]. So it can be concluded that acetyl-LDL follows an endocytotic route which involves the lysosomal compartment. Fig.2 also indicates that a similar inhibition of acetyl-LDL degradation occurs if 100 μM trifluoperazine is present. With 25 μM trifluoperazine intermediate inhibition is obtained. Fig.2 indicates that trifluoperazine also influences the initial phase of the interaction of acetyl-LDL with the cells. At shorter incubation times a lower amount of radioactivity is cell-associated than in the control incubation. However, this is not observed at prolonged incubation times, probably because the initial slight interaction of trifluoperazine with the binding of acetyl-LDL is compensated by the accumulation of acetyl-LDL due to an impaired degradation.

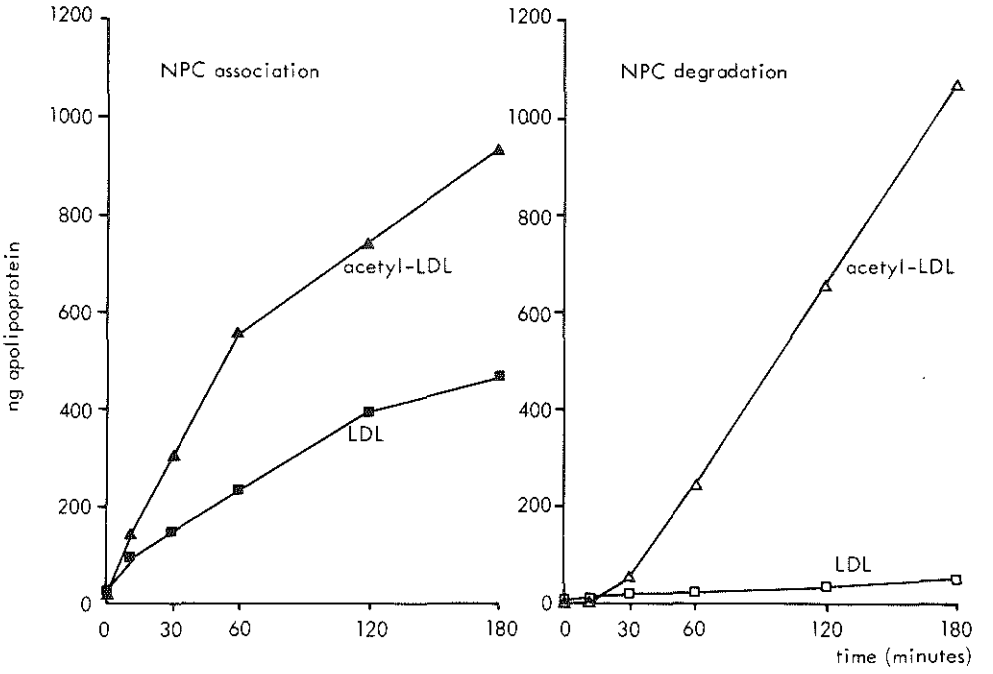


Fig.1. Time course of the cell association and degradation of LDL and acetyl-LDL by isolated non-parenchymal liver cells. The cells were incubated with 10.0 μg LDL/ml (■,□) or 10.1 μg acetyl-LDL/ml (▲,△). At the indicated time samples were withdrawn and the amount of cell-associated radioactivity (after 3 washings, left) as well as the radioactivity present in the acid-soluble water phase were determined (right). Both cell association and degradation are expressed as ng apolipoprotein/mg cell protein.

Trifluoperazine has been considered as a specific inhibitor of calmodulin, a protein which mediates the action of Ca^{2+} upon a number of cellular processes [4,13]. In [14] coated vesicles of brain were 7-fold enriched with calmodulin. The presence of calmodulin in the coated vesicles should be rationalized by a function in the endocytotic process. Fig.3 shows that the degradation of acetyl-LDL by non-parenchymal cells is hardly influenced by changes in the extracellular $[Ca^{2+}]$. In the presence of 2 mM Mg-EDTA, however, the degradation is ~50% of that measured in the absence of Mg-EDTA. From fig.3 it is also clear that this effect is not the consequence of the involvement of Ca^{2+} in the acetyl-LDL binding process, but apparently occurs at a step between binding and degradation. Fig.4 shows that the effective inhibitory concentration of trifluoperazine upon acetyl-LDL degradation is low. At 50 μM, trifluoperazine exhibits

Table 1
Effect of trifluoperazine upon the in vitro degradation of human iodine-labeled acetylated low density apolipoprotein by non-parenchymal cell homogenates

	Degradation (ng apolipoprotein . h^{-1} . mg cell $protein^{-1}$)
Acetyl-LDL	1832 ± 57
+ 100 μM trifluoperazine	1983 ± 50

The cells were homogenized by sonificating twice for 30 s at 21 kHz in a MSE ultrasonic desintegrator at 0°C. Incubations were carried out for 60 min at 37°C in acetate buffer (pH 4.2), 4 mM dithiothreitol and 10.8 μg/ml ^{125}I-labeled acetylated-LDL. The reaction was stopped by addition of 10% trichloroacetic acid and the acid-soluble water phase was obtained as indicated in section 2. For further details of the in vitro degradation measurements see [15]. The values are the mean ± SEM for 3 determinations

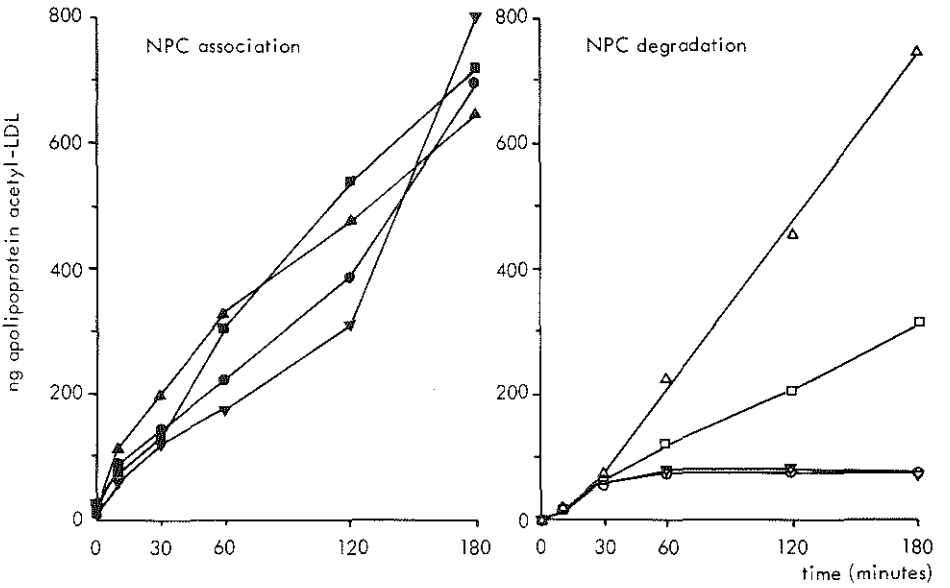
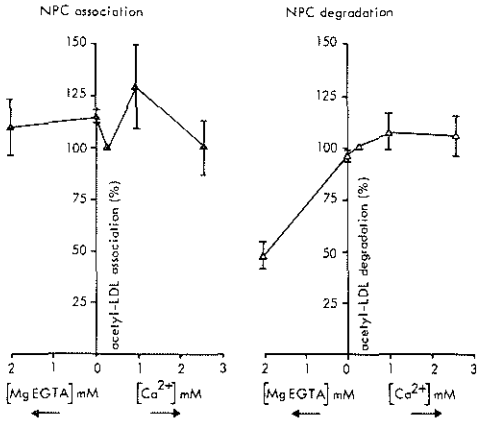


Fig.2. Effect of chloroquine and trifluoperazine upon the time course of the cell association and degradation of acetyl-LDL. The cells were incubated with 10.2 μg acetyl-LDL/ml in the absence (▲,△) or presence of: 100 μM chloroquine (▼,▽); 100 μM trifluoperazine (●,○); or 25 μM trifluoperazine (■,□). Both cell association (left) and degradation (right) are expressed as ng apolipoprotein/mg cell protein.

already a nearly complete inhibition and the half-maximal inhibitory concentration is ~20 μM. To verify whether trifluoperazine acts directly upon lysosomal enzymes we studied the effect of 100 μM trifluoperazine upon the acetyl-LDL degradation in a cell-free system at an optimal degradation pH of 4.2 (table 1). No inhibitory effect of trifluoperazine on the degradation by non-parenchymal cell homogenates was noticed.



4. Discussion

Non-parenchymal liver cells possess high affinity sites which can bind rat LDL and HDL [16]. The

Fig.3. The effect of Mg-EDTA and Ca^{2+} upon the cell-association and degradation of acetyl-LDL by isolated non-parenchymal cells. The cells were incubated 2 h with 5.5 μg acetyl-LDL/ml with the different indicated [Mg-EDTA] or [Ca^{2+}]. For the cells incubated with Mg-EDTA the last 2 washings before incubation, were also performed in the presence of 2 mM Mg-EDTA. The results were obtained with 3 different acetyl-LDL and cell preparations and are given as % of the association or degradation at 0.3 mM Ca^{2+} ± SEM. The 100% value for the cell assocation is 470 ng acetyl-LDL/mg cell protein and for the degradation 732 ng acetyl-LDL/mg cell protein.

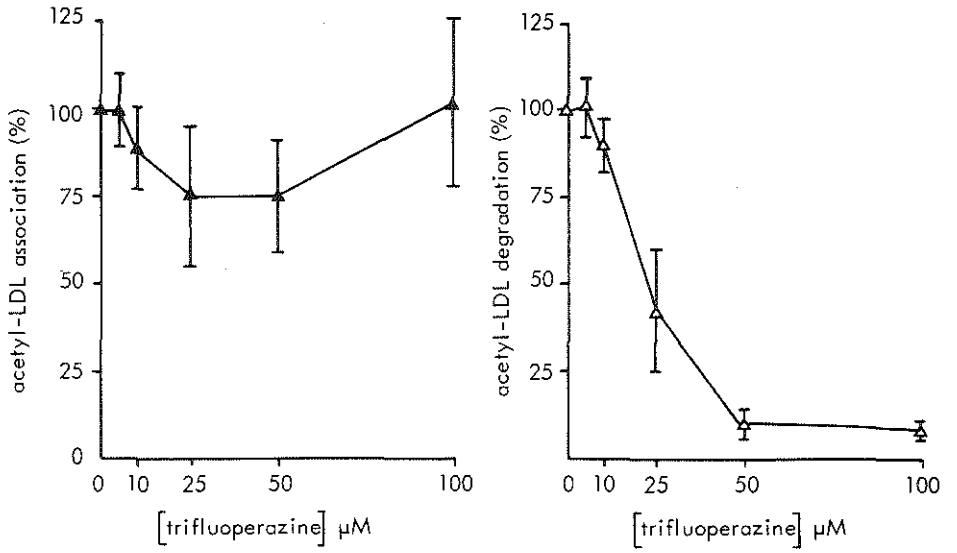


Fig.4. The effect of increasing trifluoperazine concentrations upon the cell association and degradation of acetyl-LDL by isolated non-parenchymal liver cells. The cells were incubated for 2 h with 10.3 μg acetyl-LDL/ml in the presence of the indicated amount of trifluoperazine. The results were obtained with 3 different acetyl-LDL and cell preparations and are given as % of the association or degradation in the absence of trifluoperazine ± SEM. The 100% value for the cell association is 690 ng acetyl-LDL/mg cell protein and for the degradation 844 ng acetyl-LDL/mg cell protein.

binding of rat LDL, HDL and also VLDL-remnants occurred at the same receptor. This receptor recognizes all these rat lipoproteins with a highest apparent affinity for VLDL-remnants [10]. Here we show that non-parenchymal cells bind and metabolize, in addition to the rat lipoproteins, both human LDL and acetyl-LDL. Although the cell-association of acetyl-LDL is only twice as high as for native LDL, the acetylation of native LDL increases the degradation 30–50-times. This high increase in degradation can be expected from macrophage-like cell types [2]. The degradation of acetyl-LDL by freshly isolated non-parenchymal liver cells is completely blocked by chloroquine (fig.2) or NH_4Cl (not shown). The incomplete inhibition during the first hour of incubation can be explained by the fact that the cells were not preincubated with these lysosomotropic agents. Together with the clear lag phase before degradation starts, this is strong evidence that the handling of acetyl-LDL follows the endocytotic route for receptor-mediated uptake, i.e., binding to a high affinity receptor (properties of this receptor will be reported elsewhere), uptake in endocytotic vesicles and degradation inside the lysosomes. These data show further that trifluoperazine, an inhibitor of calmodulin [34], inhibits the degradation of acetyl-LDL. The acetyl-LDL degradation is highly sensitive to trifluoperazine, with a half-maximal inhibition at 20 μM. Similar concentrations are reported to inhibit other calmodulin-mediated cellular processes [17,18]. Although this inhibition is consistent with a role of calmodulin in the endocytotic process the reported finding cannot be considered as conclusive. In [19] it was reported that the inhibitory action of trifluoperazine upon the effect of α-adrenergic agonists is due to a blockade of the binding of the α-adrenergic agonists to their receptor which is not mediated by calmodulin. For this reason, besides its action upon the degradation, we also investigated the effect of trifluoperazine upon the rate of cell-association of acetyl-LDL. It is shown that trifluoperazine indeed interacts with the initial binding (as does chloroquine), however, this does not explain the complete inhibition of the degradation of acetyl-LDL by trifluoperazine. Although a small inhibition of the cell-association occurs (at shorter incubation time), this inhibition is not observed at

prolonged incubation time probably because it is compensated by the main action of trifluoperazine, i.e., the blockade of the route to the lysosomes of acetyl-LDL, after the initial binding process. The action of trifluoperazine is probably not at the lysosomes themselves because the in vitro degradation of acetyl-LDL by cell homogenates at an acid pH is not inhibited by trifluoperazine. Further evidence that calmodulin is involved in the intracellular handling process of acetyl-LDL can also be deducted from the experiments in which the effect of Mg-EDTA and Ca^{2+} was tested. In contrast with most other ligand–receptor interactions [13,20], the association of acetyl-LDL with the cells was not Ca^{2+} or Mg-EDTA-dependent. This makes it possible to separate the cell-association process from the subsequent cellular handling and the data indicate that, in the presence of Mg-EDTA the degradation of acetyl-LDL is inhibited by ~50%. A complete inhibition was, however, not observed probably because extracellular Mg-EDTA is not able to deplete intracellular Ca^{2+} completely. In conclusion it can be stated that, in addition to the data in [14] where calmodulin was shown to be highly enriched in endocytotic vesicles, our data can be considered as evidence that calmodulin, or another still unknown target of trifluoperazine, is involved in the receptor-mediated endocytotic process.

Acknowledgements

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References

[1] Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. (1979) Nature 279, 679–685.
[2] Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) Proc. Natl. Acad. Sci. USA 76, 333–337.
[3] Levin, R. M. and Weiss, B. (1977) Mol. Pharmacol. 13, 690–697.
[4] Cheung, W. Y. (1980) Science 207, 19–27.
[5] Redgrave, T. G., Roberts, D. C. K. and West, C. E. (1975) Anal. Biochem. 65, 42–49.
[6] Basu, S. K., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. (1976) Proc. Natl. Acad. Sci. USA 73, 3178–3182.
[7] Mc Farlane, A. S. (1958) Nature 182, 53.
[8] Langer, T., Strober, W. and Levy, R. I. (1972) J. Clin. Invest. 51, 1528–1536.
[9] Van Berkel, Th. J. C. and Van Tol, A. (1978) Biochim. Biophys. Acta 530, 299–304.
[10] Van Berkel, Th. J. C., Kruijt, J. K., Van Gent, T. and Van Tol, A. (1981) Biochim. Biophys. Acta in press.
[11] Bierman, E. L., Stein, O. and Stein, Y. (1974) Circ. Res. 35, 136–150.
[12] Seglen, P. O., Grinde, B. and Solheim, A. E. (1979) Eur. J. Biochem. 95, 215–225.
[13] Means, A. R. and Dedman, J. R. (1980) Nature 285, 73–77.
[14] Linden, C. D., Dedman, J. R., Chafouleas, J. G., Means, A. R. and Roth, T. F. (1981) Proc. Natl. Acad. Sci. USA 78, 308–312.
[15] Van Berkel, Th. J. C., Van Tol, A. and Koster, J. F. (1978) Biochim. Biophys. Acta 529, 138–146.
[16] Van Berkel, Th. J. C., Kruijt, J. K., Van Gent, T. and Van Tol, A. (1980) Biochem. Biophys. Res. Commun. 92, 1002–1008.
[17] Sugden, M. C., Christie, R. M. and Ashcroft, S. J. H. (1979) FEBS Lett. 105, 95–100.
[18] Gagliardino, J. J., Harrison, D. E., Christie, M. R., Gagliardino, E. E. and Ashcroft, S. J. H. (1980) Biochem. 192, 919–927.
[19] Blackmore, P. F., El-Refai, M. F., Dehaye, J. P., Strickland, W. G., Hughes, B. P. and Exton, J. H. (1981) FEBS Lett. 123, 245–248.
[20] Kovanen, P. T., Brown, M. S. and Goldstein, J. L. (1979) J. Biol. Chem. 254, 11367–11373.

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APPENDIX PAPER IV

Processing of acetylated human low-density lipoprotein by parenchymal and non-parenchymal liver cells

Involvement of calmodulin?

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1. Modified lipoproteins have been implicated to play a significant role in the pathogenesis of atherosclerosis. In view of this we studied the fate and mechanism of uptake *in vivo* of acetylated human low-density lipoprotein (acetyl-LDL). Injected intravenously into rats, acetyl-LDL is rapidly cleared from the blood. At 10 min after intravenous injection, 83% of the injected dose is recovered in liver. Separation of the liver into a parenchymal and non-parenchymal cell fraction indicates that the non-parenchymal cells contain a 30–50-fold higher amount of radioactivity per mg of cell protein than the parenchymal cells. 2. When incubated *in vitro*, freshly isolated non-parenchymal cells show a cell-association of acetyl-LDL that is 13-fold higher per mg of cell protein than with parenchymal cells, and the degradation of acetyl-LDL is 50-fold higher. The degradation of acetyl-LDL by both cell types is blocked by chloroquine (10–50 μM) and NH_4Cl (10 mM), indicating that it occurs in the lysosomes. Competition experiments indicate the presence of a specific acetyl-LDL receptor and degradation pathway, which is different from that for native LDL. 3. Degradation of acetyl-LDL by non-parenchymal cells is completely blocked by trifluoperazine, penfluridol and chlorpromazine with a relative effectivity that corresponds to their effectivity as calmodulin inhibitors. The high-affinity degradation of human LDL is also blocked by trifluoperazine (100 μM). The inhibition of the processing of acetyl-LDL occurs at a site after the binding-internalization process and before intralysosomal degradation. It is suggested that calmodulin, or a target with a similar sensitivity to calmodulin inhibitors, is involved in the transport of the endocytosed acetyl-LDL to or into the lysosomes. 4. It is concluded that the liver, and in particular non-parenchymal liver cells, are *in vivo* the major site for acetyl-LDL uptake. This efficient uptake and degradation mechanism for acetyl-LDL in the liver might form *in vivo* the major protection system against the potential pathogenic action of modified lipoproteins.

When the concentration of circulating lipoproteins rises to high levels in the plasma of man or experimental animals, lipid is deposited in macrophages throughout the body (Wurster & Zilversmit, 1971; Fredrickson *et al.*, 1978). This is especially evident in patients with familial hypercholesterolaemia (Goldstein & Brown, 1978). The cell types involved include the hepatic non-parenchymal cells and macrophages of the spleen, kidney, bone marrow, skin tendons and other organs (Chomette *et al.*, 1971; Goldstein & Brown, 1978).

The mechanism of the lipid deposition in the cells present in the atherosclerotic plaque has not yet been elucidated (Wurster & Zilversmit, 1971). When incubated with normal lipoproteins macrophages do not accumulate large amounts of cholesterol (Goldstein *et al.*, 1979; Brown *et al.*, 1980) and it has been proposed that modification of LDL, either by acetylation (Goldstein *et al.*, 1979) or by malonaldehyde treatment (Fogelman *et al.*, 1980; Shechter *et al.*, 1981), is necessary before the lipoprotein will

Abbreviations used: VLDL, very-low-density lipoprotein ($\rho < 1.006$ g/ml); LDL, low-density lipoprotein ($\rho = 1.019$–1.050 g/ml for rat LDL or 1.019–1.063 g/ml for human LDL); HDL, high-density lipoprotein ($\rho = 1.050$–1.13 g/ml for rat HDL).

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produce a deposition of a similar magnitude as seen under pathological conditions. When modified proteins are injected into animals they are cleared mainly by the liver non-parenchymal cells, which thus serve as the major scavenger site in the body (Kooistra *et al.*, 1979; Van Berkel, 1982). In view of the possible relevance of modified lipoproteins for the development of the atherosclerotic lesion it becomes of special interest to study the fate and uptake mechanism *in vivo* of these lipoproteins.

Recently we showed that the processing of acetyl-LDL by non-parenchymal liver cells is completely inhibited by the phenothiazine tranquillizer trifluoperazine, an inhibitor of calmodulin (Van Berkel *et al.*, 1981*b*). This suggested that calmodulin, or a still unknown target of trifluoperazine, is involved in the receptor-mediated endocytotic process (Levin & Weiss, 1977; Van Berkel *et al.*, 1981*b*). This idea is extended now by studying the effect of trifluoperazine on the processing of acetyl-LDL by parenchymal cells. Furthermore its effect on the handling of the unmodified LDL is studied. To specify the target for trifluoperazine in more detail, we determined the relative ability of two other potent inhibitors of calmodulin, penfluridol and chlorpromazine on the degradation of acetyl-LDL by non-parenchymal cells.

Experimental

Materials

Acetic anhydride was obtained from Merck, Darmstadt, Germany. [^{125}I]Iodide was purchased from NEN Chemicals, Dreieich, Germany and Ham's F-10 medium from Gibco (Europe) Paisley, Scotland, U.K. Penfluridol was a product of Janssen Pharmaceutica, Beerse, Belgium, and was dissolved in dimethyl sulphoxide. Trifluoperazine was a gift from Smith, Kline and French Laboratories, Philadelphia, PA, U.S.A., and was dissolved in 20% propylene glycol. Another batch, in dihydrochloride form, was kindly donated by Rhône-Poulenc, Vitry, France. Pronase was obtained from Calbiochem, San Diego, CA, U.S.A. Collagenase (type I) and chloroquine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade.

Isolation, modification and labelling of LDL

Human LDL was isolated exactly as described by Redgrave *et al.* (1975). The density range was 1.019–1.063 g/ml. LDL was acetylated with repeated additions of acetic anhydride as described by Basu *et al.* (1976). LDL (1 ml; with a protein concentration between 1–10 mg/ml) in 0.15 M-NaCl/1 mM-EDTA/8 mM-phosphate buffer, pH 7.5, was added to 1 ml of a saturated solution of sodium acetate with continuous stirring in an ice/water bath. Next, acetic anhydride was added in multiple small portions (2 μl) over a period of 1 h. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein used, the mixture was stirred for an additional 30 min. The solution was then dialysed overnight at 6°C against buffer containing 0.15 M-NaCl, 1 mM-EDTA and 8 mM-phosphate buffer, pH 7.5. Both LDL and acetyl-LDL were iodinated at pH 10 by the ICl method (McFarlane, 1958) modified for lipoproteins by Langer *et al.* (1972). Free I^- was removed by Sephadex G-50 filtration. The iodine/protein ratio was between 0.6 and 0.8 atoms/mol for both LDL and acetyl-LDL. Of the radioactivity in the preparation 3–4% was free, 3–5% was present in phospholipids and 92–94% was protein-bound. The only apoprotein present in LDL is apolipoprotein B, as determined on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Van Berkel *et al.*, 1981*a*).

Measurement of acetyl-LDL uptake by the rat liver cells in vivo

^{125}I-Acetyl-LDL (15 μg) was injected at 09.00 h in chow-fed rats (250–350 g) under nembutal anaesthesia. After 3 min a blood sample was drawn from the retro-ocular sinus. At 10 or 30 min after injection, a liver perfusion with Ca^{2+}-free Hank's medium was started. The liver was initially perfused at a flow rate of 4 ml/min. The flow rate was increased to 14 ml/min after cannulation of the caval vein. At 8 min after the start of the perfusion, a liver lobe was tied off to determine the total hepatic radioactivity. The remaining liver was perfused with 0.05% collagenase. Subsequently, parenchymal and non-parenchymal cells were isolated as described in detail previously (Van Berkel *et al.*, 1977; Van Berkel & Van Tol, 1978). The purity, composition and integrity of the different cell preparations were tested by phase-contrast light microscopy, by Trypan Blue exclusion, by peroxidase staining and by the relative distribution and specific activities of L- and M_2-type pyruvate kinase. On the basis of light microscopy the final parenchymal-cell preparations were completely free from non-parenchymal cells and more than 95% of the isolated cells excluded Trypan Blue. The isolated cells were sensitive to glucagon in a similar way as a perfused liver system (Van Berkel *et al.*, 1978). The non-parenchymal cell preparation (termed 'NPC_1'; Pronase method) excluded Trypan Blue almost completely and no parenchymal cells were present. By peroxidase staining with diaminobenzidine about 30% of the cells were peroxidase-positive, indicating that about 30% of these cells are Kupffer cells and about 70% endothelial cells (Knook & Sleyster, 1976). The purity of every individual cell preparation was also checked by determination of the distribution and activity of L-type and M_2-type

pyruvate kinase, as described previously (Van Berkel *et al.*, 1977). In accordance with previous work (Crisp & Pogson, 1972; Van Berkel, 1974) the NPC_1 preparation contained solely M_2-type pyruvate kinase. The non-parenchymal cell preparation obtained by differential centrifugation (termed 'NPC_2') contained various amounts of parenchymal cell protein. This contamination by parenchymal cell protein can be corrected for, by measuring the pyruvate kinase activities of every individual cell preparation. The values for the uptake of acetyl-LDL *in vivo* in the NPC_2 fraction were corrected for the contamination by parenchymal cells as described by Van Berkel & Van Tol (1978). Peroxidase staining indicates that the NPC_2 preparation contains Kupffer cells and endothelial cells in a ratio of 1:7. Activity measurements of cathepsin D and peroxidase confirm the calculations mentioned above (Groot *et al.*, 1981).

Lipoprotein binding, uptake and degradation in vitro

Incubations of freshly isolated liver cells with the indicated amounts of lipoproteins and cells were performed in Ham's F-10 medium (modified), containing 5% (v/v) human lipoprotein-deficient serum (final protein concentration 2.5 mg/ml). The binding, uptake and degradation measurements *in vitro* were performed with the parenchymal- and non-parenchymal-cell preparations separated by differential centrifugation only ['PC' and 'NPC_2' (Van Berkel & Van Tol, 1978)]. No correction was made for the contamination of the NPC_2 preparation by parenchymal-cell protein. The incubations were carried out either in plastic tubes in a total volume of 1 ml or in 25 ml Erlenmeyer flasks (silicone-treated) stoppered with rubber caps with a total incubation volume and time as indicated in the legends to the Figures. At the indicated time 1 ml samples were withdrawn and the cell-associated radioactivity was determined as described by Van Berkel *et al.* (1981*a*). Degradation of the lipoproteins was measured by the method of Bierman *et al.* (1974) as described previously (Van Berkel *et al.*, 1981*a*). The degradation values represent radioactivity present in the acid-soluble water phase. In the corresponding blanks the lipoproteins were incubated in the absence of cells.

The viability of the cells during the long-term incubations was checked and remained higher than 85%. The viability was also checked after addition of chloroquine, NH_4Cl, trifluoperazine, penfluridol or chlorpromazine. It was found that at higher chloroquine or trifluoperazine concentrations (above 100 μM) the viability of the cells, especially at the longer incubation times, decreased significantly. With the concentrations reported in this paper no decrease in cell viability or cellular ATP level was observed. When effectors were added, dissolved in dimethyl sulphoxide or propylene glycol, it was checked that a similar amount of the solvent did not influence the control values.

Results

Uptake of acetyl-LDL by parenchymal and non-parenchymal liver cells in vivo

In the rat, iodinated human LDL shows a monoexponential decay curve with a half-life of about 10 h (Van Tol *et al.*, 1978). On acetylation of human LDL the decay rate is strongly influenced and Table 1 shows that at 3 min after injection only 6% of the ^{125}I is still present in serum, and at 10 min this value is further decreased to 2%. The bulk of the radioactivity is recovered in the liver. Subsequent separation of the liver cells into a parenchymal and non-parenchymal cell fraction indicates that the non-parenchymal cells contain a more than 30-fold higher amount of radioactivity per mg of cell protein than the parenchymal cells (Table 2).

When the cells are isolated 30 min after injection of acetyl-LDL, a similar distribution is found. The data obtained 30 min after injection can be compared directly with the uptake of unmodified human LDL *in vivo* by the different cell types as reported previously (Van Tol & Van Berkel, 1980). Such a comparison indicates that on acetylation of LDL the radioactivity found in the parenchymal cells is increased three times, whereas the non-parenchymal cell radioactivity is increased 14 times.

Interaction of acetyl-LDL with parenchymal and non-parenchymal cells in vitro

Fig. 1 shows the time course of the cell-association and degradation of acetyl-LDL by freshly isolated parenchymal and non-parenchymal cells. The initial cell-association rate of acetyl-LDL is rapid and slows down after 30 min of incubation.

With non-parenchymal cells the time course of trichloroacetic acid-soluble radioactivity appearing

Table 1. *Distribution of radioactivity between liver and serum 3, 10 and 30 min after intravenous injection of acetylated human LDL*

Values are means of three different experiments ± S.E.M. The liver samples were obtained after 8 min pre-perfusion with Ca^{2+}-free Hanks balanced salt solution.

Time after injection of acetyl-LDL (min)	Radioactivity distribution (% of injected dose)	
	Liver	Serum
3	—	6.0 ± 0.8
10	83.4 ± 1.7	2.2 ± 0.2
30	18.0 ± 1.2	8.4 ± 0.3

Table 2. *Distribution of acetylated human LDL apoprotein between parenchymal and non-parenchymal liver cells 10 and 30 min after intravenous injection*

Values are means of three to four different experiments ±S.E.M. The recovery values are expressed as percentages.

	10^4 × Distribution of acetylated LDL (% of injected dose/mg of cell protein)	
	10 min	30 min
Whole rat liver	450 ± 19	89 ± 2
Parenchymal cells	20 ± 6	8 ± 2
Non-parenchymal cells (method 1)	639 ± 169	454 ± 126
Non-parenchymal cells (method 2)	453 ± 125	268 ± 71
Ratio of non-parenchymal cells (method 1) to parenchymal cells	32.9 ± 5.3	55.1 ± 8.1
Recovery of radioactivity in parenchymal and non-parenchymal cells (method 1) compared with whole rat liver	14.6 ± 3.3	46.9 ± 12.5

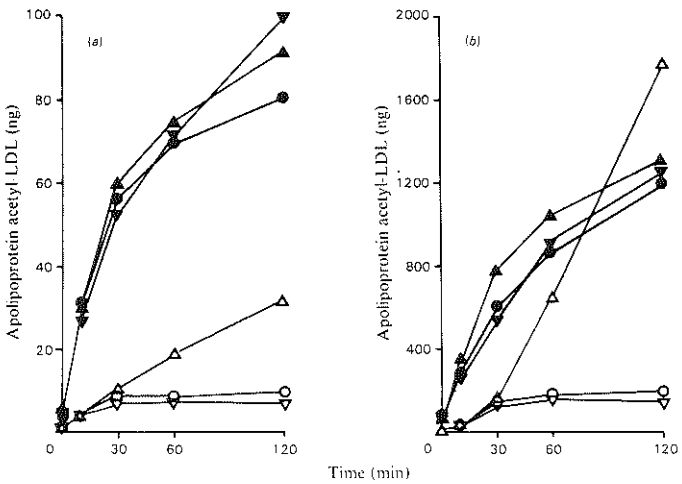

Fig. 1. *Effect of chloroquine and NH_4Cl on the time course of cell-association and degradation of acetyl-LDL by isolated parenchymal (a) and non-parenchymal (b) liver cells*

The cells were incubated with 10.1 μg of acetyl-LDL/ml in the absence (▲ and △) or presence of 100 μM-chloroquine (▼ and ▽) or 10 mM-NH_4Cl (● and ○). The cell-association of acetyl-LDL is indicated with filled symbols, whereas for the degradation open symbols are used. The results are expressed as ng of apolipoprotein associated or degraded/mg of cell protein.

in the water phase shows a clear lag phase (10–30 min) before it reaches a constant rate (up to 3 h). With parenchymal cells, such a lag phase is not readily observed. Owing to the much lower degradation rate, a short-term aspecific degradation masks such a lag phase (Van Berkel *et al.*, 1981*a*). It can be seen that the degradation of acetyl-LDL by non-parenchymal cells is about 50 times higher than by parenchymal cells, although the amount of cell-association is only 13-fold higher (per mg of cell protein). This very active degradation is totally blocked by both chloroquine (100 μM) or NH_4Cl (10 mM) as is the degradation by the parenchymal cells. (There is no increase in trichloroacetic acid-soluble radioactivity between 1 and 2 h of incubation.)

When native human LDL is incubated with parenchymal cells no high-affinity degradation is noticed (not shown). On incubation with non-parenchymal cells a high-affinity binding and degradation of human LDL is observed (Fig. 2). Both the amount of cell-associated radioactivity and the appearance of radioactivity in the trichloroacetic acid-soluble water phase is inhibited by about 50% by an excess of unlabelled human LDL. This high-affinity degradation is inhibited nearly completely by NH_4Cl (10 mM) and chloroquine (100 μM).

The specificity of the acetyl-LDL processing by non-parenchymal cells was determined by investigating the effect of an excess of unlabelled acetyl-LDL, native human LDL, rat LDL and rat HDL on the cell-association process and degradation (Fig. 3).

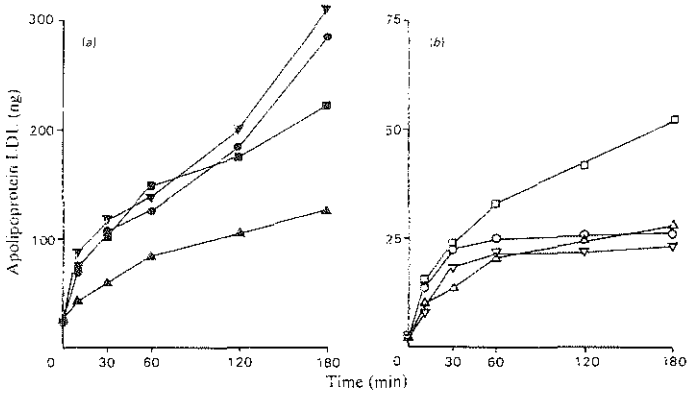

Fig. 2. *Effect of chloroquine, NH_4Cl and excess unlabelled LDL on the cell-association and degradation of native human LDL by non-parenchymal liver cells*

The cells were incubated with 10.6 μg of LDL/ml in the absence (■ and □) or presence of 100 μM-chloroquine (▼ and ▽), 10 mM-NH_4Cl (● and ○) or 103 μg of unlabelled LDL/ml (▲ and △). At the indicated times samples were drawn and the amount of cell-associated radioactivity (*a*) as well as the radioactivity present in the acid soluble water phase were determined (*b*). Both cell-association and degradation are expressed as ng of apolipoprotein/mg of cell protein.

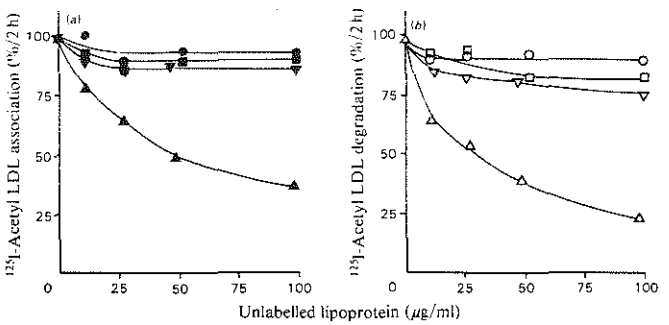

Fig. 3. *Comparison of the ability of unlabelled lipoproteins to compete with the cell association (a) and degradation (b) of ^{125}I-labelled acetylated human LDL by non-parenchymal liver cells*

Non-parenchymal cells were incubated for 2 h with 5.0 μg of ^{125}I-labelled acetyl-LDL/ml and with the indicated amounts of unlabelled human acetyl-LDL (△ and ▲), human native LDL (□ and ■), rat LDL (▼ and ▽) or rat HDL (○ and ●). ^{125}I-labelled apolipoprotein association or degradation is expressed as the percentage of the radioactivity obtained in the absence of unlabelled lipoprotein. The 100% value for the cell association is 632 ng of acetyl-LDL/mg of cell protein and for the degradation 876 ng of acetyl-LDL/mg of cell protein.

It is found that there is a competition between ^{125}I-acetyl-LDL and the unlabelled analogue both in the cell-association and degradation processes. These processes are not influenced by the presence of unlabelled native human LDL or rat LDL and HDL.

The dependency of the cell-association process on the concentration of both acetyl-LDL and unmodified LDL (Fig. 4) indicates that the non-parenchymal cells possess a high affinity for both the acetyl-LDL and native LDL with a half-maximal association rate at a concentration of about 20 μg of apolipoprotein/ml.

Effect of chloroquine, trifluoperazine and Ca^{2+} on the processing of acetyl-LDL

The relative abilities of chloroquine and trifluoperazine to influence the cell-association and degradation of acetyl-LDL by parenchymal and non-parenchymal cells are plotted in Figs. 5(*a*) and 5(*b*). Half-maximal inhibition of the degradation of acetyl-LDL occurs at 15 and 35 μM-chloroquine for non-parenchymal and parenchymal cells respectively.

The phenothiazine tranquillizer trifluoperazine appears to be a very active inhibitor of the degradation of acetyl-LDL both by parenchymal and non-parenchymal cells. A half-maximal effect is seen at about 20 μM, and with 50 μM-trifluoperazine the maximal inhibitory effect is observed. The inhibitory effect of trifluoperazine on the degradation of acetyl-LDL is not caused by a blockade of the binding of acetyl-LDL to the cells, because the cell-association is only slightly influenced (Fig. 5*a*).

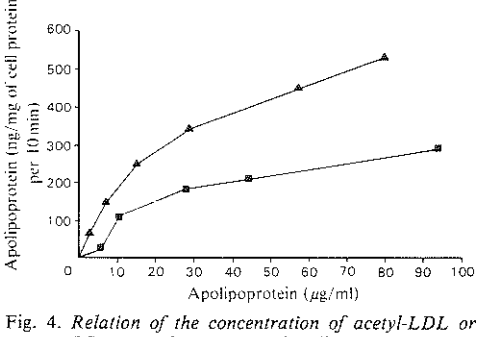

Fig. 4. *Relation of the concentration of acetyl-LDL or native LDL to the extent of cell-association with non-parenchymal liver cells*

The amount of labelled acetyl-LDL (▲) or native LDL (■) in the assay was varied as indicated and the amount of cell-associated radioactivity after 10 min of incubation was determined.

Also the high-affinity degradation of native human LDL is blocked by 100 μM-trifluoperazine (Table 3).

If calmodulin is involved in the processing of acetyl-LDL it is possible that an extracellular Ca^{2+} concentration change influences the degradation rate of acetyl-LDL. Fig. 6 shows that in the presence of Mg^{2+}–EGTA (2 mM) the degradation of acetyl-LDL is about half of that measured at 0.3 mM-Ca^{2+}. With parenchymal cells in the absence of Ca^{2+} or presence of Mg–EGTA a lower degradation rate is measured than that with 0.1–0.3 mM-Ca^{2+}.

Table 3. *Comparison of the effect of trifluoperazine, NH_4Cl and chloroquine on the high-affinity degradation of human LDL by non-parenchymal cells*

The cells were incubated for 2 h with labelled human LDL (9.9 μg/ml) and the degradation was determined as trichloroacetic acid-soluble radioactivity in the water phase. The values are means (±S.E.M.) for three to four experiments. The 100% value represents 45 ± 6 ng of acetyl-LDL degradation/mg of cell protein.

Addition	Degradation (% of control)
None	100
Trifluoperazine (100 μM)	51 ± 5
Chloroquine (100 μM)	48 ± 4
NH_4Cl (10 mM)	53 ± 3
Unlabelled LDL (103 μg/ml)	54 ± 5

Table 4. *Relative contribution of binding to the total cell-associated radioactivity after 10, 60 and 120 min incubation of non-parenchymal cells with acetyl-LDL in the absence and presence of trifluoperazine or chloroquine*

The cells were incubated for 10, 60 or 120 min with labelled acetyl-LDL (10.1 μg/ml). Subsequently the cells were centrifuged, washed and incubated in the absence and presence of collagenase (0.05%). The amount of cell-associated radioactivity was determined and the relative percentage released by collagenase was taken as cell-surface-bound and the values represent this percentage cell-surface binding. For further details see Van Berkel *et al.* (1981*a*). The values are means of at least two experiments. The 100% value for the incubation without further additions was for 10 min, 60 min and 120 min of incubation respectively 648 ± 112, 1150 ± 121 and 1694 ± 80 ng of acetyl-LDL/mg of cell protein ($n = 5$; means ± S.E.M.). These values were not significantly different when trifluoperazine or chloroquine was present.

Addition	Cell-surface binding (%)		
Incubation time (min) ...	10	60	120
None	50	32	17
Trifluoperazine	65	37	29
Chloroquine	60	33	19

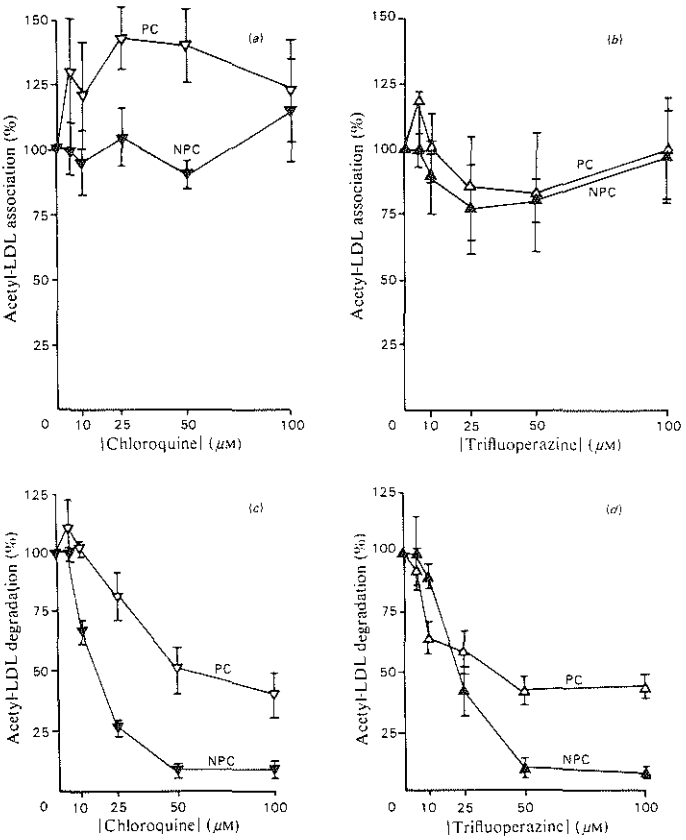

Fig. 5. *The effect of increasing chloroquine (a and c) or trifluoperazine (b and d) concentrations on the cell-association (a and b) and degradation (c and d) of acetyl-LDL by isolated parenchymal cells (PC) and non-parenchymal cells (NPC)* The cells were incubated for 2h with 10.1 μg of acetyl-LDL/ml in the presence of the indicated amount of chloroquine or trifluoperazine. The results were obtained with three different acetyl-LDL and cell preparations and are given as mean percentages of the association or degradation in the absence of the effectors ± S.E.M. (indicated by the bars). The 100% value for cell-association with PC was 59 ± 7 and for NPC was 1211 ± 220 ng of acetyl-LDL/mg of cell protein. For the degradation the 100% value for PC was 44 ± 7 and for NPC 1462 ± 180 ng of acetyl-LDL/mg of cell protein ($n = 3$; means ± S.E.M.)

To indicate more precisely the site of action of trifluoperazine we investigated to what extent the internalization of the acetyl-LDL particle is influenced by this compound.

Table 4 shows that internalization of acetyl-LDL still occurs in the presence of trifluoperazine. Furthermore we checked the possibility that trifluoperazine inhibits the excretion process of the degradation products of acetyl-LDL. The amount of cell-associated trichloroacetic acid-soluble radioactivity was, however, not increased in the presence of trifluoperazine or chloroquine as compared with control incubations.

Effect of penfluridol and chlorpromazine

As well as trifluoperazine, other antipsychotic drugs acting as inhibitors of calmodulin are available (Levin & Weiss, 1979). Among these compounds penfluridol and chlorpromazine are most effective.

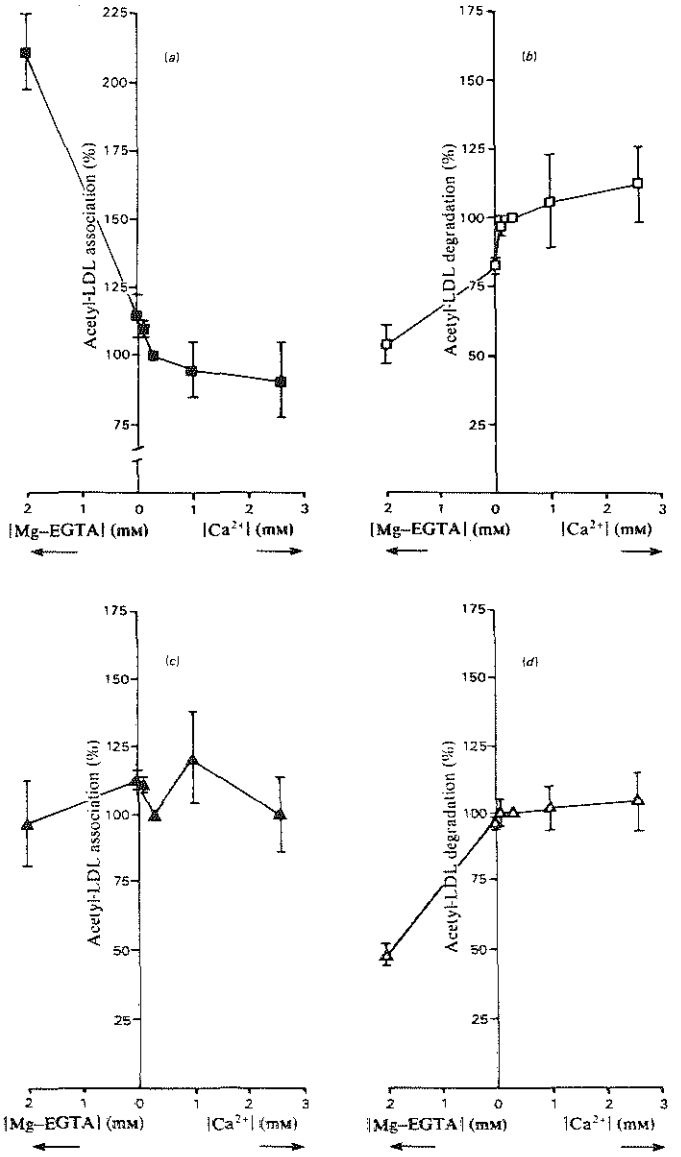

Fig. 6. *The effect of Mg–EGTA and Ca^{2+} on the cell-association (a and c) and degradation (b and d) of acetyl-LDL by parenchymal (a and b) and non-parenchymal (c and d) cells*

The cells were incubated for 2 h with 5.0 μg of acetyl-LDL/ml with the indicated [Mg–EGTA] or the different [Ca^{2+}]. For the cells incubated with Mg–EGTA the last two washings before incubation were also performed in the presence of 2 mM-Mg–EGTA. The results were obtained with four different acetyl-LDL and cell preparations and are given as mean percentages of the association or degradation at 0.3 mM-Ca^{2+} ± S.E.M. (indicated by the bars). The 100% values for the cell association were, for parenchymal cells 37.0 ± 6.3 and for non-parenchymal cells 955 ± 68 ng of acetyl-LDL/mg of cell protein. The 100% values for the degradation were, for parenchymal cells 22.5 ± 4.6 and for non-parenchymal cells 1216 ± 87 ng of acetyl-LDL/mg of cell protein (n = 4; means ± S.E.M.).

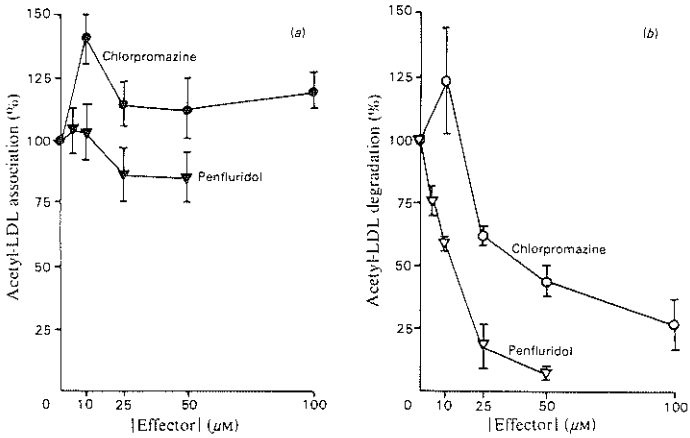

Fig. 7. *The effect of increasing penfluridol and chlorpromazine concentrations on the cell-association (a) and degradation (b) of acetyl-LDL by non-parenchymal liver cells*

The cells were incubated for 2 h with 10.1 μg of acetyl-LDL/ml in the presence of the indicated amount of effector. The results were obtained with three different acetyl-LDL and cell-preparations and are given as mean percentages of the association or degradation in the absence of effectors ± S.E.M. (indicated by the bars) The 100% value for the cell-association was 1642 ± 378 ng of acetyl-LDL/mg of cell protein and for the degradation 1947 ± 383 ng of acetyl-LDL/mg of cell protein ($n = 3$; means ± S.E.M.).

Fig. 7 shows that, besides trifluoperazine, penfluridol and chlorpromazine are effective inhibitors of the degradation of acetyl-LDL by non-parenchymal cells, with half-maximal inhibitory concentrations of 12 and 35 μM respectively.

Discussion

The present results with ^{125}I-acetyl-LDL indicate that both parenchymal and non-parenchymal cells possess a site that recognizes acetyl-LDL. The competition experiments indicate that the acetyl-LDL recognition site is specific for acetyl-LDL, as no significant competition was observed with native human LDL or with the rat lipoproteins. Furthermore 10–100 μg of unlabelled acetyl-LDL/ml was effective in showing competition, even though a 25–250-fold excess of extracellular protein was present (approx. 2500 μg of protein/ml). According to the definition of Ho *et al.* (1976) these characteristics are indicative of the presence of a specific high-affinity receptor. The binding of acetyl-LDL to its receptor is effectively coupled to uptake, and after 10 min of incubation already half of the cell-associated radioactivity is internalized. Parenchymal cells also interact with acetyl-LDL, a binding that is similarly coupled to further intracellular processing. The amount of acetyl-LDL associated with non-parenchymal cells is, however, about 13-fold higher per mg of cell protein than with parenchymal cells, with a degradation rate that is 50-fold higher. This indicates that, taking into account the relative protein contribution of non-parenchymal cells (7.5%) and parenchymal cells (92.5%) to total liver, the non-parenchymal liver cells are the major site for acetyl-LDL catabolism. That this is also the case *in vivo* can be concluded from the data on the uptake of acetyl-LDL by parenchymal and non-parenchymal cells *in vivo*.

At the moment it cannot be decided to what extent the different cell types present in the non-parenchymal cell preparations (endothelial or Kupffer cells) are responsible for the active interaction with acetyl-LDL. A further purification of the non-parenchymal cells by a procedure that does not affect the active endocytotic mechanism is therefore needed.

The present paper shows that once the acetyl-LDL is bound to its receptor on non-parenchymal cells, an efficient uptake and degradation process starts. This contrasts with previous data obtained with the native rat lipoproteins, of which the greater part (70–80%) remains extracellularly bound (Van Berkel *et al.*, 1981*a*; Ose *et al.*, 1980). The degradation of acetyl-LDL by freshly isolated non-parenchymal cells is completely blocked by low concentrations of chloroquine or NH_4Cl. These properties are consistent with a classical route for

receptor-mediated uptake, i.e. binding to a high-affinity receptor (specific for acetyl-LDL), uptake in endocytotic vesicles and degradation inside the lysosomes. A similar route can be described for the interaction of unmodified human LDL with non-parenchymal liver cells, although the amount of human LDL that is degraded relative to the amount that is cell-associated indicates a much less efficient intracellular processing. In the presence of chloroquine or NH_4Cl, the amount of acetyl-LDL associated with non-parenchymal cells at 2h of incubation is similar to the amount obtained with the incubation in the absence of these agents. Because acetyl-LDL degradation hardly occurs, this indicates that the total amount of acetyl-LDL handled by the cells is considerably decreased. This might imply that chloroquine or NH_4Cl can also exert an effect on the receptor internalization or recycling. As shown in Table 4 the internalization of acetyl-LDL is not influenced by chloroquine, indicating that this additional action of chloroquine is exerted on the receptor recycling process.

In the present study we compared the relative ability of three of the most potent inhibitors of calmodulin (Levin & Weiss, 1979) on the processing of acetyl-LDL by non-parenchymal cells. The concentrations necessary for half-maximal inhibition of acetyl-LDL degradation were 12 μM for penfluridol, 21 μM for trifluoperazine and 35 μM for chlorpromazine. The relative effectivity of these compounds to inhibit acetyl-LDL degradation corresponds to their effectivity as calmodulin inhibitors [half-maximal inhibition 2.5 μM for penfluridol, 10 μM for trifluoperazine and 42 μM for chlorpromazine (Levin & Weiss, 1979)].

The site at which trifluoperazine interferes with the degradation of acetyl-LDL was investigated in more detail. Our data indicate that its complete inhibition of degradation cannot be explained by an effect on the initial binding or internalization process. The action of trifluoperazine is probably not at the level of the lysosome itself because the degradation of acetyl-LDL *in vitro* by cell homogenates at an acid pH is not inhibited by trifluoperazine (Van Berkel *et al.*, 1981*b*). Furthermore there is no accumulation of trichloroacetic acid-soluble radioactivity inside the cells, so that trifluoperazine does not exert its action on the secretion of the degradation products of acetyl-LDL. The action of trifluoperazine is then restricted either to the intracellular route from the internalization site of acetyl-LDL to the lysosomes or to the fusion process with the lysosomes. Although the relative effectivity of penfluridol, trifluoperazine and chlorpromazine on acetyl-LDL degradation can be considered as further evidence for the involvement of calmodulin, it must be stressed that these compounds do bind to calmodulin on a single site. Probably hydrophobic regions in calmodulin are involved in this binding (Tanaka & Hidaka, 1980). Therefore it remains possible that the inhibition of acetyl-LDL degradation is either exerted at calmodulin or at a still unknown target with an active site similar to that of calmodulin.

As mentioned in the introduction section, the fate of the acetylated LDL *in vivo* was studied, in view of its possible relevance in the pathogenesis of atherosclerosis (Henriksen *et al.*, 1981). Both the uptake data *in vivo* and the data on the interaction of acetyl-LDL with the isolated liver cells *in vitro* indicate that the liver, and in particular the non-parenchymal liver cells, are the major site for acetyl-LDL uptake. Recently Henriksen *et al.* (1981) showed that native LDL can be converted by aortic endothelial cells into a form that is recognized by the macrophage receptor for acetyl-LDL. The presence of the highly active acetyl-LDL receptor in liver, as shown here, might form *in vivo* the major protection system against the potential pathogenic action of these modified lipoproteins.

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References

Basu, S. K., Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3178–3182

Bierman, E. L., Stein, O. & Stein, Y. (1974) *Circ. Res.* **35**, 136–150

Brown, M. S., Ho, Y. K. & Goldstein, J. L. (1980) *J. Biol. Chem.* **255**, 9344–9352

Chomette, G., De Gennes, J. L., Delcourt, A., Hammon, J. C. & Perie, G. (1971) *Ann. Anat. Pathol.* **16**, 233–250

Crisp, D. M. & Pogson, C. I. (1972) *Biochem. J.* **126**, 1009–1023

Fogelman, A. M., Shechter, I., Seager, J., Hokom, M., Child, J. S., & Edwards, P. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2214–2218

Fredrickson, D. S., Goldstein, J. L. & Brown, M. S. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wijngaarden, J. B. & Fredrickson, D. S., eds.), 4th edn., pp. 604–665, McGraw-Hill Book Co., New York

Goldstein, J. L. & Brown, M. S. (1978) *Johns Hopkins Med. J.* **143**, 8–16

Goldstein, J. L., Ho, Y. K., Basu, S. K. & Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333–337

Groot, P. H. E., Van Berkel, Th. J. C. & Van Tol, A. (1981) *Metab. Clin. Exp.* **30**, 792–797

Henriksen, T., Mahoney, E. M. & Steinberg, D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6499–6503

Ho, Y. K., Brown, M. S., Bilheimer, D. W. & Goldstein, J. L. (1976) *J. Clin. Invest.* **58**, 1465–1477
Knook, D. L. & Sleyster, E. Ch. (1976) *Exp. Cell Res.* **99**, 444–449
Kooistra, T., Duursma, A. M., Bouma, J. M. W. & Gruber, M. (1979) *Biochim. Biophys. Acta* **587**, 282–298
Langer, T., Strober, W. & Levy, R. I. (1972) *J. Clin. Invest.* **51**, 1528–1536
Levin, R. M. & Weiss, B. (1977) *Mol. Pharmacol.* **31**, 690–697
Levin, R. B. & Weiss, B. (1979) *J. Pharmacol. Exp. Ther.* **208**, 454–459
McFarlane, A. S. (1958) *Nature (London)* **182**, 53
Ose, T., Berg, T., Norum, K. R. & Ose, L. (1980) *Biochem. Biophys. Res. Commun.* **97**, 192–199
Redgrave, T. G., Roberts, D. C. K. & West, C. E. (1975) *Anal. Biochem.* **65**, 42–49
Shechter, I., Fogelman, A. M., Haberland, M. E., Seager, J., Hokom, M. & Edwards, P. A. (1981) *J. Lipid Res.* **22**, 63–71
Tanaka, T. & Hidaka, H. (1980) *J. Biol. Chem.* **255**, 11078–11080
Van Berkel, Th. J. C. (1974) *Biochem. Biophys. Res. Commun.* **61**, 204–209
Van Berkel, Th. J. C. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 437–482, Academic Press Inc., London
Van Berkel, Th. J. C. & Van Tol, A. (1978) *Biochim. Biophys. Acta* **530**, 299–304
Van Berkel, Th. J. C., Kruijt, J. K., Slee, R. G. & Koster, J. F. (1977) *Arch. Biochem. Biophys.* **179**, 1–7
Van Berkel, Th. J. C., Kruijt, J. K., Van den Berg, G. B. & Koster, J. F. (1978) *Eur. J. Biochem.* **92**, 553–561
Van Berkel, Th. J. C., Kruijt, J. K., Van Gent, T. & Van Tol, A. (1981*a*) *Biochim. Biophys. Acta* **665**, 22–33
Van Berkel, Th. J. C., Nagelkerke, J. F. & Kruijt, J. K. (1981*b*) *FEBS Lett.* **132**, 61–66
Van Tol, A. & Van Berkel, Th. J. C. (1980) *Biochim. Biophys. Acta* **619**, 156–166
Van Tol, A., Van't Hooft, F. M. & Van Gent, T. (1978) *Atherosclerosis* **29**, 449–457
Wurster, N. B. & Zilversmit, D. B. (1971) *Atherosclerosis* **14**, 309–322

In Vivo Catabolism of Biologically Modified LDL

J. Fred Nagelkerke, Louis Havekes, Victor W.M. van Hinsbergh, and Theo J.C. van Berkel

Incubation of human low density lipoprotein (LDL) at 37°C in the presence of human umbilical vein endothelial cells (EC) caused a time-dependent shift in the charge and density of LDL. The physical changes of the human LDL occurred parallel with an increase in its clearance from the serum and uptake in the liver when injected into rats. The serum decay of the EC-modified LDL (44 hours incubation) was 20 times faster than for control LDL. EC-modified LDL, cleared from the blood, was quantitatively recovered in the liver. Isolation of the different liver cell types (parenchymal, Kupffer, and endothelial cells) after in vivo injection of ^{125}I-EC-modified LDL showed that approximately 30 times more radioactivity was associated with the endothelial cells than with the parenchymal cells (per milligram of cell protein). In vitro experiments indicated that EC-modified-LDL was processed by the rat liver endothelial cells via a high affinity, saturable pathway related to the pathway by which these cells processed acetyl-LDL. We concluded that, if EC-modified LDL is generated in vivo, the liver, and in particular the endothelial cell, forms the major protection system against the occurrence of atherogenic particles in the blood.
(Arteriosclerosis 4:256–264, May/June 1984)

The mechanism by which low density lipoprotein (LDL) is cleared from the blood circulation has been a subject of intensive research for the last decade. The best known mechanism is the classical LDL-receptor pathway, initially described by Goldstein and Brown.[1]

However, in vivo studies indicate that the LDL clearance from the blood is not quantitatively mediated by the classical receptor.[2] Therefore, other mechanisms have been proposed, generally called "nonreceptor" pathways. This name was based upon the observation that LDL, in which the recognition site for the classical LDL receptor is blocked, is nevertheless cleared from the circulation, although at a lower rate.[2] One such additional clearance pathway, operating via a different receptor site, is the scavenger pathway. Its existence was proposed on the basis of the findings of Brown et al.[3] that accumulation of cholesterol esters in macrophages, observed in patients with increased LDL levels, could only be provoked in vitro by incubation of macrophages with acetylated low density lipoprotein (acetyl-LDL), while native LDL was ineffective. The scavenger pathway is predominantly present in cell types belonging to the mononuclear phagocyte system.[4] Furthermore, the receptor site is present in the foam cell,[5] which is thought to be derived from cells belonging to this system.

Recently we demonstrated[6] that human acetyl-LDL is almost completely cleared from the circulation by the liver within 3 minutes after injection into rats. Moreover, it was demonstrated that the rat liver endothelial cell is by far the most active liver cell type in the uptake of acetyl-LDL. In vitro studies indicated that a high affinity saturable receptor site for acetyl-LDL is present on the liver endothelial cell at a relatively high concentration.

In vivo acetylation of LDL is, however, doubtful and, therefore, the physiological importance of the scavenger pathway was unclear. Recently a more relevant biological modification of LDL, leading toward in vitro recognition by the scavenger receptor on macrophages, was reported by Henriksen et al.[7,8] The modification was achieved by incubating the LDL with rabbit aortic or human umbilical vein endo-

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thelial cells (EC-modified LDL). The purpose of this study was to investigate the behavior of EC-modified LDL in vivo and to correlate this with receptor studies in vitro. In addition, the in vivo and in vitro uptake and degradation rates of EC-modified LDL were compared with those of acetyl-LDL.

Methods

Isolation and Culture of Umbilical Vein Endothelial Cells

Human umbilical cords were kept after delivery in ice-cold cord buffer (140 mM NaCl, 4 mM KCl, 11 mM D-glucose, 10 mM Hepes, pH 7.3, 100 IU/ml penicillin, 0.10 mg/ml streptomycin). They were collected once a day from the hospital. Endothelial cells were isolated from veins as described by van Hinsbergh et al.[9] Briefly, the vein was rinsed and incubated for 15 minutes at 37°C with 0.1% collagenase in M-199 medium at 37°C. The collected venous cells were centrifuged (5 minutes 200 *g*) and suspended in M-199 medium containing 20% human serum and 15 mM Hepes buffer. They were seeded rather densely ($1–5 \times 10^4$ cells/cm^2) in six 2 cm^2 wells coated with a crude fibronectin solution. The coating was performed at 37°C for 30 minutes. The fibronectin solution was removed by aspiration immediately before the cells were seeded. The cells were cultured at 37°C in M-199 medium supplemented with 20% pooled human serum (not inactivated), 15 mM Hepes buffer, 100 IU/ml penicillin and 0.1 mg/ml streptomycin under 5% CO_2 in air. The medium (0.2 ml/cm^2) was refreshed every 2 or 3 days. At confluency, the cells were used, or released with trypsin/EDTA and passaged with a 1:3 split ratio to obtain subcultures.

Low Density Lipoproteins

LDL was isolated from freshly prepared human serum by density gradient ultracentrifugation according to the method of Redgrave et al.[10] followed by tube slicing. SDS-polyacrylamide gel electrophoresis showed only the presence of apo B. The LDL was immediately used for iodination by the ^{125}I-iodine monochloride method described by Bilheimer et al.[11] After iodination, LDL was dialyzed against phosphate-buffered saline (PBS), without EDTA, for 4 hours (4 × 500 volumes). Thereafter, it was stabilized by the addition of 10 mg of bovine serum albumin (BSA) per ml and further dialyzed overnight against 500 volumes of PBS at 4°C. The specific activity ranged from 80 to 150 cpm/ng of LDL protein. The acid-soluble (noniodine) fraction was less than 0.1%. This ^{125}I-labelled LDL was used for endothelial cell modification within 2 days of storage at 4°C.

Endothelial Cell-Modified LDL

EC-modified LDL was prepared by incubating confluent endothelial cell cultures at 37°C with medium M-199 supplemented with 10 g/l BSA, instead of 20% human serum, and with 100 μg of LDL protein per ml medium. After the indicated time intervals, the medium was aspirated and stored at 4°C. The morphology of the cells was then examined by phase contrast light microscopy. At all time intervals, no morphological changes could be detected. Incubations of LDL in the same medium at 37°C but without cells were used as controls.

EC-modified LDL and control LDL preparations were, without prior isolation from the culture media, subjected to agarose electrophoresis according to the method of Demacker.[12] After electrophoresis, the agarose plate was dried by a stream of hot air and subjected to autoradiography. Simultaneously, 100 μl of the culture media were subjected to density gradient ultracentrifugation according to the method of Redgrave et al.[10] After 14 hours of ultracentrifugation (4°C, mean RCF is 200,000 *g*) density fractions of 0.5 ml were collected using the method described by Groot et al.[13] The fractions were measured for density using a DMA 602 M densitometer and counted for radioactivity. The acid soluble (noniodine) fractions of the EC-modified and control LDL samples were measured as described by Bierman et al.[14]

Acetylation of LDL

LDL was acetylated with acetic anhydride as described by Basu et al.[15]

Rat Liver Cells

Throughout this study 3-month-old male Wistar R1 rats (Centraal Proefdiezen Bedzyf, Rotterdam, The Netherlands) were used. Rat liver endothelial, Kupffer, and parenchymal cells were prepared as previously noted.[6] After injection of the indicated lipoprotein preparations, the cells were isolated with either pronase or collagenase by a low temperature procedure. Earlier studies[6] showed that in vivo endocytosed material was not degraded during the applied isolation procedures, which led to a reliable determination of the contribution of each cell type to total liver uptake in vivo.

For in vitro studies cells were isolated with collagenase at 37°C as described earlier.[6,16] We found that this procedure gives the best preservation of lipoprotein receptor activity.

Freshly isolated endothelial cells were incubated at 37°C in Hams F-10 medium containing 2% BSA and the indicated amounts of lipoproteins. Incubations were carried out in siliconized Sorvall tubes. At the indicated times, 1 ml samples were withdrawn, transferred to plastic Eppendorff tubes, and centrifuged for 2 minutes at 600 *g*. The cell pellets were suspended in 1 ml of medium, containing 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl and 2 mg BSA; incubated for 5 minutes at 4°C; and centrifuged again. This washing procedure was repeated twice. The last washing was performed with similar medium without BSA for a reliable cell protein determination.

Degradation of the lipoproteins was measured according to the method of Bierman et al.[14] To 0.5 ml of the first supernatant, 0.2 ml of 35% trichloroacetic acid was added, followed by incubation for 15 minutes at 37°C; subsequently the mixture was centrifuged for 2 minutes at 15,000 *g*. To 0.5 ml of the supernatants, 5 μl of 40% potassium iodide and 25 μl of 30% hydrogen peroxide were added. After 5 minutes at room temperature, 0.8 ml chloroform was added and the mixture was shaken for another 5 minutes. After centrifugation for 2 minutes at 15,000 *g*, 0.4 ml of the aqueous phase (containing iodinated degradation products) and 0.5 ml of the chloroform phase (containing free iodine) were sampled.

Radioactivity was counted in a LKB-Wallace ultragamma counter.

The viability of the cells before and after incubation was more than 95% as judged by the Trypan blue exclusion test. The purity of the rat liver endothelial cell preparations was checked by a light microscope as described earlier[6] and was always more than 95% pure.

Protein Determination

Protein determination was according to the method of Lowry et al.[17] using BSA as a standard.

Chemicals

Type I collagenase and BSA, Fraction V, were from Sigma Company, St. Louis, Missouri; B-grade pronase was obtained from Calbiochem-Behring Corporation, La Jolla, California; metrizamide was from Nyegaard and Company, A/S, Oslo, Norway; Ham's F-10 was from Gibco-Europe, Hoofddorp, The Netherlands; and ^{125}I (carrier-free) in NaOH was from New England Nuclear, Dreieich, West Germany.

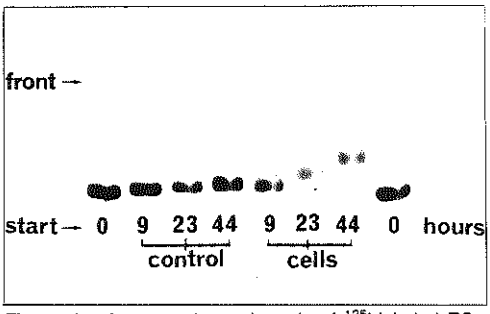

Figure 1. Agarose electrophoresis of ^{125}I-labeled EC-modified LDL and ^{125}I-labeled control LDL. After incubation in the presence or absence of cells at 37°C for the indicated time, 2 μl of medium was subjected to agarose electrophoresis according to the method of Demacker et al.[12] After 3 hours of electrophoresis, the agarose plate was dried by hot air and subjected to autoradiography for 18 hours.

Results

Characterization of the LDL Preparations

Human LDL incubated with human umbilical vein endothelial cells for increasing periods of time showed an increasing electrophoretic mobility on agarose gels (Figure 1). This effect was already observed after 9 hours of incubation. The mobility increased further with prolonged incubation times without reaching a plateau during the time studied. Note that LDL incubated for 44 hours at 37°C in the absence of cells also migrated slightly faster than LDL that was not incubated.

Density gradient ultracentrifugation demonstrated that after 44 hours of incubation, the buoyant density had shifted from the normal LDL range toward a mean density of d = 1.080 g/ml (Figure 2). The incubation of LDL in the absence of cells did not lead to a significant shift in buoyant density. The acid soluble (noniodine) radioactivity in all lipoprotein fractions was less than 1% of the acid-precipitable radioactivity (Table 1). The modification was a result of a direct interaction between LDL and the umbilical vein endothelial cells. LDL that was incubated for 44 hours in medium in which endothelial cells were previously incubated for 44 hours was not significantly altered (data not shown).

In Vivo Studies

Serum decay and liver uptake after intravenous injection into rats were determined with the same lipoprotein preparations that are characterized in Figures 1 and 2 and Table 1. The LDL preparations were injected into the recipient animals without prior isolation from the media. Figure 3 shows the serum decay of the ^{125}I-labeled EC-modified and control lipoproteins after injection into rats. (Routinely, a lipoprotein preparation containing 40–60 μg of apoprotein, specific activity 80–150 dpm/ng apoprotein was injected.) There was a strong effect of prolonged incubation of the LDL with the umbilical vein endothelial cells on the clearance from serum. Also the LDL that was incubated for 44 hours at 37°C in the absence of cells was cleared slightly faster from the circulation than LDL that was not incubated. This

Table 1. Water-Soluble (Noniodine) Radioactivity in the Lipoprotein Fractions

Incubation time (hrs)	Presence of umbilical vein endothelial cells	Absence of umbilical vein endothelial cells
0	0.05	0.05
9	0.27	0.06
23	0.39	0.07
44	0.91	0.17

Acid-precipitable and acid-soluble (noniodine) radioactivity was determined as in reference 14. Values are expressed as percentage of the acid-precipitable radioactivity.

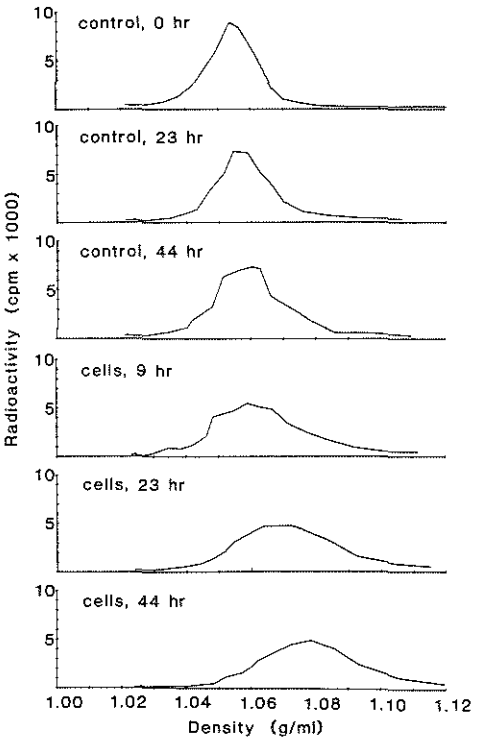

Figure 2. Density gradient ultracentrifugation of ^{125}I-labeled EC-modified LDL and control LDL. After incubation in the presence or absence of cells at 37°C for the indicated time, 100 μl of medium was mixed with 4 ml KBr salt solution (density 1.21 g/ml) and subjected to density gradient ultracentrifugation according to the method of Redgrave et al.[10] After 14 hours of ultracentrifugation (RCF 200,000 *g*, 4°C) the density fractions (0.5 ml) were collected using the apparatus described by Groot et al.[13] connected to a fraction collector. Each fraction was measured for density and counted for radioactivity. In the figure only the fractions with densities between 1.00 and 1.12 g/ml are shown.

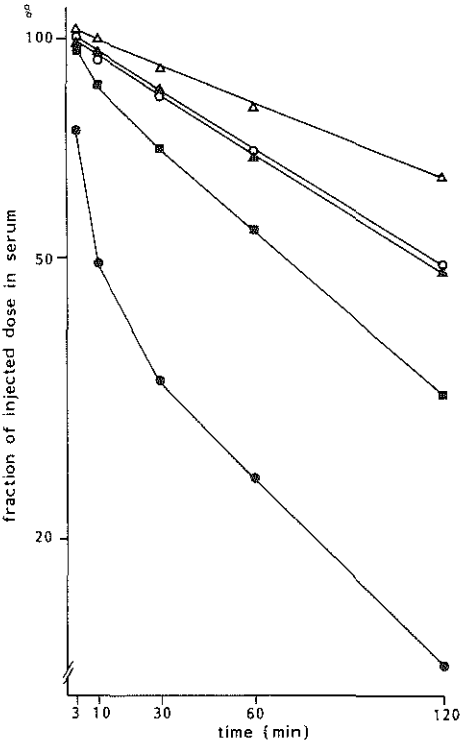

Figure 3. Serum decay of ^{125}I-labeled lipoproteins after injection into rats. Blood samples were drawn as indicated. The samples were centrifuged for 2 minutes at 20,000 *g* and radioactivity was counted in the supernatants. The values are expressed as percentages of the injected dose. LDL was incubated with human umbilical vein endothelial cells for 9 (○), 23 (■) or 44 (●) hours or in the absence of cells for 0 (△) or 44 (▲) hours. Three different batches of LDL were modified for different time periods. The results shown are from one batch. Experiments with the other two batches gave similar results.

clearance is, however, much slower than that of LDL incubated for 44 hours in the presence of cells.

To determine the kinetics of the liver association in vivo, the ^{125}I-labeled lipoproteins (40–60 μg apoprotein) were injected into rats. At different time intervals after injection, a liver lobule was tied off and excised. After weighing the lobule and counting its radioactivity, the total liver uptake was extrapolated using the assumption that 3.75% of the total body weight is contributed by the liver.[18] Figure 4 shows that the uptake of the EC-modified LDL by the liver increased with prolonged incubation of the LDL with the endothelial cells. Also the peak values of the lipoprotein content in the liver shifted to shorter time intervals after injection.

Determination of the cell types responsible for the liver uptake was performed by isolating the various cell types 10 minutes after the in vivo injection of the ^{125}I-labelled lipoproteins (40 to 60 μg apoprotein) into rats (Figure 5). It appeared that the liver endothelial and parenchymal cells were mainly responsible for the increased liver association of EC-modified LDL. The relative contribution of these cell types to the uptake of EC-modified LDL appeared to be similar to that observed earlier for chemically modified LDL (acetyl-LDL).[6] Approximately 30 times more EC-modified LDL became associated with the endothelial cells than with the parenchymal cells (per mg cell protein). Taking into account the contribution of each cell type to the total liver protein,[18] the total rat liver

endothelial cell population was the major site for the liver uptake of EC-modified LDL, similar to acetyl-LDL (Table 2). Figure 5 also shows that LDL incubated 44 hours in the absence of cells was taken up more than the nonincubated LDL. However, the presence of umbilical vein endothelial cells during incubation resulted in an uptake by endothelial and parenchymal cells that was about 10 times as high.

To determine the cellular metabolism of EC-modified LDL associated with the liver endothelial cells in vivo, we warmed the cells, isolated at 8°C, to 37°C. At different time intervals thereafter, we drew a sample and determined the cell-bound and excreted acid-soluble (noniodine) degradation products.

Figure 6 shows that the radioactivity initially bound to the cells appeared as acid-soluble (noniodine) radioactivity in the medium, indicating the degradation of the apoprotein.

To compare the intracellular processing rate of EC-modified LDL with acetyl-LDL, we repeated the experiment. This time acetyl-LDL was injected. One-half of the acetyl-LDL, initially cell-associated, was

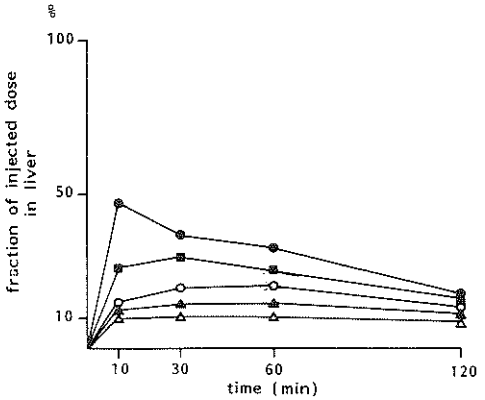

Figure 4. Liver uptake of ^{125}I-labeled lipoproteins after injection into rats. After injection, lobules were tied off and excised at the indicated times. Values are expressed as percentages of the injected doses. The lobules were not perfused; liver values here include the amount of lipoproteins present in the entrapped blood (approx. 9% of the serum value based upon ^{3}H-albumin measurements). LDL was incubated with human umbilical vein endothelial cells for 9 (○), 22 (■) or 44 (●) hours or in the absence of cells for 0 (△) or 44 (▲) hours.

Table 2. Relative Contribution of the Different Liver Cell Types to the Total Uptake of EC-Modified LDL and Acetyl-LDL by Rat Liver

Cell type	EC-modified LDL incubation time (hrs) 9	22	44	Acetyl-LDL
Parenchymal cells (%)	33	34	37	38
Endothelial cells (%)	49	49	51	53
Kupffer cells (%)	18	17	12	9

The percentage was calculated by multiplying the values from Figure 4 with the amount of protein that each cell type contributes to total liver protein. The values are expressed as a percentage of the total injected dose.

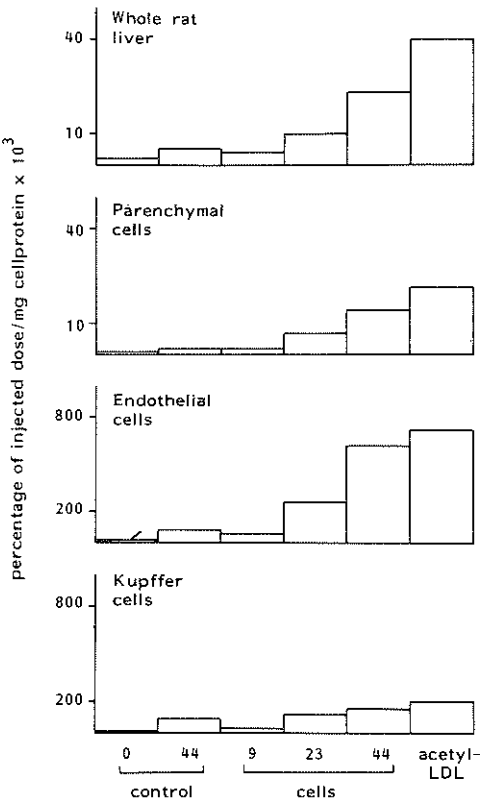

Figure 5. Cellular distribution of ^{125}I-labeled LDL in liver after intravenous injection into rats. Ten minutes later perfusion was started by cannulation of the vena porta. The perfusion and cell isolation was performed at 8°C to prevent degradation of endocytosed LDL.[6] Eight minutes after perfusion began a lobule was tied off and excised; then different cell types were isolated and separated. Values are expressed as percentages of the injected dose per mg liver or cell protein. From each lipoprotein fraction tested two independent total liver uptake values were obtained. The averages are shown at the top (left to right): 1.55 ± 0.15; 5.95 ± 1.15; 3.7 ± 0.1; 10.35 ± 0.35; 22.3 ± 4.4; 39.3 ± 2.25.

degraded within 10 minutes, although it took 30 to 40 minutes of incubation at 37°C before one-half of the cell-associated EC-modified LDL was degraded.

Nature of the Recognition Site

The time course of in vitro cell-association of the EC-modified LDL, (incubated for 44 hours) with isolated liver endothelial cells, indicates a linear increase with time up to 2 hours (Figure 7 A). In the insert the results of a similar experiment with acetyl-LDL as a substrate are shown. Here cell association was more rapid and reached a plateau after 30 minutes of incubation. The degradation rate of EC-modified LDL showed a lag-phase of 30 minutes before TCA-soluble (noniodine) degradation products were detected (Figure 7 B). In the insert the degradation rate of acetyl-LDL is shown, with degradation detectable between 10 and 30 minutes. Addition of the lysosomotropic agent chloroquine (50 μM) led to a virtually complete blockade of the degradation of EC-modified LDL, suggesting the involvement of the lysosomes (Figure 7 B).

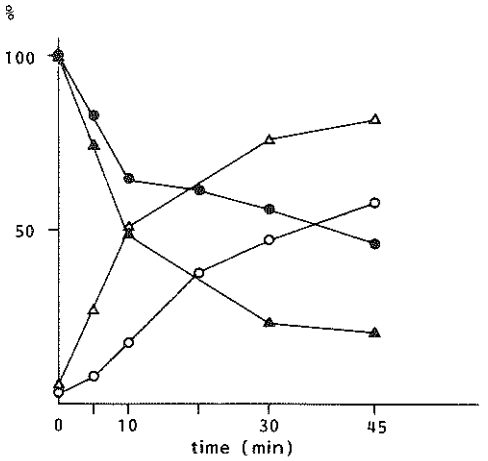

Figure 6. Time course of intracellular processing of EC-modified LDL and acetyl-LDL by isolated liver endothelial cells after in vivo injection of lipoproteins. Rat liver endothelial cells were isolated from rats injected with 40–60 μg ^{125}I-labeled lipoproteins 10 minutes before starting liver perfusion. The isolated pure endothelial cells were resuspended in buffer at 37°C. Samples were drawn at indicated times, the cells were centrifuged, and the amount of cell-associated and acid-soluble radioactivity in the supernatant was determined. Closed symbols represent cell-associated radioactivity; open symbols, acid-soluble (noniodine) radioactivity. Triangles indicate acetyl-LDL, circles indicate LDL coincubated with umbilical vein endothelial cells for 44 hours. Values are per mg/cell protein. The 100% value represents the amount of cell-bound radioactivity from the freshly prepared cells.

The slower interaction of EC-modified LDL with the endothelial cells as compared with acetyl LDL at 10 μg/ml could be due to the lowered affinity of EC-modified LDL for the receptor site, a lowered maximal cell binding, or to differences in intracellular processing. With increasing concentrations of ^{125}I-EC-modified LDL, the saturation of the cell-association (Figure 8 A) and of the degradation (Figure 8 B) indicates a high affinity association that is half-maximal at 10–20 μg of EC-modified LDL apoprotein.

To test whether the EC-modified LDL binds to the same site as acetyl-LDL on the rat liver endothelial cell, we performed a competition experiment. Endo-

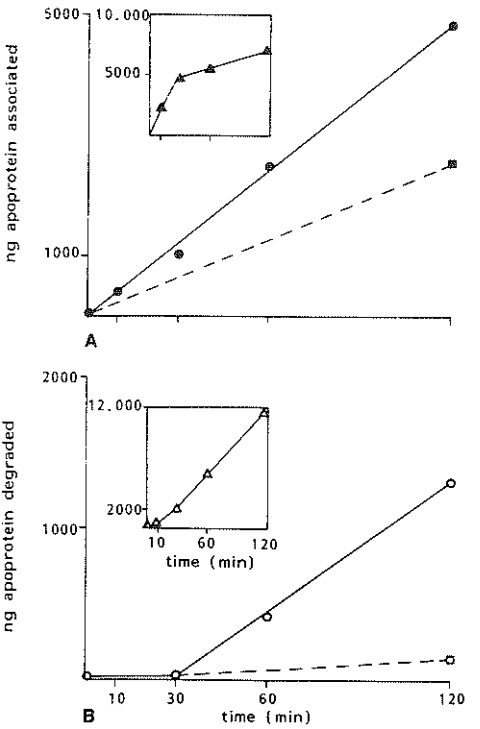

Figure 7. In vitro time course of cell association **(A)** and degradation **(B)** of EC-modified LDL (44 hours incubation) by isolated rat liver endothelial cells. Isolated cells were incubated at 37°C with 20 μg/ml EC-modified LDL. Values are the ng apoplipoprotein cell-associated **(A)** or degraded (acid-soluble radioactivity) **(B)** per mg cell protein. Cells were incubated in the absence (circles) or presence (squares) of 50 μM chloroquine. **Inserts** show the time course of in vitro association **(A)** and degradation **(B)** of acetyl-LDL by rat liver endothelial cells in vitro. Data is the same as in the main figures.

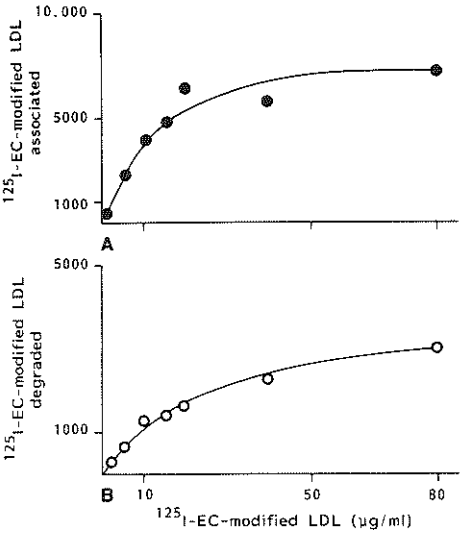

Figure 8. Cell association **(A)** and degradation **(B)** of ^{125}I-modified LDL (44 hours incubation) by rat liver endothelial cells as a function of the apolipoprotein concentration. Values are expressed as ng apolipoprotein per mg cell protein. Incubation time was 2 hours at 37°C.

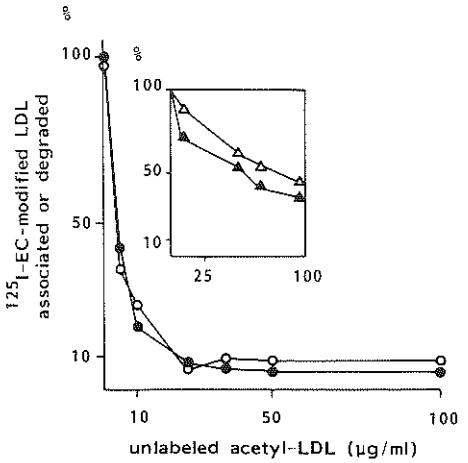

Figure 9. The effect of increasing concentrations of unlabeled acetyl-LDL on the cell association (●) and degradation (○) of ^{125}I-labeled EC-modified LDL by rat liver endothelial cells. The concentration of ^{125}I-EC-modified LDL was 10 µg/ml apolipoprotein. Cells were incubated for 2 hours at 37°C. Values are given as percentages of the control incubated in the absence of unlabeled acetyl-LDL. **Insert** shows a similar experiment for ^{125}I-acetyl-LDL and unlabeled acetyl-LDL. Data is the same as in the main figure. ▲ = association, △ = degradation.

thelial cells were incubated for 2 hours with 9.4 µg/ml ^{125}I-EC-modified LDL and increasing amounts of unlabeled acetyl-LDL. Figure 9 shows that the binding and degradation of EC-modified LDL is about 80% inhibited at equimolar amounts of both lipoproteins. The insert shows a similar competition experiment between labeled and unlabeled acetyl-LDL. Comparison of the insert and the main figure show that unlabeled acetyl-LDL competes more effectively with labeled EC-modified LDL than with labeled acetyl-LDL. A competition experiment using labeled EC-modified LDL and native LDL showed that a 30-fold excess of native LDL had no effect on either association or degradation of EC-modified LDL by endothelial cells (data not shown).

Discussion

This study was undertaken to determine the in vivo behavior of biologically modified LDL. Recent studies by Henriksen et al.[7, 8] indicated that incubation of LDL with endothelial cells induces changes in structure that resulted in recognition by macrophages and subsequent degradation at three to five times the rate of unmodified LDL. This biologically modified LDL may be pathophysiologically significant. Earlier studies with macrophages indicated that the uptake and degradation of EC-modified LDL is exerted by a pathway that is shared to some extent by acetyl-LDL. The present study shows that in vivo EC-modified LDL was processed at similar cellular sites as acetyl-LDL. EC-modified LDL was rapidly cleared from the blood and recovered quantitatively in the liver (Figures 3 and 4) at 10 minutes after injection of the lipoproteins. At longer time intervals after injection, the uptake of the disappeared EC-modified LDL was no longer quantitative, probably because iodine-labeled degradation products of EC-modified LDL left the liver. This explanation is supported by the data on the in vitro degradation of in vivo recognized EC-modified LDL. Endothelial cells that were isolated 10 minutes after injection of EC-modified LDL, actively degraded the cell-associated radioactivity to acid-soluble products, which were subsequently released from the cells.

The contribution of the different cell types to total liver uptake was similar to that observed with acetyl-LDL. The rat liver endothelial cell was approximately 30 times more active per milligram of cell protein than the parenchymal cell. Prolonged coincubation of umbilical vein endothelial cells with LDL strengthened the acetyl-LDL character of the EC-modified LDL, i.e., a faster serum decay and increased liver uptake (up to 20-fold), although the clearance and liver uptake rate of the chemically generated acetyl-LDL was never reached. In the case of acetyl-LDL 80% of the injected dose is present in the liver, after 10 minutes, indicating a high capacity of the liver acetyl-LDL receptor. Because similar concentrations of EC-modified LDL and acetyl-LDL were injected, the lower uptake of EC-modified LDL as compared

with acetyl-LDL could only be the result of a slower association rate.

These findings can be explained by the in vitro cell-association data. The dose response curves for EC-modified LDL and acetyl-LDL were similarly dependent on the substrate concentration; half-maximal cell-association and degradation occurred at about 10–20 μg apoprotein/ml for both modified forms of LDL. However, the maximal amount that became cell-associated was clearly different: 20,000 ng/mg cell protein for acetyl-LDL[6] and 7500 ng/mg cell protein for EC-modified LDL at 2 hours after incubation. This results in a higher association rate for acetyl-LDL than for EC-modified LDL at comparable concentrations of EC-modified LDL and acetyl-LDL. This difference in maximal cell association rate is reflected in the longer time needed for EC-modified LDL to reach a steady-state level (determined by the cell-association rate versus the degradation rate). The competition experiments indicated that acetyl-LDL was a more efficient competitor for ^{125}I-EC-modified LDL than for ^{125}I-acetyl-LDL. With this model, one should expect that concentrations of EC-modified LDL higher than acetyl-LDL should be necessary to compete for ^{125}I-acetyl-LDL cell-association, a finding presented earlier by Henriksen et al.[7]

In conclusion, it is evident that EC-modified LDL binds to the receptor on endothelial cells which also bind acetyl-LDL. Also the in vivo association of EC-modified LDL with parenchymal cells can be explained by the presence of an acetyl-LDL binding site on these cells as demonstrated earlier.[19]

It should be noted that we have injected human lipoproteins into rats, which could be a potential problem due to the species difference. However, it was reported[20] that upon acetylation, both rat and human LDL were processed in a similar fashion after injection into rats.

During incubation of LDL with umbilical vein endothelial cells, two physical characteristics (charge and density) of the LDL particle change. Which, if either, of these changes induces the enhanced serum decay and liver uptake is not clear at the moment. Modification of the LDL by incubation with umbilical vein endothelial cells is a continuous process.[8] The electrophoretic mobility and buoyant density gradually increases and there is a gradual increase in serum decay and liver uptake.

Besides the changes induced by incubation of LDL with umbilical vein endothelial cells, we also found that incubation of LDL for 44 hours at 37°C in the absence of cells slightly altered its behavior. The electrophoretic mobility and serum decay, as compared with native LDL were increased. The in vivo association was enhanced, but in contrast with EC-modified LDL, the live Kupffer cells were mostly responsible for the increased liver association of the 37°C incubated LDL.

Modified lipoproteins are considered potentially atherogenic because uptake of these particles can lead to accumulation of cholesterol esters in cells of the mononuclear phagocyte system.[4] The uptake of these abnormal lipoproteins by macrophages in vivo might explain the formation of foam cells in the arterial wall.[5] If EC-modified LDL is generated in vivo and enters the general circulation, it would very rapidly be removed by the liver. Therefore the liver, and in particular the liver endothelial cell, forms an important protection system against these pathophysiological lipoproteins. It is possible that a delicate balance between formation of these particles and the capacity of the liver to entrap them determines their pathological action. In this view, a small disturbance of the balance could give rise to prolonged circulation of these particles, thereby allowing the slow formation of atherosclerotic lesions.

Acknowledgments

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References

1. **Goldstein JL, Brown MS.** Low density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem 1977; 46:897–930
2. **Mahley RW, Weisgraber KH, Melchior GW, Innerarity TL, Holcombe KS.** Inhibition of receptor-mediated clearance of lysine and arginine-modified lipoproteins from the plasma of rats and monkeys. Proc Natl Acad Sci USA 1980;77:225–229
3. **Brown MS, Goldstein JL, Krieger M, Ho YK, Anderson RGW.** Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J Cell Biol 1979;82:597–613
4. **Brown MS, Basu SK, Falck JR, Ho YK, Goldstein JL.** The scavenger cell pathway for lipoprotein degradation. Specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. J Supramol Struct 1980; 13:67–81
5. **Pitas RE, Innerarity TL, Mahley RW.** Foam cells in explants of atherosclerotic rabbit aortas have receptors for β-very low density lipoproteins and modified low density lipoproteins. Arteriosclerosis 1983;3:1–12
6. **Nagelkerke JF, Barto KP, van Berkel TJC.** In vivo and in vitro uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer and parenchymal cells. J Biol Chem 1983;258:12221–12227
7. **Henriksen T, Mahoney EM, Steinberg D.** Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoprotein. Proc Natl Acad Sci USA 1981;78:6499–6503
8. **Henriksen T, Mahoney EM, Steinberg D.** Enhanced macrophage degradation of biologically modified low density lipoprotein. Arteriosclerosis 1983;3:149–159
9. **van Hinsbergh VWM, Havkes L, Emels JJ, van Corven E, Scheffer M.** Metabolism of low density lipoprotein (LDL) and acetylated LDL by subconfluent and confluent endothelial cells from human umbilical cord arteries and veins. Arteriosclerosis 1983;3:547–559
10. **Redgrave TG, Roberts DCK, West CE.** Separation of plasma lipoproteins by density gradient ultracentrifugation. Anal Biochem 1975;65:42–49
11. **Bilheimer DW, Eisenberg S, Levy RI.** The metabolism of very low density lipoproteins. I: Preliminary in vitro and in vivo observations. Biochim Biophys Acta 1972;260:212–221

12. **Demacker PNM.** Hyperlipoproteinemia — a study on diagnostic methods. [Dissertation]. Nijmegen, The Netherlands: University of Nijmegen, 1978
13. **Groot PHE, Scheek LM, Havekes L, van Noort WL, van't Hooft FM.** A one-step separation of human serum high density lipoproteins 2 and 3 by rate-zonal density gradient ultracentrifugation in a swinging bucket rotor. J Lipid Res 1982; 23:1342–1353
14. **Bierman EL, Stein O, Stein Y.** Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue-culture. Circ Res 1974;35:136–150
15. **Basu SK, Goldstein JL, Anderson RGW, Brown MS.** Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc Natl Acad Sci USA 1976; 73:3178–3182
16. **van Berkel TJC, Nagelkerke JF, Kruijt JK.** The effect of Ca^{2+} trifluoperazine on the processing of human acetylated low density lipoprotein by non-parenchymal liver cells. FEBS Lett 1981;132:61–66
17. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ.** Protein measurement with the Folin phenol reagent. J Biol Chem 1951;93:265–275
18. **Blouin A, Bolender RP, Weibel ER.** Distribution of organelles and membranes between hepatocytes and non-hepatocytes in the rat liver parenchyma. J Cell Biol 1977;72:441–455
19. **Van Berkel TJC, Nagelkerke JF, Harkes L, Kruijt JK.** Processing of acetylated human low-density lipoprotein by parenchymal and non-parenchymal liver cells. Biochem J 1982;208:493–503
20. **Mahley RW, Weisgraber KH, Innerarity TL, Windmueller HG.** Accelerated clearance of low-density and high-density lipoproteins and retarded clearance of E apoprotein-containing lipoproteins from the plasma of rats after modification of lysine residues. Proc Natl Acad Sci USA 1979;76:1746–1750

Index Terms: rat liver endothelial cell • modified LDL • umbilical vein endothelial cell

In vivo and in vitro catabolism of native and biologically modified LDL

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Incubation of human low density lipoprotein (LDL) at 37°C in the presence of human umbilical-vein endothelial cells (EC) causes a time-dependent shift in the charge and density of LDL. After intravenous injection into rats, native LDL is merely cleared from the circulation by Kupffer cells while EC-modified LDL is rapidly cleared by endothelial liver cells. The uptake of native LDL by Kupffer cells and EC-modified LDL by endothelial cells in vivo can be explained by the presence of two different specific receptors on these cell types. It is concluded that the liver endothelial cells form an important protection against a possible atherogenic action of EC-modified LDL.

Human LDL *Biologically modified LDL* *Acetylated LDL* *Human umbilical-vein endothelial cell* *Rat liver endothelial cell* *Rat liver Kupffer cell*

1. INTRODUCTION

In the past decade much progress has been made in the elucidation of the mechanism by which cells endocytose low density lipoprotein (LDL). Although the importance of the receptor-mediated uptake of LDL is indicated in vitro [1] the quantitative contribution of this system to the in vivo turnover of LDL ranges from 33 to 66% [2].

Additional uptake mechanisms for LDL are therefore proposed including the involvement of a so-called scavenger receptor [3]. As a substrate for this receptor acetylated LDL (acetyl-LDL) has been used. However, the in vivo occurrence of such a chemically modified form of LDL is questionable. A more relevant biological modification of LDL can be achieved by incubation of LDL with umbilical-vein endothelial cells [4]. To assess the relative importance of the different liver cell types (parenchymal, Kupffer and endothelial cells) in the in vivo uptake of native LDL and biologically modified LDL (EC-modified LDL), we injected the radiolabelled lipoproteins into rats and determined the cellular uptake. Additional in vitro studies were performed to determine to what extent the relative importance of the various cell types for the in vivo uptake of LDL and EC-modified LDL can be explained by the presence of specific receptors for these lipoproteins.

Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

LDL was isolated by ultracentrifugation and tube slicing from the blood of healthy volunteers, who had fasted overnight [5]. The LDL was immediately iodinated as in [6]. After extensive dialysis against PBS it was stabilized by addition of 10 mg BSA per ml and dialysed again. Specific activity ranged from 80 to 150 cpm/ng LDL protein. The acid-soluble (non-iodine) fraction was less than 0.1%.

EC-modified LDL was prepared by incubating confluent umbilical-vein endothelial cells (isolated and cultured as in [7]) in medium M-199, 10 g per 1 BSA and 100 μg ^{125}I-labelled LDL per ml

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medium. After the indicated time intervals the medium was aspirated and stored.

EC-modified LDL, non-incubated LDL and LDL incubated in the absence of cells were subjected to agarose electrophoresis as in [8]. After electrophoresis the plate was dried and subjected to autoradiography. Fig.1 shows that the LDL incubated with umbilical-vein endothelial cells for increasing periods of time showed increasing electrophoretic mobility indicating an increase in net negative charge. The buoyant density of the LDL preparations after incubation with umbilical vein endothelial cells shifted from 1.050 g/ml toward a mean density of 1.080 g/ml.

In some experiments LDL was acetylated with acetic anhydride as in [9].

Rat liver endothelial, Kupffer and parenchymal cells were isolated from 3-month-old male Wistar rats. For in vivo uptake studies cells were isolated 10 min after intravenous injection of the lipoprotein fractions. The entire isolation procedure was performed at a low temperature to prevent degradation of endocytosed lipoprotein during cell isolation. For in vitro studies, cells were isolated at 37°C. Both techniques have been detailed in [10].

Incubation of cells was performed in Hams F-10 medium with the indicated amounts of lipoproteins. Determination of the amount of cell-associated lipoprotein was done as in [11]. The viability of cells before and after incubation was higher than 95% as judged by the trypan blue exclusion test. Purity of the cell preparations was checked by light microscopy: the endothelial cell preparation was more than 95% pure and the

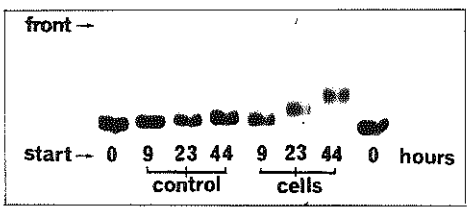


Fig.1. Agarose electrophoresis of ^{125}I-labelled EC-modified LDL and ^{125}I-labelled control LDL. LDL was incubated in the presence or absence of cells for increasing time periods. Electrophoresis and autoradiography were performed as in [8].

Kupffer cell preparation approx. 85%, the rest being endothelial cells.

Protein was determined according to [12] with BSA as a standard.

Collagenase (type I) and BSA, fraction V, were from Sigma (St. Louis, MO), pronase (B-grade) from Calbiochem-Behring (La Jolla, CA), metrizamide from Nyegaard & Co A/S (Oslo), Ham's F-10 from Gibco-Europe (Hoofddorp, The Netherlands), ^{131}I and ^{125}I (carrier-free) in NaOH from New England Nuclear (Dreieich).

3. RESULTS AND DISCUSSION

Native LDL and LDL incubated with umbilical-vein endothelial cells for increasing time periods were injected into rats. Subsequently serum decay and liver uptake were determined. Fig.2A shows that the serum decay of LDL is accelerated upon prolonged exposure of LDL to umbilical-vein endothelial cells.

The kinetics of liver uptake was determined by excising a liver lobule at different time intervals after injection. After weighing the lobule and counting its radioactivity the fraction of the injected dose present in the liver was calculated using the assumption that 3.75% of the total body weight is contributed by the liver [13]. It is evident that incubation of LDL for increasing periods of time with umbilical-vein endothelial cells increases the amount that becomes associated with the liver (fig.2B). Furthermore at 10 min after injection the amount of lipoprotein cleared from the blood is quantitatively recovered in the liver.

Determination of the cell type(s) responsible for the in vivo liver uptake was done by isolating the various liver cell types 10 min after injection by a cold procedure. ^{125}I-labelled EC-modified LDL (incubated for 44 h) was injected simultaneously with ^{131}I-labelled native LDL. Fig.3 shows the percentage of the injected dose of lipoprotein present in the different isolated cell fractions expressed per mg cell protein. It can be seen that the endothelial cell fraction contains much more EC-modified LDL than the Kupffer cell fraction. This becomes even more evident as the approx. 15% contamination of the Kupffer cell fraction with endothelial cells is taken into account. It can be calculated that at least 20-times more EC-modified LDL becomes associated with the endothelial cells

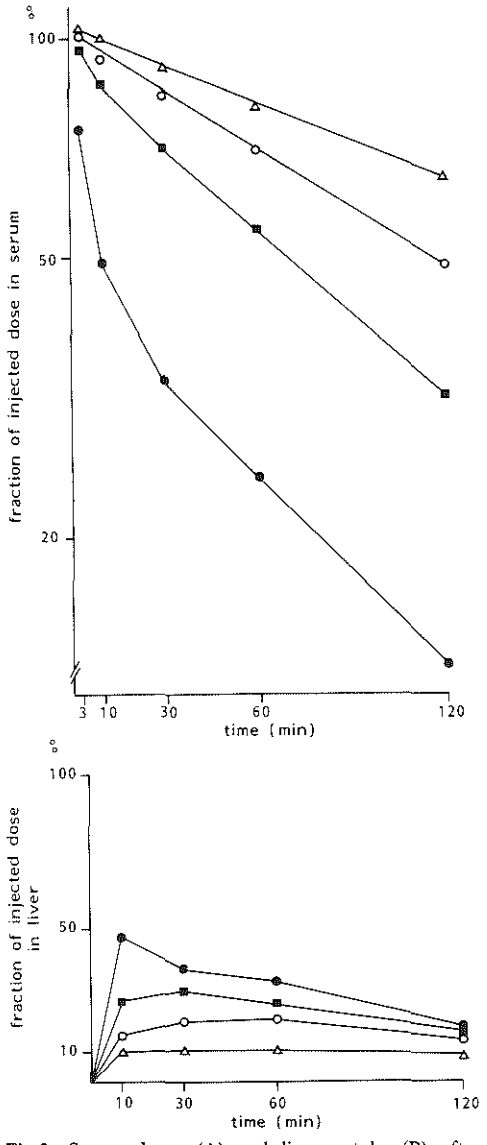


Fig.2. Serum decay (A) and liver uptake (B) after injection into rats of control (△) LDL and LDL incubated with umbilical-vein endothelial cell for 9 (○), 23 (■) or 44 h (●). Serum values were determined in the 20000 × *g* supernatant of blood samples. Liver uptake was determined by excising successively the different lobules of the liver followed by weighing of the lobule and counting of its radioactivity.

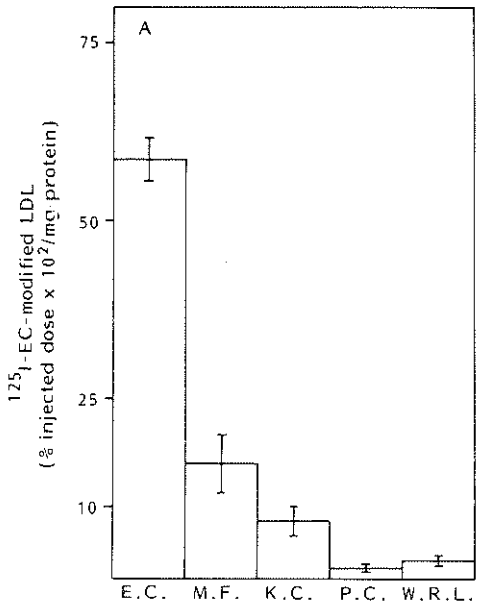
than with Kupffer cells. Fig.3B shows the in vivo uptake of native LDL by the different liver cell types. In contrast to EC-modified LDL, the Kupffer cell fraction contains much more radioactivity per mg cell protein than do the endothelial cells.

With both EC-modified LDL and native LDL the endothelial and Kupffer cells contain approx. 30-times more radioactivity per mg cell protein than do the parenchymal cells. This indicates that the non-parenchymal cell types have an important function in the clearance of both these lipoproteins from the blood.

To determine the respective specificity of the interaction of EC-modified LDL and native LDL with endothelial and Kupffer cells in vitro competition experiments were performed. Freshly isolated endothelial cells were incubated with ^{125}I-labelled EC-modified LDL and increasing amounts of unlabelled acetyl-LDL or native LDL. Fig.4 shows that the binding and degradation of EC-modified LDL are 80% inhibited at equimolar amounts of EC-modified and acetyl-LDL. A similar experiment but with a 30-fold excess of native LDL showed no competition (not shown). This indicates that EC-modified LDL is specifically recognized by a scavenger receptor on liver endothelial cells which also recognizes acetyl-LDL. The presence of this receptor was indicated earlier with acetyl-LDL as a substrate [10].

The specificity of the interaction of native LDL with Kupffer cells was determined by incubating this cell type with ^{125}I-labelled native LDL and increasing amounts of unlabelled acetyl and native LDL. Fig.5 shows that while the native LDL inhibits cell association by 50%, acetyl-LDL is completely ineffective.

Because it is known that uptake of modified (denatured) proteins is likely to occur in the reticuloendothelial system (including Kupffer cells) we performed experiments to determine to what extent denaturation of a part of native human LDL during the iodination or preparation procedures could be responsible for this uptake. It was found that previous screening of LDL did not influence the serum decay or relative involvement of the different liver cell types in the liver uptake. A similar lack of screening upon liver uptake and serum decay of rat LDL was reported in [11]. Secondly, by simultaneous injection of ^{125}I-labelled LDL and ^{131}I-labelled reductively



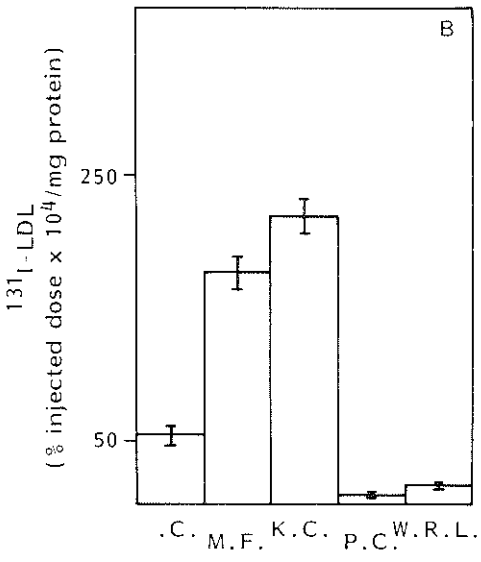


Fig.3. In vivo uptake of ^{125}I-labelled EC-modified LDL (incubated 44 h) (A) and ^{131}I-labelled native LDL (B). Both lipoproteins were injected intravenously into rats; 10 min later a cold liver perfusion was started. After 8 min perfusion a lobule was tied off and excised to determine the whole rat liver value (W.R.L.). Subsequently endothelial (E.C.), Kupffer (K.C.) and parenchymal cells (P.C.) were isolated. (M.F., mixed fraction of endothelial and Kupffer cells.) Values are expressed as percentage of injected dose per mg cell protein × 10^2 (A) or × 10^4 (B).

methylated LDL it was found that half of the Kupffer cell uptake can be defined as receptor-mediated (after reductive methylation of LDL Kupffer cell uptake was half of that obtained with native human LDL) (not shown). These results show that the Kupffer cell has a specific recognition site for LDL which plays an important role in the liver uptake of LDL.

The increased decay of LDL upon incubation with umbilical-vein endothelial cells can be adequately explained by the increased interaction of

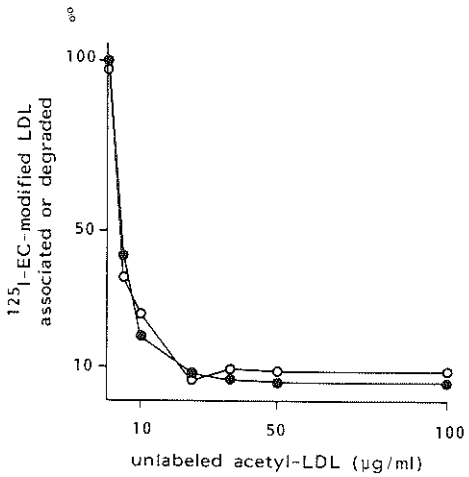


Fig.4. Competition between ^{125}I-labelled EC-modified LDL and unlabelled acetyl-LDL for cell association (●) and degradation (○) by rat liver endothelial cells. The concentration of ^{125}I-labelled EC-modified LDL was 10 µg/ml. Values are given as percentage of the control incubated in the absence of acetyl-LDL.

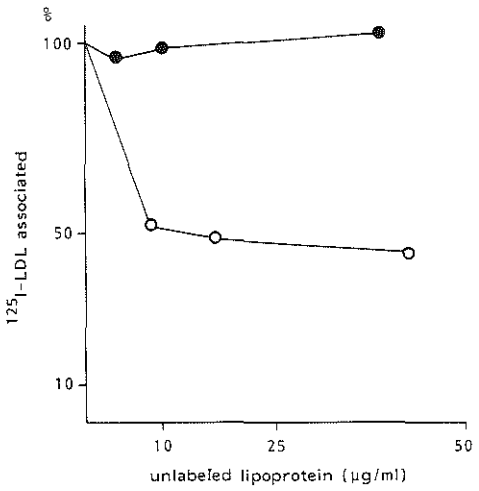


Fig.5. Competition between ^{125}I-labelled LDL and unlabelled acetyl-LDL (●) or unlabelled LDL (○) for cell association with rat liver Kupffer cells. The concentration of ^{125}I-labelled LDL was 6 μg/ml. Values are given as percentage of the control incubated solely with ^{125}I-labelled LDL.

EC-modified LDL with the scavenger receptor on endothelial cells. Modified lipoproteins are considered as potentially atherogenic because uptake of the particles can lead to accumulation of cholesterol esters in cells of the mononuclear phagocyte system [3]. The uptake of these abnormal lipoproteins by macrophages in vivo might explain the formation of foam cells in the arterial wall [15]. If EC-modified LDL is generated in vivo and the modified LDL enters the general circulation then the liver and in particular liver endothelial cells will entrap them. In this way the occurrence of potentially atherogenic particles in the blood will be prevented.

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REFERENCES

[1] Goldstein, J.L. and Brown, M.S. (1977) Annu. Rev. Biochem. 46, 897–930.
[2] Brown, M.S. and Goldstein, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 3330–3337.
[3] Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K. and Goldstein, J.L. (1980) J. Supramol. Struct. 13, 67–81.
[4] Henriksen, T., Mahoney, E.M. and Steinberg, O. (1981) Proc. Natl. Acad. Sci. USA 78, 6499–6503.
[5] Redgrave, T.G., Roberts, D.C.K. and West, C.E. (1975) Anal. Biochem. 65, 42–49.
[6] Bilheimer, D.W., Eisenberg, S. and Levy, R.I. (1972) Biochim. Biophys. Acta 260, 212–221.
[7] Van Hinsbergh, V.W.M., Havekes, L., Emeis, J.J., Van Corven, E. and Scheffer, M. (1983) Arteriosclerosis 3, 547–559.
[8] Demacker, P.N.M. (1978) Thesis, University of Nijmegen, Nijmegen, The Netherlands.
[9] Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1976) Proc. Natl. Acad. Sci. USA 73, 3178–3182.
[10] Nagelkerke, J.F., Barto, K.P. and Van Berkel, T.J.C. (1983) J. Biol. Chem. 258, 12221–12227.
[11] Van Berkel, T.J.C., Nagelkerke, J.F. and Kruijt, J.K. (1981) FEBS Lett. 132, 61–66.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 93, 265–275.
[13] Blouin, A., Bolender, R.P. and Weibel, E.R. (1977) J. Cell Biol. 72, 441–455.
[14] Chao, Y., Windler, E.E., Chen, G.C. and Havel, R.J. (1979) J. Biol. Chem. 254, 11360–11366.
[15] Pitas, R.E., Innerarity, T.L. and Mahley, R.W. (1983) Arteriosclerosis 3, 1–12.

APPENDIX PAPER VII

in vivo intrahepatic processing of radiolabeled cholesterylester, biologically incorporated into acetylated low density lipoprotein.

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Cholesteryloleate doubly radiolabeled in the cholesterol and oleate-moiety was biologically incorporated into human low density lipoprotein (LDL). First high density (HDL) was delipidated and relipidated with the radiolabeled cholesteryloleate. Subsequently the HDL was incubated with LDL in the presence of cholesterylester transferprotein (CETP) for 18 hours at 37°C. The re-isolated LDL contained routinely 20 % of the initially added cholesteryloleate. Serum decay of the LDL after injection into rats was the same as for the un-incubated ^{125}I-LDL.

The LDL was acetylated and injected into rats. At different times after injection liver parenchymal and endothelial cells were isolated by a low temperature procedure. At 10 min after injection of the [^{3}H] cholesteryl [^{14}C]oleate labeled acetyl-LDL the isolated endothelial cells contained 20-40 times more radioactivity than the parenchymal cells (per mg protein) while the radioactivity was mainly present as cholesteryloleate.

At longer time-intervals after injection the amount of cholesterylesters in the endothelial cells rapidly decreased. The [^{14}C]oleate radioactivity was secreted from the endothelial cells while the [^{3}H]cholesterol radioactivity remained present as free cholesterol up to two hours after injection. The amount of cholesterylester in the parenchymal cells was also rapidly hydrolysed but, in contrast to the endothelial cells, the [^{14}C]oleate was incorporated into triglycerides and phospholipids. In addition the increase in ^{14}C-radioactivity in parenchymal cells between 10 and 30 min after injection of the [^{3}H]cholesteryl [^{14}C]oleate labeled acetyl-LDL indicates that[^{14}C]oleate was transfered from the endothelial cells to the parenchymal cells. The [^{3}H]cholesterol radioactivity in the parenchymal cells remained, similarly as with endothelial cells present as free cholesterol. These data indicate that the *in vivo* uptake of acetyl-LDL by the liver cells does not lead to hepatic cholesterylester deposition. It is concluded that the liver, and in particular the liver endothelial cell, is fully capable to catabolize modified lipoproteins which strenghtens the hypothesis that these cells form the major protection system against the atherogenic action of these lipoproteins.

abbreviations used:

LDL	low density lipoprotein
acetyl-LDL	acetylated low density lipoprotein
HDL	high density lipoprotein
LPDS	lipoprotein deficient serum
CETP	cholesterylester transferprotein
FFA	free fatty acid
BSA	bovine serum albumin

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Th.J.C. van Berkel is an established investigator of the Dutch Heart Foundation.

INTRODUCTION

The scavengerpathway is a route whereby chemically or biologically modified lipoproteins are taken up into cells of the mononuclear phagocytotic system (1-4). Recently we demonstrated that acetylated low density lipoprotein (acetyl-LDL), iodine-labeled in the apoprotein moiety, is cleared from the blood within 3 minutes after intravenous injection into rats. This clearance is quantitatively exerted by the liver (4). The highest concentration of acetyl-LDL accumulates in the liver endothelial cell, approximately 30 times more than in the parenchymal cell (when expressed per mg cellprotein). The contribution of the endothelial cell to total liver protein is however 3,3%, while the parenchymal cell contributes 92,5% (5). By taking into account these relative protein contributions, it can be calculated that *in vivo* both cell populations are responsible for approximately 50% of the liver uptake.
In vitro studies with isolated parenchymal (6) and endothelial cells (4) indicated that the degradation of the protein moiety of acetyl-LDL is inhibited by lysosomotropic agents, suggesting that the uptake of acetyl-LDL is effectively coupled to a lysosomal degradation pathway. Studies with isolated macrophages in culture have indicated that besides the apoprotein also the cholesterylesters present in acetyl-LDL are hydrolysed in the lysosomes. Subsequently the liberated cholesterol is transported to cytoplasm of the cell where approximately 50% is re-esterified with exogenous free fatty acid (FFA). *In vitro* and maybe under pathological conditions *in vivo* this results into cellular accumulation of cholesterylesters. This mechanism of cholesterolester-deposition was called the two-compartmentmodel (1), because it involves both the lysosomal and cytosolic compartment.
The aim of the present study is to determine the fate of the cholesterylesters from acetyl-LDL *in vivo*. In order to compare the results from this study with a former one (4) in which the fate of the apoprotein was followed we took as a criterion that the LDL containing the radiolabeled cholesterylesters should have the same halflife in serum as LDL which was labeled in the apoprotein. Because, in our hands, the method described in the literature (7) did not meet this criterion we developed a new technique for the incorporation of cholesterylesters into LDL, which is also described in this paper.

MATERIALS AND METHODS

Cell isolation

Throughout this study, 12 weeks old male Wistar R_1 rats, (CPB, Centraal Proefdieren Bedrijf, Rotterdam, The Netherlands) were used who had free access to standard labchow and water. For determination of the *in vivo* uptake, liver endothelial cells were isolated with a low temperature (8°C) pronase method, and liver parenchymal cells with a low temperature collagenase technique. These techniques have been developed in order to obtain a quantitative recovery of *in vivo* endocytosed substrates in the isolated cells. For *in vitro* studies endothelial cells are isolated with a 37°C-collagenase method. The cell isolation methods described extensively earlier (4).

Cellpurity

Biochemical and light microscopical determination of the purity of the isolated cell fractions was done as described before (4). Parenchymal cell purity was always 100%, endothelial cell preparations contained more than 95% endothelial cells.

In vivo - in vitro experiments

In vitro incubation of isolated cells, preloaded *in vivo* with radiolabeled acetyl-LDL, was performed by transferring the cells, isolated at 8°C, to a Hams F-10 medium at 37°C. After different time intervals aliquots were drawn and cells and medium were separated by centrifugation for 2 min 400 g. The lipids were extracted from the cell pellet and the supernatant and after thin-layer chromatography the different spots were assayed for radioactivity.

In vitro incubations

Endothelial cells were isolated with collagenase at 37°C (4). The cells were incubated in Hams F-10 medium with 25 μg/ml ^{125}I-acetyl-LDL and [^{14}C] oleate-albumin complex (molar ratio 5 : 1 - final concentration FFA, 0,12 mM). At different time intervals, cell-association and degradation of the protein moiety of acetyl-LDL was determined according to (8). Thereafter the cells were homogenised, the lipids were extracted and characterized by thin layer chromatography.

Isolation, delipidation and relipidation of the lipoproteins

Lipoproteins were isolated according to (9) from the blood of healthy volunteers who had fasted over night. After 20 hours ultracentrifugation the tubes were sliced and low density lipoprotein (LDL $1{,}024 < d < 1{,}055$ g/ml) high density lipoprotein (HDL, $1{,}06 < d < 1{,}21$ g/ml) and lipoprotein deficient serum (LPDS, $d > 1{,}21$ g/ml) were collected. In initial experiments the LDL was brought to a density of 1,21 g/ml and re-isolated according to (9). The LDL was delipidated and relipidated exactly as described by Krieger et al. (7). This includes dialysis, lyophilisation in the presence of potato starch, extraction of neutral lipids with heptane, introduction of cholesteryl [^{14}C]oleate, and solubilisation in an aqueous buffer. In the section Results is discussed why, in our hands, this technique was unsatisfactory. Therefore we combined and modified the techniques of Krieger et al. (7) and Craig et al. (10). LDL, HDL and LPDS were isolated according to (9), the LDL and HDL were brought to a density of 1,21 g/ml and re-isolated. HDL, 20 mg protein, was repetitively dialysed against 0,3 mM EDTA at pH 7,5. Potato starch was added to HDL in a final concentration of 10 mg/ml. After overnight lyophilisation the HDL was stored in an exicator for 4 hours and subsequently the neutral lipids were extracted with 5 ml heptane at -10°C for 30 min. The HDL was centrifuged, 5 min 400g, the supernatant was removed and the procedure was repeated twice. In a siliconised tube a solution of 8 mg cholesteryllinoleate in heptane was evaporated, 25 μCi cholesteryl [^{14}C] oleate (56,6 mCi/mmol) in benzene and 25 μCi[^{3}H] cholesteryl -1, 2, 6, 72- oleate (82,7 Ci/mmol) in toluene were added and the solvent was evaporated. Finally 500 μl heptane was added and the solution was sonicated for 2 min is an ultrasonic waterbath. The delipidated HDL was solubilized in 500 μl heptane and added to the cholesterylester solution. The mixture was kept at -10°C for 1 hour followed by evaporation of the heptane at -10°C for 1 hour. The dry relipidated HDL was suspended in 1 ml 10 mM Tris-HCl pH 8,0 immediately vigorously vortexed and stirred overnight at 4°C. The next morning the starch was removed by centrifugation, 10 min 1400 g.

The LPDS obtained after the first ultracentrifugation was brought to $d = 1{,}25$ g/ml and recentrifuged. After 22 hours, 40.000 rpm centrifugation, the tube was sliced and the LPDS between $d = 1{,}268$ g/ml and $d = 1{,}290$ g/ml was collected.

The relipidated HDL, the native LDL and the LPDS were extensively dialysed against 10 mM Tris-HCl pH 8,0. 1 ml HDL (19,2 mg protein) + 2,7 ml LDL (1,95 mg protein) + 4,3 ml LPDS (150 mg protein) were mixed and incubated at 37°C for 19 hours. After the incubation the mixture was brought to $d = 1{,}21$ g/ml and the LDL was reisolated according to Redgrave (9). The final LDL contained solely apo B as judged by gelelectroforesis. Routinely 20% of the cholesterylester added initially to the HDL was finally incorporated into the LDL. The ratio protein: total cholesterol: free cholesterol in the LDL was 1 : 2,2 : 0,5. Finally the LDL was acetylated according to Basu (11).

Iodination of acetyl LDL

Native LDL and acetylated LDL were iodinated according to Bilheimer (12). Specific activity of the LDL was 43 dpm/ng and of the acetylated LDL 68 dpm/ng.

In vivo experiments

Injection of lipoproteins and blood sampling was done in the same way as in (4).

Determination of cholesterylesters and metabolic products

Total radioactivity in the liver, the isolated cells and in serum was determined by digesting the samples with Soluene ® and counting in Tricarb-scintillation counter (Packard 2680). As scintillation solution dimilumen was used in order to prevent chemiluminisence.

The molecular forms in which the radioactivity is present in the liver in the isolated cells and in serum was determined by thin-layerchromatography (13). Samples (800 μl) were sonicated for 15 seconds, 2 ml CH_3OH, 2 ml $CHCl_3$ and 1 ml 150 mM NaCl pH = 1,0 were added. After centrifugation, 10 minutes 600 g, the lower phase was transferred to another tube and evaporated to dryness. 100 μl of a solution ($CHCl_3 : CH_3OH = 2 : 1$) containing 200 μg unlabeled cholesteryloleate and 200 μg cholesterol was added. After vortexing, the solution was applied to precoated TLC-silica plates. The developing solvent was 200 ml of heptane-dietylether -acidic acid (60 : 40 : 1). After development, the plates were dried by air and the spots were visualized by exposure to I_2-vapor. The spots were scraped from the plates, put into counting vials, dissolved into 5 ml H_2O by sonication, and finally 10 ml Instagel was added. The radioactivity was counted in the Packard 2680, programmed to correct for quenching.

Protein determination

This was done as described by Lowry (14) with BSA as a standard.

Chemicals

Type I collagenase and BSA, Fraction V, were from Sigma Company, St. Louis, Missouri; B-grade pronase was obtained from Calbiochem-Behring Corporation, La Jolla, California; Metrizamide was from Nyegaard and Company A/S, Oslo, Norway: Ham's F-10 was from Gibco-Europe, Hoofddorp, The Netherlands; ^{125}I (carrier-free) in NaOH, [^{3}H] cholesteryloleate and cholesteryl [^{14}C]oleate were from New England Nuclear Dreieich, West-Germany; TLC silica gel 60 plates were obtained from Merck, Darmstadt, West-Germany.

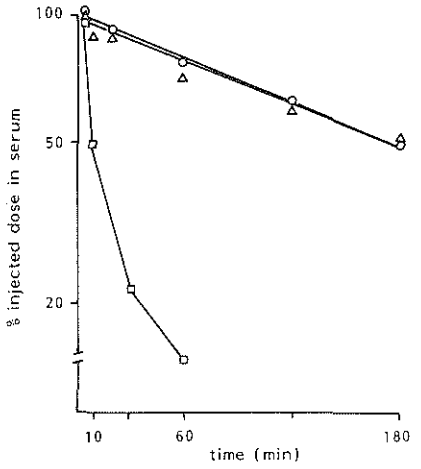


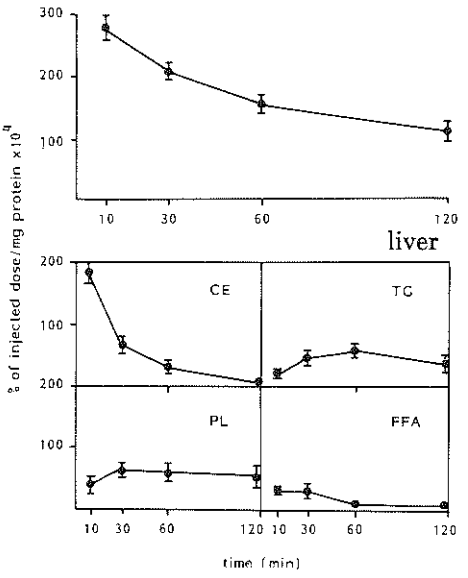
FIGURE 1.

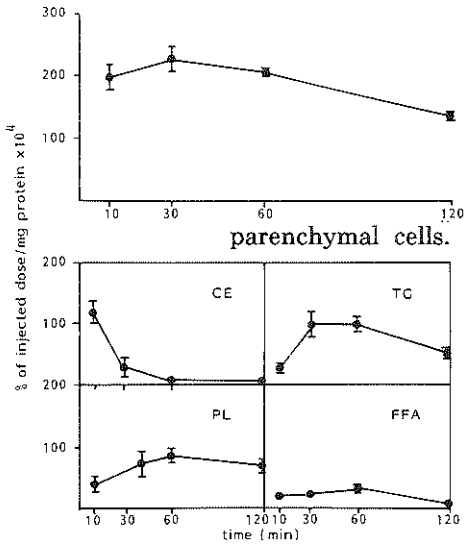
Serum decay of radiolabeled lipoproteins after injection into rats.
Blood samples were drawn as described before(4). The samples were centrifuged for 2 minutes at 15.000 g and radioactivity was counted in the supernatants. The amount present at 3 minutes after injection was taken as 100% (O) represent ^{125}I-LDL (Δ), obtained by transfer of the cholesterylester from HDL to LDL (□) LDL obtained by direct incorporation of the cholesteryloleate in LDL (according to ref. (7)).

RESULTS

Characterisation of the LDL radiolabeled in the cholesterylester moiety

The serumdecay of the radioactive cholesteryloleate, which was incorporated into LDL by the procedure of Krieger et al. (7), is much faster than the decay of untreated LDL iodine-labeled in the apoprotein (Fig.1). A more physiological incorporation of radioactive cholesterylesters into LDL can be achieved with the aid of the cholesterylester transfer protein. With this technique, described fully in the section Materials & Methods, the radioactive cholesterylesters are introduced into HDL and subsequently transfered into LDL by the cholesterylester transfer protein, which is present in the LPDS fraction. The serum decay of these biologically incorporated cholesterylesters is identical with the decay of the iodine-labeled apoprotein moiety of untreated LDL (Fig.1).





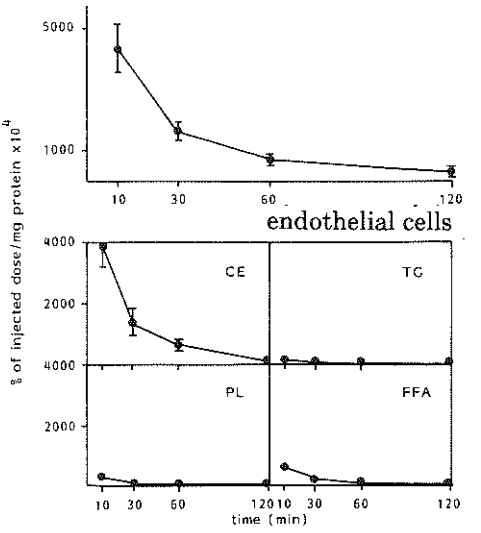


FIGURE 2A, 2B, 2C.

The amount and form of ^{14}C-radioactivity in the liver and parenchymal and endothelial cells at various times after injection of [^{3}H]cholesteryl[^{14}C]oleate - acetyl-LDL into rats.

The upper figures represent total ^{14}C-radioactivity contents. The lower figures indicate the molecular form of the ^{14}C-radioactivity. CE is cholesterylester, TG is triglyceride, PL is phospholipid and FFA is free fatty acid.

In vivo uptake of [^{3}H]cholesteryl [^{14}C]oleate from acetyl-LDL

The LDL, from which the cholesteryloleate was labeled by transfer of the esters from HDL to LDL, was acetylated and injected into rats. The protein moiety of acetyl-LDL is cleared from the serum within 3 minutes and quantitatively recovered in the liver (4). The [^{3}H]cholesteryl [^{14}C]oleate decay of acetyl-LDL is equally rapid as that of the protein moiety and within 3 min the label is totally removed from serum (not shown). At different time intervals after *in vivo* injection a liverperfusion was started, at 8°C and after 8 min perfusion a liver lobule was tied off and excised. Subsequently the different liver cell types were isolated by the low temperature procedures. In the isolated cell fractions and in the liver lobule total radioactivity was determined, and a lipid extraction followed by TLC was performed.

The fate and form of the ^{14}C-oleate of the cholesteryloleate is shown in Fig. 2. Ten minutes after injection of acetyl-LDL the endothelial cells contain 20 times more

^{14}C-radioactivity than the parenchymal cells (per mg cell protein). However, thirthy minutes after injection the amount of ^{14}C-radioactivity in total liver is slightly decreased, in endothelial cells it is sharply decreased, but in contrast parenchymal cells show an increased amount. Because there is no new influx of acetyl-LDL from the serum possible between 10 and 30 min after injection, this indicates that an intrahepatic redistribution of ^{14}C-radioactivity has occurred. A characterization of the label after lipidextraction indicate that the initial cholesterylesters are rapidly hydrolysed in the endothelial cells. Only a small part of the ^{14}C-radioactivity in the cells is present as free oleic acid, so upon hydrolysis it must be secreted. Also in the parenchymal cell the major part of the cholesterylesters are hydrolysed within 30 minutes. However in this cell type the ^{14}C-radioactivity is at 30 min after acetyl-LDL injection predominantly present in triglycerides and in phospholipids. Between 30 and 120 minutes after injection of the acetyl-LDL also in the parenchymal cells the amount of ^{14}C-radioactivity decreases. The release of ^{14}C-radio-activity from the parenchymal cell is accompanied by the appearance of varying amounts (0,5 - 2% of the injected dose, of [^{14}C]-triglyceride in the serum (not shown).

The fate and form of the [^{3}H] cholesterol part of the cholesteryloleate from acetyl-LDL is shown in Fig. 3. In whole liver, parenchymal and endothelial cells the rate of [^{3}H]cholesteryloleate hydrolysis is completely identical with the rate determined with the cholesteryl [^{14}C]oleate. However it appears that the formed cholesterol does not leave the cells and remains as free cholesterol inside the cell. Probably the relatively small amount of labeled cholesterylester that is injected, approx. 200 μg, is entrapped into a preexisting pool.

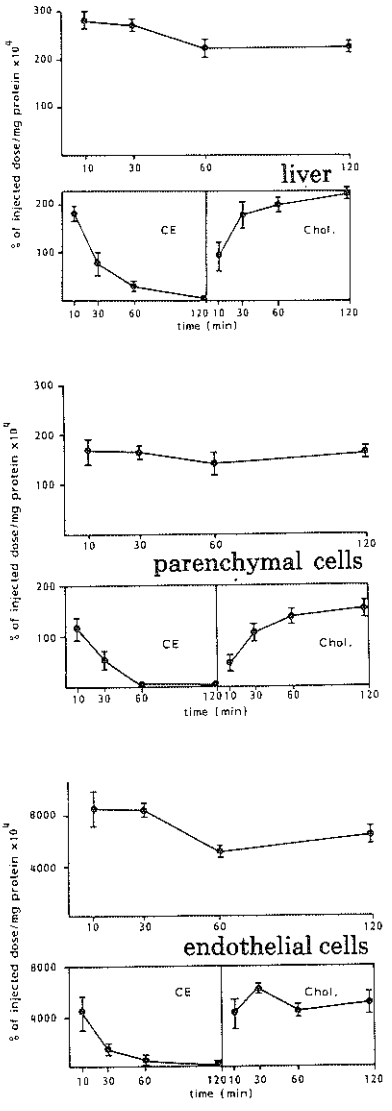


FIGURE 3A, 3B, 3C.

The amount and form of ^{3}H-radioactivity in the liver and parenchymal and endothelial cells at various times after injection of [^{3}H]cholesteryl[^{14}C]oleate - acetyl-LDL into rats.

The upper figures represent total ^{3}H-radioactivity. The lower figures indicate the molecular form of the ^{3}H-radioactivity. CE is cholesterylester, chol. is free cholesterol.

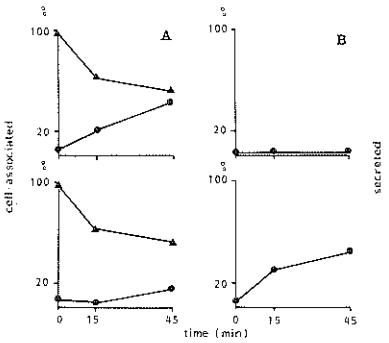


FIGURE 4.

Time course of the *in vitro* intracellular processing of the cholesterylester introduced *in vivo* by acetyl-LDL into liver endothelial cells.
Cholesteryl [^{14}C] oleate labeled acetyl-LDL was injected into rats before the cell isolation procedure was started. The isolated endothelial cells were incubated at 37°C in Hank's buffer (4A,B) or in Hams F-10 supplemented with 2% LPDS (4C,D). Samples were drawn at the indicated times, the cells were centrifuged and cells and supernatant were subjected to a lipid extraction procedure, whereafter the amount and the molecular form of the ^{14}C-radioactivity was determined by TLC. A and C represent cell-bound radioactivity B and D radioactivity secreted into the medium. Triangles is cholesteryloleate, circles free fatty acid. The 100% value represent the amount of cholesterylester present in the freshly isolated cells.

***In vitro* processing of in *in vivo* internalized acetyl-LDL**
The processing of the *in vivo* internalized acetyl-LDL can be followed *in vitro* by incubating the cells, isolated at 8°C, at a temperature of 37°C (4).
Ten minutes after the injection of the double labeled cholesteryloleate incorporated into acetyl-LDL, a cell isolation procedure was started and the amount of cell-associated and secreted label was determined at different times after *in vitro* incubation (Fig.4.). The incubation of the isolated cells at 37°C was performed in Hanks' buffer (fig. 4A, 4B) and in HAMS F-10-medium supplemented with 1% v/v LPDS (fig. 4C, 4D). The left panels show that under the applied incubation conditions the intracellular hydrolysis of the cholesterylester is similar. However the *in vivo* determined secretion of radioactivity in the form of oleic acid, is only observed *in vitro* when the isolated cells are incubated with HAMS + LPDS.

The incorporation of radioactive oleate into cellular cholesterylesters and triglycerides in the presence of acetyl-LDL.

Incubation time	30 min	60 min	120 min
Acetyl-LDL apoprotein cell-associated	6120	9900	12.060
Acetyl-LDL apoprotein degraded	2740	5640	14.200
Cholesterylester formed	22	50	122
Triglyceride formed	5	8	50
Non-esterified oleic acid	130	190	382

TABLE 1:
Isolated endothelial cells were incubated with 25 μg/ml ^{125}I-acetyl-LDL and 0,12 mM [^{14}C]oleate-albumin complex. At the indicated times a sample was drawn. The amount of cell-associated and degraded acetyl-LDL apoprotein was determined while simultaneously the cellular [^{14}C]radioactivity was characterized by TLC. The values represent the amount of protein or oleate in ng per mg cell protein.

***In vitro* processing of acetyl-LDL**
In the two-compartment model for the processing of cholesterylesters in acetyl-LDL, exogeneous added oleate is used for the cytosolic re-esterification of cholesterol (1). Because freshly isolated liver endothelial cells actively internalize and degrade the protein moiety of acetyl-LDL it was determined to what extent exogeneous added ^{14}C-oleate complexed with albumin was used for re-esterification of the free cholesterol (Table I). The ratio protein: total cholesterol in LDL or acetyl-LDL is 1:2,2. Therefore it can be calculated that the degradation of 14.200 ng apoprotein of acetyl-LDL is equivalent with about 30.000 ng cholesterol. For reason that only 122 ng of cholesterylester are formed from ^{14}C-oleate it can be calculated that only 0,3% of the cholesterol

from the degraded acetyl-LDL was re-esterified. This was not the consequence of an inadequate supply of oleate inside the cells because 9 times as much ^{14}C-oleate was incorporated into di- and triglycerides than in cholesterolesters.

DISCUSSION

This paper describes the *in vivo* processing of the cholesterylesters of acetyl-LDL by rat-liver parenchymal and endothelial cells. At a short time (10 min) after intravenous injection into rats the cholesterylester is recovered at the same hepatic sites as the apoprotein i.e. the endothelial and the parenchymal cells. In the endothelial cell the hydrolysis of the cholesterylester is followed by a direct secretion of the FFA. Rapid hydrolysis of the cholesterolester also occurs in the parenchymal cell but in this celltype the fatty acid is immediately reutilized for the biosynthesis of phospholipides and triglycerides. The *in vivo* uptake and processing of the acetyl-LDL by the liver is rapid, when compared to cells in culture (1). Within three minutes after injection the lipoprotein is completely cleared from the circulation. At ten minutes after injection already a part of the cholesterolesters are hydrolysed and secreted by the endothelial liver cells (indicated by the difference between ^{3}H-cholesterol and ^{14}C-oleate radioactivity. The increase in ^{14}C-fatty acid radioactivity in the parenchymal cell between 10 and 30 min after injection, which contrasts the decrease in total liver and endothelial cell radioactivity, indicates that a substantial amount of the fatty acid, secreted from the endothelial cell is transferred to the parenchymal cell (remember that later than 3 minutes after injection there is no more influx of acetyl-LDL from the blood into the cells).

The transfer of fatty acid from the endothelial cell to the parenchymal cell may be direct or mediated by a carrier in the blood. The *in vitro* experiments with isolated endothelial cells, loaded *in vivo* with acetyl-LDL, indicate that under these conditions an extracellular acceptor (albumin) for the fatty acid must be present in order to allow secretion. However *in situ* there is direct contact between the liver endothelial cell and the parenchymal cell and contacts between the transfertubules from the endocytotic-lysosomal system in the endothelial cell with the microvilli of the parenchymal cell have been demonstrated (15). Further experiments under various metabolic conditions are therefore necessary to prove that also *in vivo* a mediating step for the blood compartment is necessary.

It should be emphasized that the mechanism by which the endothelial cell processes the fatty acids from the cholesterolesters *in vivo* is predicted by the two-compartmentmodel developed by Brown & Goldstein (1). They have shown that the cholesterolester in acetyl-LDL which was taken up by macrophages *in vitro* is hydrolysed in the lysosomes and that subsequently the cholesterol is re-esterified in the cytoplasm. However re-esterification can only be achieved by addition of exogeneously fatty acid, so that the intracelled present unesterified. Probably it is captured into the cellular free cholesterol pool. When the preparation of this manuscript was in its final stage Blomhoff et al. (17) reported, that cholesterol can be transported from endothelial cells to parenchymal cells 3 to 4 h after cell uptake of acetyl-LDL. However, the FFA were not followed and it was not possible to characterise the kinetics of the hydrolysis of the cholesterolesters in vivo because 37°C liver perfusion and cell separation procedures were applied, hence hydrolysis and possibly redistribution of free cholesterol may have occurred during the cell isolation procedures. Our data clearly show that upon hydrolysis of the cholesterylesters in the endothelial cells the FFA is almost instanteneously transported to the parenchymal cells. Upto two hours after uptake of acetyl-LDL the movement of cholesterol is insignificant and the mechanism of the redistribution of free cholesterol at longer times certainly deserves more investigations before it can be decided if it follows a similar specific route as the FFA. However both the work of Blomhof and ours indicate that the cholesterol once converted to a free form is not re-esterified in the cells. The difference in cellular pro-

cessing between isolated macrophages and liver cells is probably not solely explained by the *in vitro* vs *in vivo* circumstances because also *in vitro* with isolated endothelial liver cells only 0.3 % of the cholesterol was found to be re-esterified. We suggested earlier (2, 4) that the liver uptake of modified lipoproteins forms a protection system of the body which prevent excessive accumulation of cholesterolesters in macrophages at other sites in the body (and their possible conversion to foam cells). Of course such a protection function will only be efficient if the uptake is coupled to a complete procesing of the modified lipoproteins without local pathological implications. The observation that the liver cells do not accumulate cholesterylesters after the rapid uptake and processing of acetyl-LDL, adds further strength to the hypothesis that these cells indeed might form the major protection system against the possible atherogenic action of the modified lipoproteins.

REFERENCES

1. Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K. and Goldstein, J.L. (1980). J. Supramol. Struct. *13*: 67-81.
2. Nagelkerke, J.F., Havekes, L., Van Hinsbergh, V.V.M. and Van Berkel, Th.J.C. (1984). Arteriosclerosis. *4*: 256-264
3. Henriksen, T., Mahoney, E.M. and Steinberg, D. (1983). Arteriosclerosis *3*: 149-159.
4. Nagelkerke, J.F., Barto, K.P. and Van Berkel, Th.J.C. (1983). J. Biol. Chem. *258*: 12221-12227.
5. Blouin, A., Bolender, R.P. and Weibel, E.R. (1977) J. Cell Biol. *72*: 441-455.
6. Van Berkel, Th.J.C., Nagelkerke, J.F., Harkes, L. and Kruijt, J.K. (1982). Biochem. J. *208*: 493-503.
7. Krieger, M., Brown, M.S., Faust, J.R. and Goldstein, J.L. (1978). J. Biol. Chem. *253*: 4093-4101.
8. Bierman, E.L., Stein, O. and Stein, Y. (1974). Circ. Res. *35*: 136-150.
9. Redgrave, T.G., Roberts, D.C.K. and West, C.E. (1975) Anal. Biochem. 1975 *65*: 42-49.
10. Craig, I.F., Via, D.P., Sherrill, B.C., Sklar, L.A., Mantulin, W.W., Gotto, A.M. and Smith, L.C. (1982) J. Biol. Chem. *257*: 330-335.
11. Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1976). Proc. Natl. Acad. Sci. U.S.A. *73*: 3178-3182.
12. Bilheimer, D.W., Eisenberg, S., Levy, R.I. (1972) *260*: 212-221.
13. Bligh and Dyer (1959) Can. J. Biol. Physiol. *37*: 911-917.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. *93*: 265-275.
15. De Bruyn, P.P.H., Michelson, S. and Becker, R.P. (1977). J. of Ultrastr. Res. *58*L: 87-95.
16. Scow, R.O., Blanchette-Mackie, E.J., Smith, L.C. (1980) Fed. Proc. *39*: 2610-2617.
17. Blomhoff, R., Drevon, C.A., Eskild, W., Norum, K.R. and Berg, T. (1984) J. Biol. Chem. *259*: 8898-8903.

CURRICULUM VITAE

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publicaties

1. Th.J.C. van Berkel, G.B. van den Berg, J.F. Nagelkerke, J.K. Kruijt and J.F. Koster
   Is there any hormonal regulation of metabolism in non-parenchymal cells?
   In: Short-term Regulation of Liver Metabolism (Hue, L. and Van den Werve, G., eds.), Chapter 22. Elsevier/North Holland Biomedical Press, Amsterdam, 1981, pp. 379-388

2. Th.J.C. van Berkel, J.F. Nagelkerke and J.K. Kruijt
   The effect of Ca^{2+} and trifluoperazine on the processing of human acetylated low density lipoprotein by non-parenchymal liver cells
   FEBS Letters **132** (1981) 61-66
3. Th.J.C. van Berkel, J.F. Nagelkerke, L. Harkes and J.K. Kruijt
   Extralysosomal and lysosomal processing of native and acetylated lipoproteins by parenchymal and non-parenchymal liver cells. Involvement of calmodulin?
   Biochem. J. **108** (1982) 493-503
4. Th.J.C. van Berkel, J.F. Nagelkerke and L. Harkes
   Liver sinusoidal cells and lipoprotein metabolism
   In: Sinusoidal Liver Cells (Knook, D.L. and Wisse, E., eds.)
   Elsevier Biomedical Press, Amsterdam, 1982, pp. 305-318
5. Th.J.C. van Berkel, J.F. Nagelkerke, L. Harkes and J.K. Kruijt
   Influence of antipsychotic drugs and local anesthetics upon the endocytotic processing system of non-parenchymal cells
   In: Sinusoidal Liver Cells (Knook, D.L. and Wisse, E., eds.)
   Elsevier Biomedical Press, Amsterdam, 1982, pp. 497-498
6. J.F. Nagelkerke, K.P. Barto and Th.J.C. van Berkel
   Receptor-mediated endocytosis in rat-liver non-parenchymal cells. An evaluation of two recently developed isolation procedures
   In: Sinusoidal Liver Cells (Knook, D.L. and Wisse, E., eds.)
   Elsevier Biomedical Press, Amsterdam, 1982, pp. 319-326
7. J.F. Nagelkerke, K.P. Barto and Th.J.C. van Berkel
   Isolation of parenchymal cell-derived particles from non-parenchymal rat liver cell preparations
   Exp. Cell Res. **138** (1982) 183-191
8. V. Lenaerts, J.F. Nagelkerke, Th.J.C. van Berkel, P. Couvreur, L. Grislain, M. Roland and P. Speiser
   In vivo uptake and cellular distribution of biodegradable polymeric nanoparticles
   In: Sinusoidal Liver Cells (Knook, D.L. and Wisse, E., eds.)
   Elsevier Biomedical Press, Amsterdam, 1982, pp. 343-352
9. J.F. Nagelkerke, K.P. Barto and Th.J.C. van Berkel
   In vivo and in vitro uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer and parenchymal cells
   J. Biol. Chem. (1983): **258**: 12221-12227
10. V. Lenaerts, J.F. Nagelkerke, Th.J.C. van Berkel, P. Couvreur, L. Grislain, M. Roland and P. Speiser
    In vivo uptake of poly-isobutylcyanoacrylate nanoparticles by rat liver Kupffer, endothelial and parenchymal cells
    J. Pharm. Sci. **73** (1984): 980-982
11. J.F. Nagelkerke, L. Havekes, V.W.M. van Hinsbergh and Th.J.C. van Berkel
    In vivo catabolism of biologically modified LDL
    Arteriosclerosis, **4** (1984): 256-264
12. J.F. Nagelkerke, L. Havekes, V.W.M. van Hinsbergh and Th.J.C. van Berkel
    In vivo and in vitro catabolism of native and biologically modified LDL
    FEBS Letters **171** (1984): 149-153
13. A. Amar-Costesec, M. Prado-Figueroa, H. Beaufay, J.F. Nagelkerke and Th.J.C. van Berkel
    Analytical study of microsomes and isolated subcellular membranes from liver. IX Association of NAD glycohydrolase with the plasma membrane of Kupffer cells.
    J. Cell. Biol. (1984). In press

14.Th.J.C. van Berkel, J.K. Kruijt, H.H. Spanjer, J.F. Nagelkerke, L. Harkes and H.M. Kempen
The effect of a water-soluble tris-galactoside terminated cholesterol derivative on the fate of low-density lipoproteins and liposomes.
J. Biol. Chem. In press